UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Biological Sciences

The Genetic Basis of Salad Leaf Processability

by

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Thesis presented for the degree of Doctor of Philosophy

December, 2006

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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THE GENETIC BASIS OF SALAD LEAF PROCESSABILITY

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Baby salad has become a more popular and profitable product as a healthy and convenient food, but it is a highly perishable commodity. In order to improve salad leaves and increase shelf life, it is necessary to determine the genetic basis of leaf processability traits, which are related to leaf characteristics and development. The quantitative genetic approach used in this study is a multi-gene approach to improve the leaf quality with longer shelf life.

The recombinant inbred lines (RILs) mapping population (F_9/F_{10}) used in this study was derived from a cross between cultivated lettuce (*Lactuca sativa* cv. Salinas) and wild lettuce (*L. serriola* acc.UC96US23). Significant correlations were found between shelf life and leaf area, weight, chlorophyll content, stomatal index and cell number. For the first time, the link was found between pre-harvest leaf growth characteristics and post-harvest shelf life, for an important leafy crop, lettuce. A total of 51 quantitative trait loci (QTL) for the traits of interest were identified in this study, while 13 QTL were common in both field trials (Portugal and the UK) and they were proposed to be environment independent QTL. LG1: 90 cM-100 cM and LG2: 80 cM-90 cM were 'hot-spot' areas, as several QTL co-located in these two regions. QTL for leaf processability and leaf development traits were co-located at LG1: 90 cM-100 cM. The candidate-gene strategy using single nucleotide polymorphisms (SNP) detection was explored for identification of candidate genes for leaf processability. The superior long-life line 112 and line 1 were selected for further potential commercial application. The identification of QTL and candidate genes for leaf development and processability will lead to a better understanding of leaf processability at a genetic and cellular level, and allow improvement of salad leaf quality through commercial breeding.

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ACKNOWLEGEMENTS

I am extremely grateful to Prof. Gail Taylor and Dr. Mark Dixon for supervision and support during this project and Dr. Steve Rothwell and at Vitacress Salads Ltd. for providing advice and the Industrial CASE funds for this study. This study was also supported financially by BBSRC industrial CASE studentship. I would like thank Dr. Graham Clarkson at Vitacress Salads Ltd. for all his advice and help during this project. Thanks go to Prof. Richard Michelmore for the gift of seed material and allow me to use the genetic map and to Dr. M. Truco for teaching QTL cartographer software. I would like to thank Dr. L. Knerr at Shamrock Seed Company for bulking up the extreme lines.

I am grateful for the help of the staff at Vitacress Salads Ltd., in particularly, Keston Williams, Andy Elworthy & Erik Wiersma for the arrangement and maintenance of the field trials. I am indebted to Lucy, Duncan, Fabian, Tina, Ningying, Matt, Laura, Mike and Angelina, Oswaldo for their hard work to help planting and harvesting. I am also thankful to Katy Cherry who did the initial parent experiment during her undergraduate project and Jacques Bertrand who did the whole population experiment in the glasshouse during his undergraduate project. I was also appreciated to have Dr. Anne Rae for advice on QTL analysis and help on the California field trip, Dr. Penny Tricker for her advice and encouragement, Dr. Carol Wagstaff for her help with the transgenic study, and Mike Cotton and for his help to look after the plants in glasshouse. I would also like to thank Caroline, Maricela, Matt, Laura, Nat, James, Nicole, Herriet, all the members of Plants and Environment lab.

Finally, I would also like to thank my husband, Fulong Wu, and all my family for their helpful advice, assistance, encouragement and support during this PhD study.

List of abbreviations and symbols

A_1	Leaf area for the first point in the growth rate measurement experiment
A_2	Leaf area for the second point in the growth rate measurement experiment
AFLP	Amplified fragment length polymorphism
AGR	Absolute growth rate
ANOVA	Analysis of variance
BC	Backcross population
^{0}C	Degrees Celsius
CaMV35S	Cauliflower mosaic virus 35S promoter
cDNA	Copy DNA
cM	Centimorgans
cv	Cultivar
d	Days
CAP	Cleaved amplified polymorphism
CCI	Chlorophyll content index
CCM	Relative chlorophyll content meter
CHL	Chlorophyll content
CIM	Composite interval mapping
C_t	Total chlorophyll concentration
DH	Double haploid population
DHPLC	Denaturing high performance liquid chromatography
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DW	Dry weight
DWP	Dry weight as a percentage of fresh weight
E	Elasticity
ECA	Epidermal cell area
ECN	Epidermal cell number per leaf
EST	Expressed sequence tag
FW	Fresh weight
G x E	Genotype by Environment
gDNA	Genomic DNA

Definitions and Abbreviations

h	Hour
LOD	Likelihood of odds
LPI	Leaf plastochron index
MAS	Marker assisted selection
MIM	Multiple interval mapping
min	Minute
mRNA	Messenger RNA
ML	Maximum load
NIL	Near isogenic lines population
ns	Not significant
Ψ_S	Solute potential
OSM	Osmolality
P	Plasticity
PCR	Polymerase chain reaction
PI	Plastochron Index
PR	Photosynthesis rate
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphism
RFLP	Restrict fragment length polymorphism
RGR	Relative growth rate
RIL	Recombinant inbred line
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
SAT	*Lactuca sativa* cv. Salinas
SD	Stomatal density
sec *or* s	Second
SEM	Scanning electron microscope
SER	*Lactuca serriola* (US96US23)
SC	Stomatal conductance
SI	Stomatal index
SL	Shelf life

Definitions and Abbreviations

SLA	Specific leaf area
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats
T	Time gap for the growth rate measurement experiment
T_a	Primer annealing temperature
TEM	Transmission electron microscope
TILLING	Targeting induced local lesion in genomes
TRA	Transpiration rate
WT	Wild type
XET	Xyloglucan endotransglucosylase
XTH	Xyloglucan endotransglucosylase/hydrolase

CHAPTER 1

Introduction

1.1 Background

1.1.1 Industry demand

During the last decade, consumers have become more interested in eating healthy and more convenient foods (Masih et al., 2002). The modification of eating habits includes the consumption of more vegetables and fruits and less dietary fat. Baby salad leaves contribute to the 'five-a-day' intake of fresh fruits and vegetables, as recommended by health professionals. Pre-packed baby salads, consisting of lettuces, beets, herbs and spinach, fit this consumer demand perfectly. Sales of bagged salads are growing vigorously and supermarkets are devoting increased space to the baby leaf salad as a profitable and popular product. The demand for fresh, minimally processed vegetables has led to an increase in the quantity and variety of products available to the consumer (Jacxsens et al., 2002). In the UK, the prepared salad sector was estimated to be worth £494 million in 2004 in a Mintel report (http://www.preparedfoods.com/FILES/HTML/Mintel_Reports/PF_mintel_reports/). The most rapid increase in the market has been in the 1990s and was growing at a rate of 23% a year from 2003-2004 (DEFRA statistics, 2005, http://statistics.defra.gov.uk/esg/default.asp), one of the strongest growing food sectors in the UK. Internationally, the market for pre-prepared salads in the USA exceeded $2 billion in 2002 (http://www.freshcut.com/freshcut/fcstories.htm). However, prepared salad leaves often do not last long enough once in the fridge, and their nutritional content could be better. The commercial value of these crops is affected by a relatively short shelf life (3-5 days). There is a need for crop improvement to extend shelf life.

1.1.2 The process

The processing of salad leaves includes the necessary harvesting, washing, sanitisation, de-watering and packaging (Figure 1.1). The term "processability" is defined here as the ability of leaves to withstand the whole process (Clarkson et al., 2003). The processes cause bruises, rendering leaves susceptible to desiccation and wilting. The fresh leaves are vulnerable to discoloration because of damaged cells and tissues, and the processes also expose internal tissues to microbes and potentially deleterious endogenous enzymes (Watada & Qi, 1999). Weak leaves are easily bruised and damaged with consequential impact upon shelf life and saleability. A key factor in the commercial success of this sector lies in the ability of salad leaves to withstand the whole process.

1.1.3 Shelf life

Among the limitations to the shelf life of salad leaf products are microbial spoilage, desiccation, discolouration or browning, bleaching, textural changes and development of off-flavour or off-odour (Barrett et al., 1998). At present, most products offer quite a short term, usually little more than 2 or 3 days, between purchase and end of life. A minimum five-day shelf life is required, consequently, there is a strong demand from retailers for longer shelf lives of seven days or more. It is predicted that the sales of baby salad would increase if the produce remained in good condition over a significant period, as customers are demanding more bags a week for a healthy diet. Consumers expect salad leaf products to be without defects, of optimum maturity and in fresh condition (Watada & Qi, 1999). Highly processable leaves are hence desirable, as they would presumably exhibit extended shelf life. To develop salad leaves with higher processability will benefit the commercial industry.

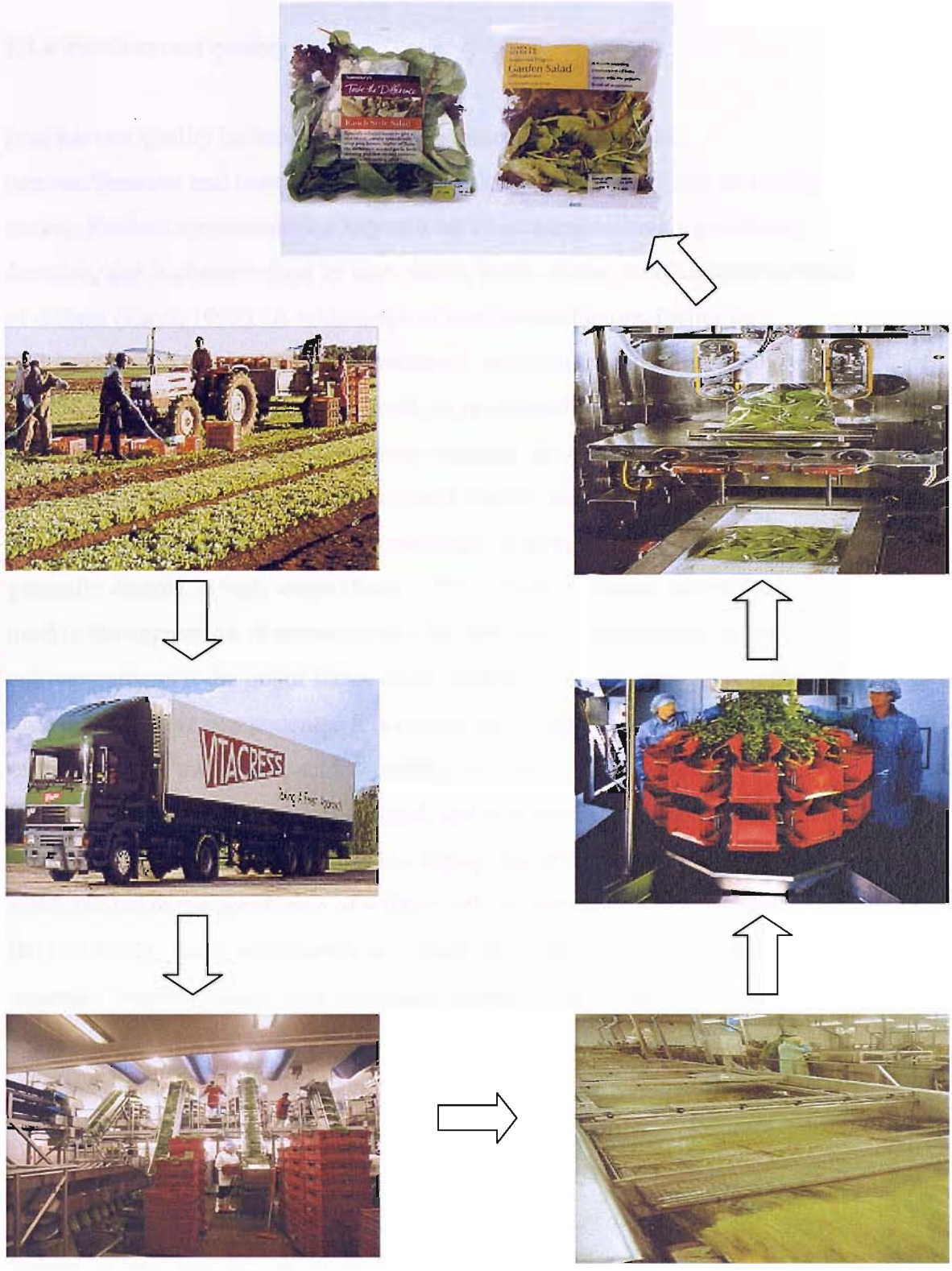


Figure 1.1 The process of baby leaf salad from harvest to market product. The process includes harvest, transport, inspection, washing, drying, and packaging as final supermarket product, shown in the flowchart order (modified from Clarkson, 2004).

1.1.4 Post-harvest quality

Post-harvest quality includes general appearance, sensory quality (texture/firmness and taste), nutrient quality, longevity of shelf life, and safety issues. Product appearance is a key trait for consumers making a purchasing decision, and is characterised by size, shape, form, colour, condition and absence of defects (Kays, 1999). A wide range of pre-harvest factors, including biological, physiological and environmental factors, mechanical damage and genetic variation, can change the product's appearance (Kays, 1999). Textural traits are very important in determining consumer acceptability. Biochemical components such as lipid content, cell wall content and composition, particle size and shape, and moisture content all contribute to texture. Crisp firmness is generally desired in leafy crops (Sams, 1999). Environmental factors may modify the expression of textural traits, but the genetic background of the cultivar variants is the major factor controlling texture. Flavour is a complex of taste and aromatic components. It is critical for repeat purchases. Key taste components are sweetness, acidity, astringency and bitterness (Aked, 2002). Taste is not readily defined or measured, and is assessed differently by individual preference. Longevity of shelf life is a highly desirable quality in baby leaf salad, related to the prevention of wilting, off-flavours and rotting disease (Ryder, 2002). Baby salad leaves are important contributors of vitamins and minerals. Nutrient quality is of increasing interest to the consumer, with special interest in anti-carcinogenic effects. In leafy lettuces, there are well-recognised nutrition contents such as Vitamin A and C and calcium.

1.1.5 Improvement approach

Extending leaf shelf life can be approached from two angles: modifying the leaf product using genetic approaches including breeding and transformation, or modifying the process using different post-harvest treatments. To date, numerous studies have addressed the latter, investigating ways in which the preparatory process may be modified to minimize the damage to salad leaves in order to improve product quality. These process studies include: modified atmosphere packaging (MAP) (e.g. Rai et al., 2002; Jamie & Saltveit, 2002); pre-

packaging chemicals (Fukumoto et al., 2002, Wills et al., 2002); heat-shock (Delaquis et al., 2000; Saltveit, 2001); diurnal time of harvest (Clarkson et al., 2005). One study has modified the expression of several xyloglucan endotransglucosylase/hydrolase (*XTH*s) in transgenic lettuce, significantly altering several leaf properties (Clarkson, 2004). So far, few studies have addressed the link between pre-harvest leaf growth characteristics and post-harvest leaf processability, and understanding of the genetic basis of post-harvest shelf life in leafy crops is rather limited, although likely to be linked to pre-harvest leaf traits. Investigating the genetic basis of leaf processability is a quantitative genetic approach to improve leaf processability. Compared with the single-gene approach of transformation, the quantitative genetic approach applied in this study is a simultaneous multi-gene approach.

1.1.6 Lettuce as a target

1.1.6.1 History of lettuce

The earliest records for cultivated lettuce date back to as far as 2500 BC in Egypt, with images of lettuce with long thick stems and long, narrow, pointed leaves (Ryder, 1999). Stem lettuce went to China as early as the 5th century. Lettuce with either green or red leaves was quite popular in Ancient Greece and Rome and moved north into Western Europe. It was introduced to America by Columbus in the 15th century. The first true iceberg-type lettuce, "Great Lakes", was released in 1941 (Ryder, 2002). Cultivated lettuce (*Lactuca sativa* L.), a diploid rosette plant, exists in a large genus of over 100 species of the Composite family. It comprises 9 pairs of chromosomes. Wild lettuce, *L. serriola* L. (prickly lettuce), is the best-studied wild species of lettuce, and thought to be the progenitor of *L. sativa* (Kesseli et al., 1994). Microsatellite fingerprinting showed a close relationship between the two species of lettuce; however there are differences in morphological traits, which directly relate to the domestication that separates the wild and cultivated forms (Van de Wiel et al., 1998). These two species are capable of free interchange of genes, with little, if any, reduction in fertility (Ryder, 1999).

1.1.6.2 Lettuce breeding

Over the centuries, traditional selective breeding has developed the immense intraspecific and interspecific lettuce variation that we see today (Ryder, 1999). Variation within *L. sativa* accessions occurs predominantly in leaf length, shape, colour, texture and size, presumably as these are economically important traits. Cultivar selection is of primary importance in achieving the desired product. While cultivars range widely in shape, size, and colour, they also vary in their ability to achieve the desired phenotype under differing production conditions (Kays, 1999).

In past decades, successful cultivars have dominated the production on the basis of their resistance to various diseases, including down-mildew (caused by the fungus *Bremia lactucae*). Disease resistance in cultivated lettuce was achieved through exploitation and introgression of elements of wild lettuce, including *L. serriola* germplasm (Lebeda & Pink, 1998). Now, there is more interest in breeding for consumer/end user orientated output traits. Traditional plant breeding programmes tend to be costly in both time and money, as it usually needs 6-8 generations to achieve the homozygous desired cultivar. However, by using molecular markers, it is possible to identify the superior genotype for the trait of interest from a small quantity of sample tissue in the relatively short term. To date, genomic approaches have been generating large amounts of data on plants and their pathogens. High-throughout genotype technologies that are being developed for genomics research will have significant impacts on breeding for crop improvement (Michelmore et al., 2003).

1.1.6.3 Lettuce demand

Lettuce is a popular fresh salad vegetable in many countries. Johnston et al. (2000) reported that lettuce is the most commonly consumed vegetable in the American diet. About 40% of Americans consumed lettuce during their study period and lettuce alone is valued 1.86 billion dollars per year in the USA (Johnston et al., 2000). Lettuce is increasingly being traded to food service and retail customers as a baby leaf salad, either alone or in combination with other

salad leaf types (Desphande & Salunkhe, 1998). Owing to the inherent perishability of lettuce and its market demand, lettuce was chosen to be the target for improvement of leaf processability in this project.

1.2 Modern genetic modification methods

Transformation is the process of introducing foreign DNA into a new host species; a direct introduction of a small number of genes by transformation seems to be a reliable approach to engineering crops with desired traits (Turner et al., 2000). Plant transformation has become an important approach for crop quality improvement (Bhat & Srinivasan, 2002).

1.2.1 Transformation studies in lettuce

In 1987, Michelmore et al. (1987) developed the first successful transformation of lettuce (*L. sativa*) using plasmids of *Agrobacterium tumefaciens* containing a chimeric kanamycin resistance gene. In order to optimize the transformation protocol for a broad range of germplasms, Curtis et al. (1994) developed a genotype-independent *Agrobacterium*-mediated protocol. Thirteen lettuce cultivars, representing the main lettuce types, have been successfully transformed. The genotype-independent transformation procedure has been successfully utilised in the following transgenic research in lettuce (Curtis et al., 1999a, 1999b; Yang & Li, 1999; McCabe et al., 2001).

Several transgenic studies have reported recently on the expression of the *ipt* gene (which encodes isopentenyl phosphotransferase). A direct effect of cytokinin on senescence was shown by expression of the *ipt* gene under the control of a SAG12 promoter in *Arabidopsis* (Gan & Amasino, 1995). This construct was designed to express *ipt* only at the onset of senescence. Transgenic plants have delayed senescence and produce more flowers with no apparent effect on morphology. This gene was also transferred into lettuce. The transgenic lettuce showed increased cytokinin and chlorophyll contents (Curtis et al., 1999a). McCabe et al. (2001) reported that the *ipt* gene under control of

senescence-specific SAG12 (P_{SAG12}-IPT) significantly delayed developmental and post-harvest leaf senescence in mature heads of transgenic lettuce. The *ipt* gene is suggested to be of benefit to long-term storage of leafy vegetables.

1.2.2 Anti-sense *XTH* transgenic study

Xyloglucan is a major structural polysaccharide in the expanding cell walls of higher plants (Fry et al., 1992). Xyloglucan endotransglucosylases/hydrolases (*XTH*s) cleave and re-join xyloglucan chains and then contribute to both wall-assembly and wall loosening (Thompson & Fry, 2001). They suggested *XTH*s serve important roles in both the assembling and loosening of the cell wall, together enabling long-term plant cell expansion with minimal loss of wall strength. The anti-sense *XTH* transgenic lollo rosso type lettuce was generated with the potential for being more processable. The previous preliminary study from primary *XTH* transformants in the lab suggested that *XTH* plays an important role in leaf processability, and three transgenic lines were suggested for further study (Clarkson, 2004). Further study would determine the fundamental impact of cell wall *XTH* on leaf traits and the application of this technology to improve processability. The anti-sense approach could be used to reduce cell expansion, reducing translation of a gene or group of genes involved in the loosening of the cell wall to permit expansion by the incorporation of new xyloglucan chains. This is a single gene approach to manipulate the cell wall so as to improve leaf processability.

1.2.3 Stability of regenerated plants

Since the first report of *Agrobacterium*-mediated transformation of lettuce (Michelmore et al., 1987), there have been several reports on the production of transgenic lettuce (Gilbertson, 1996; Dinant et al., 1997; Curtis et al., 1999b; Frugis et al., 2001; McCabe et al., 1999a, 1999b, 2001). However, industry breeders have not benefited from the genetic engineering, as transgenic lettuces have not been released onto the market. This probably is due to the high degree of transgene instability in lettuce. Gilbertson (1996) reported that 80% of transgenic first seed generation (T_1) lettuce plants resistant to lettuce mosaic

virus (LMV) lost their resistance in the T_2 generation. The principal causes of low transgene expression in plants have been reported to be the position of transgene integration into the plant genome (Curtis et al., 1999a), integrity (McCabe et al., 1999a), the promoter selection (McCabe et al., 1999b), copy number (Hobbs et al., 1993) and silenced genes (Pang et al., 1996).

1.2.4 Targeting induced local lesions in genomes (TILLING)

Targeting induced local lesions in genomes (TILLING), a reverse genetic strategy, was first described by McCallum et al. (2000), providing an allelic series of induced point mutations from a population of chemically mutagenised individuals. Transformation such as the anti-sense *XTH* lettuce discussed above is a reliable approach to achieve a particular gene for the trait of interest, but it has led to the complication of genetic modification (GM) food, as it is usually difficult to have GM food accepted by consumers. On the other hand, the introduced gene may be silenced after a few generations, which increases the complication of the transgenic technology application. Thus, a non-GM alternative is highly desirable for the commercialization of any novel lettuce lines. The reverse genetic TILLING approach overcomes these disadvantages. It applies the findings gained from transformation to commercial application. For example, a TILLING population of a legume has been developed to screen the *SYMRK* mutant line in the UK by Perry et al. (2003).

To apply this TILLING technology to crop improvement, the Anawah Company has been established in Seattle, WA (http://www.anawah.com). There are 2 mutation libraries consisting of over 9,000 lettuce plants in this company. These libraries consist of M3 lettuce seed, and a corresponding DNA sample is accessible to screen for mutant lines for genes of interest. It takes only a few months to achieve the TILLED mutation set (Henikoff et al., 2004). After further investigating T_3/T_4 *XTH* transgenic lettuce to confirm the role of *XTH* in leaf processability, this TILLING technology will be applied in the future project to develop non-GM lettuce to improve quality with regard to extended shelf life.

1.3 Mapping lettuce populations and modern molecular breeding

1.3.1 Quantitative trait loci (QTL)

In quantitative genetics, if the trait is determined by many genes, the population segregation will be a continuous distribution. A large proportion of morphological and physiological traits are controlled quantitatively, exhibiting continuous variation from extreme to extreme in a segregating population (Kearsey & Pooni, 1996). Such quantitative traits cannot be studied individually by the principles of Mendelian genetics in the same way as qualitative traits. The quantitative traits are under the more complex control of several genes of additive effect, which may be also affected by the environment to varying degrees (Kearsey & Farquhar, 1998). Quantitative trait loci (QTL) are 'chromosomal locations of individual genes or groups of genes which influence complex traits' (Paterson, 1998). QTL analysis has become an important tool to dissect the genetics of complex characters (Yin et al., 2003).

1.3.2 Mapping population

There are several types of mapping population, depending on the mating system of the crop species, including F_2 plants, backcross (BC) to the parental lines, homozygous double haploid (DH) lines, and recombinant inbred lines (RIL), derived from an initial cross between two inbred lines (Kearsey & Pooni, 1996). Plants enjoy especially great latitude for different populations. The most common types of population are listed below.

1.3.2.1 F_2 population

Traditionally, genetic linkage mapping relied heavily upon intercrossing two homozygous genotypes to produce a heterozygous F_1, and selfing or intercrossing of heterozygous F_1s creates a population that shows segregation at any given locus where parents show allelic variation in the traditional 1:2:1 ratio. The phenotypic distributions between a dominant homozygous and a

heterozygous genotype are often impossible to distinguish. It cannot be replicated in the F_2 population, as heterozygosis prevents their maintenance by self-mating, so the material is finite and can only be used once. It is impossible to use the same population to repeat the experiment in several environmental conditions, different years, locations etc.

1.3.2.2 Backcross population (BC)

Crossing the heterozygous F_1 back to one of its parents creates 1:1 segregation for polymorphic alleles from the donor parent. This population remains useful for a number of purposes, especially introgression of exotic germplasm from a wild relative into a domestic type (Kearsey & Pooni, 1996). It is easier and quicker to obtain, and at low cost. However, the accuracy of using this population is often poor, as a large population size is needed, which is time-consuming from the point of view of fieldwork and genotype analysis. It has the same problem of non-reproducibility as F_2 populations.

1.3.2.3 Recombinant inbred lines (RILs)

As RILs are produced by repeated selfing of F_2 individuals to produce a homozygous segregated population, the lines undergo several rounds of meiosis before the homozygosity is reached; there is a higher probability of recombination between linked genes. There are fewer heterozygous individuals with each passing generation, and the homozygous RILs may be reached usually after 6-8 generations of selfing. QTL can be identified much more accurately using replicates of true breeding populations (i.e. near homozygous or completely homozygous lines). These RIL populations are highly informative but at the cost of time to produce. The mapping population used in this project consists of F_9 and F_{10} generations, and are assumed to be homozygous. The biggest advantage of RIL populations is they are comprised of homozygous genotypes, which can be replicated in different environments or different years. The main disadvantages are the time and cost of producing such populations, but once produced these populations are more effective to use than basic generations.

1.3.2.4 Double haploid population (DH)

DH population is an attempt to combine the advantage of homozygosity with the speed at which an early generation population can be made. Production from cultured cells with subsequent chromosome doubling can obtain homozygous lines from a hybrid in a single generation. DH populations exist for barley, rice, *Brassica oleracea*, and a number of other major crops (Paterson, 2002). Genetically, DH individuals contain two identical chromosomes, and therefore each contains only one uniquely informative gamete, equivalent to first-generation backcross individuals as recombinant in terms of information content. The advantage of the use of DH lines is that when several genes control a trait, recessive alleles at all loci can be seen in the DH population, while only a small proportion of homozygous recessive alleles can be seen in the F_2 population and cannot been seen in a backcross population if the recessive allele is from the donor genotype (Paterson, 2002).

1.3.2.5 Near-isogenic lines (NILs)

It may be possible to map QTL more precisely by specifically selecting for recombinant events in a particular region. QTL that cannot be resolved in early generation studies may be detected by using large-scale backcrossing strategies. Near-isogenic lines are derived from two genotypes that are identical apart from a relatively small defined region on a particular chromosome. The differences in phenotype between these two genotypes must be due to genes in the defined region. NILs have been used in plants to validate and fine-map QTLs, since all phenotypic variation can be associated with the lone introgressed allele (Monforte &Tanksley, 2000). Since each line carries only a small fraction of the original two genotypes' genomes, NILs have also been employed to identify multiple genetic factors contributing to a single QTL (Eshed & Zamir, 1995), to indicate pleiotropic effects and the physiological basis of QTL effects (Paterson, 2003), and to separate genes for desirable traits from linked genes for undesired traits (Monforte & Tanksley, 2000).

1.3.3 Phenotypes of mapping populations

For QTL mapping, the parents of the population should have contrasting phenotypes for the traits of interest, as QTL can only be mapped if polymorphism is observed in the segregating population. Once the traits of interest have been identified, every individual of the mapping population must be scored for the traits of interest, which is called phenotyping the population. In general, a large sample of individual genotypes has to be assessed to represent the variation of the population, to provide observable effects of a number of recombinants, and to allow a thorough assessment of the traits of interests. In the RILs population, a few replicates of each line were assessed for each trait measurement to obtain precise phenotypic means for QTL analysis.

1.3.4 Molecular markers

The knowledge of the structure and function of plant genomes is rapidly expanding due to the fast development of techniques in molecular biology such as automated sequencing, DNA library construction and screening, and DNA marker technologies (Jeuken et al., 2001). Genes contributing to the variation in crosses and populations can be counted and located using numerous techniques such as RFLP and AFLP. Various marker technologies differ in the inheritance of information content (dominant or co-dominant) and the polymorphism of information content. Several molecular marker analyses are listed below.

1.3.4.1 Restriction fragment length polymorphism (RFLP) markers

Restriction fragment length polymorphism (RFLP) analysis employs cloned sequences to probe specific regions of the genome for sequence variations at the DNA level (Landry et al., 1987). These variations are seen as changes in the length of DNA fragments produced by digestion with restriction endonucleases. The segregation of many polymorphic RFLP markers can often be followed in a single cross. In Landry et al.'s study on lettuce, the construction of a linkage map using RFLPs had three phases: development of probes, choice of parental lines and identification of polymorphic probes and segregation analysis. RFLPs

provide the opportunity to develop detailed genetic maps from a limited number of crosses. The advantages of RFLPs are locus-specificity and co-dominant inheritance. RFLPs are used in the estimation of genetic diversity in plant species (Pejic et al., 1998). The disadvantage is that the technology is time-consuming, laborious and costly. It requires large amounts of genomic DNA and often uses radioactivity.

1.3.4.2 Random amplified polymorphic DNA (RAPD) markers

Following Landry et al.'s map, random amplified polymorphic DNA (RAPD) markers were developed to develop the map (Kesseli et al., 1994). While RFLPs have been used to identify the majority of loci in most of the map, RAPD markers are being used with increasing frequency, and their reliability as markers is being determined. RFLP and RAPD markers have been found to show similar distributions through the genome and similar levels of polymorphism have been identified. RAPD loci were much quicker to identify but more difficult to order. Typically, RAPDs are dominant markers and RFLPs are co-dominant. The map intervals calculated between dominant markers vary greatly in precision according to whether linkages are in coupling or repulsion phase, whereas linkages between co-dominant markers are more accurate, as recombinants are more readily detected (Waycott et al., 1999). Despite these differences, they found that gene orders and linkage distances were fairly well maintained between the two maps.

1.3.4.3 Microsatellite or simple sequence repeats (SSR) markers

It was reported that microsatellite fingerprinting is capable of distinguishing all accessions of a series covering all known lettuce crop types using only one $(TCT)_{10}$ probe / *Taq*I restriction enzyme combination (Van de Wiel et al., 1998). They developed microsatellite fingerprinting, also called simple sequence repeats (SSRs) to be molecular markers for the study of genetic diversity (Van de Wiel et al., 1999). SSRs are reliable markers with potentially many alleles and hence a co-dominant inheritance. These are mainly used as easy applicable markers for specific loci. The information contents (the amount of polymorphism), measured

by the expected heterozygosity and the average number of alleles, were higher for the SSRs. They have become more popular because of higher degrees of information (Rafalski, 2002). Their disadvantage is the *a priori* sequence information that is required to design the locus-specific primers (Jeuken et al., 2001).

1.3.4.4 Amplified fragment length polymorphism (AFLP) markers

AFLP (Amplified fragment length polymorphism) analysis was developed as a PCR-based method for detecting differences in restriction fragments (Vos et al., 1995). The technique involves the restriction of genomic DNA with two endonucleases, ligation of adapters of known sequence to each end, selective PCR of a subset of fragments followed by polyacrylamide gel analysis of the amplified fragments. Most AFLP markers are dominant. Up to 200 loci can be analysed in a single reaction, depending on the genome size, the enzyme combination, the number of selective nucleotides, and the resolution of the electrophoresis system (Witsenboer et al., 1997). The AFLP technique does not require *a priori* sequence information and combines the advantages of RFLP markers with the advantages of PCR. AFLP markers are efficient and reliable and can be used across species (Qi et al., 1998; Vuylsteke et al., 2000). These new marker technologies allow the efficient construction of high-density maps, which have several applications in genetics and breeding (Jeuken et al., 2001). AFLPs have become more popular due to their higher efficiency.

Witsenboer et al. (1997) explored a method of selectively amplified microsatellite polymorphic locus (SAMPL) for identification, genetic localization and allelic diversity in lettuce. SAMPL analysis uses one AFLP primer in combination with a primer complementary to microsatellite sequences. They explored the potential of SAMPL analysis in lettuce to detect PCR-based co-dominant microsatellite markers. Fifty-eight SAMPLs were identified and placed on the genetic map. SAMPL analysis is more applicable to intra-specific than to inter-specific comparisons. The analysis based on SAMPLs resulted in a dendrogram similar to those based on RFLP and AFLP markers in their study (Witsenboer et al., 1997).

1.3.4.5 Cleaved amplified polymorphism (CAP) markers

Cleaved amplified polymorphism (CAP), also known as PCR-RFLP, markers utilize amplified DNA fragments digested with a restriction endonuclease to display restriction site polymorphisms (Konieczny & Ausubel, 1993). It is similar to AFLPs, but the difference is that DNA fragments in CAPs are first amplified and subsequently cleaved with restriction enzymes. In comparison with other molecular markers, CAP markers have several advantages: quick and easy to assay, small amount of DNA required due to PCR amplification and co-dominant information content (Iwata et al., 2001). CAP markers are developed from sequenced-tagged-site (STS) primers with designs based on cDNA (i.e. expressed-sequence-tags (ESTs)). They therefore present real functional genes and are more useful as genetic markers than those based on anonymous non-functional sequences such as SSRs.

1.3.5 Genetic linkage maps

Detailed genetic linkage maps are fundamental tools for studies on selection, identification and organization of plant genomes. Genetic studies in lettuce have identified many morphological genes controlling leaf, flower, seed characteristics and numerous genes for disease resistance (e.g. Waycott & Taiz, 1991; Ryder, 1996; Witsenboer et al., 1997; Shen et al., 1998; Jeuken & Lindhout, 2002). Some of these characters have been shown to be linked (Kesseli et al., 1994; Waycott et al., 1999).

Landry et al. (1987) constructed the first linkage map for lettuce with RFLP, isozyme, disease resistance and morphological markers. The genetic markers were distributed into nine linkage groups and cover 404 centimorgans (cM), which may be about 25-30% of the lettuce genome. The flanking markers were used to study the source of variation in downy mildew resistance genes (*Dm*) and are also part of a strategy to clone resistance genes. Based on the intra-specific cross "*L. sativa* cv. Calmar x *L. sativa* cv. Kordaat", a genetic map, comprising 319 loci, including 152 RFLP, 130 RAPD, 7 isozyme, 19 disease resistance and 11 morphological markers, was produced by Kesseli et al. (1994). In this map,

thirteen major and four minor linkage groups, as well as several unlinked markers, were identified for this genome, which is estimated to be approximately 1950 cM. It consists mainly of RFLP and RAPD markers with an average spacing of 6.1 cM and major gaps of up to 28 cM. It has been used to map *Dm* genes and other disease resistance genes (e.g. Okubara et al., 1994; Witsenboer et al., 1995).

The above two genetic mapping studies on lettuce were focused on population segregation for disease resistance (Landry et al., 1987; Kesseli et al., 1994). Waycott et al. (1999) mapped 10 morphological genes of lettuce using two different mapping strategies (RFLP and RAPD). Seven morphological loci were placed on the genetic map. The morphological traits in their study included seed colour, flower colour and leaf colour. Markers linked to additional horticultural characteristics such as leaf shape and head formation as well as colour determinants would be useful. Usually, the expression of many of these commercially important genes cannot be determined during the early stages of growth. The early and rapid determination of superior genotypes through marker-assisted selection prior to field maturity would be of benefit to the breeder (Waycott et al., 1999).

Jeuken et al. (2001) made an integrated inter-specific AFLP map of lettuce based on two *L. sativa* x *L. saligna* F_2 populations. Due to the high similarity in marker order and map distances of 124 markers common to both populations, the two independent genetic maps of the two F_2 populations were integrated. They used special software, JoinMap 2.0 (Stam & Van Ooijen, 1995), to construct the linkage map. This integrated map consisted of 476 AFLP markers and 12 SSRs on nine linkage groups spanning 854 cM. It has been reported that AFLP markers using the *Eco*RI/*Mse*I restriction enzyme combination tend to cluster around centromeric regions in other crops (Qi et al., 1998 in barley; Young et al., 1999 in soybean). However, the AFLP markers did not show severe clustering in Jeuken et al.'s map. This provides good opportunities for use in QTL mapping and marker-assisted selection in lettuce. Recently, lettuce backcross inbred lines (BILs) have been developed in which chromosome segments of wild lettuce (*Lactuca. saligna*) were introgressed into cultivated lettuce (*L. sativa*) (Jeuken &

Lindhout, 2004). The set of BILs covers the complete genome of *L. saligna*, while each BIL contains only one homozygous introgression of *L. saligna*. These BILs can be a useful alternative if the basis of segregation populations does not meet the prerequisites that allow QTL mapping. The genetic map (Jeuken et al., 2001) previously developed was immediately used for MAS in this BILs population.

1.3.6 QTL mapping methods

QTL mapping is the process of finding and estimating associations between a continuous quantitative trait and a set of molecular markers that have been previously placed on a genetic map, with the aim of identifying markers that are linked to the control of the phenotypic trait that has been measured. QTL mapping works on the principle that if a locus on the genome is causing variation in a trait, and data are obtained from a cross in which the QTL is segregating, then values of the trait will be correlated with markers linked to that locus (Mackay, 2001). Once the phenotypic data have been collected on each individual, statistical associations between markers and quantitative traits are established through the following statistical approaches.

1.3.6.1 Single-marker tests

Single-marker tests such as t-test and general linear regression assess the segregation of a phenotypic trait with the marker genotype. Typically, a marker that is linked to a locus controlling the phenotypic trait will generate a stronger regression than one that is less linked. This method has the potential to identify numerous significant markers. However, the strength of the regression between markers and loci will decrease as the genetic distance between markers and loci increases. This method loses accuracy if the QTL does not lie exactly at the marker position. The approach cannot distinguish between a QTL with a small effect tightly linked to the marker gene and a QTL with a large effect that is loosely linked.

1.3.6.2 Interval mapping

Interval mapping, made popular by Lander & Botstein (1989), uses an estimated genetic map as the framework for the location of QTL. The intervals are defined by ordered pairs of markers, and a single QTL is tested across a marker interval. For each interval in a genome, the most likely phenotypic effect of a putative QTL associated with the trait of interest is computed, maximising the likelihood of the observed phenotypic data. The odds ratio is calculated by dividing the chance that the data would arise from a QTL with the effect by the chance that the data would arise given on linked QTL. Interval mapping calculates the likelihood of the odd (LOD) score, which is the logarithm of the likelihood of the odds ratio. LOD is the probability of a QTL being located within a marker interval measured against the probability of the result occurring by chance. If the LOD score exceeds a predetermined threshold, the presence of a QTL is assumed. Interval mapping is certainly more powerful than single-marker approaches for detecting QTL. However, it is limited as a single QTL method by being a one-dimensional search that does not allow interactions between multiple QTL to be detected.

1.3.6.3 Composite interval mapping

Composite interval mapping (CIM) is an extension of interval mapping to include additional markers as cofactors in a multiple regression analysis (Jansen & Stam, 1994; Zeng, 1994). The purpose is to remove the variation that is associated with other (linked) QTL in the genome. The principle of CIM has been extended to multiple traits, enabling the evaluation of the main QTL effects as well as QTL by trait and QTL by environment interactions (Kulwal et al., 2003). Strictly speaking, CIM methods are not multiple QTL methods, as the model for evaluating the effects of each interval depends on the marker cofactors included, which vary across intervals.

1.3.6.4 Multiple interval mapping

A true multiple interval mapping (MIM) method has been developed that uses multiple marker intervals simultaneously to construct multiple putative QTL (Kao et al., 1999). MIM has been shown to be powerful and precise in detecting QTL. The MIM model can readily search for and analyse epistatic QTL and estimate the individual genotypic value and the heritabilities of quantitative traits. QTL interactions are of particular interest as these QTLs indicate regions of the genome that might not otherwise be associated with the quantitative trait using a one-dimensional search (Doerge, 2002). The marker cofactors are under the control of the investigator, as the order of factors entered into a multiple regression model must be determined by the investigator.

1.3.7 QTL mapping and marker-assisted selection (MAS)

Genetic maps are essential to locate the loci that are involved in the expression of traits. This can be easily done for simple heritable traits based on a single gene, but it is also possible for complex traits that are based on more loci (Kearsey & Pooni 1996). QTL mapping is very useful to locate multi-loci. In this case, large segregating populations (n >100) are required to unravel the number of loci involved in the trait (Jeuken et al., 2001). After characterizing the phenotype and genotype of the population, a marker-by-marker ANOVA may be used to identify 'significant differences between the phenotypic means for the genetic classes at a particular marker locus' (Prioul et al., 1997). When significant values are achieved, the specific molecular marker used to define this genotypic class is considered to be associated with a QTL for the trait of interest. However, this method fails to discriminate between QTLs of strong effect distant from the marker and QTLs of relatively weak effect positioned closer to the marker (Prioul et al., 1997). The interval map with multiple markers is now employed to overcome these constraints (Kearsey & Farquhar, 1998). When the map positions of important loci are known, indirect selection of plants bearing the useful loci can take place at the DNA level on the basis of markers linked to the loci of interest.

QTL were identified for differences between wild (*L. serriola*) and cultivated lettuce (*L. sativa*) for root architectural traits and water acquisition (Johnson et al., 2000). Thirteen QTL were detected, each accounted for 28-83% of the phenotypic variation. In this experiment, co-dominant AFLP markers were used for composite interval mapping. The 513 markers fell into ten linkage groups spanning 1342 cM and provided markers throughout the genome. Then a subset of 109 markers spaced approximately 10-15 cM apart was used as a framework map in QTL analysis. The results provided the opportunity for marker-assisted selection via the introgression of wild alleles into cultivated lettuce to improve soil water and nutrient acquisition from deeper soil zones, thereby minimizing water and fertilizer inputs and ultimately enhancing water quality. Recently, lettuce backcross inbred lines have been developed for exploration of the *L. saligna* (wild lettuce) germplasm (Jeuken & Lindhout, 2004). These studies show the potential value of the wild lettuce germplasm in improving existing cultivars and developing a new cultivar towards the 'optimal design' or the 'ideal ideotype' (Kearsey & Farquhar, 1998). To our knowledge, there are only three QTL studies on lettuce, reporting the improvement of root water use efficiency (Johnson et al., 2000), seed traits (Argyris et al., 2005), and disease resistance (Jeuken & Lindhout, 2002).

In the last decade, QTL mapping has been a useful tool in the study of the genomic nature of traits, not only for providing DNA markers linked to agronomic traits, but also for elucidating the fundamental mechanisms of genetic control, for example, for 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 (Frary et al., 2000; Fridman et al., 2002; Seymour et al., 2002); bean disease resistance (Kelly et al., 2003); fibre strength in cotton (Zhang et al., 2003) and maize stalk quality (Krakowsky et al., 2005). Recently, the use of marker-assisted selection has shown high potential in plant breeding (Knapp, 1998; Zamir, 2001; Asíns, 2002; Moose et al., 2004).

1.3.8 Genotype and environment interaction (G × E)

The successful application of QTL mapping depends on the reliability and accuracy of the QTL analysis from which information has been obtained. The accuracy of the QTL mapping relies on many factors: the ability of the statistical method to detect the QTL, the experimental design, its size, the heritability of the traits, QTL distribution over the genome, the number and distance between consecutive markers, the percentage of co-dominant markers, the reliability of the order of markers in the linkage map, the evaluation of the trait, etc. (Asíns, 2002). There are two interaction effects that may affect the QTL: genotype by genotype interaction (epistasis) and genotype by environment interaction (Mackay et al., 2001). The robustness of QTL can be tested by assessing QTL in different environments.

As genes, QTL effects may be environmentally sensitive, and this sensitivity results in phenotypic plasticity (Maloof, 2003). In maize, QTL effects for grain yield and plant height were largely independent of the environment, while QTL for grain moisture were highly environment dependent (Mackay, 2001). The number and contribution of each quantitative QTL that has significantly different effects across environments will be associated with substantial genotype by environment (G x E) interaction effects (Moreau et al., 2004). Such G x E interaction effects can be indicative of QTL that are specific to a particular environment; lack of a G x E interaction can suggest that a QTL is a more general growth regulator (Maloof, 2003). G x E interaction is of critical importance to genetic breeding (Van Kleunen & Fischer 2005). In breeding practice, it is a risk to apply the superior genotype to different environments when it is achieved based on QTL information obtained in only one environment. If the superior genotypes predicted in different environments are very different, their superiority may be dramatically reduced in different environments. Thus, in order to develop broadly adaptable cultivars, it is necessary to partition the main QTL effects and G x E interaction effects, and to base genetic improvement only on the QTL with the main effects (Yang & Zhu, 2005). If a QTL shows G x E interaction then selection of genotypes adapted to specific environments may well be achieved.

1.4 Functional genomics to determine and map candidate genes

1.4.1 EST data-mining for identifying candidate genes for leaf processability

Expressed sequence tag (EST) databases are currently the fastest growing and represent the largest proportion of the available DNA sequence databases. They are potentially significant resources for the detection of single nucleotide polymorphisms (SNPs) (Lopez et al., 2005). ESTs have become an important tool to understand plant genome structure and gene expression and function. These resources have been used in large-scale identification of SNPs in humans (Picoult-Newberg et al., 1999), *Arabidopsis* (Schmid et al., 2003), maize (Batley et al., 2003) and rice (Ren et al., 2004). EST-data mining will be a new strategy to identify the candidate genes associated with phenotypic characteristics. The current Compositae Genomics Programme, funded by USDA and NSF, has identified in excess of 50,000 lettuce ESTs, providing a unigene set of 19,523 (http://cgpdb.ucdavis.edu) (Table 1.1). This genomic resource is fully available to this project. Many of the leaf development characteristics are tractable in model plants such as *Arabidopsis* (Kessler & Sinha, 2004), and it is now possible to utilize information from model systems linked to emerging genomic resources in crops to the development of informed molecular plant improvement programmes (Zamir, 2001).

Table 1.1 Summary of available functional genomic resource in lettuce.

Database	**Lettuce**
Total number of reads	76,593
Average read length (nt)	528
Number of ESTs in contigs	56,853
Total number of contigs	8,179
Number of singletons	11,344
Unigene set	19,523
Average unigene length (nt)	848

1.4.1.1 Cell wall

The plant cell wall is an important structure that determines cell size and shape, glues cells together, provides essential mechanical strength and rigidity, and protects against pathogens and dehydration (Cosgrove, 2001). The cell wall is a complex network of cellulose, hemicelluloses, pectins and structural proteins (Campell & Braam, 1999). The cell wall has two conflicting characteristics: tensile strength, resilience and stability versus structural plasticity in response to developmental signals and adaptation to environmental conditions (Rose & Bennett, 1999). Processability is likely to be governed by cell wall thickness, stiffness and cell wall biochemistry. *XTH*s have been suggested to play an important role in both the assembling and the loosening of the cell wall, together enabling long-term plant cell expansion with minimal loss of wall strength (Fry, 1997; Thompson & Fry, 2002). It is becoming clear that all plant species probably contain large families related to *XTH*s with a total of 46 *XTH*s revealed from 14 plant species. Thirty-three *XTH*s have been found in *Arabidopsis* and these have been sorted into three phylogenetic groups based on gene structure and organisation (Rose et al., 2002).

Expansins are cell wall proteins that induce pH-dependent wall extension and stress relaxation in a characteristic and unique manner. Two families of expansins are now well established, named α-expansins (*EXP*) and β-expansins (*EXPB*) (Cosgrove et al., 2002). In *Arabidopsis* the *EXP* family has 26 members and the *EXPB* family has five members (http://www.bio.psu.edu/expansins/) (Li et al., 2002). The biological roles of expansins are related to the action of expansins in loosening cell walls and thereby stimulating plant cell enlargement. Wall loosening by expansins is very different from that caused by an endoglucanase that hydrolyses xyloglucan. This endoglucanase causes walls to extend only after a significant lag period (minimum of 6 minutes) and this was accompanied by a large increase in wall plasticity, while *EXP*s induced wall extension immediately and did not increase wall plasticity (Cosgrove et al., 2002). *XTH*s and e*xpansins* have been reported to be involved in cell wall loosening and breakdown during fruit ripening (Brummell & Harpster, 2001). Hazen et al. (2003) reported that QTL for sugar composition of the cell walls

were detected in maize. They identified a few candidate genes involved in the essential process of cell wall biosynthesis. Investigating the role of the cell wall is likely to lead to identification of the candidate genes involved in leaf processability.

1.4.1.2 Cell division

Cell division and expansion both contribute to final leaf size and shape (Dengler & Kang, 2001; Tsukaya, 2003; Taylor et al., 2003). To date, the co-ordination 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). Cell division plays a role both in the developmental processes that create plant structure and in the modulation of plant growth rate in response to the environment (Cockcroft et al., 2000). The plant cell cycle is regulated at multiple points, involving a number of key regulators such as cyclin-dependent kinases (CDKs), CDK inhibitor genes, cyclins, retinoblastoma (Rb) protein homologs, and E2F transcription factors (Dewitte & Murray, 2003). Menges et al. (2002) identified about 500 cell-cycle-regulated genes in *Arabidopsis*. Cell division control was shown to be a consequence of interaction between plant hormones, developmental regulators as well as cell wall metabolism. Leaf size and cell wall are critical for leaf processability, and thus a number of genes that control the cell cycle may serve as further candidate genes for leaf processability.

1.4.1.3 Leaf surface – wax

Plants develop a cuticle as an efficient barrier against uncontrolled water loss. The cuticle is a thin (0.1-10 μm thick) continuous membrane consisting of a polymer matrix (cutin), polysaccharides and associated solvent-soluble lipids (cuticle wax) (Riederer & Schreiber, 2001). The cuticle has to accommodate the plasticity of the cell wall (Carpita & Gibeaut, 1993). The cuticle wax is divided into surface (epicuticular) and embedded (intracuticular) wax. The mechanical properties of the cuticle depend on the amount of hydroxylated fatty acids (Marga et al., 2001). The elasticity of the cuticle impacts plant development and

consequently influences fruit and vegetable storage times (Roy et al., 1999). The wax cuticle prevents water loss and its thickness is proposed to be one factor to protect leaf material that is more processable. Thus the genes involved in wax synthase and accumulation are considered as putative candidate genes for leaf processability.

1.4.1.4 Senescence, cytokinin and ethylene

Leaf development ends with senescence, consisting of deteriorative events that lead to cell death (Guarente et al., 1998). During leaf senescence, the cells experience dramatic changes in metabolism and cellular structure (Oh et al., 1997). Because of the biological importance and potential for improvement of crop characteristics such as plant productivity and post-harvest storage, there have been some physiological and biochemical studies about plant leaf senescence during the past decade (e.g. Gan & Amasino, 1997; Buchanan-Wollaston, 1997; Guarente et al., 1998). However, molecular and genetic analyses of the mechanism of leaf senescence have only recently been investigated (Nam, 1997; Quirino et al., 2000; Buchanan-Wollaston et al., 2003; Lim et al., 2003; Yoshida, 2003). A number of genes, referred to as senescence-associated genes (SAGs), are up-regulated in leaves before or during senescence in different species (Gepstein et al., 2003; Lin & Wu, 2004). More recently, transcriptome associated with leaf senescence was examined by large-scale single-pass sequencing of ESTs prepared from senescing *Arabidopsis* leaves (Gepstein, 2004; Guo et al., 2004). This information about senescence associated genes (SAGs), well studied in *Arabidopsis*, is very useful in the investigation of the role of SAGs in leaf processability in baby salad leaves.

Cytokinins play an important role in plant growth and development. A direct effect of cytokinin on senescence was shown by expression of the *ipt* gene (encoding isopentenyl phosphotransferase) under control of a SAG12 promoter in *Arabidopsis* (Gan & Amasino, 1995). Transgenic plants had delayed senescence and produced more flowers with no apparent effect on morphology (Curtis et al., 1999b). McCabe et al. (2001) reported that the *ipt* gene under control of senescence-specific SAG12 promoter from *Arabidopsis* (P_{SAG12}-IPT)

significantly delayed developmental and post-harvest leaf senescence in mature heads of transgenic lettuce. The *ipt* gene is suggested to be of benefit in long-term storage of this leafy vegetable (McCabe et al., 2001). Delayed leaf senescence has also been achieved in transgenic lettuce, using the *Arabidopsis knl*-like homologue gene, *KNAT*, under the promoter PetE. It was shown that the over-expression of *KNAT1* in the aerial tissues of lettuce (*L. sativa*) induced profound changes in plant architecture (Frugis et al., 1999). They found that *KNAT1* over-expression is associated with an overproduction of specific types of cytokinins (Frugis et al., 2001). They suggested that the involvement of *knox* genes involved in the control of cell fate is possible through the modification of cytokinin metabolism.

Ethylene is a naturally occurring plant growth substance that has many effects on the growth, development and storage life of vegetables (Hackett et al., 2000). Ethylene has been demonstrated to induce the expression of SAGs (Hajouj & Gepstein, 2000). Ethylene production is stimulated when plant tissues are injured and it can accumulate in packages of fresh-cut product, which can lead to undesirable effects (Kim et al., 1995). Removal of C_2H_4 or inhibition of its action can delay colour changes in storage and prolong the storage of selected commodities (Saltveit et al., 2003). An ethylene-insensitive mutant of *Arabidopsis* has been shown to exhibit a 30% increase in longevity compared with wild-type leaves on the plant. This delay in senescence coincides with a delay in the expression of SAGs and higher expression levels of photosynthesis-associated genes (Grbic & Bleecker, 1995). Costa et al. (2005) recently suggested two genes (*Md-ACO*1 and *Md-ACO2*) controlling the ethylene biosynthetic pathway as candidate genes for the shelf life of apples.

1.4.2 Detection of single nucleotide polymorphisms (SNPs)

1.4.2.1 SNPs

SNPs are a single pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals in some populations, where the frequency is higher than 1% (Brookes 1999). Single variants in cDNAs (cSNPs)

are usually classed as SNPs since most of these will reflect underlying genomic DNA variants. The typical frequency with which one observes single base differences in human genomic DNA from two equivalent chromosomes is of the order of 1/1000 bp (Wang et al., 1998). SNP markers could be used in many applications including the construction of high resolution genetic maps, mapping traits, genetic diagnostics, analysis of the genetic structure of populations, phylogenetic analysis, etc. (Rafalski, 2002). The major advantage of SNPs is their abundance in the genome.

The interest in SNPs in plants, stimulated in part by the progress of human SNP discovery, is rapidly increasing. It has been reported that over 1.4 million SNPs have been identified in the human genome (Sachidanandam et al., 2001). Cereon Corp. (http://www.arabidopsis.org/cereon/) has identified over 56,000 DNA polymorphisms in *Arabidopsis*. They have produced a very large SNP collection available to academic researchers (Buckler & Thornsberry, 2002). No comparable data set exists in any other plant species (Rafalski, 2002). The current Compositae Genomics Programme, funded by USDA and NSF, has identified in excess of 50,000 lettuce expressed sequence tag (EST) sequences (http://cgpdb.ucdavis.edu). It should be possible to discover the SNPs in lettuce using this database. Since the ESTs are derived from a number of different genotypes, many polymorphisms are expected to exist between multiple EST representations of a gene (Rafalski, 2002). There are some reports that identify the SNPs using the special computer programme POLYBAYES (Marth et al., 1999; Picoult-Newberg et al., 1999; Buetow et al., 1999).

1.4.2.2 Methods for SNP genotyping

The most direct approach to the discovery of DNA polymorphisms is direct sequencing of PCR products from a number of diverse individuals. PCR primers are designed on the basis of known DNA sequences. If the individuals to be genotyped by sequencing are homozygous, as in the recombinant inbred lines, it is easy to detect the DNA polymorphisms. But the haplotype, where a combination of alleles at closely linked loci tends to be inherited together, could be unambiguously determined (Rafalski, 2002). A large genome will need

extremely large expenditures for sequencing. Directed re-sequencing of gene segments distributed throughout the genome appears to be much more practical.

A number of methods have recently been developed for genotyping SNPs (Wang et al., 1998; Hoogendoorn et al., 1999; Lindroos et al., 2002; Yoshida et al., 2002; Lee et al., 2004). SNPs do not require separation of DNA fragments by size and thus can be performed in 96- or 384-well high-density assay plates. The principles of these methods include oligonucleotide hybridization, nuclease cleavage of mismatches, oligonucleotide ligation, primer extension and direct sequencing (Gupta et al., 2004). Low assay cost throughout and automation are very important to develop a high-multiplex, robust SNP detection technology.

Denaturing high performance liquid chromatography (DHPLC)

Denaturing HPLC was initially developed for large-scale SNP detection in the human genome (Wang et al., 1998). DHPLC allows the automated detection of single base substitutions as well as small insertions and deletions. The underlying principle of the technique is a slightly altered melting behaviour of heteroduplexes versus homoduplexes, leading to a difference in retention time on ion-pair reverse-phase HPLC columns. On a chromatogram, DNA homoduplexes are generally eluted in one peak, whereas DNA heteroduplexes produce one or more additional peaks. The sequence difference is thus translated into an altered chromatogram, which eliminates the need to obtain information about the change at the DNA sequence level (Nairz et al., 2002). DHPLC is a very efficient technique for genotyping SNPs without the necessity of determining the nature of the SNPs. The analysis time is 6-10 min per sample, depending on the configuration of the instrument, and heteroduplex profiles are easily distinguished from homeoduplices. The sensitivity and specificity values range from 96% to 100% (Wagner et al., 1999).

Two other significant advantages of DHPLC are the low cost of analysis and the minimal labour involved in loading crude PCR products into an auto-sampler and reviewing the elution profiles. DHPLC costs approximately $1 versus $15 for sequencing (Wagner et al., 1999). DHPLC has been recently used in numbers of

human disease studies (Nairz et al., 2002; Yoshida et al., 2002). It has been used for SNP mapping in plant science (Kota et al., 2003), but in this case, it was only introduced as an assay system to detect an already established sequence-verified SNP. DHPLC could be very powerful for plant genetic SNP mapping and also could be applied to the screening of candidate genes.

Oberacher et al. (2002) demonstrated the ability of completely denaturing ion-pair reverse-phase HPLC to purify the different alleles and to determine their sequence on-line by electrospray ionization mass spectrometry (ESI-MS). This method is a fully automated, computer-aided data interpretation and inexpensive. It may have applicability in population genetics (Oberacher et al., 2002).

Multiplex SNP analysis using capillary DNA sequencer

The advanced genetic analysis systems, such as the Beckman CEQ8000, enable a capillary electrophoresis system to perform automated sizing and allele calling from multiplex SNP products. A single-base primer extension is applied to detect the precise location of an SNP site. It utilizes the inherent accuracy of DNA polymerase to determine the presence or absence of the specific nucleotide at the SNP site. A specially synthesized DNA primer is used to anneal to the SNP site of interest. The primer anneals one base short of the target SNP. DNA polymerase inserts the complementary dideoxy nucleotide terminator into the SNP site. This technology provides a simple multiplexable SNP-genotyping solution with high accuracy and reproducibility.

Real-time PCR

Polymerase chain reaction (PCR) is a diagnostic method widely used for gene detection. The PCR product of the amplification is usually loaded onto an electrophoretic gel and separated by means of an electric field. The amplified bands are recognized to be the right ones because of their molecular weight compared with an appropriate standard. This is called "end-point PCR" (Giovannini & Concilio, 2002). Real-time PCR is a modified PCR technique that uses two primers and an additional dual-labelled fluorescent probe to allow

the continuous monitoring of amplicon synthesis during thermocycling, and requires no post-PCR sample handling for target quantification (Cullen et al., 2002). The first fluorescent method allows specific sequence detection because this fluorescent is used to label the sequence-specific hybridization probes. The probes include TagMan, Molecular Beacons, LightCycler and Amplifluor. The second fluorescent method uses the DNA binding dye, SYBR green I. This dye binds in the minor groove of double-stranded DNA in a sequence-independent way (Deprez et al., 2002).

In a real-time PCR assay, depending on the number of amplification cycles applied, even a very small initial DNA template (few picograms) is amplified million/billion-fold and is detectable. For this reason, real-time PCR is used as a quantitative method, while end-point PCR is usually considered a qualitative method only (Giovannini & Concilio, 2002). Real-time PCR has been proved to be a sensitive and accurate quantitative PCR diagnostic assay. It has been recently applied in many studies such as gene expression (Charrier et al, 2002); disease diagnostics (Cullen et al., 2002); gene detection (Giovannini & Concilio, 2002) and stress study (Svensson et al., 2002). However, few studies have as yet reported its application in quantitative genetics. Real-time PCR assay offers the potential for efficient and accurate SNP mapping. But real-time PCR is more expensive than DHPLC. Thus, it is better to use DHPLC to screen the identified candidate ESTs, and to use real-time PCR to obtain high throughout information for the selected 10 best ESTs for identifying candidate genes involved in leaf processability.

1.4.3 Co-location of QTLs and candidate genes for the traits of interest

It is possible to detect and locate QTLs only by the joint analysis of segregation of marker genotypes and of phenotypic values of individuals or lines (Asíns, 2002). Recently, several studies have been carried out to co-located QTL and candidate gene mapping. Once the putative candidate genes derived from EST-data mining have been mapped at the same location as the QTL for the traits of interest, these co-located candidate genes will be identified for the traits of

interest. Several studies have successfully identified major co-location QTL, and suggest the candidate genes for further research (Causse et al., 2002; Hazen et al., 2003; Bert et al., 2003; Kulwal, 2003; Zhang et al., 2005).

Jeuken & Lindhout (2002) showed a good model for exploring an interesting gene via QTL mapping. Because of major yield losses in lettuce (*L. sativa*) cultivation due to downy mildew, lettuce breeders have put a large effort into obtaining resistance to this pathogen. *Dm* genes that confer race-specific resistance have been identified and these genes have been introgressed into commercial cultivars from cultivated germplasm sources or closely related species like *L. serriola*. However, these genes were soon rendered ineffective by adaptation of the pathogen (Lebeda & Pink, 1998). *L. saligna* is resistant to all downy mildew races and can be consider as a non-host. Jeuken & Lindhout (2002) developed an F_2 population based on a *L. saligna* x *L. sativa* cross. QTL mapping with AFLP markers revealed a qualitative gene (*R39*) involved in the race-specific resistant and three QTLs (*RBQ1*, *RBQ2* and *RBQ3*) involved in quantitative resistance. *R39* will be an alternative resistance gene that is non-race specific.

Another significant application of QTL analysis may be in transgenic technology. Once the candidate genes for the traits of interest have been identified, these genes can be cloned for use in gene functional research. QTL analysis not only provides DNA markers for efficient selection, it is also of particular value in connecting plant genomics functions to the agronomic traits with which they are involved (Frary et al., 2000; Asíns 2002; Reymond et al., 2003). Several QTL have been cloned by map-based cloning in *Arabidopsis*, rice and tomato (Prioul et al., 1999; Frary et al., 2000). QTL cloning is becoming quite feasible (Remington et al., 2003; Salvi & Tuberosa, 2005). Cloned QTL provide valuable information to evolutionary biologists and population geneticists about the molecular nature of quantitative traits, adaptation and evolution.

To date, there are only two QTL studies for candidate genes in lettuce (Johnson et al., 2000; Jeuken & Lindhout, 2002). Recently, lettuce backcross inbred lines

have been developed for exploration of the *L. saligna* (wild lettuce) germplasm (Jeuken & Lindhout, 2004). Using the QTL approach, it should be possible to develop a new 'ideal ideotype' lettuce with high processability to meet the needs of the growing market. QTL mapping has proved to be a strategy to improve crops. A summary of QTL for this study is shown in Figure 1.2. In the study of lettuce leaf processability, the development of a population of two different types of lettuce cross and analysis of the population with molecular markers will be a possible way to detect useful genes or interesting QTLs for leaf processability through QTL mapping, thereby improving salad leaf processability.

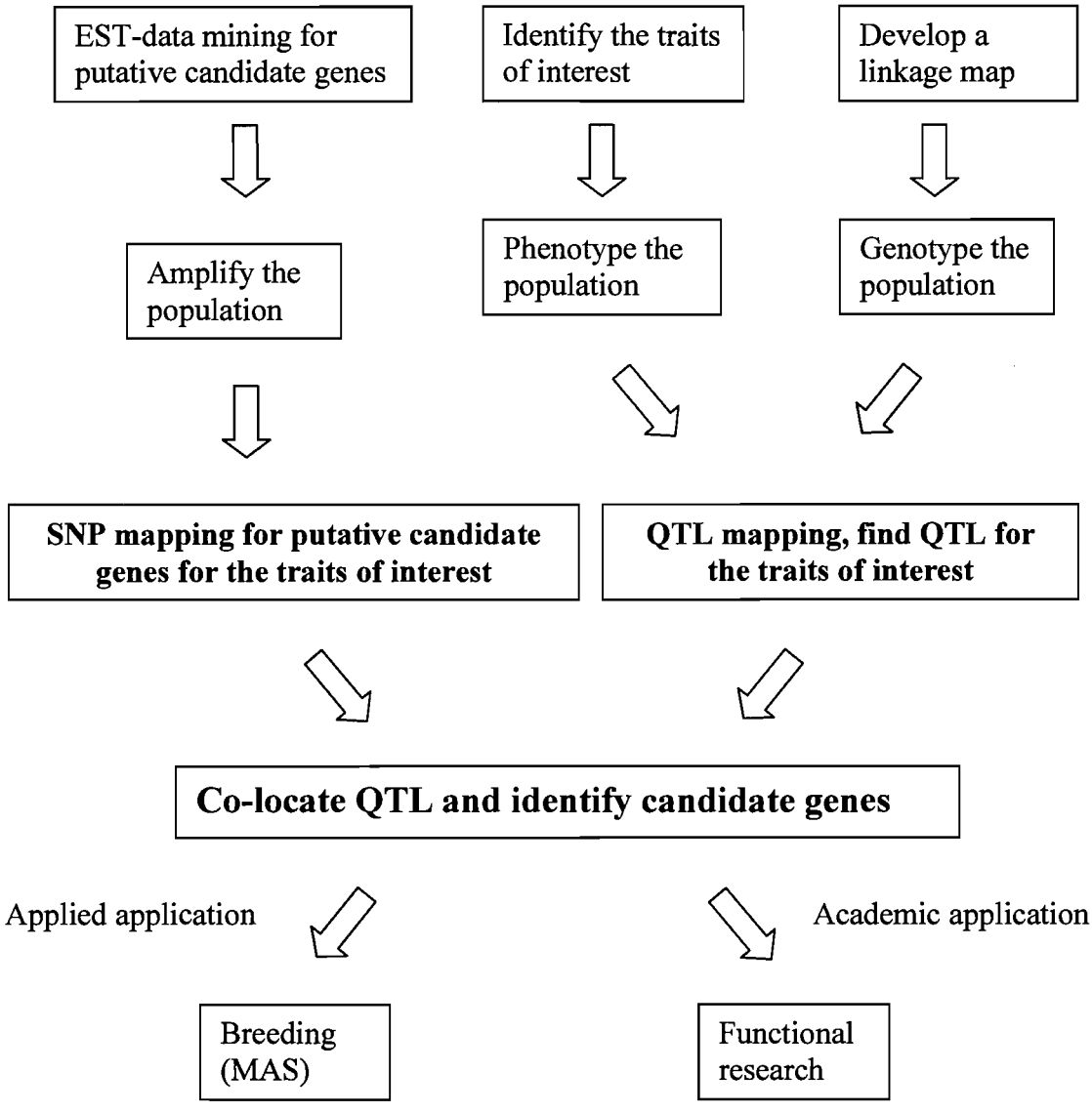


Figure 1.2 Summary of QTL mapping strategy for exploring the genetic basis of the traits of interest.

Aims:

I. **To identify the QTL for leaf development and processability, and to test the robustness of QTL in different environments.**

II. **To identify the long-lived superior genotype line and to explore its commercial application.**

III. **To explore the SNP strategy to identify the candidate genes involved in leaf development and processability using a quantitative genetic approach.**

CHAPTER 2

Material and Methods

2.1 Plant material

2.1.1The parents of RIL mapping population

The parents of the mapping population are wild lettuce (*Lactuca serriola* US96US23) (SER) and cultivated lettuce (*L. sativa* cv. Salinas) (SAT). The seed material was supplied by Professor R. W. Michelmore (University of California, Davis). Seeds were planted in 125 cm^3 cell seed trays (Homebase) in a 2:1 mixture of vermiculite (Avon Crop, Blacknell) and Levingtons F2 compost, watered and held in the dark at 2°C for 72 h. Germination and growth took place in a glasshouse and the trays were placed in a randomised arrangement and watered from the base every day. The growth conditions in the glasshouse were day temperature of 18°C, night temperature of 14°C and 12h day-length.

After 2 weeks of growth, 15 plants of each species were randomly selected and labeled for pre-harvest leaf length and leaf area measurements as well as the harvest experiment. After five weeks from germination, 15 of the labeled plants of each species were sampled for harvest measurements. The leaf number is based on the order in leaf development stages (leaf 1 was the youngest leaf and leaf 6 was the oldest leaf). Leaf 7 and 8 were the first two true emerged leaves and were discarded because of wilting. The growth of the two species was observed visually and recorded photographically at various stages of development.

2.1.2 Recombinant Inbred Lines (RIL) population

The mapping population includes 113 recombinant inbred lines (RILs). The seed material of the whole population was a gift from Professor R.W. Michelmore (University of California, Davis). Sixty highly informative recombinant lines from the whole population were selected for the field trial study. In the field

trials, the 60 F_9 RILs and two parents were grown for phenotype at two commercial farms with standard industry maintenance. The extended F_{10} mapping population (113 RILs) was grown in the glasshouse for DNA and RNA extraction and phenotypes of some traits which were difficult to assess in the field.

2.1.3 The parents of HRI mapping population

The parents of HRI mapping population are *Saladin* and *Iceberg*. The seed material was supplied by Prof. D. Pink (Horticulture Research International, UK). Saladin is a standard American iceberg type crisp bred by Dr. E. Ryder at Salinas, California. Most of the modern iceberg types grown in Europe are derived from it. Saladin is the European name of *L. sativa* cv. Salinas. Iceberg is not an iceberg type. Actually it was taken to the US by immigrants and the iceberg types are derived from it. It is an old Batavian variety bred in France in the mid 1800s. Its original name is Batavia blonde bord rouge. It has much softer pale green leaves with red edges.

2.1.4 Transgenic lettuce

Seeds for transgenic study were collected from T_2 *XTH* anti-sense transgenic lettuce, produced previously in this laboratory (Clarkson, 2004). Lollo rosso red leaf types *L. sativa* were targeted for transformation. Three T_2 transgenic lines (7-25; 11-9; 19-3) showed the most interesting results from the previous study. T_3 trait investigation will be the focus on these three lines (Appendix 1).

2.2 Trial experiments

2.2.1 Field trials of selected mapping population (60 RILs)

Two separate field sites were used in this study (Figure 2.1). The first field trial was taken at Boavista farm, near Odemira, Portugal (Latitude 37°C 36'N, longitutde 1°C 10'N) during 10th April, to 27th May, 2003. The experiment was

undertaken in Portugal because the weather is usually better and there are longer hours of daytime in Portugal than the UK. But there were heavy rains during the first two weeks in Portugal after sowing the material, which was not the ideal environment condition for this experiment. The second field trial was taken at Pinglestone Farm, near Winchester, UK (Latitude 51° 6'N, longitude 1° 10'N) during 15th July to 29th August, 2003. It was possible to grow the material in 2003 in the UK because it was the hottest summer in 30 years. The temperature during the growing period at Pinglestone farm was around 25°C to 35°C.

The field trials were designed with three blocks (Appendix 2.1 & 2.2). In each block, three replicates of the 62 lines were planted in a randomized pattern, generated with the statistical software package Minitab 13.0 for Windows (Minitab Inc., Philadelphia). There were four plants in each replicate line plot. In each field trial, there were three to nine replicates, with six replicates as an average for each trait measurement, depending on the seed germination.

In the UK field trial, there was one extra HRI block, which comprised of 30 plants of the two parents (Iceberg and Saladin) of HRI mapping population. This trial was to test the suitability of this HRI mapping population to meet the project objective to map QTL for leaf processability (Appendix 3).

2.2.2 Field trial of extended mapping population (113 RILs)

In year 2004, a field trial was undertaken at the field site of the USA National Agricultural Research Station at Salinas, California, USA (Figure 2.1). The environment conditions (temperature, light and rain) of Salinas, California are considered to be the most suitable for growing lettuce in the world. Three replicates of 113 F_{10} RILs (whole mapping population) were randomized in this field trial. There were 20 plants in each replicate line plot with a 20cm gap between plants (Appendix 2.3). The field site was at the research station and plants received standard maintenance. At eight weeks after sowing, the leaf samples were collected for soluble sugar content measurement and xyloglucan endotransglucosylase/hydrolase (XTH) activity assessment.

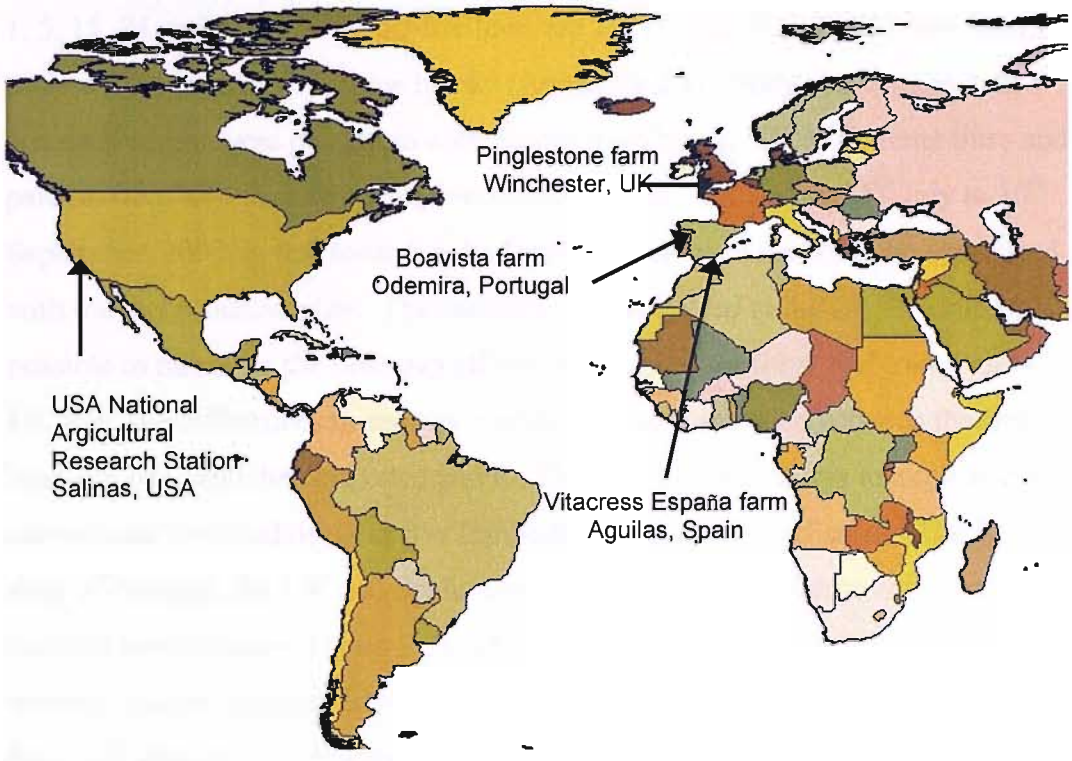


Figure 2.1 Locations of four field trial sites used in the study.

2.2.3 Field trials of extreme shelf life lines

Five long-life lines and short-life lines were selected from the previous two field trials, the Portugal trial and the UK trial. These extreme lines and two parents were planted in a new site, Vitacress España farm (Figure 2.1), Aguilas, Spain (Latitude 37° 41', Longitude -1° 58') during 20th January to 7th April, 2005 to test the reliability of these extreme lines in terms of shelf life. Five long-life lines are 1, 5, 15, 74 and 112. Five short-life lines are 13, 19, 32, 89 and 105. The field trials were designed with three blocks (Appendix 2.4). Sixty plants of each line and each parent were planted in each randomized block. These extreme lines and parents were also planted at Pinglestone farm, in the UK during 15th July to 10th September, 2005, to test some key leaf traits, which were shown to be associated with the leaf processability. The samples were collected at the similar time as possible to minimise the time gap effects on the time-sensitive leaf traits. In this UK trial, the differences in sensory profile were also assessed between the five long-life lines and the cultivated parent. The aim of this trial was to explore the commercial potential of these five long-life lines for baby leaf salad. The field sites of Portugal, the UK and Spain were in the commercial farms with standard industry maintenance (Figure 2.2), while the USA site is a national agricultural research station, maintained to industrial standard. The photographs of these four field sites at harvest were shown in Figure 2.3.

2.2.4 Glasshouse experiments of extended mapping population

Two glasshouse trials with the extended F_{10} mapping population of 113 RILs were untaken in the glasshouse in May and October 2004, respectively. The growth conditions were the same as described in the previous parent experiment in section 2.2.1. There were 12 plants in each RIL tray and the trays were randomized on the bench in the glasshouse (Figure 2.4).

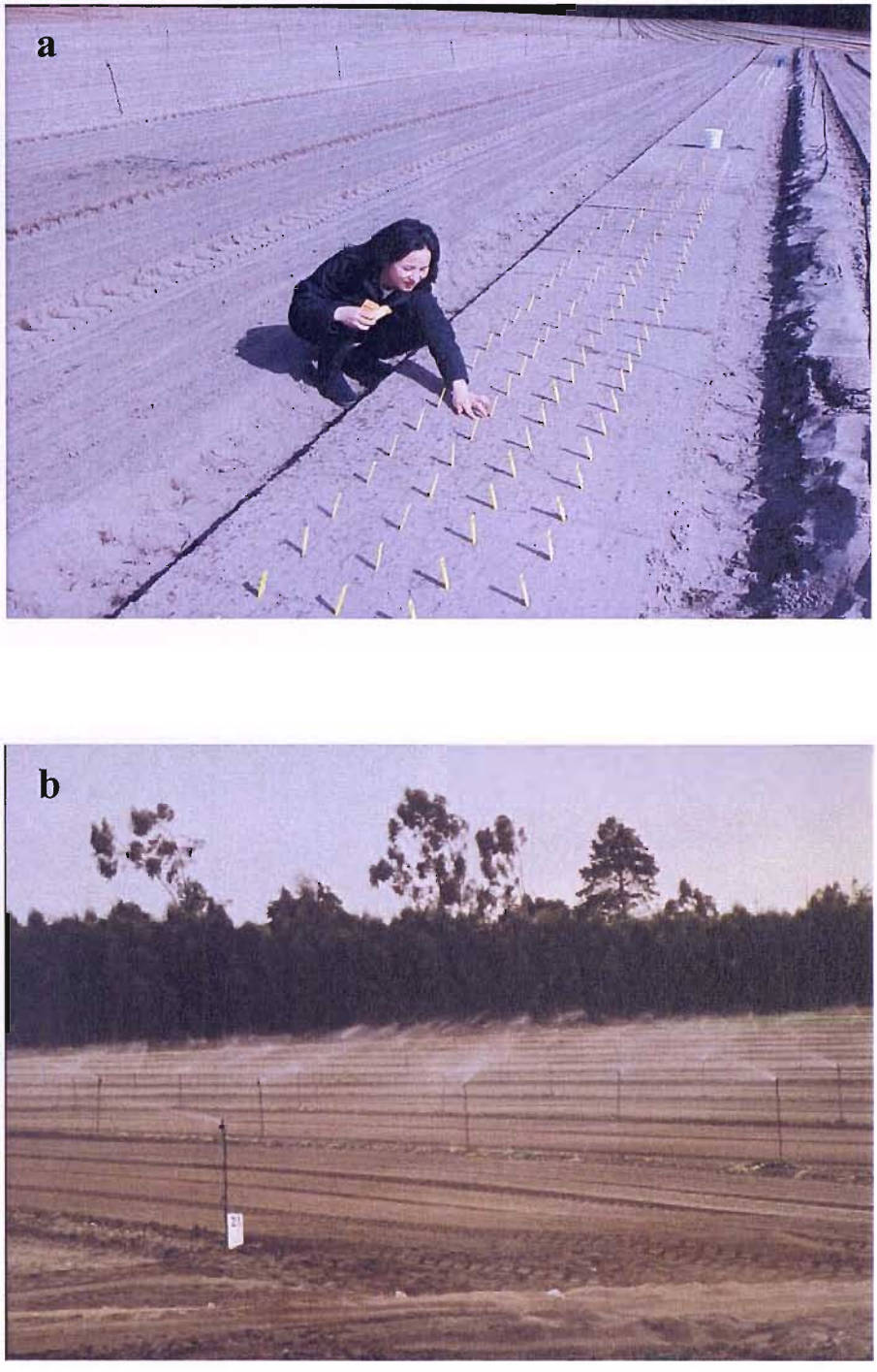


Figure 2.2 Portugal field trial at the stages of planting (a) and watering with standard industrial maintenance (b).

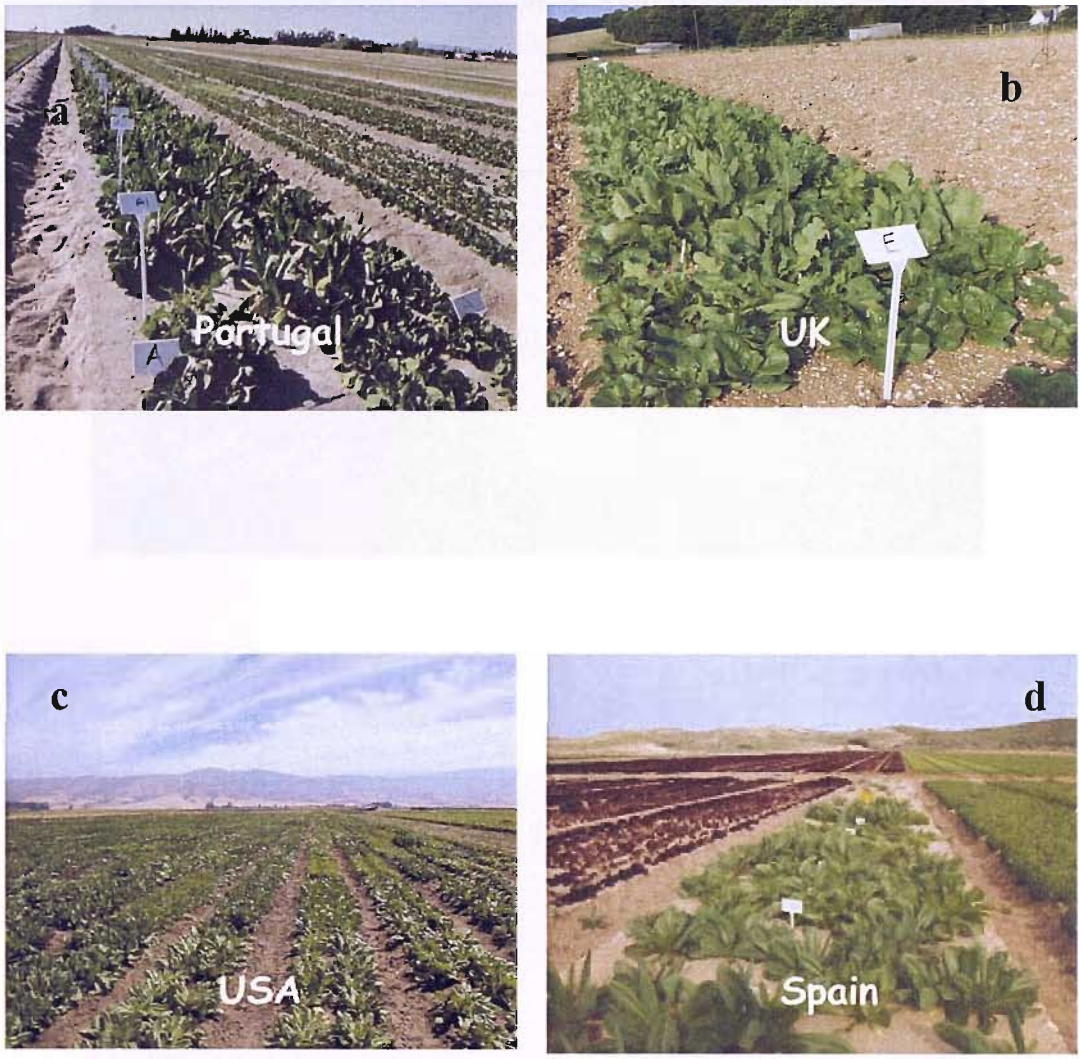


Figure 2.3 The four field sites at harvest. Portugal field site (a); the UK field site (b); USA field site (c) and Spain field site (d).

Figure 2.4 The recombinant inbred line (RIL) mapping population in the glasshouse at the stages of planting (a) and harvesting (b).

2.2.5 Growth room experiment of transgenic lettuce

The seeds of T_2 transgenic lines and wild type lettuce were planted in 125 cm^3 cell seed trays (Homebase) in a 2:1 mixture of vermiculite (Avon Crop, Blacknell) and Levingtons F_2 compost. The plants were grown in the controlled environment growth room and regularly watered from beneath. The growth conditions were day temperature of 20°C, night temperature of 14°C and 12h day-length. At five weeks after germination, five replicates of each line were sampled for trait assessments.

2.3 Trait measurements

2.3.1 The parent population experiment

2.3.1.1 Leaf development

The Plastochron Index (PI) was introduced to compare the difference of the leaf development between species (Erickson & Michelini, 1957). The PI was calculated using leaf length measurements taken at three different times, according to the following formula:

$$PI = n + \frac{LogL_{n+1} - Log10}{LogL_n - LogL_{n+1}}$$

Where: L_{n+1} was the length in millimeters of a leaf just shorter than 10mm, L_n was the length of the next leaf which was longer than 10mm and n was the serial number of the leaf. The PI was therefore equivalent to the distance in the time between two successive leaves reaching 10mm. Two youngest leaves of the plant were measured using a digital caliper, at time intervals depending on the growth rate. Leaf plastochron Index (LPI) was calculated by this formula:

$$LPI = PI - a$$

Where: α is the serial number of the individual leaf. Therefore leaves of exactly 10mm will have a LPI = 0, those shorter will have negative LPI values and those longer than 10mm will have positive LPI values.

Labeled leaves were photographed using a digital camera (Nikon Coolpix 5000), against a scaled white background. These photographed leaf areas were used to determine growth rate ($mm^2\ h^{-1}$). At harvest, six leaves from leaf 1 to leaf 6 were removed from the 15 replicates of each parent and their surface scanned on a flat bed scanner (Hewlett Packard Scan Jet 6100C) with a scaled marker. Leaf area (LA) measurements were taken using computer software "Metamorph" (Metamorph version 5, Universal Image Corporation, Philadelphia, USA).

2.3.1.2 Transverse sections

A leaf disc (10mm diameter) was collected from leaf 4, to the right of the mid-rib vein, approximately 10mm from the leaf tip, avoiding major veins. Leaf samples were placed in fixative buffer (Formalin: glacial acetic acid: 70% (v/v) ethanol [1:1:18, v/v]) (Taylor et al., 2003). For light microscopy, the leaf disc was cut into 1 – 2 mm squares and fixed in 1% (w/v) buffered osmium tetroxide . The specimens were then rinsed and dehydrated in an ethanol series. A section of about 0.5μm was cut using an OMU 3 Ultramicrotome (Leica Microsystems, Milton Keynes, UK), mounted on a slide and stained with 1% (v/v) toluidine blue in 1% borax (v/v). Images were then captured at x100 magnification using a digital camera attached to a light microscope (Zeiss axiophot 2). Leaf thickness (μm) was measured in 'Scion Image' with 6 replicate of each parent.

2.3.1.3 Scanning electron microscope (SEM) section

A leaf disc (10mm diameter) was collected from leaf 7 of two parents from the Portugal field trial. The leaf samples were stored in the primary fixative buffer (3% (v/v) glutaraldehyde in 0.1 M phosphate buffer pH 7.2). The specimens were then rinsed in 0.1 M phosphate buffer (pH 7.2) and dehydrated in an ethanol series. A section was cut using an OMU 3 Ultramicrotome (Leica Microsystems, Milton Keynes, UK), mounted on stubs and embedded in spur resin. The section was viewed on a Hitachi 5800 scanning electron microscope. The stomatal and wax concentration was observed for the two leaf surfaces.

2.3.1.4 Transmission electron microscope (TEM) section

The leaf discs were collected as described above and stored in the primary fixative buffer at 4°C to be processed back in the laboratory. Specimens were then rinsed in 0.1 M phosphate buffer, postfixed in 1% (w/v) buffered osmium tetroxide for 1 h, rinsed in buffer, dehydrated and embedded in Spurr resin in the normal way. Silver section were cut on an OMU 3 Ultramicrotome, stained with uranyl acetate followed by Reynolds lead stain and viewed on a Hitachi H7000's transmission electron microscope. The wax thickness was measured from the TEM images.

2.3.1.5 Epidermal cell area

At harvest, a leaf disc of 10 mm diameter was taken from the adaxial surface, parallel to the mid-rib vein, at approximately 10 mm from the leaf tip. Epidermal cell imprints were made by painting the leaf disc with clear nail varnish (Boots, No. 17) and left to dry for about 15 min. Once dry, a 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 x 200 magnification. The images were imported into "Metamorph" program for analysis. Ten cells per image were chosen at random, with the exception of those bordering stomata complex; from each mean epidermal cell area (ECA) for each sample leaf was calculated.

An estimation of epidermal cell number (ECN) per leaf could be calculated by dividing leaf area by the 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. Stomatal index (SI) was calculated using the following formula:

SI (%) = [stomata]/ [total cells + stomata] x 100

2.3.1.6 Cell wall properties: plasticity and elasticity

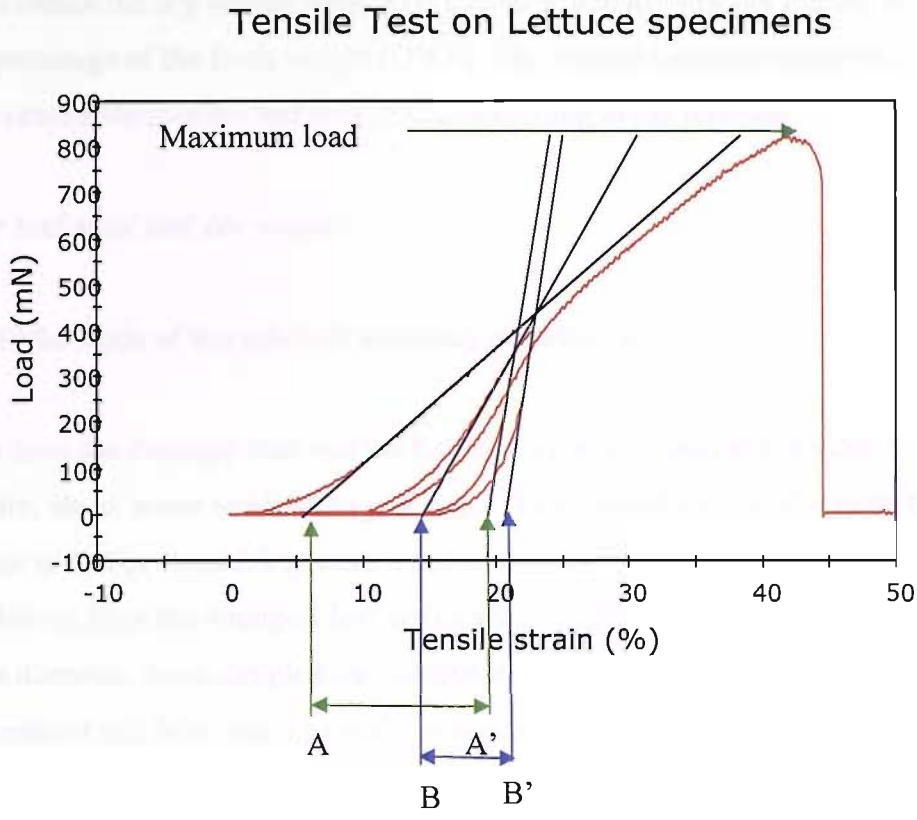
At harvest, leaf 4 of each species was stored in 20 ml vials of methanol solution at 4°C. The biophysical properties of leaf cell wall were determined using an in-house produced instron apparatus. This technique was described by Van Volkenburgh et al. (1983). The whole leaves were fully re-hydrated in 250 ml distilled water for 10 min on a shaker (Mini Orbital Shaker, SO5, Stuart Scientific) at 50 rev/min. Following removal from the water, leaves were blotted dry, and a precise leaf strip (5 x 15mm) was cut, from the right side, parallel to the mid-rib vein at approximately 10mm from the tip, avoiding major veins. Leaf strips were attached between two clamps set 5mm apart and stretched twice by a 5g load (Ferris et al., 2001). The irreversible extension (% plasticity) and reversible extension (% elasticity) were calculated from the chart record. The gradient of the first slope is equivalent to the total extensibility of the leaf sample, including plasticity and elasticity (P+E). The gradient of the second slope is equivalent to the elastic extensibility (E) only. Plastic extensibility (P) of the leaf tissue is calculated from these two slopes,
P = (P+E) – (E) (Figure 2.5).

2.3.1.7 Chlorophyll content

The leaf disc (10mm diameter) was taken from leaf 4, 6 and 8 of each plant, parallel to the mid-rib vein to the left, at approximately 10mm from the leaf tip, and put in a Ependorf with 0.5ml of dimethylformamide (DMF) and kept dark at 4°C for over 48 h. Chlorophyllous pigment quantity was measured by absorbance at 647 and 664nm in a spectrophotometer (U-2001, Hitachi). Total chlorophyll content (CHL) was calculated according to the following formula (Moran, 1982):

$$C_t = 7.04\, A_{664} + 20.27\, A_{647}$$

Where C_t is total chlorophyll concentration in $\mu g\ ml^{-1}$.



$$\%\,P + \%\,E = ((A' - A) / A) \times 100$$

$$\%\,E = ((B' - B) / B) \times 100$$

$$\%\,P = (\%P + \%E) - (\%E)$$

Figure 2.5 Cell wall plasticity and elasticity values obtained from Instron equipment. The example is a trace from a *Lactuca sativa* cv. salinas, leaf harvested into methanol, re-hydrated and subjected to consecutive loads of 400 mN, two curves, A and B, were recorded. Extensibility values were calculated from the gradient of the slope and tangents to the curve at the load peak, using the equations shown. Maximum load was shown until breakage at 800 mN in this example.

2.3.1.8 Leaf fresh weight and dry weight

The fresh weight (FW) of leaf discs (mg) was measured on a top pan balance (Satorius, Borolabs, Kent, UK). Then the discs were dried in oven at 85 °C for 72 h to obtain the dry weight (mg) (DW), from which to work out the dry weight as a percentage of the fresh weight (DWP). Dry weight measurements were also used to calculate specific leaf area (SLA) according to the formula:

SLA = leaf area/ leaf dry weight.

2.3.2 Field trials of the selected mapping population

Plants from the Portugal trial and the UK trial were harvested at a similar maturity, about seven weeks after planting. The assessed leaf development traits relevant to leaf processability were listed in Table 2.1. In the Portugal field trial, eight leaves from the youngest leaf which was big enough to take two discs of 10mm diameter, were sampled and labeled in accordance with the leaf development age from leaf 1 to leaf 8 in series. In the UK field trial, only leaves 3, 6, 7, 8 were sampled for the further measurements.

Labeled leaves from leaf 1 to leaf 8 in the Portugal trial were photographed using a digital camera when harvesting all samples. The traits including leaf area, leaf thickness, epidermal cell area, cell wall properties, chlorophyll content, were assessed using a similar method as described in the parent experiment. The methods for other trait measurements were listed as following.

2.3.2.1 Leaf growth rate

During the growing period in the UK field trial, at about five weeks after planting, digital images of a labeled leaf from each replicate plant were photographed twice with a four day gap. Pairs of leaf images were used to determine absolute growth rates (AGR) ($mm^2\ h^{-1}$) and relative growth rates (RGR) (h^{-1}). The absolute growth rate of leaves (AGR) ($mm^2\ h^{-1}$) 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.

Relative growth rate (RGR) (h^{-1}) was calculated as (note as above)

RGR = $(LA_f - LA_0)/LA_0/h$.

2.3.2.2 Leaf fresh weight and dry weight

After two disc samples for chlorophyll content and transverse section measurement were taken, the fresh weight of leaves 1, 4, and 7 were measured on a portable balance (AM/ACB150, Camlab Ltd, Cambridge, UK). These leaves were stored in a paper bag (12"x 10"), and sent back to the UK, dried in an oven at 85°C to obtain the dry weight. From these two measurements, the percentage of dry weight of fresh weight can be calculated and this indicates the water content percentage. The dry weight measurements were also used to calculate specific leaf area (SLA), according to the following formula:

SLA = (leaf area-2 x disc area)/ leaf dry weight

2.3.2.3 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 freezer 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 centrifuge for 15 s at 16, 000 x g. The centrifuge tubes were placed on ice after spinning. A 10 ul sap sample was taken from the top of the supernatant and tested following the manufacturer's instructions of Wescor 5100C vapor pressure osmometer (Wescor Inc., Logan, USA).

2.3.2.4 Shelf life (leaf processability)

The leaves of each line were harvested after the above experimental samples were collected and sent back to the lab. The leaf samples were collected from Portugal and transported back to the UK in a commercial refrigerated lorry (Vitacress Salads Ltd.) which took 3 days. For the UK trial, the leaves were washed and packaged the next day after harvesting. Leaves were washed in 20 L distilled water in a Hotpoint twin tub washing machine (Model 9404, General Domestic Appliances Ltd, Peterborough, UK) for 1min on the lowest setting and dried for 20 s in the spin compartment. Five grams of the leaves were packed in a zip sealed polythene bags. Where sufficient leave 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 everyday. When breakdown, bruising or damage was seen in the pack, this bag was rejected. This trait is named as shelf life and noted as SL_H in QTL analysis, accounting the day value from harvesting to being rejected. The process including washing, drying, and packing causes the most damage to leaf material, which mainly affected the shelf life value. To compare two trials consistently, we also counted the shelf life day value from the day the material was processed to the day when the material bag was rejected, noted as SL_P. The whole period from the harvest was truncated individually everyday to assess each line condition. The data on each RIL condition were treated as binary in nature and were recorded as good condition (score1) and bad condition (rejected) (score 0). These binary data sets were used for further QTL analysis.

Table 2.1. The list of the leaf traits assessed in the selected F_9 RIL mapping population in Portugal and the UK field trials.

Leaf traits	**Portugal field trial**	**UK field trial**
Leaf area	Leaf 1 to 8	Leaf 3,6, 7, 8
Leaf fresh weight	Leaf 1, 4, 7	Leaf 7
Leaf dry weight	Leaf 1, 4, 7	Leaf 7
Dry weight as a percentage of fresh weight	Leaf 1, 4, 7	Leaf 7
Specific leaf area	Leaf 1, 4, 7	Leaf 7
Leaf thickness	Leaf 1, 4, 7	Leaf 7
Chlorophyll content	Leaf 1, 4, 7	Leaf 7
Epidermal cell area	Leaf 2, 5, 8	Leaf 8
Epidermal cell number per leaf	Leaf 2, 5, 8	Leaf 8
Stomatal density	Leaf 2, 5, 8	Leaf 8
Stomatal index	Leaf 2, 5, 8	Leaf 8
Cell wall properties	Leaf 2, 5, 8	Leaf 8
Cell sap osmolality	Leaf 6	Leaf 6
Shelf-life	Leaf 6,7, 8	Leaf 6,7, 8
Growth rate	N/A	Leaf 3 to 6

2.3.3 The USA field trial of extended mapping population

2.3.3.1 Soluble carbohydrate content

At harvest, about eight weeks after sowing, eight leaf disc samples were harvested from young leaves which are big enough to take eight disc samples. One set of four discs (7 mm diameter) were taken from a young leaf using 0.5 ml microfuge tube, parallel from both sides of the mid-rib vein, about 10 mm from the tip. The microfuge tubes with four discs samples were put straight into liquid nitrogen, sent back to the UK in a cryogenic ship, and then stored -80°C freezer for carbohydrate analysis. Three replicates of each line sample were collected for this analysis. The carbohydrate analysis protocol refers to the method described in Clarkson (2005).

2.3.3.2 Xyloglucan endotransglucosylase/hydrolase (XTH) activity

The second set of four discs (7mm diameter) was taken from the above leaf, parallel from both side of mid-rib vein, about 25 mm from the tip. The discs were put into liquid nitrogen, sent back to the UK in cryogenic ship, and then stored in - 80°C freezer for XTH enzyme activity analysis. Three replicates of each line sample were collected for this analysis. The XTH activity analysis protocol was described in Clarkson (2004).

2.3.4 Glasshouse experiment of extended RIL mapping population

2.3.4.1 Relative chlorophyll content measurement

Two separate glasshouse experiments were taken relatively in May and October, 2004 to measure leaf relative chlorophyll content of 112 F_{10} RILs mapping population using Chlorophyll Content Meter (CCM-200, Opti-Sciences Inc., Tyngsboro, USA). The leaves with similar maturity (3^{rd} leaf from the young leaf) were chosen for this measurement. Three replicates of each line were assessed to obtain the mean value of each RIL. The measurements were taken at

7 weeks after sowing the seeds in the first experiment and at 10 weeks after sowing the seeds in the second experiment.

Laboratory methods for determination of chlorophyll content are both time consuming and destructive to the sample. Typically a sample must be detached, ground up in a solvent, and then assayed in a spectrophotometer. A sample can be measured only once precluding the monitoring of trends in chlorophyll content over the growing cycle. The Chlorophyll Content Meter provides non-destructive, rapid measurements of relative chlorophyll content without the need to detach and grind a sample. Chlorophyll has several distinct optical absorbance characteristics that the CCM-200 exploits to measure relative chlorophyll concentration without destructive sampling (Richardson et al., 2002). Strong absorbance bands are present in the blue and red but not in the green or infrared bands, hence the green appearance of a leaf. By measuring the amount of energy absorbed in the red band an estimate of the amount of chlorophyll present in the tissue is possible. Measurements in the infrared band show absorbance due to cellular structure materials. By using this infrared band to quantify bulk leaf absorbance, factors such as leaf thickness can be taken into account in chlorophyll content index (CCI) value. On the second experiment, thirty leaf disc samples were collected for the actual chlorophyll content measurement as described in section 2.3.1.7 to calibrate the chlorophyll content meter.

2.3.4.2 Leaf photosynthesis rate and stomatal conductance

Gas exchange measurement, photosynthesis rate (PR), transpiration rate (TR) and stomatal conductance (SC) were assessed in three replicates of each RIL using an infrared gas analyzer (Licor 6400 portable photosynthesis system, Lincoln, Nebraska, USA) (Figure 2.6a). The exposed chamber conditions were as the following: PAR 300 μmol m^{-2} s^{-1}, CO_2 400 μmol mol^{-1} and constant fixed flow CO_2 of 400 μmol mol^{-1}. Leaf temperature was 20 °C. Three replicates of each RIL with similar developmental stage leaves (semi-mature leaf) were chosen for this measurement.

2.3.4.3 Cell wall properties: plasticity, elasticity and maximum load.

The sample preparation was described in section 2.3.1.6. But the measurements were determined using new Instron equipment (Instron 5542, Instron Ltd., UK) (Figure 2.6b). Leaf strips were stretched twice between two small brass clamps to a load of 400 mN. After stretching twice to obtain the measurements of plasticity and elasticity, the leaf strips were stretched again to breakpoint to obtain the maximum load value of the leaf material (ML). The results were expressed as percentage plasticity (% P; % irreversible extension per 0.4 N loads) and percentage elasticity (% E; % reversible extension per 0.4 N loads), % total extension, and maximum load (ML) (Figure 2.5).

2.3.5 The Spanish field trial of extreme lines

2.3.5.1 Shelf life

At harvest, leaf materials of the extreme lines and two parents were collected in the morning and flown back to Southampton University, then processed in the lab the next day. The process and assessment was described previously at session 2.3.2.4. At least 10 replicates of each line were assessed in this shelf life experiment.

2.3.5.2 Harvesting material for DNA and RNA extraction

At harvest, the young leaves which were big enough to take four sample discs were chosen for sampling. Four discs (7 mm diameter) were taken from young leaf using 0.5 ml microfuge tube, parallel from both sides of mid-rib vein, about 10 mm from the tip. The microfuge tubes with the four discs samples were put straight into liquid nitrogen, brought back to the UK in dry ice, and stored in -80°C freezer for further DNA or RNA extraction.

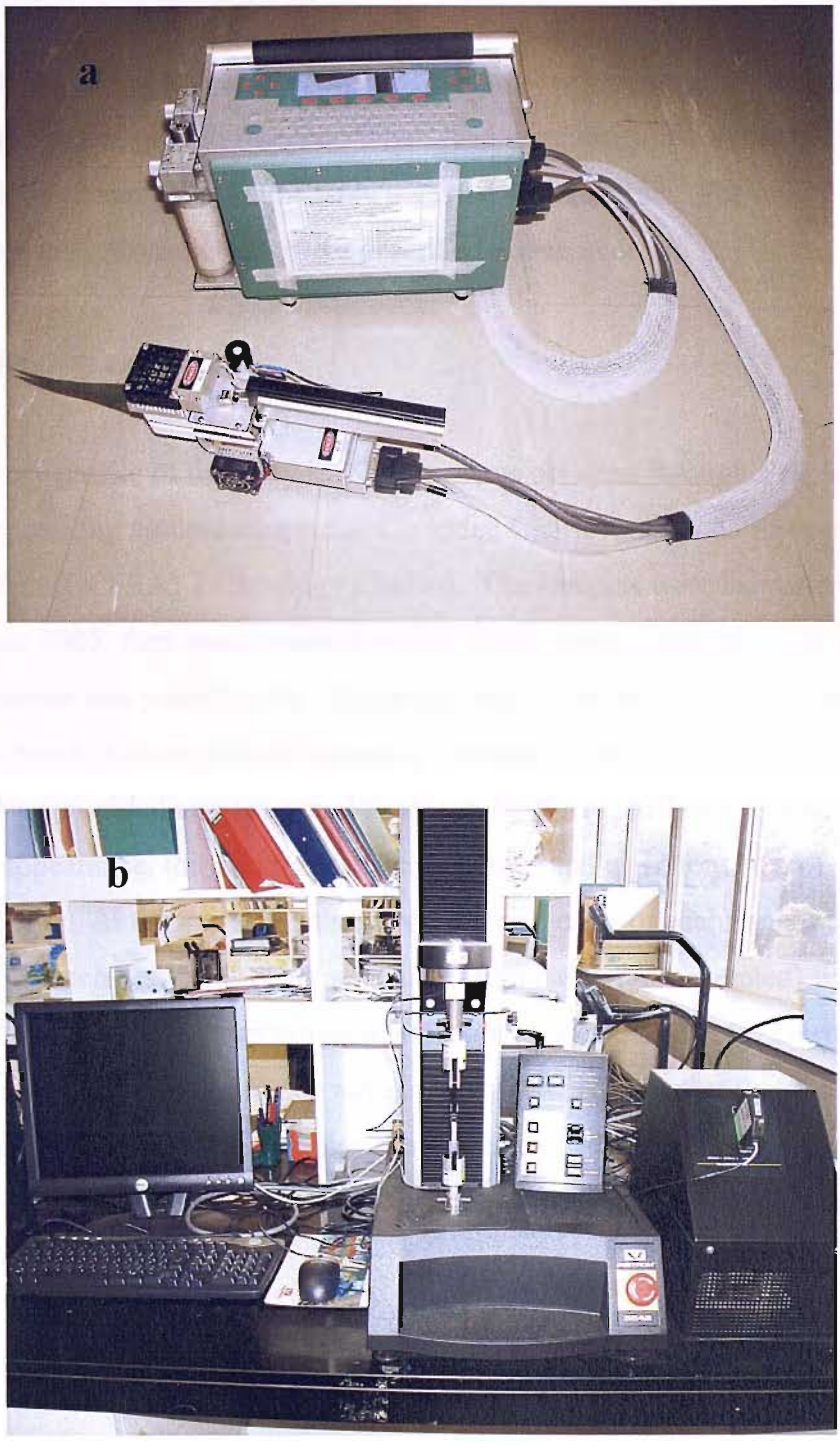


Figure 2.6 The equipment of Licor 6400 used for measuring photosynthesis rate, transpiration rate and stomatal conductance (a) and Instron used for measuring elasticity, plasticity and breakthrough (b).

2.3.6 The UK field trial for extreme lines in 2005

2.3.6.1 Leaf trait comparison between long-life lines and short-life lines

Leaf traits including leaf area, epidermal cell area, cell number per leaf, stomatal density and index, growth rate, maximum load were measured in the UK trial in 2005. Six replicates of each line were assessed for each trait. The trait collections and measurements were described above in detail.

2.3.6.2 Sensory assessment

The sensory profile of the baby leaf lettuces was obtained through a rapid attribute profiling method adopted at Campden Chorlewood Food Research Association (CCFRA) Technology Limited. The samples were harvested on 8th September 2005, then were stored in sealed plastic bags to eliminate any cross-contamination and placed in the refrigerator until assessment on 13th September, 2005. A panel of seven trained assessors evaluated both the stems and leaves of the samples provided and generated feedback for thirty attributes overall (i.e. nine for appearance, thirteen for taste and flavour, and eight for texture) (Appendix 4). At the assessment session, two replicates of each sample were assessed by each assessor. A latin-square design (software-generated) was used in order to minimize any carry-over and presentation order effects. During the training and assessment, samples were initially screened to meet the standard supermarket requirements. Samples were then washed approximately five minutes prior to assessment. Filtered water and water crackers were given as palate cleaners.

2.3.7 Growth room experiment of transgenic lettuce

2.3.7.1 Cell wall properties: plasticity, elasticity and maximum load

Five replicates of each transgenic line and wild type lettuce from young and mature leaves were sampled for this trait measurement, as described at sections 2.3.1.6 and 2.3.4.3.

2.3.7.2 Shelf life

Five replicates of the first young leaf (about 2 mm^2) were sampled for RNA extraction. After the samples for the above experiment were collected, all the leaves left were collected for shelf life assessment, as described in section 2.3.2.5. The weight of each replicate leaf bag was around 5g. There were 17 replicates for Line 7-25; 9 replicates for Lines 9-11 and 19-3, and only 2 replicates for wild type due to low germination rate.

2.4 QTL analysis

Sixty 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 has nearly as many recombinant breakpoints and was therefore as informative as a population of ~ 90 RILs. A saturated molecular maker linkage map had been recently improved from the previous map (Johnson et al. 2000), using mostly co-dominant AFLP markers and EST markers (Argyris et al., 2005). Over 600 markers fell into ten linkage groups spanning 1342 cM and provided markers throughout the genome. A subset of 229 markers was chosen spaced approximately 5 cM apart to use as a framework map for QTL analysis. Framework markers were selected to maximise genome coverage and marker information content. Forward stepwise regression and backward elimination regression methods were used to choose significant markers for each trait.

Composite interval mapping method was employed to increase resolution and reduce background marker effects (Zeng, 1994). QTLs were detected using the computer program QTL Cartographer 2.0 (Basten et al., 2002), all of the significant markers were selected to control the genetic background, and window size (10 cM) was used to control for the genetic background. The thresholds for logarithm of odds (LOD) score for declaring QTL significance at $P \leq 0.05$ were estimated by permutation analysis for each trait using 1000 iterations (Churchill & Doerge 1994). If no significant QTL for the traits of interest was detected,

LOD of 2.5 was set for declaring QTL as putative QTL in the glasshouse experiments. The graphical representation of the linkage maps and QTL were prepared using the MapChart 2.1 software (Voorrips, 2002).

2.5 SNP mapping

2.5.1 DNA extraction

2.5.1.1 DNeasy Plant Mini Kit (Qiagen method)

The genomic DNA of two parents was extracted using DNeasy Plant Mini Kit (QIAGEN Ltd, UK). Two discs (10 mm diameter) of leaf material were collected from the young leaf of each parent into micro centrifuge tubes, frozen in liquid nitrogen until later extraction. The extraction was according to manufacturer's protocol. The extracted DNA was suspended in 20 µl 1x TE (10x TE: 100 mM Tris HCL pH 8.0, 10 mM EDTA) and stored at -20°C for the future experiment. The gDNA was checked on an agarose gel with a ladder and a known weight standard DNA to estimate the gDNA concentration. This Qiagen method uses a column to purify DNA.

2.5.1.2 Plant DNA miniprep (CTAB method)

The genomic DNA of all the 60 RILs was extracted using the CTAB protocol. The detailed steps were described as following:

1) Three discs (10mm diameter) of the young leaf tissue were harvested from the 60 RILs and two parents into 1.5 ml tubes.
2) The leaf tissue were ground to a powder using a pestle with a small amount of ground glass and 500 µl of nuclear extraction buffer was immediately added. (120 mM Tris HCL pH 7.5, 30 mM EDTA, 1.2 M NaCl, 1.2% (w/v) CTAB). Prior to use the extraction buffer, 0.38% (w/v) sodium bisulphite was added.
2) 100 µl of 5% (w/v) sarkosyl was added, inverted several times and incubated at 65°C for a minimum of 20 min.

3) 500 µl of phenol/chloroform was added, inverted 20 times and the phases separated by centrifuging at a maximum speed in a micro centrifuge for 2 min.
4) The upper phase was transferred to a new 1.5 ml tube and 300 µl of isopropanol was added, inverting several times to mix them.
5) The genomic DNA was recovered by centrifuging at full speed for 1 min. The supernatant was removed; the pellet of DNA was washed with 500 µl 70% (v/v) ethanol and centrifuged as before.
6) The supernatant was carefully removed and the DNA pellet dried at room temperature. The dry pellet of gDNA was resuspended in 100 µl of 1x TE, and checked on an agarose gel with 1 Kb ladder and a known weight standard of DNA to estimate the gDNA concentration. The extracted gDNA was stored at -20°C.

2.5.2 DNA gel electrophoresis

The extracted DNA products were determined on agarose gels. 0.7 -1.4 % (w/v) Agarose (Sigma, UK), depending the size of the products, was melted in 50 ml or 100 ml 1x TAE buffer (10 mM Tris Acetic acid pH 8.0, 10mM EDTA) using a microwave and cooled to 50°C. 1 µl of ethidium bromide stock solution was added; the gel solution was poured in the gel tank and allowed to set. 1 x TAE was used as the gel running buffer. The extracted DNA samples were diluted 5 times with 2 µl template DNA product, 1 µl 10x orange loading dye (0.25% (w/v) Orange G, 15% (w/v) ficoll in water), and 7 µl of H_2O. The diluted 10 µl product was carefully loaded in the gel well with 1 Kb DNA ladder (Promega, UK) and known concentration standard DNA were run when needed. The gel runs with the setting of 120 V, until the orange dye reached the end of the gel. The gel images were captured using an Alpha Imager System (Flowgen, Shenstone).

2.5.3 Primer design

2.5.3.1 Manual design

Initially, wax synthase and two genes of xyloglucan endotransglucosylase/hydrolase (XTH) were selected for this practice. The primers for these three genes were designed, based on publicly available sequence data, and described as following:

1) Search the index by gene product name (i.e. wax) in TIGR *Lettuce* Gene Index (LsGI) website. (http://www.tigr.org/tdb/tgi/lsgi/)
2) The searched gene sequence was listed (i.e.TC15225) with the open reading frame information. The amino acid sequence was copied into the Arabidopsis database BLAST program (http://www.arabidopsis.org/Blast/). When inputting the query sequence from the ORF, choose BLASTP: AA query, AAdb as the Blast program, AGI Proteins (Protein) as the datasets.
3) The list of BLAST query on *Arabidopsis* sequences of the interested gene was shown. The most matched sequence (i.e. At3g51970.1) was viewed via quick search in the website (http://www.arabidopsis.org/).
4) Using the information regarding intron/exon structure in Arabidopsis, primers were designed to regions of the lettuce sequence avoiding possible conserved splice sites. The primer length could be around 22-24 bases and the location could be adjusted in order to get the close annealing temperature for both forward primer and reverse primer.

The primers for gene of wax synthase were (5' to 3'):
TC15225F: ATGAAAATGGCTCTGTTTTGTG
TC15225R: CAATAGTAAGGGCAAGATACGT
The primers for two genes of *XTH* were (5' to 3'):
TC12535F: ATATTTGTTTGGACGTGTGAGC
TC12535R: TGGAAAGGTAACACCTTTTGC
TC9490F: GTACATGTTTGGCAAAGTAAATG
TC9490R: TTTAGGGAACGGGATGCCCTT

2.5.3.2 Software design

Fifteen pairs of primers for the candidate genes were produced using the primer design software "primer 3" (Table 2.2). Actin gene was chosen for standard control gene. The candidate genes include 7 *XTHs*, 1 *CycD3* (D-type Cyclin), 2 *CDKA* (A-type Cyclin-Dependent Kinases), 2 *Wax* (Wax synthesis), and regulators *E2F* and *RBR* (Retinoblastoma protein related gene). The sequence of *XTH 3B1* was from the previous research in this lab and other gene information was from the lettuce sequence database based on the Michelmore lab, Davis, CA, USA http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=lettuce. The annealing temperature for all of these primers was about 60 °C, the PCRs for all these primers could be done together.

2.5.4 Polymerase chain reaction (PCR)

PCR was performed using a Peltier thermal cycler, the PTC-225 DNA Engine Tetrad Cycler (Genetic Research Instrumentation, Braintree). The primer annealing temperature was estimated by the following formula:

$$T_a = 4 \times N_{G/C} + 2 \times N_{A/T}$$

Where: T_a was the annealing temperature, $N_{G/C}$ was the total number of G or C and $N_{A/T}$ was the total number of A or T.

The PCR reaction program was: hot start at 96°C for 2 min, 35 cycles of [96°C (15 s); 55°C (15 s); 72°C (3 min)], extended at 72°C for 5 min, suspended at 16°C and the lid temperature was 106°C. The typical 20 µl of the reaction mixture were: 10 µl 2 x Biomix (Bioline); 1 µl forward primer (20 µM); 1µl Reverse primer (20 µM); 2 µl template DNA (~ 10ng gDNA) and 6 µl H_2O. The PCR products were determined visually by agarose gel electrophoresis

Table 2.2 List of primers for the putative candidate genes involved in leaf processability.

Gene	Sequence size (intron + exon)	Forward primer (F)	Reverse primer (R)	Product size
XTH 3B1	506	AACAGCAGTTCCACCTTTGG	ATCATGTGCTTGGTTTGCAC	400
XTH TC9490	1052	TGATGGAGATTCGGTGAACA	ACCGGACATTCACAACCATT	549
XTH TC11389	735	TTCTGGATTCGGGTCAAAAG	TGAGCCCAATTAGCTTTCGT	448
XTH TC12535	1135	CGGGACACAGAAACACACAC	CCCATTGGTTGAAATTTTGG	615
XTH TC10683	1016	AAAATGGTTCCCGGAGATTC	CCGTCGATGTGGAAACTCTT	437
XTH TC14434	867	TTTGAGCCTTCCAAAGCCTA	CGATGATGTCTCCGTTCAAA	515
XTH TC11662	738	TGGATGATGTGCCGATAAGA	CCCCATCTCAACACACCCTA	478
Actin TC8011	1760	AGAGCTGAGGGCTAGGGTTC	GATCCAAACGGAGGATAGCA	661
CycD3 QGG13J16.yg.ab1	738	TCTAGAAAGATGCGCGAACA	GCCTGCTGCTGCTACTTCTT	455
CDKA TC9313	770	TCGGAAATGGAGAATTGAGG	AACCCATTCGAAACGACCTT	464
CDKA TC13033	710	GGATAATTCGGGAGCAGTGA	TTCCTGGCGTTAGTTCAACC	527
E2F QGB17J02.yg.ab1	548	AGGGTGGTTGTTGAGAGGTG	GGGTTTGGGGGTTTTGTT	530
RBR QGH6D15.yg.ab1	704	GGCAAATTTTGTGCATCTCA	CATTCTGATGCTGAGCAAGG	438
Wax TC13934	802	TTCTTGGCGCTATTTCCAGT	CAGGGAACCAGATGGCTAAA	581
Wax TC15225	846	TCGAACCACAGTTCGATGAG	TCCACGAACAACAAGTTCCA	473

2.5.5 DNA purification

PCR products need to be purified to remove free primers and nucleotides before sequencing. This was performed using Micro CLEAN (Microzone, UK) according to manufacturer's protocol.

2.5.6 BigDye Cycle-sequencing

Sequencing PCR reactions were set up for forward or reverse primer for each DNA preparation. This is performed according to manufacturer's protocols. The sequencing reaction products were run on an automated ABI Prism377 DNA sequencer (Applied Biosystems, California, USA). The sequence data were listed using ABI Sequencing Analysis 3.3 and the sequences with the forward primer and reverse primer were assembled using ABI Auto Assembler 2.1 software. From the designated sequences of the parents, the differences in polymorphisms between two parents were analysed.

2.5.7 Restriction enzyme reaction

After running gradient PCR with wax synthase primers, the PCR fragments were digested with the restriction enzyme (*Nae* I) (Promega Ltd, Southampton, UK). The enzyme reaction mixture was: 10 µl PCR product, 1 µl Promega buffer A, 1 µl Orange G loading buffer, 0.25 µl enzyme *Nae* I and 7.75 µl H_2O to a final reaction volume of 20 µl. The enzyme reaction mixtures were incubated at 37°C for over 1 h or overnight. The reaction products were checked in the 1.4% (w/v) agarose gel electrophoresis.

2.5.8 RNA extraction (Qiagen RNeasy Plant Mini Kit method)

The RNA of the whole RIL mapping population was extracted using DNeasy Plant Mini Kit (QIAGEN Ltd, UK). 50-100 mg of fresh weight leaf material of each RIL was harvested and immediately frozen in liquid nitrogen. The material was then ground to a fine powder using mixer mill (MM300, Retsch ltd, Leeds, UK) at the speed of $30s^{-1}$ for 30s, chilled in liquid nitrogen and mixed again for

three times. RNA extraction from the powdered material was according to manufacturer's protocol. Buffer RLT was used and RNA was eluted in 30 µl Rnase-free H_2O. 2 µl of the RNA was quantified using Nanodrop (ND-1000 Spectrophotometer, NanoDrop Technologies Inc., Rockland, USA).

2.5.9 RNA purification

The DNA contamination was removed from the extracted RNA using the DNA-*free* Kit (Ambion (Europe) Ltd., UK) following the manufacturer's instructions. 1 µl of DNA-*free* treated RNA RNA was checked on an agarose gel and quantified using Nanodrop ND-1000 Spectrophotometer (Nanodrop Inc. USA) and Agilent 2100 Bioanalizer (Agilent Technologies, Inc., USA) according to the manufacturer's instructions.

2.5.10 Non-denaturing RNA gel electrophoresis

The purified RNA products were determined on agarose gels. 1 % (w/v) agarose was dissolved in 100 ml 1x MOPS buffer (Sigma, UK) using a microwave and cooled to 50°C. The gel solution was poured in the gel tank and allowed to set. 1 x MOPS was used as the gel running buffer. 1 µl of RNA sample, 1 µl 10x orange loading dye and 8 µl of ethidium bromide MOPS stock solution were mixed properly, then carefully loaded in the gel well. The gel runs with the setting of 110 V for about 45 min. The gel images were captured using an Alpha Imager System (Flowgen, Shenstone).

2.5.11 First-Strand cDNA synthesis

cDNA synthesis was generated by a reverse transcriptase (RT) reaction. 10 µg of the DNA-*free* treated RNA with 2 µl of oligo$(dT)_{20}$ (50 µM), 2 µl of 10 mM dNTP Mix and H_2O to 26 µl was denatured at 65°C for 5min and quenched on ice. To this 8 µl 5X first-strand buffer; 2 µl 0.1 M DTT; 2 µl RnaseOUT recombinant RNase inhibitor (40 U μl^{-1}) and 2 µl of superscript III RT (200 U μl^{-1}) (Invitrogen Ltd., UK) was added and incubated at 50°C for 60 min. Then the reaction was inactivated by heating at 70°C for 15 min. 2 µl (4 U) of *E. coli*

RNase H was added in the above reaction and incubated at 37°C for 20 min. The synthesized cDNA was cleaned up with QIAquick PCR Purification Kit (QIAGEN Ltd, UK) using the manufacturer's instructions. The purified cDNA was stored at -80°C for the future experiments.

2.5.12 DNA sequencing using CEQ 8000

Sequencing PCR reactions were set up for forward or reverse primer for each DNA preparation. This is performed according to the manufacturer's protocols. The dried pellet was re-suspended in Loading Solution supplied with a CEQ sequencing kit. The sequencing reaction products were transferred to appropriate wells of the CEQ sample plate with one drop of mineral oil overlaid on top of each sample. The sequencing products were run on an automated CEQ 8000 DNA sequencer (Beckman Coulter Inc. CA, USA). The sequence data were collected from CEQ 8000 Sequence Analysis software. The sequences with the forward primer and reverse primer were assembled using software Vector NTI Advance 10 (Invitrogen Life Technologies, UK). From the designated sequences of the parents, the single nucleotide polymorphisms between two parents were analysed for the set of putative candidate genes.

2.6 Statistical analysis

The relationship between leaf length and leaf area for the two parents was analysed using a linear regression in the statistical software package Minitab 13.0 for Windows (Minitab Inc., Philadelphia, USA). All other measured and calculated values were analyzed with means of a one-way analysis of variance (ANOVA) in Minitab to test the significant difference between two parents *L. serriola* (US96US23) and *L. sativa* cv. Salinas. Significant effects are shown as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, and ns, no significant. All the measured trait values were analyzed with a one-way ANOVA in Minitab to test the significant genotype variation among all RILs before further QTL analysis.

The normal distribution of all the traits was analyzed using the Anderson-Darling normality test. The normal distribution graphical representation was prepared using Minitab 13.0 or SigmaPlot 8.0 for Windows (Systat Software Inc., CA, USA). The correlation among all the traits was analysis using the statistical software package SPSS 12.0 for Windows (SPSS Inc., Chicago, USA).

In the sensory assessment, a two-way analysis of variance was used to test if there was a significant difference between the means of samples for each variable (attribute). The Neuman Keuls (NK) multiple comparison test was used to determine whether the samples were significantly different for each attribute at the specified 5% level of significance.

CHAPTER 3

Identifying the leaf traits for leaf processability in the parent population

3.1 Introduction

In quantitative genetics, if the trait is determined by many genes, the population segregation will be a continuous distribution. For QTL mapping, the parents of the mapping population should have contrasting phenotypes for the traits of interest as QTL can only be mapped if polymorphism is observed in the segregating population. The parent population experiment was to assess the suitability of this particular mapping population in meeting the object to map QTL for leaf processability in this study. Leaf processability traits are likely related to leaf cell water relations and the biochemistry of the cell wall. The aim of this preliminary experiment was to identify the traits relevant to leaf processability in parents.

In this study, leaf processability is likely to involve traits related to leaf cell water relations and cell wall biochemistry, as well as to rates of cell expansion and production. Epidermal cell size, cell production and cell wall properties were selected as traits associated with leaf processability, due to their contribution to maintaining water status. 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).

Water relations (water, pressure and osmotic potential) are a critical factor affecting the post-harvest quality of salad leaves. After harvest, water can no longer be taken up from the soil, while transpiration water losses cannot be prevented. The increasing water deficit may induce a chain of physiological

reactions to maintain water status (Galindo et al., 2004). It is very common in the literature to define the water potential (ψ_w) by the following formula:

$\psi_w = \psi_p + \psi_\pi$

Where ψ_p is the pressure potential (tugor pressure) and ψ_π is the osmotic potential (Boyer, 1995).

The resistance to cell wall stretching causes the tugor pressure. The extent to which the cells can stretch depends on the elasticity of their cell walls. The relationship between the pressure potential and water volume is determined by the cell wall and the overall elastic properties of the leaf. Osmotic potential can be directly related to the concentration of the solutes. As solutes lower the chemical potential, ψ_π has a negative value (Galindo et al., 2004). Maintaining water status is tightly related to cell wall properties and cell size. Relatively high water content may reduce premature wilting or senescence of leaves, is likely to give greater resistance to compression forces during the salad process, and generally contributes to a better taste and crisper texture of the leaf.

Aim:

To test the suitability of the mapping population to identify leaf traits for leaf processability.

3.2 Results

3.2.1 Leaf development

The seed germination rate of the cultivated lettuce, *L. sativa* cv. Salinas (SAT), is 97%, 9% higher than that of wild lettuce, *L. serriola* (US96US23) (SER) (88%). Figure 3.1 illustrated the growing period of the parent lettuces. The wild lettuce *L. serriola* (left) was much taller than cultivated lettuce *L. sativa* (right). The leaves of *L. serriola* are longer, narrower, more flaccid than that of *L. sativa*. The wild lettuce has spines on the leaf margin and the underside of the midrib. Insignificant difference in the Leaf Plastochron Index (LPI) between two parents, in a one-way ANOVA ($P = 0.36$) suggest that leaves of the same emergent number are of equivalent developmental stage and thus can be directly compared. The mean LPI of leaf n (L_n) was 0.64 and 1 Plastochron equated to 7.5 days. Due to the time-consuming measurement of LPI and its insignificant difference between two parents, the leaves were directly compared according to the emergent number between two parents in further experiments.

There was a strong relationship between leaf length and leaf area for the first and second emerged leaves in two parents (Figure 3.2). The regression between leaf area and leaf length were shown in this figure. The R^2 values were similar between *L. serriola* ($R^2 = 0.8025$) and *L. sativa* ($R^2 = 0.7747$). These data were only collected at two weeks after sowing, where the leaves of *L. sativa* were longer than those of *L. serriola.* After two weeks, the leaves of *L. sativa* were broader with curly leaf margin. At harvest, about 4 weeks after germination, the wild lettuce has long and narrow leaves (Figure 3.3). From the leaf images, the leaf area was measured (Figure 3.4). Leaf 4 is the mature leaf while the leaf area reaching the maximum (mean ± SD), around 1710 (± 345) mm^2 in *L. sativa* and 3256 (± 449) mm^2 in *L. serriola.* Leaf area of *L. serriola* was significantly larger than that of *L. sativa* at different developmental stages except leaf 6. There was much difference between two parents in the young leaf than in the old leaf. *L. serriola* was 156% bigger than *L. sativa* in leaf 1, 90% bigger in leaf 4, but only 5% bigger in leaf 6.

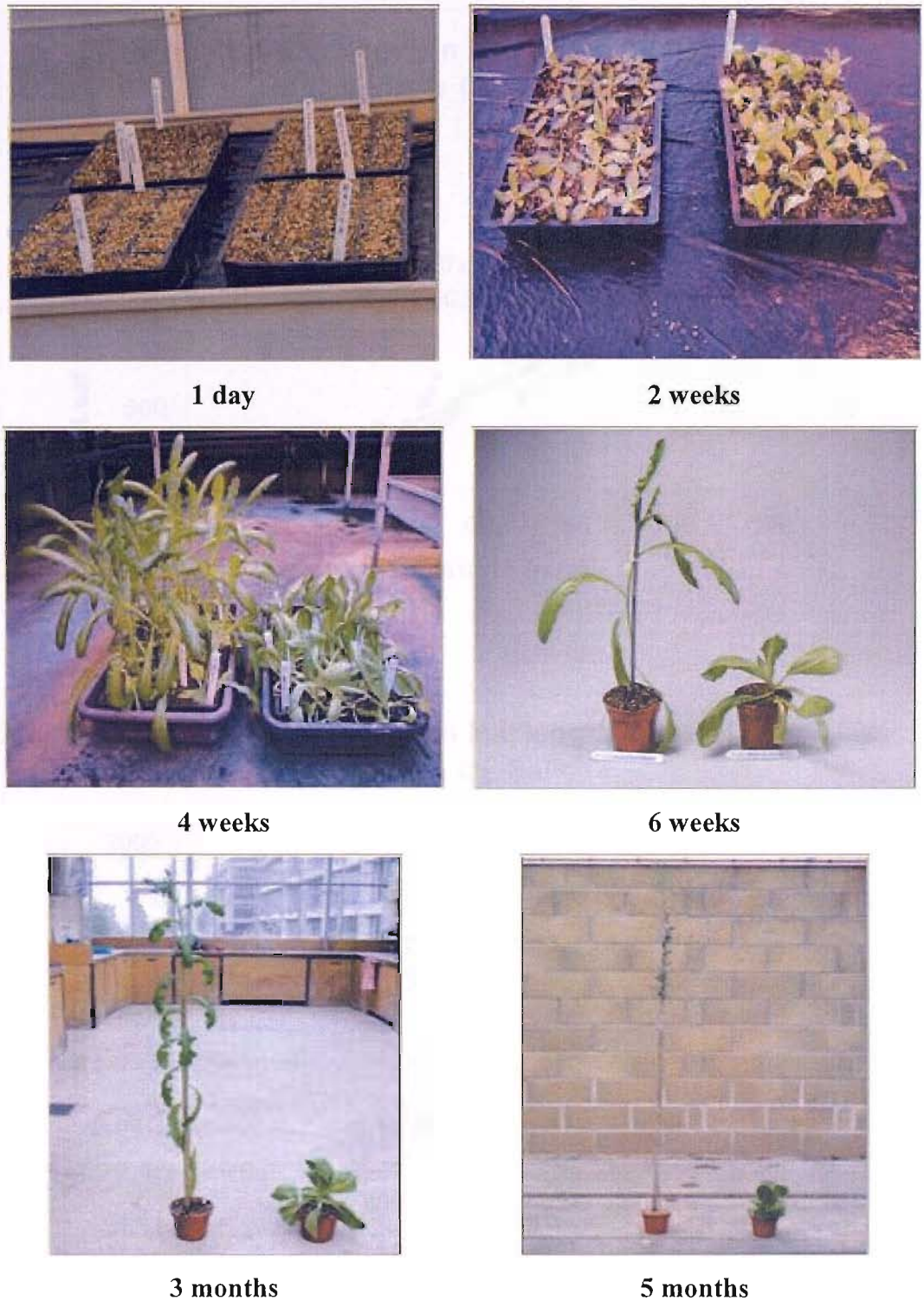
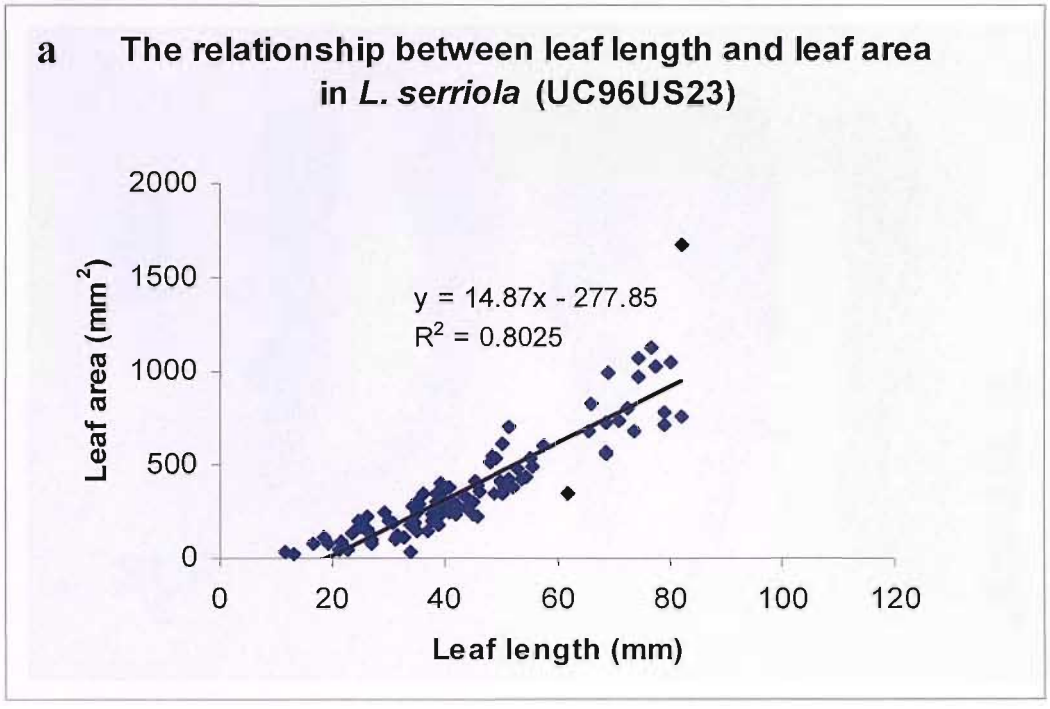


Figure 3.1 The parent lettuce images at the different development stages. *L. serriola* (US96US23) was pictured to the left and *L. sativa* cv. Salinas to the right. The plants were grown in the glasshouse with a minimum of 12 h day-length, at a day temperature of 18°C and night temperature of 14°C, with automatic ventilation set at 20°C.



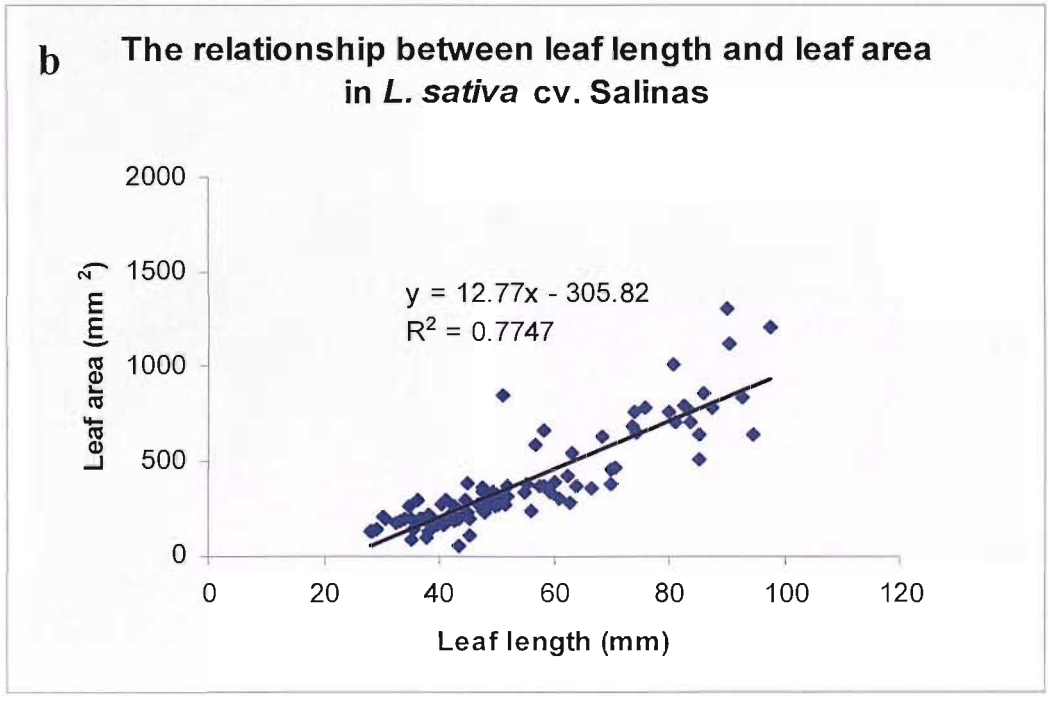


Figure 3.2 The relationship between leaf length and leaf area in the parents. *L. serriola* (UC96US23) (a) and *L. sativa* cv. Salinas (b). Linear regression equations are displayed on the charts. The measurement was taken at two weeks after germination and the leaves were the first and the second true leaves of the plants.

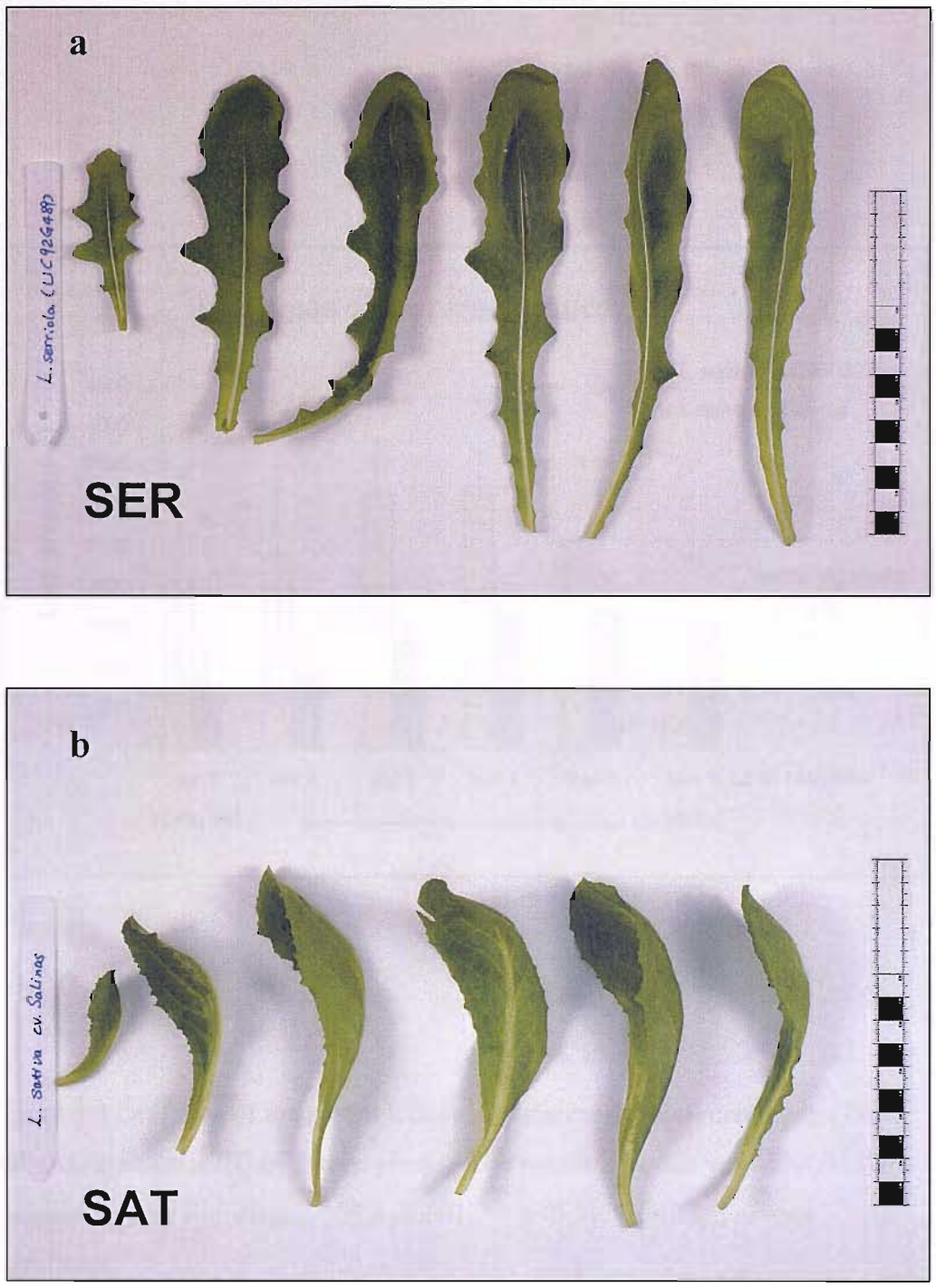


Figure 3.3 Leaf images of the parent lettuce at harvest. *L. serriola* (UC96US23) (SER) (a) and *L. sativa* cv. Salinas (SAT) (b). The youngest leaf of each plant (leaf 1) is displayed on the left to the oldest leaf (leaf 6) on the right in series in each picture.

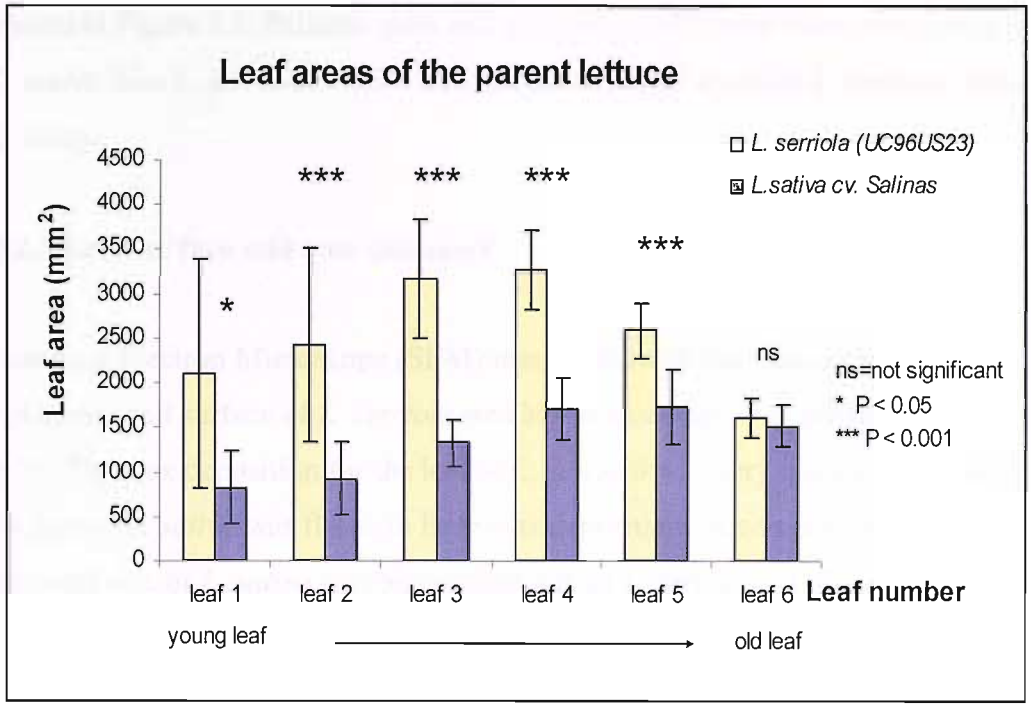


Figure 3.4 Leaf area of the parent lettuce at different development ages. (Data points are means ± SD of fifteen plants. The results of a one-way ANOVA are indicated where significant: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns=not significant)

3.2.2 Leaf thickness

The transverse leaf sections measurement showed wild lettuce *L. serriola* was significantly thinner than the cultivated lettuce *L. sativa* ($P < 0.05$, Figure 3.5), where the mean (± SD) of thickness were 142μm (± 20μm) for *L. serriola* and 170μm (± 23 μm) for *L. sativa*. The examples of the transverse leaf section were shown in Figure 3.6. Palisade cells and mesophyll cells were more compacted in *L. sativa* than *L. serriola*. There was more internal air space in *L. serriola* than *L. sativa*.

3.2.3 Leaf surface and wax thickness

Scanning Electron Microscope (SEM) images showed wax concentration in epidermal cell surface of *L. serriola* was higher than that of *L. sativa* (Figure 3.7). The wax deposition for the leaf of *L. serriola* was very intense while the surface of *L. sativa* was flat with little wax deposition. It was also observed that stomatal size of *L. sativa* was bigger than that of *L. serriola*. TEM images also showed that the cuticle of *L. serriola* was about 250% thicker than that of *L. sativa* (6.5 μm vs 1.7 μm) (Figure 3.8). It was a surprise to see that no internal structure difference along the cuticle layer between the two parents.

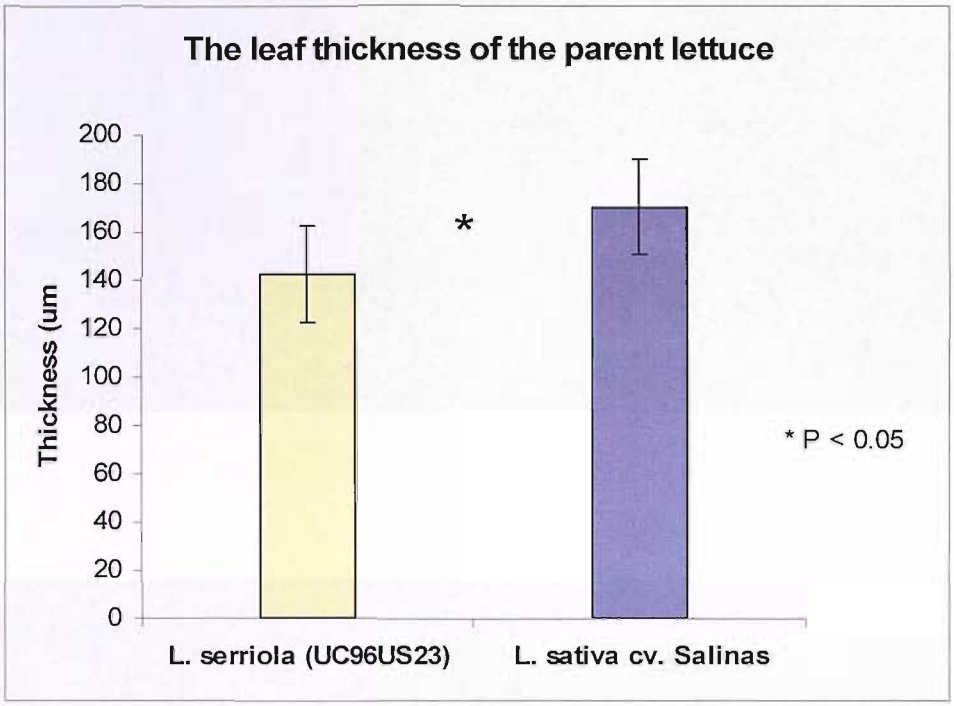
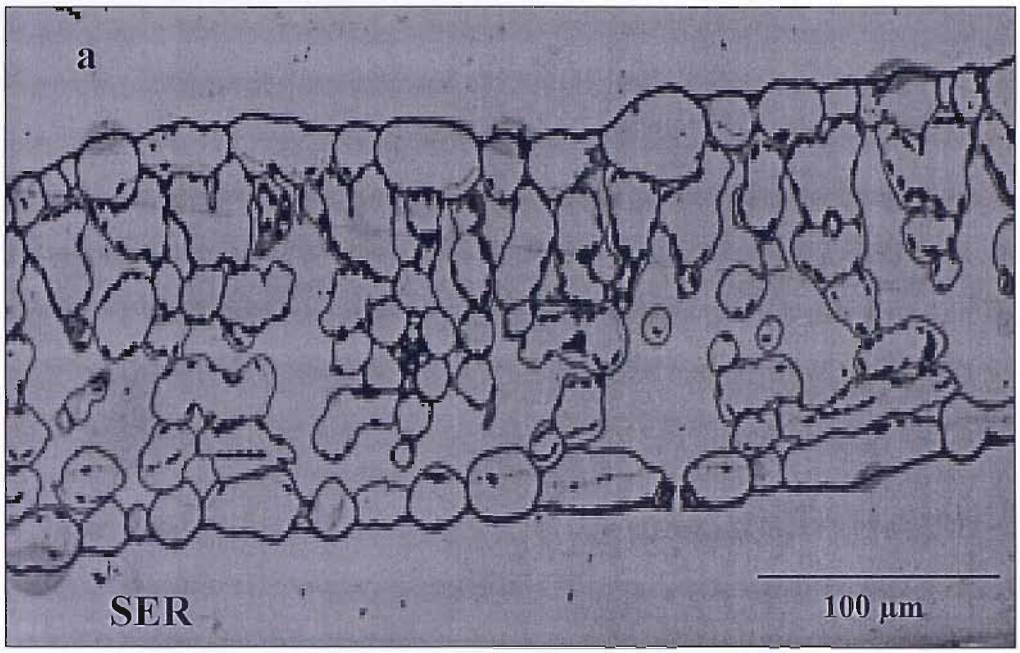


Figure 3.5 The leaf thickness of the parent lettuce. Data points are means ± SD of six replicates. The results of a one-way ANOVA are indicated where significant: * $P < 0.05$.



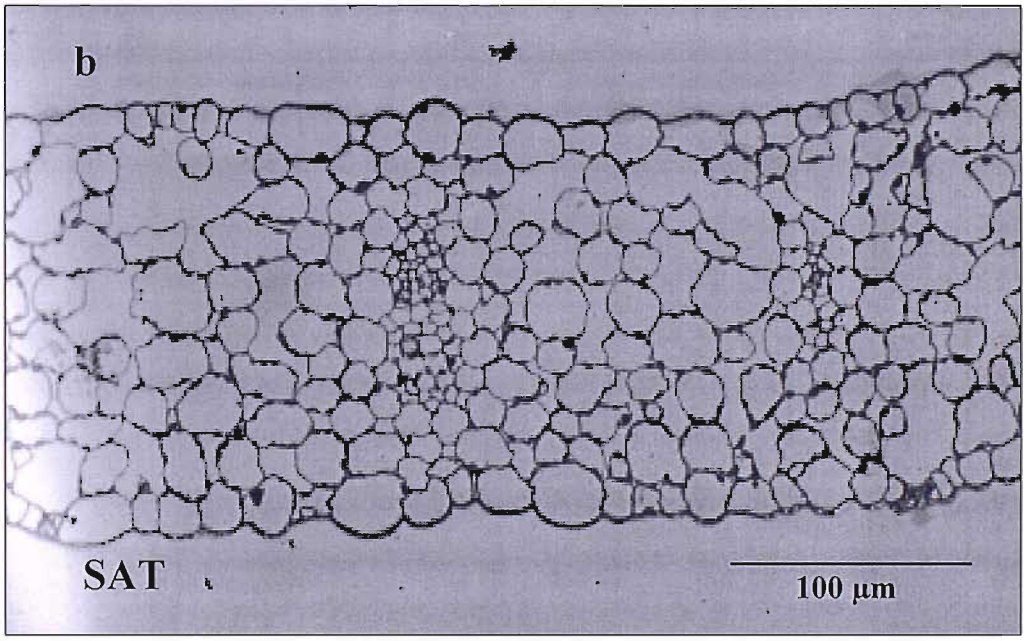


Figure 3.6 Transverse sections of leaf 5 of the parent lettuce. *L. serriola* (UC96US23) (a) and *L. sativa* cv. Salinas (b), taken at x100 magnification with a scale bar representing 100 µm.

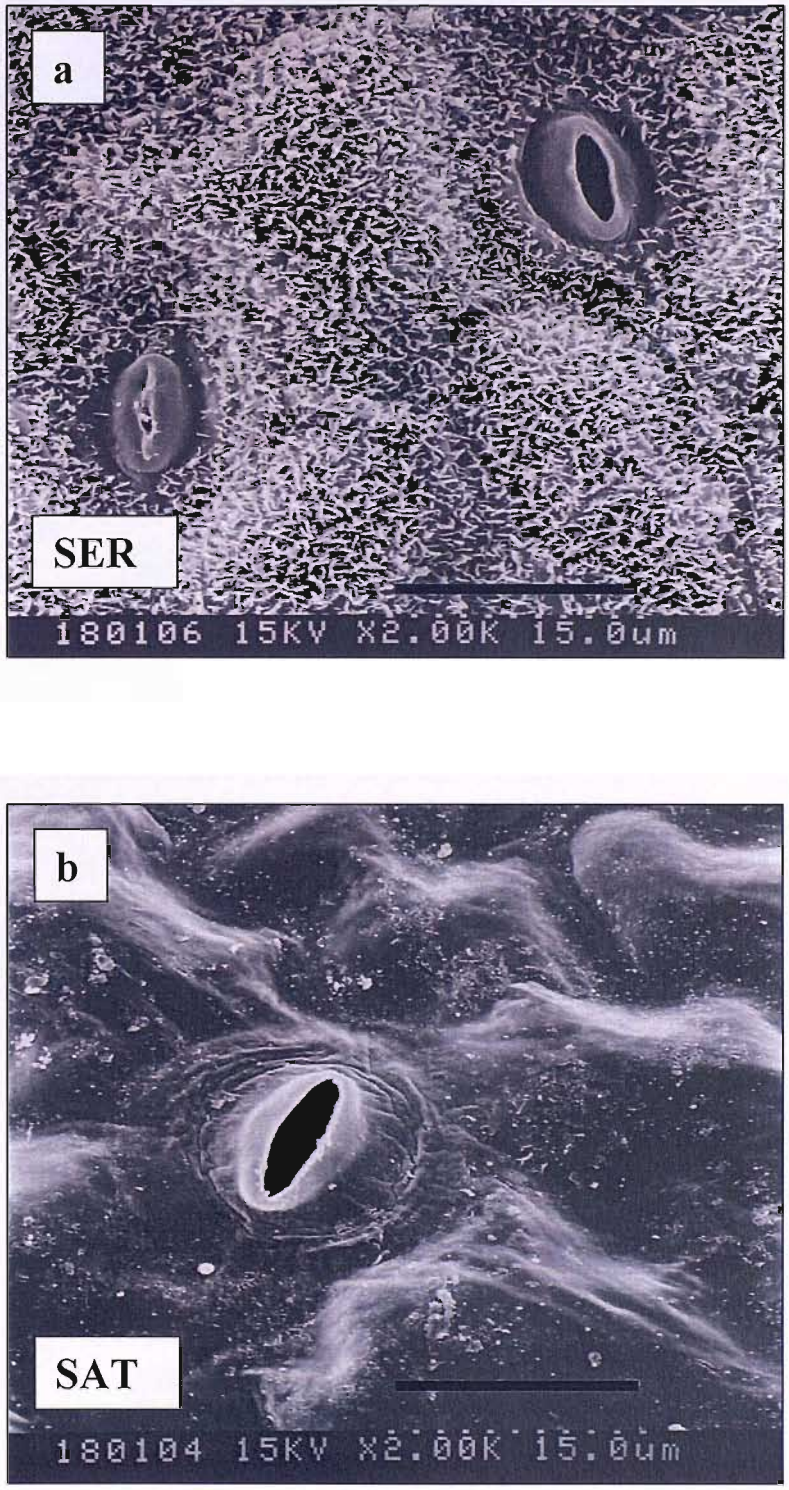


Figure 3.7 The SEM images of the leaf epidermis of the parent lettuce. *L. serriola* (UC96US23) (a) and *L. sativa* cv. Salinas (b), taken at x2K magnificent with a scale bar representing 15 um.

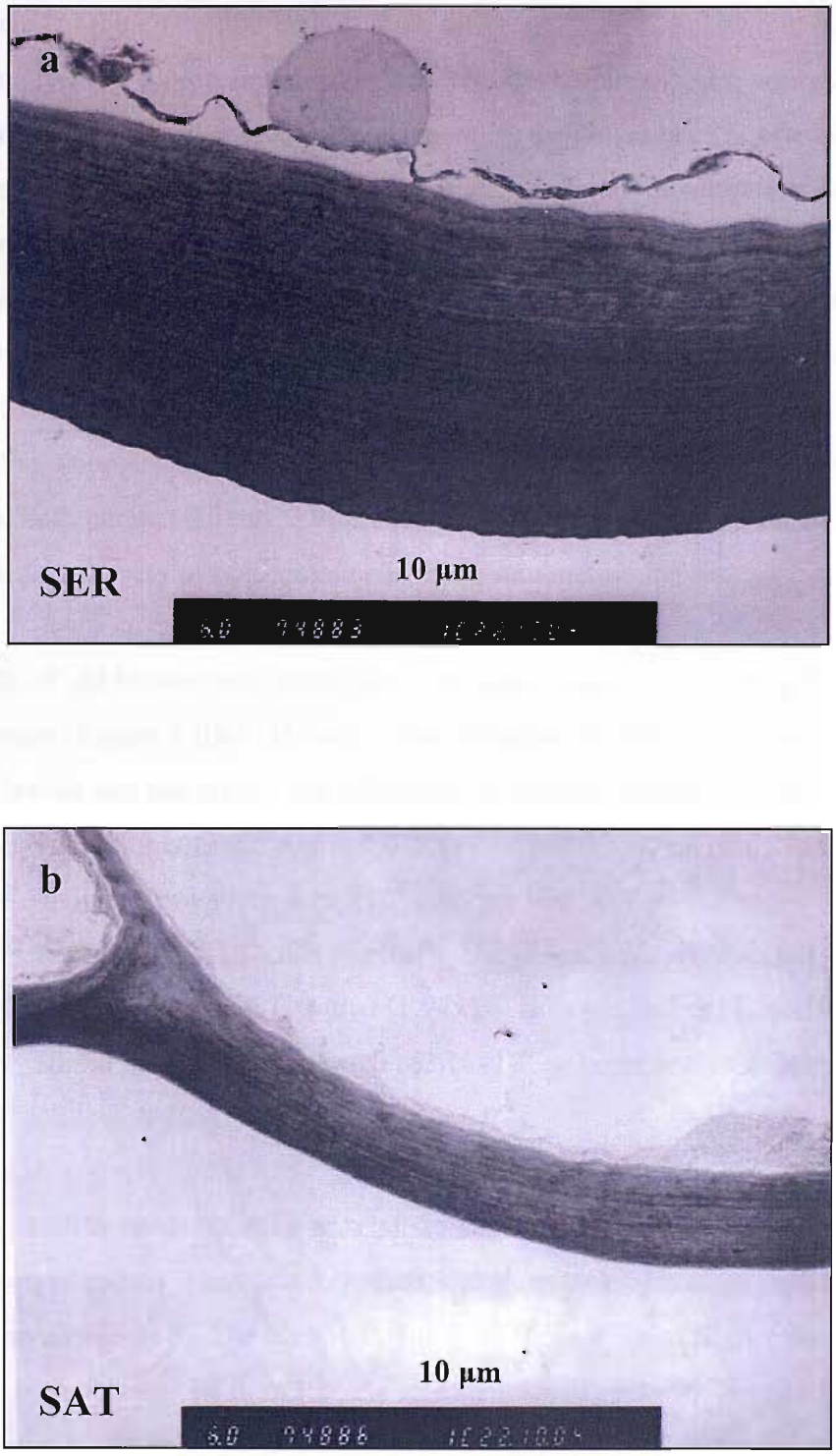


Figure 3.8 The TEM images of the cell cuticle of the parent lettuce. *L. serriola* (UC96US23) (a) and *L. sativa* cv. Salinas (b), taken at x6K magnificent with a scale bar representing 10 µm.

3.2.4 Epidermal cell area and stomatal traits

The images of the epidermal cells at three different stages are shown in Figure 3.9. Leaf 1, 3 and 5 represented three different development ages, young, semi-mature and mature leaf, respectively. Generally, the cell area of *L. serriola* was significantly greater than that of *L. sativa* at each different development stage ($P < 0.05$ at leaf 1 and $P < 0.001$ at leaf 3 & 5). In *L serriola*, mean (±SD) epidermal cell area at leaf 1, 3, 5 were respectively 3175 (± 367) μm^2, 1645 (± 277) μm^2 and 977 (± 297) μm^2, while means cell area were 1837(± 352) μm^2, 1091(± 313) μm^2 and 582 (± 316) μm^2 in *L. sativa,* respectively (Figure 3.10a). In addition, younger leaves were composed of smaller cells than more mature leaves in both parents (Figure 3.9), indicating that more recently initiated leaves have a higher density of epidermal cells than more mature leaves.

The cells of old leaves were about three fold larger than that of young leaves in both parents (Figure 3.10a). However, the difference in leaf area between young and old leaves was not great. The difference in the cell number per leaf between two parents was not consistent with the development (Figure 3.10b). In the old leaf (leaf 5), there were about 1.16×10^6 cells per leaf of *L. sativa*, more cells than that of *L. serriola* (0.82×10^6 cells per leaf). But in leaf 3, *L. serriola* has significantly more cells than *L. sativa* (1.94×10^6 cells per leaf vs. 1.30×10^6 cells per leaf). So did the young leaf (leaf 1) (2.11×10^6 cells per leaf of *L. serriola*, vs. 1.46×10^6 cells per leaf of *L. sativa*).

Significant differences between adaxial stomatal density of the two parents were only observed in leaf 3 and leaf 5 (Figure 3.11a), as lack of enough replicates was achieved in leaf 1. The stomatal density of the leaf 3 was higher than that of leaf 5 in both parents. In *L. sativa*, the stomatal density was 96.74 (±23.28) mm^{-2} in leaf 3 and 52.95 (±15.98) mm^{-2} in leaf 5, while 67.21 (±19.30) mm^{-2} and 38.23 (±11.25) mm^{-2} for leaf 3 and leaf 5 of *L. serriola*, respectively. For stomatal index, there was significant difference between two parents in leaf 5 (Figure 3.11b) ($p < 0.01$), but not significant difference in leaf 3 ($P = 0.123$). However, the overall mean of the adaxial stomatal index (%) of *L. serriola* (10.25% ±1.24%) was significantly higher than *L. sativa* (8.27% ±1.33%).

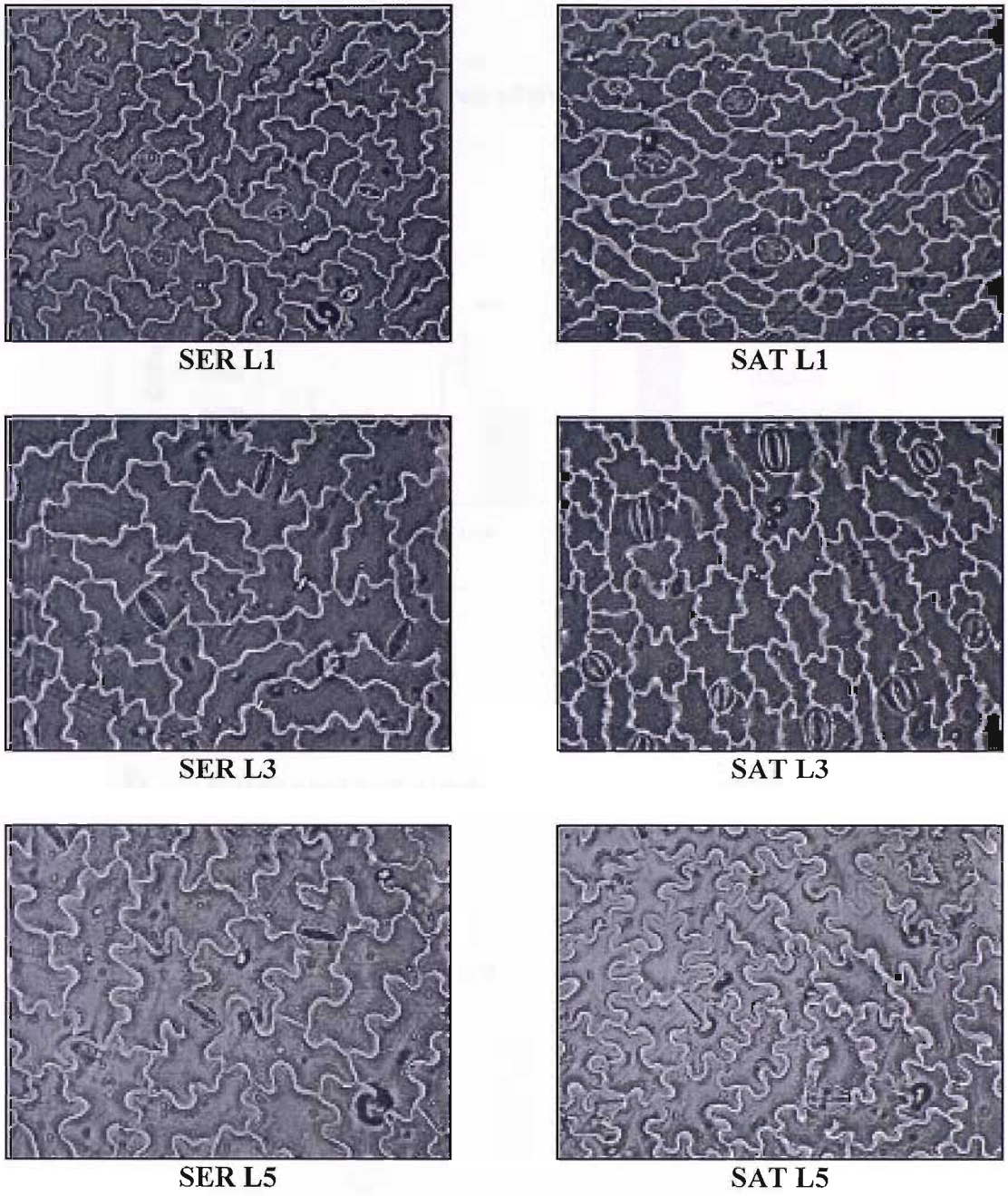
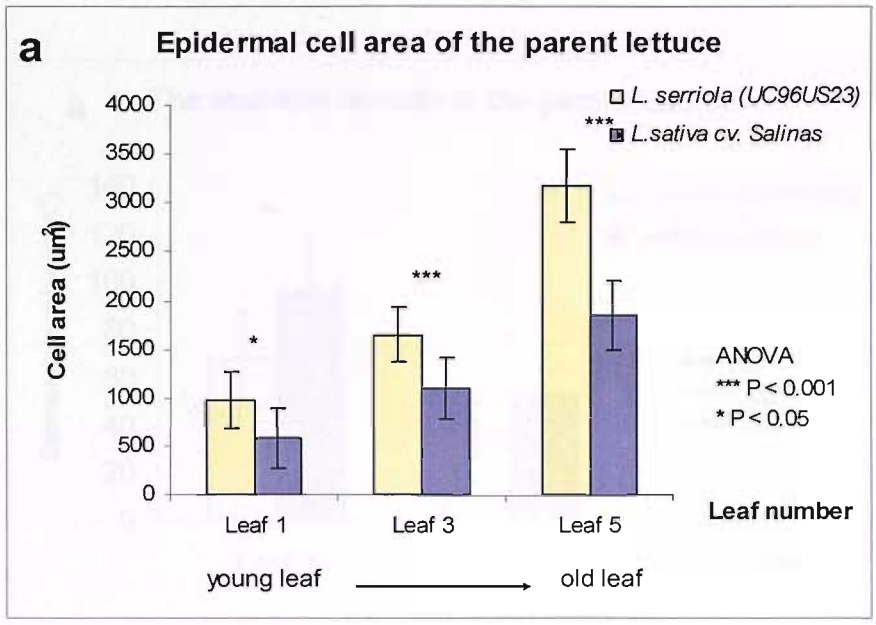


Figure 3.9 Epidermal cell images of the parent lettuces at different development ages. Two parents are *L. serriola* (US96US23) (SER) and *L.sativa* cv. Salinas (SAT). The images were taken at x200 magnification, with a scale bar representing 100 µm. L1, L3, L5 represents three different development ages of the plants (young, semi-mature, mature leaf).



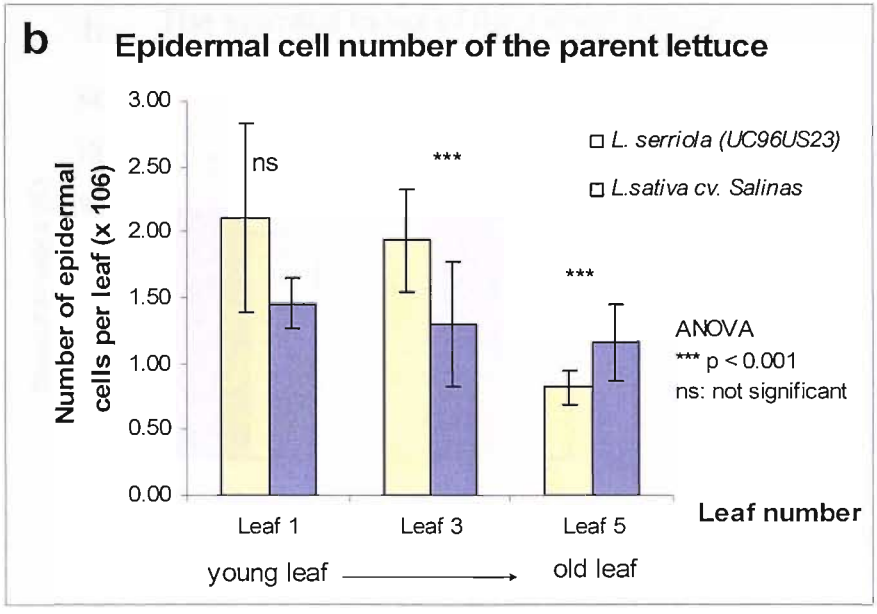
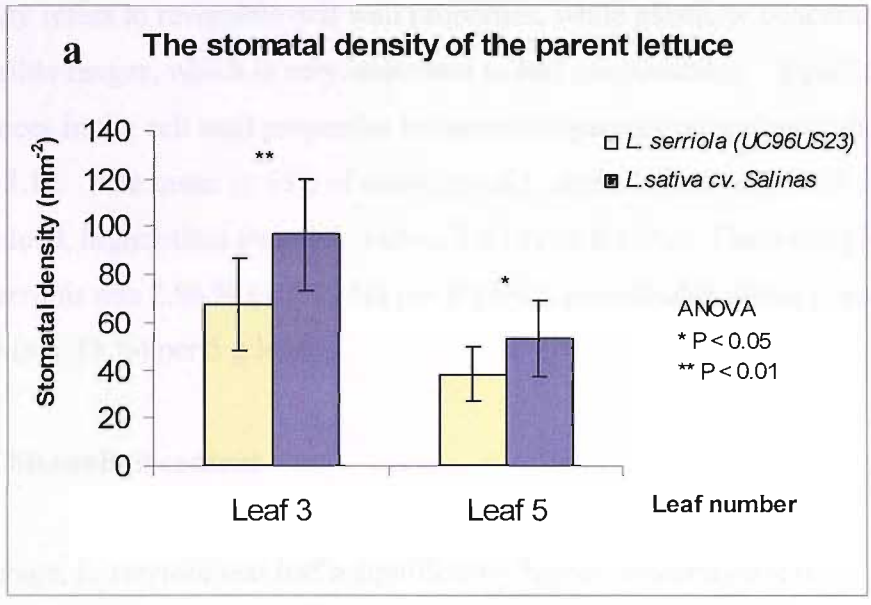


Figure 3.10 Epidermal cell (a) and epidermal cell number per leaf (b) of the parent lettuce at different developmental stages. Data points are means ± SD of fifteen plants. The results of a one-way ANOVA are indicated where significant: *** $p<0.001$, ns=not significant.



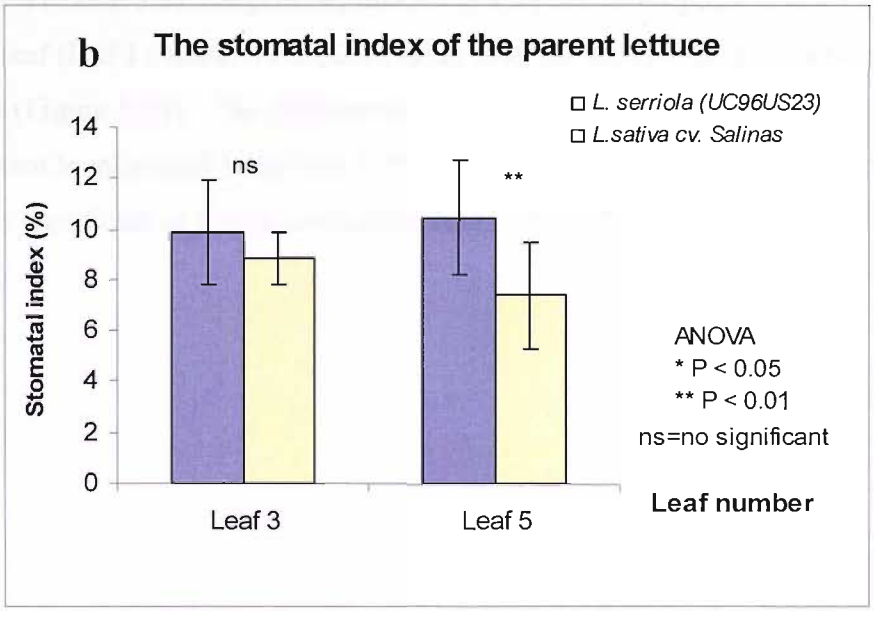


Figure 3.11 Stomatal density (a) and stomatal index (b) of the parent lettuce at different development ages. Data points are means ± SD of fifteen plants. The results of a one-way ANOVA are indicated where significant: ** $p<0.01$, * $p<0.05$, ns=not significant.

3.2.5 Cell wall properties: plasticity and elasticity

Plasticity and elasticity indicates the cell wall properties of the leaf material. Elasticity refers to reversible cell wall properties, while plasticity concerns irreversible ranges, which is very important to leaf processability. Significant differences in the cell wall properties between two parents were clearly shown in Figure 3.12. The mean (± SD) of elasticity of *L. serriola* was 4.97 % (± 0.91%) per 5 g load, higher than that of *L. sativa*, 9.83 % (± 0.46%). The mean plasticity of *L. serriola* was 2.96 % (± 1.50 %) per 5 g load, over double of the *L. sativa*, 1.29 % (± 1.13 %) per 5 g load.

3.2.6 Chlorophyll content

On average, *L. serriola* leaf had a significantly higher concentration of chlorophyllous pigments than that of *L. sativa* ($P < 0.001$, 0.23 μg mm^{-2} vs. 0.19 μg mm^{-2}) (Table 3.1). In general, there was a higher chlorophyll content in young leaf (leaf 1), about 40%-50% higher than the old leaf (leaf 5) in two species (Figure 3.13). The differences between the two parents were at significant level in leaf 1 and leaf 5 ($P < 0.05$). But in the leaf 3, the difference was not significant at a 95% confidence level, ($P < 0.16$).

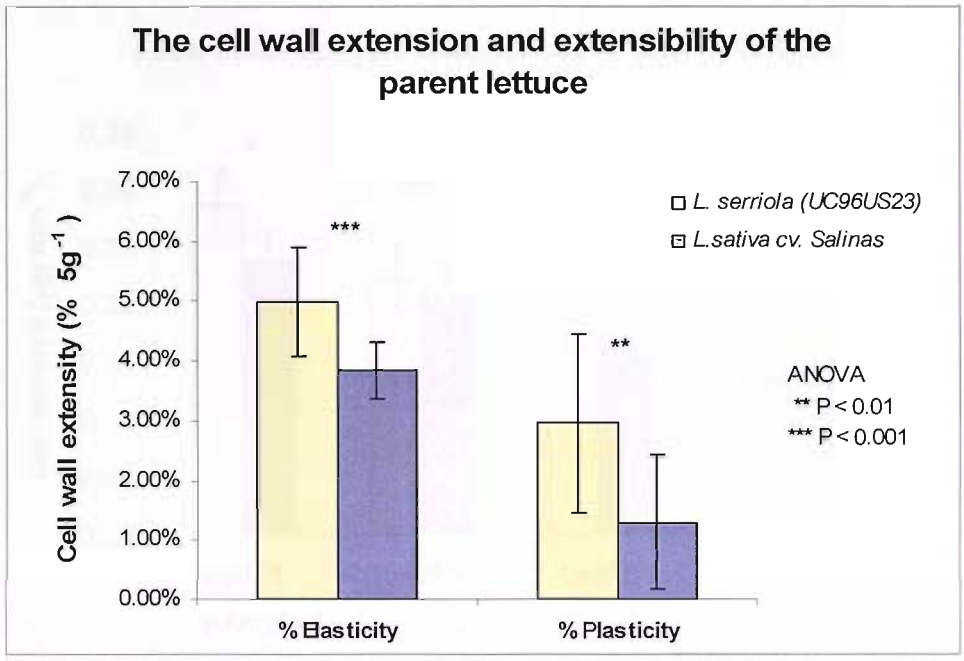


Figure 3.12 Cell wall properties (elasticity and plasticity) of the parent lettuce. Leaf material was measured from Instron apparatus. Data points are means ± SD of fifteen plants. The results of a one-way ANOVA are indicated where significant: ** $p<0.01$.

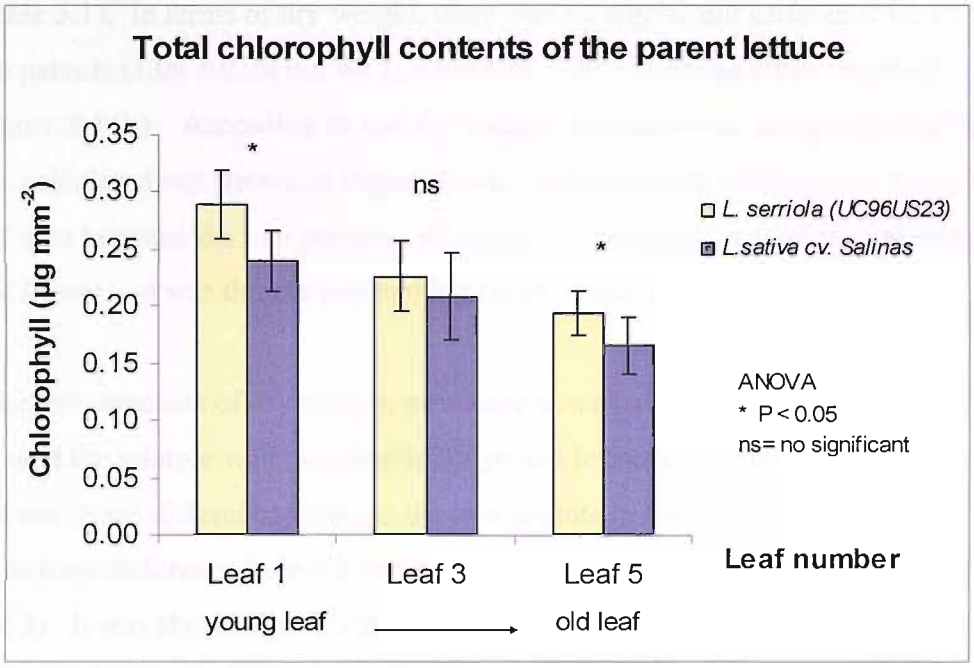


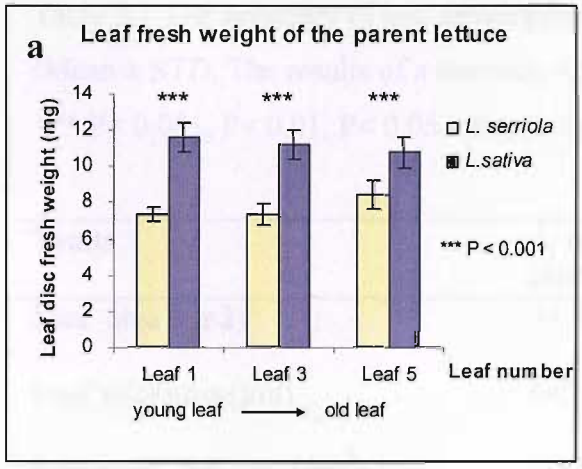
Figure 3.13 Total chlorophyll contents of the parent lettuce at different development ages. Data points are means ± SD of fifteen plants. The results of a one-way ANOVA are indicated where significant: * $p<0.05$, ns = not significant.

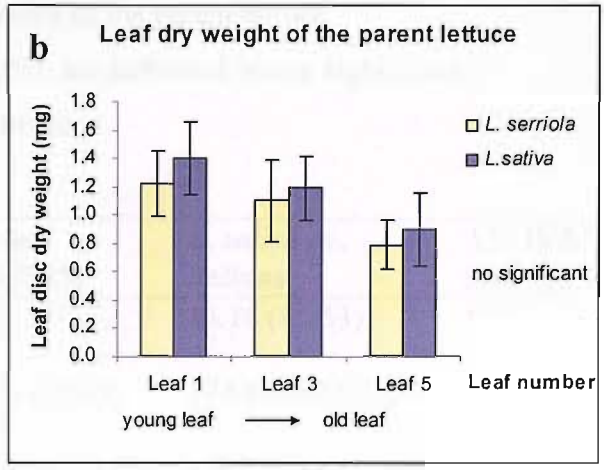
3.1.7 Leaf fresh weight and dry weight

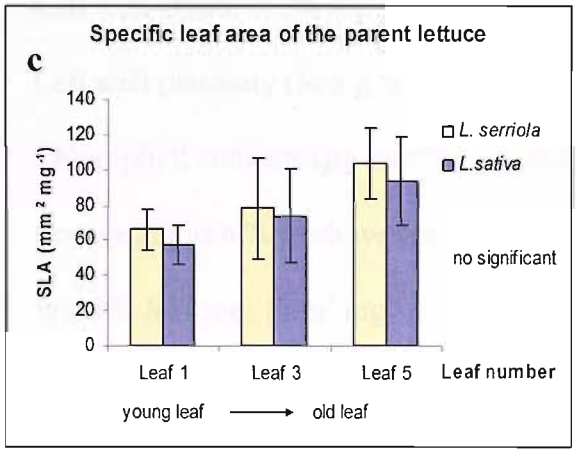
A highly significant difference in leaf fresh weight was found between the two parents (Figure 3.14a, $P < 0.001$). There was not a big difference in leaf disc fresh weight among three of the leaf development ages in both species. The mean fresh disc weight of *L. sativa* was about 45% higher than that of *L. serriola* (Table 3.1). In terms of dry weight, there was no significant difference between two parents (1.09 ± 0.20 mg for *L. sativa* vs. 1.00 ± 0.16 mg for *L. serriola*) (Figure 3.14b). According to leaf dry weight measurement, the specific leaf area was calculated and shown in Figure 3.14c. No significant difference in specific leaf area between the two parents. However, the traverse cross section showed that *L. serriola* was thinner and had higher SLA that *L. sativa*.

The measurements of dry weight, presented as a percentage of fresh weight showed the relative water content in the parent lettuces (Figure 3.14d). There was not much difference between the two parents in leaf 5, but there was significant difference in leaf 1 and leaf 3 ($P < 0.05$ for leaf 1 and $P < 0.001$ for leaf 3). It was shown that *L. sativa* leaves have higher water content than *L. serriola* ($P < 0.001$, Table 3.1). Figure 14d also demonstrated that older leaves have a lower value of dry weight percentage of the fresh weight and thus hold more water than relatively newly initiated leaves.

All the trait measurements were summarised in Table 3.1. For multi-stages trait measurement, the mean of three different stages were calculated to test the difference between two parents. All the traits were significantly different between two parents, except the specific leaf area.







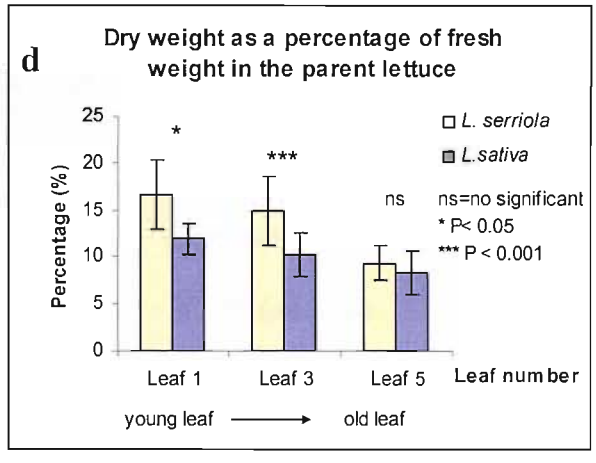


Figure 3.14 Leaf fresh weight (a), dry weight (b), specific leaf area (c) and dry weight as a percentage of fresh weight (d) of the parent lettuce at different development ages. Data points are means ± SD of fifteen plants. The results of a one-way ANOVA are indicated where significant: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

Table 3.1 The summary of leaf development traits of the parent lettuce.

(Mean ± STD, The results of a one-way ANOVA are indicated where significant: *** P< 0.001, P< 0.01, P< 0.05, ns=not significant.)

Traits	***L. serriola* (US96US23)**	***L. sativa* cv. Salinas**	**ANOVA (*n*=30)**
Leaf area (cm2)	24.55 (±3.07)	14.16 (±1.53)	***
Leaf thickness (μm)	142.39 (±19.85)	170.40 (±23.29)	*
Epidermal cell area (μm^2)	1988.65 (±323.94)	1332.52 (±220.64)	***
Epidermal cell no. per leaf (x 10^6)	1.54 (±0.32)	1.27 (±0.28)	*
Stomatal density (mm^{-2})	55.17 (±15.65)	81.13 (±23.66)	**
Stomatal index (%)	10.25 (±1.24)	8.27 (±1.33)	***
Cell wall elasticity (% 5 g^{-1})	4.97% (±0.91%)	3.83% (±0.46%)	***
Cell wall plasticity (% 5 g^{-1})	2.96% (±1.50%)	1.29% (±1.13%)	**
Chlorophyll contents (μg mm^{-2})	0.23 (±0.02)	0.19 (±0.03)	***
Dry weight as a % fresh weight (%)	13.18% (±2.06%)	9.64% (±1.93%)	***
Specific leaf area (mm^2 mg^{-1})	85.68 (±15.18)	77.98 (±15.54)	ns

3.3 Discussion

3.3.1 Leaf development

The germination of cultivated lettuce seeds was higher than that of wild lettuce. *L. sativa* is a crisp head cultivar, which is very popular in the super market. It was likely to be a selection of high germination by seed companies to achieve seed of the highest quality. *L. serriola* usually grows in the natural environment and maybe has adapted to drought habitats with long, narrow and hairy leaves. Further studies to compare germination need to be carried out under controlled condition.

There was a strong relationship between leaf length and leaf area in the early stages of leaf development in two parents (Figure 3.2), which could be used in the future experiment to measure the leaf length to convert to the leaf area. No significant difference in LPI between two parents was found in this study. It suggested that leaves of the same emergent number are of equivalent developmental stage and can thus be directly compared. But LPI is more accurate to compare the leaf development between two species (Erickson & Michelini, 1957). There was no significant difference in LPI between two species in this study.

In the early stages of growth, *L. sativa* leaves were larger than *L. serriola*, but by harvest the difference had reversed and *L. serriola* leaves were significantly larger (Figure 3.3). The latter result agreed with Gallardo's study (Gallardo et al., 1996). In their study, the same species were measured after seven weeks of growth in a glasshouse. The growth rate of leaf area in lettuce showed periodic fluctuation in the early stage. The result that *L. sativa* leaf was thicker than *L. serriola* (Figure 3.5) suggested that the larger leaf area in *L. serriola* did not necessarily represent a greater growth rate in this species. Thus, it suggested that 3D analysis of leaf area and leaf thickness should be considered in the leaf development measurement in two different species.

3.3.2 Morphological and physiological leaf traits

The study of the parents of the RIL population has shown that cultivated lettuce parent differs greatly from the wild lettuce in leaf morphology and physiology (Table 3.1). From the transverse section, it showed there was less internal air space throughout the leaf in *L. sativa* than in *L. serriola* (Figure 3.4). The composition of *L. sativa* is denser than *L. serriola*, with smaller epidermal cells (Figure 3.6). It was reported that the leaves with bigger cells were less likely to withstand the whole process of baby leaf salad preparation than leaves with smaller cells (Clarkson et al., 2003). It is therefore predicted that the leaves of *L. serriola* with bigger cells are less likely to withstand the whole process than leaves of *L. sativa* with smaller cells.

The mature leaves have bigger cells than young leaves in both parents (Figure 3.9). Taylor et al. (2003) also reported similar result that the epidermal cell area is smaller in the young leaves in Poplar. This finding suggests the abaxial epidermal cells are denser in recently emerged leaves, and later leaf expansion occurs predominantly by cell expansion, as well as cell production, and thus young baby salad leaves would be more processable than the mature leaves. This means baby salad leaves could have longer shelf life and this would benefit the baby salad industry. It would be interesting to investigate the cell expansion and cell production rate in leaf development, and explore how cell expansion or cell production has an impact on the leaf processability. Recent study has proved that cell expansion and cell production act together in a coordinated manner to determine leaf area and shape (Kim et al., 2002). Leaf area and shape are considered to be important factors in leaf processability. The relative importance of cell production and expansion varies among species and is under genetic control (Fiorani et al., 2000). It would be worth investigating the genetic basis of cell expansion and cell production in two parents.

L. sativa parental line has a significantly higher stomatal density than *L. serriola* parent line. When the cell number was accounted, the stomatal index was significantly different in leaf 5, but not significant in leaf 3. The stomatal index

remained constant with the leaf development. When the samples with two different leaf development ages are pooled together, it was found that *L. serriola* leaves had a significantly higher proportion of stomata relative to total epidermal cells (Table 3.1). This result confirmed the study of Gallardo et al. (1996). They suggested that a higher net photosynthesis rate observed in *L. serriola* were matched by a higher stomatal conductance. Our results suggested a link between stomatal initiation and epidermal cell production. The non-significant difference between two parents in leaf 3, which conflicts with the average results that stomatal index significantly differed between two parents, could be explained by not enough replicates for this leaf development age. It was reported that stomatal traits often differ greatly between adaxial and abaxial leaf surface (Ferris et al., 2001), analysis of both surfaces would give us more information about the stomatal initiation and cell production.

The cell wall has two important properties: elasticity and plasticity. The elastic part of the cell wall is reversible; it will return to its original tense after exerted force load. Plasticity is the irreversible extensibility of the cell wall. The cell wall stiffness is indicated from these two biophysical properties, plasticity and elasticity (Cosgrove, 1999). The leaf with a higher plasticity indicates a less extensible cell wall and thus would have lower deformability. This experiment indicated that *L. serriola* was more extensible than *L. sativa* (Figure 3.12), as *L. serriola* had a higher elasticity and plasticity. In this study, the old leaves of *L. serriola* could not be assessed in the Instron apparatus as they irreversibly deformed before reaching 50 % extension with 5 g load. This analysis suggested that leaves of *L. sativa* had an advantageous rigidity, and resistance to breakage and damage caused by the forces exerted in processing and packaging and are hence more processable. The plant cell wall is an important structure to provide essential mechanical strength and rigidity, and to protect against pathogens and dehydration (Cosgrove, 2001). The cell wall has two conflicting characteristics: tensile strength and stability versus structural plasticity. To date, there are few studies about the role of the cell wall in the leaf processability and the shelf-life of leafy vegetables. The study of Kaku et al. (2002) indicated that XET enzyme increased the cell wall extensibility of epidermal tissues by hydrolysing

xyloglucans within the native cell wall architecture. XET activity will be a potential trait relevant to leaf processability.

The lower percentage of dry weight of fresh weight in *L. sativa* leaves indicated that there was higher water content in the leaves of *L. sativa* than *L. serriola* (Cherry, 2003). The small cells of *L. sativa* would presumably have a higher turgidity, and thus more closely packed. This configuration makes cell walls of adjacent cells contact and thus supports the leaves to withstand the whole process. This could suggest that *L. sativa* appears more processable than *L. serriola*. In addition, high water content is likely to contribute to a more favourable taste and texture of leaves. It also looks more attractive to the customer in the super market as the leaves with higher water contents usually appear more shining and fresher. The visual appearance has a great impact on the sale of the products (Watada & Qi, 1999). The same mapping population has been studied for water use efficiency and root architecture at University of California (Johnson et al., 2000). Their results provide the opportunity for marker-assisted selection via the introgression of wild alleles into cultivated lettuce to improve soil water and nutrient acquisition.

The result of higher chlorophyll contents in *L. serriola* than *L. sativa* supported the previous report of lower net photosynthesis rates in *L. sativa* than in *L. serriola* in a closed system (Gallardo et al., 1996). *L. serriola* may therefore be a source of alleles to breed darker green leaf and higher yield. QTL analysis of sunflower (*Helianthus annuus*) RILs under glasshouse conditions successfully identified three QTL associated with chlorophyll concentration, speculated to contain genes involved in chlorophyll turnover (Herve et al., 2001). This suggested that there is a possibility for mapping QTL for chlorophyll content in lettuce. In addition, lower chlorophyll concentration was measured in the oldest leaves rather than the newly emerged leaves in both parents. This suggested the older leaves were in the process of senescence. Recently, Mathas et al (2003) planned to map QTL for shelf life in Broccoli, based on the rate of postharvest senescence. Several genetic mutants and potential regulatory components have been identified in leaf senescence in *Arabidopsis* (Lim et al., 2003). So, the analysis of senescence rate in lettuce and its genetic basis would be beneficial to improve the shelf life of baby leaf salad.

3.4 Conclusions

1. The cultivated lettuce (*L. sativa* cv. Salinas) appears to be more processable than *L. serriola* (US96US23) from the analysis of certain traits.

2. The leaf development traits, including leaf area, leaf thickness, leaf fresh weight and dry weight, epidermal cell area, cell wall extensibility and chlorophyll contents, are significantly different between parents. Thus, QTL detection for leaf processability in this population should be possible.

CHAPTER 4

Mapping QTL in a selected RIL population

4.1 Introduction

From the preliminary experiments of the parent population, it was shown that *L. serriola* and *L. sativa* have contrasting leaf development traits. The traits thought to be relevant to leaf processability have been identified in the parental material. A new genetic linkage map, which was used to map QTL for leaf processability in this study, was developed in University of California at Davis. This study focused on the phenotype of the selected recombinant inbred lines (60 lines). These 60 F_9 RILs were selected as the lines containing the most genetic information from a 113 RIL mapping population. Two large scale field trials were conducted in two contrasting environments, Portugal and the UK. Leaf development traits were assessed at three different developmental stages in the Portugal field trial, while the leaf traits were only assessed at one development stage in the UK field trial. QTL mapping software, QTL cartographer, was used to map QTL for leaf development and leaf processability. Once the QTL for leaf development traits and processability were identified, the effect of QTL by environment interaction was compared in two field trials to test the robustness of QTL for the traits of interest.

Aims:

i. **To map QTL for leaf development traits at three different developmental stages in the Portugal field trial.**

ii. **To test the robustness of QTL detected in two different environments to explore the genotype by environment interaction.**

4.2 Results

4.2.1 Trait performance

4.2.1.1 Leaf morphology

The average germination rate over the whole population was 50% in the Portugal field trial and 67% in the UK field trial. The weather during the growing period in Portugal was not ideal; there were many rainy days during the first two weeks in Portugal, while the weather in the UK was good and the temperature was around 30 °C. Only two blocks (D and E) were harvested for the samples collected for ten leaf traits because 6 replicates of each line were obtained from two blocks. Three blocks were all used for the leaf growth rate experiment. Leaf shape varied among the 60 RIL lines. Figure 4.1 showed some examples of these leaf images. It was clear to see the segregation among these lines. Some leaves are smooth and round shape while the others are hairy or prickly, with curly edges.

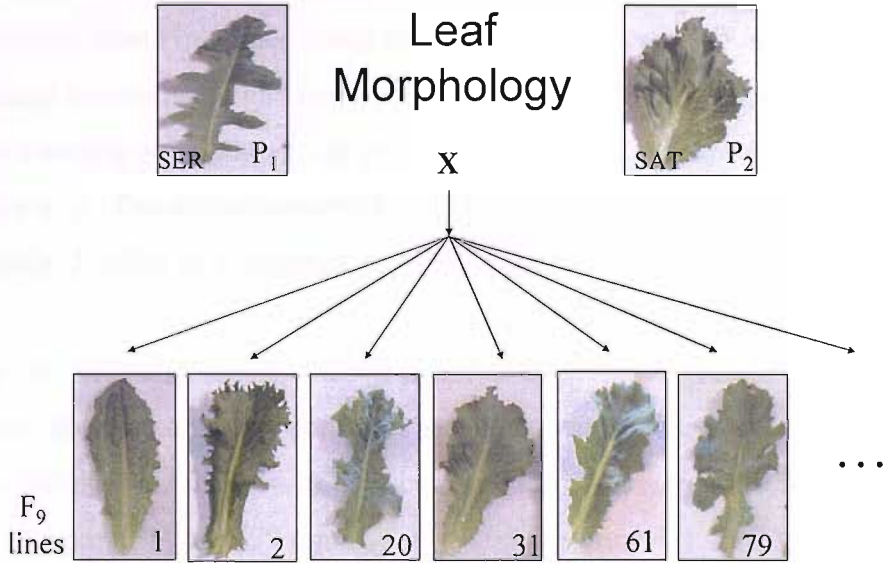
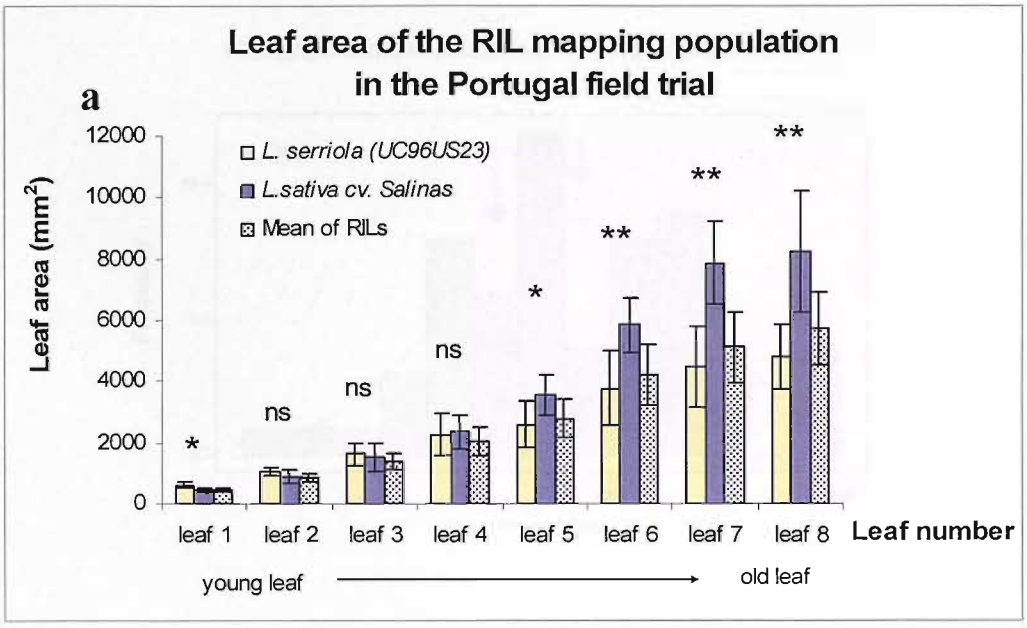


Figure 4.1 Examples of contrasting leaf morphology of the RIL mapping population. Wild lettuce *L. serriola* (SER, P_1) and cultivated lettuce *L. sativa* (SAT, P_2) are the parent lettuce. Only six images are shown as an example of the segregation in the F_9 lines.

4.2.1.2 Leaf area and growth rate

In the Portugal field trial, eight leaves were collected for different traits from the youngest leaf (leaf 1) to eldest leaf (leaf 8) in each plant to test the difference between the leaf development stages. Leaf 1 to 3 represented young leaves, leaf 4 to leaf 6 represented semi-mature leaves, and leaf 7 and 8 were mature leaves. The leaf area of *L. sativa* (leaf 1: 474 ± 83 mm^2) was smaller than leaf area of *L. serriola* (leaf 1: 613 ± 90 mm^2) in the early developmental stage (from leaf 1 to leaf 3). Then *L. sativa* leaves gradually increased in size to become larger than *L. serriola* leaves from leaf 4 (Figure 4.2a). With the leaf developing, the difference of leaf area between two parents became significantly bigger ($P < 0.01$). At each developmental stage, means of the F_9 RILs were approximately equal to intermediate values between two parents. The range of RILs for mean of leaf area from leaf 1 to leaf 8 were from 1480 mm^2 (line 59) to 3978 mm^2 (line 2) in Portugal and its normal distribution curve was shown in Figure 4.3a. In the UK, leaves of 3, 6, 7 and 8 were measured for leaf area (Figure 4.2b). Compared with the Portugal trial, there was a similar trend for the difference between two parents in the UK trial. No significant difference for leaf area between two parents was found in young leaves (leaf 3), but significant difference was found in mature leaves (leaf 7 and leaf 8). Mean leaf area (MLA) of leaf 3, 6, 7 and 8 of the mapping population in the two trials are shown in the normal distribution (Figure 4.3). The differences of MLA between two parents were significant in two trials ($P < 0.01$ in Portugal, $P < 0.05$ in UK).

In the UK field trial, the absolute growth rate and relative growth rate were assessed during the growth period. These traits were not assessed in Portugal. *L. sativa* had doubled the growth rates compared to *L. serriola* (absolute growth rate; 11.36 $mm^2\ h^{-1}$ vs 5.79 $mm^2\ h^{-1}$, $P < 0.05$ (Figure 4.4a); relative growth rate; 16.02 $x10^{-3}\ mm^2\ mm^{-2}\ h^{-1}$ vs 8.55 $x10^{-3}\ mm^2\ mm^{-2}\ h^{-1}$, $P < 0.05$ (Figure 4.5a)). The absolute growth rate of the F_9 RILs ranged from 3.61 $mm^2\ h^{-1}$ to 17.79 $mm^2\ h^{-1}$ and the mean of the RILs for absolute growth rate (9.43 $mm^2\ h^{-1}$) was close to *L. sativa* (Figure 4.4b). But for *L. sativa,* the relative growth rate (16.02 $x10^{-3}\ mm^2\ mm^{-2}\ h^{-1}$) was out of the RIL variable range (4.84 to14.09 $x10^{-3}\ mm^2\ mm^{-2}\ h^{-1}$) (Figure 4.5d).



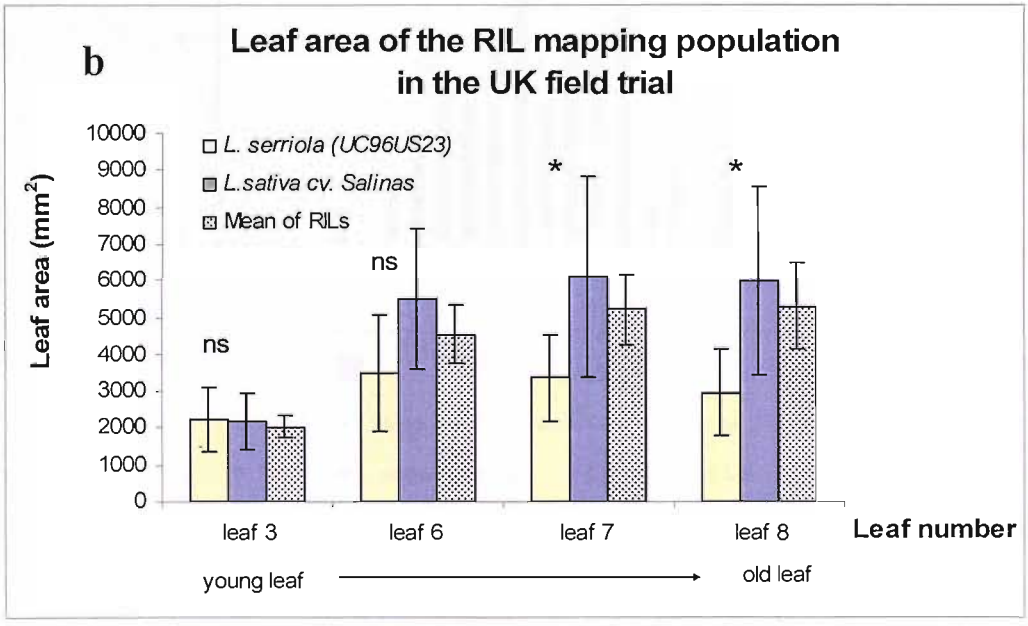


Figure 4.2 Leaf area of the selected F_9 RIL mapping population at different developmental ages in two field trials. Portugal trial (a) and the UK trial (b). Data points are means ± SD, n=6. The results of a one-way ANOVA t-test between two parents are indicated where significant: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns=not significant.

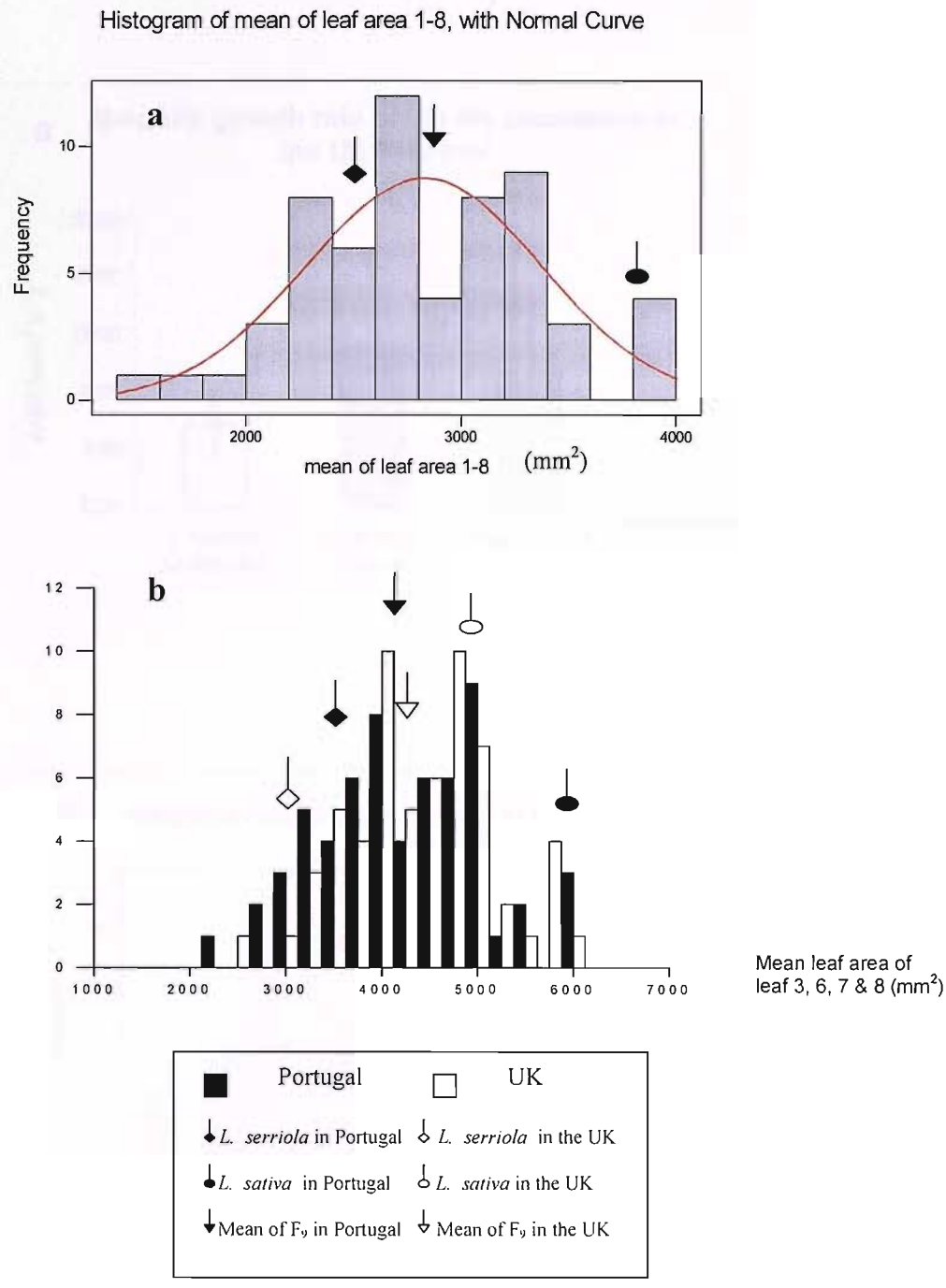
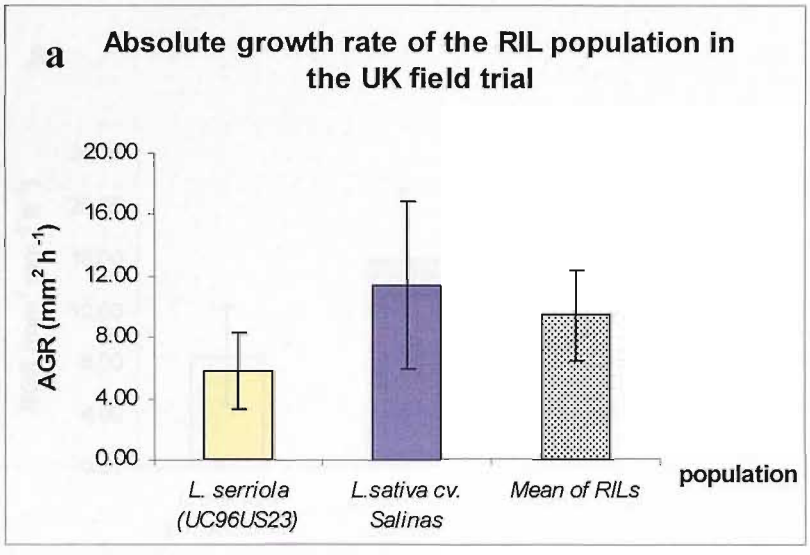


Figure 4.3 Distribution of leaf area in the selected F_9 RIL mapping population. Mean leaf area from leaf 1 and leaf 8 in the Portugal field trial with the normal curve (a) and mean leaf area from leaf 3, 6, 7 and leaf 8 in two field trials (b). The mean values of two parents *L. serriola*, *L. sativa* and of the RILs are indicated by arrows.



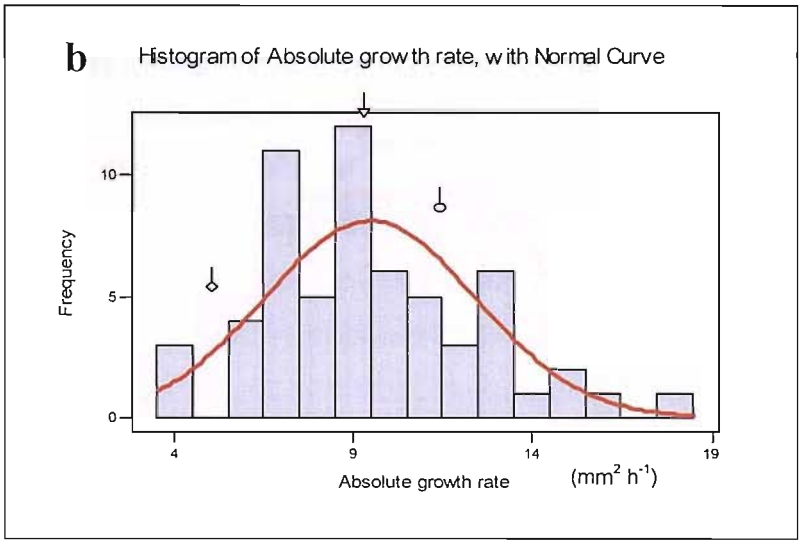
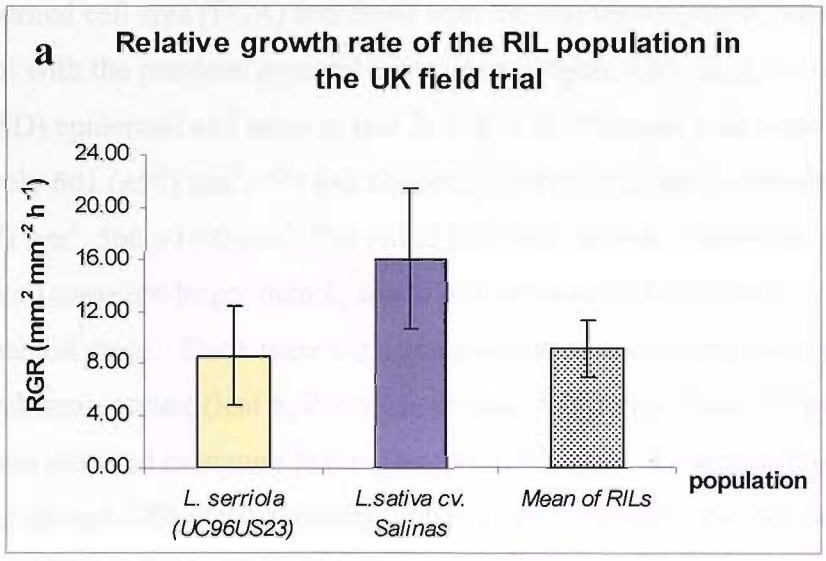


Figure 4.4 Leaf absolute growth rate (a) and its distribution (b) of the F_9 RIL mapping population in the UK field trial. Data points are means ± SD, n=6. The mean values of two parent *L. serriola* (⇩), *L. sativa* (⇩) and of the RILs (⇩) are indicated by arrows.



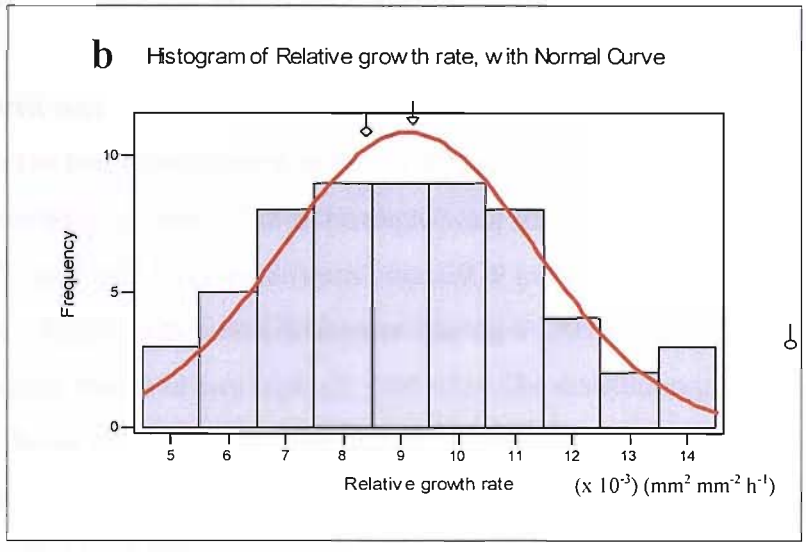


Figure 4.5 Leaf relative growth rate (a) and its distribution (b) of the F_9 RIL mapping population in the UK field trial. Data points are means ± SD, n=6. The mean values of two parent *L. serriola* (⇩), *L. sativa* (⇩) and of the RILs (⇩) are indicated by arrows.

4.2.1.3 Epidermal cell area, cell number and stomatal traits

The epidermal cell area (ECA) increased with the leaf development, which is consistent with the previous parental experiment (Figure 4.6). In *L. serriola*, the mean (±SD) epidermal cell areas at leaf 2, 5, 8 in the Portugal trial were respectively 601 (±97) μm^2, 973 (±223) μm^2, 1294 (±352) μm^2, where they were 182 (±37) μm^2, 568(±169) μm^2, 970 (±97) μm^2 in *L. sativa*. Generally, *L. serriola* cell areas are larger than *L. sativa* cell areas at each different developmental stage. There were significant differences in young (leaf 2, $P < 0.001$) and semi-mature (leaf 5, $P < 0.01$) leaves, but no significant difference ($P = 0.14$) was detected in mature leaves (leaf 8) in Portugal. However, there was a highly significant difference in mature leaves (leaf 8) between the two parents in the UK trial ($P < 0.001$). The cell area in Portugal was about one third of the corresponding cell area in the UK and about half of the corresponding cell area of the parental trial in the glasshouse, with the exception of the young leaves from *L. sativa*, where the differences among the trials were greater.

The cell area was variable among the different trials. But there was a similar trend with the leaf development in the F_9 RIL mapping population. The mean cell areas of RILs at leaf 2, 5, 8 in Portugal were respectively 348 (±136) μm^2, 729 (±179) μm^2 and 1082 (±223) μm^2, and 2939 (±545) μm^2 at leaf 8 in UK. There was a highly significant difference among 60 RILs at three different developmental stages in two trials ($P < 0.001$). The distributions of ECA in two trials are shown in Figure 4.7. The ranges of cell area at leaf 2, 5, 8 in Portugal were respectively from 122 $\mu m^2$to 857 μm^2, 372 μm^2 to 1203 μm^2, 602 μm^2 to 1626 μm^2, and 1772 μm^2 to 4177 μm^2 at leaf 8 in the UK. Some lines performed relatively consistent in the RILs in the two trials. For example, Line 3 had the smallest cell area at leaf 5 (372 μm^2) and leaf 8 (602 μm^2) in Portugal and was one of the smaller cell area groups at leaf 2 in Portugal (153 μm^2) and at leaf 8 in the UK (1856 μm^2). The lines with the biggest cell area at leaf 2, 5, 8 in Portugal were Line 74 (857 μm^2), Line 63 (1203 μm^2), and Line 51 (1626 μm^2) respectively. Comparing all RILs, these lines (74, 63, 51) all had relatively big epidermal cell area in the UK (4129 μm^2, 3519 μm^2, 2791 μm^2). Line 59 was the

line with the biggest cell area at leaf 8 in the UK (4177 μm^2) and was one of lines with the big cell area in Portugal.

The epidermal cell number per leaf (ECN) was significantly different between two parents, consistently at three different developmental stages (Figure 4.8). *L. sativa* had about 2.5 fold higher epidermal cell number per leaf compared to *L. serriola* at each leaf developmental stage. There was a similar difference between the two parents in the UK. The means of the F_9 RIL mapping population at leaf 2, 5, 8 were respectively 2.84 $x10^6$ cells per leaf, 4.10 $x10^6$ cells per leaf, 5.75 $x10^6$ cells per leaf in Portugal. The range of the F_9 RILs at leaf 8 was from 0.88 x 10^6 cells per leaf to 3.95 x 10^6 cells per leaf, with the mean value of 2.0 x 10^6 cells per leaf in the UK (Figure 4.9). The mean of the F_9 RILs in the Portugal trial had about two times higher cell number than that of the UK trial. The ECN of the mean of F_9 RILs was close to mean of two parents in Portugal, but it was close to the value of *L. sativa* in the UK.

Stomatal density (SD) decreased with the leaf development from young to mature leaf (Figure 4.10). *L. serriola* had significantly higher stomatal density than *L. sativa*, except at leaf 8 of the Portugal trial. This difference between two parents was bigger in young leaves (*L. serriola* vs *L. sativa*: 475 mm^{-2} vs 356 mm^{-2}) than that in the mature leaves (195 mm^{-2} vs 161 mm^{-2}) in Portugal. The mean of RILs for stomatal density was close to the value of *L. serriola* in both trials (Figure 4.11).

In contrast to stomatal density, stomatal index of *L. serriola* was relatively similar with the leaf development, around 20% stomata of total cell number (Figure 4.12). It varied from 6.05% in young leaves to 15.08% in mature leaves for *L. sativa*. The stomatal index of the RILs at leaf 8 in Portugal ranged from 11.63% (Line 43) to 21.11% (Line 92) and varied between 15.17% (Line 53) to 13.70% (Line 59) in the UK (Figure 4.13). *L. serriola* had significantly higher stomatal index than *L. sativa* at each developmental stage, consistently in both trials. The difference of stomatal index between two parents was significant in both trials ($P < 0.01$).

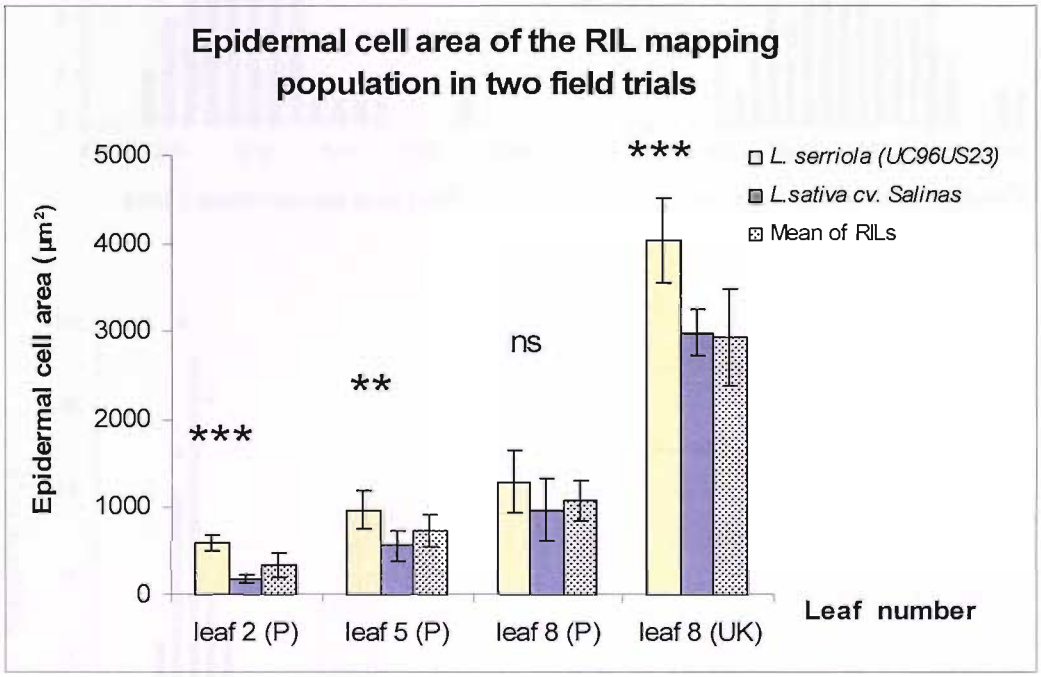


Figure 4.6 Epidermal cell area of the F_9 RIL population at different developmental ages in two field trials. The Portugal trial is noted as P and the UK trial is noted as UK. Data points are means ± SD, n=6. The results of a one-way ANOVA t-test between two parents are indicated where significant: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

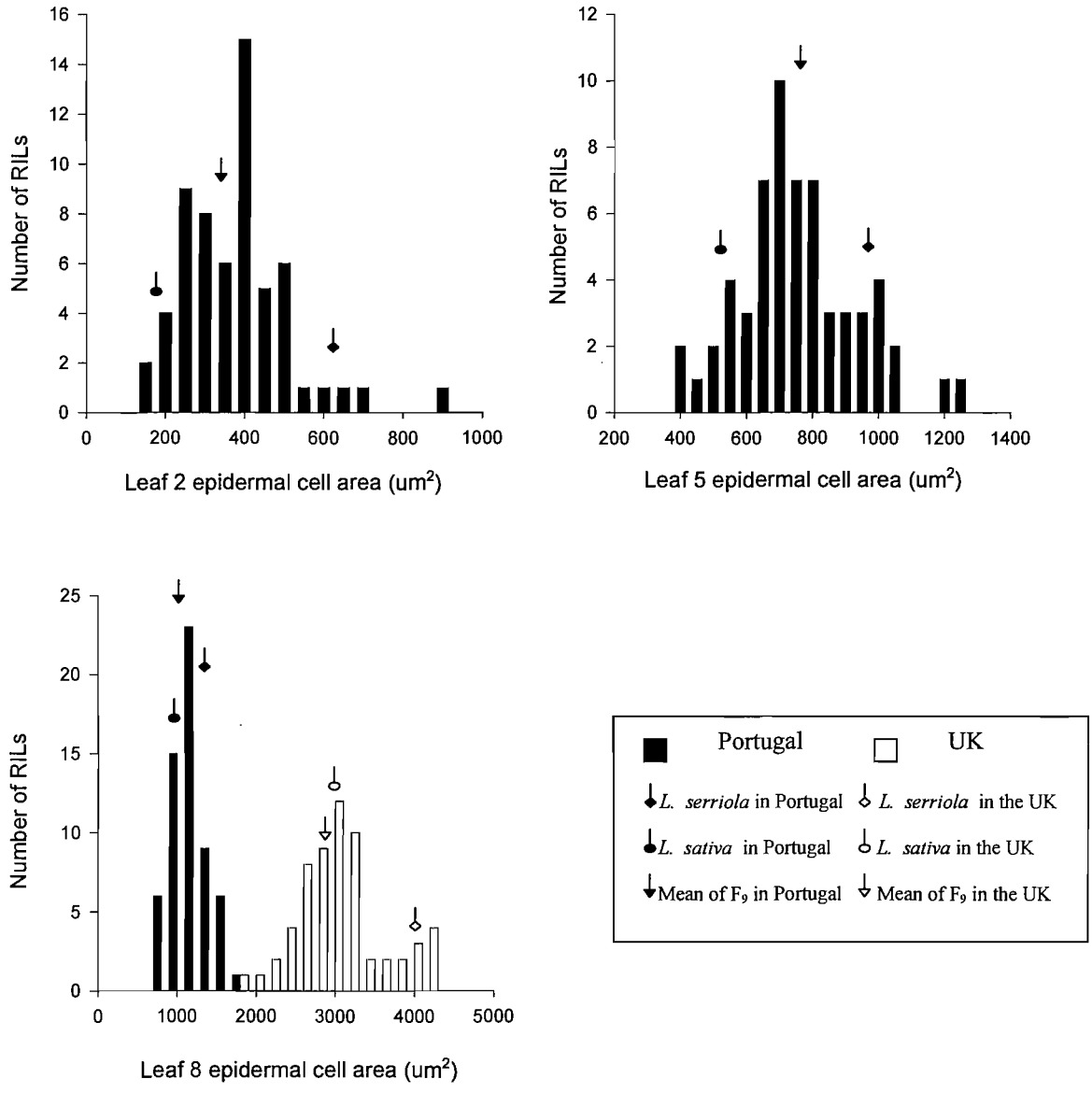


Figure 4.7 Distributions of epidermal cell area in the F_9 RI population. Leaf 2 epidermal cell area in the Portugal field trial (a), leaf 5 epidermal cell area in the Portugal field trial (b), and leaf 8 epidermal cell area in both field trials (c). The field sites are indicated as either Portugal (closed bar) or UK (open bar). The mean values of two parents *L. serriola*, *L. sativa* and of the RILs are indicated by arrows.

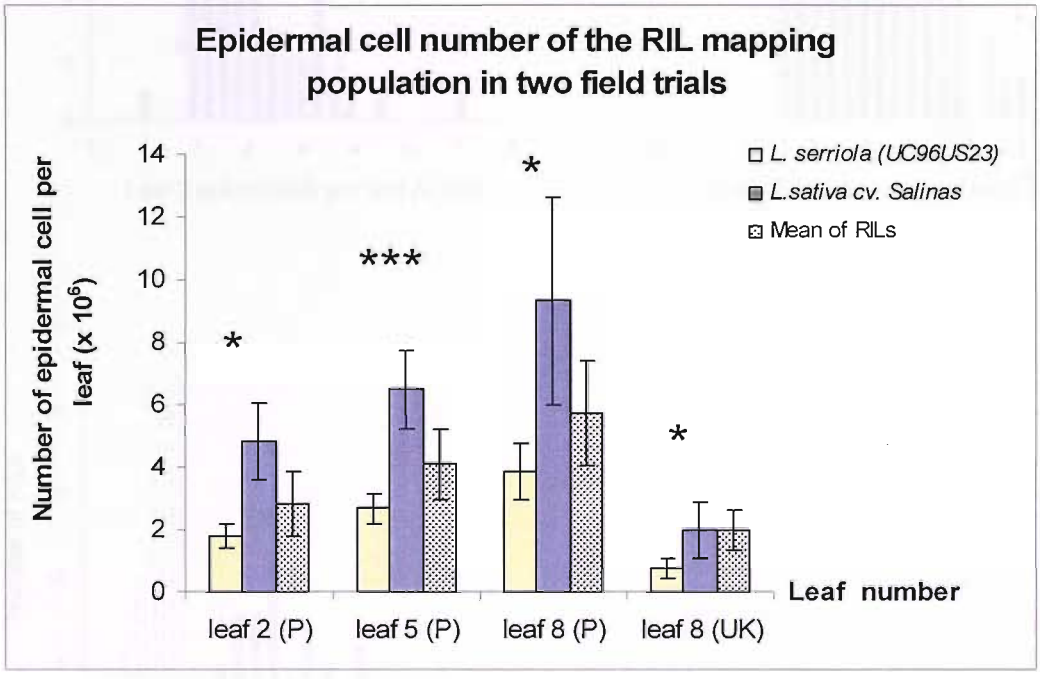


Figure 4.8 Epidermal cell number per leaf of the F_9 RIL population at different development ages in two field trials. The Portugal trial is noted as P and the UK trial is noted as UK. Data points are means ± SD, n=6. The results of a one-way ANOVA t-test between two parents are indicated where significant: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

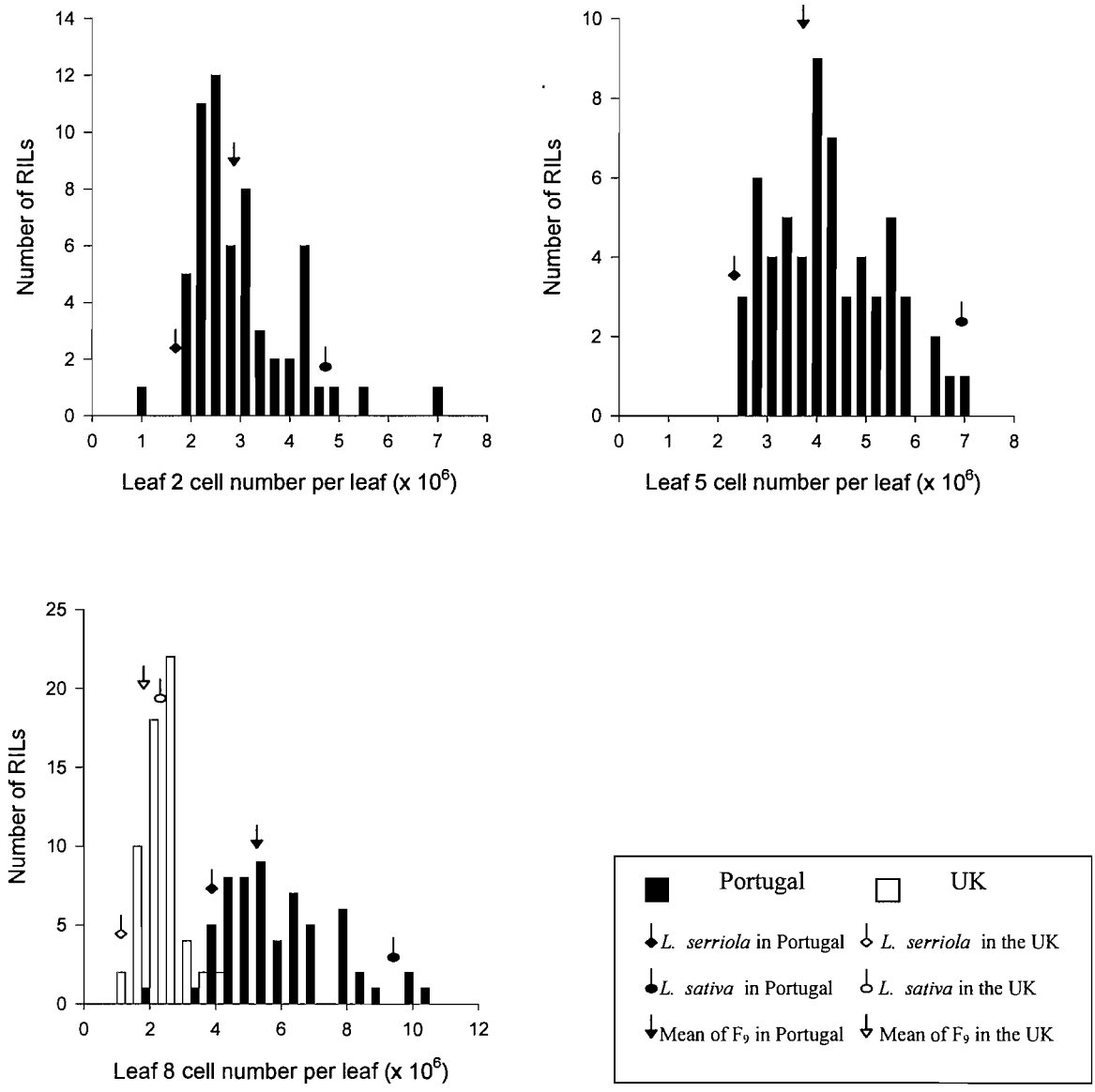


Figure 4.9 Distributions of epidermal cell number per leaf in the F_9 RIL mapping population: leaf 2 epidermal cell number per leaf in the Portugal field trial (a), leaf 5 epidermal cell number per leaf in the Portugal field trial (b), and leaf 8 epidermal cell number per leaf in both field trials (c). The field sites are indicated as either Portugal (closed bar) or UK (open bar). The mean values of two parents *L. serriola*, *L. sativa* and of the RILs are indicated by arrows.

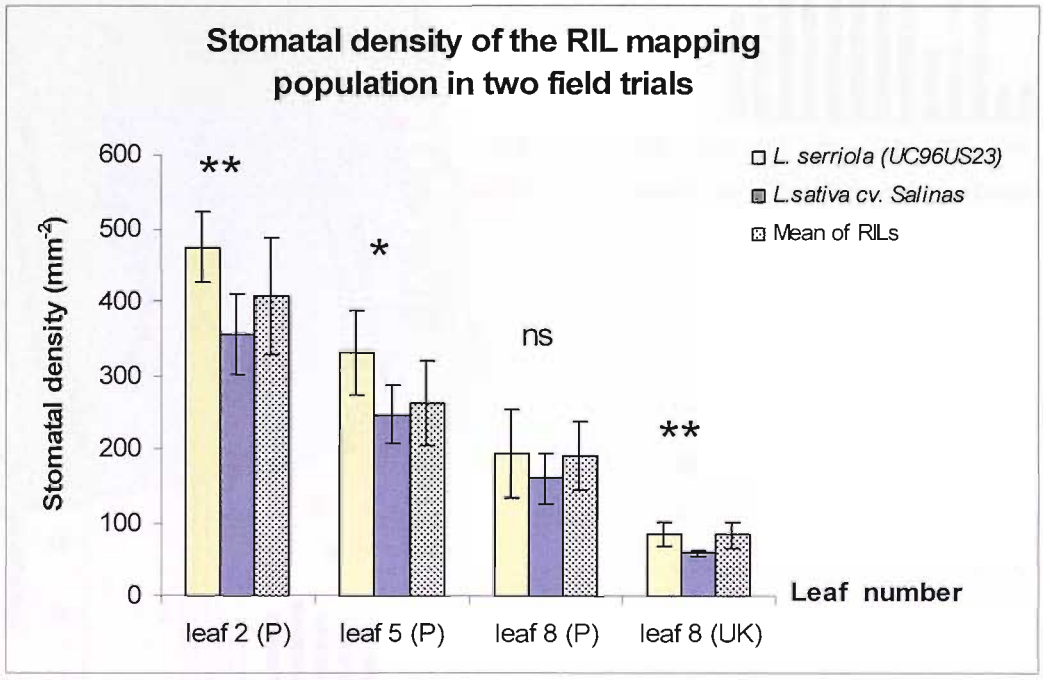


Figure 4.10 Stomatal density of the F_9 RIL population at different development ages in two field trials. The Portugal trial is noted as P and the UK trial is noted as UK. Data points are means ± SD, n=6. The results of a one-way ANOVA t-test between two parents are indicated where significant: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

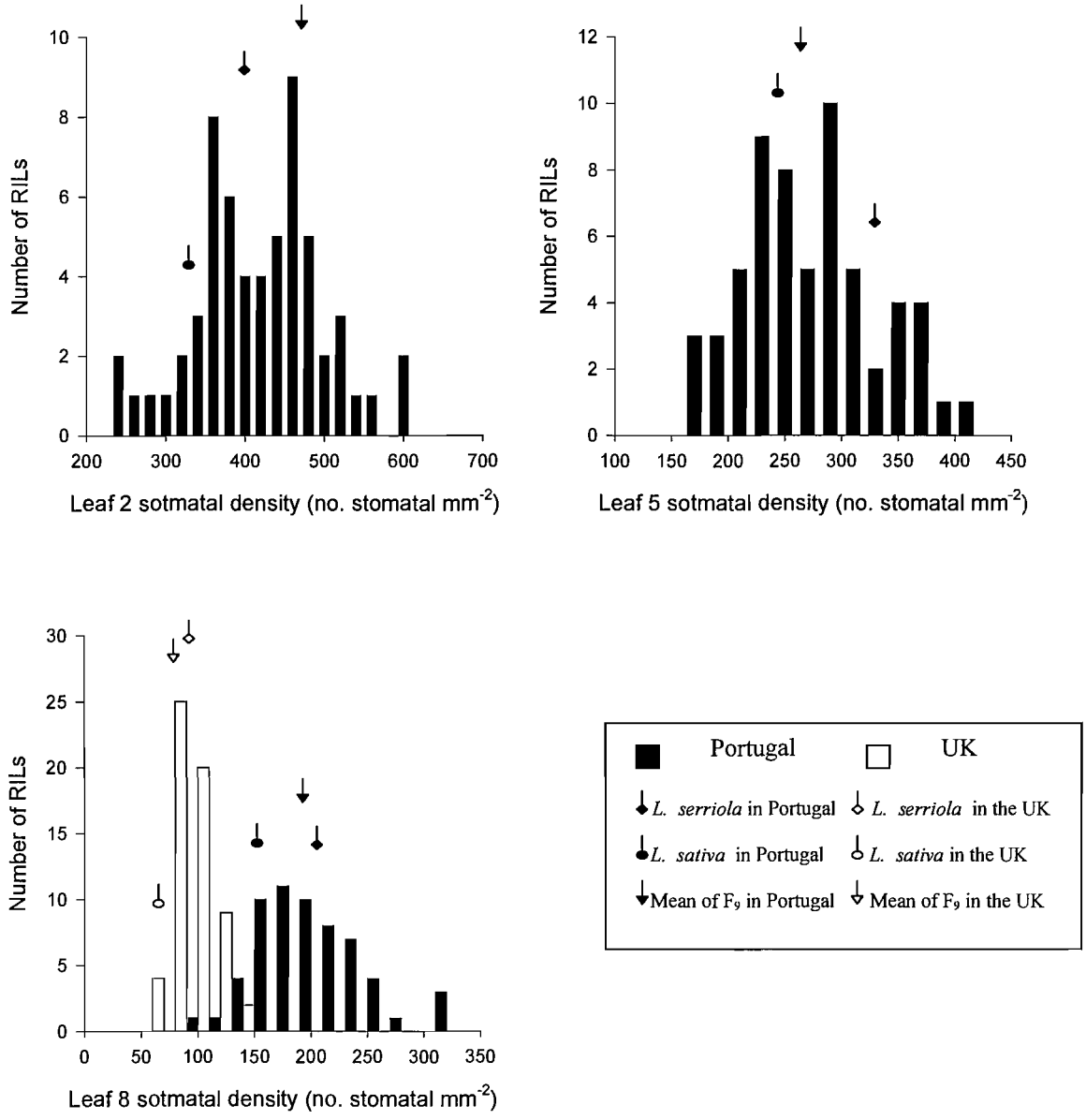


Figure 4.11 Distributions of stomatal density in the F_9 RIL population. Leaf 2 stomatal density in the Portugal field trial (a), leaf 5 stomatal density in the Portugal field trial (b), and leaf 8 stomatal density in both field trials (c). The field sites are indicated as either Portugal (closed bar) or UK (open bar). The mean values of two parents *L. serriola*, *L. sativa* and of the RILs are indicated by arrows.

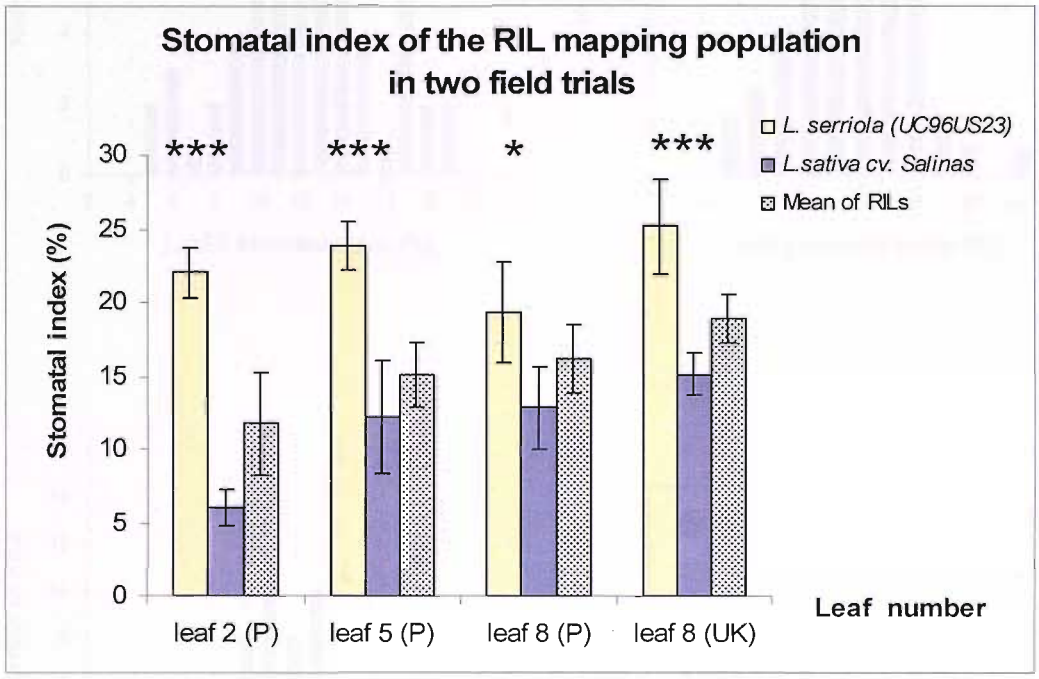


Figure 4.12 Stomatal index of the RIL population at different development ages in two field trials. The Portugal trial is noted as P and the UK trial is noted as UK. Data points are means ± SD, n=6. The results of a one-way ANOVA t-test between two parents are indicated where significant: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

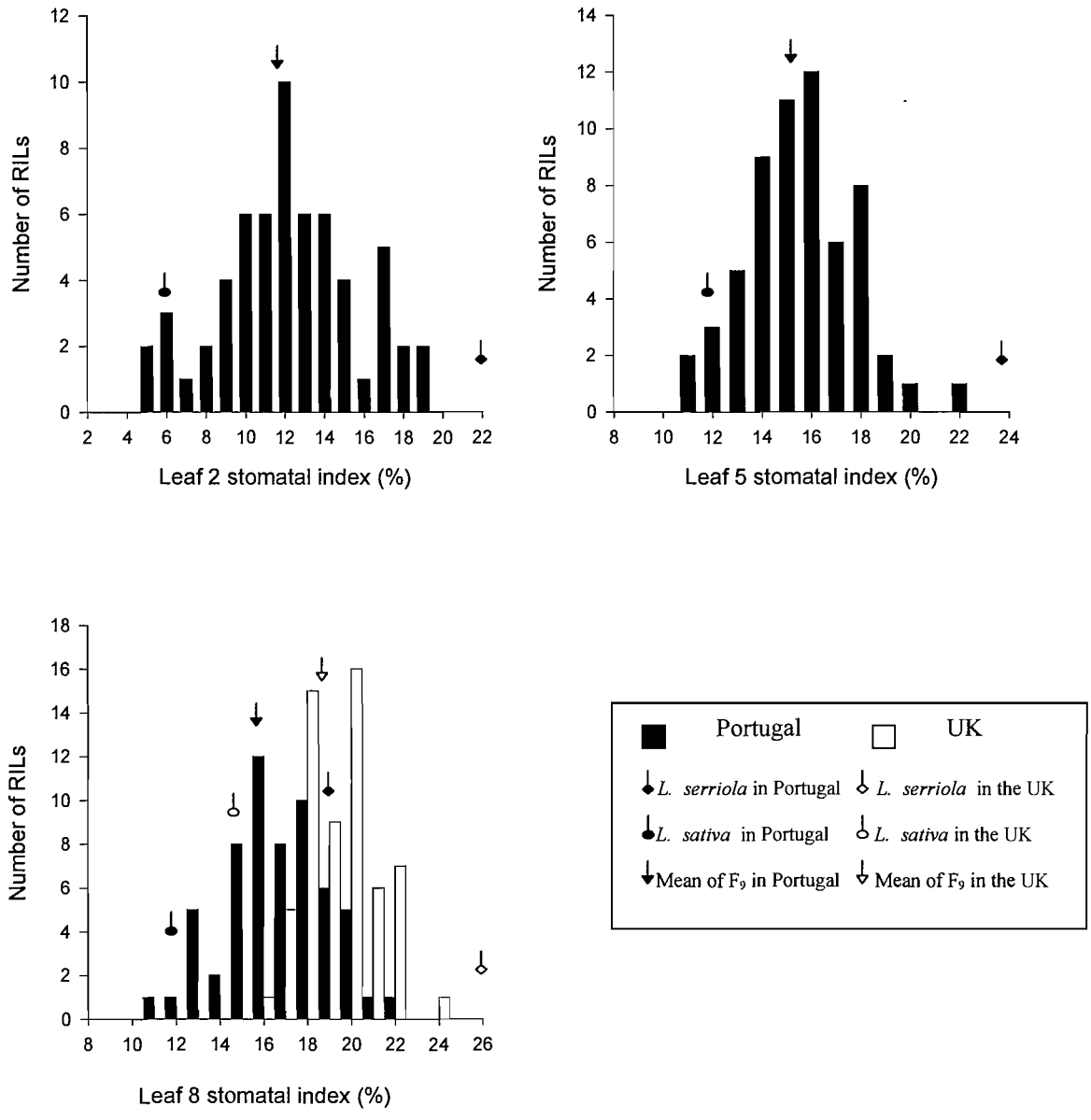


Figure 4.13 Distributions of stomatal index in the F_9 RIL mapping population. Leaf 2 stomatal index in the Portugal field trial (a), leaf 5 stomatal index in the Portugal field trial (b), and leaf 8 stomatal index in both field trials (c). The field sites are indicated as either Portugal (closed bar) or UK (open bar). The mean values of two parents *L. serriola*, *L. sativa* and of the RILs are indicated by arrows.

4.2.1.4 Chlorophyll content

L. serriola leaf had higher chlorophyll content than *L. sativa* at three different developmental stages in the Portugal trial, but it only showed significance at the 95% confidence level at young leaf ($P < 0.05$), as the standard deviation of *L. sativa* for this trait was high at semi-mature leaf and mature leaf (Figure 4.14). In Portugal, total chlorophyll content of *L. serriola* increased about 70% from young leaves (0.278 $\mu g\ mm^{-2}$) to mature leaves (0.469 $\mu g\ mm^{-2}$), while that of *L. sativa* increased more than 3 times from young leaves (0.084 $\mu g\ mm^{-2}$) to mature leaves (0.364 $\mu g\ mm^{-2}$). The mean of F_9 RILs increased 150% with the leaf development (Figure 4.15). In the UK, no significant difference between two parents (SER vs SAT: 0.200 $\mu g\ mm^{-2}$ vs 0.217 $\mu g\ mm^{-2}$, $P = 0.10 > 0.05$).

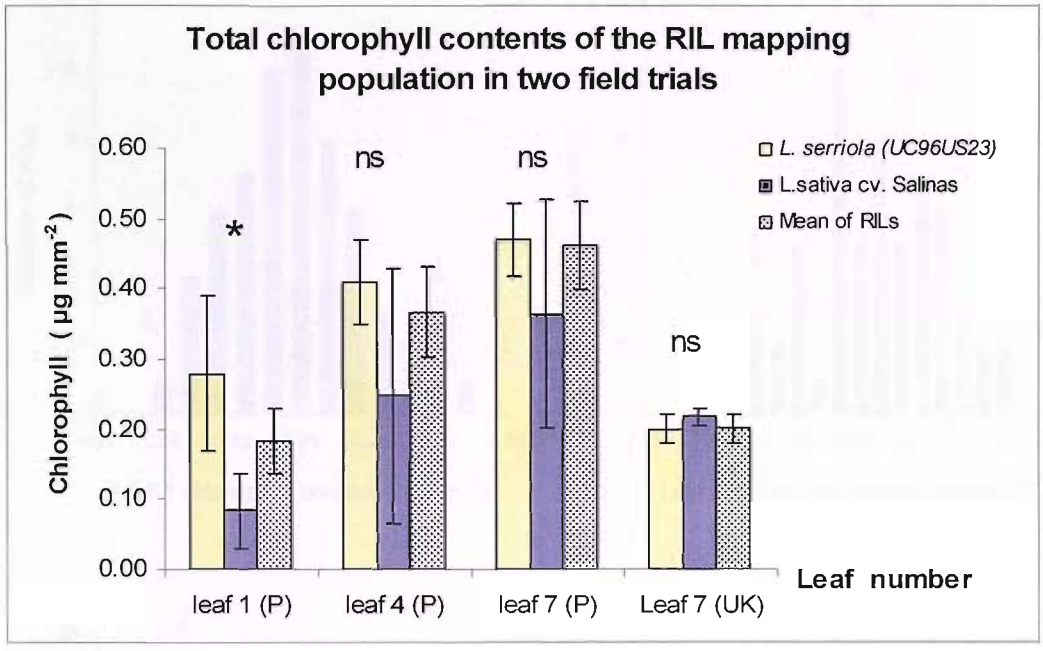


Figure 4.14 Total chlorophyll contents of the F_9 RIL population at different development ages in two field trials. The Portugal trial is noted as P and the UK trial is noted as UK. Data points are means ± SD. The results of a one-way ANOVA t-test between two parents are indicated where significant: * $p<0.05$, ns = not significant.

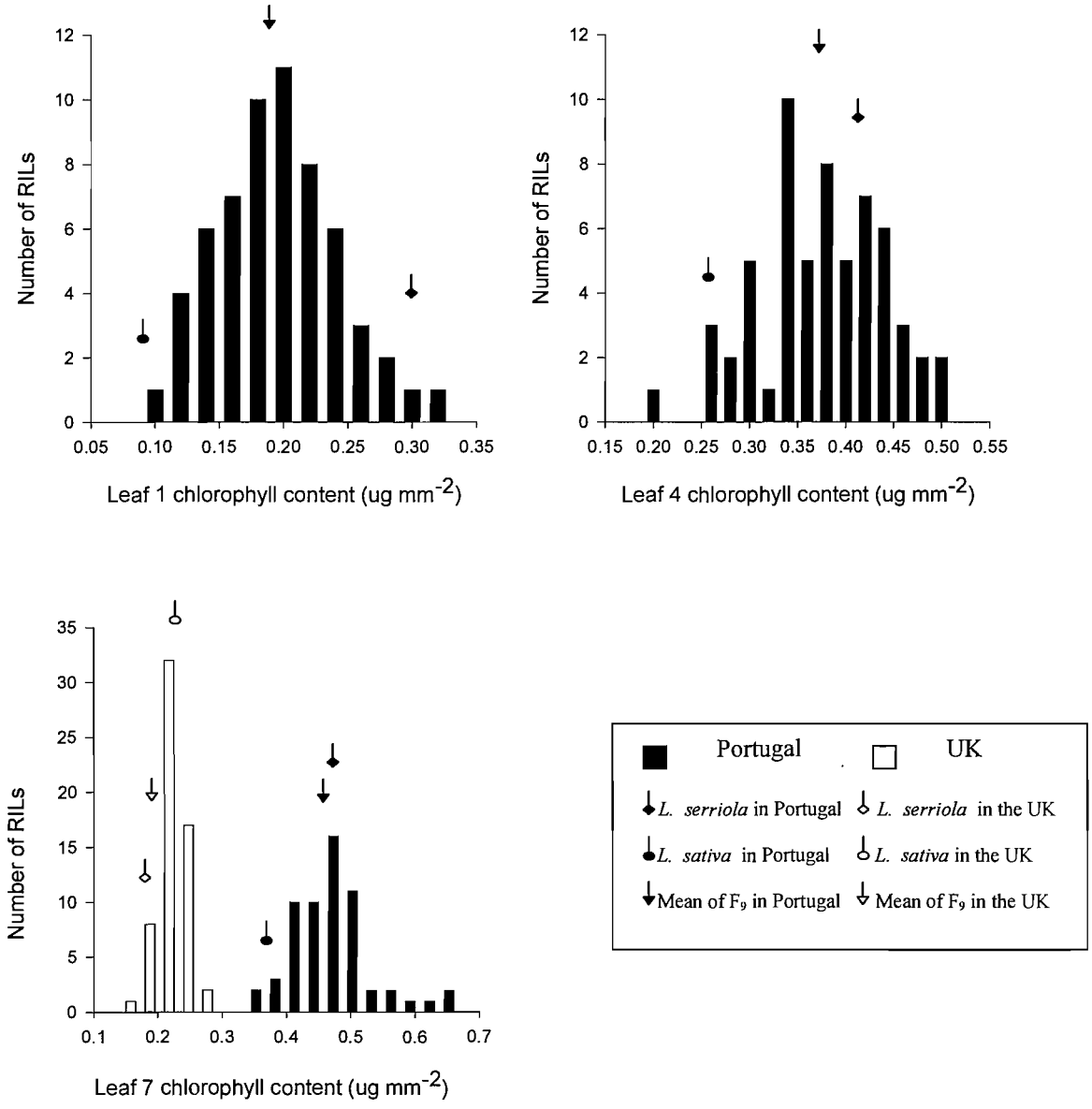


Figure 4.15 Distribution of chlorophyll content in the F_9 RIL population. Leaf 2 chlorophyll content in the Portugal field trial (a), leaf 5 chlorophyll content in the Portugal field trial (b), and leaf 8 chlorophyll content in both field trials (c). The field sites are indicated as either Portugal (closed bar) or UK (open bar). The mean values of two parents *L. serriola*, *L. sativa* and of the RILs are indicated by arrows.

4.2.1.5 Leaf fresh weight, dry weight, thickness and specific leaf area

There was a significant difference in leaf fresh weight (FW) and leaf dry weight (DW) in mature leaves between two parents in both trials ($P < 0.05$), but no difference in young leaves in the Portugal trial (Figure 4.16a and 4.16b). For fresh weight, a significant difference was found between the two parents in both field trials, especially in parent *L. serriola*, but no significant difference was found in mean of RILs between two trials (Portugal vs UK: 2.19 mg vs 2.08 mg). *L. serriola* had significantly higher fresh weight than *L. sativa* in the semi-mature leaves, but no significant difference for dry weight in the same age leaf in Portugal. The dry weight of mean of RILs in the Portugal (0.26 mg) was 50% higher than that in the UK (0.17 mg).

Specific leaf area (SLA) did not change much with the leaf development and only one significant difference was found between two parents in the semi-mature leaves (Figure 4.16c). Mean of the RILs for SLA in Portugal (30.66 mm mg^{-1}) was significantly higher than that in the UK (19.76 mm mg^{-1}) ($P< 0.05$).

There were significant differences for dry weight as a percentage of fresh weight (DWP) between two parents, and DWP did not change with the leaf development in the Portugal trial (Figure 4.16d). DWP of mean of RILs in Portugal (12.11%) was significantly higher than that in the UK (8.40%). Distributions of these traits for leaf 7 for the F9 RIL mapping population in two field trials are shown in Figure 4.17.

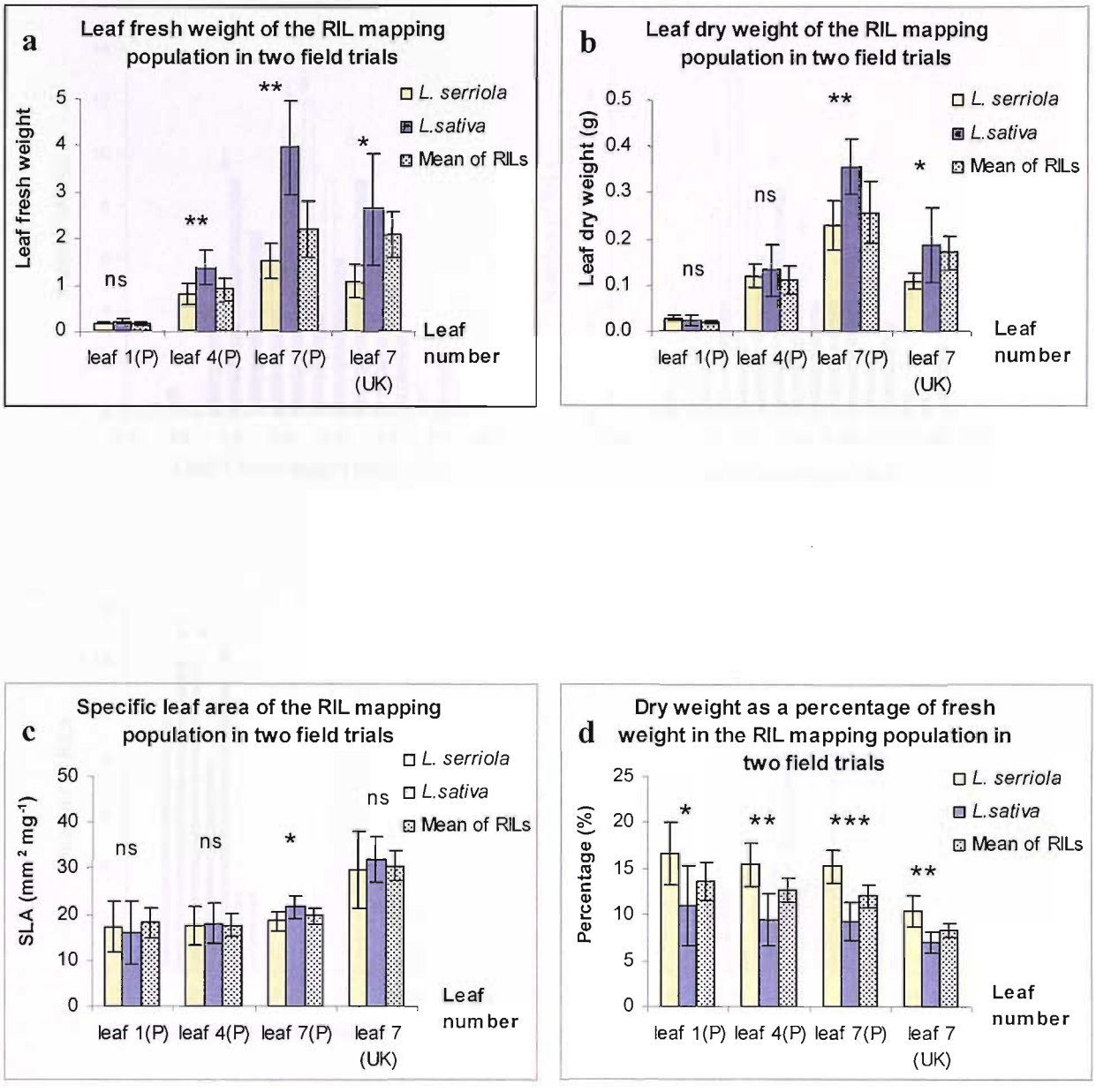


Figure 4.16 Leaf fresh weight (a), dry weight (b), specific leaf area (c) and dry weight as a percentage of fresh weight (d) of the F_9 RIL population at different development ages in two field trials. (The Portugal trial is noted as P and the UK trial is noted as UK. Data points are means ± SD, n=6. The results of a one-way ANOVA t-test between two parents are indicated where significant: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant).

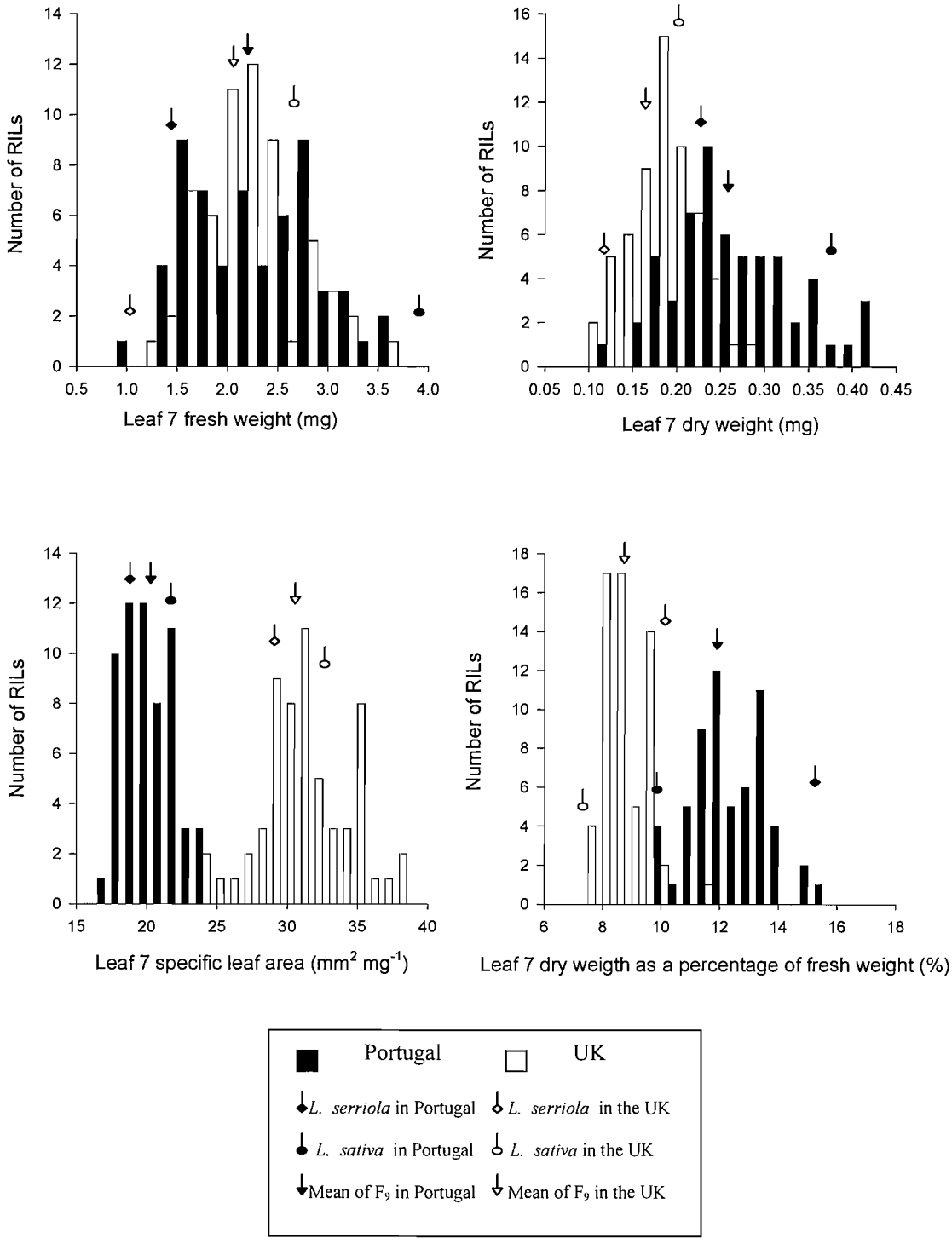


Figure 4.17 Distributions of Leaf 7 fresh weight (a), dry weight (b), specific leaf area (c) and dry weight as a percentage of fresh weight (d) of the F_9 RIL population in two field trials.

4.2.1.6 Osmolality

Only one leaf developmental stage (leaf 6) was assessed for cell sap osmolality (OSM) in both trials. The mean OSM of RILs in Portugal (464 mmol kg^{-1}) was significantly higher than that in the UK (412 mmol kg^{-1}) (Figure 4.18a, $P < 0.001$). But there was no significant difference in OSM between two parents in each trial. OSM in Portugal ranged from 389 mmol kg^{-1} to 625 mmol kg^{-1}, while it was variable, from 367 mmol kg^{-1} to 469 mmol kg^{-1} in the UK. The distribution of OSM in the UK was shown to be a normal distribution; however, the genotype variation of osmolality among the RILs was not significant in the UK field trial ($P > 0.05$) (Figure 4.18b). It was a clear normal distribution in Portugal, permitting the further QTL analysis.

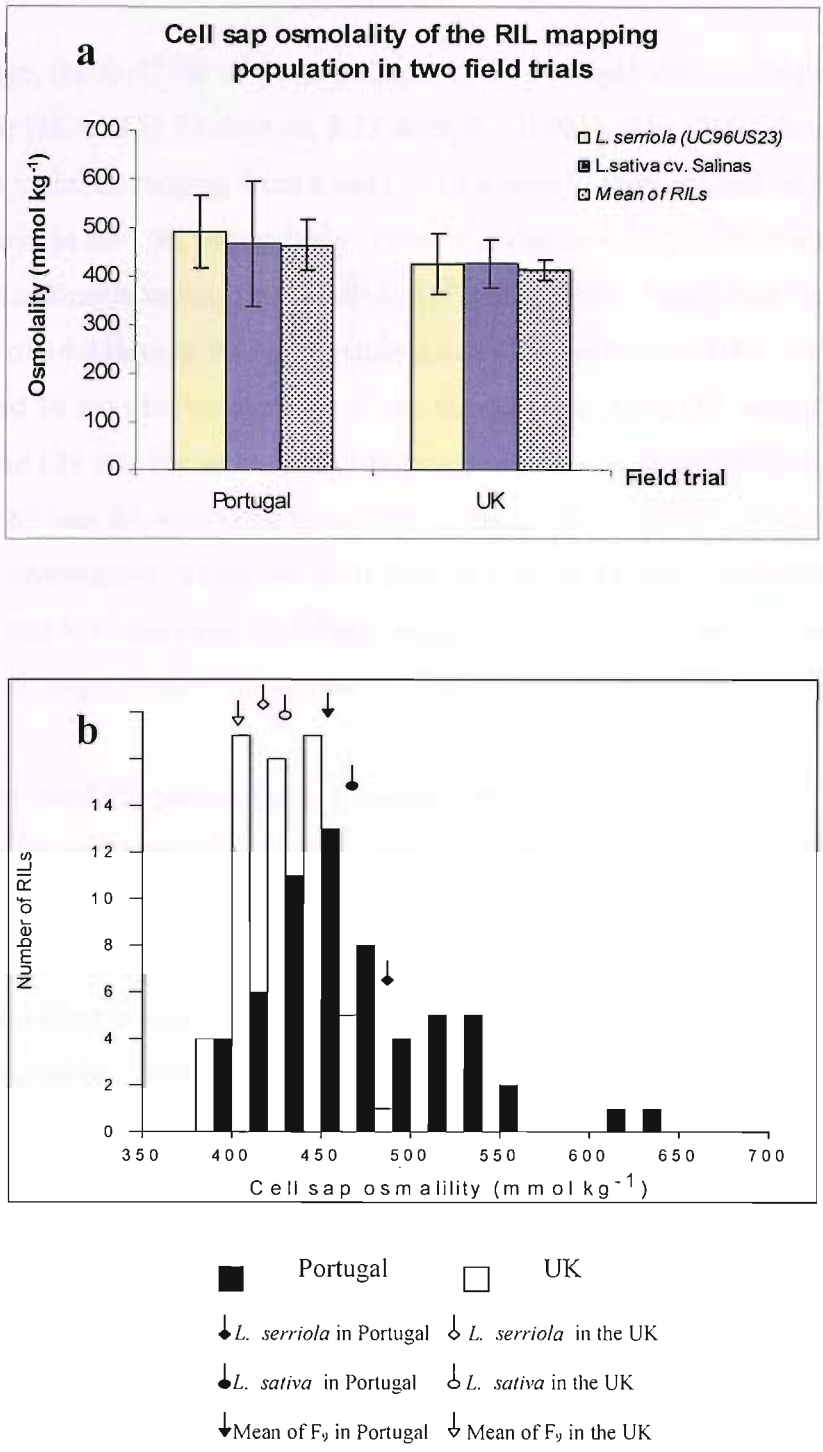


Figure 4.18 Cell sap osmolality (a) and its distribution (b) of the F_9 RIL mapping population in two field trials. Data points are means ± SD, n=6. The result of a one-way ANOVA t-test indicates no significant difference for the cell sap osmolality between two parents.

4.2.1.7 Shelf life

On average, the shelf life of the population in the Portugal trial was higher than that of the UK trial (9.73 days vs. 8.37 days, $P < 0.001$). The shelf life of F_9 RIL lines was variable, ranging from 6 days to 14.4 days in Portugal and from 5 days to 13.4 days in the UK, respectively (Table 4.1, Figure 4.19a). There was a normal distribution among the population (Figure 4.19b). Line 1 had the longest shelf life of 14.4 days in Portugal, while it had 12.8 days in the UK. Line 112 only lasted 10 days in Portugal, but it was the best line in the UK, lasting 13.4 days. Line 121 was the worst line in Portugal in terms of the shelf life (6 days) and Line 89 was the worst line in the UK (5 days). The shelf life of two parents was also investigated, whilst the shelf lives of *L. serriola* and *L. sativa* in Portugal was 9.33 days and 12.6 days, respectively, and they were much shorter in UK trial, respectively 7.2 days and 9.2 days.

During the shelf life period, the leaf material of each line was assessed for good condition (score 1) or bad condition (score 0) everyday. The total number of lines in good condition each day is shown in Figure 4.20. Due to the lack of sufficient leaf samples for some lines, only 56 lines were assessed in the Portugal trial and 60 lines in total were assessed in the UK trial. The truncated binary data sets were used for further QTL analysis.

All the trait measurements of the selected F_9 mapping population at different developmental stages in Portugal are summarized in Table 4.2. The comparison of the trait measurements at the same developmental stage between the Portugal and the UK are listed Table 4.3. Analysis of variance for RILs showed significant genotype variation for all the traits measured except the osmolality in the UK trial.

Table 4.1 The records of shelf life experiments of the F_9 RIL mapping population in the Portugal and the UK field trials.

RILs	Portugal trial		UK trial	
	Replicates	shelf life (days)	Replicates	shelf life (days)
1	5	14.4	5	12.8
2	5	10.8	5	8.6
3	5	10.6	5	9.0
4	5	8.6	5	7.6
5	5	12.6	5	12.4
9	5	7.6	5	8.6
11	5	7.8	5	9.2
12	5	12.0	5	12.4
13	4	7.25	5	6.2
14	0		5	7.2
15	5	12.4	5	12.0
19	1	8.0	5	5.6
20	5	12.0	5	8.2
21	1	10.0	5	6.6
22	5	7.2	5	6.8
31	5	10.6	5	8.4
32	5	8.2	5	5.6
34	0		5	7.2
40	5	8.8	5	7.6
41	5	9.2	5	7.0
43	5	10.6	5	10.6
47	2	9.0	5	7.4
51	5	11.3	5	6.2
53	5	9.0	5	7.0
55	5	9.8	5	8.4
58	0		5	8.6
59	0		1	6.0
61	5	11.2	5	9.0
63	4	8.0	5	6.6
68	5	11.4	5	10.0
70	5	10.6	5	8.2
71	5	9.8	5	8.0
74	5	13.4	4	11.4
75	5	11.2	5	8.8
76	5	10.2	5	8.6
78	5	8.2	5	9.0
79	5	11.8	5	9.6
80	5	10.4	5	8.8
83	3	8.7	5	5.0
85	5	9.4	5	8.4
89	5	8.4	5	5.0
90	5	10.0	5	10.6
91	3	10.0	4	8.5
92	2	8.5	5	7.2
93	3	8.3	5	5.8
94	5	8.6	5	8.0
96	5	10.8	5	10.2
98	5	10.4	5	6.0
99	5	10.8	5	11.2
104	5	12.4	5	9.0
105	4	7.5	5	5.4
106	4	8.5	5	7.0
107	2	7.0	5	11.4
109	4	9.5	5	8.2
111	5	10.8	5	10.2
112	5	10.0	5	13.4
114	5	10.8	5	9.6
121	1	6.0	5	10.2
122	5	8.4	5	6.4
123	5	9.4	5	7.8
L. serriola	3	9.3	5	7.2
L. sativa	5	12.6	5	9.2

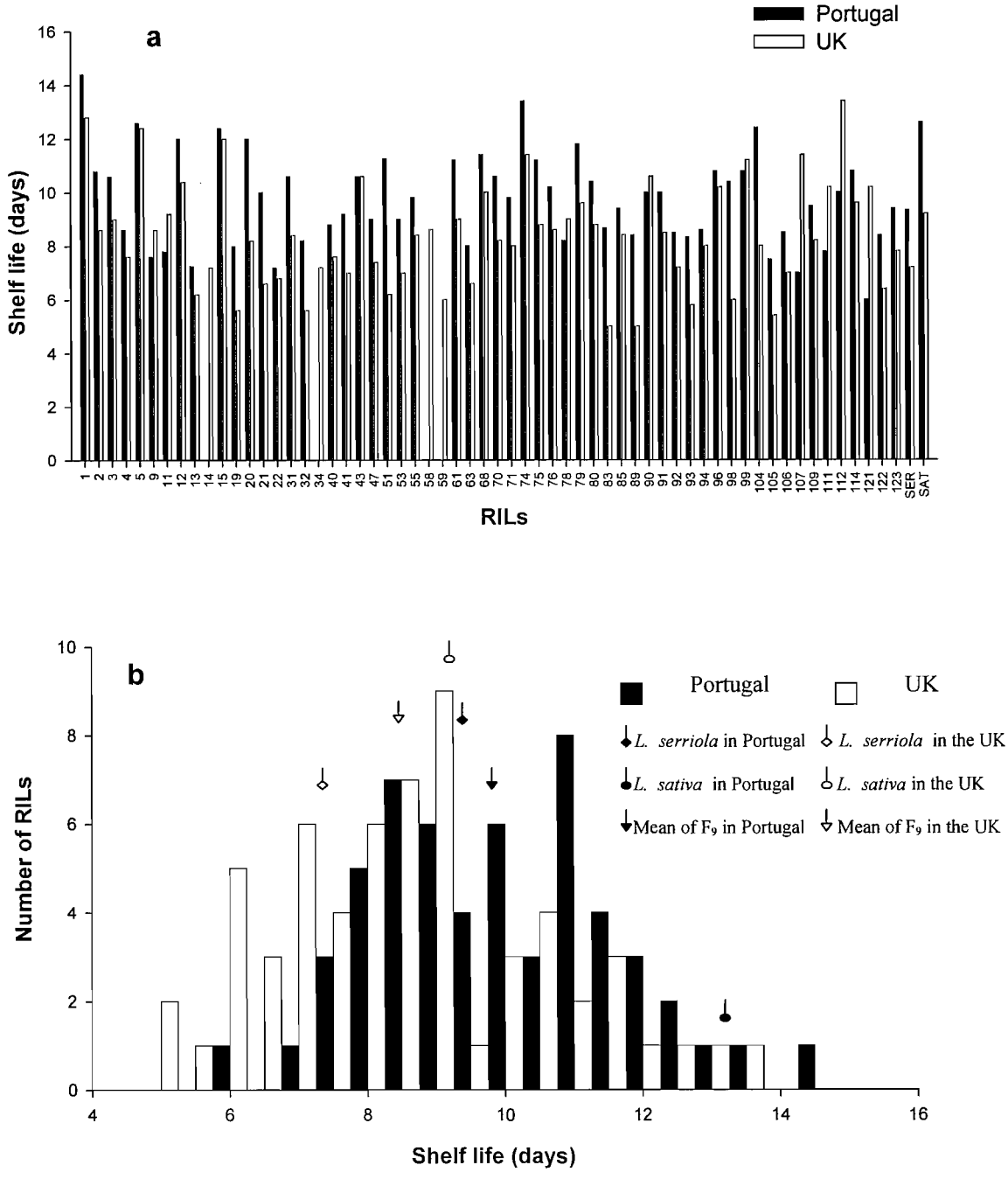


Figure 4.19 Shelf life (a) and its distribution (b) of the F_9 RIL mapping population in two field trials: Portugal (closed bar) and UK (open bar). Average of five replicates of most lines was kept at 7°C fridge and shelf life was determined through visual assessment. When breakdown, bruising or damage was seen in the pack, the bag was rejected.

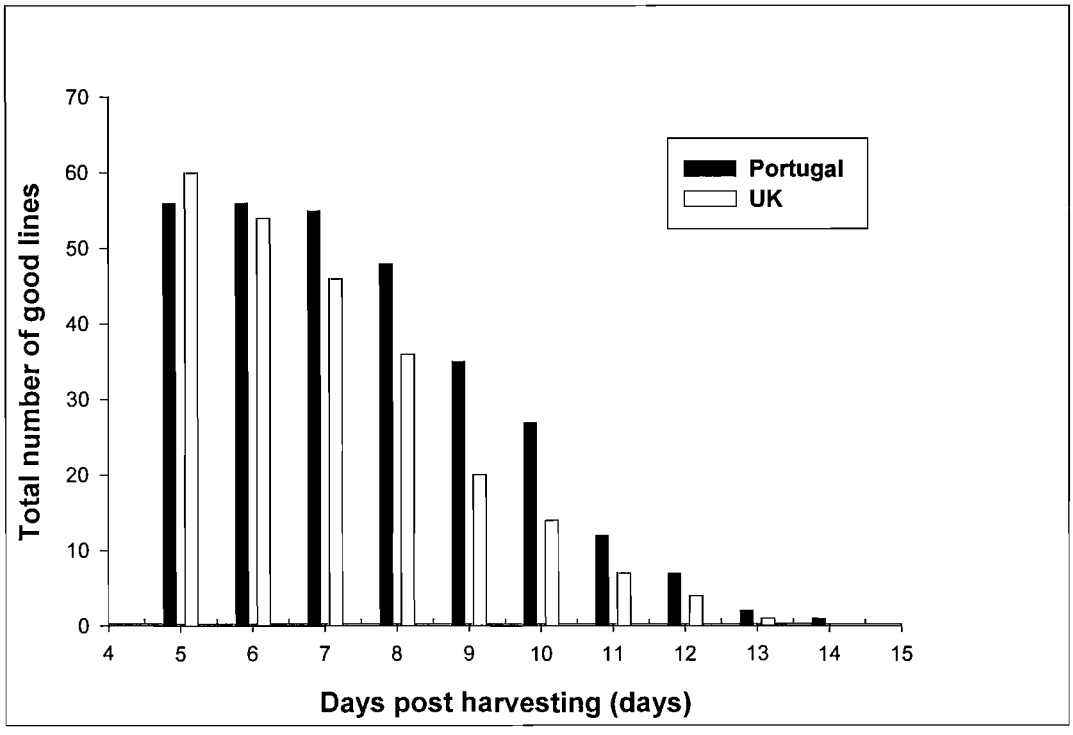


Figure 4.20 Post-harvest rejection of the F_9 mapping population during the shelf life period in two field trials.

Table 4.2 Mean and range values for measured traits of the F_9 mapping population and parents at different leaf developmental stages in the Portugal trial.

Trait[a]	Leaf no.	Parents			RILs		
		L. serriola	*L. sativa*	Mean	Minimum	Maximu	Mean
LA (mm^2)	1	613	474	544	334	646	479
	2	1053	869	961	561	1172	838
	3	1632	1531	1581	859	2238	1370
	4	2267	2358	2313	1162	3606	2040
	5	2602	3548	3075	1395	4492	2776
	6	3787	5858	4823	2180	6450	4218
	7	4471	7870	6171	2366	7826	5112
	8	4817	8254	6536	2599	8379	5734
FW (mg)	1	0.168	0.205	0.187	0.080	0.214	0.143
	4	0.795	1.381	1.088	0.528	1.712	0.897
	7	1.523	3.973	2.748	0.990	3.550	2.193
DW (mg)	1	0.028	0.023	0.026	0.012	0.028	0.019
	4	0.120	0.132	0.126	0.062	0.215	0.110
	7	0.229	0.356	0.293	0.117	0.413	0.257
DWP (%)	1	16.62	11.05	13.84	9.36	18.93	13.62
	4	15.44	9.48	12.46	10.01	15.63	12.63
	7	15.25	9.32	12.28	9.74	15.05	12.11
SLA (mm^2 mg^{-1})	1	17.26	16.02	16.64	7.29	29.21	18.28
	4	17.51	17.98	17.74	12.56	24.30	17.71
	7	18.64	21.72	20.18	16.94	23.21	19.76
CHL (μg mm^{-2})	1	0.278	0.084	0.181	0.097	0.317	0.183
	4	0.410	0.247	0.329	0.186	0.497	0.367
	7	0.469	0.364	0.416	0.332	0.653	0.462
ECA (μm^2)	2	601	182	392	121	857	348
	5	973	568	770	372	1203	729
	8	1294	970	1132	602	1626	1082
ECN (x 10^6 cell per leaf)	2	1.80	4.82	3.31	0.99	6.86	2.84
	5	2.68	6.49	4.58	2.23	6.76	4.10
	8	3.87	9.32	6.60	1.77	10.04	5.75
SD (no. stomata mm^{-2})	2	475	355	415	225	590	406
	5	332	248	290	155	392	263
	8	195	161	178	87	315	191
SI (%)	2	22.03	6.05	14.02	4.81	18.97	11.71
	5	23.82	12.21	18.02	10.09	21.41	15.06
	8	19.32	12.84	16.08	10.84	21.11	16.16

Note: [a]Trait abbreviation: LA: leaf area (mm^2); FW: leaf fresh weight (mg); DW: leaf dry weight (mg); DWP: leaf dry weight as a percentage of fresh weight (%); SLA: specific leaf area; CHL: chlorophyll content (μg mm^{-2}); ECA: epidermal cell area (μm^2); ECN: epidermal cell number per leaf (x 10^6); SD: stomata density (no. stomata mm^{-2}); SI: stomata index (%).

Table 4.3 Mean and range values for measured traits of the selected F_9 RIL mapping population and parents in the Portugal and the UK field trials.

Trait[a]	Field site	Parents			RILs		
		L. serriola	*L. sativa*	Mean	Minimum	Maximum	Mean
LA (mm^2)	Portugal	3677	5878	4778	2001	5956	4108
(mean of Leaf 3,6,7,8)	UK	3016	4954	3986	2317	5910	4283
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
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
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
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
CHL (ug 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
ECA (μm^2)	Portugal	1294	970	1132	602	1627	1082
	UK	4034	2988	3511	1772	4177	2939
ECN (x 10^6 cell 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
SD (no. stomata mm^{-2})	Portugal	195.23	160.72	177.98	87.50	315.31	191.3
	UK	84.53	59.52	72.02	53.57	134.52	84.45
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
OSM (mmol kg^{-1})	Portugal	492	467	480	389	625	464
	UK	423	425	424	367	469	412
SL_H (days)	Portugal	9.33	12.60	10.97	6.0	14.4	9.73
	UK	7.2	9.2	8.2	5.0	13.4	8.38
AGR ($mm^2\ h^{-1}$)	Portugal	-	-	-	-	-	-
	UK	5.79	11.36	8.58	3.61	17.79	9.43
RGR ($x10^{-3}\ mm^2\ mm^{-2}\ h^{-1}$)	Portugal	-	-	-	-	-	-
	UK	8.55	16.02	12.29	4.84	14.09	9.12

Note: [a]Trait abbreviation: LA: leaf area (mm^2); FW: leaf fresh weight (mg); DW: leaf dry weight (mg); DWP: leaf dry weight as a percentage of fresh weight (%); SLA: specific leaf area; CHL: chlorophyll content ($\mu g\ mm^{-2}$); ECA: epidermal cell area (μm^2); ECN: epidermal cell number per leaf (x 10^6); SD: stomata density (no. stomata mm^{-2}); SI: stomata index (%). OSM: osmolality (mmol kg^{-1}); SL_H: shelf life, accounting form harvest to be rejected (days); AGR: absolute growth rate ($mm^2\ h^{-1}$) and RGR: relative growth rate ($x10^{-3}\ mm^2\ mm^{-2}\ h^{-1}$).

4.2.2 Correlation among the traits

Correlations between the traits in both field trials were calculated using Pearson's correlation coefficient analysis (Table 4.4-4.6). In Portugal, LA had a highly significant positive correlation with FW and DW and a lower significant correlation with ECA. LA had positive significant correlation with ECN in semi-mature and mature leaves and the correlation value became higher with the leaf age ($P < 0.001$). FW was positively correlated with DW and negatively correlated with DWP. SD was negatively correlated with ECA and positively correlated with ECN, while SI showed a strong positive relationship with ECA and a negative one with ECN in the young leaves but not in the mature leaves. No significant relationship between SL with the traits assessed in the young leaves was detected.

Comparing the leaves with similar maturity (mature leaves) in two field trials, there is a consistent correlation relationship between most pairs of traits in the two field trials (Table 4.6). For example, LA had a highly significant positive phenotypic correlation with FW (0.87; 0.86, 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, SL showed a highly significant positive correlation with LA (0.41), FW (0.36), DW (0.36) ($P < 0.01$), and ECN (0.28) ($P < 0.05$), and significant negative correlation with CHL (-0.29) and SI (-0.27) ($P < 0.05$). But no significant correlations between SL with the above traits were detected in the Portugal trial. Only one trait OSM was found to have significant correlation with SL in the Portugal trial, but it was not detected in the UK trial.

Table 4.4 Pearson's Correlation coefficient of the traits assessed in the young leaves of the F_9 RIL population in the Portugal field trial.

Trait[a]	LA-1	LA-2	LA-3	FW-1	DW-1	DWP-1	SLA-1	CHL-1	ECA-2	ECN-2	SD-2	SI-2
LA-1	1											
LA-2	0.66***[b]	1										
LA-3	0.44***	0.79***	1									
FW-1	0.56***	0.35**	0.23	1								
DW-1	0.59***	0.46***	0.28*	0.73***	1							
DWP-1	0.12	0.22	0.07	-0.17	0.51***	1						
SLA-1	0.24	0.11	0.15	0.28*	-0.55***	-0.53***	1					
CHL-1	0.23	0.13	0.07	-0.16	0.16	0.42**	-0.03	1				
ECA-2	0.53***	0.58***	0.46***	0.06	0.25*	0.31*	-0.78***	0.36**	1			
ECN-2	-0.16	-0.20	-0.16	0.22	0.02	-0.27*	-0.07	-0.35**	-0.78***	1		
SD-2	-0.08	-0.18	-0.24	-0.01	0.14	0.22	-0.31*	0.13	-0.32*	0.16	1	
SI-2	0.49***	0.52***	0.34**	0.41*	0.31**	0.42**	0.001	0.39**	0.82***	-0.75***	0.23	1
SL	-0.003	-0.001	0.09	0.17	0.08	-0.16	0.06	-0.21	0.06	0.16	-0.17	-0.10

Note: [a]Trait abbreviation: LA: leaf area (mm^2); 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 ($x\ 10^6$); SD: stomata density (no. stomata mm^{-2}); SI: stomata index (%); SL: shelf-life period, count from harvesting to the day before being rejected (days). The number indicates the leaf developmental stage of the trait assessed.

[b] * Correlation is significant at the 0.05 level (2-tailed); **, correlation is significant at the 0.01 level; and ***, correlation is significant at the 0.001 level.

Table 4.5 Pearson's Correlation coefficient of the traits assessed in the semi-mature leaves of the F_9 mapping population in the Portugal field trial.

Trait[a]	LA-4	LA-5	LA-6	FW-4	DW-4	DWP-4	SLA-4	CHL-4	ECA-5	ECN-5	SD-5	SI-5	OSM-6
LA-4	1												
LA-5	0.91***[b]	1											
LA-6	0.80***	0.92***	1										
FW-4	0.73***	0.80***	0.87***	1									
DW-4	0.82***	0.82***	0.83***	0.92***	1								
DWP-4	0.14	-0.05	-0.22	-0.26*	0.10	1							
SLA-4	0.21	0.10	-0.08	-0.34*	-0.34**	-0.04	1						
CHL-4	0.18	0.15	0.05	0.49	0.17	0.28*	0.001	1					
ECA-5	0.37**	0.34**	0.13	0.08	0.20	0.28*	0.25	0.09	1				
ECN-5	0.41**	0.49***	0.58***	0.59***	0.48***	-0.34**	-0.13	-0.01	-0.59***	1			
SD-5	-0.35**	-0.43***	-0.38**	-0.23	-0.31*	-0.14	-0.11	-0.03	-0.71***	0.29*	1		
SI-5	0.14	0.01	-0.15	-0.10	-0.03	0.24	0.16	0.14	0.48***	-0.44***	0.22	1	
OSM-6	0.11	-0.06	-0.14	-0.13	0.14	0.75***	-0.14	0.26*	0.25	-0.22	-0.13	0.131	1
SL	0.08	0.10	0.10	0.003	0.04	0.04	0.07	-0.06	0.17	0.02	-0.09	-0.004	0.27*

Note: see Table 4.4.

Table 4.6 Pearson's Correlation coefficient of the traits assessed in the mature leaves of the mapping population in two field trials.

Field site	Trait[a]	LA-7	LA-8	FW-7	DW-7	DWP-7	SLA-7	CHL-7	ECA-8	ECN-8	SD-8	SI-8
Portugal	LA-7	1										
UK		1										
Portugal	LA-8	0.95***[b]	1									
UK		0.95***	1									
Portugal	FW-7	0.89***	0.85***	1								
UK		0.87***	0.83***	1								
Portugal	DW-7	0.92***	0.83***	0.90***	1							
UK		0.87***	0.85***	0.93***	1							
Portugal	DWP-7	-0.17	-0.25*	-0.46***	-0.06	1						
UK		-0.19	-0.16	-0.41**	-0.07	1						
Portugal	SLA-7	-0.06	0.03	-0.27*	-0.41**	-0.22	1					
UK		0.07	0.04	-0.26*	-0.40**	-0.29*	1					
Portugal	CHL-7	-0.16	-0.23	-0.14	-0.02	0.26*	-0.35**	1				
UK		-0.30*	-0.37**	-0.02	-0.08	-0.15	-0.41**	1				
Portugal	ECA-8	0.014	-0.06	-0.12	0.01	0.32	-0.04	-0.09	1			
UK		-0.08	-0.19	-0.16	-0.18	0.07	0.21	-0.28*	1			
Portugal	ECN-8	0.61***	0.69***	0.62***	0.51***	-0.42**	0.09	-0.20	-0.69***	1		
UK		0.68***	0.78***	0.67***	0.69***	-0.15	-0.12	-0.09	-0.70***	1		
Portugal	SD-8	-0.26*	-0.22	-0.17	-0.28*	-0.18	0.06	0.14	-0.65***	0.32*	1	
UK		-0.11	-0.05	-0.03	-0.01	0.04	-0.20	0.24	-0.79***	0.48***	1	
Portugal	SI-8	-0.30*	-0.31*	-0.32*	-0.30*	0.17	-0.02	0.12	0.31*	-0.42**	0.48***	1
UK		-0.29*	-0.36**	-0.29*	-0.22	0.28*	-0.09	-0.02	0.19	-0.30*	0.41**	1
Portugal	SL	0.13	0.16	0.07	0.07	-0.01	0.158	-0.19	0.14	0.11	-0.06	0.03
UK		0.38**	0.49***	0.36**	0.36**	-0.10	0.07	-0.29*	0.04	0.28*	-0.20	-0.27*

4.2.3 QTL analysis

4.2.3.1 QTL for leaf area and growth rate

Three QTL for leaf area were identified in Portugal and these mapped on LG1, 88-102 cM, LG2, 82-88 cM and LG4, 159-168 cM (Figure 4.21). The QTL for LA on LG1 was identified in all leaf development stages from leaf 1 to leaf 8 and was common in both field trials (Figure 4.21a). In Portugal, the phenotypic variation explained by this QTL from leaf 1 to leaf 8 were 10.32%, 12.06%, 12.97%, 13.04%, 14.80%, 14.22%, 17.12% and 17.23%, respectively. The percentage of the explained variation increased from young to mature leaves. This QTL also explained 15.43% of phenotypic variation for leaf 7 and 15.67% for leaf 8 in the UK (Table 4.7). The nearest marker for this QTL is L2222. The QTL for LA on LG 2 (Figure 4.21b) was only detected in the mature leaves (leaf 7 and leaf 8) in Portugal, which explained 10.39% and 11.83% of phenotypic variations. This QTL was not detected in any stage leaf in the UK field trial. The nearest marker is 1A20-178. QTL for LA on LG 4 was only identified from leaf 1 (young leaves) to leaf 6 (semi-mature leaves), but not identified in leaf 7 and leaf 8 (mature leaves) in Portugal (Figure 4.21c). The phenotypic variations explained by this QTL from leaf 1 to leaf 6 were 11.33%, 11.94%, 12.99%, 13.08%, 12.27% and 14.70%, respectively (Table 4.8). E45/M48-F-249 was the nearest marker for this QTL for LA. The QTL allele effects on LG 1 and LG 2 were from the parent *L. sativa*, but allele effect on LG 4 was from *L. serriola*. Figure 4.21 only shows the example plots of the QTL for LA identified in this study. The detailed individual QTL data for different leaf development stages were listed in Table 4.7. The data from one leaf developmental stage was chosen to compare the results in two trials (Leaf 7 for traits LA, FW, DW, DWP, SLA, CHL, Leaf 8 for traits ECA, ECN, SD, SI and Leaf 6 for trait OSM, Leaf 6-8 for SL) (Table 4.8). In Portugal, the total variability explained by the identified QTL for LA were from 22% (Leaf 1) to 29% (Leaf 8) in Portugal, while only one QTL was identified in the UK, accounting about 15% of phenotypic variance. AGR and RGR were only measured in the UK. QTL for AGR and RGR co-located with QTL for LA on LG 1 and LG2, respectively (11.85%; 12.77%).

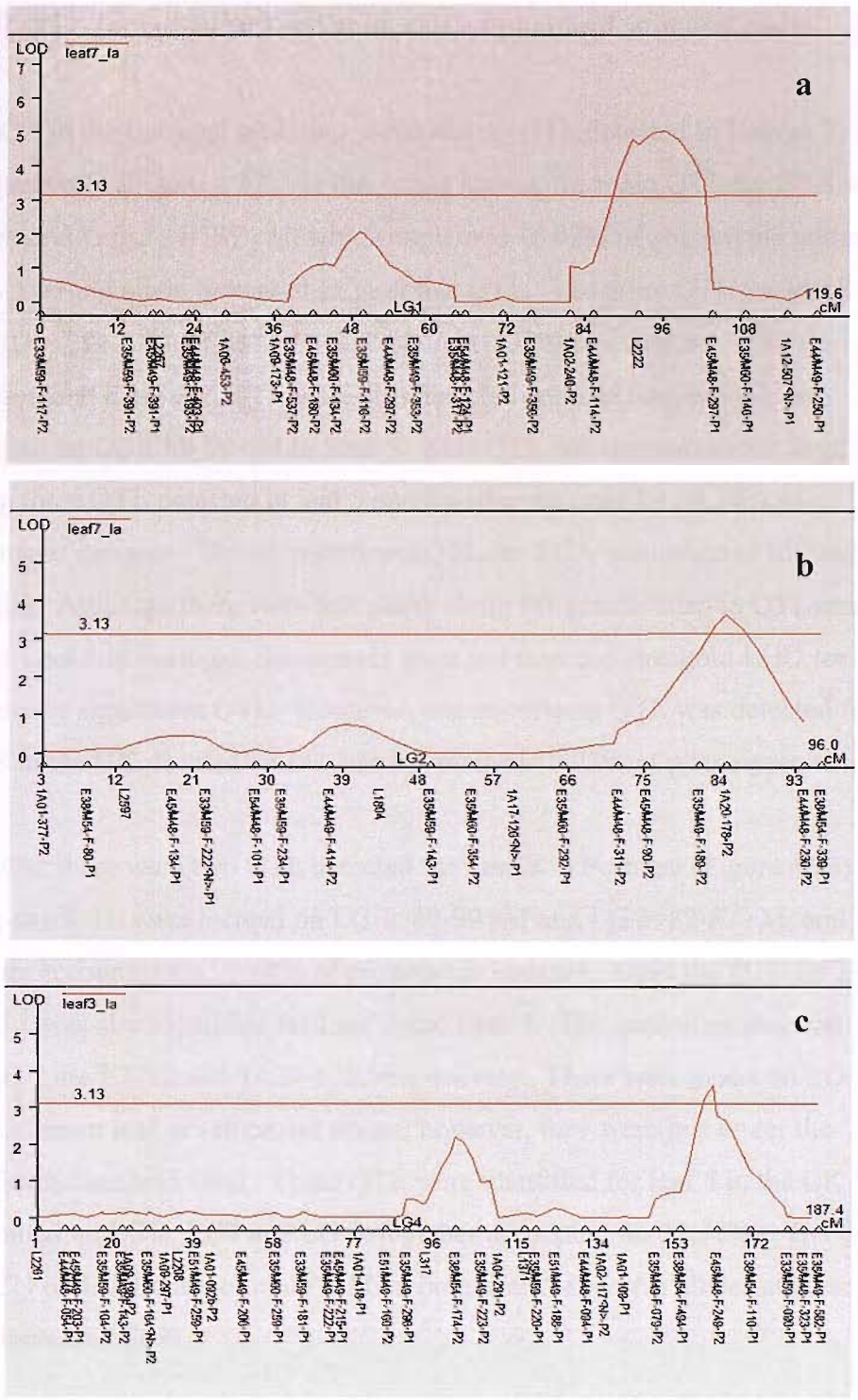


Figure 4.21 QTL cartographer plots for leaf area at three linkage groups in the selected F_9 RIL mapping population using composite interval mapping analysis. Notes: QTL for LA at LG1:88-102 cM was identified at all different developmental stages and was common in both field trials (a); QTL for LA at LG 2: 80-88 cM was only identified in the mature leaves in the Portugal (b) and QTL for LA at LG 4: 159-168 cM was only identified in the young leaves in Portugal (c). LOD score is indicated on the Y axis. The X axis represents each marker map position (cM) along the linkage group.

4.2.3.2 QTL for epidermal cell area, cell number and stomatal traits

For ECA in the Portugal trial, two, three and no QTL detected in Leaves 2, 5 and 8, respectively (Figure 4.22). In the young leaves, the main QTL for ECA was located on LG 6, 127-137 cM, which explained 16.02% of phenotypic variance. The *L .serriola* allele increased ECA at this QTL. The other QTL for leaf 2 was located on LG 2, 83-87 cM, which explained 9.33% of variance. With the leaves development, these two QTL were also detected and one further QTL was identified on LG 1 89-99 cM in Leaf 5. This QTL had comparatively large effect among three QTL detected in leaf 5 and together account for 35.18% of phenotypic variance. But no significant QTL for ECA was detected for leaf 8 in Portugal. Although there were few peaks along the genetic map in QTL analysis plot of Leaf 8 in Portugal, these peaks were not over the threshold LOD for statistically significant QTL. However, one significant QTL was detected for Leaf 8 in the UK, located on LG 2 and explained 12.73% of phenotypic variance.

For ECN, there were two QTL detected for Leaf 8 in Portugal (Figure 4.23). These two QTL were located on LG 1: 89-99 cM and LG 2: 82-87 cM, and together accounted for 25.68% of phenotypic variance. Only the QTL for ECN on LG 1 was also identified for Leaf 2 and Leaf 5. The nearest markers on LG 1 and LG 2 are L2222 and 1A20-178, respectively. There were peaks on LG 7 at three different leaf development stages; however, they were just under the significant threshold level. Three QTL were identified for leaf 8 in the UK trial, distributed on LG 1, LG7 and LG 9, together accounted for 35.31%. The QTL for ECN on LG 1 was common QTL in both trials. *L. sativa* alleles at these four QTL increased ECN.

One QTL for stomatal density (SD) was detected on LG 2: 83-90 cM at all three different developmental stages in Portugal. It explained 11.20% phenotypic variation of leaf 8 of this population (Table 4.7 & 4.8). Two QTL for stomatal index (SI) were detected in the young leaf, located on LG 2: 83-86 cM, and LG 6: 132-136 cM. Only one QTL for SI was detected in the mature leaf (LG 2) and none was detected in the semi-mature leaf in Portugal. No QTL was detected for SD or SI in the UK.

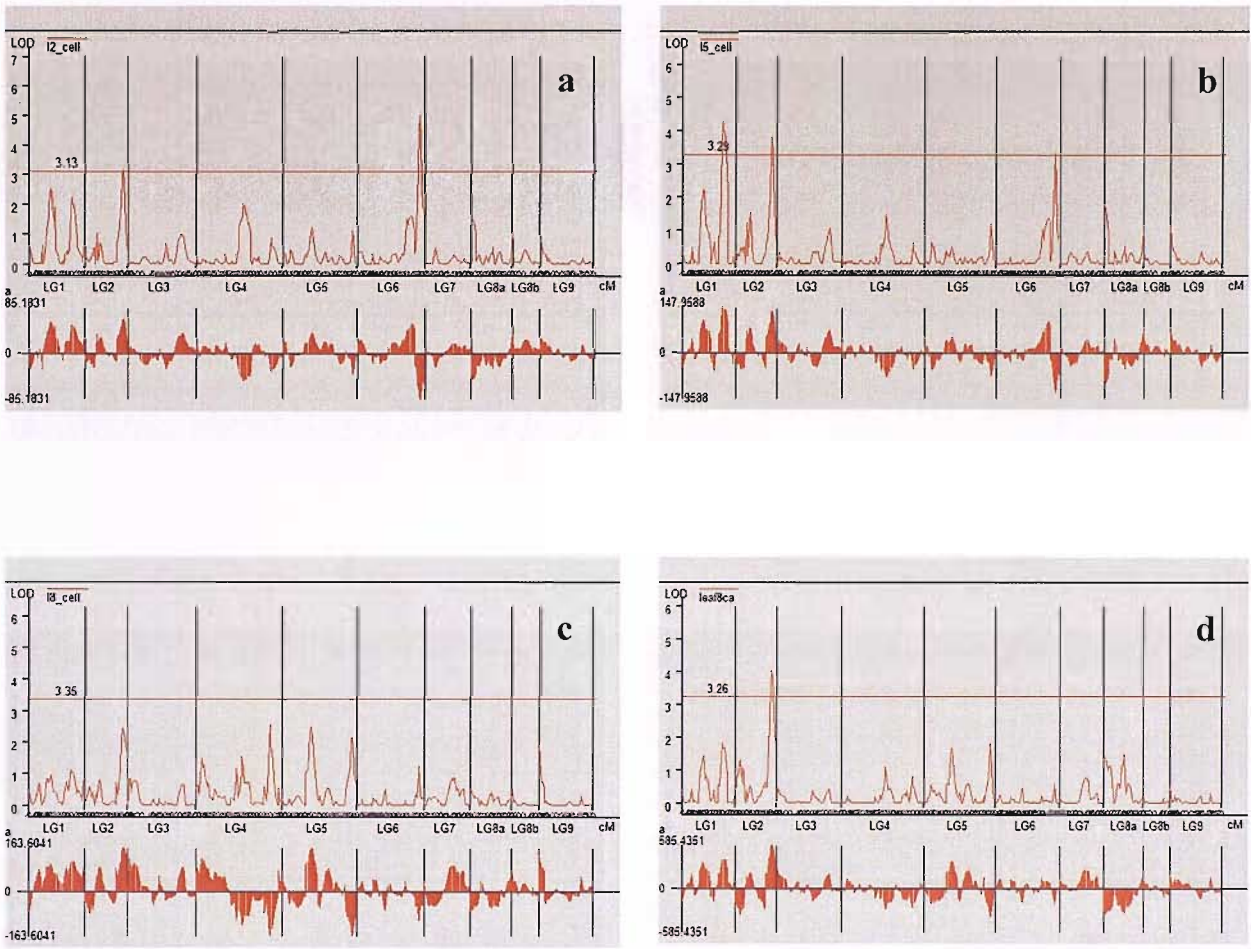


Figure 4.22 QTL Cartographer plots for epidermal cell area (ECA) at different leaf development stages. Notes: ECA of leaf 2 in the Portugal field trial (a), ECA of leaf 5 in the Portugal field trial (b), ECA of leaf 8 in the Portugal field trial (c) and ECA of leaf 8 in the UK field trial (d). LOD score was indicated on the upper Y axis. The X axis represents the ten linkage groups arranged end-to-end as shown with vertical grid lines. The LOD threshold value for each QTL was shown as a horizontal line in the upper graph of each plot. The contribution of two parent alleles for each QTL is shown in the lower graph of each plot. Positive value peaks indicate the allele of the corresponding QTL was from parent *L. sativa* and the negative peaks indicate the QTL is from the parent *L. serriola*.

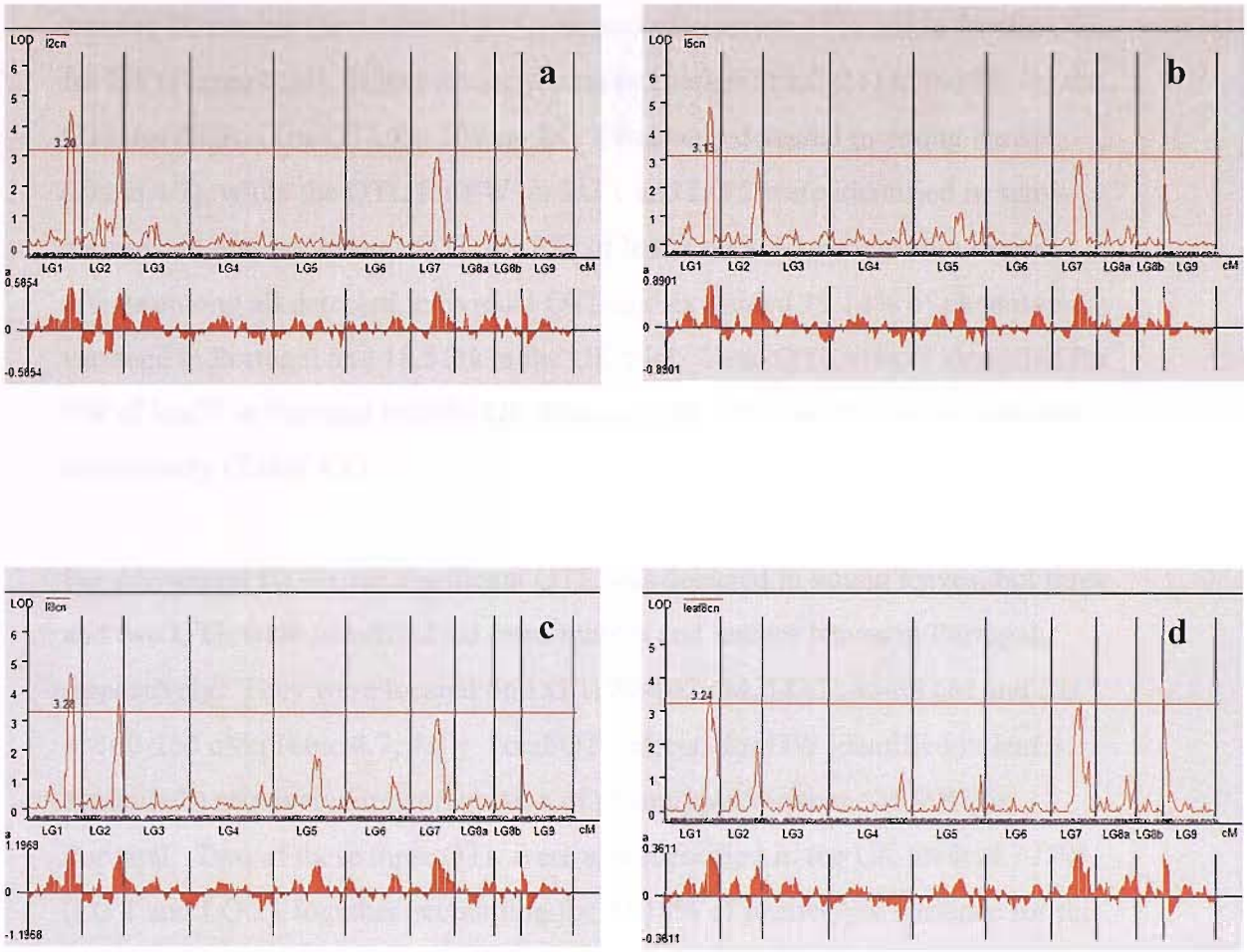


Figure 4.23 QTL Cartographer plots for epidermal cell number per leaf (ECN) at different leaf development stages. ECN of leaf 2 in the Portugal field trial (a), ECN of leaf 5 in the Portugal field trial (b), ECN of leaf 8 in the Portugal field trial (c) and ECN of leaf 8 in the UK field trial (d). Notes see Figure 4.22.

4.2.3.3 QTL for leaf fresh weight, dry weight, specific leaf area

For leaf fresh weight (FW), three and two QTL were resolved in Portugal and the UK, respectively. Two common QTL were detected in both trials, which co-located with the QTL for LA on LG 1 and 2, but the nearest marker on LG 1 was number 20 marker (E44/M48-F-114), instead of number 21 (L2222) for the QTL for LA (Figure 4.24). It had the same nearest marker in LG 2 (1A20-178) for the QTL for RGR. The QTL for FW on LG 7 was only detected in young leaves (Table 4.7), while the QTL for FW on LG 1 and LG 2 were identified in semi-mature and mature leaves. QTL for FW of leaf 7 on LG 1 showed the largest effects among all detected individual QTL and explained 19.14% of phenotypic variance in Portugal and 18.51% in the UK trial. Total QTL effects identified for FW of leaf 7 in Portugal and the UK explained 29.17% and 29.13% of variance, respectively (Table 4.8).

For dry weight (DW), no significant QTL was detected in young leaves, but three and two QTL were identified for semi-mature and mature leaves in Portugal, respectively. They were located on LG 1: 89-102 cM, LG 2: 83-86 cM and LG 4: 160-163 cM (Table 4.7, 4.8). Total QTL effects for DW identified in leaf 4 explained a relatively large proportion of phenotypic variance (35.99%) in Portugal. Two of these three QTL were also identified in the UK for leaf 7 DW (LG 1 and LG 2), together accounting for 30.18% of phenotypic variance for this mapping population.

For dry weight as the percentage of fresh weight (DWP), one common QTL was identified on LG 5: 61-65 cM in both trials, accounting for 10.15% and 12.00% of phenotypic variance in Portugal and the UK trial, respectively (Table 4.7, 4.8). *L. sativa* alleles at this locus contributed to the increase in DWP. The nearest marker is E33/M59-F-176-P1. There were two additional QTL detected in Portugal, one was located on LG 2: 81-86 cM, which was only detected in young leaves, and the other one was located on LG 4: 160-169 cM, which was detected in the semi-mature and mature leaves.

In young leaves, three QTL for SLA were detected, respectively located on LG 1: 92-100 cM; LG 2: 82-87 cM and LG 4: 158-168 cM (Table 4.7). With the leaf development, two QTL for SLA were identified in the semi-mature leaves (LG 2 and LG 4), and only one was detected in the mature leaves in Portugal (LG2). But none of these QTL for SLA was identified in the UK. Three QTL for SLA all co-located with the QTL for LA and the nearest markers were the same as the ones for LA (L2222; 1A20-178 and E45/M48-F-249-P2). *L. sativa* increased SLA trait value at QTL on LG 1 and LG 2, while *L. serriola* increased the SLA trait value at QTL on LG4.

4.2.3.4 QTL for chlorophyll content and osmolality

Two QTL for chlorophyll content were identified for leaf 7 in Portugal, located on LG 1: 91-94 cM and LG 2: 81-86 cM, together accounted for 22.95% of phenotypic variance (Figure 4.25a). The nearest markers were E44/M48-F-114 on LG 1, which co-located QTL for AGR, and 1A20-178, which co-located with QTL for some traits such as LA and RGR. The QTL for CHL on LG 1 was only detected in mature leaves and the QTL for CHL on LG 2 was detected in young leaves and mature leaves. No significant QTL for CHL was identified in the semi-mature leaves in Portugal. Only QTL on LG 2 was detected and contributed to 10.40% of the observed phenotypic variance in UK (Figure 4.25b).

For osmolality (OSM), two QTL were identified for leaf 6 in Portugal. The QTL for OSM on LG 2: 83-84 cM explained 10.31% phenotypic variance and the positive allele was from *L. sativa*. While the other QTL on LG 4: 161-165 cM contributed 11.89% of the observed phenotypic variance and this positive allele was from *L. serriola* (Table 4.7). No significant QTL for OSM was identified in the UK.

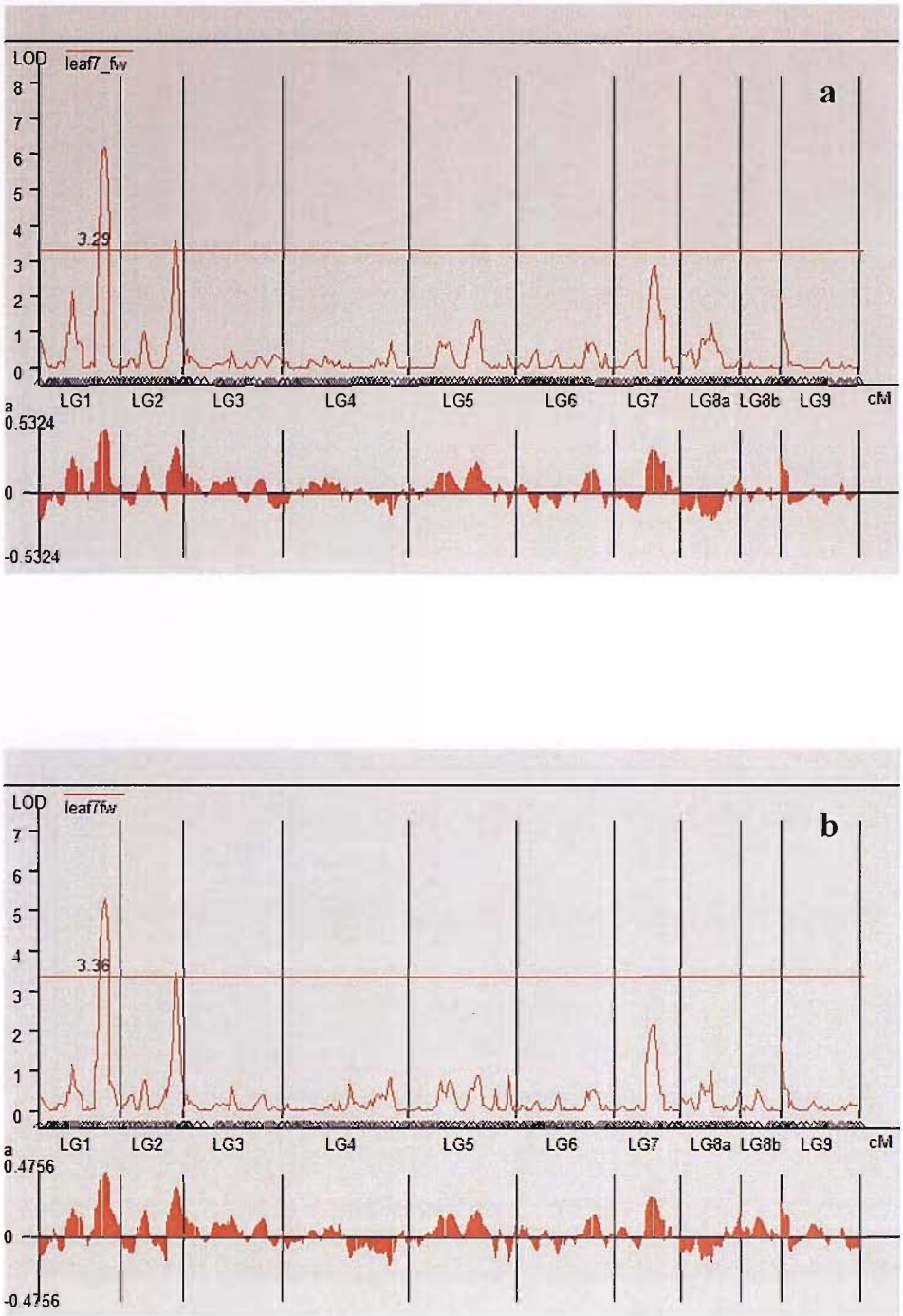


Figure 4.24 QTL Cartographer plots for leaf 7 fresh weight (FW) of the F_9 RIL mapping population in two field trials. Notes: the Portugal field trial (a) and the UK field trial (b). Other notes see Figure 4.22.

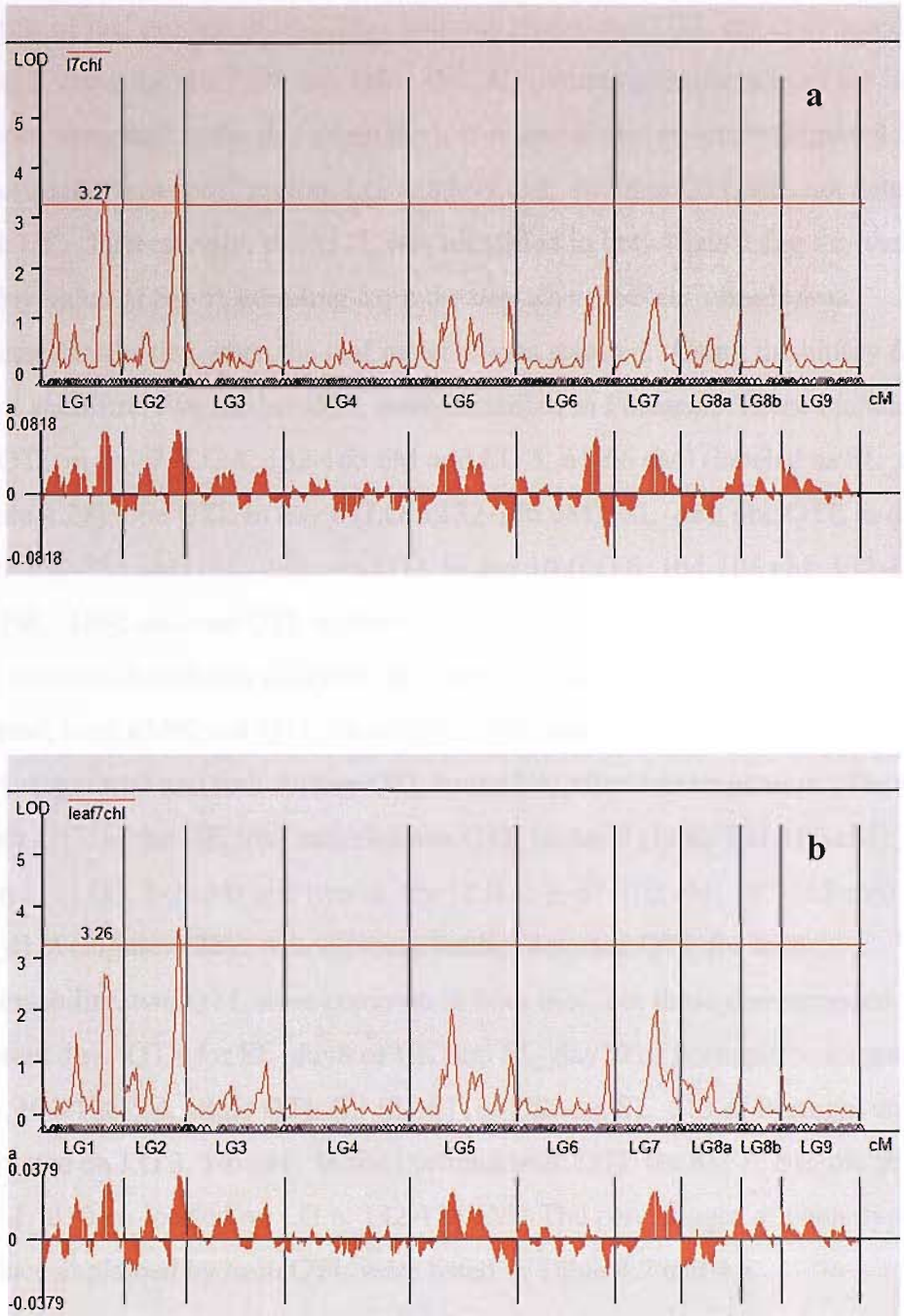


Figure 4.25 QTL Cartographer plots for leaf 7 chlorophyll content (CHL) of the F_9 RIL mapping population in two field trials. Notes: the Portugal field trial (a) and the UK field trial (b). Other notes see Figure 4.22.

4.2.3.6 QTL for processability

In terms of leaf processability, there was one significant QTL effect detected in Portugal using the shelf life day value (SL_H) (counting the days from the leaf material harvested to the day when the leaf material was rejected) (Figure 4.26). It was on the "hot-spot" region, LG 1, 89-99 cM. But this QTL was not detected in the UK. Interestingly, this QTL was identified in both trials using the shelf life day value of SL_P, counting from the day when the leaf sample was processed to the day when the leaf material was rejected. Using the binary data sets of shelf life, five further QTL were identified in Portugal. These included two QTL in day 7 (LG 4, 162-165 cM and LG 5, 61-66 cM) (labeled as SL_d7) (Figure 4.27); one QTL in day 8 (LG 6,132-136 cM) (SL_d8); one QTL in day 9 (LG6, 132-136 cM) (SL_d9); two QTL in day 10 (LG 6, 104-106 cM; 132-136 cM) (SL_d10); and one QTL in day 13 (LG 3, 1-6 cM) (SL_d13). As the same QTL location detected at different days was counted as one further QTL detected, total additional QTL identified in the analysis were five further QTL in the Portugal trial and four further QTL in the UK after data truncation. The four further QTL in the UK trial included one QTL in day 8 (LG6, 104-106 cM); one in day11 (LG3, 1-2 cM) and two in day 12 (LG 5, 87-102 cM; 127-132 cM) (SL_d12) (Figure 4.28). Among these further detected QTL for leaf processability, two QTL were common in both trial, but these corresponded to different day. QTL for SL_day8 of UK and SL_day10 of Portugal co-located on LG6, 104-106 cM, while QTL for SL_d11of UK and SL_d13 of Portugal were co-located on LG 3, 1-6 cM. In the Portugal trial, QTL for SL_P, SL_d8, SL_d9 and SL_d10 co-located on LG 6, 132-136 cM. The percentages of phenotypic variance explained by each QTL were listed in Table 4.7 and 4.8.

In summary, a total of 44 QTL with significant effects were detected for the fourteen traits, distributed on eight out of the ten linkage groups (Table 4.8, Figure 4.28). Each QTL accounted for 8.88% -19.14% of the phenotypic variation in this population. Among these detected QTL, 36 and 18 significant QTL were identified for the leaf traits measured in Portugal (Figure 4.29, red bar) and the UK (Figure 4.29, green bar), respectively. Twelve QTL were

common to both field trials (Figure 4.29, blue bar), including the QTL for the same trait detected at different stages or different days during the shelf life in two trials. They were QTL for LA, FW, DW, ECN and SL_P on LG 1, QTL for FW, DW, CHL and ECA on LG 2, QTL for SL on LG 3 and LG 6, and QTL for DWP on LG5. The total number of QTL identified in the UK trial is less than that in Portugal due to the fact that fewer leaf developmental stage samples were collected in the UK trial. Three different developmental stage samples were assessed for the most traits in Portugal, while only one leaf sample was assessed in the UK. Three of a total of 8 QTL for leaf processability from this study were common in both trials, located on LG 1, 3 and 6.

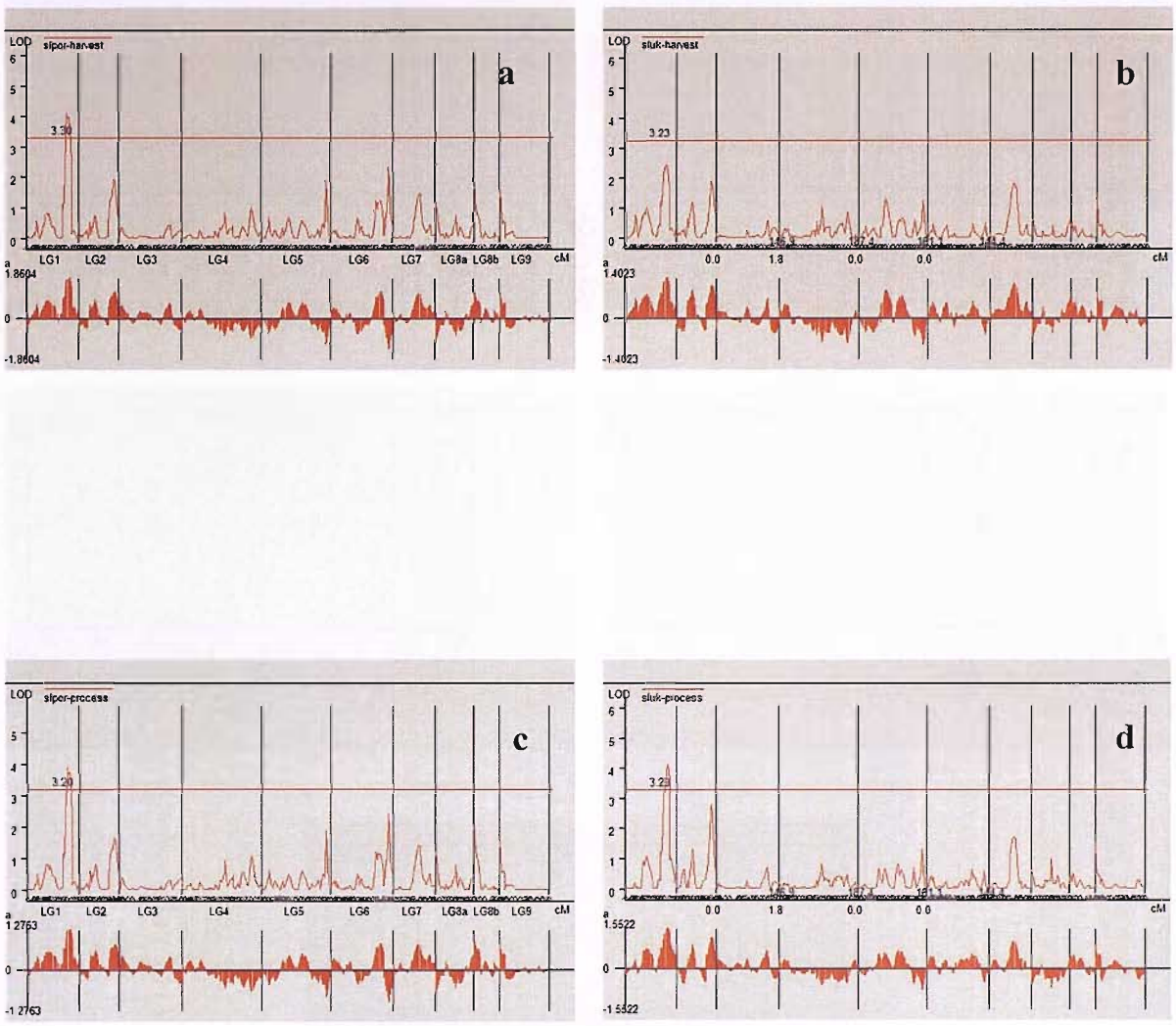


Figure 4.26 QTL Cartographer plots for shelf life of the F_9 RIL mapping population in two field trials. Notes: SL_H (shelf life day value counting from harvesting to the day when leaf material was rejected) in the Portugal trial (a), SL_H in the UK field trial (b), SL_P (shelf life day value counting from leaf material was processed to the day when leaf material was rejected) in the Portugal field trial (c) and SL_P in the UK field trial (d). Other notes see Figure 4.22.

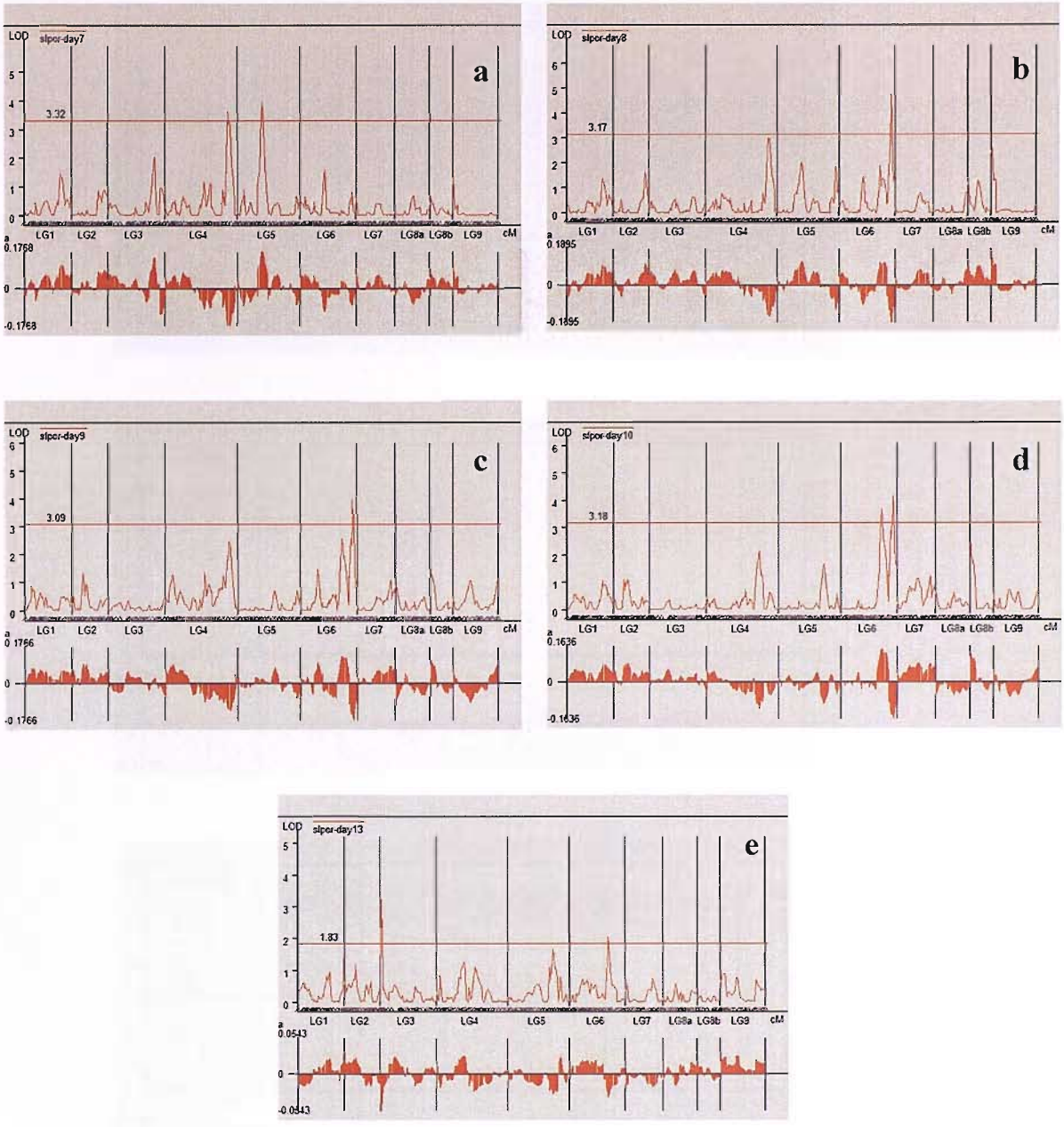


Figure 4.27 QTL Cartographer plots for leaf processability detected daily during the shelf life period in the Portugal field trial. Notes: QTL for leaf processability were analysis based on each RIL condition on each day after harvest. RIL condition was treated as binary in nature and were recorded as score 1 for good condition and score 0 for bad condition (being rejected). QTL for SL_d7 was detected on Day 7 after harvest (a); QTL for SL_d8 (b); QTL for SL_d9 (c); QTL for SL_d0 (d) and QTL for SL_d13 (e). Other notes see Figure 4.22.

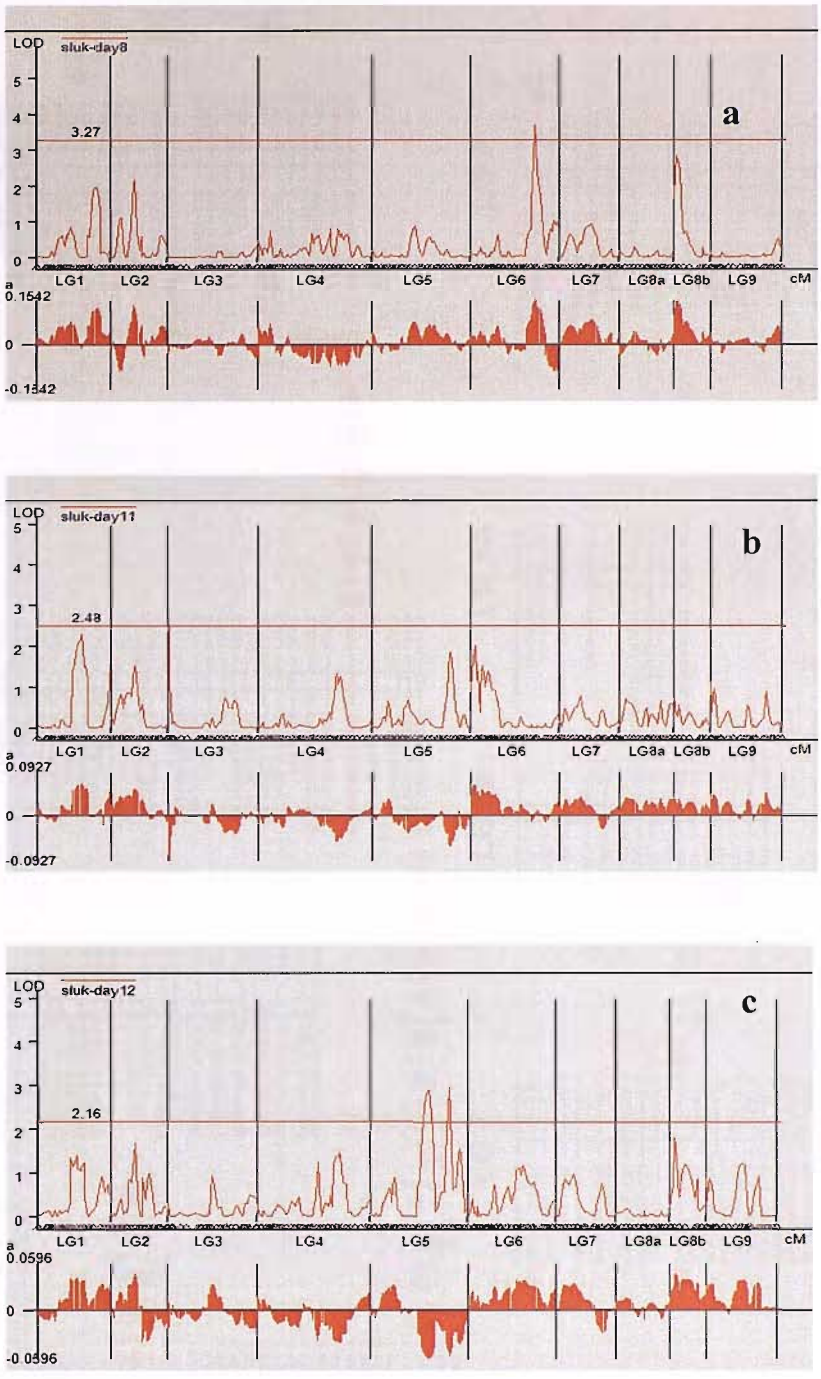


Figure 4.28 QTL Cartographer plots for leaf processability detected daily during the shelf life period in the UK field trial. Notes: QTL for SL_d8 (a); QTL for SL_d11 (b) and QTL for SL_d12 (c). Other notes see Figure 4.22 and 4.27.

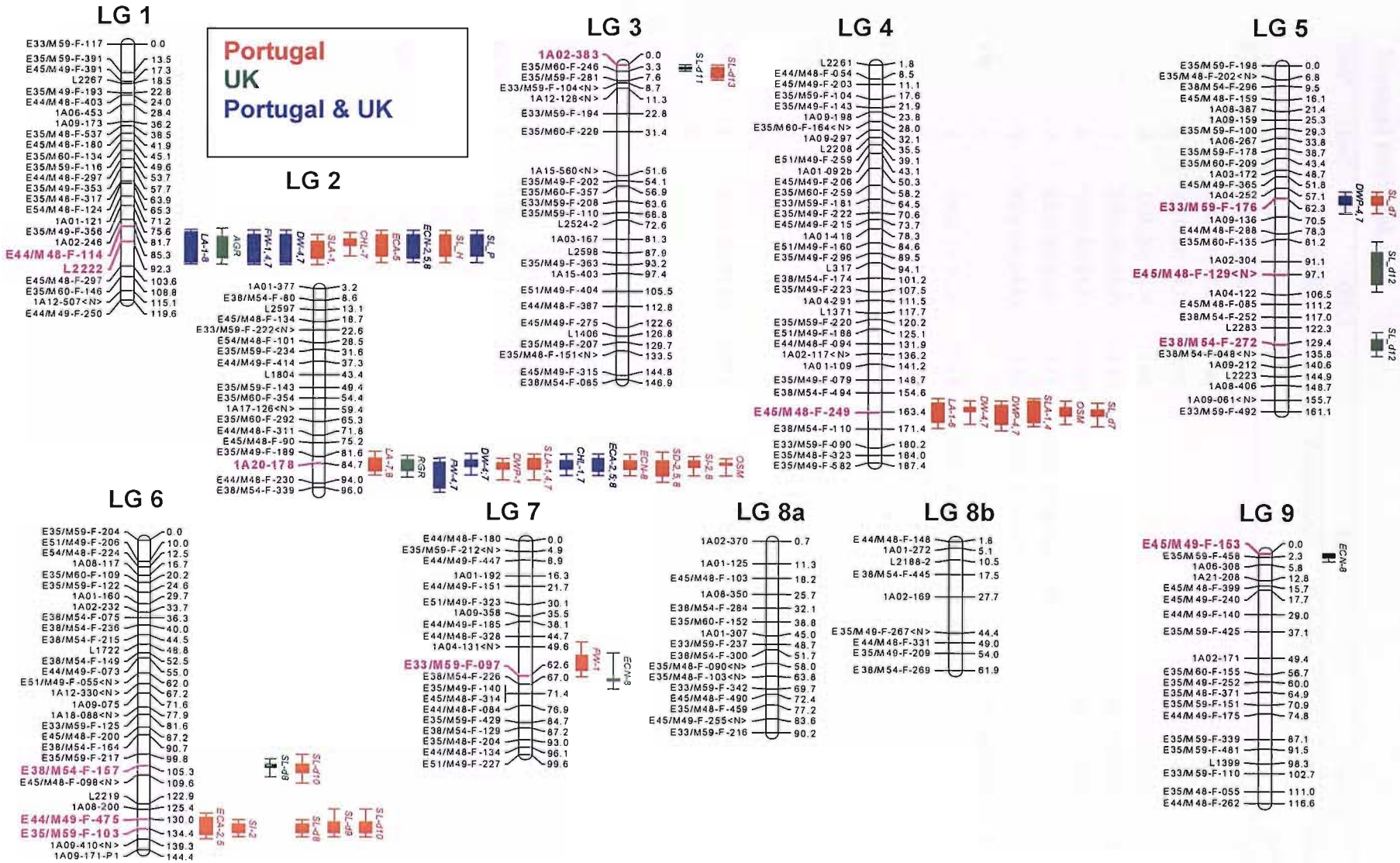


Figure 4.29 Chromosome distribution of QTL in the molecular linkage map in the RIL mapping population based on composite interval mapping. Notes: Map positions were given in cM. The length of the bars indicates the LOD interval over significant threshold for each QTL, and the line extensions of each bar indicates a confidential interval of one LOD lower than significant LOD threshold for each QTL. The markers in pink indicate the nearest marker to QTL for the trait of interest. The red bars represent the QTL detected in Portugal; the green bars represent the QTL detected in the UK; and the blue bars represent QTL detected in both trials. The trait abbreviation see note in Table 4.7 and the number after trait indicates the leaf development age.

Table 4.7 QTL detected for leaf traits at different leaf development stages in the Portugal field trial.

Trait[a]	Leaf	QTL1		QTL 2		QTL 3		Total
	no.[b]	Position[c] (cM)	Variance[d] (%)	Position (cM)	Variance (%)	Position (cM)	Variance (%)	Variance (%)
LA	1	LG1: 21; 94.31	10.30			LG4: 32; 163.61	11.33	21.63
	2	LG1: 21; 94.31	12.06			LG4: 32; 163.61	11.94	24.00
	3	LG1: 21; 94.31	12.97			LG4: 32; 163.61	12.99	25.96
	4	LG1: 21; 94.31	13.04			LG4: 32; 163.61	13.08	26.12
	5	LG1: 21; 94.31	14.80			LG4: 32; 163.61	12.27	27.07
	6	LG1: 21; 94.31	14.22			LG4: 32; 163.61	14.70	28.92
	7	LG1: 21; 94.31	17.12	LG2: 17; 81.51	10.39			27.51
	8	LG1: 21; 94.31	17.23	LG2: 17; 81.51	11.83			29.06
FW	1					LG7: 10; 57.61	12.41	12.41
	4	LG1: 21; 94.31	15.14	LG2: 17; 81.51	10.48			25.62
	7	LG1: 21; 94.31	19.14	LG2: 17; 81.51	10.03			29.17
DW	4	LG1: 21; 94.31	12.79	LG2: 17; 81.51	11.15	LG4: 32; 163.61	12.05	35.99
	7	LG1: 21; 94.31	18.10					18.10
DWP	1	LG2: 17; 81.51	10.89					10.89
	4			LG4:32; 163.61	14.58	LG5: 14; 62.31	10.46	25.04
	7			LG4:32; 163.61	14.86	LG5: 14; 62.31	10.15	25.01
SLA	1	LG1: 21; 94.31	12.63	LG2: 17; 81.51	12.88	LG4: 32; 163.61	12.78	38.29
	4			LG2: 17; 81.51	12.47	LG4: 32; 163.61	13.20	25.67
	7			LG2: 17; 81.51	10.50			10.50
CHL	1			LG2: 17; 81.51	10.67			10.67
	7	LG1: 20; 91.31	11.05	LG2: 17; 81.51	11.90			22.95
ECA	2			LG2: 17; 81.51	9.33	LG6: 27; 134.01	16.02	25.35
	5	LG1: 20; 91.31	14.03	LG2: 17; 81.51	11.15	LG6: 27; 134.01	10.00	35.18
ECN	2	LG1: 21; 94.31	13.33					13.33
	5	LG1: 21; 94.31	15.78					15.78
	8	LG1: 21; 94.31	14.49	LG2: 17; 81.51	11.19			25.68
SD	2	LG2: 17; 81.51	11.94					11.94
	5	LG2: 17; 81.51	11.84					11.84
	8	LG2: 17; 81.51	11.20					11.20
SI	2	LG2: 17; 81.51	10.59	LG6: 27 134.01	12.06			22.65
	8	LG2: 17; 81.51	9.96					9.96
OSM	6	LG2: 17; 81.51	10.31	LG4: 32; 163.61	11.89			22.20
SL_H	6-8	LG1: 20; 91.31	12.67					12.67
SL_P	6-8	LG1: 20; 91.31	11.90					11.90
SL-d7	6-8	LG4:31; 160.81	11.47	LG5:14; 62.31	12.08			23.55
SL-d8	6-8	LG6:28; 134.41	14.04					14.04
SL-d9	6-8	LG6:28; 134.41	12.54					12.54
SL-d10	6-8	LG6:23; 105.31	10.17	LG6:28; 134.41	12.98			23.15
SL-d13	6-8	LG3:1; 2.01	11.42					11.42

Notes:

[a]Trait abbreviation: LA: leaf area; AGR: Absolute growth rate (AGR) ($mm^2\ h^{-1}$); Relative growth rate (RGR) ($x10^{-3}\ mm^2\ mm^{-2}\ h^{-1}$); 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 ($x\ 10^6$); SD: stomata density (no. stomata mm^{-2}); SI: stomata index (%); OSM: cell sap osmolality ($mmol\ kg^{-1}$); SL-H: shelf-life period, count from harvesting to the day before being rejected (days). SL_d: shelf life data as binary for QTL analysis at individual day, accounting from harvesting.

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

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

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

Table 4.8 QTL detected by composite interval mapping for all leaf traits in two field trials.

Trait[a]	Position[b] (cM)	Marker[c]	Portugal field trial				UK field trial			
			Leaf no.[d]	LOD[e]	Additive[f]	Var.[g] (%)	Leaf no.	LOD	Additive	Var. (%)
LA	LG1: 88-102	L2222	1-8	3.13	1118.325	17.12	3,6,7,8	3.22	1071.134	15.43
	LG2: 82-88	1A20-178	7-8	3.13	885.5957	10.39	-	-	-	-
	LG4: 159-168	E45/M48-F-249	1-6	3.10	-941.0803	14.71	-	-	-	-
AGR	LG1: 90-100	E44/M48-F-114	NA				3 to 6	3.37	1.7861	11.85
RGR	LG2: 83-91	1A20-178	NA				3 to 6	3.22	1.7812	12.77
FW	LG1:89-102	E44/M48-F-114	4,7	3.29	0.5202	19.14	7	3.36	0.4756	18.51
	LG2: 81-86	1A20-178	4,7	3.29	0.3752	10.03	7	3.36	0.3584	10.62
	LG7: 53-60	E33/M59-F-097	1	3.13	0.0244	12.41	-	-	-	-
DW	LG1: 89-102	E44/M48-F-114	4,7	3.13	0.0590	18.10	7	3.29	0.0303	17.70
	LG2: 83-86	1A20-178	4	3.18	0.0202	11.15	7	3.29	0.0303	11.48
	LG4: 160-163	E45/M48-F-249	4	3.18	-0.0217	12.05	-	-	-	-
DWP	LG2: 81-86	1A20-178	1	3.48	2.3285	10.89	-	-	-	-
	LG4: 160-169	E45/M48-F-249	4,7	3.33	-2.3693	14.58	-	-	-	-
	LG5: 61-65	E33/M59-F-176	4,7	3.33	1.9864	10.15	7	3.32	1.4712	12.00
SLA	LG1: 92-100	L2222	1	3.32	3.3819	12.63	7	-	-	-
	LG2: 82-87	1A20-178	1,4,7	3.33	3.4641	10.50	-	-	-	-
	LG4: 158-168	E45/M48-F-249	1,4	3.32	-3.6246	12.78	-	-	-	-
CHL	LG1: 91-94	E44/M48-F-114	7	3.27	0.0791	11.05	-	-	-	-
	LG2: 81-86	1A20-178	1,7	3.27	0.0817	11.90	7	3.24	0.0334	10.40
ECA	LG1: 89-99	E44/M48-F-114	5	3.29	147.9588	14.03	-	-	-	-
	LG2: 81-88	1A20-178	2,5	3.29	133.9457	11.15	8	3.26	575.1725	12.73
	LG6: 129-137	E44/M49-F-475	2,5	3.29	-126.9219	10.00	-	-	-	-
ECN	LG1: 89-100	L2222	2,5,8	3.28	1.1857	14.49	8	3.30	0.3456	11.55
	LG2: 83-87	1A20-178	8	3.28	1.0064	11.19	-	-	-	-
	LG7: 64-65	E33/M59-F-097	-	-	-	-	8	3.30	0.3536	11.72
	LG9:0-2	E45/M49-F-153	-	-	-	-	8	3.30	0.3611	12.04
SD	LG2: 83-90	1A20-178	2,5,8	3.20	33.7928	11.20	-	-	-	-
SI	LG2: 83-86	1A20-178	2,8	3.26	2.0840	9.96	-	-	-	-
	LG6: 132-136	E44/M49-F-475	2	3.29	-2.2672	12.06	-	-	-	-
OSM	LG2: 84-85	1A20-178	6	3.33	77.1386	10.31	-	-	-	-
	LG4: 161-165	E45/M48-F-249	6	3.33	-79.3150	11.89	-	-	-	-
SL_H	LG1: 89-99	E44/M48-F-114	6,7,8	3.30	1.8054	12.67	-	-	-	-
SL_P	LG1: 89-99	E44/M48-F-114	6,7,8	3.20	1.2462	11.90	6,7,8	3.28	1.5522	15.11
SL_d7	LG4: 162-165	E38/M54-F-494	6,7,8	3.32	-0.1742	11.47	-	-	-	-
	LG5: 61-65	E33/M59-F-176	6,7,8	3.32	0.1768	12.08	-	-	-	-
SL_d8	LG6: 104-106	E38/M54-F-157	6,7,8	-	-	-	6,7,8	3.27	0.1542	10.96
	LG6: 132-136	E35/M59-F-103	6,7,8	3.17	-0.1895	14.04	-	-	-	-
SL_d9	LG6: 132-136	E35/M59-F-103	6,7,8	3.09	-0.1693	12.54	-	-	-	-
SL_d10	LG6: 104-106	E38/M54-F-157	6,7,8	3.18	0.1449	10.17	-	-	-	-
	LG6: 131-136	E35/M59-F-103	6,7,8	3.18	-0.1564	12.98	-	-	-	-
SL_d11	LG3: 1-2	1A02-383	-	-	-	-	6,7,8	2.48	-0.0927	10.64
SL_d12	LG5: 87-102	E45/M48-F-129<N>	-	-	-	-	6,7,8	2.16	-0.0593	9.65
	LG5: 127-132	E38/M54-F-272	-	-	-	-	6,7,8	2.16	-0.0561	8.88
SL_d13	LG3: 1-6	1A02-383	6,7,8	1.83	-0.0543	11.42				

Notes:

[a]Trait abbreviation: see Table 4.7.

[b]Position indicated 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 which leaf development stage 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 (likelihood ratio)

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

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

4.3 Discussion

4.3.1 Leaf processability and leaf characteristics

This study is the first to report QTL for leaf processability, and links this post-harvest trait to pre-harvest leaf development characteristics. It showed that leaf processability and leaf development QTL are apparent as "clusters", linked to several leaf traits. There appears to be an association of longer shelf life with larger leaf, lower chlorophyll content, higher cell number per leaf and lower stomatal index (Table 4.4-4.6). After the data truncation, there were two common QTL identified in the two trials, located on LG 3 and LG 6, despite the two day gap between the two sites that accounted for transportation to the UK (Figure 4.29). These data suggest that genes in these two regions are associated with the leaf material strength in a specific stage during the shelf life period. *L. sativa* alleles increased the shelf life value in QTL for leaf processability detected on LG 1 and LG 6, while *L. serriola* alleles increased the shelf life value in QTL detected on LG 3, 4, and 5. One explanation for this result is that some alleles from *L. serriola* for leaf processability were being expressed only at certain specific stages, and then were suppressed at the transition stage while the alleles from *L. sativa* were still being expressed at the further stages.

QTL for leaf processability also co-located with QTL for OSM and DWP on LG 4, QTL for DWP on LG 5, and QTL for ECA and SI on LG 6. The lower percentage of dry weight compared to fresh weight in *L. sativa* leaves indicates that there was higher water content in *L. sativa* than in *L. serriola* leaves. The small cells of *L. sativa* with a higher percentage of water had higher turgidity, which agreed with the result that *L . serriola* had higher osmolality than *L. sativa* in the Portugal trial. Thus, it showed significant correlation with shelf life in the Portugal trial. Stomatal index shows the configuration of a leaf to adjust stomatal opening and closure with water concentration. 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 leaf shelf life also associated with cell wall biochemistry, as well as rates of cell expansion and

production. Increased leaf cell expansion relates to the stimulation in cell wall loosening (Crosgrove, 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; Brummell et al., 2004). The study of Kaku et al. (2002) indicated 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 several tissues assayed and correlates closely with growth rates in many cell types (Thompson & Fry, 2001). Anti-sense *XTH* lettuce plants show a reduced XET activity which correlate with changes in traits related to processability, for example, reducing epidermal cell areas, increasing epidermal cell number and altered leaf irreversible extensibility (Clarkson, 2004).

4.3.2 Clusters of QTL

If two QTL peaks are situated very close to each other and the 1-LOD support interval completely or mostly overlap, these two QTL can be regarded potentially as a single QTL with pleiotropic effects. However, in most studies, each QTL has been counted independently to simplify the number of QTL detected for each individual specific trait (Xu et al., 2004). Several clusters of QTL were detected in this study (Fig. 4.29), mainly on linkage group (LG) 1, 2, 4, and 6. For example, QTL for traits of LA, AGR, FW, DW, SLA, CHL, ECA, ECN and SL all map to a similar position on LG 1, 88-102 cM, while QTL for traits of LA, FW, DW, SLA, CHL, ECA, ECN, SD, SI and OSM mapped to a region on LG 2, 81-88 cM. In fact, most of common QTL detected in both trials were located in these two 'hot-spots' of the genome. In the Portugal trial, several additional QTL also clustered to the region on LG 4 and LG 6, but these two clusters were not identified in the UK trial. Similarly clustered QTL have been reported in other studies for poplar leaf traits (Rae et al., 2004), rice seed traits (Xu et al., 2004), and fruit quality traits (Causse et al., 2002). The results showing the clustering of QTL is consistent with the high correlation coefficients among the traits (Table II). 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 heavier (FW or DW) leaves, with greater thickness, usually had higher chlorophyll content. Therefore, it is not surprising that one QTL for CHL was clustered with QTL for FW and DW leaf traits. It is consistent with the recent report that growth traits (plant area, dry weight, and relative growth rate) collocated at five genomic regions in an *Arabidopsis* RIL population (El-Lithy et al., 2004). They also found the co-location of QTL for leaf area, specific leaf area and chlorophyll fluorescence. This clustering of QTL has important implications for any plant breeding program. The selection of the ideal genotype for one trait should be very carefully considered to avoid reducing other important domestic trait value.

Transgressive segregation among the RILs was shown in all measured traits. Big pleiotropic effects of two "hot-spots" indicated their potential importance in commercial breeding. But in most cases, beneficial alleles derived from the cultivated lettuce parent. The effects of QTL detected in this study are of similar size, only explained 10%-20% phenotypic variation among the RILs. Quantitative trait dispersed across the genome would make it difficult for breeders to handle.

4.3.3 Leaf development specific QTL

In the Portugal field trial, QTL effects for ten leaf traits (LA, FW, DW, DWP, SLA, CHL, ECA, ECN, SD and SI) were analysed in three different developmental stages (young, semi-mature, mature). Only 5 of the 25 QTL were identified at all three different developmental stage leaves. These 5 QTL were QTL for LA, FW, and ECN on LG1, and QTL for SLA and SD on LG 2. For these 5 QTL, *L. sativa* alleles caused an increase in all corresponding traits. However, *L. serriola* showed an increase in trait values for QTL detected on LG 4 and 6. The QTL for LA on LG 1 was identified in all leaves of different developmental stages, however, the QTL on LG 4 for LA was only detected in leaves 1 to 6 (young and semi-mature leaves), while the QTL for LA on LG 2 was only identified in leaf 7 and 8 (mature leaf). These data suggest that QTL effects for LA on LG 2 and 4 depend on the leaf development stage (Table 4.7), 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, QTL for growth-related traits in *Arabidopsis* were found mainly for the earlier phase of growth (El-Lithy et al., 2004).

In this study, QTL for ECN co-located with QTL for LA in all different leaf developmental stages; however, the co-location of QTL for ECA and LA was only detected at some developmental stages. This suggests that the leaf size was determined by a combination of cell production and cell expansion. The contribution of cell expansion to increased leaf growth was limited to a specific time in development, but the cell production showed a contribution to leaf growth throughout the whole leaf development. The co-location of QTL for AGR and RGR with LA further supports the above point. Research on *Arabidopsis* mutants also demonstrates the importance of both cell production and cell expansion in leaf growth (Kim et al., 2002). However, Donnelly et al., (1999) pointed out that cell division occurs only after cells have reached a certain size, which is in contrast with this study. The relative importance of cell division and cell expansion varies among plant species and is under genetic control (Taylor et al., 2003). Our results show the cell expansion is highly responsive to environmental conditions, as the epidermal cell area was significantly different between two trials (Table 4.3). Other studies have reported that leaf cell expansion appears to be extremely sensitive to environment conditions and QTL for leaf cell expansion was identified in response to different environments (Ferris et al. 2002). Leaf area appears to be driven by leaf cell production through leaf development. The cell-division regulator cyclin-dependent kinase A (CDKA) correlates significantly with the rate of cell production and root growth rate (Beemster et al., 2002). Granier et al. (2000) showed similar evidence that cell division and CDKA activity co-varied in maize leaves growing under both control and water-stressed conditions. The study of the cell cycle may lead to a more detailed and mechanic understanding of plant growth.

4.3.4 Genotype x Environment interaction

QTL effects may be environmentally sensitive and this sensitivity results in phenotypic plasticity (Gurganus et al., 1999). In this study, QTL for leaf

development and shelf life have been studied in two different environments. 36 QTL were detected in the Portugal trial and 18 in the UK trial. Of these, 12 QTL were common to both trials and assumed to be independent of the environment (Table 4.8, Figure 4.29). However, QTL for SI, SD and OSM must be largely environment dependent, as no common QTL for these traits was detected. The number and contribution of each QTL that have significantly different effects across the environments would be associated with substantial genotype by environment (G x E) interaction effects.

The G x E interaction can be estimated from a complete analysis of variance (ANOVA) ($G_i + E_j + G_i \times E_{ij}$) (Kang, 2002). The robustness of QTL is more interesting to breeders. Researchers have applied some models such as the Linear-Bilinear model and the AMMI model to analyse G x E interaction (Kang, 2002). The G x E interaction for this study will be analysed further after assessment of this mapping population in another different environment (i. e. Spain or Kenya field trial). However, G x E interaction have been predicted by comparing QTL detected separately in different environments in many crops, including lettuce and rice (Johnson et al., 2000; Xing, 2002). Until further analysis, it is hard to distinguish whether a QTL detected in one environment but not in the other, could result from experimental noise, sample error or experimental error, and thus does not indicate a real G x E interaction. For example, there was a QTL peak detected on LG 7 for ECN in all three different developmental stage leaves in the Portugal trial, but none of them was over the significant threshold. However, it was over the significant threshold in the UK trial. This could result from experimental noise or sample error in the Portugal trial. Further research to test the robustness of QTL and G x E interaction is planned.

For clustered QTL, in theory, the ideal genotypes could be obtained in a modest breeding population, although breaking close genetic linkage is often expensive. The relatively small population size and the significant level of this study only allow the detection of QTL of large effect (Kearsey & Farquhar, 1998). Only 60 RILs of the most recombinant lines were analysed in this study, although they are informative as a population of about 90 lines. The fact that much of the

variation was left unexplained in this study suggested that there might be more QTL with smaller effects that could not be detected. Increasing the population size would increase the possibility of detecting the QTL with small effects. It would be necessary to plant the whole population of 113 RILs for any such experiment. Recently, with the development of new molecular markers, such as single nucleotide polymorphisms (SNP) detection (Rafalski, 2002), and improved statistical methods, it is possible to identify the candidate genes involved in leaf processability as discussed above.

4.4 Conclusions

1. A total of 44 QTL were identified for leaf traits, 12 stable QTL were identified in both environments. The 'hot-spots' were LG1: 90-100 cM and LG2: 82-88 cM.

2. QTL for leaf processability and leaf development traits were co-located at LG1: 90-100 cM.

3. The effects of QTL detected in this study are of similar size, only explained 10%-20% phenotypic variation.

CHAPTER 5

Mapping QTL for cell wall properties and photosynthesis and detailed analysis of extreme lines

5.1 Introduction

The previous mapping experiment showed that QTL for shelf life co-located with the clustered QTL for several leaf development traits such as leaf area, leaf growth rate and leaf weight. There appears to be an association of longer shelf life with larger leaves, lower chlorophyll content and lower stomatal index (Table 4.4-4.6). To study leaf photosynthesis and the stomatal regulation mechanisms would help in understanding the relationship between processability and leaf development traits. Leaf water relations are also reported to associate with leaf processability and turgor pressure is a factor that contributes to leaf processability (Clarkson et al., 2005).

Stomata control leaf gas exchange, especially CO_2 uptake for photosynthesis and water vapour loss via transpiration (Nejad & Van Meeteren, 2005). Stomata play a critical role in regulating water status. Characteristics such as the relative size of stomatal aperture, stomatal density and stomatal index form a complex mechanism to control water loss and photosynthetic rate (Buckley, 2005). It was found that post-harvest cooling at 4°C inhibited stomatal closure in harvested Brassica leaves (Thomson, 2005). Genetic manipulation of the stomata, altering its opening and closing, could boost the gaseous exchange ability of crop plants, thus improving the yield (Mann, 1999). In cut flowers of *Anthirrhinum majus*, a long-lived genotype was reported to have 53% fewer leaf stomata than a short-lived genotype (Schroeder & Stimart, 2005). A gas exchange study will explore the role of photosynthesis rate in plant growth and stomatal response, so as to investigate their effects on leaf processability.

Initial QTL analysis (Chapter 4) suggested that more small QTL effects would be detected if the population size was increased. Therefore, the extended population with 113 F_{10} RILs was used for further analysis of a few selected traits in the glasshouse. The leaf traits in this glasshouse experiment include chlorophyll content index, photosynthesis rate, transpiration rate, stomatal conductance and cell wall properties.

Analysis of the parents suggested that cell wall properties relate to leaf processability from the previous parent experiment. Cultivated lettuce *L. sativa* with lower elasticity and plasticity had longer shelf life than wild type *L. serriola* (Chapter 3). To date, none of the study has explored the genetic control of cell wall properties. Cell wall extensibility reflects changes in cell wall structure and the genetic control of this is an important aspect to understand the basis of leaf processability. Samples for cell wall properties were also collected from the previous two field trials, in Portugal and the UK. A new Instron apparatus made it possible to measure the cell wall properties in three trials, Portugal, UK and glasshouse experiments. QTL mapping for cell wall properties was compared in different population sizes and different replicates to test the efficiency of QTL detection.

Five long-life and five short-life recombinant lines were selected from the previous two field trials, in Portugal and the UK. These extreme lines were planted in Spain to test the shelf life of these lines in a new environment. These extreme lines were also planted in the UK again in 2005 to test some key leaf traits, which showed association with leaf processability. The samples were collected at the same time to minimise the effects of the time gap on the time-sensitive leaf traits. Further on, in the second UK trial, the differences in the sensory profile between the selected long-life lines and the cultivated control parent were examined. This experiment explored the commercial potential of these five long-life lines for baby leaf salad.

Aims:

i. **To map QTL for photosynthesis and cell wall properties.**

ii. **To test efficiency of QTL detection by increasing mapping population size and reducing the replicate of each line.**

iii. **To test the reliability of the extreme lines selected from the mapping population in terms of shelf life in a different environment.**

iv. **To assess the sensory profile of the long-life lines for commercial potential.**

5.2 Results

5.2.1 QTL mapping for photosynthesis and cell wall properties

5.2.1.1 Trait performances

Chlorophyll content index

Chlorophyll content index (CCI) showed relative chlorophyll content in the leaves of the RIL mapping population. The higher CCI indicates the leaf had relatively higher chlorophyll content. *L serriola* leaf had higher chlorophyll content index (CCI) than *L. sativa* in both glasshouse experiments, which were carried out in May and October of 2004. The difference of CCI between the two parents showed significance at a 95% confidence level only in the first experiment (labeled as CCI-1) (Figure 5.1a). The CCI of *L. serriola* were respectively 16.07 and 11.53 in the first and second experiments, while they were 13.00 and 8.33 respectively for *L. sativa* in two experiments. Although the difference in CCI between two parents in the second experiment (3.20) was higher than that in the first experiment (3.06), this did not show a significance level in the second experiment. This results from the relatively higher standard deviation of *L. serriola* for this trait. The mean CCI of 113 RILs was respectively 17.01 and 13.12 in two experiments. The CCI range showed normal distribution among the 113 lines in both experiments (Figure 5.1b). Generally, the CCI for the mapping population was higher in the first experiment (8.83-30.80) than that in the second experiment (6.23-22.73).

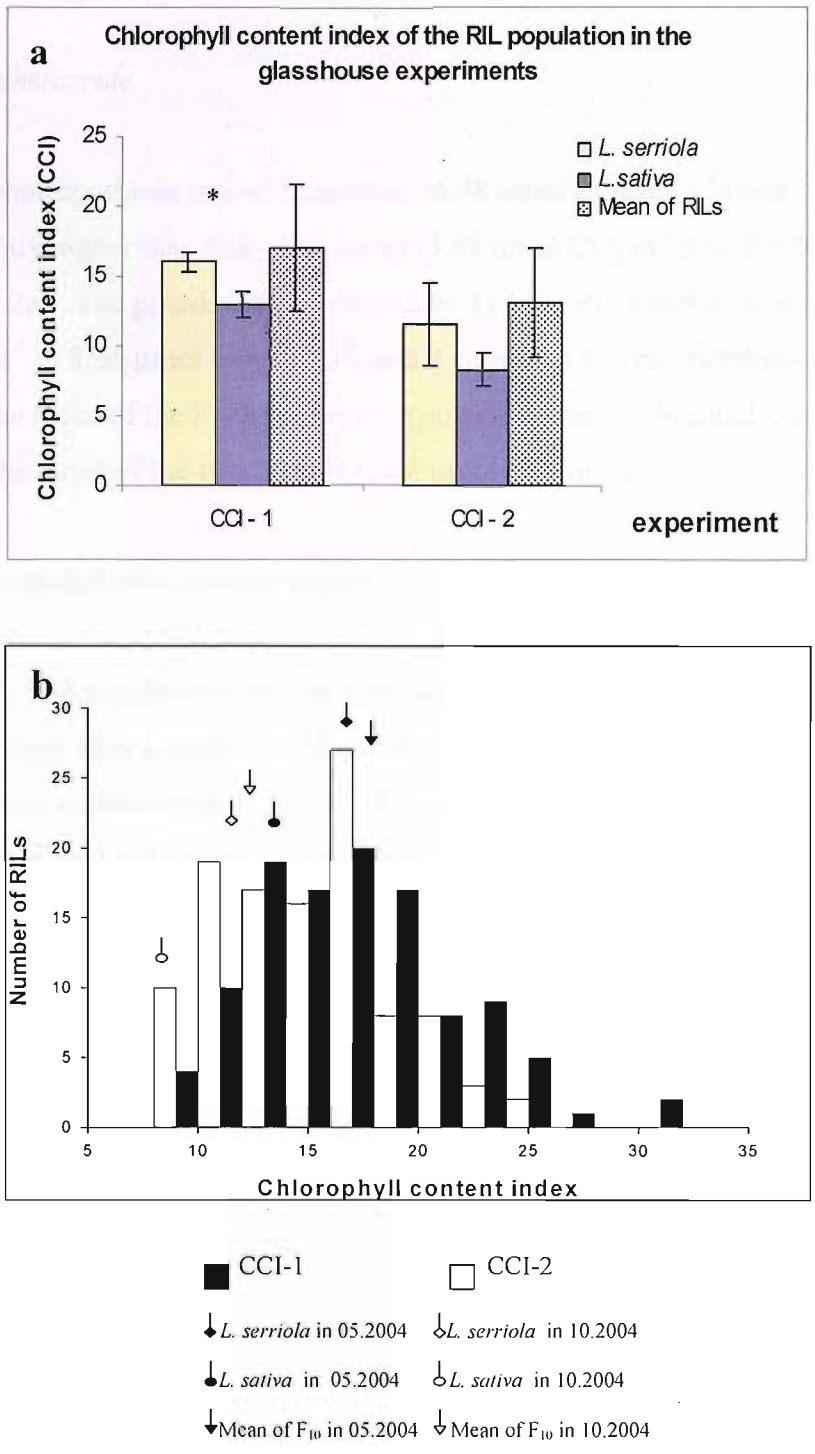


Figure 5.1 Chlorophyll content index (CCI) (a) and its distribution (b) of the F_{10} RIL mapping population in two glasshouse experiments (CCI-1 and CCI-2). Data points are means ± SD, n=3. The result of a one-way ANOVA t-test indicates where there was significance difference between the two parents: * $p < 0.05$.

Photosynthetic CO_2 assimilation

Photosynthetic rate

The net photosynthesis rate of *L. serriola* (6.48 μmol CO_2 m^{-2} s^{-1}) was significantly higher than that of *L. sativa* (3.68 μmol CO_2 m^{-2} s^{-1}) ($P < 0.001$) (Figure 5.2a). The photosynthetic rate of the 113 F_{10} RILs varied from 1.67μmol CO_2 m^{-2} s^{-1} to 8.35 μmol CO_2 m^{-2} s^{-1}, and it showed a normal distribution (Figure 5.2b). The mean of the F_{10} RIL mapping population was 5.04 μmol CO_2 m^{-2} s^{-1}, close to the mean of the two parents (5.08 μmol CO_2 m^{-2} s^{-1}).

Stomatal conductance to water vapor

L. serriola had significantly higher stomatal conductance (0.58 mol H_2O m^{-2} s^{-1}) to water vapor than *L. sativa* (0.26 mol H_2O m^{-2} s^{-1}) ($P < 0.01$) (Figure 5.3a). The stomatal conductance of *L. serriola* was out of the range of 113 F_{10} RILs (0.05 to 0.46 mol H_2O m^{-2} s^{-1}). The mean stomatal conductance of this population was close to that of the parent *L. sativa* (Figure 5.3b). The distribution was not normal for the F_{10} RIL population for this trait.

Transpiration rate

L. serriola had a significantly higher stomatal conductance (5.28 mmol H_2O m^{-2} s^{-1}) to water vapor than *L. sativa* (3.19 mmol H_2O m^{-2} s^{-1}) ($P < 0.01$) (Figure 5.4a). The stomatal conductance of the 113RILs varied from 0.87 to 4.5 mmol H_2O m^{-2} s^{-1}, with mean of 2.74 mmol H_2O m^{-2} s^{-1}. Its distribution in the RIL population was not normal and the mean of transpiration rate of this population was close to that of the parent *L. sativa* (Figure 5.4b), similar to the data for stomata conductance.

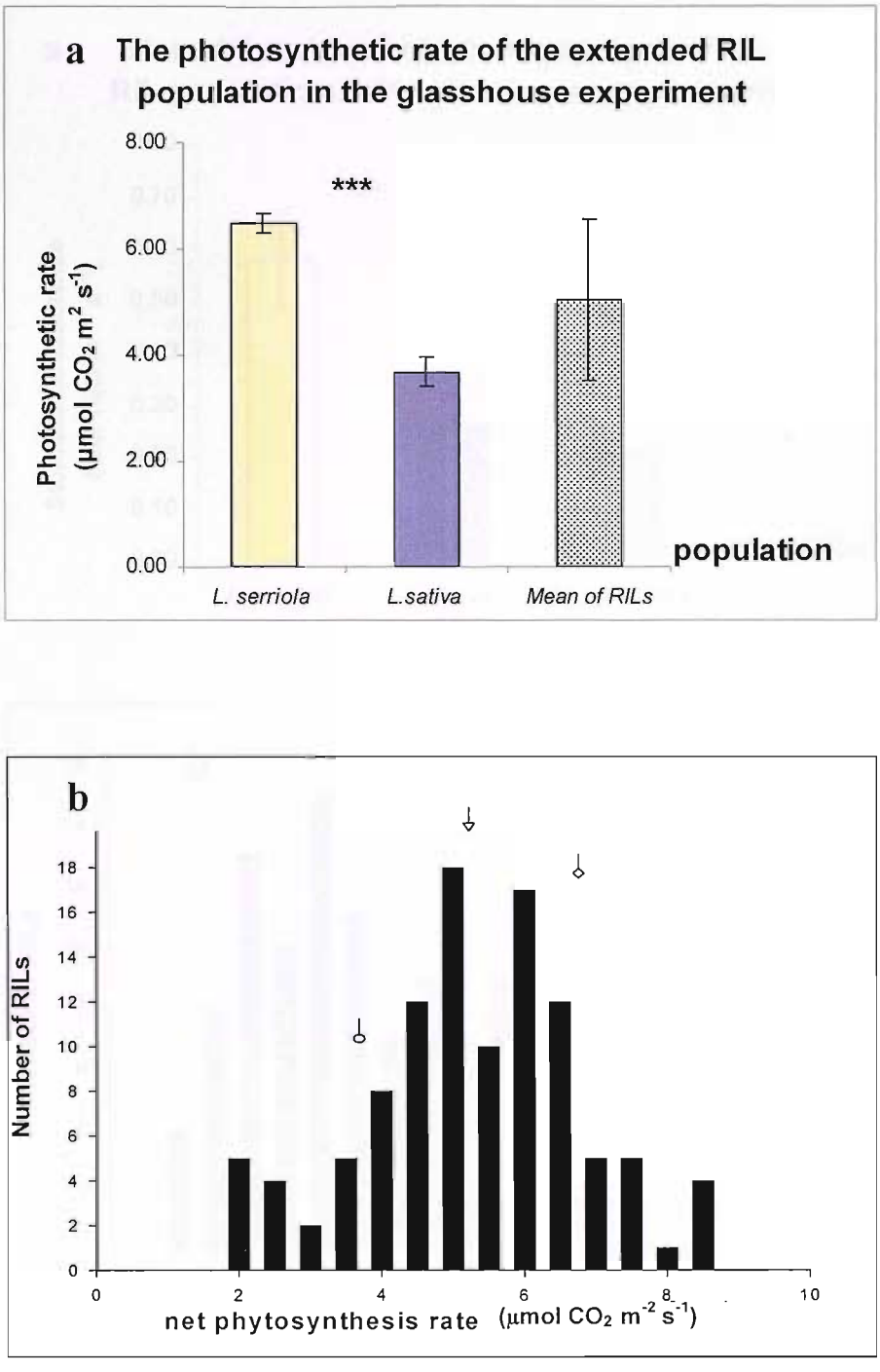


Figure 5.2 Net photosynthesis rate (a) and its distribution (b) for the F_{10} RIL mapping population in the glasshouse experiment. Data points are means ± SD, n=3.The mean values of two parent *L. serriola* (⇩), *L. sativa* (⇩) and of the RILs (⇩) are indicated by arrows. The result of a one-way ANOVA t-test indicates where there was significant difference between the two parents: *** $p < 0.001$.

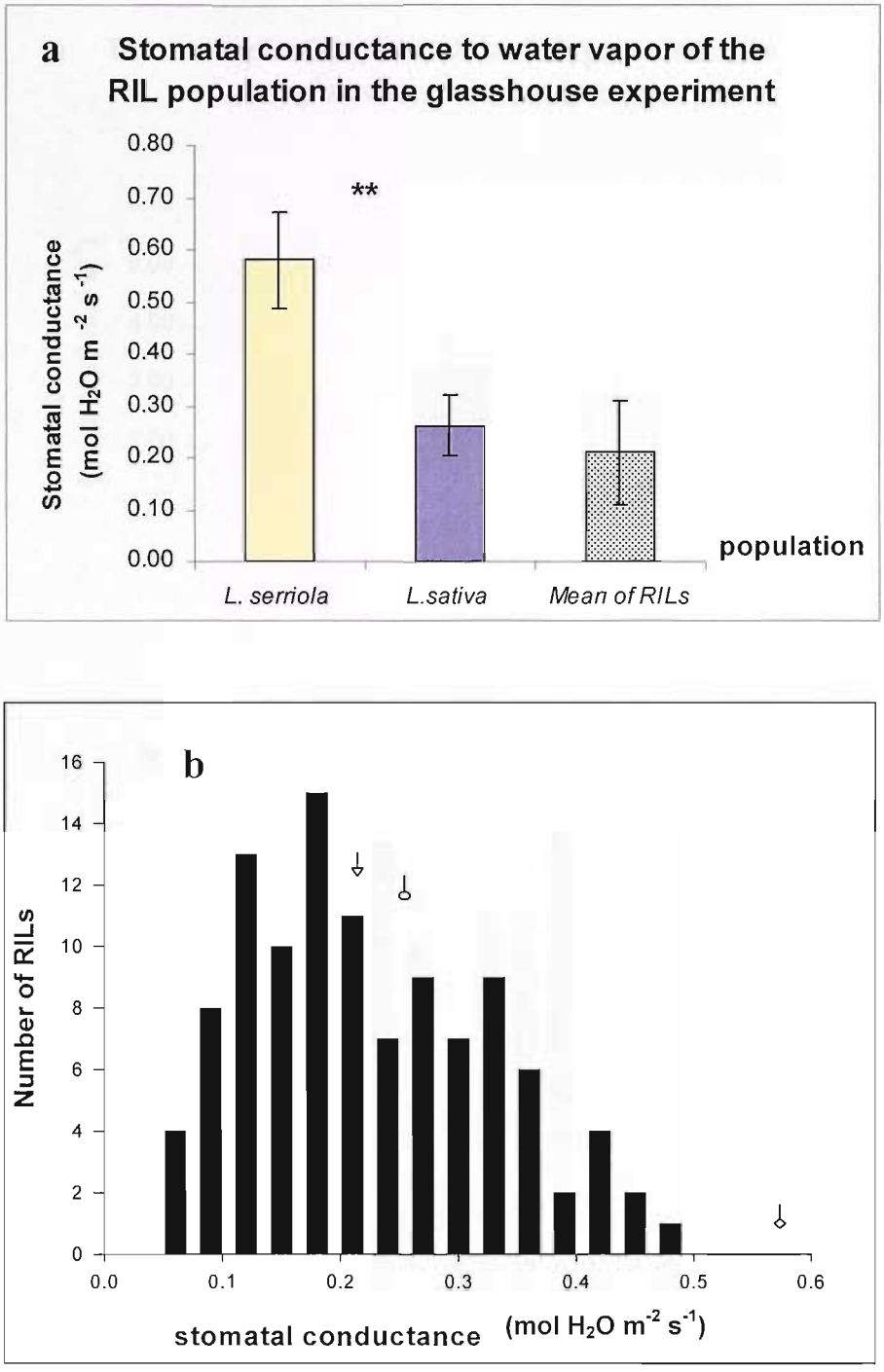


Figure 5.3 Stomatal conductance (a) and its distribution (b) of the F_{10} RIL mapping population in the glasshouse experiment. Data points are means ± SD, n=3.The mean values of two parent *L. serriola* (↓), *L. sativa* (↓) and of the RILs (↓) are indicated by arrows. The result of a one-way ANOVA t-test indicates where there was significant difference between the two parents: ** $p < 0.01$.

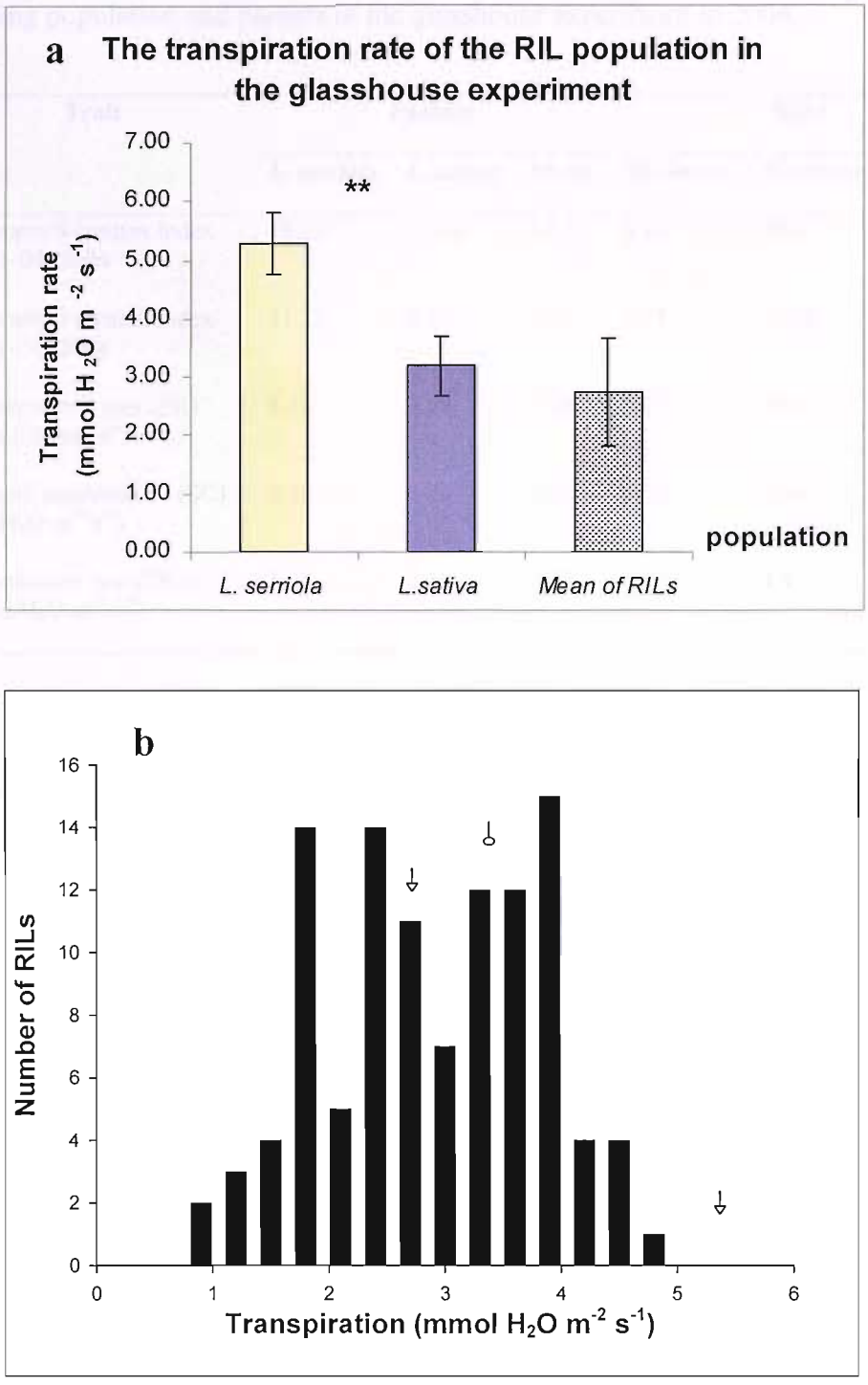


Figure 5.4 Transpiration rate (a) and its distribution (b) of the F_{10} RIL mapping population in the glasshouse experiment. Data points are means ± SD, n=3.The mean values of two parent *L. serriola* (⇩), *L. sativa* (⇩) and of the RILs (⇩) are indicated by arrows. The results of a one-way ANOVA t-test indicated where there was significant difference between the two parents: ** $p < 0.01$.

Table 5.1 Mean and range values for measured photosynthesis traits of 113 F_{10} mapping population and parents in the glasshouse experiment in 2004.

Trait	Parents			RILs		
	L. serriola	*L. sativa*	Mean	Minimum	Maximum	Mean
Chlorophyll content index (CCI) -05, 2004	16.07	13.00	14.53	8.83	30.8	17.01
Chlorophyll content index (CCI) -10, 2004	11.53	8.33	9.93	6.23	22.73	13.13
Photosynthesis rate (PR) (μmol $C0_2$ m^{-2} s^{-1})	6.48	3.68	5.08	1.67	8.35	5.04
Stomatal conductance (SC) (mol H_2O m^{-2} s^{-1})	0.58	0.26	0.42	0.05	0.46	0.21
Transpiration rate (TRA) (mmol H_2O m^{-2} s^{-1})	5.28	3.19	4.23	0.87	4.5	2.7

Leaf cell wall properties

Maximum load

The maximum loads required to break the leaf material of *L. serriola* in three trials were 0.75 N (Portugal); 0.57 N (UK) and 0.51 N (Glasshouse); while for leaf material of *L. sativa*, they were respectively 1.04 N (Portugal), 1.03 N (UK) and 0.83 N (Glasshouse) (Fig. 5.5). The difference in maximum load between the two parents was significant in the UK and glasshouse trials ($p < 0.01$), but not significant in the Portugal trial. The maximum load of the RIL population ranged from 0.53 N to 1.29 N in the Portugal trial; while it was between 0.55 N to 1.40 N in the UK trial and 0.43 N to 1.08 N in the glasshouse trial (Table 5.2). The mean maximum load of the RILs was between the two parents in each trial. They were respectively 0.80 N for the Portugal trial, 0.82 N in the UK trial and 0.69 N in the glasshouse trial. The segregation among the RILs was not normal distribution in any trial.

Elasticity and plasticity

Significant differences in the cell wall properties between two parents were found in the Portugal and glasshouse trials (Figure 5.6a). Comparing the three trials, the UK field trial had the lowest value of the cell wall properties, while the glasshouse trial had the highest value (Table 5.2). The elasticity of *L. serriola* was respectively 3.98% per 0.4 N load for the Portugal trial; 3.00% per 0.4 N load for the UK trial and 9.22% per 0.4 N load for the glasshouse. It was higher than that of *L. sativa* in each trial: 2.39% for Portugal; 1.72% for the UK and 4.53% per 0.4 N load for glasshouse experiment, respectively. There were significant differences in elasticity between two parents in the Portugal and glasshouse trials, but the difference was not at a significant level in the UK trial. The mean elasticity of the RIL population was respectively 4.28% (Portugal); 2.10% (UK) and 6.87% per 0.4 N load (glasshouse). The variable among the RIL population in the glasshouse trial is the highest (from 1.48% to 10.78% per 0.4 N load) and only varied between 1.15% and 3.88% per 0.4 N load in the UK trial. There was a normal distribution of the mapping population for this trait in

the glasshouse trial, while no normal distribution was found for elasticity in either Portugal or the UK trial.

Significant differences in plasticity between two parents were clearly shown in Figure 5.6b. The value range of plasticity had the similar trend as the elasticity among the three trials (Table 5.2). The plasticity of *L. serriola* was respectively 8.17% per 0.4N load for the Portugal trial; 4.14% per 0.4 N load for the UK trial and 21.61% per 0.4N load for the glasshouse. It was higher than that of *L. sativa* in each trial: 2.56% for Portugal; 1.80% for the UK and 4.66% per 0.4 N load for glasshouse experiment, respectively. The mean plasticity of the RIL population was respectively 9.44% (Portugal); 2.81% (UK) and 14.08% per 0.4 N load (glasshouse). The variation among the RIL population in the glasshouse trial is the highest, ranging from 1.58% to 32.85% per 0.4 N load, while it ranged from 4.02% to 20.17% in the Portugal trial and only varied between 1.21% and 6.56% per 0.4 N load in the UK trial. There was a normal distribution of the mapping population for this trait in the glasshouse trial, while no normal distribution was found for elasticity in either Portugal or the UK trial. But the plasticity of each line was quite variable and showed higher value of standard deviation with three replicates of leaf material in the glasshouse trial, whilst there was an average of six replicates of each line in the Portugal trial and the UK trial. The mapping population in the glasshouse includes 113 F_{10} RILs, while only 60 F_9 RILs were selected in the Portugal trial and the UK trial.

The total cell wall extensibility including elasticity and plasticity also showed the similar pattern. All cell wall properties measurements for the RIL mapping population in the three trials are summarized in Table 5.2.

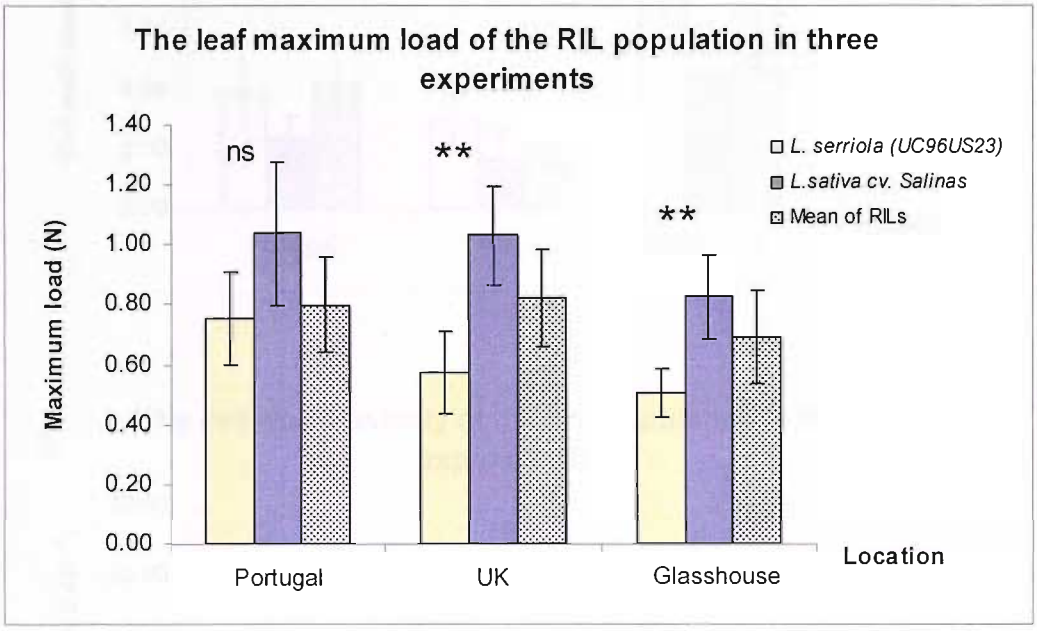


Figure 5.5 The leaf maximum load of the RIL mapping population in three experiments. Data points are means ± SD, the RIL population size is 60 and six replicates of each line in the Portugal trial and the UK trial, while the population size is 112 and three replicates of each line in the glasshouse experiment. The result of a one-way ANOVA t-test indicates where there was significant difference between the two parents: ns, not significant; ** $p < 0.01$.

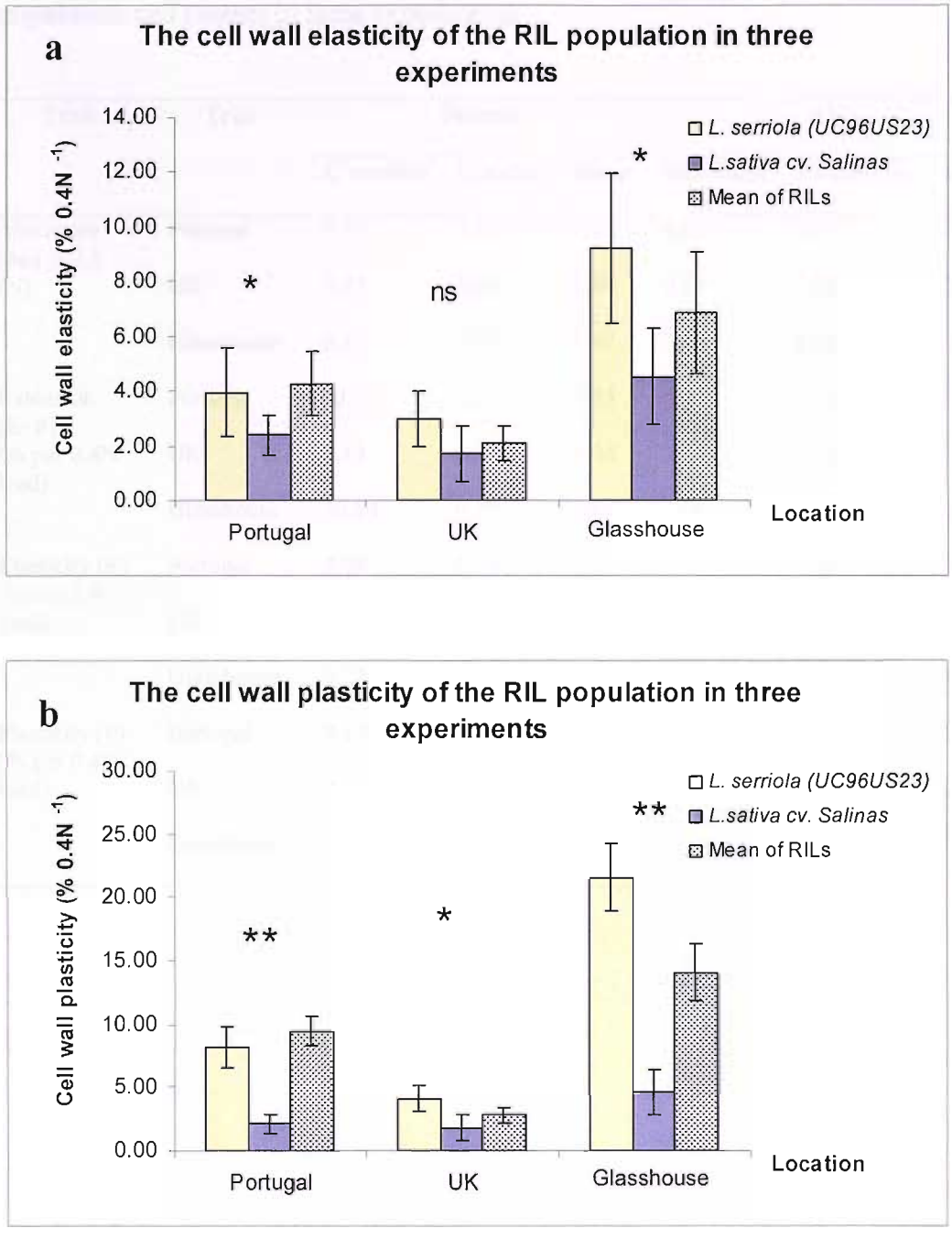


Figure 5.6 The cell wall properties of the RIL population in three experiments: elasticity (reversible extension) (a) and plasticity (irreversible) (b). Data points are means ± SD, the RIL population size is 60 and six replicates of each line in the Portugal trial and the UK trial, while the population size is 113 and three replicates of each line in the glasshouse experiment. The result of a one-way ANOVA t-test indicates where there was significant difference between the two parents: ns, not significant; * $p < 0.05$; ** $p < 0.01$.

Table 5.2 Mean and range values for cell wall properties of the RIL mapping population and parents in three experiments.

Trait	Trial	Parents		RILs			
		L. serriola	*L. sativa*	Mean	Minimum	Maximum	Mean
Maximum load (ML) (N)	Portugal	0.75	1.04	0.89	0.53	1.29	0.80
	UK	0.57	1.03	0.80	0.55	1.40	0.82
	Glasshouse	0.51	0.83	0.67	0.43	1.08	0.69
Extension (E+P) (% per 0.4N load)	Portugal	12.14	4.55	8.35	6.60	27.43	13.70
	UK	7.14	3.53	5.34	2.54	10.15	1.73
	Glasshouse	30.84	9.19	20.01	3.06	43.32	20.95
Elasticity (E) (% per 0.4N load)	Portugal	3.98	2.39	3.19	2.37	8.08	4.28
	UK	3.00	1.72	2.36	1.15	3.88	2.10
	Glasshouse	9.22	4.53	6.87	1.48	10.78	6.87
Plasticity (P) (% per 0.4N load)	Portugal	8.17	2.56	5.37	4.02	20.17	9.44
	UK	4.14	1.80	2.97	1.21	6.56	2.81
	Glasshouse	21.61	4.66	13.14	1.58	32.85	14.08

5.2.1.2 Correlation among the traits

There was significant genotypic variation among the 113 RILs in all the traits measured ($P<0.05$). Correlations between the traits in the glasshouse experiment were calculated using Pearson's correlation coefficient analysis (Table 5.3). The chlorophyll content index had a significant correlation in two experiments ($p < 0.001$). CCI in the second experiment (CCI-2) showed positive significant correlation with cell wall properties ($P < 0.05$), but a slightly negative correlation with maximum load without being at a significant level ($P < 0.05$ level). The photosynthesis rate (PR) had a higher significant positive correlation with stomatal conductance (SC) ($P < 0.001$) and transpiration rate (TR) ($P < 0.001$). SC had a strong correlation with TR; the correlation value is 0.971 ($P< 0.001$). SC and TR both showed positive significant correlations with cell wall properties (elasticity (E) and plasticity (P)) ($P < 0.01$). There was a strong significant relationship between E and P (0.873, $P < 0.001$). Maximum load (ML) negatively correlated with E or P ($P < 0.001$). No direct correlation was found between all the above traits and shelf life because the shelf life of the mapping population has not been done in the glasshouse experiment. If the average shelf life of the population in the two field trials was used, no significant correlation was found probably because of the different environments and population sizes used in the study. However, the parent experiment did show that *L. sativa*, with longer shelf life, had lower plasticity than the wild type *L. serriola*, with shorter shelf life. The relationship between cell wall properties and leaf processability was also reported by Clarkson et al. (2003).

Table 5.3 Pearson's correlation coefficient of the assessed traits of the extended mapping population in the glasshouse experiment.

Trait[a]	CCI-1	CCI-2	PR	SC	TRA	ML	E+P	E
CCI-1	1							
CCI-2	0.412***[b]	1						
PR	-0.093	0.077	1					
SC	-0.084	0.125	0.456***	1				
TR	-0.114	0.124	0.466***	0.971***	1			
ML	0.016	-0.172	0.028	-0.094	-0.060	1		
E+P	0.047	0.252**	0.043	0.408***	0.374***	-0.618***	1	
E	0.084	0.232*	0.002	0.309**	0.279**	-0.690***	0.923***	1
P	0.035	0.250**	0.054	0.425***	0.391***	-0.578***	0.993***	0.873***

Notes:

[a]Trait abbreviation: CCI-1: chlorophyll content index measured in the first glasshouse experiment in 05.2004; CCI-2: chlorophyll content index measured in the second glasshouse experiment in 10.2004; PR: net photosynthetic rate (μmol CO_2 m^{-2} s^{-1}); SC: stomatal conductance to water vapour (mol H_2O m^{-2} s^{-1}); TR: transpiration rate (mmol H_2O m^{-2} s^{-1}); ML: maximum load to break down the leaf material (N); E+P: total cell wall extension, including elasticity and plasticity; E: elasticity, reversible cell wall properties (% per 0.4N load); P: plasticity, irreversible cell wall properties (% per 0.4 N load).

[b] * Correlation is significant at the 0.05 level (2-tailed); **, correlation is significant at the 0.01 level; and ***, correlation is significant at the 0.001 level.

5.2.1.3 QTL analysis

QTL for chlorophyll content index

One significant QTL was detected for chlorophyll content index in the first experiment (LOD = 3.26), accounting for 14.66% of phenotypic variance. It located on LG 3: 63-66 cM (Table 5.4) and the nearest marker is E33/M59-F-208. QTL cartographer plot (Figure 5.7) shows a similar trend along the linkage groups for CCI in the two experiments. There was no significant QTL identified in the second experiment, but a peak was detected in a similar location LG 3: 56-63 cM (LOD =2.5). The nearest marker is E35/M60-F-357, next to the marker identified in the first experiment. Here, QTL is defined as putative (possible) QTL when its LOD is over 2.5, but below the 3.26 significance threshold ($P < 0.05$). Three putative QTL were detected in the second experiment. They located respectively at LG3: 56-63 cM; LG3: 72-80cM and LG4: 34-37 cM. They explained 10.13%, 9.98% and 8.31% of phenotypic variation, individually. The cultivated (*L. sativa*) allele on the QTL region on LG 2 increased CCI values in both experiment, but the wild type allele on the putative QTL region on LG 3 increases the trait value in the second experiment.

QTL for stomatal conductance and transpiration

Neither significant QTL nor putative QTL were detected for photosynthesis rate in this experiment. Two significant QTL for stomatal conductance were identified, located on LG 4: 39-48 cM and LG 6: 137-140 cM (Figure 5.8a). The QTL respectively contributed to explain 12.16% and 10.64% of phenotypic variance of the mapping population. The nearest markers were E51/M49-F-259 and 1A09-410<N>, respectively (Table 5.4). The QTL for transpiration rate had similar trends along the linkage group to QTL for stomatal conductance. Only one QTL over the significant threshold level was identified for plant transpiration rate, located on LG 4: 35-42 cM (Figure 5.8b). About 11% phenotypic variance of this population was explained by this QTL. The cultivated allele in these detected QTL increase stomatal conductance and transpiration values in this experiment.

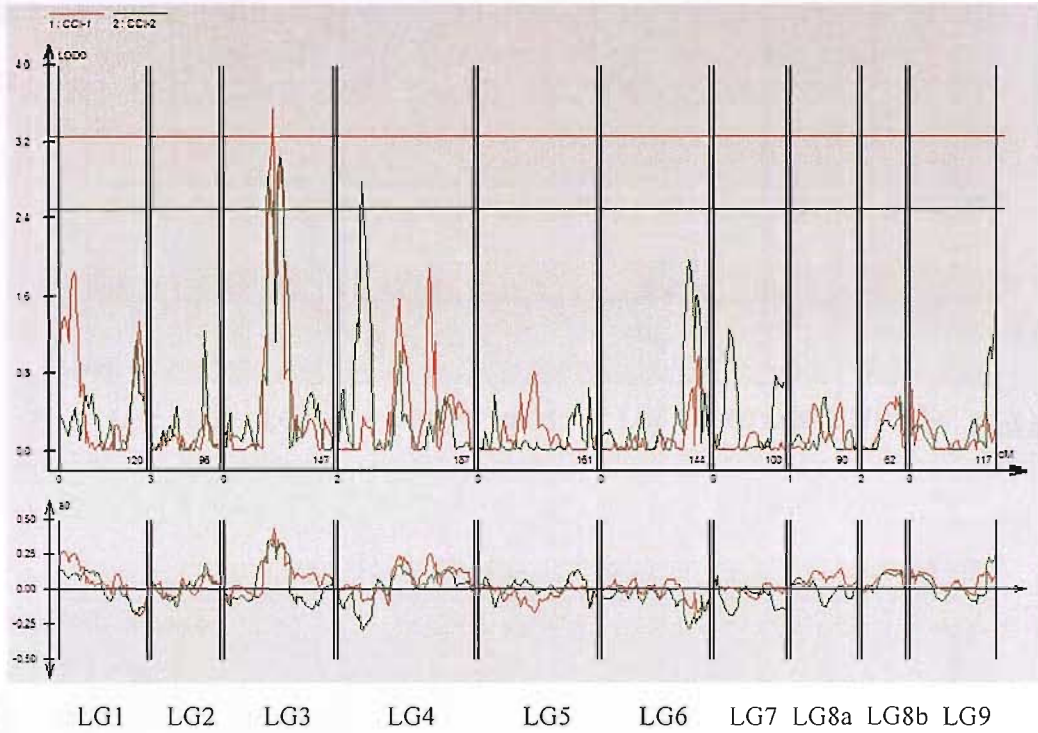


Figure 5.7 QTL cartographer plots for chlorophyll content index of F_{10} RIL mapping population. The LOD score is indicated on the upper Y axis. The X axis represents the ten linkage groups of F_{10} recombinant inbred line map arranged end-to-end. The red line (LOD = 3.26, $P < 0.05$) represent the significant threshold for CCI-1. The green line (LOD=2.5) represent the putative QTL threshold for CCI-2, as no significant QTL detected for CCI-2. The lower graph in the plot shows the additive effect from which parental allele that causes an increase in the trait value. Positive values indicate the cultivated (*L. sativa*) allele increased trait values and negative values indicate wild type (*L. serriola*) allele increased trait values.

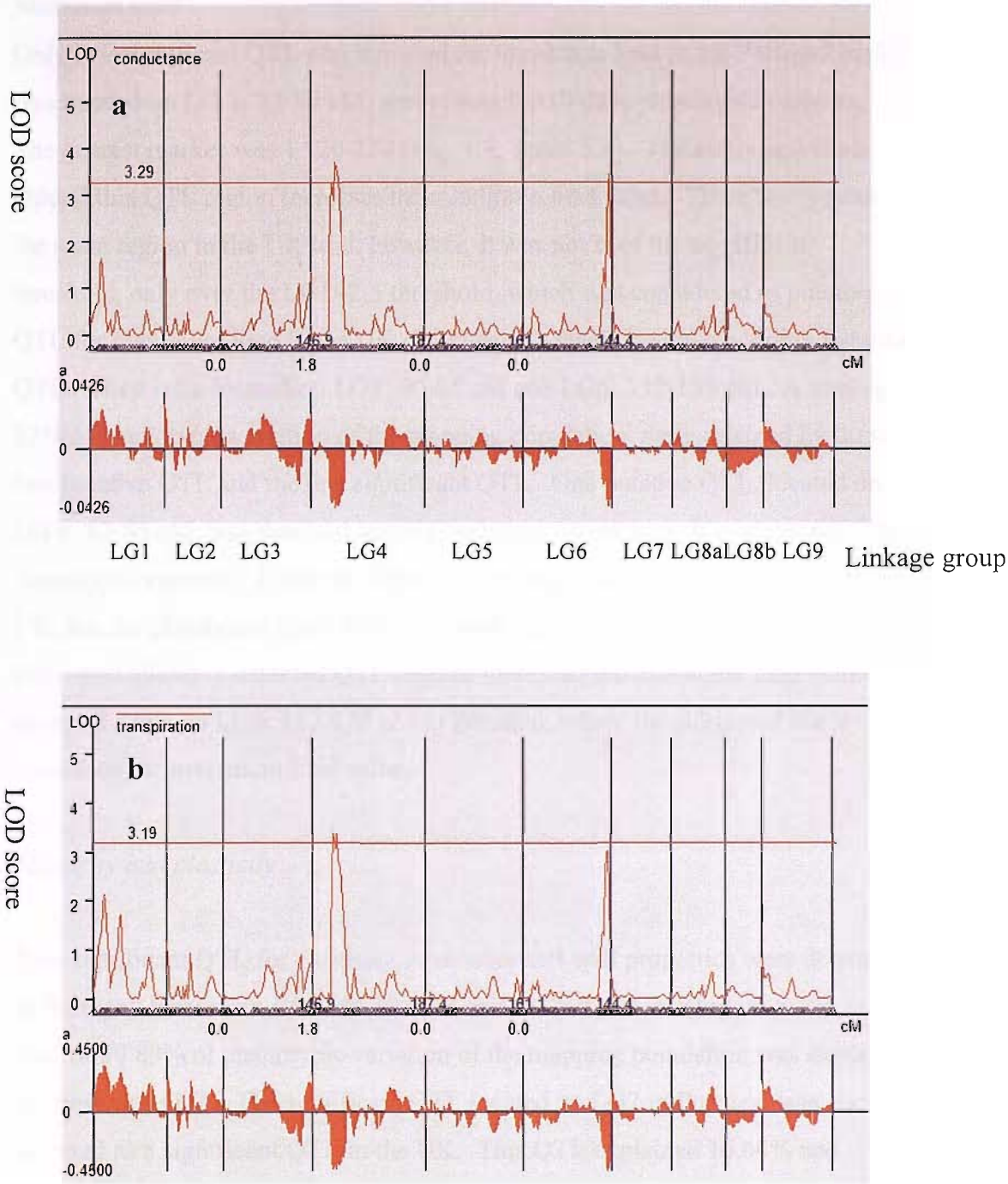


Figure 5.8 QTL cartographer plots for transpiration rate (a) and stomatal conductance (b) of F_{10} RIL mapping population. Notes see Figure 5.7.

QTL for cell wall properties

Maximum load

Only one significant QTL was detected for maximum load in the Portugal trial. It was located on LG 2: 82-88 cM, accounting for 13.08% phenotypic variance. The nearest marker was 1A20-178 (Fig. 5.9, Table 5.4). The cultivated allele around this QTL region increases the maximum load value. There was a peak in the same region in the UK trial; however, it was not over the significant threshold, only over the LOD-2.5 threshold, which was considered as putative QTL for maximum load in the UK. In Portugal, there were two addition putative QTL. They were located on LG1: 90-96 cM and LG6: 132-135 cM. A total of 33% of phenotypic variation of the mapping population was explained by these two putative QTL and the one significant QTL. One putative QTL, located on LG 4: 82-85 cM, was detected in the glasshouse experiment. It explained 8.92% phenotypic variance. From the Figure 5.9, Portugal has similar QTL trend as the UK, but the glasshouse has a different trend than the above two trials. The cultivated alleles at detected QTL regions increased the maximum load values except the one on LG6: 132-135 cM in Portugal, where the cultivated allele decreased the maximum load value.

Elasticity and plasticity

Two significant QTL for elasticity, reversible cell wall properties were detected in Portugal, located on LG1: 84-103 cM and LG2: 82-91cM (Figure 5.10). A total of 30.89% of phenotypic variation of the mapping population was explained by these two QTL. The significant QTL located on LG2 in Portugal was also detected as a significant QTL in the UK. This QTL explained 16.64% and 12.04% of phenotypic variance of the mapping population in Portugal and the UK, respectively. The nearest marker was 1A20-178-P2 for this QTL. The other significant QTL on LG1 in Portugal was only considered as putative QTL in the UK, accounting for 12.04% of phenotypic variation (Table 5.4) in the UK. There was one more putative QTL on LG 7: 82-85 cM in the UK. In the glasshouse, only one putative QTL was detected on LG 5: 134-136 cM, accounting for about 10% of phenotypic variance. This putative QTL was

identified neither in Portugal nor the UK. The cultivated alleles at these detected QTL increased the elasticity value.

In terms of plasticity, irreversible cell wall properties, one significant QTL and one putative QTL were respectively identified on LG1: 86-102 cM and LG 2: 83-90 cM in Portugal. These two QTL explained about 27.77% of phenotypic variance of the mapping population in Portugal. These two QTL positions were the same as the QTL for elasticity in Portugal, but they were not detected in the UK or glasshouse trials. In the UK, one significant QTL at LG 5: 59-62 cM and two putative QTL at LG 3: 119-121 and LG 4: 161-170 cM were identified. They contributed together to explain 36.37% phenotypic variance. No QTL was detected in the glasshouse experiment. The cultivated allele increases the plasticity value at most of QTL regions except the *L. sativa* alleles at LG 4: 161-170 cM decreased the plasticity value. One significant QTL for total extensibility was located on LG 1 at 86-102 cM in Portugal and at 49-51 cM in the UK. The nearest common putative QTL at LG 2: 84-90. There is an additional putative QTL on LG 7: 84-85 cM in the UK. No QTL for total extensibility was detected in the glasshouse.

A total of four QTL with significant effects and six putative QTL with lower LOD threshold (LOD = 2.5) were identified for all leaf traits assessed in the glasshouse experiment. They were located on LG3, 4, 5 and 6 (Table 5.3). Each QTL accounted for 8.31% - 14.66% of the phenotypic variation in this population. The distributions of these significant QTL (closed bar) and putative QTL (open bar) are indicated in the molecular genetic linkage map (Figure 5.11). For cell wall properties, a total of 8 with significant effects distributed on seven out of the ten linkage groups (Table 5.4). Each QTL accounted for 10.28% - 18.98% of the phenotypic variation in this population. Thirteen putative QTL were also identified. The distributions of these significant QTL (closed bar) and putative QTL (open bar) are indicated in the molecular genetic linkage map (Figure 5.12).

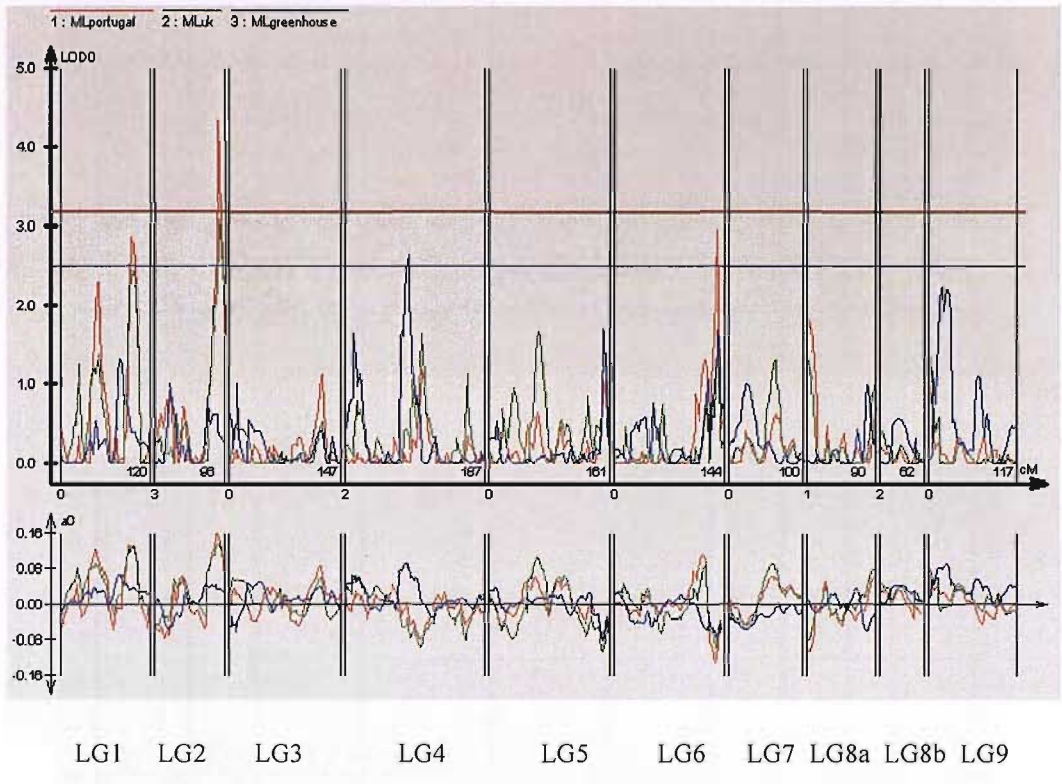


Figure 5.9 QTL cartographer plot for leaf material maximum load of the RIL mapping population detected in three trials. The log likelihood ration scale is indicated on the Y axis. The X axis represents the ten linkage groups of the recombinant inbred line map arranged end-to-end as shown in the vertical grid lines. The red line represents the QTL detected in Portugal; the green line represents the QTL detected in the UK; and the blue line represents the QTL detected in the glasshouse experiment. The horizontal red and green lines represent the significant threshold for the QTL detected in the corresponding field trial, Portugal and the UK, respectively. The horizontal green line represents the putative QTL threshold (LOD=2.5). The lower graph in the plot shows the additive effect from which parental allele that causes an increase in the trait value. Positive values indicate the cultivated (*L. sativa*) allele increased trait values and negative values indicate wild type (*L. serriola*) allele increased trait values.

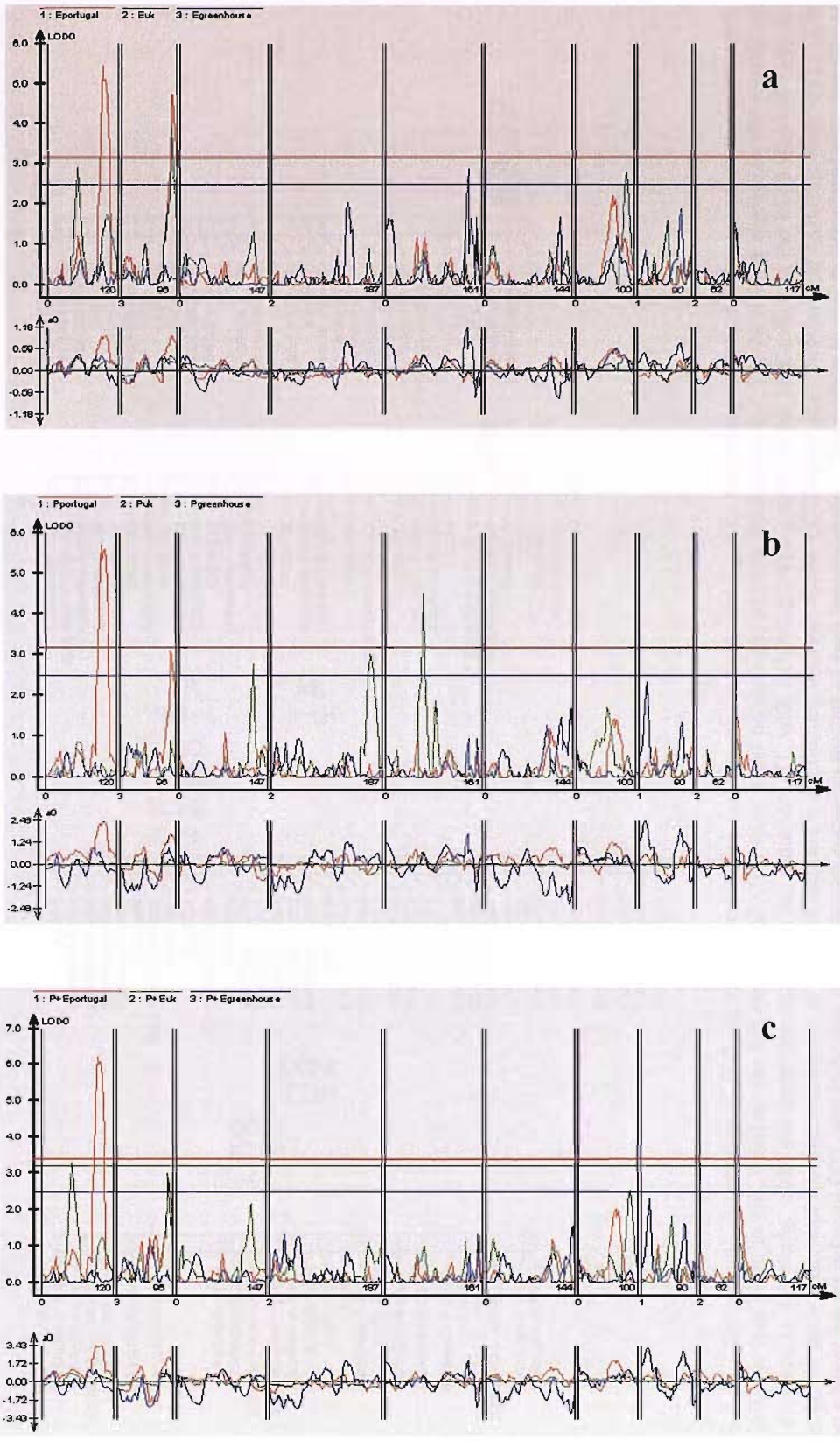


Figure 5.10 QTL cartographer plot for leaf cell wall properties of the RIL mapping population detected in three trials: Elasticity (a); Plasticity (b) and total cell wall extensibility (elasticity + plasticity) (c). Note: see Figure 5.9.

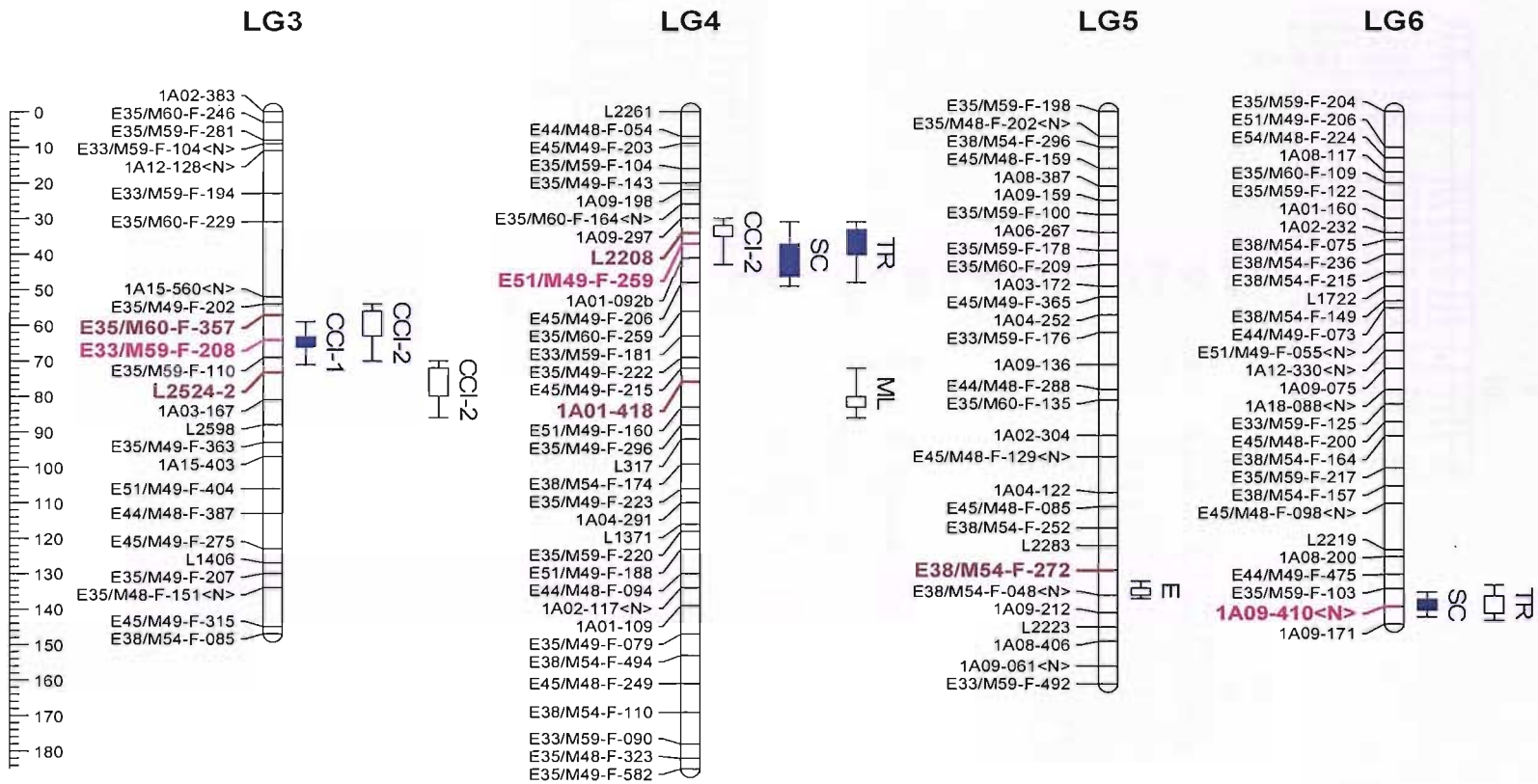


Figure 5.11 Chromosome distribution of QTL in the molecular linkage map of the extended F_{10} RIL mapping population in the glasshouse experiments. Map position was given in cM, listed on the left margin and QTL were listed on the right of each linkage group. The closed bar represents the significant QTL detected in this study, while the open bar represents the putative QTL with LOD=2.5. The length of the bars indicates the LOD interval over the threshold for each QTL, and the line extensions of the bars indicate a confidence interval of one LOD lower than threshold of LOD for each QTL. The nearest marker for the relevant QTL was indicated in the relevant colour on the left of each linkage group. The marker in pink is associated with significant QTL and the marker in brown is associated with putative QTL. For trait abbreviations see Table 5.3.

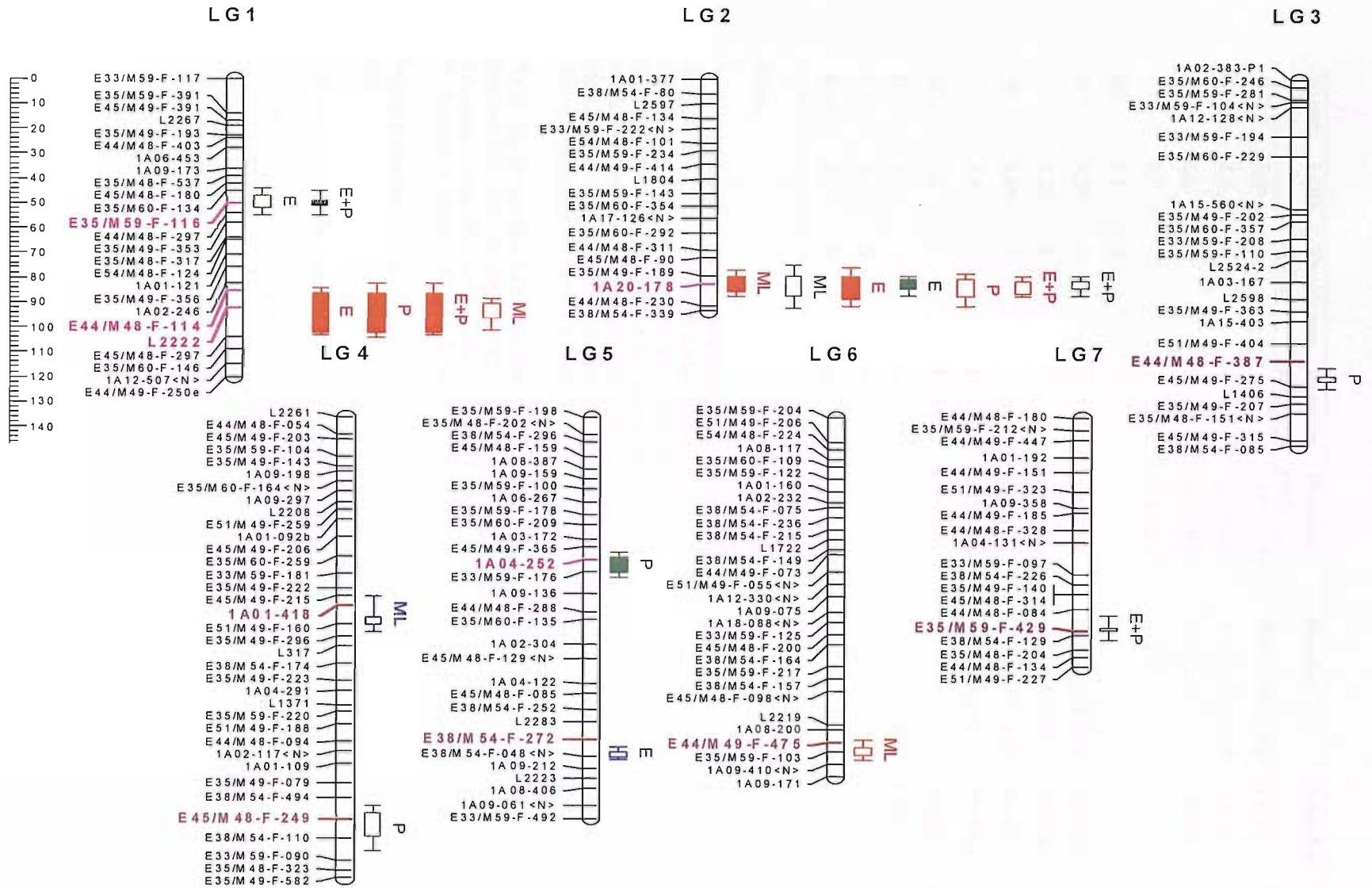


Figure 5.12 Chromosome distribution of QTL for leaf cell wall properties in the molecular linkage map of the RIL mapping population in three trials. The red bars represent the QTL detected in Portugal; the green bars represent the QTL detected in the UK; and the blue bars represent QTL detected in the glasshouse. The trait abbreviation see note in Table 5.4 and the other notes see Fig. 5.10.

Table 5.4 Significant and putative QTL detected by composite interval mapping for all leaf traits in the glasshouse experiment.

Trait[a]	LOD[b]	Significant QTL	Putative QTL	Position[c] (cM)	Marker[d]	Additive[e]	Variance[f] (%)
CCI-1	3.26	1		LG3:63-66	E33/M59-F-208	2.9171	14.66
CCI-2	2.5		1	LG3:56-63	E35/M60-F-357	1.9832	10.13
	2.5		2	LG3:72-80	L2524-2	1.8620	9.98
	2.5		3	LG4:34-37	L2208	-1.7108	8.31
PR	2.5	0	0				
SC	3.29	1		LG4:39-48	E51/M49-F-259	-0.0426	12.16
	3.29	2		LG6: 137-140	1A09-410<N>	-0.0418	10.64
TR	3.19	1		LG4:35-42	E51/M49-F-259	-0.4425	10.97
	2.5		1	LG6: 136-141	1A09-410<N>	-0.4358	9.90
ML	2.5		1	LG4: 82-85	1A01-418	0.0893	9.17
E	2.5		1	LG5: 134-135	E38/M54-F-272	1.180	9.55
P	2.5	0	0				
E+P	2.5	0	0				

Notes:

[a]Trait abbreviation: CCI-1: chlorophyll content index measured in the first glasshouse experiment in 05.2004; CCI-2: chlorophyll content index measured in the second glasshouse experiment in 10.2004; PR: net photosynthetic rate (μmol $C0_2$ m^{-2} S^{-1}); SC: stomatal conductance to water vapour (mol H_2O m^{-2} s^{-1}); TR: transpiration rate (mmol H_2O m^{-2} s^{-1}); ML: maximum load to break down the leaf material (N); E+P: total cell wall extension, including elasticity and plasticity; E: elasticity, reversible cell wall properties (% per 0.4N load); P: plasticity, irreversible cell wall properties (% per 0.4 N load).

[b]LOD: log of the Odds score. To convert LR to LOD values, LOD= 0.217 LR. If LOD > 2.5, the detected QTL list is significant QTL (denoted as Sig. QTL in the table), which is over the threshold estimated by permutation analysis of each trait using 1000 iterations, while if LOD =2.5, the QTL listed in the table is putative QTL (denoted as Sug. QTL in the table).

[c]Position indicated the significant QTL interval over the threshold estimated by permutation analysis of each trait using 1000 iterations or putative QTL interval over the threshold of 2.5.

[d]Markers that are the nearest marker to the QTL

[e]Additive: Additive effect indicate which parental allele that cause an increase in the trait value. Positive values indicate the cultivated (*L. sativa*) allele increased trait values and negative values indicate wild type (*L. serriola*) allele increased trait values.

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

Table 5.5 Significant (Sig.) and putative (Put.) QTL detected by composite interval mapping for leaf cell wall properties in three trials.

Trait[a]	Trial	LOD[b]	Sig. QTL	Put. QTL	Position[c] (cM)	Marker[d]	Add[e]	Var[f] (%)
ML	Portugal	3.24	1		LG2: 82-88	1A20-178	0.1556	13.08
		2.5		1	LG1:90-96	E44/M48-F-114	0.1290	9.29
		2.5		2	LG6: 132-135	E44/M49-F-475	-0.1289	8.96
	UK	2.5	0	1	LG2: 82-90	1A20-178	0.1291	8.92
	Glasshouse	2.5	0	1	LG4: 82-85	1A01-418	0.0893	9.17
E	Portugal	3.24	1		LG1: 84-103	E44/M48-F-114	0.9333	16.64
		3.24	2		LG2: 82-91	1A20-178	0.8794	14.25
	UK	3.16	1		LG2: 83-87	1A20-178	0.4070	12.04
		2.5		1	LG1: 47-52	E35/M59-F-116	0.3641	9.10
		2.5		2	LG7: 82-85	E35/M59-F-429	0.3585	8.55
	Glasshouse	2.5		1	LG5: 134-136	E38/M54-F-272	1.180	9.55
P	Portugal	3.23	1		LG1: 86-102	L2222	2.4066	18.98
		2.5		1	LG2: 83-90	1A20-178	1.6205	8.77
	UK	3.19	1		LG5: 59-62	1A04-252	0.7558	15.72
		2.5		1	LG3: 119-121	E44/M48-F-387	0.5311	10.20
		2.5		2	LG4: 161-170	E45/M48-F-249	-0.5433	10.45
	Glasshouse		0	0				
E+P	Portugal	3.35	1		LG1: 86-102	L2222	3.3413	18.71
		2.5		1	LG2: 84-90	1A20-178	2.2060	7.99
	UK	3.21	1		LG1: 49-51	E35/M59-F-116	0.9296	10.28
		2.5		1	LG2: 84-87	1A20-178	0.8491	8.99
		2.5		2	LG7: 84-85	E35/M59-F-429	0.8133	7.64
	Glasshouse		0	0				

Notes:

[a]Trait abbreviation: ML: maximum load to break down the leaf material (N); E: elasticity, reversible cell wall properties (% per 0.4N load); P: plasticity, irreversible cell wall properties (% per 0.4 N load); E+P: total cell wall extension, including elasticity and plasticity. Other notes see Table 5.3.

5.2.2 Detailed analysis of extreme lines

5.2.2.1 Shelf life

The average germination rate over the extreme lines was only about 20% in the Spanish field trial. There was snow two weeks after sowing the seeds. It was the coldest winter in Spain in the 20 year weather record. The temperature ranged from 0°C to 22 °C and the precipitation varied between 5.6 – 34 mm during the growing period (http://weather.co.uk). Although the germination rate was low compared to the Portugal trial and the UK trial, 180 plants per line were planted in the Spanish trial to secure enough material for the shelf life experiment. The individual plant images are shown in Figure 5.13. The leaf shape of Line 112 was more like cultivated lettuce with a curly and folded shape, while that of Line 15 and 105 appeared more like wild lettuce with a dark green and flat shape. Line 13 had picky edge as wild lettuce.

The shelf life of RIL lines was variable, ranging from 6 days to 14.4 days in Portugal, from 5 days to 13.4 days in the UK, and from 7.3 days to 14.6 days in Spain, respectively (Table 5.6, Figure 5.14). Line 112 had the longest shelf life of 14.6 days in Spain and 13.4 days in the UK, but it only lasted 10 days in Portugal. Line 1 was the best line in Portugal, lasting 14.4 days, and it lasted 12.8 days in the UK and 11.8 days in Spain, respectively. The cultivated parent, *L. sativa*, had a shelf life of 12.60 days in Portugal; 9.20 days in the UK; and 11.40 days in Spain respectively. It consistently lasted longer than the wild type parent, *L. serriola*, which had a shelf life of 9.33 days in Portugal; 7.20 days in the UK; and 9.11 days in Spain, respectively. Line 13 was the worst line in Portugal, only lasting 7.25 days and Line 89 was the worst line in the UK and the Spain field trials, lasting 5 days and 7.33 days, respectively. Generally, the shelf life of the population in the Portugal and Spain trial was higher than that of the UK trial. The difference between the long-life lines and short-life lines was significant in all three trials. Among the three trials, the difference between long-life and short-life lines was the smallest in Spain and the highest in Portugal. On average of the three trials, Line 1 was the best one, lasting 13.01 days, followed by Line 15, 112, 5 and 74 in order.

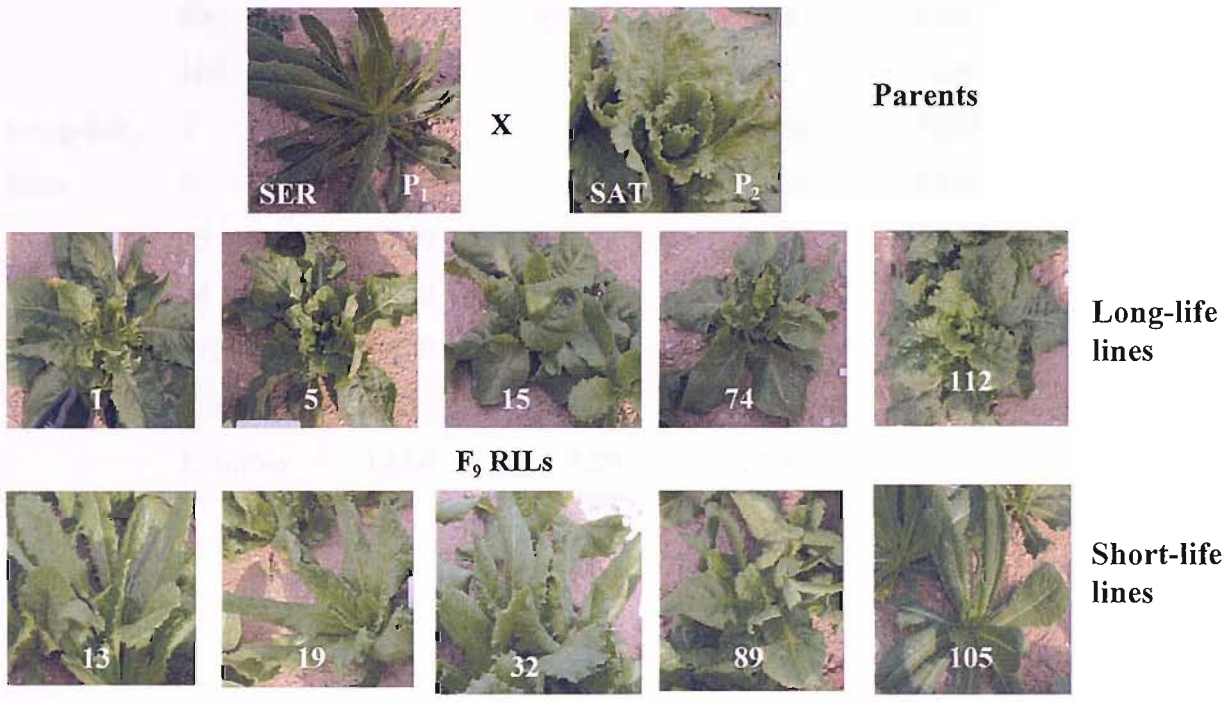


Figure 5.13 Leaf images of the extreme lines and two parents in the Spanish field trial. Wild lettuce *L. serriola* (SER, P_1) and cultivated lettuce *L. sativa* (SAT, P_2) are the parent lettuce. The extreme RIL number is indicated in each image.

Table 5.6 Shelf life of the extreme lines and two parents in three field trials

Line		**Portugal (days)**	**UK (days)**	**Spain (days)**	**Mean (days)**
Short-life	13	7.25	6.20	9.07	7.51
lines	19	8.00	5.60	7.80	7.13
	32	8.20	5.60	8.30	7.36
	89	8.40	5.00	7.30	6.91
	105	7.50	5.40	8.90	7.26
Long-life	1	14.40	12.80	11.80	13.01
lines	5	12.60	12.40	12.90	12.63
	15	12.40	12.00	13.60	12.68
	74	13.40	11.40	10.30	11.71
	112	10.00	13.40	14.60	12.68
Parents	*L. serriola*	9.33	7.20	9.10	8.54
	L. sativa	12.60	9.20	11.40	11.07

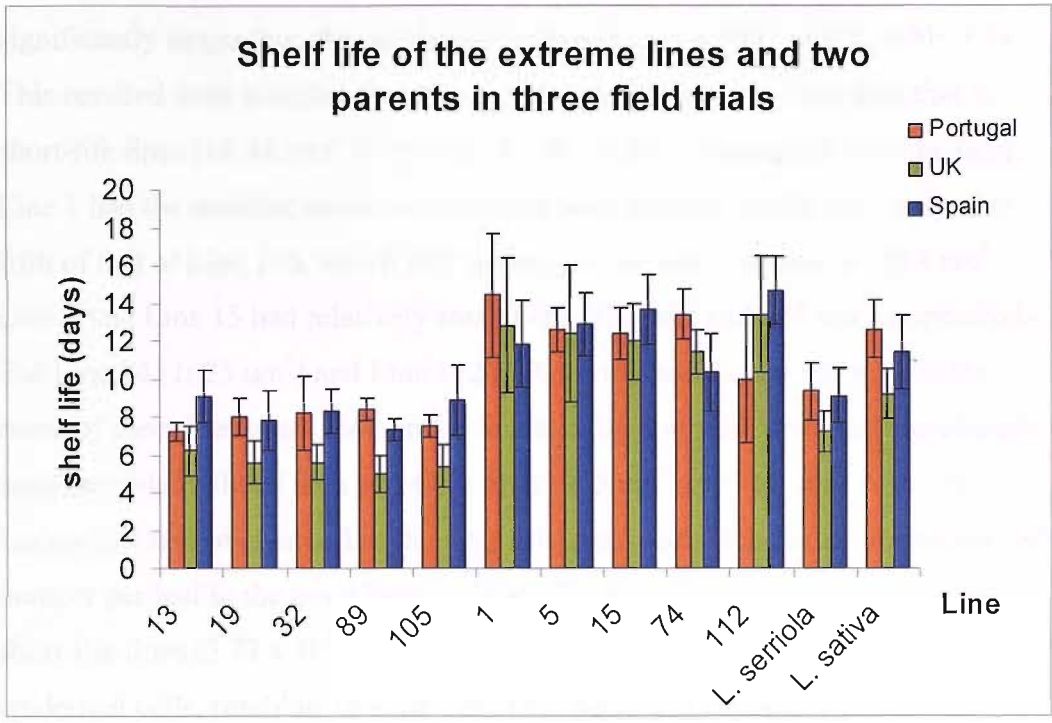


Figure 5.14 Shelf life of the extreme lines and two parents in three field trials. Data points are means ± SD, n=5 (Portugal and UK trials), n=10 (Spain trial). The leaf material was kept in pack at 7 °C fridge and shelf life was determined through visual assessment. When breakdown, bruising or damage was seen in the pack, the bag was rejected.

5.2.2.2 Leaf trait measurements

All the trait measurements with a significant difference between short-life and long-life lines are summarised in Table 5.6. Although Line 89 had a relatively big leaf area, Line 13 and Line 105 were much smaller than the average of leaf area. On average, leaf area of long-life lines (mean = 5510 mm^2) was significantly larger than that of short-life lines (mean = 5093 mm^2) (Table 5.7). This resulted from a higher absolute growth rate in long-life lines than that in short-life lines (14.44 $mm^2\ h^{-1}$ vs $mm^2\ h^{-1}$, $P < 0.01$). Among the extreme lines, Line 1 had the smallest epidermal cell area with average of 422 um^2, only one-fifth of that of Line 105, which had the largest cell with average of 2188 um^2. Line 5 and Line 15 had relatively small cells, 792 um^2 and 635 um^2, respectively. But Line 74 (1623 um^2) and Line 112 (1404 um^2) had similar cell area as the mean of short-life lines (1540 um^2). Statistically, long-life lines had significantly smaller epidermal cell than short-life lines (975 um^2 vs 1540 um^2, $P < 0.001$). Larger leaf and smaller cell in the long-life lines resulted in higher epidermal cell number per leaf in the good lines (7.36×10^6 cells per leaf), about twice as the short-life lines (3.77×10^6 cells per leaf). Line 1 had the highest number of epidermal cells, reaching 14×10^6 cells per leaf, which is more than six times of Line 105 (2.15×10^6 cells per leaf).

Stomatal density of long-life lines was very variable, ranging from 65 mm^{-2} to 319 mm^{-2}, while stomatal density of short-life lines was less variable as long-life lines, ranging between 85 mm^{-2} and 143 mm^{-2}. When the difference in epidermal cell area was accounted for, stomatal index was less variable among the lines. The mean of stomatal index of short-life lines (12.82%) was significantly higher than that of long-life lines (10.40%).

In terms of cell wall maximum load to break down the leaf material, long-life lines had a significantly higher value than short-life lines (0.89 N vs 0.76 N). Line 74 had the highest value of maximum load of 0.96 N, while Line 89 had the lowest value of 0.64 N. No significant difference in cell wall elasticity or plasticity between short-life lines and long-life lines (data not shown).

Table 5.7 Leaf trait measurements of the extreme lines in the UK field trial in 2005.

Type	RIL	Trait[a]							
		LA	**ECA**	**ECN**	**SD**	**SI**	**AGR**	**ML**	**SL**
Short-life lines	13	4780	1201	4.34	143	14.28	6.53	0.72	7.51
	19	5086	1720	3.44	85	10.44	8.67	0.77	7.13
	32	5050	1309	3.98	112	12.58	9.27	0.8	7.36
	89	6081	1283	4.91	105	11.44	9.95	0.64	6.91
	105	4470	2188	2.15	85	15.33	6.78	0.88	7.26
	Mean	5093	1540	3.77	106	12.82	8.24	0.76	7.23
Long-life lines	1	5624	422	14.06	319	11.57	17.79	0.73	13.01
	5	5202	792	6.7	193	13.09	13.06	0.92	12.63
	15	4993	635	8.11	151	8.63	16.04	0.83	12.68
	74	5545	1623	3.42	73	10.32	9.81	0.96	11.71
	112	6253	1404	4.49	65	8.38	15.49	0.86	12.68
	Mean	5510	975	7.36	160	10.4	14.44	0.89	12.54
t-test[b] (n=30)		*	***	***	**	**	**	*	***

Note:

[a]Trait abbreviation: LA: leaf area (mm^2); AGR: Absolute growth rate (AGR) ($mm^2\ h^{-1}$); ECA: epidermal cell area (um^2); ECN: epidermal cell number per leaf ($x\ 10^6$); SD: stomata density (no. stomata mm^{-2}); SI: stomata index (%); ML: maximum load to break down the leaf material (N) SL: shelf-life period, counted from harvesting to the day before being rejected (days). This value is the mean of three field trials and was not assessed in the second UK field trial in 2005.

[b]t-test: the result of one-way ANOVA t-test are indicated where significant: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

5.2.2.3 Sensory assessment

The sensory profile of the baby leaf lettuces was obtained through a rapid attribute profiling method adopted at CCFRA Technology Limited (Chapter 2). Five long-life lines with similar maturity were evaluated using the cultivated lettuce as a control in this study. Three major aspects of sensory were examined, including appearance, flavour / taste and texture/mouth feel. The significant comparison of the sensory profile between test line vs control were not shown individually. Line 112 was an example to show the comparison in detail in Table 5.7. All the attribute profiles were also illustrated in the spider plot (Figure 5.15), where each attribute is a spider axis to show the overview the difference between Line 112 and control and also indicated the most different attribute between them. All attribute assessment values of the five test lines and the control are listed in Table 5.8.

Appearance

The control sample was relatively large in size, the leaves were very curly and crinkled, and it had significantly the thickest stem. The colour was relatively uneven and pale across the leaf, and it was rated the least firm in appearance. Line 112 is paler green, firmer in body firmness, and thinner in stem thickness than the control. Only 3 of 9 in appearance were significantly different between Line 112 and the control. Line 1, 74 and 112 had the similar curly leaf as the control (Table 5.8). Line 15 had flat, even dark green and the firmest leaf, and the thinnest stem among the five lines.

Flavour/Taste

The comparison of leaf flavour among cultivated lettuce and five test lines are shown in Figure 5.16. In overall leaf strength and leaf flavour, five test lines all had slightly higher value than the control, but not significant ($P > 0.05$) (Table 5.9). The leaf of the control sample was perceived significantly the strongest in lettuce flavour and sweetness, and it was rated the least in leaf flavour, bitter and acid taste. Comparing 112 vs control, 6 out of 13 attributes showed no

significant difference, including leaf flavour and acidic in leaf and stem, overall strength in leaf and earthy in stem, while 7 out of 13 attributes showed significant difference, including weaker lettuce, weaker sweet and stronger bitter in both leaf and stem, stronger earthy in leaf (Table 5.8). But Line 112 had the highest value of lettuce and sweet flavour and lowest value of bitter among all test lines (Table 5.9). Line 112 had stronger earthy than control (3.5 vs 0.7), whereas all the other lines had very weak earthy (0.1). Line 1 had the lowest value of acidic (15.8) among the test lines. Line 74 had the weakest sweet (1.7), strongest bitter (54.8) and acidic (24.3) among the five lines.

Texture and Mouthfeel

There were significant differences in four texture attributes between the control vs the test lines ($P < 0.001$) (Figure 5.17, Table 5.9). The control leaf was perceived to be significantly crispier, crunchier, juicer and the least chewy versus all test lines. Line 112 was shown to have a less crisp, crunch, juicy leaf than the control, but it had the crispest, most crunch and juiciest leaf among the test lines and the stem of Line 112 was similar crisp as the control and only slightly more fibrous or stringy than the control ($P > 0.05$). Line 1 was least chewy among the test lines, similar to the control.

The number of significant differences detected across the sensory characteristics for each of the lettuce lines versus the control sample was summarised in Table 5.10. The results showed that the appearance of Line 112 was perceived to have the least significant differences versus the control, whereas Line 15 showed the most differences. For the flavour/taste; Line 5 was rated as having the least significant differences to control, whereas Line 74 showed a large degree of significant differences. For texture, both 112 and 1 had the least significant differences to control and Line 15 and 74 showed the most differences.

Table 5.8 Sensory assessment comparison between Line 112 and cultivated lettuce (refer to Appendix 4).

Sensory	Part of lettuce	Attribute	Significance	
			Not Significant	**Significant**
Appearance	Leaf	Amount of curling	Similar to control	
		Depth of colour (green)		Paler than control
		Evenness of green	Slightly more than control	
		Surface smoothness	Slightly more than control	
		Type of leaf edges	Similar to control	
		Size	Similar to control	
		Maturity	Similar to control	
		Body firmness		Firmer than control
	Stem	Thickness		Thinner than control
	Total		**6 out of 9 attributes**	**3 out of 9 attribute**
Flavour/	Leaf	Overall strength	Similar to control	
Taste		Leaf/Grassy/green	Similar to control	
		Lettuce		Weaker than control
		Earthy		Stronger than
		Sweet		Weaker than control
		Bitter	Slightly more than control	
		Acidic	Slightly more than control	
	Stem	Leaf/Grassy/green	Similar to control	
		Lettuce		Weaker than control
		Earthy	Similar to control	
		Sweet		Weaker than control
		Bitter		Stronger than
		Acidic	Similar to control	
	Total		**7 out of 13 attributes**	**6 out of 13**
Texture/	Leaf	Crisp		Less than control
Mouthfeel		Crunch		Less than control
		Moistness/juicy		Less than control
		Chewy		More than control
	Stem	Crisp	Similar to control	
		Crunch		Less than control
		Moistness/juicy		Less than control
		Fibrous/stringy	Slightly more than control	
	Total		**2 out of 8 attributes**	**6 out of 8 attributes**

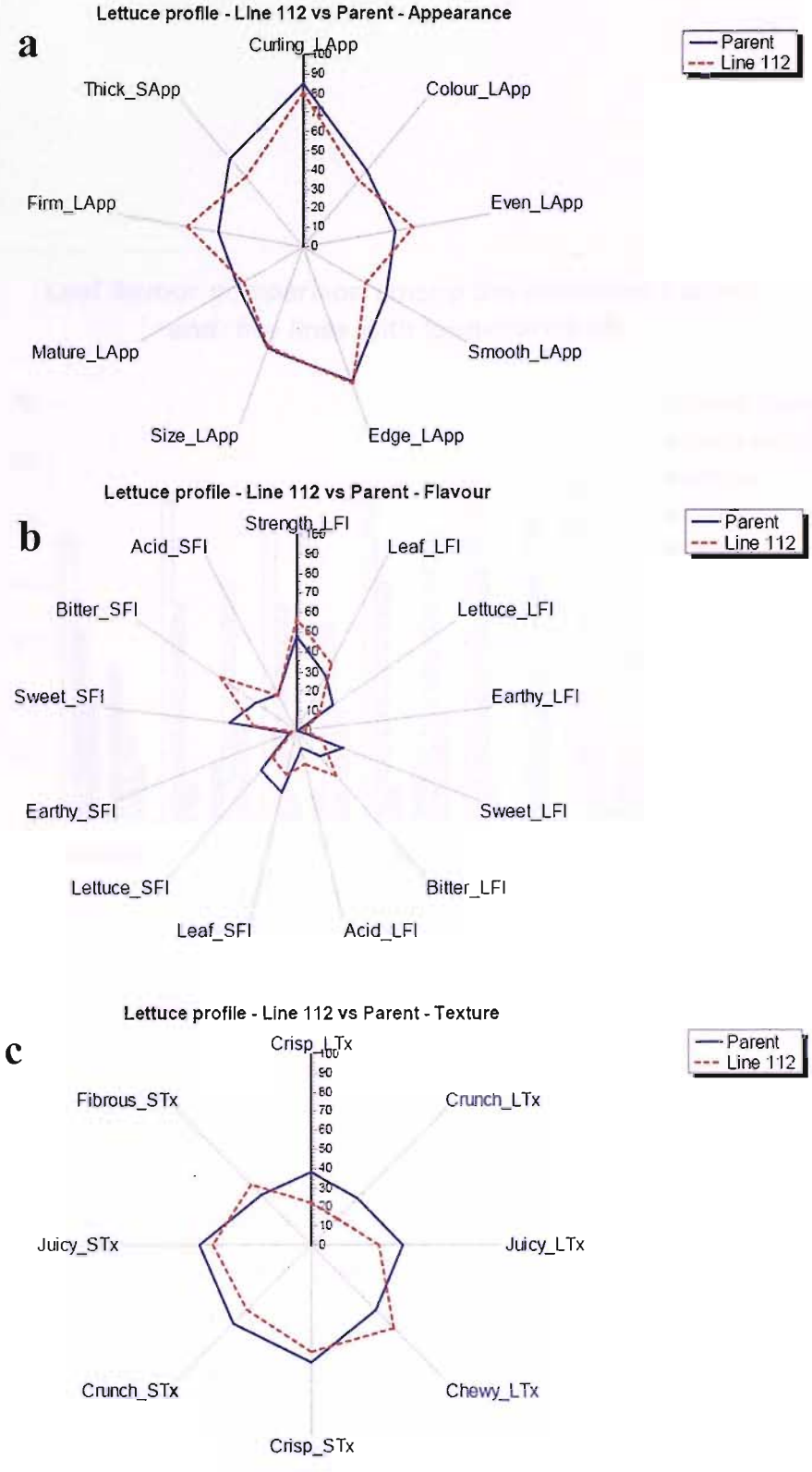


Figure 5.15 Spider plots of the sensory profile comparison between Line 112 and the cultivated parent. The sensory groups are appearance (a), flavour (b) and texture (c). The abbreviation of each group indicated after each attribute as LApp: leaf appearance, SApp: stem appearance, LF: leaf flavour, SF: stem flavour, LTx: leaf texture and STx: stem texture.

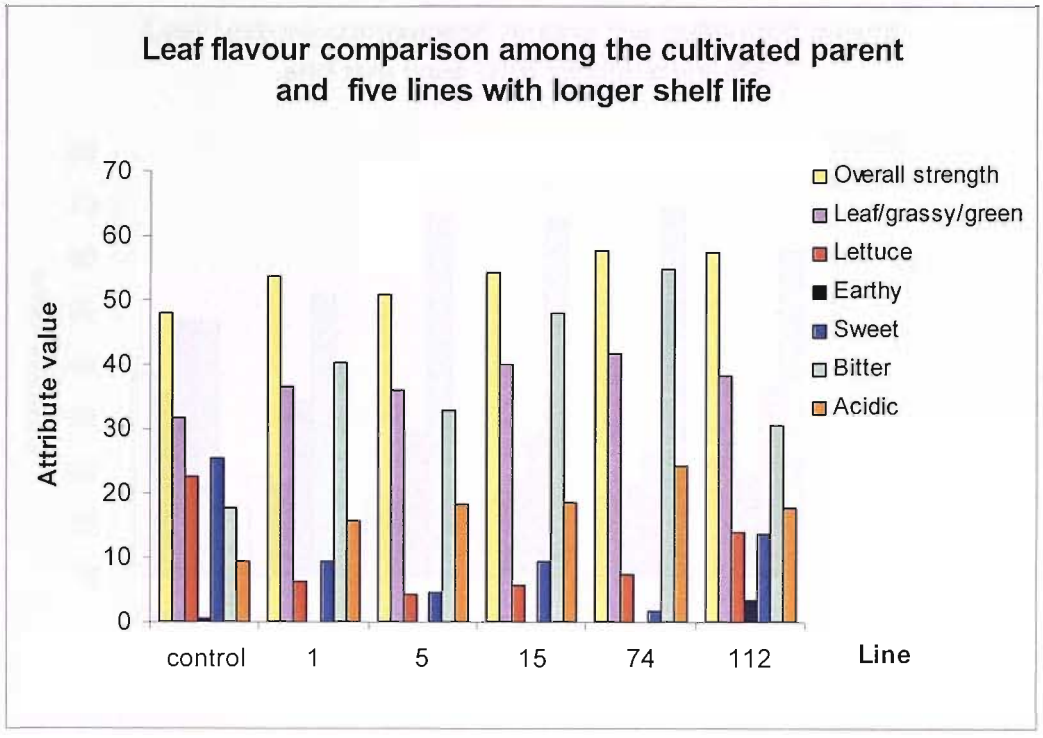


Figure 5.16 The leaf flavour comparisons among the cultivated parent and five long-life lines. Data points are means of each attribute value of individual line, n=7. The two-way ANOVA analysis showed no significant differences among the cultivated parent *L. sativa* and five RILs for the flavour attributes of overall strength and leaf/grassy/green; whereas significant differences for the flavour attributes of lettuce, earthy, sweet, bitter and acidic.

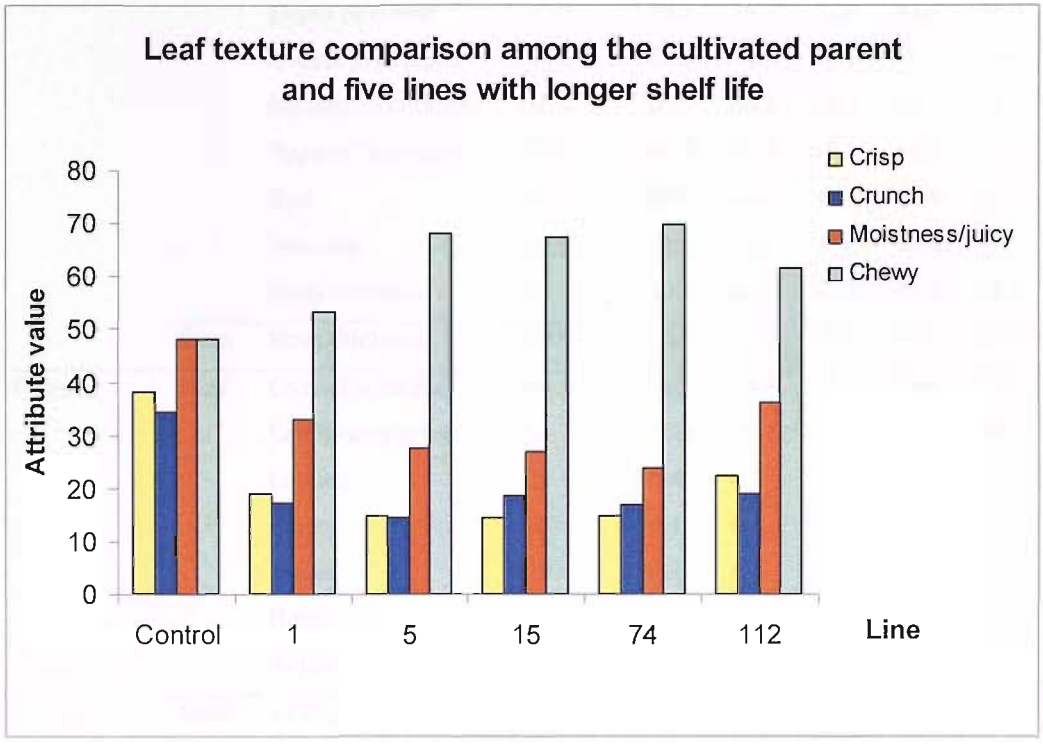


Figure 5.17 Leaf texture comparisons among the cultivated parent and five long-life lines. Data points are means of each attribute value of individual line, n=7. Two-way ANOVA analysis showed the significant differences ($P < 0.001$) among the cultivated lettuce and five RILs in all the texture attributes assessed in this study.

Table 5.9 Sensory assessment score of the cultivated lettuce and five long-life lines.

Sensory	Part	Attributes	Control	1	5	15	74	112	ANOVA
Appearance	Leaf	Amount of curling	85.4	88.3	49.3	5.6	80.5	80.4	***
		Depth of colour	52.2	79.2	76.2	77.6	54.3	44.9	***
		Evenness of green	48.7	80.6	74.6	80.0	59.1	58.9	***
		Surface smoothness	50.4	61.1	44.3	39.7	58.1	37.7	***
		Type of leaf edges	74.9	49.1	36.8	45.0	40.9	76.1	***
		Size	56.2	42.8	46.9	44.4	38.9	55.1	***
		Maturity	39.9	39.7	41.6	42.0	49.6	36.7	ns
		Body firmness	45.6	50.6	66.3	71.9	60.4	61.4	***
	Stem	Stem thickness	59.9	45.8	47.9	38.1	44.3	47.1	**
Flavour	Leaf	Overall strength	48.1	53.7	50.9	54.2	57.6	57.3	ns
		Leaf/grassy/green	31.7	36.5	36.1	39.9	41.6	38.4	ns
		Lettuce	22.6	6.4	4.2	5.6	7.3	13.9	**
		Earthy	0.7	0.1	0.1	0.1	0.1	3.5	**
		Sweet	25.4	9.3	4.7	9.3	1.7	13.7	**
		Bitter	17.8	40.2	32.9	47.9	54.8	30.6	**
		Acidic	9.4	15.8	18.4	18.5	24.3	17.6	*
	Stem	Leaf/grassy/green	32.7	25.2	27.6	28.6	23.4	23.4	ns
		Lettuce	27.4	12.7	9.4	7.8	4.1	18.5	***
		Earthy	3.6	0.4	0.6	0.6	1.9	2.5	***
		Sweet	35.0	17.1	26.1	17.9	16.9	21.9	**
		Bitter	25.2	46.5	40.4	46.6	57.6	48.1	***
		Acidic	20.9	24.6	18.9	22.9	29.0	20.5	ns
Texture	Leaf	Crisp	38.0	18.9	14.7	14.4	14.7	22.3	***
		Crunch	34.2	17.0	14.5	18.6	16.9	19.0	***
		Moistness/juicy	48.1	32.9	27.3	26.8	23.8	36.0	***
		Chewy	47.9	53.1	67.9	67.2	69.6	61.4	***
	Stem	Crisp	61.2	53.6	48.1	46.6	47.8	56.0	**
		Crunch	44.7	41.6	40.4	42.4	47.8	58.4	***
		Moistness/juicy	58.6	48.6	40.0	37.4	40.4	51.5	***
		Fibrous/stringy	36.7	49.1	46.2	49.1	49.6	44.6	*

Table 5.10 The summary of the number of significant differences detected across the sensory characteristics for each of the long-life lines versus the control parent.

Attributes	**Appearance (9 Attributes)**	**Taste/Flavour (13 Attributes)**	**Texture (8 Attributes)**
Control vs Line 1	5	7	6
Control vs Line 5	6	5	7
Control vs Line 15	7	7	8
Control vs Line 74	4	8	8
Control vs Line 112	3	6	6

5.3 Discussion

5.3.1 Photosynthesis mechanism

The results of chlorophyll content index of the extended mapping population were consistence in two glasshouse experiments (Bertrand, 2005). *L. sativa* had lower CCI than *L. serriola* and the mean of RILs was out of the two parent range in two experiments. Lower chlorophyll content was observed in *L. serriola* than *L. sativa* in the Portugal field trial. This resulted in lower net photosynthesis rates in SAT than SER in this glasshouse study (Figure 5.2), confirming the report from Gallardo et al. (1996). QTL for CCI mapped to similar position at LG3: 60cM in two experiments. This putative QTL for CCI at LG3 was robust, as the results were consistent in two experiments. It indicated that CCI meter measurement is stable. The QTL for CCI location was different from the QTL for chlorophyll content identified in the field trials (LG 1: 91-94 cM and LG 2: 81-86 cM). This might be a result of the different locations of the experiments taken to measure CCI and real chlorophyll content. Another possibility is that CCI meter measurement could not represent the real chlorophyll content. But it was reported that CCI has a strong correlation with chlorophyll content (Richardson et al., 2002). In this experiment of chlorophyll content calibration, it was not possible to be sure that the leaf area tested with CCM-200 apparatus was the exact same leaf area taken for spectrophotometer calibration and thus the regression between CCI and chlorophyll content was not significant. It would be better to convert the CCI value to chlorophyll content if the significant correlation was found between CCI and chlorophyll content. Then map QTL for chlorophyll content based on the calibrated chlorophyll content value. One putative QTL for CCI was found co-located with QTL for stomatal conductivity and transpiration. As the leaf photosynthesis mechanism is usually linked to leaf transpiration, the above co-location suggests that CCI relatively represented the leaf chlorophyll content and indicated that the region might be associated with the photosynthesis mechanism.

The chlorophyll content of a leaf is symptomatic of its physiological status and leaf colour is an important agricultural trait of crops (Xu et al., 2000). Leaf colour turns yellow during the senescence and is due to the degradation of chlorophylls and photosynthetic protein. A "stay-green" locus has been reported for rice (Cha et al., 2002). The photosynthetic carbon assimilation rate for mean of the mapping population was similar to that for the mean of the two parents. The photosynthesis rate of this mapping population showed normal distribution. However, no significant or putative QTL for PR was detected in this study. This might result from few markers related to PR being mapped in this genetic map or the accurate mean photosynthesis rate of each RIL was not achieved by limited replicates.

5.3.2 Leaf water relations

Leaf water relations are influenced in two ways by processes related to photosynthesis: osmotic pressure changes due to the photosynthetic accumulation of carbohydrate (Ψs) and transpiration, which depend on the behaviour of stomata (Koroleva et al., 2002). Stomatal conductance changes could be trigged by the accumulation of carbohydrate or changes in the hydrostatic pressure of epidermal cells. Open stomata might hasten water loss and contribute to deterioration in quality. It was reported that a long-lived genotype of the cut flower, *Antirrhinum majus*, had 50% fewer stomata than a short-lived genotype (Schroeder & Stimart, 2005). Investigation was also made by Thomson (2005) on the effects on stomatal aperture of harvested *Brassica* leaves during the post-harvest handling.

Our result showed that stomata density of *L. sativa* with longer shelf life was lower than that of *L. serriola* with shorter shelf life in both the Portugal trial and the first UK trial (Table 4.2), which is consistent with the report by Schroeder & Stimart (2005). However, no significant correlation between shelf life and stomata density was found in the mapping population in the Portugal trial and the first UK trial. In contrast, longer-shelf life lines had higher stomatal density than short-shelf life lines in the extreme line comparison experiment in the second UK trial, 2005 (Table 5.6, 160 stomata mm^{-2} vs 106 stomata mm^{-2}). As shown in this

study, stomata density was changed with the leaf development and varied between different environment trials (Figure 3.11 & Figure 4.10). But, when the cell number was taken into account, stomatal index was shown to remain constant with the leaf development and stable in different field trials (Figure 4.12). It was proved to be negatively significant correlated with shelf life in whole mapping population in the first UK trial ($P < 0.05$). This relationship was also confirmed in the extreme lines experiment in the second UK trial (Table 5.6). Stomatal index would be more accurate to represent the contribution of stomatal in water loss as the epidermal cell is also accounted in stomatal index. Due to the stability of stomatal index and its significant relationship with shelf life, this finding may provide the breeder as a rapid and non-destructive indirect selection method for long shelf life. QTL for stomatal index were also detected on LG 2: 83-86 cM (the beneficial allele was from cultivated lettuce, *L. sativa*) and LG 6: 132-136 cM (the beneficial allele was from wild lettuce, *L. serriola*). The nearest markers, 1A20-178 and E35/M49-F-475, could be applied in the agricultural breeding for shelf life in the future crop breeding.

Higher osmolality was found to be associated with longer shelf life for baby salad leaves (Clarkson et al., 2003). In this study, a higher photosynthetic rate was found in parent *L. serriola* than *L. sativa*; however, no significant difference in osmolality between two parents was found. The shelf life of *L. serriola* was found lower than that of *L. sativa*. This might be due to the higher transpiration and stomatal conductance observed in *L. serriola* than *L. sativa*. It suggested that the two factors, photosynthesis and stomatal conductance, co-regulated the leaf water relations. A balance between the rate of water lost from the leaf through transpiration and the amount of solutes potentially gained from photosynthesis may determine the actual turgidity of individual cells and the overall rigidity of the leaf. *L. sativa* leaves lose far less water to the environment through transpiration, yet also photosynthesis produced solute potential, the balance between these two factors might account for *L. sativa* had higher rigidity and therefore longer shelf life. Stomatal conductance and transpiration rate had similar trends in the QTL analysis, and their QTL mapped to the same locations (LG 4: 35-45 cM and LG 6: 136-141 cM). This confirmed the strong correlation relationship between SC and TR in the correlation analysis (Table 5.2). Also,

these QTL co-located with QTL for ECA, SI, and SL_d8, SL_d9, and SL_d10, which were detected in the Portugal field trial in the previous study.

5.3.3 Cell wall properties

The maximum load to break the leaf material is an indicator of leaf material strength. The leaf with higher maximum load results in stronger leaf material to withstand the whole leaf process, so as to have longer shelf life. The extreme line comparison confirmed this hypothesis (Table 5.6). The locations of two detected QTL for maximum load on LG1 and LG2, respectively, were collocated with the two "hot-spots" as identified in the previous study. QTL for elasticity, plasticity and total extensibility were also identified in the same location of cluster QTL for the leaf development trait. These would suggest that a group of genes at these two regions are involved in *L. sativa* leaf development, cell wall extensibility and shelf life.

As elasticity decreases, cell walls become more rigid, and this reduces both water potential and turgor per unit change in volume (Clifford et al., 1998). Irreversible plastic cell walls do preclude turgor maintenance of low water contents and have several potential advantages over elastic cell walls. A rigid cell wall may be better to maintain cell integrity during the salad leaf washing process. The cell wall stiffness is indicated by plasticity and elasticity. The leaf with a higher plasticity indicates a less extensible cell wall. It showed that *L. serriola* with shorter shelf life had consistently higher elasticity and plasticity than *L. sativa* with longer shelf life in the field trials and glasshouse experiments. But neither elasticity nor plasticity was found to be significantly different between extreme long-life lines and short-life lines (data not shown). This may be resulting from limited replicates used in this extreme line experiment (n=6). However, the collocation between plasticity with shelf life and the parent comparison strongly suggests that plasticity could be served as an indicator for shelf life. The importance of the cell wall was discussed in the previous chapters. Further investigation of the cell wall enzymes is needed to explain how it regulates the leaf processability.

5.3.4 QTL detection efficiency

Sixty informative F_9 recombinant inbred lines were used in the Portugal trial and the first UK trial. A total of 41 and 21 QTL for the leaf traits of interest were detected in these trials, respectively. One hundred and thirteen F_{10} RILs were tested in the glasshouse trial. Only 4 significant QTL were detected in the glasshouse experiment, including one for chlorophyll content index, two for stomatal conductance and one for transpiration. The cell wall property traits were undertaken in the field trials and the glasshouse to test the efficiency of QTL detection. No QTL was identified as significant QTL for leaf cell wall properties in the glasshouse experiment, while 4 QTL for these traits were detected in the Portugal trial and 3 QTL were identified in the UK trial (Table 5.4). This might result from the environmental conditions or the experiment design.

In this study, the QTL for ML, elasticity and plasticity were clustered on LG 1 and LG 2 was common in both field trials. These putative QTL were environmentally independent, but none of them was identified in the glasshouse experiment. The difference in experiment design may explain the above result. The bigger mapping population with 113 RILs was used in the glasshouse experiment, which is predicted to detect more QTL with small effects. It was surprising to see no QTL detected in the glasshouse experiment. Due to time-constraints for data collection of the cell wall traits, only three replicates of each line were practised in the glasshouse, while six replicates of each lines were analysed in both the Portugal trial and the UK trial. The reduction of replicate increased the variable error of each line, thus reducing the accuracy of the mean value. The increase of the mapping population size and the increase replicates of each genotype will be benefit QTL detection. Based on this study, it was suggested that the replicate number of each genotype was critical to achieve genuine mean value, which could represent the individual genotype for further QTL anaylysis. If the time and labour cost is limited, the increase of population size and reduction of the replicate will not be effective to detect QTL for the traits of interest. "This experiment also indicated the additional 53 lines brought

the additional genetic information (recombinant), but they were not increasing the power of the analysis".

5.3.5 Long-life lines

In terms of shelf life, the extreme lines were tested in the new environment, Spain. Significant difference in shelf life between the long-life lines and short life lines was confirmed in three field trials, Portugal, the UK and Spain. In this study, the long-life lines have relatively larger leaves; smaller cells; higher cell number; higher stomatal index, higher growth rate and higher maximum load, compared to short-life lines (Table 5.6). This is the most important finding from this study, which linked the pre-harvest leaf characteristics to post-harvest leaf quality. Also, the long-life lines were selected to have at least 2.5 days longer than average of the mapping population. The baby leaf salad in the supermarket usually lasts only 2-3 days. If the shelf life of baby leaf salad was extended to a further 2 days, the salad industry will have great financial benefit. It would be worth investing in the commercial potential of these long-life lines.

The sensory result showed the comparison between control samples versus five long-life lines. The identified control sample was an iceberg-type lettuce, which is a cultivated parent of the mapping population and already widely accepted by the customer. The sensory profile showed that the leaf of the control parent was perceived significantly strongest in lettuce flavour and sweetness and it was rated the least in overall strength, leaf flavour, bitter and acid taste. However, the sensory attribute may vary in importance based on consumer like/dislike. Actually, there are not many iceberg-type lettuces in the baby leaf salad mix pack in the supermarket. In contrast, there are some very strong flavour herb leaves. Crisp firm leaves are generally desired in baby salad leaves. A relative firm leaf will not be easily broken and able to withstand the salad process and thus maintain a fresh appearance, which is an important factor to attract the customers. The result shows that Line 112 is the closest line to the cultivated lettuce among the five selected long-life lines, viewing the product as a whole. It will have commercial potential with a long shelf life; high value of sweet flavour and low value of bitter among the test lines.

5.4 Conclusions

1. QTL for cell wall properties were co-located with shelf life and other leaf traits at the two "hot-spots": LG 1: 90-100 cM and LG2: 82-88 cM.

2. The shelf life of the extreme lines was confirmed in the new environment. The long-life lines have larger leaves, smaller cells, and a lower stomatal index and a higher maximum load to breakdown the material.

3. Line 112 has a sensory profile close to the cultivated lettuce and could be selected as superior genotype for future commercial application.

CHAPTER 6

EST data mining and candidate gene SNP detection

6.1 Introduction

QTL for leaf traits has been identified and the relationship between leaf development and leaf processability was explored in previous QTL mapping studies. Further expansion of this QTL work has been complicated by the lack of a simple, direct strategy for characterizing QTL for leaf processability in lettuce. As genome information has become available, more groups for QTL study have turned to identification of new genes using candidate-gene strategy, where QTL map positions are compared with genomic sequences and used to select candidate genes, which are then tested for the possible association with the traits of interest between them using extreme lines (Ren et al., 2004). Genetic mapping with markers derived from ESTs or known genes whose functions are possibly related to the phenotypic traits is an effective method for candidate genes associated with QTL for the traits of interest (Zhang et al., 2005). The ESTs become candidates if their map positions coincide with those significant QTL. SNP detection has recently developed to efficiently map candidate ESTs in genetic maps with high density molecular markers (Schmid et al., 2003, Gupta et al, 2004). Thus, a combinatorial approach using SNP mapping and candidate gene strategy seems appropriate for the candidate genes identification of QTL for leaf processability in this study.

EST data mining strategy begins in the *Arabidopsis* genome, using a comparative genomics approach, whereby candidate genes for the following leaf development traits are an indication of those that will be considered in this data-mining exercise. *Arabidopsis* and lettuce BLAST searches are possible on the compositae database site and these will enable lettuce ESTs to be identified for the genes of interest already well studied in *Arabidopsis* (Table 6.1).

Table 6.1 List of EST data-mining for putative candidate genes involved in leaf development and processability using information available from A*rabidopsis*.

Trait	**Putative Candidate gene**	**Gene no. in *Arabidopsis***
Cell wall	*Xyloglucan endotransglycosydases/hydrolase(XTHs)*	33
	α-Expansin	26
	β-Expansin	5
Cell division	A-type *Cyclin-Dependent Kinases* (*CDKA*)	1
	B-type *Cyclin-Dependent Kinases* (*CDKB*)	4
	A-type *cyclins* (*CYCA*)	10
	B-type *cyclins* (*CYCB*)	2
	D-type *cyclins* (*CYCD*)	10
	Retinoblastoma-related protein (RBR)	1
	E2F (transcription factors)	6
Leaf surface	*Wax synthase gene*	2
	CER	30
Senescence	*SAG*	12

Aims:

i. To identify putative candidate genes involved in leaf processability for SNP detection.

ii. To detect SNP for candidate genes for SNP mapping.

6.2 Results

6.2.1 PCR products for candidate genes

The genomic DNA of the two parents was extracted using the Qiagen method. With the designed primers, wax synthase (TC15225) were visible in agarose gel in both parents. There was no difference between the two parents in gene size, around 650 bp. Two primers were designed to amplify xyglucan endotransglucosylase/hydrolase (*XTH*) genes (TC12535 and TC9490), only one XTH gene (TC9490) was successfully amplified, where another *XTH* gene (TC12535) was not amplified with the specific primer in this practice (Figure 6.1). No difference in the gene size was found between the two parents. From the designated sequences, there were some polymorphism differences between two parents. There are 14 bases differences in the wax syntheses gene between two parents, while there are only two bases differences in *XTH* gene between two parents (Table 6.2). The sequencing reactions were run on an automated ABI Prism 377 DNA sequencer.

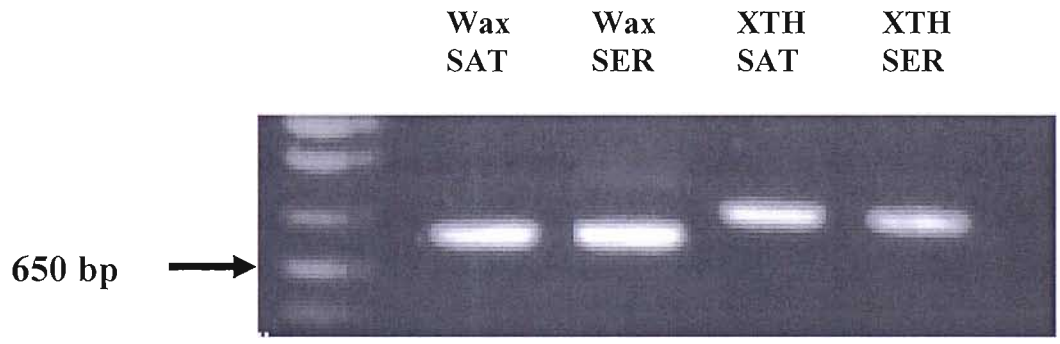


Figure 6.1 Detection of wax synthase and XTH genes with the designed primers in the two parents. The first lane was a standard DNA marker. The PCR products showed there was no difference in the gene size between the two parents.

Table 6.2 List of polymorphism differences between two parents in the sequence of wax synthase gene and XTH gene using the ABI- DNA sequencer.

Gene	**Base location**	***L. serriola* (US96US23)**	***L. sativa* cv. Salinas**
Wax synthase (*Wax*) (TC15225)	62	A	T
	92	T	C
	167	A	T
	176	G	C
	188	C	A
	200	T	C
	209	G	A
	230	T	C
	242	G	A
	308	G	A
	411	C	G
	414	G	T
	425	C	T
	523	T	C
Xyglucan endotransglucosylase/hydrolase (*XTH*) (TC9490)	39	A	G
	274	T	G

6.2.2 Development of a Cleaved Amplified Polymorphism (CAP) assay for the wax synthase locus

In the wax synthase gene sequence, there was a base difference at position 412. GCCGGC was for *L. sativa*, while GCCGCC was for *L. serriola* (Figure 6.2). Using restriction enzyme reaction (*Nae* I), in theory, there would be a difference between the two parents in PCR products. There would be two bands for *L. sativa* and one band for *L. serriola*, which gene would not be digested. Presumably, if F_9 population is genotyped, there should be two bands in some lines, while only one band in other lines (Figure 6.3). However, the preliminary experiment result (Figure 6.4) showed that there were two clear bands and another minor band for *L. sativa* (SAT). There were one major band and extra two minor bands for *L. serriola* (SER). In the DNA mixture sample (SAT/SER), there were three even bands.

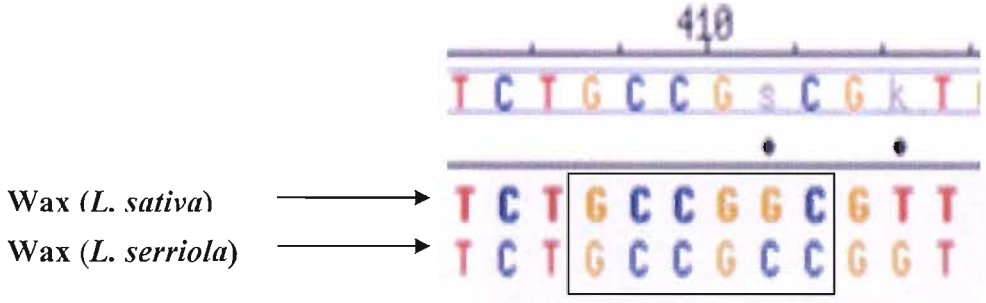


Figure 6.2 An example of polymorphism difference in the sequence of wax synthase gene between the two parents. There was a base difference at position 412, while GCCGGC was for *L. sativa*, while GCCGCC was for *L. serriola*. This region could be the restriction enzyme (*Nae*I) reaction site.

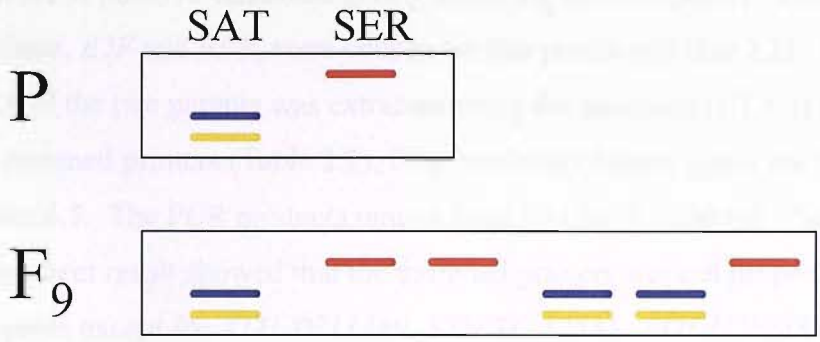


Figure 6.3 The theoretical polymorphism map for the gene with the restriction enzyme reaction. There would be two bands for *L. sativa* after the restriction enzyme reaction and one band for *L. serriola*. In the F_9 RIL population, there would be two bands in some lines, while only one band in other lines.

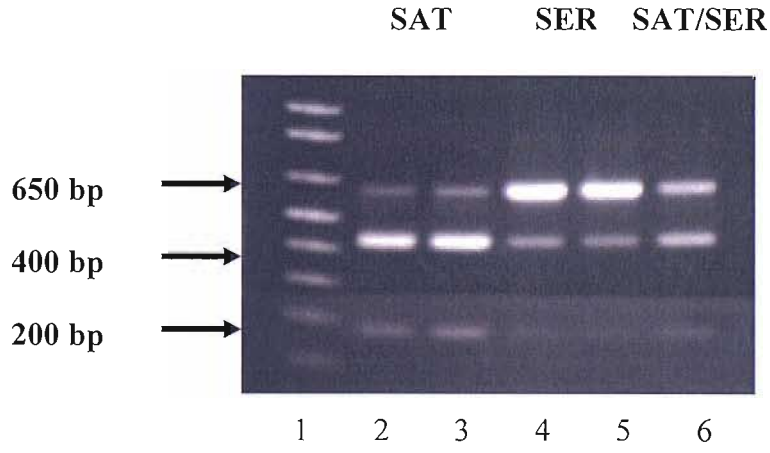


Figure 6.4 Detection of restriction enzyme (*Nae*I) digestion products in the two parents. The lane 1, standard DNA marker; lane 2 & 3, *L. sativa* (SAT); Lane 4& 5, *L. serriola* (SER); lane 6, DNA mixture of SAT and SER.

6.2.3 EST data mining for putative candidate genes

First set of putative candidate genes, including *XTH*s, *CycD3*, *CDKA*, *Wax synthase*, *E2F* and *RBR*, were chosen for this practice (Table 2.2). The genomic DNA of the two parents was extracted using the miniprep (CTAB) method. With the designed primers (Table 2.2), PCR products of these genes are shown in Figure 6.5. The PCR products ranged from 500 bp to 1200 bp. The preliminary experiment result showed that the designed primers worked properly for most of the genes except for *XTH TC11389*, *XTH TC 12535*, *XTH TC14434* and *E2F*. However, there was PCR product difference between two parents for *XTH TC11389* and *XTH TC12535*. There was no band for gene *XTH TC11389* for SAT, but one band for SER. There were three bands for *XTH TC12535* for SAT and two bands for SER. These differences between two parents need to be confirmed. There were two bands for both parents in PCR product of *XTH TC14434*. No PCR product of *E2F* was shown in the gel.

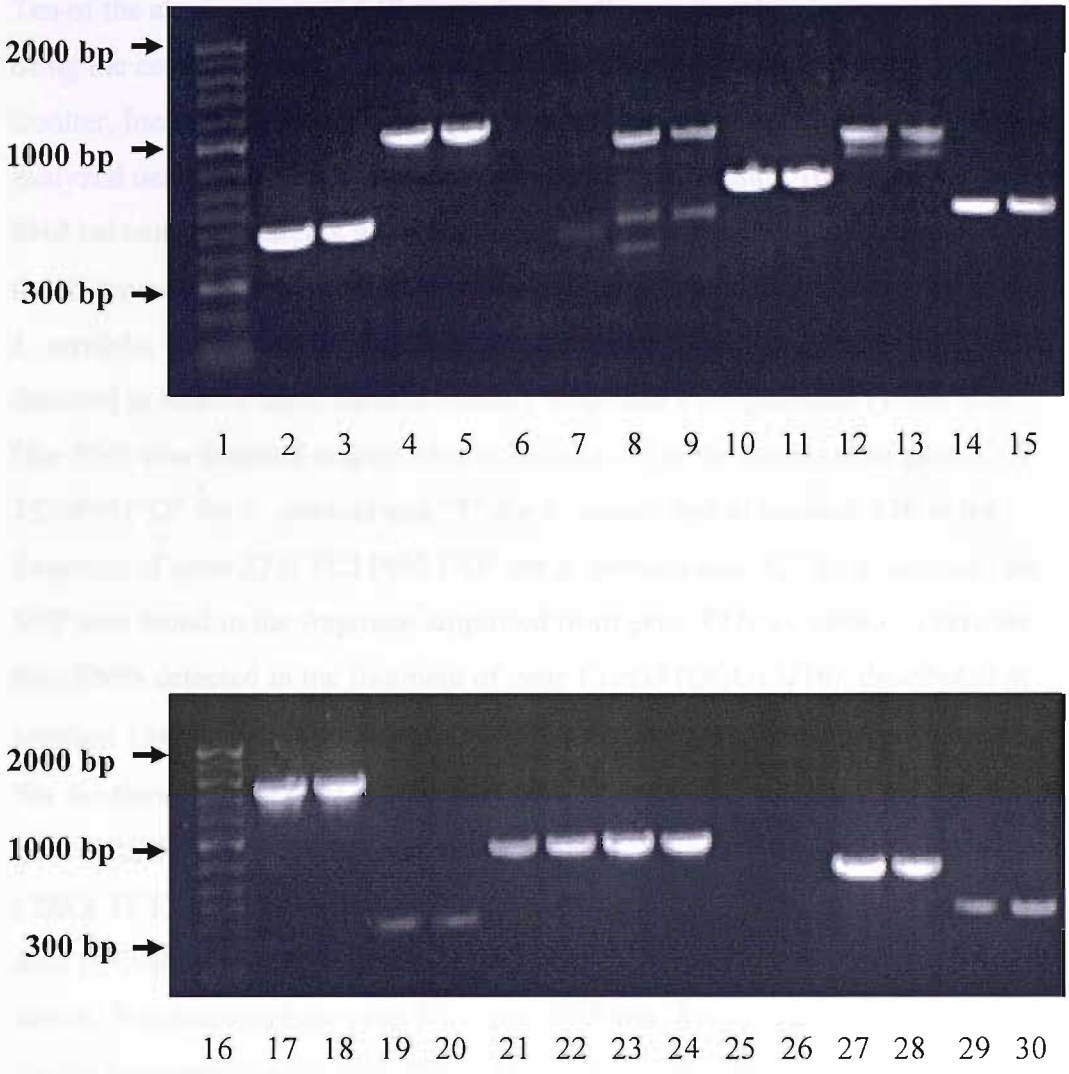
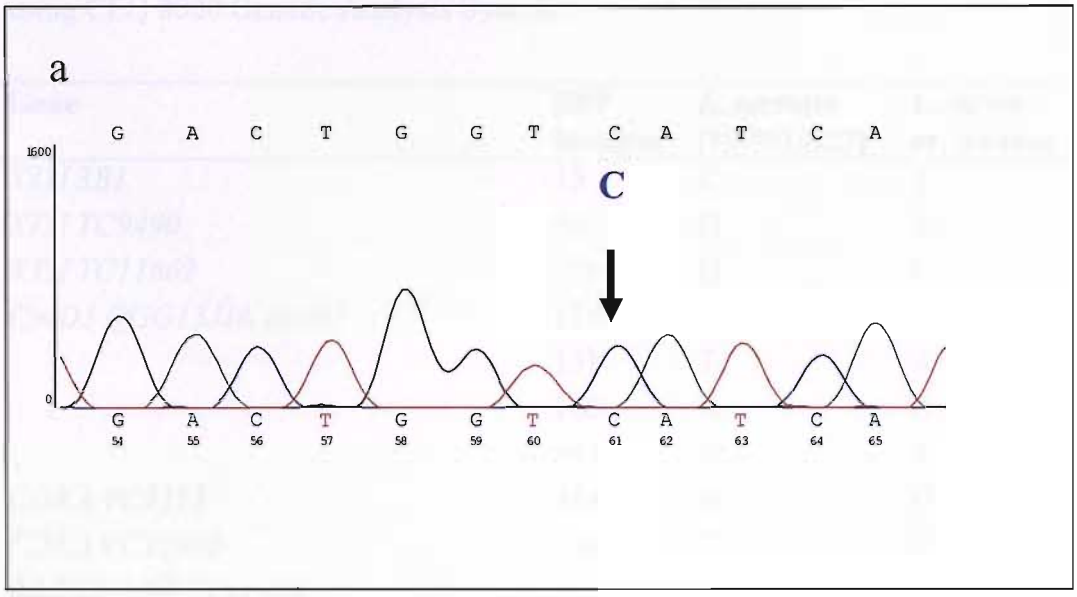


Figure 6.5 Detection of putative candidate genes with the designed primers in the two parents. Lane 1 & 16: standard DNA markers. Lane 2 & 3: *XTH 3B1*, *L. sativa* (SAT) & *L. serriola* (SER); Lane 4 & 5: *XTH TC9490*, SAT & SER; Lane 6 & 7: *XTH TC11389*, SAT & SER; Lane 8 & 9: *XTH TC12535*, SAT & SER; Lane 10 &11: *XTH TC10683*, SAT & SER; Lane 12 & 13: *XTH TC14434*, SAT & SER; Lane 14 & 15: *XTH TC11662*, SAT & SER; Lane 17 & 18: *Actin TC8011*, SAT & SER; Lane 19 & 20: *CycD3* SAT & SER; Lane 21 & 22: *CDKA TC9313*, SAT & SER; Lane 23 & 24: *CDKA TC13033*, SAT & SER; Lane 25 & 26: *E2F*, SAT & SER; Lane 27 & 28: RBR, SAT & SER; Lane 29 & 30: *Wax TC15225*, SAT & SER.

6.2.4 SNP detection

Ten of the above successful PCR products with one clear band were sequenced using the new equipment, CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc. USA). The polymorphism detections between two parents were analyzed using the advance program, "Vector NTI" (version 10). An example of SNP between two parents was shown in Figure 6.6. One SNP was detected in the fragment of gene *XTH* 3B1 between two parents. It is "C" in the wild parent, *L. serriola*, where it is "T" in the cultivated parent, *L. sativa*. At least one SNP detected in nine of these ten successfully amplified PCR products (Table 6.3). One SNP was detected respectively at location 50 in the fragment of gene *XTH* TC9490 ("G" for *L. serriola* and "T" for *L. sativa*) and at location 176 in the fragment of gene *XTH* TC11662 ("G" for *L. serriola* and "C" for *L. sativa*). No SNP was found in the fragment amplified from gene *XTH* TC10683. There are four SNPs detected in the fragment of gene *CycD3* (QGG13J16), distributed at location 136 (*L. serriola* /*L. sativa*: T/C); 151 (T/A); 166 (A/G) and 345 (G/T). For the family genes *CDKA*, one SNP was respectively found for the fragment of gene *CDKA* TC9313 at location 424 (A/G) and at location 334 (T/C) for the gene *CDKA* TC13033. One SNP at location 118 was found in the fragment of gene *RBR* (QGH6D15) at location 118, where "A" is for *L. serriola* and "G" is for *L. sativa*. For wax synthase gene *Wax*, one SNP was detected at location 69 (G/T) for the fragment of gene *Wax TC13934* and at location 423 for *Wax TC15225* (T/A), respectively.



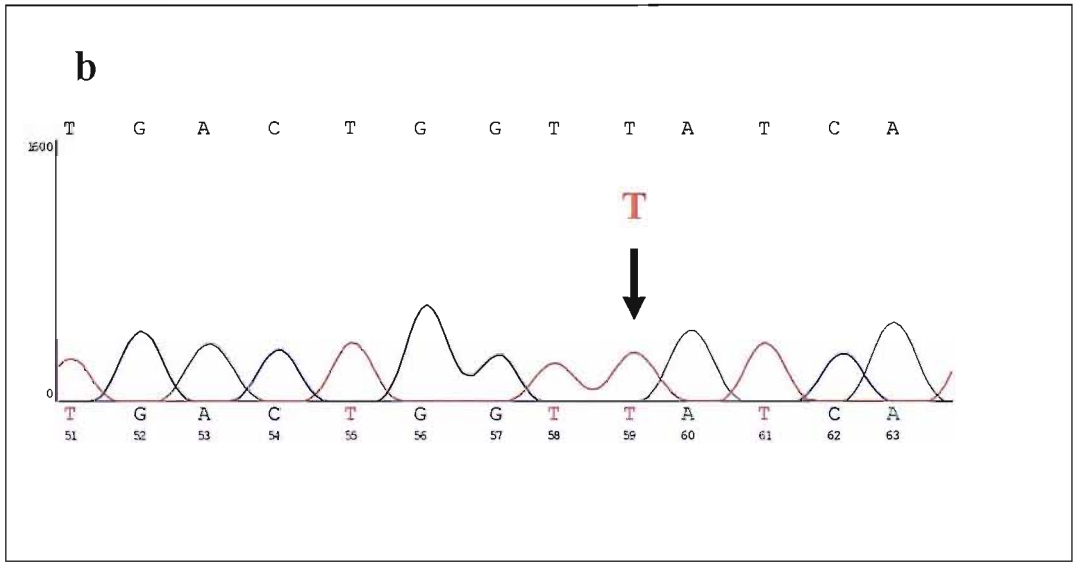


Figure 6.6 An example of single nucleotide polymorphism in the sequence of *XTH 3B1* between two parents. There was base "C" for *L. serriola* (a), while there was base "T" for *L. sativa* (b).

Table 6.3 List of single nucleotide polymorphism (SNP) detected in this study using CEQ 8000 Genetic Analysis System.

Gene	**SNP location**	***L. serriola* (US96US23)**	***L. sativa* cv. Salinas**
XTH 3B1	15	C	T
XTH TC9490	50	G	T
XTH TC11662	176	G	C
CycD3 QGG13J16.yg.ab1	136	T	C
	151	T	A
	166	A	G
	345	G	T
CDKA TC9313	424	A	G
CDKA TC13033	334	T	C
RBR QGH6D15.yg.ab1	118	A	G
	489	T	A
Wax TC13934	69	G	T
Wax TC15225	423	T	A

6.3 Discussion

6.3.1 PCR amplification

Initially, one wax synthase (TC 15225) and two XTH genes (TC12535 and TC9490) were selected to explore EST data-mining to identify the candidate genes involved in leaf processability. Two primers were designed to amplify XTH genes, but only one XTH gene (TC9490) was successfully amplified. In the second experiment, the primers for the set of 14 genes, including the above three genes, were designed using program "Primer 3". The amplified PCR products for the same gene were different with the different primers. Among the set of 14 putative candidate genes, ten genes were successfully amplified with one clear band of the PCR product. Most likely, the reason for unsuccessful PCR amplification is that the primer designed to an EST of the genes was amplified from gDNA introns, which make the region too large to be amplified efficiently. It was interesting to see the difference of PCR products between two parents for two XTH genes. There was no band for gene *XTH TC11389* for *L. sativa*, but one band for *L. serriola*. There were three bands for *XTH TC12535* for *L. sativa* and two bands for *L. serriola* with the secondly designed primers. These differences of PCR products between two parents need to be confirmed. If the difference is confirmed in the RIL population, it could be useful to map the whole RIL population based on the different bands of the PCR products. It would be a very efficient method to map the RIL population. It is worth further investigation to explore the possibility of mapping the population based on theses difference of PCR products.

6.3.2 CAPs application

Cleaved Amplified Polymorphism (CAP), utilize the amplified DNA fragments digested with a restriction endonuclease to display restriction site polymorphisms. It was reported as informative co-dominant markers to develop the linkage map of Sugi, an important forestry tree species (Iwata et al., 2001). In the wax synthase gene restriction enzyme digestion, main wax synthase gene

was digested and showed the two clear bands in the gel in *L. sativa*. However, the minor band was detected at the original gene size in *L. serriola*, which was supposed to be digested into two small fragments. In *L. serriola*, there was a major band at the original gene size, which suggested only a very small amount of genes were cut and show two thin bands in the gel. This might be explained if more than one wax synthase gene was being amplified by the primers, or if the *Nae* I enzyme was exhibiting star activity due to the non-optimal buffer conditions. More practice will be needed to test whether it will be possible to map the whole population using this CAPs assay. However, CAPs assay needs to optimize each gene restriction enzyme reaction individually and it is difficult to detect multi-genes in the same time. It is necessary to explore a new efficient and reliable mapping method such as SNP detection.

6.3.3 SNP detection

Fourteen putative candidate genes were selected for the SNP detection practice. Ten of these putative candidate genes were successfully amplified with clear PCR products. These PCR products were sequenced for polymorphism detection between two parents. There were at least one SNP detected in nine of these ten putative candidate genes except the fragment of the gene *XTH* TC9490. The size of these PCR products in the practice ranged from 500 bp to 1200 bp. The result showed the similar SNP frequency predicted by Wang et al. (1998). The typical frequency with which one observes single base differences in genomic DNA from two equivalent chromosomes is of the order of 1/1000 bp. Four SNPs were detected in the 500 bp size fragment of the gene CyD3 (QGG13J16). More SNPs were found between two parents in the first PCR practice with 14 SNPs found in one Wax synthase gene (TC3421) and 3 SNPs found in one XTH gene (TC5489) using an ABI DNA sequencer. The SNP frequency was relatively higher when the sequences were analysed by ABI-DNA sequencer, which might result from the accuracy of the older equipment of the ABI sequencer. All these SNP locations needs to be confirmed by further sequencing using new CEQ 8000 genetic analyser system.

SNPs are the most abundant type of DNA polymorphism in the genome, and have quickly become useful markers in agricultural research and have recently been applied in for marker-assisted selection for plant improvement (Zhang et al., 2005; Lopez et al., 2005). The most direct approach to discovery of SNP is direct sequencing of PCR products from the whole population. If the individuals are to be genotyped by sequencing, these involve extremely large expenditures of sequencing. Directed re-sequencing of gene segments distributed throughout the genome appears to be much more practical. A number of methods have been developed for genotyping SNPs (Gupta et al., 2004; Lee et al., 2004). The new advance genetic analyzer system provides a simple multiplex SNP genotyping solution with high accuracy and reproducibility. Once the SNP location was identified, a specially synthesized set of primers will be designed to anneal to the SNP site of interest. The primer anneals one base short to the target SNP. DNA polymerase inserts the complementary dideoxy nucleotide terminator to the SNP site. A full set of cDNA from the whole RIL population (113 lines) have been synthesised and ready for multiplex SNP genotyping using the CEQ genetic analyzer system. This is now undertaken in the Taylor's lab, University of Southampton.

6.3.4 SNP mapping strategy

SNP are a new class of molecular markers that have recently attracted much interest. The candidate gene mapping using SNP marker has become possible as a result of the dramatic advance in sequencing technology. Genetic mapping with markers derived from ESTs or known genes whose functions are possibly related to the phenotypic traits is an effective method for identifying QTL-associated genes. It is a direct and efficient approach without fine structure mapping. EST databases are currently the fastest-growing and largest proportion of the available DNA sequence databases. Recently, it has been reported to acquire a large number of SNP by computer analysis of available EST (Picoult-Newberg et al., 1999; Ren et al., 2004). This SNP mapping strategy would be powerful and 300 hundred of candidate genes are targeted to map in this project to identify the co-located candidate gene for leaf processability.

6.4 Conclusions

1. SNPs were detected in nine putative candidate genes including three XTH genes, two wax synthase genes and one CycD3, CDKA, RBR gene. These nine genes will be used for further candidate gene mapping.

2. Multi-SNPs were detected in gene *XTH* TC9490, *Wax* TC15225 and *RBR*. Only one SNP each gene will be targeted for multiplex SNP detection using the advanced genetic analyzer system, CEQ 8000. SNP will provide a promising molecular marker for plant improvement.

CHAPTER 7

General discussion and conclusions

A complex trait such as leaf processability is regulated by many genes and must be dissected into a number of component traits in order to understand the underlying principles. Comparing phenotypic correlations and their QTL locations should make it possible to improve the understanding of the genetic basis of leaf processability. This research presented is to improve the leaf processability using a quantitative genetic approach, which is a multi-gene approach.

In a quantitative genetic study, generally, the parents of the mapping population should have contrasting phenotypes for the traits of interest, as QTL can only be mapped if a polymorphism is observed in the segregating population. The traits associated with leaf processability were identified in the parental experiment (Chapter 3). Two mapping populations were tested for their suitability to meet the object to map QTL for leaf processability in this study. The mapping population from University of California at Davis was selected for further study as there were significant differences between the two parents in most of the traits of interest, which had previously been identified as being relevant to leaf processability (Clarkson et al., 2003). The amount of genetic variation indicated that QTL mapping was likely to reveal QTL for most of the traits of interest in this mapping population. There were some differences in morphology between the parents of the population from HRI (Appendix 3). However, these differences were not sufficiently significant to meet the criteria for QTL analysis (Kearsey & Pooni, 1996). Salvi & Tuberosa (2005) pointed out that the limited genetic variability might result from using only two parental lines. The population would only segregate for a fraction of the many more QTL segregating for the same trait in other populations, no matter how carefully the parental lines were chosen. They suggested using the multi-parental intercross populations to overcome the limitation. This approach will increase the efficiency of QTL detection and thus exploring other lettuce populations to meet the objective is needed in the future.

The detection of QTL analysis does not depend only on the genetic variation of the parents of the mapping population, but also on other factors such as the population size, the heritability of the trait, the evaluation of the trait, gene by environment interactions, the accuracy of the genotype information and reliability of the position of markers in the linkage map. Usually, a large segregation population is required to unravel the number of loci involved in the trait of interest. The larger number of genotypes tested, the more QTL with small effects would be detected (Kearsey, 1998). But there are practical limits to this, particularly in an agricultural context. In this study, QTL for cell wall properties were detected in the experiments (Portugal or the UK field trial) with six replicates of 60 RILs, but no significant QTL for cell wall properties was detected in the greenhouse experiment with three replicates of 113 lines (Chapter 5). This suggests that the replicate number of each phenotype was critical to achieve a genuine mean value for further QTL analysis. The ability of QTL analysis is determined by the size of the standard error of the mean of each line relative to the size of individual gene effects. The more individuals scored, the smaller the difference that can be detected between two means of genotypes (Kearsey & Ponni, 1996). Only sixty highly informative recombinant lines were used in this study, but they were selected from the genetic map using MapPop (Vision et al., 2000) and GenoPlayer. This provided a population that has nearly as many recombinant breakpoints and was therefore as informative as a population of ~90 RILs. This selected RIL population has proved suitable to detect significant QTL for the traits of interest in this study (Chapter 4).

Several clusters of QTL were detected in this study (Chapter 4 and 5), mainly on linkage group 1, 90-100 cM and linkage 2, 80-90 cM. These multiple QTL can be regarded as potentially single QTL with pleiotropic effects. The associated traits (leaf area, growth rate, weight, specific leaf area, chlorophyll content, epidermal cell area and number, stomatal density and index, and shelf life) are likely to be regulated by the same group of genes in a similar physiological pathway. The results showing the clustering of QTL is consistent with the high correlation coefficients among the traits (Table 4.4-4.6). Co-locations of growth traits (leaf area, specific leaf area and chlorophyll fluorescence) were also reported in an Arabidopsis RIL population (El-Lithy et al., 2004). This

clustering of QTL has important implications for plant breeding programs. If the clustered QTL showed pleiotropic effects positively or negatively on the associated traits in the same direction, 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 QTL or a single QTL with pleiotropic effects. If the latter is the case, it would be difficult to select for an improved genotype. Big pleiotropic effects of two "hot-spots" indicated their potential importance in commercial breeding. But in most cases, beneficial alleles derived from the cultivated lettuce parent. The effects of QTL detected in this study are of similar size, only explained 10%-20% phenotypic variation among the RILs. Quantitative trait dispersed across the genome would make it difficult for breeders to handle"

The results of this study showed an association of longer shelf life with larger leaves, lower chlorophyll content, higher cell number per leaf and lower stomatal index in the UK trial (Chapter 4), suggesting that these pre-harvest leaf development traits should be targets for future shelf life improvement through selection and breeding. Stomata control the water loss via its opening and closing mechanism and control the transpiration via CO_2 fixation (Brownlee, 2001). Stomatal number has been reported to be related to shelf life of the cut flowers (Schroeder & Stimart, 2005). In this study, stomatal density was shown to change with the leaf development and vary between different environments (Chapter 4). Changes in stomatal density are often paralleled by changes in the ratio of stomata to epidermal cells (stomatal index). Stomatal index showed a negatively significant correlation with shelf life in four experiments (Chapter 3, 4 and 5). It could serve as an indicator for leaf processability and provide the breeder with a rapid and non-destructive indirect method for cultivar selection with longer shelf life. The nearest markers of QTL for stomatal index, 1A20-178 and E44/M49-F-475, could be applied in marker assisted selection for crop improvement breeding.

Epidermal cell number per leaf also positively correlated with leaf processability, indicating the leaves with higher numbers of small epidermal cells would latter withstands the salad preparation process. Recent research has suggested that the 'ideal' leaf for processability is likely characterized by small cells and limited cell wall extensibility (Clarkson et al. 2003). However, epidermal cell size depends on leaf development and is environment sensitive (Ferris et al., 2002). Cell number per leaf is more controlled by genetic variation, rather than environment. QTL for cell wall properties co-located with QTL for leaf growth traits on LG 1 and 2, indicating a group of genes at these two regions are involved in leaf development, cell wall extensibility and leaf processability. The maximum load to break the leaf material showed a significant difference between the extreme long-life and short-life lines (Chapter 5). The irreversible cell wall extensibility (plasticity) was found to be different between the two parents with different shelf life (Chapter 3). This agrees with other report (Clarkson et al. 2003). Higher leaf plasticity indicates a less extensible cell wall. A rigid cell wall may be better to maintain cell integrity during the process. Recently, some work has been done on cell wall extension, a complex process involving maintaining cell wall structure, turgor pressure and flexibility (Kaku et al., 2005). However, to my knowledge, no one has explored the genomic control of cell wall properties. The cell wall extensibility reflects significant changes in the cell wall structure and the genomic control of this is an important aspect to understand the genetic basis of leaf processability

A QTL for chlorophyll content correlated with shelf life on LG1. Lower chlorophyll content was found in the older leaves compared to newly emerged leaves in this study. The post-harvest senescence is a result of a rather rapid loss of chlorophyll (Mathas et al., 2003). However, shelf life was found to be negatively correlated with chlorophyll content in this study. In appearance, dark green leaves of *L. serriola* contain higher chlorophyll content than the pale green leaves of *L. sativa*. *L. sativa* leaves have relatively longer shelf life than *L. serriola*, as a result of small cell and lower cell wall extensibility, but not as a result of lower chlorophyll content. Lower chlorophyll content is not a causal factor for longer shelf life. This suggestion was supported by the HRI population experiment. Saladin contained about double the chlorophyll content of Iceberg,

but there was no significant different in shelf life between them (Appendix 3). To investigate the change of chlorophyll content during the shelf life period would enable a better understanding of the mechanism of leaf post-harvest senescence. Several genetic mutants and potential regulator components have been identified for leaf senescence in *Arabidopsis* (Lim et al., 2002), which will benefit the future analysis of the senescence rate and its genetic basis in lettuce.

QTL for ten leaf growth traits were analysed in three different developmental stages. Only 5 of the 25 QTL were identified at all three different developmental stage leaves (Chapter 4). QTL effects for the traits depend on the leaf developmental stages, indicating that some loci may have overall effects on plant growth, while others only specifically regulate certain processes during a specific phase of growth. Similarly, QTL for growth related traits on chromosome 3 of Arabidopsis were found mainly for the earlier phase of growth (El-Lithy et al., 2004). The co-location of QTL for leaf area, cell number and cell area at different leaf developmental stages, suggests that leaf area appears to be driven by leaf cell production through leaf development, while the contribution of cell expansion to increased leaf growth was limited to a specific time in development. It was suggested that local expression of expansin induces the entire process of leaf development and modifies leaf shape (Pien e al., 2001). Kim et al. (2002) also reported the importance of both cell production and cell expansion in leaf growth. However, Donnelly et al. (1999) pointed out that cell division occurs only after cells have reached a certain size, which is in contrast with this study. Further study of the candidate genes involved in cell expansion and cell production may lead to a more detailed and mechanic understanding of plant growth.

Two large-scale field trials were under taken in two contrasting environments to examine the genotype by environment interaction (G x E). In total, 51 QTL distributed on eight out of the ten linkage groups, were detected from the eighteen traits (Chapter 4 & 5). Only 11 QTL were common in both environments and assumed to be independent of the environment. Three of 8 QTL for shelf life from this study were common in both trials. The cultivated lettuce alleles increased trait values in most of the QTL. The number and

contribution of each QTL that has significantly different effects across the environments are associated with substantial G x E effects (Moreau et al., 2004). Genotype by environment interaction is crucial for any program of plant improvement. The success application of QTL depends on the reliability and accuracy of the QTL analysis where information has been achieved. In breeding practice, it is a risk to apply the superior genotype to various environments when it is environment dependent, as its superiority may be dramatically reduced across environments. In order to develop environment-independent cultivars, only the information of QTL with main effects can be the base for genetic improvement (Yang & Zhu, 2005).

Two 'hot-spot' areas, LG1: 90 cM-100 cM and LG2: 80 cM-90 cM, were identified in this study, as several QTL co-located in these two regions. In most cases, the beneficial alleles were from cultivated lettuce parent. A few were from the wild lettuce parent. Near isogenic lines (NILs) can be developed at these two regions. QTL cloning is becoming quite feasible through further research on these regions and the development of NILs for gene cloning for leaf processability. So far, several QTL have been successfully cloned by map-based cloning in *Arabidopsis* (Werner et al., 2005), rice (Doi et al., 2004) and tomato (Frary et al., 2000, Fridman et al., 2004). QTL cloning, originally developed from plant genetic studies, is rapidly advancing in human and animal research (Andersson & Georges, 2004). The confidence interval of significant QTL usually ranges from 10-30 cM. It is about 10 cM for the QTL identified in this study, which is a relatively short distance compared to other QTL studies. However, this chromosome interval (10 cM) usually contains several hundreds of genes. It was estimated that there would be an average of 440 genes in a 10 cM chromosome interval of *Arabidopsis* (Salvi & Tuberosa, 2005). The increase in mapping resolution is essential to reduce the number of the genes associated with the QTL for the trait of interest. A combined QTL- microarray approach was reported to identify the pattern of gene expression associated with QTL appear to be particularly promising for identification of candidate genes (Kirst et al., 2004) for the trait of interest. Pooling cDNA from extreme lines (long-life lines vs short-life lines) to run microarray would be a possible route to identify candidate

genes for leaf processability in the future. QTL cloning for leaf processability should be very interesting in theory and useful for crop breeding improvement.

Besides fine structure mapping, candidate gene mapping is another approach to verify genes associated with the traits of interest. In this project, SNP mapping for candidate genes was explored (Chapter 6). A group of selected genes with known functions related to leaf processability were selected for this practice. If candidate genes reside within a QTL interval for the trait of interest, it is possible to hypothesise that this candidate gene may be associated with the trait of interest. It is a direct and efficient approach without the need for fine structure mapping. Dramatic advances in sequencing technology have resulted in the detection of SNPs. SNP mapping in plants was stimulated by the progress of human SNP discovery. It should be possible to acquire a large number of SNP by computer analysis of available EST. Over 1.4 million SNPs have been identified in human genome and over 56,000 DNA polymorphisms in *Arabidopsis* are available in the database (Rafalski, 2002). No comparable data set exists in lettuce yet. However, the developing lettuce EST database at University of California at Davis will provide very useful information for SNP mapping for leaf processability.

Eventually, independent proof is needed to validate the role of the identified candidate genes on the observed phenotypic effects. The functional testing of a candidate gene can be performed by overexpressing or down-regulating the target gene through transformation or new technology, RNAi. Anti-sense *XTH* transgenic lettuces were investigated to explore the role of *XTH* in leaf processability (Appendix 1). Samples for XTH activity analysis were also collected in California field trial. As it is impracticable to test XTH activity for all RILs due to time constraints and a lot of radioactive involvement, selective lines from long-life lines vs short-life lines will be tested to confirm the function of XTH genes. The *XTH* anti-sense lettuce has been shown to have an extended shelf life. This gene information will be exploited using TILLING technology. Transgenic technology results in the generation of GM, which is not always broadly acceptable to consumers. The TILLING, reverse genetic approach for mutation generation and discovery will be able to produce non-GM lettuce with

improved quality. This technology has successfully applied in wheat for crop improvement recently (Slade et al., 2005). Currently, two lettuce mutation libraries from Anawah Company, consisting of over 9,000 lettuce plants, are available to screen *XTH* mutation lines for further commercialization. Also, Eco-Tilling is another approach to identify the ideal genotype from natural resource.

The superior long-life genotypes, such as line 112 and line 1, were identified through three field trials in different environments. They can be marketed directly as natural resources. Thirty sensory attributes were compared between the long-life lines with the cultivated lettuce, *L. sativa* cv. *Salinas*, which has already been widely accepted by the consumer (Chapter 5). The results showed that the long-life lines are not as sweet or crispy as the cultivated lettuce. However, the control cultivated lettuce is an iceberg type lettuce, which is quite sweet and juicy compared to most of the baby leaf salad material on the market. The flavour, taste and texture are accepted by peoples' preference and depend on individuals. The long-life lines which are most relevant to cultivated lettuce would be still accepted by customers. Further sensory comparison is needed between these long-life lines with Cos type lettuce, which is one of the most popular leaf in baby leaf salad. With health diet trends, the nutrient quality has attracted more attention and has a good impact on the market sale. QTL for nutrient content is ongoing as part of another PhD project using the same population. Leaf samples of 113 lines for carbohydrate contents have been collected from California field trial and stored in -80°C freezer. The leaf sample will be used to determine the concentration of sucrose and glucose using enzyme-linked assay. Once this trait data analysis is completed, QTL for carbohydrate content will be mapped using the same genetic map and QTL cartographer software to explore the genetic basis of sucrose and glucose.

The work could have a global impact on the salad market. The market has been undergoing a rapid increase in the last decade and is growing at a rate of 23% per year from 2003-2004. Market research has indicated that the shelf life is the key constrain for baby salad marketing and a shelf life increase of a couple of days could result in a 20% to 30% sales increase (Vitacress Salads Ltd., UK). The improvement of shelf life will benefit the industry and consumers. More

processable leaves can be bred through the marker assisted selection. QTL cloning and candidate gene strategies are feasible approaches for the future development of more processable leaves. The knowledge obtained from this project can also be applied to other crop improvement.

In conclusion, this work contributes not only to the accelerated application in crop improvement, but also to a better understanding of fundamental science on plant growth. For the first time, the link was found between pre-harvest leaf growth characteristics and post-harvest shelf life, for an important leafy crop, lettuce. The identification of QTL for leaf 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. QTL for leaf development traits have been identified in this project and these will enable a better understanding of the plant growth mechanisms. Secondly, the superior long-life lines were selected for further commercial potential application. Thirdly, the candidate-gene strategy using the single nucleotide gene approach was explored for identification of candidate genes for leaf processability. Further work both within and beyond the framework of this project has been developed for crop improvement and fundamental science.

APPENDICES

Appendix 1 *XTH* transformation study

A1.1 Introduction

XTHs have a proposed role in the biogenesis of the cell wall and in strengthening as a response to a mechanical stress. For example, the *TCH4* gene in *Arabidopsis thaliana*, an *XTH* that is up-regulated following touch (Xu et al. 1996) with a third physiological role for *XTHs* is in the degradation of the cell wall with *XTH* levels increased in fruit ripening. An *XTH* gene (*VXET1*) has been demonstrated in grapes at the end of life to soften the fruit (Ishimaru & Kobayahi et al. 2002). *XTH* has been targeted for transformation study to improve the baby salad leaf processability in this lab. *XTH* genes are a multi-gene family and there are 33 members in *Arabidopsis* and 46 in Rice (Rose et al., 2002). Many have very distinct and highly responsive transcriptional patterns which correlate with cell wall properties.

A suitable system to transform lettuce has been established in the previous study in this laboratory (Clarkson, 2004), modified from Curtis et al. (1994) using *Agrobacterium tumefaciens*. An *XTH* fragment was cloned and sequenced from young *Lollo Rosso* leaves using degenerate primers designed to two distinct conserved domains in all described *XTHs*. This XTH fragment was transformed back into the plant in anti-sense orientation under the control of a constitutive promoter (CaMV35S). A preliminary analysis of the T_2 plants from this system has revealed some promising findings. *XTH* had been co-localized in the cell wall with a fluorescence dye (Figure A1.1), showing that *XTH* activity is higher in wild type versus one anti-sense *XTH* line (Clarkson, 2004). This strongly suggested that *XTH* play a very important role in the leaf processability. We now need to confirm these findings in T_3/T_4 anti-sense *XTH* lettuce and apply a commercial approach to take this discovery forward.

Aim:

To further characterise T_3/T_4 anti-sense *XTH* lettuce, using biochemical, physiological and morphological traits and confirm leaf shelf life.

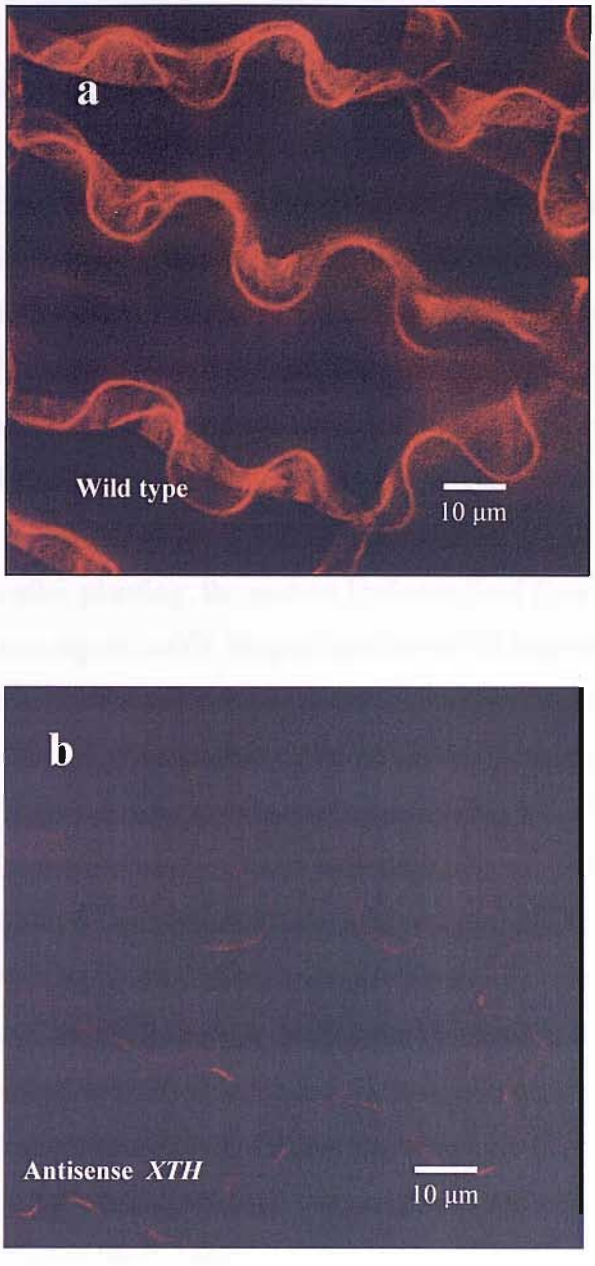


Figure A1.1 Confocal microscope sections through the epidermal layer of wild type (a) and antisense *XTH* plants (b) at the same development stage. The tissue had been vacuum infiltrated with a sulforhodamine conjugate of xyloglucan oligosaccharide (XGO-SR) into leaf sections to act as an artificial acceptor substrate. Fluorescence indicates the co-localisation of endogenoue donor substrate. XET action is localized within the cell wall in leaf tissue, with fluorescence intensity dependent on the rate of action of the enzyme on its wall-bound xyloglucan substrate. Scale=10 µm.

A1.2 Results

A1.2.1 Morphological and physiological traits of the transgenic lettuces

T_3 anti-sense lettuce plants were produced through self-fertilising in a glasshouse. Three most interesting transformed lines (7-25, 9-11 and 19-3), which from our preliminary analysis have shown to be most promising, were selected for this study. There was no visual phenotype difference between transgenic plants and wild type lettuce (Figure A1.2). However, in Line 19-3, there was dwarf segregation with ratio of 3:1 (Figure A1.3).

At harvest, six week after planting, the mature leaf area (leaf 6) of the wild type (7709 mm^2) lettuce was significantly bigger than that of all three transgenic lines (Figure A1.4a). The leaf area was reduced about 28% in line 7-25; 23% in Line 19-11; and 17% in Line 19-3, respectively. However, epidermal cell area was only significantly different (smaller) than wide type in Line 19-3 ($P=0.03$, data not shown). The fresh weight was analysed at six different leaf development stages (Figure A1.4b). In young leaves, (leaf 1 and leaf 2), leaf fresh weights of wild type were significantly lower than that of all three transgenic lines, while the comparisons were contrast in the later developmental stages (leaf 4 to leaf 6). It showed that leaf expansion in the transgenic lines were more rapid than wild type in early developmental stages, then it was stable in later developmental stages (leaf 4 to leaf 6), while the wild type lettuce continued to expand to reach bigger leaf area and higher fresh weight. A two-way ANOVA showed a highly significant interaction between line and leaf age, indicating the effect of anti-sense is dependent on leaf development stages.

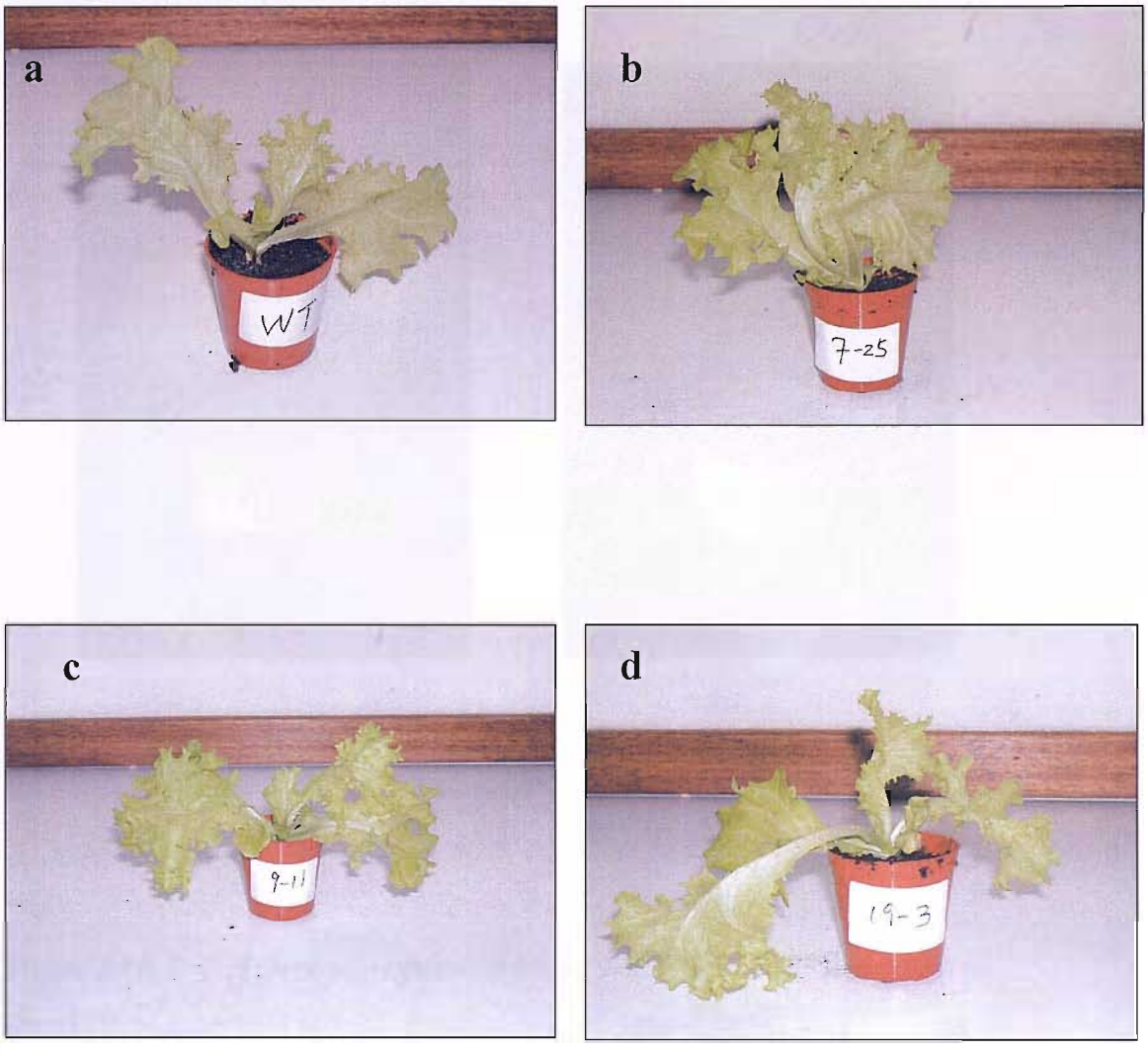


Figure A1.2 The plant images of the wild type and transgenic lettuce taken at harvest. Wild type (a), Line 7-25 (b), Line 9-11 (c) and Line 19-3 (d). The leaf samples for the traits associated with leaf processability were collected at six weeks after planting.

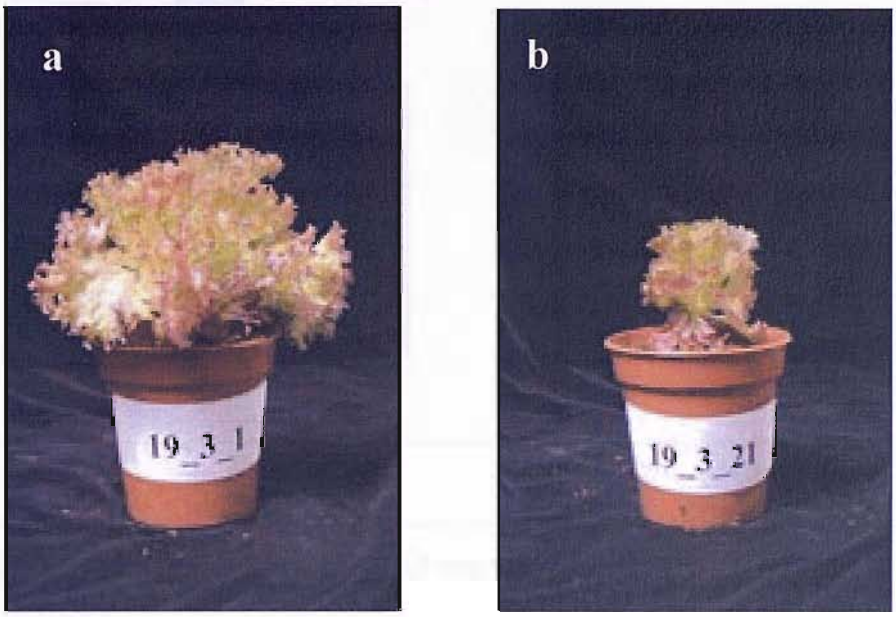
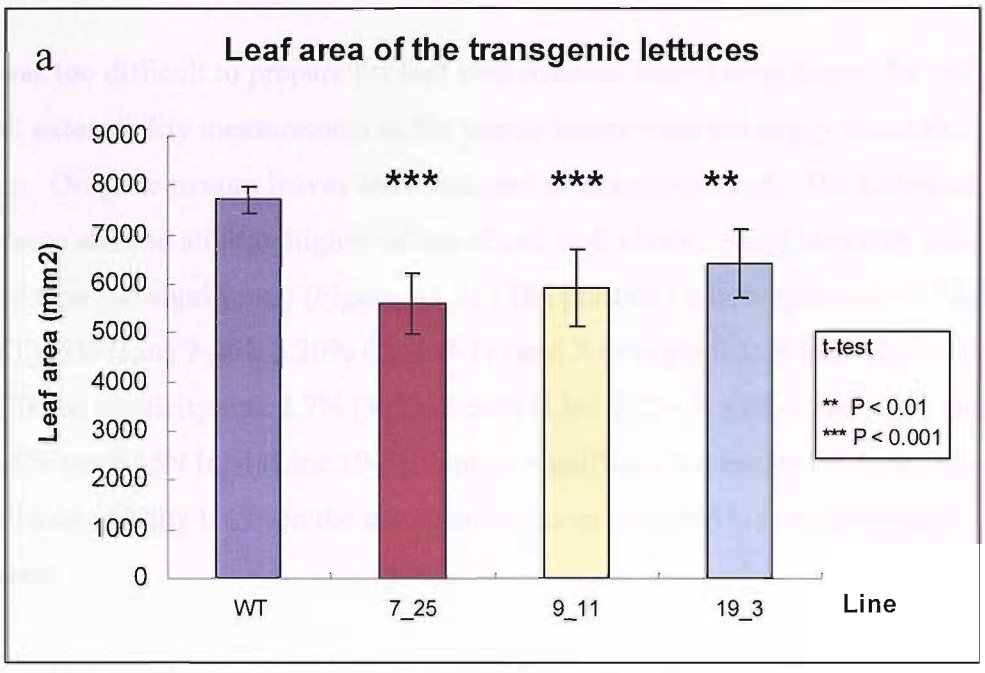


Figure A1.3 Dwarf plant segregation in Line 19-3. The ratio of normal (a) to dwarf (b) of Line 19-3 is 3:1.



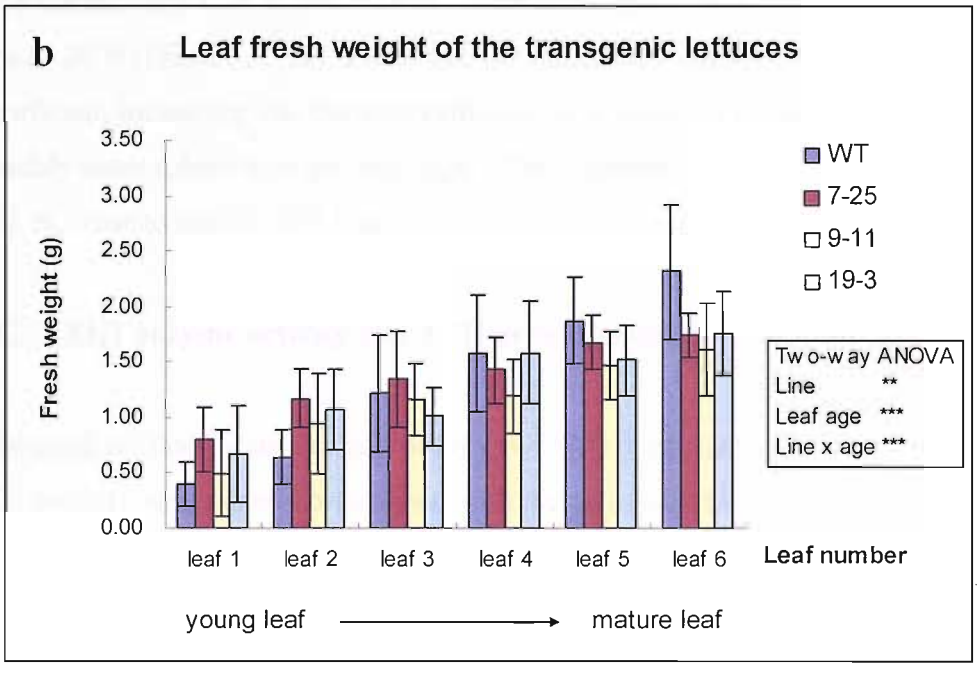


Figure A1.4 Leaf physiological traits of the transgenic lettuce and wild type. Leaf 6 leaf area (a) and leaf fresh weight at different development ages (b). Leaf 1 to leaf 6 is labelled according to the leaf development ages from young to mature stages. Data points are means ± SD, n=6. The results of t-test in leaf area between wild type and the transgenic line are indicated where significant: ** $P < 0.01$; *** $P < 0.001$. Two-way ANOVA showed a significant difference between lines and leaf age and interaction between line and leaf age, indicating the effect of line is dependent on leaf development stage (data collected by Dr. C. Wagstaff).

A1.4.2 Cell wall properties in the transgenic lettuces

It was too difficult to prepare the leaf strip material from young leaves for cell wall extensibility measurement as the young leaves were too soggy to cut the strip. Only the mature leaves were assessed in this experiment. The transgenic lettuces showed slightly higher values of cell wall elasticity and plasticity than wild type (no significant) (Figure A1.5). The plasticity was respectively 4.38% (WT), 5% (Line 7-25), 6.20% (Line 9-11) and 5.44% per 0.15N load (Line 19-3); while the elasticity was 2.7% (WT), 3.64% (Line 7-25), 4.13% (Line 9-11) and 3.26% per 0.15N load (Line 19-3). But no significant difference exists in the cell wall extensibility between the transgenic lettuces and wild type in the mature leaves.

The maximum load to break down the leaf material of Line 7-25 and Line 19-3 were respectively 0.24 N and 0.26 N. They were higher than that of the wild type (0.20 N) (Figure A1.6). However, the increases were not statistically significant, indicating that the transgenic leaves of Line 7-25 and Line 19-3 were possibly more robust than the wild type. The maximum load of Line 9-11 was 0.21 N, close to that of wild type.

A1.2.3 XET enzyme activity and XTH gene expression

The confocal laser scanning microscopy (CLSM) images again confirmed the XET activity was indeed co-localised with the cell wall only and was reduced in Line 7-25 and Line 19-3 as they showed less fluorescence that the equivalent wild type leaf (data not shown, images similar to Figure A1.1). The *XTH* gene expression of Line 19-3 was showed down-regulated when the gene expression signal normalised to *Actin* expression (data not shown), while the *XTH* gene expression of the other two transgenic lines (7-25 and 9-11) were about the same level as that of wild type lettuce.

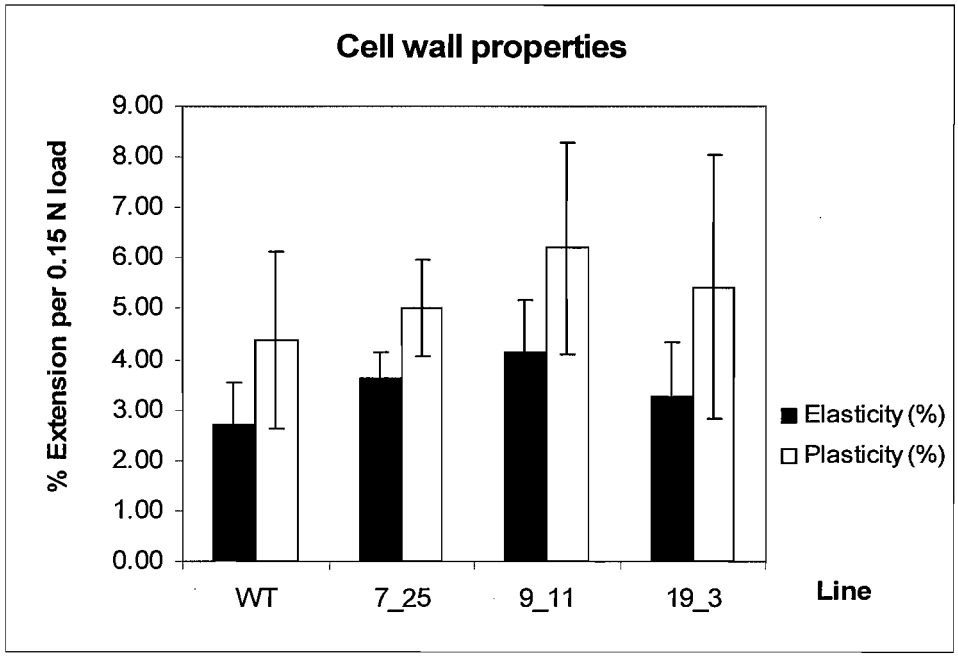


Figure A1.5 Cell wall properties (elasticity and plasticity) of the wild type lettuce and the transgenic lettuce. Data points are means ± SD, n=5. No significant difference between the transgenic lines and the wild type lettuce is shown in the one-way ANOVA t-test.

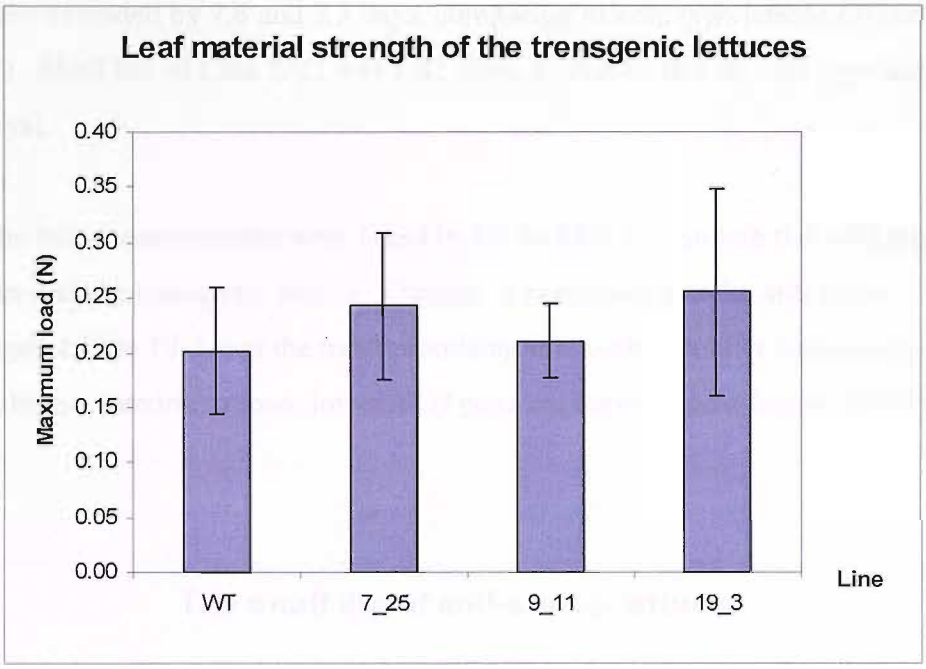


Figure A1.6 Leaf material maximum load of the wild type lettuce and the transgenic lettuce. Data points are means ± SD, n=5. No significant difference between the transgenic lines and the wild type lettuce is shown in one-way ANOVA t-test.

A1.2.4 Shelf life of transgenic lines is extended

Six-week old plants were harvested, and processed using simulated mini-process equipment in the laboratory. The sealed bags were kept at 7 °C fridge and shelf life was determined through visual assessment. When breakdown, bruising or damage was seen in the pack, the bag was rejected. Preliminary data from T_3 transgenic plants showed that the shelf life of the transgenic plants (9-11 and 19-3) were extended by 2.6 and 2.3 days, comparing to wild type lettuce (Figure A1.7). Shelf life of Line 7-25 was 7.82 days, similar to that of wild type lettuce (8 days).

All the trait measurements were listed in Table A1.1 to compare the wild type lettuce and the transgenic lettuce. Overall, it suggested that the anti-sense transgenic Line 19-3 was the most promising line with a smaller leaf area, cell area, higher maximum load, lower *XTH* gene expression and a longer shelf life.

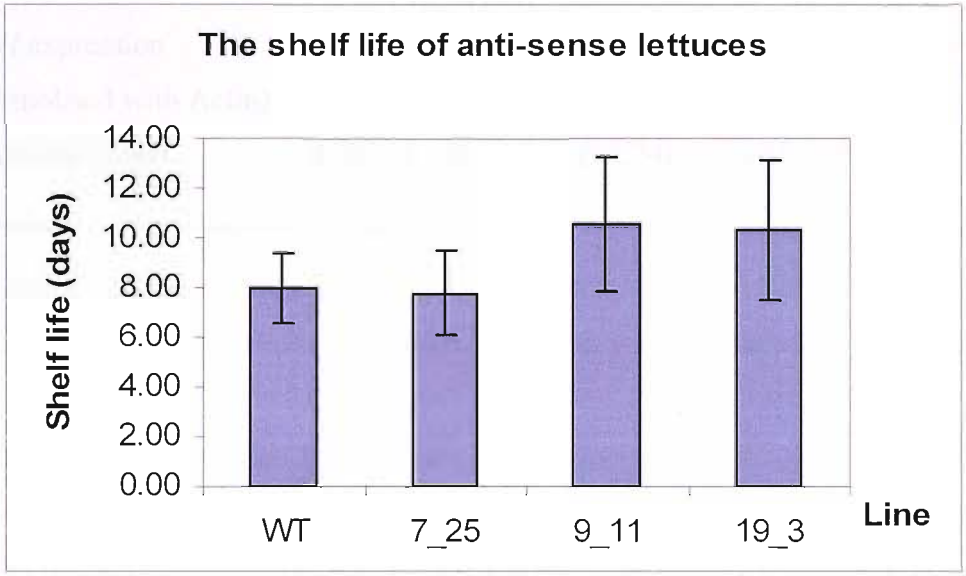


Figure A1.7 Shelf life of T_3 anti-sense *XTH* lettuce and wile type lettuce. Data points are means ± SD of replicates. The replicates of each line are: 2 (wild type); 17 (7-25); 9 (9-11) and 9 (19-3). For shelf life assessment see note on Figure 4.19.

Table A1.1 The trait measurement comparisons between the wild type lettuce and the transgenic lettuce (Mean ±SD).

Line	**Wild type**	**7-25**	**9-11**	**19-3**
Leaf area (mm^2)	7709 (±263)	5571(±619)	5930 (±783)	6412 (±705)
Fresh weight (Leaf 6)	2.32 (±0.61)	1.74 (±0.20)	1.61 (±0.42)	1.77 (±0.38)
Elasticity (E) (% per 0.15 N load)	2.70 (±0.83)	3.64 (±0.49)	4.13 (±1.04)	3.26 (±1.08)
Plasticity (P) (% per 0.15 N load)	4.38 (±1.75)	5.00 (±0.96)	6.20 (±2.09)	5.44 (±2.59)
Total extension (P+E) (% per 0.15 N load)	7.08 (±2.45)	8.64 (±1.09)	10.33 (±2.91)	8.69 (±3.56)
Maximum load (N)	0.20 (±0.06)	0.24 (±0.07)	0.21 (±0.03)	0.26 (±0.09)
XTH expression (normolised with Actin)	1.14	1.15	1.00	0.40
Shelf-life (days)	8.00 (±1.41)	7.82 (±1.74)	10.56 (±2.70)	10.33 (±2.83)

A1.3 Discussion

Overall, Line 19-3 showed as the most promising transformed plant with higher break strength (maximum load), longer shelf life and lower *XTH* gene expression. It suggested that reduction of XTH activity in the anti-sense *XTH* leaves increased rigidity of cell wall, therefore, improved resistance to damage from process and resulted in longer shelf life in transgenic line 19-3. No significant difference between transgenic lettuce and wild type lettuce was observed in this study. This might be due to an insufficient number of replicates measured in this experiment. Repeat experiment of shelf life with more replicates of wild type lettuce is planned to do. Further analysis will be done on in-vivo co-localization of XTH activity to quantify XTH as leaves develop and cells expand, to provide further fundamental insight on the role of this enzyme in the control of cell growth. The molecular weight distribution of xyloglucan hemicelluloses and cellulose in the cell wall fraction of these plants will be assessed to explore which cell wall fraction may be altered by the genetic modification.

Young transgenic leaves showed a greater fresh weight than the wild type leaves, which implied that young transgenic leaves have more water taken than wild type leaves (Figure A1.4). But the fresh weight plateaus midway through the growth period such that older leaves have a reduced fresh weight in transgenic lines compared to wild type. Older leaves also have a reduced leaf area in the transgenic lines, indicating the reduction of XTH activity is developmental specific, only at young developmental stage. This explained the result that the cell wall plasticity of transgenic lines was not reduced in the transgenic plants. A similar result was observed in the previous study which found that only cell wall plasticity was reduced in the young leaves of the transgenic lettuce rather than mature leaves of the transgenic lettuce (Clarkson et al, 2004). A repeat experiment, with more replicates of different leaf development stages (not too young to prepare leaf strip), is planned to assess the developmental effect pattern of anti-sense *XTH* on cell wall properties. An important part of this will be the acquisition of adequate seed supplies and this will be achieved using a commercial and confidential technique developed by Shamrock Seed Company, one of the biggest lettuce seed companies in the world. To further investigate the

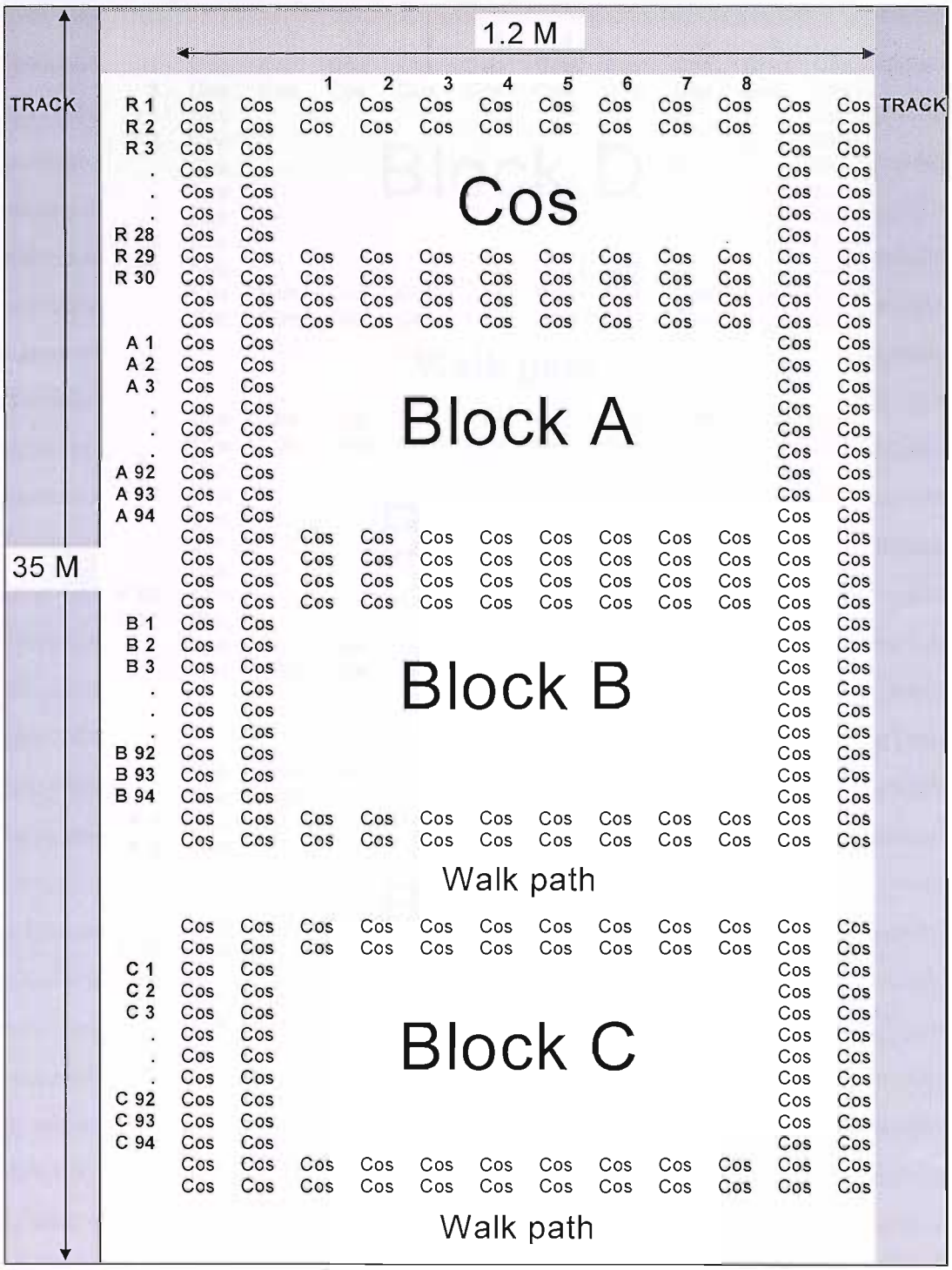
shelf life in T_3 and T_4 transgenic plants with a larger number of replicates salad bags assessed for shelf life if enough material is available.

The introduced gene may be silent in Line 7-25 and Line 9-11 after a few generations. Recently, silenced genes have received much attention. Studies have shown that silenced, or low transgene expression, is caused by transgene methylation. Transgene silencing in lettuce was reported in other studies (McCable et al., 1999b). The dwarf segregation observed in Line 19-3 suggested that there might be multiple insertion events in Line 19-3, as the second insertion in this line had a pleiotropic effect due to the gene it has gone into. Hobbs et al., (1993) showed the transgene copy number could negatively be associated with transgene expression. On contrast, a higher transgene copy number has been reported to cause a high transgene expression in tobacco (Van der Hoeven et al., 1994). It would be interesting to do southern analysis on dwarf Line 19-3 to reveal any correlation between plant phenotype and transgene gene copy number.

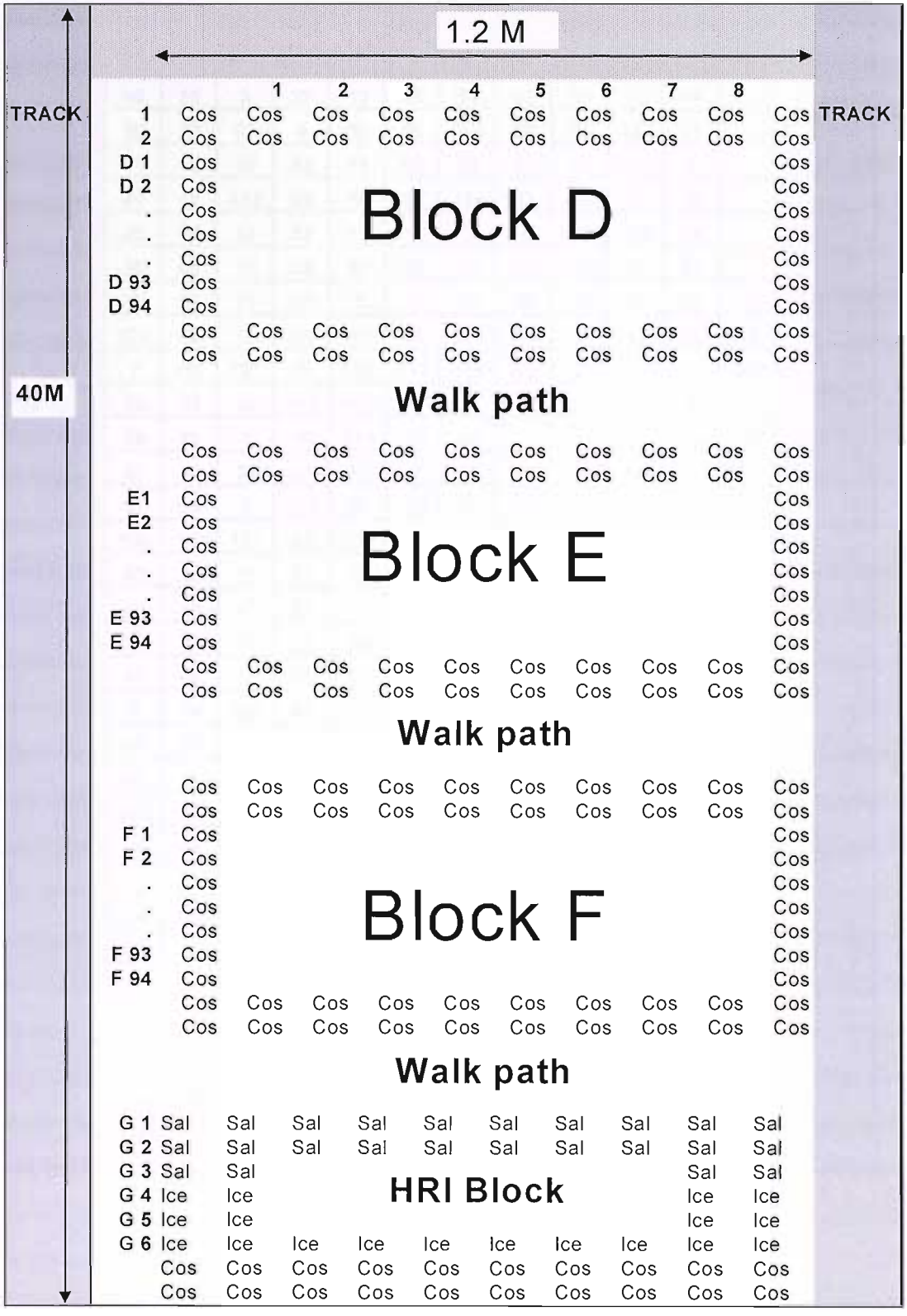
The instability of transgenic plants significantly increases the cost for the transgenic technology application commercially. The traditional transformation such as anti-sense *XTH* lettuce discussed before offers a precise function of a particular gene of interest. But it has led to questions about the use of transgenic technologies for food. The new technology, TILLING, has recently developed to produce a target gene mutant line without GM complication. Once the important role of the XTH gene on leaf shelf life is confirmed by further investigation, this non-GM, reverse genetic approach, TILLING, is planned to take this finding in transgenic study forward for the commercial breeding.

Appendix 2 The field trial maps

Appendix 2.1 The field map of the Portugal field trial. Three randomised blocks (A, B, C) of three replicates of 62 lines were planted in this site. The plant material growing period was from 10/04/2003 to 27/05/2003 in this field trial.



Appendix 2.2 The field map of the UK field trial. It included three randomised blocks (D, E, F) of three replicates of 62 lines and one HRI block of two parents for another population from Horticulture Research Institute (HRI), UK. The plant material period was from 15/07/2003 to 29/08/2003.



Appendix 2.3 The field map of the extended F_{10} RIL mapping population in the American field trial (Califonia, USA). Three replicates of 112 lines were randomised in one block. The plant material period was from 05/04/2004 to 02/06/2004.

96	55	3	27	19	45	31	63	67	12	94	
99	25	61	9	52	56	106	56	64	127	23	
31	72	29	82	78	69	58	51	77	20	6	
40	35	132	93	36	80	114	23	92	11	50	
26	47	57	62	71	12	3	115	70	118	74	
36	62	18	23	97	59	56	132	120	8	63	
77	95	75	103	8	14	98	88	131	43	48	128
125	64	15	80	102	41	110	131	2	12	102	85
7	111	121	6	128	117	109	130	52	112	69	104
22	83	98	105	105	20	57	30	30	15	39	76
96	81	7	76	117	121	44	17	119	9	110	107
45	90	94	101	50	17	99	59	11	83	13	19
91	68	3	125	95	127	86	108	114	77	70	119
104	87	121	25	103	24	70	16	112	99	79	5
93	40	59	43	33	16	123	11	96	64	1	1
24	48	95	83	6	112	116	116	72	30	21	97
25	81	57	29	88	42	90	1	92	104	41	109
91	89	37	102	98	85	91	69	40	106	106	53
4	74	128	84	33	58	79	26	94	67	17	66
66	27	79	87	118	68	14	75	29	15	7	101
93	8	18	71	19	85	13	111	45	87	22	51
123	47	4	18	108	32	84	89	78	103	53	55
39	42	105	84	21	107	118	111	115	41	43	66
2	92	21	109	24	42	76	132	5	50	16	32
130	127	34	26	22	13	86	107	123	78	89	110
130	33	74	47	120	58	27	34	88	4	122	67
75	44	71	119	32	120	20	37	34	80	63	35
97	53	55	101	9	82	37	14	39	44	36	116
90	35	61	5	125	81	114	52	82	122	62	48
51	86	61	108	117	115	72	131	2	31	68	122

Appendix 2.4 The field map of the extreme RILs in the Spain field trial. Three blocks of 12 lines randomized in each block (5 good and 5bad lines in terms of shelf life and two parents). The plant material period was from 20/01/2005 to 7/04/2005.

1.2 M

		1	2	3	4	5	6	7	8	9	10	11	12	
TRACK	R 1-6	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	TRACK
	J1-6	32	32	32	32	32	32	32	32	32	32	32	32	
	J7-12	1001	1001	1001	1001	1001	1001	1001	1001	1001	1001	1001	1001	
	J13-18	15	15	15	15	15	15	15	15	15	15	15	15	
	J19-24	19	19	19	19	19	19	19	19	19	19	19	19	
	J25-30	1002	1002	1002	1002	1002	1002	1002	1002	1002	1002	1002	1002	
	J31-36	13	13	13	13	13	13	13	13	13	13	13	13	
	J37-42	1	1	1	1	1	1	1	1	1	1	1	1	
	J43-48	112	112	112	112	112	112	112	112	112	112	112	112	
	J49-54	74	74	74	74	74	74	74	74	74	74	74	74	
	J55-60	105	105	105	105	105	105	105	105	105	105	105	105	
	J61-66	5	5	5	5	5	5	5	5	5	5	5	5	
Block J	J67-72	89	89	89	89	89	89	89	89	89	89	89	89	
	R7-12	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	
	G1-6	105	105	105	105	105	105	105	105	105	105	105	105	
	G7-12	19	19	19	19	19	19	19	19	19	19	19	19	
	G13-18	89	89	89	89	89	89	89	89	89	89	89	89	
	G19-24	1001	1001	1001	1001	1001	1001	1001	1001	1001	1001	1001	1001	
	G25-30	112	112	112	112	112	112	112	112	112	112	112	112	
Block G	G31-36	1	1	1	1	1	1	1	1	1	1	1	1	
	G37-42	5	5	5	5	5	5	5	5	5	5	5	5	
	G43-48	32	32	32	32	32	32	32	32	32	32	32	32	
	G49-54	74	74	74	74	74	74	74	74	74	74	74	74	
	G55-60	13	13	13	13	13	13	13	13	13	13	13	13	
	G61-66	5	5	5	5	5	5	5	5	5	5	5	5	
	G67-72	1002	1002	1002	1002	1002	1002	1002	1002	1002	1002	1002	1002	
	R 13-18	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	
	H1-6	15	15	15	15	15	15	15	15	15	15	15	15	
	H7-12	112	112	112	112	112	112	112	112	112	112	112	112	
	H13-18	89	89	89	89	89	89	89	89	89	89	89	89	
	H19-24	32	32	32	32	32	32	32	32	32	32	32	32	
	H25-30	1	1	1	1	1	1	1	1	1	1	1	1	
	H31-36	1001	1001	1001	1001	1001	1001	1001	1001	1001	1001	1001	1001	
Block H	H37-42	105	105	105	105	105	105	105	105	105	105	105	105	
	H43-48	5	5	5	5	5	5	5	5	5	5	5	5	
	H49-54	13	13	13	13	13	13	13	13	13	13	13	13	
	H55-60	1002	1002	1002	1002	1002	1002	1002	1002	1002	1002	1002	1002	
	H61-66	74	74	74	74	74	74	74	74	74	74	74	74	
	H67-72	19	19	19	19	19	19	19	19	19	19	19	19	
	R18-24	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	Tango	

Appendix 3 The parental experiment of HRI mapping population

The parents of the HRI mapping population are Saladin and Iceberg. The seed material was supplied by Prof.. D. Pink (Horticulture Research International, UK). Saladin is a standard American iceberg type crisp bred by Dr. E. Ryder at Salinas, California. Most of the modern iceberg types grown in Europe are derived from it. Iceberg is not an iceberg type. Actually it was taken to the US by immigrants and the iceberg types are derived from it. It is an old Batavian variety bred in France in the mid-1800s. Its original name is Batavia blonde bord rouge. It has much softer pale green leaves with red edges.

In 2003, the parental experiment was taken in Pinglestone farm, Winchester, UK to test the suitability of this population to identify leaf traits for leaf processability. Thirty plants of each parent were planted in this field trial and 10 replicates of each parent were sampled for the leaf traits of interest. The results showed there were no significant differences between two parents of the HRI mapping population in most of the traits tested, except chlorophyll content and leaf fresh weight and epidermal cell number per leaf (Table A3.1). The leaves of *L. saladin* had a slightly longer shelf life than those of L. iceberg (7.8 days vs 6.5 days), however, it was not significant ($P = 0.14 > 0.05$). No further experiment was taken on this mapping population in this study.

Table A3.1 The summary of leaf development traits of parent lettuce (Mean ± STD, The results of a one-way ANOVA are indicated where significant: *** P < 0.001, ** P< 0.01, * P< 0.05, ns=not significant.)

Trait	**Saladin**	**Iceberg**	**ANAVA (*n*=10)**
Mean leaf area of L3, 6, 7 & 8 (mm^2)	5564 (± 1124)	6386 (± 948)	ns
Epidermal cell area (μm^2)	2164 (± 328)	1897 (± 433)	ns
Epidermal cell no. per leaf (x 10^6)	2.62 (± 0.65)	3.58 (± 1.15)	*
Chlorophyll contents ($\mu g\ mm^{-2}$)	0.20 (± 0.02)	0.10 (± 0.02)	***
Leaf fresh weight (g)	3.78 (±0.55)	4.55 (± 0.64)	**
Leaf dry weight (g)	0.25 (± 0.05)	0.27 (± 0.05)	ns
Dry weight as a % fresh weight (%)	6.51 (± 0.58)	6.02 (± 1.83)	ns
Specific leaf area ($mm^2\ mg^{-1}$)	1.87 (± 0.32)	1.74 (± 0.25)	ns
Osmolality ($mmol\ kg^{-1}$)	328 (± 38.66)	361 (± 65.82)	ns
Shelf life (days)	7.80 (± 1.75)	6.5 (± 0.25)	ns

Appendix 4 The list of attribute and definition in sensory assessment.

Sensory	Attribute	Definition	Anchors
Appearance	Amount of Curling	Degree in which the leaf is curled over	Flat - folded
	Depth of colour (green)	Depth of overall colour, ranging from pale to dark green	Pale - dark
	Evenness of green	Degree in which the green leaf colour is consistent across the entire surface	Not at all - very strong
	Surface smoothness	Leaves having a smooth surface, not crinkled	Not - very
	Type of leaf edges	The shape of the edges of the leaf from smooth rounded to crinkled/serrated	Rounded - crinkled
	Size	The overall size of the entire leaf sample	Small- large
	Maturity	Sample having an over-mature/aged appearance	Not - very
	Body firmness	The degree in which the leaf can maintain its structure when held from the stem	Not - very
Flavour	Thickness	Thickness of stem	Thin - thick
	Overall strength	Overall strength of flavour related to the leaf	Not - very
	Leaf/grassy/green	Flavour reminiscent of fresh green leafy vegetables	Not - very
	Lettuce	Flavour reminiscent of fresh iceberg lettuce	Not - very
	Earthy	Flavour reminiscent of clean dirt	Not - very
	Sweet	Associated with sucrose	Not - very
	Bitter	Associated with quinine	Not - very
	Acidic	Associated with citric acid	Not - very
Texture	Crisp	The initial bite of the leaf using front teeth accompanied with a characteristic sound	Not - very
	Crunch	The texture of the leaf when using the back molars to break down product accompanied with a characteristic sound	Not - very
	Moistness/juicy	The amount of moisture released when chewed	Not - very
	Chewy	The amount of mastication required to break down the product	Not - very

Appendix 5 Publications

Journal papers

Taylor, G., Tricker, P. J., **Zhang, F. Z.**, Alston, Victoria, J., Miglietta, F., & Kuzminsky, E., (2003) Spatial and temporal effects of free-Air CO_2 enrichment (POPFACE) on leaf growth, cell expansion, and cell production in a closed canopy of Poplar, Plant Physiology **131**: 177-185.

Zhang, F.Z., Dixon, M.S., Rothwell, S. D., Clarkson, G.J., Michelmore, R.W, Truco, M. J. & Taylor, G., Identification of QTL for leaf development in lettuce and their relationship to post-harvest leaf longevity. (Accepted by Journal of Experimental Botany).

Wagstaff G., Clarkson, G.J., **Zhang F.Z.,** Rothwell, Fry, S.C., Taylor, G. & Dixon, M., Modification of cell wall properties in lettuce improves shelf life. (Under review).

Zhang, F.Z., Wagstaff, G., Dixon, M.S., Rothwell, S. D., Michelmore, R.W, Truco, M. J. & Taylor, G., Identification of QTL for leaf cell wall properties related to post-harvest leaf longevity in lettuce and their linkage to candidate genes (In preparation).

Conference paper presentations

Zhang, F.Z., Cherry, K., Clarkson, G.J.J., Dixon, M.S., & Taylor, G., Leaf development in lettuce: identifying the processability traits in parents of a mapping population. Comparative Biochemistry and Physiology Part A. 134, S167. Society for Experimental Biology conference in Southampton, UK, 31st March – 4th April, 2003 (Awarded the 2003 Irene Mante Prize for best poster in Plant Section, SEB Annual General Meeting, Southampton, 2003).

Zhang, F.Z., Clarkson, G.J.J., Michelmore, R.W, Dixon, M.S., & Taylor, G., Elucidating QTL for leaf development in lettuce and their linkage to candidate genes. Comparative Biochemistry and Physiology Part A. 137, S200. SEB conference in Edinburgh, UK, 29th March – 2nd April, 2004.

Zhang, F.Z., Rothwell, S. D., Clarkson, G. J. J., Michelmore, R. W., Dixon, M. S. & Taylor, G., Can we find the perfect salad leaf? Invited to present research poster at Britain's Top Younger Scientists, Engineers and Technologists (SET) evening reception held at House of Common on 14th March, 2005.

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APPENDIX 6 Poster presented at House of Commons

CAN WE FIND THE PERFECT SALAD LEAF?

University of Southampton

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Introduction

Perfect salad leaf traits:

- High nutritional content
- Good taste
- Long shelf life
- Contributes to 'five-a-day'

Long life salad leaf has:

- Small cells
- Strong cell walls
- High leaf thickness
- High cell solute concentration
- More cuticular waxes

Methods

➢A gene involved in cell wall loosening (XTH) was selected to make transgenic lettuce as a research tool to improve baby salad leaf.

➢A lettuce mapping population was assessed for leaf quality in the quantitative genetic study.

Aim

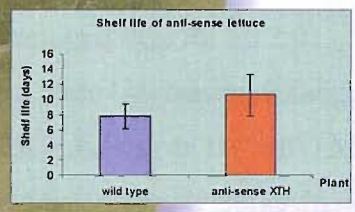
The aim of this research is to improve the baby leaf salad quality with longer shelf life.

Fig. 1 Pre-packed baby leaf salad

Results (1) – transgenic study

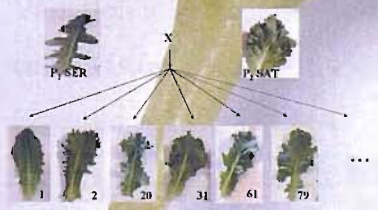
The XTH activity is co-localised to the cell wall with a fluorescence dye. The XTH activity was dramatically reduced in the anti-sensed plant (Fig. 2).

The shelf life of leaves from anti-sense XTH was 2.5 days longer than wild type (Fig. 3). This strongly suggests that XTH plays a very important role in the leaf shelf life.



Results (2) - genetic study

Leaf images of different plants in the mapping population show very different leaf shapes (Fig. 4).



The leaf genome areas that are responsible for a whole variety of salad leaf quality traits were identified in this study.

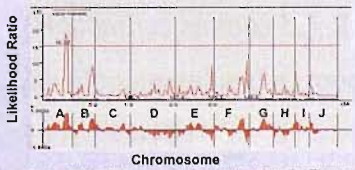


Fig. 5. An example of DNA area associated with leaf quality. The peak in the graph indicates the location of genome area associated with leaf shelf-life is on chromosome A: 90 -100cM.

Conclusions

➢**Anti-sense XTH extends the baby leaf salad shelf life by 2.5 days.**

➢**Areas of the lettuce genome (DNA) responsible for leaf quality were identified.**

Application

Acknowledgements

BBSRC and Vitacress Salad Ltd for the financial support.
Professor S. Fry (University of Edinburgh) for XTH activity assessment.
Dr. C. Wagstaff (University of Southampton) for the transgenic lettuce planting.
Dr. M. Truco (University of California) for helping with genetic analysis.

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