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Department of Warwick HRI

Genetic Characterisation of Post Harvest Spoilage in Lettuce

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Thesis presented for the degree of Doctor of Philosophy

in Plant and Environmental Sciences

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Declaration

I declare that the material contained in this thesis is obtained from my own work which has not been submitted to another university for a degree and has not been published previously. Any data, materials and information obtained from external sources, published or unpublished, are identified as such and referenced accordingly.

Laura D Atkinson

Summary

Post harvest discolouration in lettuce is an increasingly important problem due to the shift in the market for prepacked processed salads. Variation in post harvest discolouration was recorded in a lettuce diversity set of 28 accessions representative of the lettuce genepool. The parents of the WHRI lettuce mapping population, Saladin and Iceberg were included in the diversity set. They showed significantly different responses for discolouration and the difference between them was representative of a major part of the variation seen in the diversity set. F_7 RILs derived from a cross between Saladin and Iceberg were suitable for genetic analysis of post harvest discolouration. As a precursor to the genetic analysis, a good quality linkage map based on the F_7 Sal x Ice population was generated. Significant genetic variation in the post harvest response was demonstrated for these RILs. Twenty-one significant QTL were identified for post harvest discolouration traits, and the markers linked to the QTL can be used for marker assisted selection. Significant but weak correlations were recorded between discolouration and important agronomic traits, however as these were not highly correlated this means that post harvest discolouration and agronomic traits can generally be independently selected for by breeders without having to compromise on other traits. Research was also initiated to understand the metabolic changes underlying the phenotype change. Significant variation in levels of metabolites related to post harvest discolouration including phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and total phenolic content (TPC) was observed in RILs with extreme phenotypes. The differences in metabolite levels were significantly correlated with the discolouration phenotypes. Work was also initiated to identify candidate genes associated with the biosynthetic pathway responsible for discolouration (phenylpropanoid pathway) in an attempt to identify genes underlying QTL. Twenty-three genes have been placed on the Sal x Ice map using comparative genomic approaches. Some of these co-locate within the region of a discolouration QTL and are therefore candidate genes for the QTL effect. Mutants with altered post harvest discolouration phenotypes were also confirmed in this study; however the point of mutation could not be identified although it is thought to be downstream of PPO. This indicates that a desired phenotype with reduced levels of post harvest discolouration can be achieved by two approaches to breeding; using natural and induced variation. This study has provided the tools and knowledge to do this.

CHAPTER 1

Introduction

1.1. Background

1.1.1. Pre-packed cut salad industry

In recent years there has been encouragement by governments for consumers to eat more healthily. In the UK, this has been encouraged by the Food Standards Agency and The Department of Health via the 'five-a-day' initiative relating to servings of fruit and vegetables. Ready-to-eat food products provide consumers with a convenient way to meet these targets (Masih *et al.* 2002). Pre-prepared salad packs fit the criteria of being both healthy and convenient with sales reflecting this worldwide.

There is an escalating demand for 'ready-to-eat' salad products with minimal preparation times due to the increasingly busy lifestyles of consumers (Soliva-Fortuny and Martin-Belloso 2003). In an independent report, MINTeL oxygen (2007) conveyed that the UK processed bag and dressed salad sector is worth approximately £798 million per annum. The provisional value of UK lettuce production alone marketed for 2008 exceeded £98 million, with imports of lettuce in excess of £155 million (Defra 2009). An annual growth of 15-20% has been reported to cater for the increased consumer demand for prepared fresh produce from multiple retailers. Despite a recent levelling of sales in Europe, the European salads market has been predicted to reach €7 billion by 2012 (Schene 2007). The increased demand for fresh processed produce has simultaneously led to an increase in quantity and variety of produce available to the purchaser (Francis *et al.* 1999).

However, substantial variation in product quality and consequential losses have been reported. In today's ever-increasing market of 'food perfection', any alteration to the visual characteristics of a product is likely to incite an unfavourable consumer response; with a prime example being pre-packed cut salads. It has recently been suggested that almost 50% of salads purchased in the UK are thrown away (WRAP 2008). Of this 22% is lettuce and 13% being mixed salads (lettuce is a major component of these mixed salads) which are thought to cost £230 million. The main reason for wastage (48%) was that the product had passed its 'sell by date'. Wastage levels of up to 30% have been recorded for lettuce during processing of raw material due to post harvest discolouration (causing a loss of quality) costing the UK industry an estimated £2.5 million per annum. This does not however include wastage during transport, packaging, hosting and service costs (Soliva-Fortuny and Martin-Belloso 2003; Wurr *et al.* 2003). Seed needs to be purchased by growers, the mature crop harvested and packaged, transported for sale and then sold. If the salad pack does not last its required shelf life then previous processes have been uneconomical, with the cost still needing to be accounted for by the grower. In addition, fresh cut products have a limited life on the shelf before the 'best before' time expires. Pre-packed salads generally have a relatively short shelf life of 3-5 days and there is a need to extend post harvest quality and the resultant shelf life in order to reduce waste and deliver a product of consistently good quality to the consumer.

1.1.2. Lettuce as a commercial crop

Consumer demand

In many countries lettuce has become an extremely popular leafy vegetable. It is estimated that lettuce is the most frequently consumed vegetable in America, with approximately 37-42% of Americans consuming lettuce within their diet (Johnston *et al.* 2000). Lettuce is progressively being sold to consumers through retail and/or food service either alone or in salad mixes, with consumption increasing due to its perception as being amongst healthier foods (Desphande and Salunkhe 1998; Dupont *et al.* 2000). In particular, minimally processed lettuce has become common due to the fast food industry and prepared salads market (Altunkaya and Gökmen 2008). The consumption of lettuce is predicted to increase as the adult population aim to consume five or more servings of fruit and vegetables per day (Johnston *et al.* 2000). As result of the increased market demand coupled with the restricted shelf life of lettuce, post harvest quality (specifically discolouration) of lettuce was selected for targeted improvement.

Processing and handling

Good quality raw materials are initially required in order to produce a high quality prepacked processed product (Sandhya 2010). The processing operations applied to commercial ready to use lettuce generally include cleaning/washing, trimming, coring, either cutting (typically to 5-8cm^2 (Hilton *et al.* 2009)), slicing or shredding, washing (in good quality, in terms of microbial content and pH processing water with mild additives (for disinfection and prevention of discolouration)), centrifugation and packaging (with the most appropriate materials and conditions) in low temperatures and under strict hygiene and good manufacturing practices (King *et*

al. 1991; Sandhya 2010). The product then needs to be handled carefully under the correct temperature and humidity during distribution and retailing (Sandhya 2010). Processing can damage lettuce leaves and reduce post harvest quality. The commercial success of the ready-to-eat product industry relies heavily on the ability of the lettuce tissue to withstand each process. From harvest, there is usually a period of one to four days for the processing, packaging and transport procedure in order to maintain post harvest quality (Wagstaff *et al.* 2010).

1.1.3. Post harvest quality

Post harvest quality relates to many aspects including appearance, sensory quality (including texture, taste and aroma), nutrient value and shelf life longevity (Kader 2002). Appearance is considered as the decisive factor which results in a purchase. Product appearance is categorised by numerous factors including size, shape, form, colour, condition and presence/absence of defects from the 'normal' perception (Kays 1999). Various pre harvest factors can alter the appearance of a product including genetic variation, biological factors, physiological factors, agronomic and or environmental factors, extraneous matter and mechanical damage (Kays 1999; Hilton *et al.* 2009).

Texture is also an extremely important factor for consumer acceptability for any fresh produce. In leafy vegetables a crisp firmness is generally desired. The texture of vegetables can be affected by many parameters including cellular organelles, water content or turgor and cell wall composition. The genetic background of the cultivar is the main factor controlling texture, however agronomic and environmental factors modify the expression of traits (Sams 1999).

Although flavour has no impact on the initial purchase decision, it is the critical reason for repeat purchasing (Aked 2002; Kader 2002). Flavour is an individuals' response to a product and is a complex mixture of taste (sweetness, bitterness, acidity and astringency) and aromatic components (Aked 2002).

The health promoting benefit of eating particular types of food is becoming increasingly important for consumers. Lettuce differs from other vegetables by its content of several types of fibres and micronutrients. It is a key contributor of vitamins and minerals in the human diet, and has been shown to be an important source of dietary antioxidants, in addition to having high vitamin A, C and E content and high levels of calcium and potassium (Ryder 2002; Caldwell 2003; Nicolle *et al.* 2004a). Lettuce consumption can lead to lowering of cholesterol levels and provide protection against cardiovascular disease (Nicolle *et al.* 2004b). High folate levels have been recorded in romaine lettuce which impact on human diseases such as birth defects, cancer and heart disease (Kader 2002).

Shelf life

The minimum requirement for the shelf life of prepacked lettuce to the consumer is currently five to six days (Wagstaff *et al.* 2010); although salad packs generally only survive three days from time of purchase until 'end of life'. There is therefore a high demand from both retailer and consumer for products with longer shelf life. Additionally, consumers expect produce to be fresh, visually uniform, without detrimental change to organoleptic (sensory) characteristics and to be at the correct stage of maturity depending on salad type (Watada and Qi 1999). Shelf life of salad products can be limited by microbial spoilage, discolouration, textural changes and the development of 'off-flavour' or 'off-odour' characteristics (Barrett *et al.* 1998).

The Phenylpropanoid Pathway

Cut salad packs often suffer from discolouration on leaf surfaces within a few days after harvest, limiting their shelf life. Enzymatic and non-enzymatic oxidative processes cause 'browning' and 'pinking' which results in the emergence of coloured pigments (brown and pink/red respectively) at cut surfaces produced via the phenylpropanoid pathway (see figures 1.1. and 1.2.) (Martinez and Whitaker 1995; Payne *et al.* 2006). The alteration in visual appearance results in rejection by the consumer and quality loss due to a decrease in beneficial phenols (Tomás-Barberán and Espín 2001). The occurrence of enzymatic browning in many food products has been reported as a severe problem for the food industry as a whole (Chiesa 2003).

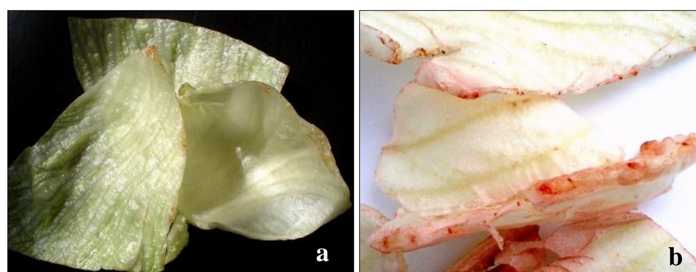

Figure 1.1. Post harvest browning (a) and pinking (b) of lettuce tissue.

The phenylpropanoid pathway is a secondary metabolic pathway producing non essential metabolites. Phenylpropanoid plant products are derived from *trans*-cinnamic acid formed by the deamination of phenylalanine by phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) (Wanner *et al.* 1995). PAL catalyses the conversion of phenylalanine to *trans*-cinnamic acid in the initial and core step of the pathway thus controlling the flux of primary metabolites into this secondary metabolic pathway (Wanner *et al.* 1995). There are subsequent specific branch pathways for the formation of all major classes of phenylpropanoid compounds including monolignols/lignin, sinapate esters, condensed tannins, anthocyanins, coumarins, benzoic acids, flavonoids/isoflavonoids and stilbenes (Dixon *et al.* 2002).

PAL is normally induced upon plant tissue wounding, increasing activity in vascular tissue and therefore increasing downstream biosynthesis of polyphenols for oxidation (Nicholas *et al.* 1993; Lopez-Galvez *et al.* 1996; Peiser *et al.* 1998; Hisaminato *et al.* 2001). PAL genes occur in small families of 2-6 members in the majority of plant species (Cramer *et al.* 1989; Lois *et al.* 1989; Minami *et al.* 1989; Tanaka *et al.* 1989; Ohl *et al.* 1990; Frank and Vodkin 1991; Gowrier *et al.* 1991; Lee *et al.* 1992; Minami *et al.* 1993; Subramaniam *et al.* 1993).

The synthesis of phenolic compounds is associated with the endoplasmic reticulum, with the proteins involved in their synthesis either incorporated or loosely associated with it. Once the phenolic compounds have been formed they are glycosylated and then transported from the endoplasmic reticulum in transport vesicles formed from its membrane. Phenolic compounds are then transported to the vacuole or into the apopolast or cell wall compartment. Phenolic compounds may be found at low levels in chromoplasts, cytoplasm and the mitochondria although these are normally associated with specialised functions (Hrazdina and Wagner 1985).

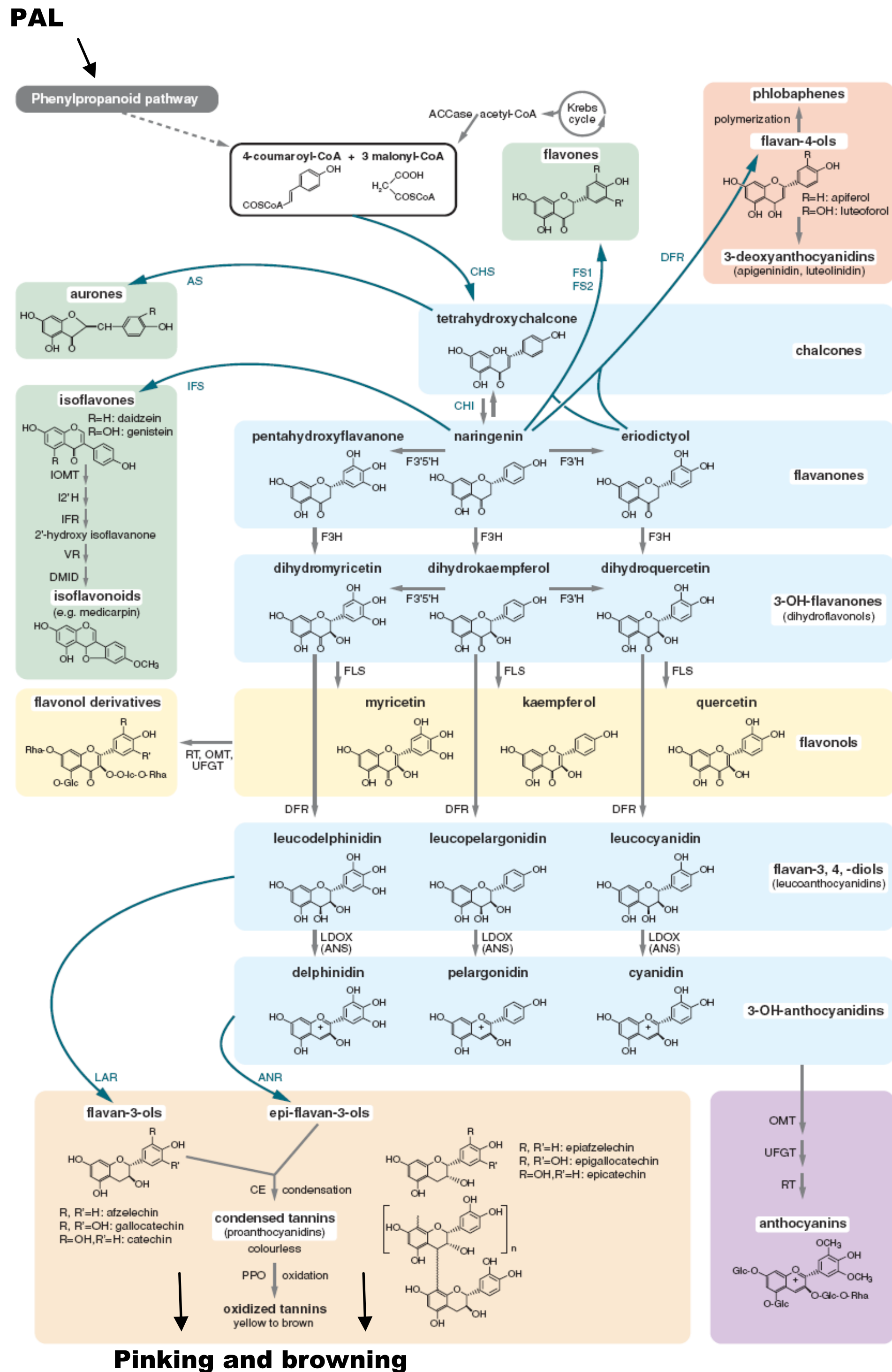

Figure 1.2. The Phenylpropanoid pathway resulting in post harvest pinking and browning. Where (*ACCase*) acetyl CoA carboxylase; (*ANS*) anthocyanidin synthase; (*ANR*) anthocyanidin reductase; (*AS*) aureusidin synthase; (*DFR*) dihydroflavonol 4-reductase; (*DMID*) 7,2_-dihydroxy, 4_-methoxyisoflavanol dehydratase; (*F3H*) flavanone 3-hydroxylase; (*F3_H*) flavonoid 3_-hydroxylase; (*F3_5_H*) flavonoid 3_5_ hydroxylase; (*FLS*) flavonol synthase; (*FSI/FS2*) flavone synthase; (*I2_H*) isoflavone 2_-hydroxylase; (*IFR*) isoflavone reductase; (*IFS*) isoflavone synthase; (*IOMT*) isoflavone *O*-methyltransferase; (*LAR*) leucoanthocyanidin reductase; (*LDOX*) leucoanthocyanidin dioxygenase; (*OMT*) *O*-methyltransferase; (*PAL*) phenylalanine ammonia lyase; (*RT*) rhamnosyl transferase; (*UFGT*) UDP flavonoid glucosyl transferase; (*VR*) vestitone reductase. Figure from Lepiniec *et al.* (2008).

Discolouration is initiated by the breakdown of membranes within cells of plant tissues (Toivonen 2004). Upon physical stress cub-cellular compartmentalisation is disrupted at the wounded surface. This results in the mixing of substrates and enzymes which are normally separated commencing reactions which would not normally occur (see figure 1.3.) (Degl'Innocenti *et al.* 2005; Toivonen and Brummell 2008). Polyphenol oxidase (PPO) is the main agent responsible for discolouration; oxidising polyphenols synthesised via the phenylpropanoid pathway (Hisaminato *et al.* 2001). However a synergistic effect between PPO and phenol peroxidase (POD) (EC 1.11.1.7) is possible (Padiglia et al., 1995; Bouwens et al., 1999; Tomás-Barberán and Espín 2001).

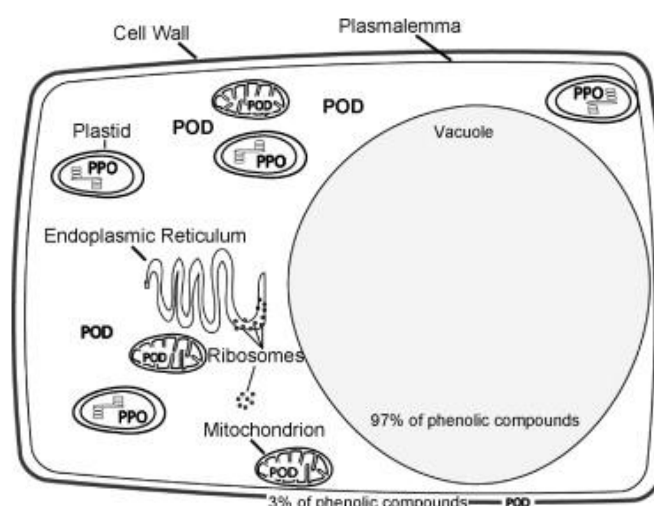

Figure 1.3. The internal and external localisation of phenolic compounds and phenolic oxidising enzymes. Where *PPO* (polyphenols oxidase); *POD* (phenol peroxidise). Figure from Toivonen and Brummell 2008.

PPO interacts with phenolic substrates in the presence of oxygen and catalyses two reactions; the hydroxylation of monophenols to diphenols via monophenol monooxygenase or tyrosinase (EC1.14.18.1) and the oxidation of diphenols to quinones via diphenolase (EC1.10.3.1) (Webb 1992; Lopez-Galvez *et al.* 1996). The hydroxylation reaction is slow and results in colourless products, while the oxidation reaction is rapid and results in coloured quinones (see figure 1.4.)

(Toivonen and Brummell 2008). Quinones undergo subsequent reactions (non enzymatic polymerisation with amino acids or proteins) leading to melanin accumulation, these occur as mainly brown pigments but can also occur as red pigments which are associated with 'browning' and 'pinking' in plant tissues (the formation of black pigments has also been recorded) (Joslin and Pointing 1951; Zawistowski *et al.* 1991; Martinez and Whitaker 1995; Solomon *et al.* 1996; Toivonen and Brummell 2008; Van Vliet *et al.* 2009). The reaction resulting in the pigments is dependent upon the structure of the phenolic substrate, with pinking more susceptible to phenolic composition (Wurr *et al.* 2003; Toivonen and Brummell 2008). It has also been suggested that pink pigments will gradually turn brown after prolonged storage (Van Vliet *et al.* 2009).

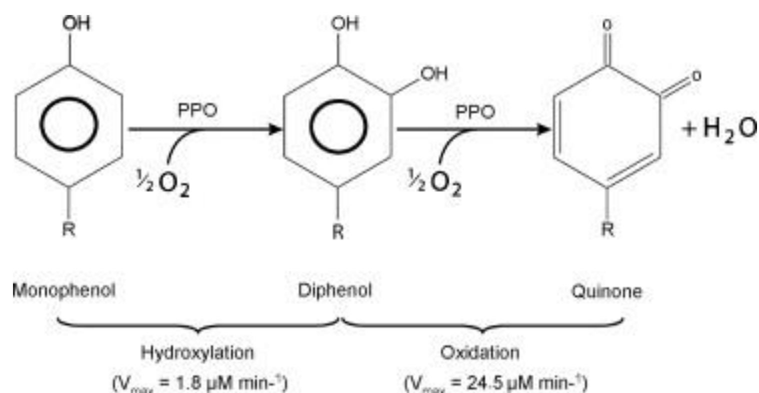

Figure 1.4. The mechanism for polyphenol oxidase action on monophenols and diphenols. The process of hydroxylation is slower than the process of oxidation (see Vmax). Figure from Toivonen and Brummell 2008.

The role of POD is difficult to define as one of its main substrates; hydrogen peroxide (H_2O_2) is present at low cell concentrations (Veljovic-Jovanovic et al., 2002). However H_2O_2 is associated with oxidative injury and is thus tightly regulated within cells (Jiang and Miles 1993; Mittler 2002). PPO mediated production of quinones could lead to H_2O_2 accumulation, increasing the free radical species concentration enabling POD mediated browning (Jiang and Miles 1993).

The structure of the phenolic substrate will also affect the potential for POD mediated browning due to differences in potential H_2O_2 yield (Cantos *et al.* 2002). However the mechanism of PPO mediated H_2O_2 generation has only been recorded *in vitro* (Jiang and Miles 1993). It has been suggested that POD could enhance browning reactions in the presence of PPO mediated browning (Richard-Forget and Gauillard 1997). Although it remains unclear whether POD mediated browning can occur in the absence of PPO mediated browning and therefore whether POD is actually a significant consistent component in the browning response of fresh produce (Toivonen and Brummell 2008).

Cell disruption leads to de-compartmentalisation involving a cascade of events, including PPO activation and promotion of PAL synthesis. PPO activation and both PAL and POD induction has been observed in six lettuce cultivars following cutting. However, there appeared to be no simple correlation between browning susceptibility and any of the parameters (Cantos *et al.* 2001). Although the degree of discolouration appears to have a genetic component, with 'Iceberg' types having lower documented PAL activity than 'Butterhead' ones, while 'Romaine' green and red leaf types appear to have intermediate PAL activity (Lopez-Galvez *et al.* 1996).

PPO appears to be constantly present in the cell, compartmentalised within the chloroplast and other plastids at supraoptimal levels, only being released upon tissue damage. This suggests that the degree of discolouration observed may be determined by absolute substrate availability driven by PAL activity rather than PPO activity (Wurr *et al.* 2003). Therefore the regulation of polyphenol biosynthesis could be a source for effective control of discolouration post processing in lettuce (Hisaminto *et al.* 2001).

Understanding the processes which lead to these changes is essential in developing better approaches to minimise them, which would result in improved quality and an extended shelf life. The biochemical processes causing changes in appearance and texture have generally been studied in the whole plant; the consequences of processing cut leaves have not been studied as extensively (Toivonen and Brummell 2008). The full determination of the heritability of post harvest discolouration would allow development of improved varieties for trait combinations (including organoleptic and metabolomic characteristics) by breeders. Therefore it is important to link gene function and gene regulation to phenotype. Hypothetically it should be possible to link metabolomic changes in biochemical pathways to the enzymes involved and consequently the underlying genetic variation.

1.1.4. Increasing shelf life

Pre-harvest factors including climatic conditions, cultural practice, produce maturity, harvesting method, storage and processing (see section 1.1.2.) can all affect the shelf life of a product (Lee and Kader 2000). In order to maintain minimally processed fresh produce of high organoleptic quality, a variety of post harvest treatments such as heating and cooling, dipping preservative chemicals and additives (hormones and physiologically active chemical exposure), high pressure and high field electric pulse treatments, irradiation and ultrasound treatments, films and edible coatings, packaging (moderate vacuum packaging (MVP) and modified atmospheric packaging (MAP)) have been developed for individual use or in combination (Tomás-Barberán and Espín 2001; Soliva-Fortuny and Martin-Belloso 2003).

However, MAP used in conjunction with various other techniques is most commonly used for minimally processed fresh vegetables (Sandhya 2010).

Modified atmospheric packaging (MAP)

The prevention of post harvest discolouration in pre-packed processed lettuce (resulting in prolonged shelf life) is currently achieved by combinations of various post harvest treatments, however the most widespread is MAP (Brecht *et al.* 2003; Hilton *et al.* 2009). Fresh produce is more susceptible to disease due to an increase in respiration rate post harvest. Respiration and transpiration continue after the product has been harvested, however as the product is now isolated from a source of water, minerals and photosynthates, it is wholly dependent on its own moisture content and food reserves. Water loss is related to saleable weight, and a loss of even 5% weight can result in the produce appearing wilted or shrivelled. Under ambient conditions this can lead to a very limited shelf life (Sandhya 2010). MAP reduces respiration rate and water loss of the enclosed product thus slowing the metabolic rate of tissue and delaying postharvest discolouration (Hilton *et al.* 2008).

In MAP the air composition surrounding the produce is altered, which can slow the natural deterioration of the product. Oxygen (O_2), carbon dioxide (CO_2) and nitrogen gas (N_2) are most commonly used in MAP, and for lettuce 1-3% O_2, 0% CO_2 and 97-99% N_2 is the recommended gas mixture. If the permeability of the packaging to O_2 and CO_2 is adapted to the product respiration, an equilibrium modified atmosphere will be created within the package and the shelf life will be extended. MAP can provide control of respiration and ethylene production, in addition to the associated visual benefits which can result in a product of high organoleptic quality. However this is dependent on temperature control. Biological

reactions normally increase two to three-fold for each 10ºC temperature rise (Sandhya 2010).

Effectively all fresh processed products are by necessity handled in MAP to receive the required commercial post harvest life span (Brecht *et al.* 2003). However as soon as the packet is opened, oxygen enters and comes into contact with the tissue, and in lettuce initiates the phenylpropanoid pathway resulting in discolouration. A genetic engineering approach, via production of genetically improved lines with reduced likelihood for discolouration would avoid the need for such treatments (Peiser *et al.* 1998; Tomás-Barberán and Espín 2001). Genetic engineering could be used to manipulate the biochemical pathways; however, currently public opinion opposes this approach in Europe. An alternative is to exploit the natural genetic variation in lettuce for post harvest discolouration through a conventional breeding approach; this is the preferred option for lettuce breeding companies. The current project aims to provide tools and resources for breeding lettuce varieties with improved 'shelf life' and an underpinning understanding of the genetics of post harvest browning and pinking.

1.2. Lettuce

1.2.1. History

The recorded history of cultivated lettuce (*Lactuca sativa* L., of the Asteraceae (or Compositae) family) began in Ancient Egypt approximately 2500 BC (Keimer 1924; Harlan 1986). Tomb paintings show lettuce with long, narrow pointed leaves and

thick stems (Ryder 1999). However it is possible that cultivation may have originated in the Middle East prior to this (Ryder 2002). Lettuce appeared in China in the 5th century where it was developed in to a different morphotype grown for consumption of its stem rather than its leaves. It was being cultivated in Western Europe by the 15th century and was introduced to America by Columbus on his second voyage at the end of the 15th century.

Cultivated lettuce is a diploid species with 9 pairs of chromosomes. It is a preferential inbreeder (with pollination occurring as the capitulum opens), existing as a crop as hundreds of uniform varieties (Watts 1980). Local selection has resulted in six edible forms of lettuce in the species *L. sativa:* crisphead (Batavia and iceberg types), butterhead, romaine, leaf, Latin and stem (Ryder 1999). Although many of these types have been cultivated for centuries, the first true iceberg type lettuce, 'Great Lakes' was not introduced until 1941 (Ryder 2002).

The wild species *L. serriola* L. (prickly lettuce) is thought to be the progenitor of *L. sativa*; a close relationship has been shown between the two species with differences in morphological traits directly relating to the process of domestication (Kesseli *et al.* 1994; Van de Wiel *et al.* 1998). *L serriola* has been used extensively as a source of beneficial alleles (particularly disease resistance) in commercial lettuce breeding.

1.2.2. Lettuce breeding

Traditional selective breeding has resulted in the huge range of inter- and intra-specific variation that is observed in lettuce today (Ryder 1999). Variation within *L. sativa* accessions is principally for leaf morphology although as already mentioned stem types exist in Asia. Cultivars also have different abilities to achieve their

desired phenotype under a range of growth and production conditions (Kays 1999). Conventionally breeders have looked at characteristics such as disease resistance, yield, colour and flavour (Kesseli *et al.* 1994; Michelmore *et al.* 1994; Jeuken *et al.* 2001; Grube *et al.* 2005; Syed *et al.* 2006). There has been limited research on post harvest discolouration of lettuce, and an understanding of the heritability of this trait would allow interaction with disease and agronomic traits to be established and facilitate breeding of varieties with improved shelf life with already established traits for growers.

Successful cultivars have generally been superseded on the basis of their disease resistance, particularly to downy mildew. This is the major disease of lettuce worldwide and is caused by the oomycete *Bremia lactucae* and there exists a gene-for gene relationship between host and pathogen which has resulted in a 'boom-bust' cycle of 'breakdown of resistance in varieties. Breeding for resistance to downy mildew has been a major priority. Introduction of new resistance genes into the cultivated crop has been achieved through intogression of wild lettuce germplasm, largely *L. serriola,* (Lebeda and Pink 1998) through classical breeding techniques.

After disease resistance, good 'field quality' characteristics have been focused on. However as the market changes to processed lettuce, this means that post harvest quality, especially discolouration has increased in importance.

1.2.3. Genetic modification methods

Plant transformation has become an important approach for crop quality improvement (Bhat and Srinivasan 2002). Genetic transformation is the process of introducing foreign DNA into a new host species (Turner *et al.* 2000), and can be

used to broaden the genetic basis of plant germplasm available for conventional breeding (Curtis *et al.* 1994). It has been shown to be a reliable approach to engineering crops with desired traits by the direct introduction of a small number of genes (Turner *et al.* 2000). It can also reduce the time it takes to introduce single gene traits into crop plants (Curtis *et al.* 1994).

Lettuce in tissue culture has been shown to be highly responsive to a range of growth regulators (Michelmore and Eash 1988). However, a variation in the responsiveness of genotypes to regeneration in tissue culture has been recorded for most plant species (including lettuce) (Xinrun and Conner 1992; Curtis *et al.* 1994). Shoot regeneration has been achieved from many cultivars representing diverse genotypes (Xinrun and Conner 1992). The introduction of foreign genes into lettuce has been successful using *Agrobacterium* mediated transformation (Michelmore *et al.* 1987; Torres *et al.* 1993; Curtis *et al.* 1994; Dinant *et al.* 1997).

A genetic engineering approach would allow regulation of the pathways controlling the trait of interest (post harvest discolouration), therefore avoiding treatment use (Peiser *et al.* 1998; Tomás-Barberán and Espín 2001). However, since current widespread public opinion opposes this type of modification in Europe, exploitation of natural allelic variation through traditional breeding programmes is the preferred option for lettuce breeding companies. However, lettuce breeding programmes are costly and time consuming as on average they take 6-8 generations to achieve the desired homozygous cultivar. By using molecular markers it is possible to identify the superior genotype for a trait of interest which can be used for marker assisted selection to accumulate beneficial alleles independently of phenotype. Although lettuce is not an ideal model crop species, most cultivars are highly inbred and reveal extensive genetic homozygosity allowing for genetic studies (Michelmore *et al.*

1994). Significant progress has recently been made in the identification of genes and molecular markers and in their physical and functional relationship to each other. Numerous research groups have cooperated to develop an integrated genetic map of *L. sativa* using various phenotypic and molecular markers, including commercially important genes potentially useful in breeding (Ryder 1999; Truco *et al.* 2007). High-throughput technologies are concurrently being developed for genomic research and will have significant impacts on lettuce breeding for crop improvement (Michelmore *et al.* 2003). Genomic resources are now becoming available for lettuce specifically through 'The Compositae Genome Project' at UC Davis (see http://compgenomics.ucdavis.edu), which include the Affymetrix high density GeneChip© microarray, a large scale expressed sequence tag (EST) sequencing project and most recently a new Illumina OPA (pooled oligo set).

1.3. Lettuce mapping populations and molecular breeding

Genetic mapping is only possible if there is genetic variation for the trait of interest. A successful mapping study is fully dependent on the choice of contrasting parental lines used to generate the population (Jones *et al.* 2009). Genetic variation is ultimately due to variation in DNA structure; all types of DNA variation are potentially useful as molecular markers (Jones *et al.* 2009). Those linked to detectable trait variation are useful for marker assisted selection (MAS), while those with no phenotypic effect are useful for molecular mapping.

1.3.1. Mapping populations

A number of types of experimental populations exist, which ones are applicable for a given crop depends on the mating system of the species. Mapping populations include F_2 (2nd generation plants), back cross (BC) (crossed back to a parental line), double haploid (DH) (homozygous due to 'doubling' of either male or female gametes of an heterozygous, usually an F_1 hybrid individual), recombinant inbred lines (RILs) (see below) and near isogenic lines (NILs) (genetically identical lines which the exception of an introgressed locus or chromosomal segment). These are all derived from the hybridisation of two parental genotypes with significant variation for a trait of interest (Kearsey and Pooni 1996; Abdurakhmonov and Abdukarimov 2008).

Recombinant inbred lines (RILs)

A RIL population contains fully homozygous individuals that are obtained by repeated generations of selfing of individual F_2 plants derived by selfing an individual F_1 hybrid. The population represents ~50% of each parental genome in different combinations. RIL populations are therefore extremely informative in terms of gene combinations and recombination events and because they have a high degree of homozgosity they can be grown in replicated experiments in different environments and across years. However producing a RIL population is both time consuming and costly, as they generally require a minimum of 6-8 generations of selfing to reach the required level of homozygosity.

1.3.2. Molecular markers

The first generation of molecular markers included restriction fragment length polymorphism (RFLPs), random amplified polymorphic DNA (RAPDs) and simple sequence repeats (SSRs). In the past decade there have been vast developments in molecular markers, these reflect advances in technical procedures and are more efficient and effective. Marker technologies do however differ in the information they give about inheritance (dominant or co-dominant) and the number of marker bands that can be obtained (i.e. AFLPs can produced hundreds of bands while SNPs produce one); in addition to having their own technological restraints. Several molecular marker analyses are summarised below (see table 1.1.), with those used in this study described in more detail. In recent years there has also been a surge of knowledge and in databases of genomics and bioinformatics (Jones *et al.* 2009).

Table 1.1. Classification of marker systems. From Jones *et al.* 2009

Marker system	Advantages	Disadvantages
First-generation markers based on restriction fragment detection		
Restriction fragment length polymorphism (RFLP)	Co-dominant. Highly reproducible.	Low multiplex ratio. High on time and labour.
Second-generation markers based on PCR		
Cleavage amplification polymorphism (CAP)	Insensitive to DNA methylation. Radioactivity not needed.	Produces informative PCR products.
Random amplified polymorphic DNA (RAPD)	Medium multiplex ratio. Low on time and labour.	Dominant information. Low reproducibility.
Sequence-specific amplification polymorphism (S-SAP)	Can target any gene, transposon or sequence of interest.	Sequence must be known to enable design of specific PCR primers.
Simple sequence repeat (microsatellite) (SSR)	Co-dominant information. Highly reproducible. Low on time and labour.	High cost of development. Low multiplex ratio.
Inter-simple sequence repeat (ISSR)	Technically simple. No prior genomic information needed to reveal both inter- and intraspecific variation.	Dominant information. Band staining can be weak.
Variable number tandem repeat (minisatellite) (VNTR)	Numerous multiallelic loci.	Low-resolution fingerprints in plants.
Sequence tagged sites (STS)	Co-dominant information.	Requires sequence knowledge.
Sequence characterised amplification region (SCAR)	Dominant or co-dominant information.	Difficult to reproduce.
Sequence amplification of microsatellite polymorphic loci (SAMPL)	High multiplexing. Co-dominant information. Extensive polymorphism.	Some blurred banding. Stutter bands.
Genome scanning for expressed genes		
Sequence-related amplified polymorphism (SRAP)	Simple. High throughput. High reproducibility. Targets coding sequences. Detects multiple loci without previous knowledge of sequence information. PCR products directly sequenced.	Detects co-dominant and dominant information. Null alleles detected directly.
Target recognition amplification protocol (TRAP)	Simple. Highly informative. Produces numerous markers by using existing public EST databases. Uses markers targeted to a specific gene.	Requires cDNA or EST sequence information for primer development.
Markers using array technology		
Microarrays	Whole-genome scanning. High-throughput technology. Genotype–phenotype relationship. Expression analysis of large numbers of genes.	Expensive. Requires gene sequence data. Technically demanding.

Table 1.1. continued.

Marker system	Advantages	Disadvantages
Diversity array technology (DArT)	No sequence data required. High throughput. Detects single base changes and indels. Rapid germplasm characterization.	Dominant information. Technically demanding.
Other marker systems		
Denaturing gradient gel electrophoresis (DGGE)	Separates individual sequences from a complex mixture of microbes based on sequence differences.	PCR fragment size limited to about 500 bp. Difficult to resolve fragments that differ by only one or two bases.
Temperature gradient gel electrophoresis (TGGE)	Almost identical to DGGE. More reliable. Uses temperature gradient.	Technically demanding. Little used in plants.
Methylation-sensitive PCR	Detects sites of methylated DNA.	Technically demanding. Little used in plants.

Additional second-generation markers based on PCR include amplified fragment length polymorphism (AFLP) and conserved ortholog set (COS). Third-generation markers based on DNA sequencing include single nucleotide polymorphism (SNP) and single position polymorphism (SPP) (principally the same) and genome scanning for expressed genes to identify expressed sequence tag/s (EST/s). Other marker systems including single-strand conformational polymorphism (SSCP) have been used in this study.

Amplified fragment length polymorphism (AFLP)

The AFLP method combines the use of restriction enzymes with PCR amplification fragments and detects any polymorphisms in fragment length. The technique involves the restriction of genomic DNA with two endonucleases, ligation of adapters with known sequence to each end, selective PCR of a subset of fragments with fluorescent tagging followed by sequencer analysis of the amplified fragments (Vos *et al.* 1995; Meudt and Clarke 2007). Although the AFLP system is both labour and time intensive, it can detect up to 200 loci in a single reaction (depending on genome size, enzyme combination, number of selective nucleotides and resolution of output) (Witsenboer *et al.* 1997*;* Jones *et al.* 2009). AFLP markers are generally reliable and efficient; they can also be used for many species (Qi *et al.* 1998; Vuylsteke *et al.* 2000). This technology can be used for the construction of high-density maps which have important applications in genetics and breeding (Jeuken *et al.* 2001; Jones *et al.* 2009)

Conserved ortholog set (COS)

COS markers are evolutionary conserved single copy genes that have been identified from large EST databases (Fulton *et al.* 2002). They represent orthologous genes in

many plant species and are particularly useful for comparative genomics (Castelblanco and Fregene 2006). They are also useful for constructing syntenic genetic maps amongst species (Liewlaksaneeyanawin *et al.* 2009). There are many ways of genotyping COS markers depending on the information requirements and resource constraints.

Single nucleotide polymorphism (SNP)

SNPs are single base pair variations in genomic DNA at which 2 alleles are usually present. A SNP must be present in at least 1% of individuals from a population to be classified as 'polymorphic' (Jones *et al.* 2009). SNPs are extremely common polymorphisms occurring 1/1000 bp and are generally evenly distributed through the genome (Wang *et al.* 1998). There are many ways of detecting and genotyping SNPs depending on the information requirements and resource constraints (Lörez and Wenzel 2005; Gupta *et al.* 2008). This technology has many applications including the use for the construction of high-density maps, mapping of traits and phylogenetic analysis (Rafalski 2002).

Expressed sequence tag (EST)

ESTs are short sub-sequences of 200-800 bp of cDNA sequence transcribed from mRNA that can be used to tag genes of interest and generate specific markers, allowing gene discovery, sequence determination and mapping. ESTs are generated by sequencing a single or both ends of an expressed sequence. They are subsequently used to find the equivalent gene by hybridising to homologous sequences from a genomic library. Identifying genes within genomes of different organisms is dependent on genome size, the presence/absence of introns (interrupting coding sequences of genes) which can reduce homology between the original gene

and EST (as a product of mRNA the intronic regions have been removed) (Jones *et al.* 2009). There has been rapid progress in EST identification and entries in public databases for plant species in recent years; such as the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/dbEST/) and the Compositae Genome Project database' (http://compgenomics.ucdavis.edu/compositae_index.php) (see 'Genomics and bioinformatics' below).

Single-strand conformational polymorphism (SSCP)

SSCP analysis has been used to identify single nucleotide changes within sequences. SSCP gel electrophoresis separates DNA molecules based on their conformation (the way in which single stranded DNA will fold into secondary structures) rather than by their size (Orita *et al.* 1989; Sunnucks *et al.* 2000; Celotto and Gravely 2004). In this method PCR is carried out on the DNA sample, the sample is then heat-denatured and cooled to prevent renaturation. Single-stranded fragments are separated by gel electrophoresis and detected with UV light (if fluorescent primers were used) or silver staining (Bosari *et al.* 1995; Jones *et al.* 2009). Fragments may differ by as little as a single base pair, and mutations can be detected at multiple sites (Jones *et al.* 2009). It is considered the most suitable method for detecting mutations in short DNA sequences although it is temperature and pH dependent (Schwieger and Tebbe 1998; Jones *et al.* 2009). SSCP can be used to profile individuals and populations (Jones *et al.* 2009).

Genomics and bioinformatics

'The Compositae Genome Initiative' coordinated by Professor Richard Michelmore's research group at UC Davis, CA intends to establish tools and

resources for the Compositae. The ‘Compositae Genome Project database’ (CGP) (Phase I 2000-2004; Phase II 2004-2009; Phase III 2009-2013) within this initiative is developing gene catalogues and genomic sequences for important species in the family. Within the project lettuce EST homologues of *Arabidopsis* sequences have been isolated with the majority of data accessible via CGP2 (at http://cgpdb.ucdavis.edu/cgpdb2). The initial phase of the project generated ≥132,000 ESTs from sunflower (~64,000) and lettuce (~68,000). The second phase included an additional 160,000 lettuce ESTs which are estimated to represent >80% of lettuce genes. Lettuce EST libraries were produced from 10 pools of RNA consisting of diverse tissues, developmental stages and environmental conditions from cultivated *L. sativa* cv Salinas and wild *L. serriola* (Michelmore 2006).

1.3.3. Genetic linkage maps

‘The main purpose of genetic mapping is to detect neutrally inherited markers in close proximity to the genes controlling complex or quantitative traits’ (Abdurakhmonov and Abdukarimov 2008). Marker and genotypic data for individuals from a mapping population are used to generate a linkage map. The linkage map represents the order and position of markers based on their linkage using a relative genetic distance (in cM) along linkage groups; this is achieved via analysis of recombination rates between marker loci. The theory underlying linkage mapping of markers was first available in the 1920s (Mather 1938), however slightly different algorithms are now used in the final stages of mapping to cope with multiple marker data (Lander *et al.* 1987; Stam *et al.* 1993).

Genetic studies in lettuce have identified genes controlling seed characteristics and disease resistance (Witsenboer *et al.* 1995; Witsenboer *et al.* 1997; Waycott *et al.* 1999; Jeuken and Lindhout 2002) and there has been significant progress in the development of lettuce based linkage maps in recent years (see section 4.4.). Genetic maps have been utilised for marker assisted breeding (Dekker and Hospital 2002), dissection of quantitative traits (Salvi and Tuberosa 2005) and comparative genomics (Paterson *et al.* 2000). Maps constructed from an intraspecific cross can be readily used for breeding purposes as they contain markers for a related genepool. Although interspecific crosses have a higher level of polymorphism, they often show high segregation distortion and access to wild alleles is often limited (Truco *et al.* 2007).

1.3.4. Phenotypic assessment

Phenotypic variation can be either continuous or discontinuous. Continuous variation is quantitative while discontinuous variation is qualitative. Discontinuous variation is a characteristic of traits that are controlled by a single or low number of genes that behave in a Mendelian fashion. Variation for mapping is often generated experimentally, where crossing lines with contrasting phenotypes results in a population segregating for a particular trait (Jones *et al.* 2009).

1.3.5. Quantitative trait loci (QTL)

Complex traits are usually influenced by a large number of genes in addition to environmental effects (Kearsey and Farquhar 1998). QTL are chromosomal regions of individual or groups of genes which influence complex traits (Paterson 1998).

The understanding of the genetic basis of these complex traits is based on the separation of phenotypic variation within and among individuals with a known degree of relatedness (Lynch and Walsh 1998). QTL are initially predicted based on associations between the quantitative trait and marker alleles segregating in the population (Kearsey and Farquhar 1998). Markers ordered on the linkage map are correlated with phenotypic trait data of individuals of the mapping population, identifying QTL regions affecting the associated trait with 'tag' markers (that are linked to the control of the trait measured) (Abdurakhmonov and Abdukarimov 2008). The precision of QTL mapping depends on the genetic variation covered by a population, the size of the mapping population and number of marker loci (Abdurakhmonov and Abdukarimov 2008).

QTL mapping methods

There are many techniques for QTL analysis, however the most widely used method is interval mapping (Kearsey and Farquhar 1998), which was developed by Lander and Botstein (1989). The log of the ratio of likelihoods (LOD) (probability) of there being a QTL present within a marker interval or not, is calculated by examining intervals between adjacent pairs of markers (Lander and Botstein 1989). When the LOD exceeds a predefined significance threshold (at marker intervals) a segregating QTL has been detected (Van Ooijen *et al.* 2002). However interval mapping is one dimensional and does not detect interactions between multiple QTL which are likely to be present when analysing quantitative traits. However multiple QTL models such as multiple QTL model (MQM) mapping method developed by Jansen (1993, 1994) are an extension of interval mapping that remove any residual variation caused by other QTL increasing the power of individual tests (Jansen 1993, 1994; Jansen and Stam 1994).

Alternative approaches include multiple regression developed by Haley and Knott (1992) and marker regression developed by Kearsey and Hyne (1994).

1.3.6. Gene-environment (GXE) interactions

GXE interactions can occur for QTL where the difference in phenotypic values of an allele at a locus is dependent on environmental heterogeneity (Juenger *et al.* 2005). Cultivar performance/phenotype can vary across environments due to different response to numerous biotic, climatic and edaphic factors (Dixon *et al.* 1991). If genes are environmentally sensitive this results in phenotypic plasticity (Maloof 2003). While GXE interactions indicate that a QTL is specific to an environment, the absence of an interaction would imply the QTL has a general effect independent of environment (Maloof 2003). QTL with GXE interactions are generally QTL with small effects (Collard and Mackill 2007). GXE interactions can limit the progress of crop improvement beyond the site of analysis as successful application of QTL mapping depends on the robustness of QTL. It is therefore necessary to separate main QTL and environmental QTL effects, and to base genotypic selection on main effects (Yang and Zhu 2005; Collard and Mackill 2007). However, GXE interactions can be exploited by plant breeders to benefit agriculture.

1.3.7. Marker assisted selection (MAS)

MAS is an indirect selection process where a trait of interest is selected for based on a QTL 'tagged' marker linked to the QTL (Ribaut and Hoisington 1998; Reynolds *et al.* 2001; Rosyara 2006). It is assumed that the allele linked to the marker is

associated with the QTL or underlying gene of interest. However, for markers to be used for MAS QTL must be confirmed or validated; fine/high resolution mapping may also be required (Langridge *et al.* 2001) in order to have tightly linked markers (i.e. to reduce the possibility of recombination between the marker and QTL). Desired genotypes can be effectively selected with MAS independent of phenotypic selection and hence environmental effects resulting in faster line development and therefore variety release (Collard and Mackill 2007). MAS is particularly useful for traits with low heritability and traits that are difficult to quantify or are expressed late in development.

1.3.8. Functional genomics to map candidate genes

The main objective of molecular genetics is to identify and isolate genes relating to important traits. The candidate gene approach is a strategy for identifying agronomically important genes controlling qualitative traits. It hypothesizes that genes of known function, previously sequenced and putatively involved in trait variation based on biological function may correspond to loci controlling the trait of interest. Validated candidate genes could be used for identification of genotypes in addition to marker assisted selection. The genes would act as efficient markers allowing fuller control of the introgressed region, whilst markers based on genes underlying QTLs would provide the perfect marker for the QTL with no possibility of recombination events between marker and QTL (Pflieger *et al.* 2001).

CHAPTER 2

Materials and methods

2.1. Plant material

This chapter describes more general materials and methods; materials and methods specific to a particular chapter are described within the 'experimental' chapters.

2.1.1. Parents of the Warwick HRI recombinant inbred line mapping population

Crisphead varieties 'Saladin' (syn Salinas) and 'Iceberg' (syn Batavia blonde a bord rouge) were used as parents for generation of the WHRI recombinant inbred line (RIL) mapping population (see figure 2.1.). Iceberg is a traditional Batavian variety bred in France during the late 1850's, it has pale green leaves with variable red edges (Rodenberg *et al.* 1960). Saladin is an iceberg type and has dark green leaves; it is synonymous with cv. Salinas and was bred in the 1970's at the United States Department of Agriculture, California by Dr Ed Ryder (1979). The majority of modern European iceberg type cultivars are derived from Saladin.

Figure 2.1. Iceberg and Saladin; parents of the Warwick HRI recombinant inbred line mapping population. Where *a* (Iceberg); *b* (Saladin).

2.1.2. Saladin x Iceberg recombinant inbred line population

The F_7 mapping population includes 125 recombinant inbred lines (RILs) from the Saladin x Iceberg cross with seed produced at Warwick HRI, UK. Ninety-four highly informative lines (based on recombination events) from the population were selected for the 2008 field trials and 11 lines which had extreme discolouration genotypes (and phenotype in 2008) were selected for the 2009 field trial. All RILs were used for agronomic assessment.

2.2. Experimental trials

2.2.1. Plant culture

Plant raising

In the UK seeds were planted in FP7 modular trays into a 3:1 mixture of Levington M2 compost (Levington Horticulture, UK) with a vermiculite covering (Avon Crop, Blacknell, UK). Germination took place in the dark at 5°C and lettuce transplants were then raised under the following glasshouse conditions in a randomised arrangement. Seedlings were subjected to a 16 hr day (18°C day and 16°C night) with a light threshold (High pressure sodium 400 W son-t bulbs) of 300 Wm^2 (when outside light levels >300 W/m^2 the lights turned off and resumed when the outside light dropped below the threshold). Transplants were predominantly watered with tap water at the base by hand and when needed with liquid feed Vitax on tap (where the concentrated feed was diluted to 1:200). After 21 days of growth, transplants were placed outside in a cold frame to harden off for two weeks prior to

transplanting. Plants grown in the Netherlands were raised by Rijk Zwaan under their standard growing procedures.

Field trials

Lettuce transplants were raised as described above and randomly selected from the pool available for each accession for the field. Each plot contained 12 plants (4 rows x 3 plant arrangement) of the same accession with 35 cm spacing between plants. Crops were treated with protective sprays 'Dovetail' lambda-cyhalothrin and 'Aphox' pirimicarb (1.5 L/ha and repeated as information was released on the Horticultural Development Company (HDC) Pest Bulletin (www2.warwick.ac.uk/fac/sci/whri/hdcpestbulletin/)). Plants were irrigated through an oscillating line as required for establishment (although no soil moisture deficit was recorded the land was irrigated to bring it near field capacity prior to transplanting) and irrigation stopped 7 days before harvest. As above plants grown in the Netherlands by Rijk Zwaan were subjected to their standard growing procedures.

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2.2.2. Harvest and processing

Field trials

On harvest day the central two 'guarded' replicated heads per plot were cut at soil level. Excess material from each head was trimmed by removing the outermost exposed wrapper leaves and the trimmed weight recorded. Heads were processed into ~4 cm^2 pieces by halving the head, removing the core, cutting lengthwise from butt to crown and again transversely (see figure 2.2.).

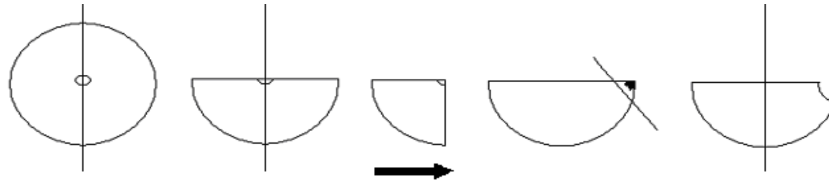


Figure 2.2. Processing protocol for lettuce heads. Each head was halved cutting lengthwise from the butt to the crown and again transversely with a sharp stainless steel knife. The core was removed by cutting a V shape, and then each quarter cut in half.

Figure 2.3. Pre-packed cut lettuce from experimental trials stored either a) hung vertically or b) stored vertically in fridge at 5°C. Bags were hung vertically for the 2007 trials and stored vertically for 2008, 2009 and 2010 trials.

Processed material from a single head was separated and mixed thoroughly. Approximately 50g (for the 2009/2010 trials), 75g (for the 2007 trial) or 100g (for the 2008/2009 trials) of unwashed processed material were sealed (removing any excessive air and ensuring the seal was not compromised by material) in a non-selective permeability film bag (P-PLUS 35PA240; 200 x 250mm; Amcor Flexibles P-Plus) with material from one head filling two bags (1 for the glasshouse trial). The use of non-selective film ensured that the atmosphere within the pack did not reduce the natural intrinsic rate of discolouration in each product. Bags were stored at 5° (see figure 2.3.).

2.2.3. Trait measurements

Phenotypic assessment of discolouration

On each assessment date bags were removed from storage and arranged under a halogen light source for assessment, ensuring that the bags had the same orientation on each assessment date. Material was scored 'blind' (so genotype was unknown at time of scoring) as not to bias results.

A 12-square 3 x 4 acetate grid (50 mm x 50 mm) was overlaid on the bag and arranged so that each square covered a representative sample of material. Discolouration in each square was then scored based on a set of photographic standards for pink and brown discolouration (Hilton *et al.* 2009) (see figure 2.4.). Pinking and browning were each split into 2 categories of severity, slight and severe. When no discolouration was observable, the 'square' was classified as clean. When there was uncertainty about whether the discolouration was brown or pink it was classified as visible. This prevented any missing data and allowed the discolouration to be included in the general discolouration classification category for analysis. As time progressed the material became easier to score so the amount of data classified as visible decreases. The different types of discolouration were measured in two ways, with mean score discolouration representing the intensity of the discolouration while percentage discolouration represented the extent of this discolouration.

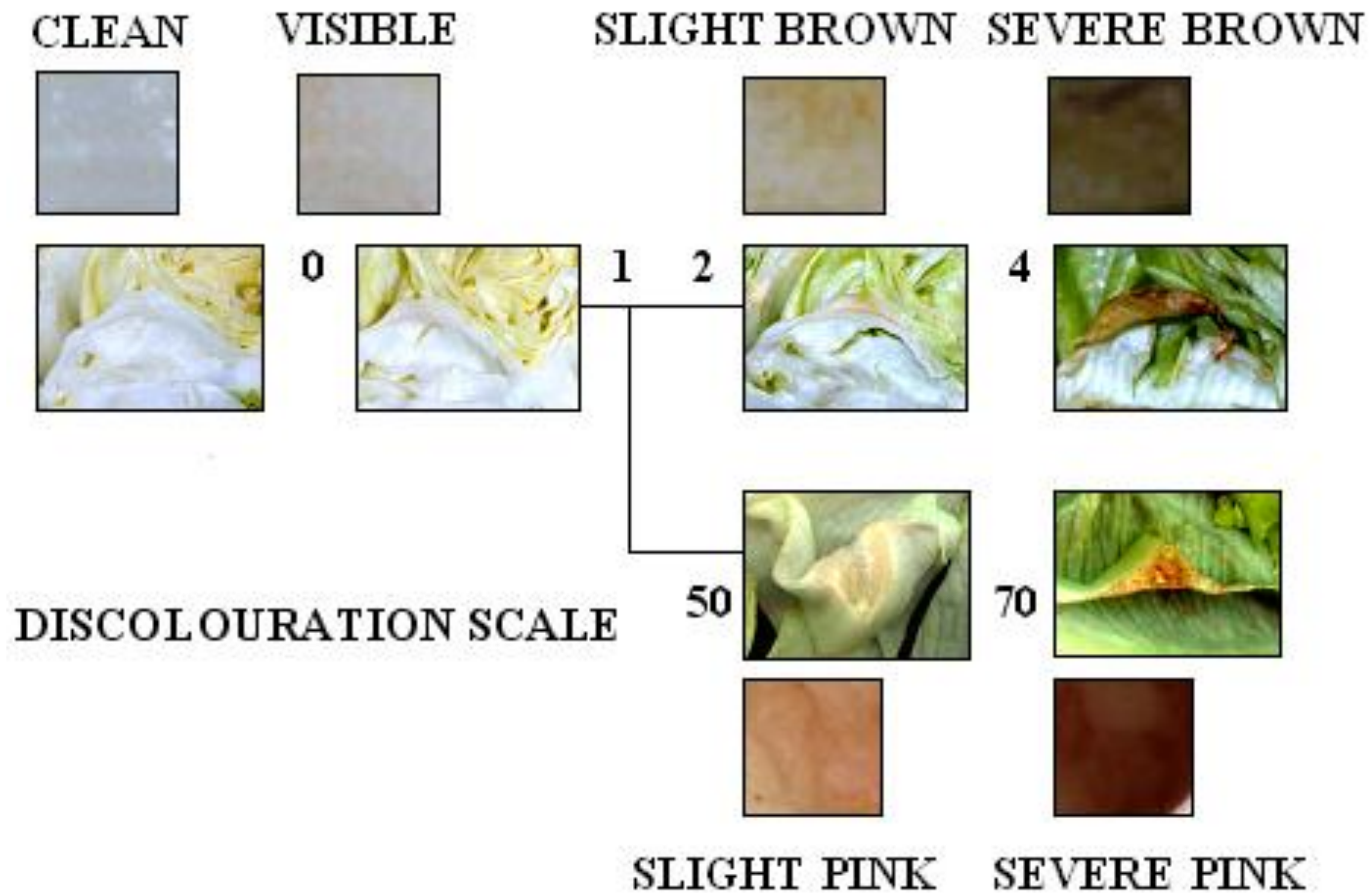


Figure 2.4. Discolouration scale for lettuce post harvest discolouration assessment. Based on Hilton *et al,* (2009).

Each grid square was given a single score as a discolouration coordinate at its most intense representation, resulting in 12 tallies per scoring grid per bag on each assessment day (see table 2.1.).

Table 2.1. Sample score grid for a single bag of lettuce representing the discolouration. Where / (impossible score); italicised text (output score).

					PINK	
Bag ID			**Clean**	**Visible**	**Slight**	**Intense**
			0	**1**	**50**	**70**
	Clean	**0**	*0*	*/*	*50*	*70*
	Visible	**1**	*/*	*1*	*51*	*71*
BROWN	**Slight**	**2**	*2*	*12*	*52*	*72*
	Intense	**4**	*4*	*14*	*54*	*74*

The percentage discolouration (= 100 x (number of grid squares with discolouration / 12)) and the intensity of discolouration (mean discolouration score / number of grid squares showing the discolouration) were determined on each assessment day for pinking, browning, visible and overall discolouration (i.e the sum of all categories).

Spectrophotometer readings were taken from tissue samples of cv. Iceberg for analysis to ensure the scoring method was consistent. Fifty readings were taken independently from leaf and midrib tissue of an Iceberg lettuce (store brought) using a Minolta CM5031 Spectrophotometer (Konica Minolta Sensing Inc.) for scores of no and visible discolouration, slight and severe brown, and slight and severe pink discolouration. Additionally, 5 readings were taken for the corresponding photographic standards (see figure 2.4.). Results were analysed via discriminant analysis (Burr and Doak 2007); the analysis showed consistency between the 2 scoring methods. However, to use the Minolta was time consuming and impractical for a large scale trial. Therefore the visual scoring method was used for all assessments.

QTL analysis

The dense molecular marker linkage map for the F_7 RILs (see Chapter 4) was used as a framework for the QTL analysis. Interval mapping was conducted using MapQTL ®4.0 (Van Ooijen *et al.* 2002; Jansen 1993, 1994 and Jansen and Stam 1994) to increase resolution and reduce background marker effects (Zeng 1994). QTL were detected with QTL significance at P <0.05 using a genome wide threshold for logarithm of odds (LOD). QTL were then confirmed by multiple QTL model (MQM) mapping to define QTL using MapQTL again with a genome wide threshold. MQM mapping increased robustness of the identification of putative QTL (by taking into account the effect of other QTL). The graphical representation of the linkage maps and QTL were prepared using MapChart®2.2 software (Voorrips 2002).

2.3. Metabolite analysis

2.3.1. Metabolite extraction

Sample material

When material was harvested from trials for bagged phenotypic assessment (including field trial of extreme discolouration lines and glasshouse trial for wild type and mutant lines) tissue was also harvested for metabolite analysis. Two x 22 mm cork borer samples were taken from the middle of each lettuce head per plot once halved (avoiding core tissue). Each sample was split where it fell naturally into

inner and outer leaf tissue (with inner tissue closest to the core of the head). One sample per plot (from the same extraction) was frozen on the day of harvest. The remaining samples were placed in semi permeable bags as used in all other trials and stored as bagged samples for phenotypic assessment; samples were then frozen on day 4. Frozen samples were subsequently freeze dried (Edwards 12K Super Modulyo Meadowrose Scientific).

Enzyme and protein extraction

Finely ground freeze dried material (0.03g per sample) was added to a pre-cooled centrifuge tube and 1 ml phosphate buffer (50 mM pH 6.5 (phosphate buffer contained 18.75 ml 0.2 M dibasic sodium phosphate, 31.25 ml 0.2 M monobasic sodium phosphate and 50 ml dH_2O per 100 ml)) at 4°C was added.

Total phenolic content extraction

Finely ground freeze dried material (0.02g per sample) was added to a pre-cooled centrifuge tube and 1 ml acetone-water (1:1, v/v) added. Samples were left at room temperature for 15 hr.

All samples were mixed for 2 min at dial adjusted to 0.25 (Retsch MM300 Mixermill) and then spun in a centrifuge (Mikro 200R, Hettich Zentrifugen) for 20 mins at 1800 rpm at 4°C. The supernatant was removed and stored at -20°C for subsequent assays.

2.3.2. Metabolite identification

All samples were quantified in 96 well plates (Immuno 96 MicroWell™ plate, with MaxiSorp F96 surface, nunc™) using a GENios fluorometer (Genesis workstation 150, Tecan).

PAL activity

PAL activity was measured as developed by H Hilton (personal communication). Thirty µl of sample was added to 150 µl L-phenylalanine (10 mM) in borate buffer (100 mM, pH 8.6 (Borate buffer was created by adding boric acid (0.2 M) to borax (sodium tetraborate) (0.2 M) until pH 8.6 was reached, and diluted to 100 mM using dH_2O)). Samples were shaken for 10 s and absorbance quantified immediately at 280 nm. Samples and blanks were subsequently incubated for 120 minutes at 35°C in dark and re-quantified.

PPO activity

PPO activity was measured as developed by Howard Hilton (personal communication). One-hundred and ninety µl catechol mix (10 mM catechol in 50 mM phosphate buffer at pH 6.5) was added to 10 µl sample. Samples were shaken for 10 s and absorbance quantified immediately at 420 nm. Samples and blanks were incubated for 2 minutes at 35°C in the dark *in situ* and re-quantified.

Total phenolic content

Total phenolic content was analysed by the Folin-Ciocalteu method using gallic acid as a standard (Yu *et al.* 2003; Liu *et al.* 2007). Each reaction contained 3 µl of sample or gallic acid standard, 134 µl dH_2O, 12 µl Folin-Ciocalteu reagent and 34 µl of Na_2CO_3 (20 g / 100 ml). Samples and blanks were incubated for 120 minutes at

35°C in the dark. Samples were shaken for 10 s and absorbance quantified at 765 nm.

Blanks of dH_2O and substrate minus extract were also read.

2.4. Statistical analysis

Preliminary statistical analyses for experiments for analysis of post harvest discolouration phenotype data (Chapters 3-8) were conducted using GenStat 10th edition (Payne *et al.* 2007). Initially the distribution of the data was plotted to determine whether the data were distributed normally. Data that were normally distributed were subjected to further analysis.

2.4.1. Restricted maximum likelihood (REML) analysis

All experiments for analysis of phenotype data were analysed using Restricted Maximum Likelihood (REML) procedure (Patterson and Thompson 1971; Thompson and Welham 2000). REML analysis is a generalised ANOVA which is suitable for unbalanced designs for both fixed and random effects. Due to poor weather conditions resulting in little or no material for some bags, balanced designs were not always possible. REML was subsequently used to analyse trial data to identify main effects due to variety/genotype; all transformed discolouration variables were analysed per day and across days for each trial. Wald statistics were used to ensure that actual and estimated means from the REML analyses were not significantly different.

2.4.2. T-Test

For normally distributed data t-tests were conducted using GenStat 10th edition to test the null hypothesis that two genotype means corresponding to the two random samples were equal (Payne *et al.* 2007). F-tests were initially conducted to test for equality of variance; t-tests were then altered accordingly. Estimates for the variance were pooled unless there was evidence of unequal variances, separate variances would then be used. Data was transformed as for the associated REML analysis for data from each of the field trials. T-tests were carried out for individual day data for each discolouration parameter between accessions of interest.

2.4.3. Analysis of Variance (ANOVA)

Experiments for analysis of metabolite data (Chapter 7 and Chapter 8) were analysed using one-way Analysis of Variance (ANOVA) (no blocking). ANOVA is suitable for balanced designs amongst 2 or more independent groups to identify whether means were drawn from the same population (Howell 2002). Metabolite data was analysed by ANOVA across and per days to identify main effects due to genotype.

2.4.4. Correlation analysis

Correlation matrices were created using GenStat 10th edition (Payne *et al.* 2007) to suggest possible causal or mechanistic relationships between all variables analysed within a chapter. R values were generated for all discolouration variables, processing data, metabolite activity and related morphological traits recorded for the associated trial which were compared to the relevant p values. Due to large data sets

only $P < 0.001$ were classified as significant enough to present a relationship between parameters.

Significant effects are shown as *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

CHAPTER 3

Diversity for post harvest discolouration within the lettuce gene pool

3.1. Introduction

In order to study the genetics of post harvest discolouration it was necessary to establish whether there is genetic variation for the trait. Previous studies (Wurr *et al.* 2003) indicated that the cvs Saladin and Iceberg differed significantly for both the degree of post harvest discolouration and its intensity, indicating that the F_7 Saladin x Iceberg RIL mapping population should be suitable for genetic analysis of post harvest discolouration. However, before embarking on such a genetic analysis it was decided to confirm this and to ascertain whether this variation between Iceberg and Saladin was representative of the range of natural variation observed in lettuce.

The aims of this experiment were to

- Demonstrate genetic variation for post harvest discolouration using a lettuce diversity set.
- Confirm that Saladin and Iceberg differ significantly from one another for post harvest discolouration traits and that the Sal x Ice F_7 RIL mapping population is suitable for genetic analysis of post harvest discolouration.

3.2. Material and methods

3.2.1. Warwick HRI lettuce diversity set

The Warwick HRI lettuce diversity set assembled over the past 10 years consists of 28 accessions representing cultivars of different crop types and geographic origin and accessions of the wild relative *Lactuca serriola* (see table 2.1.).

Table 3.1. Accessions of the WHRI lettuce diversity set. Where *L. serriola* 005095 and *L. serriola* 03050 are accessions of *Lactuca serriola.*

Accession name	Type	Accession name	Type
Adriatica 2	Summer Butterhead	Lobjoits Green Cos	Summer Cos
Ambassador	Winter Round	Lollo Biondo	Summer Curly Green
Batavia Blonde de Paris	Summer Batavia	Lollo Rossa	Summer Curly Red
Batavia Tezier	Summer French	Madras	Iceberg
Bloody Warrior	Summer Cos	Merveille des Quatre Saisons	Summer Batavia
Chinese Stem Lettuce	Stem	New Chicken	Stem
Cobham Green	Summer Round	Platinas	Iceberg
Iceberg	Summer Batavia	Red Grenoble	Summer Batavia
Imagination	Batavia	Romaine de Benicardo	Summer Cos
Jazzie	Batavia	Saladin	Summer Iceberg
L. serriola 005095	Summer Wild Type	Stoke	Summer Cos
L. serriola 03050	Summer Wild Type	Waldmanns Dark Green	Summer Leaf
Lilian	Summer Round	Webb's Wonderful	Summer Crisp
Little Gem	Summer Gem	Wunder von Stuttgart	Summer Butterhead

3.2.2. Field trial and assessment of post harvest discolouration of Warwick HRI lettuce diversity set

Plants for use in the assessment of post harvest discolouration were grown in a replicated field trial during the 2007 growing season on the experimental site Sheep Pens (west) at Warwick HRI, UK (Latitude: 52.183. Longitude: 1.583). Plants were raised and maintained as described (see section 2.2.1.), with the trial planted on 1st May 2007. The trial was designed in 3 blocks each containing a single plot of the 28 accessions of the diversity set, randomised in an alpha design to take into account any potential environmental gradient (see Appendix A for field plan and randomisation). Crop protection was as described, with the additional use of 'Greencrop Saffron FL' propyzamide herbicide (3.5 L/ha) according to good agricultural practice. Fencing and flappers also surrounded the land to provide protection from the local fauna (see figure 3.1.). Harvests occurred on 30th May, 5th June, 12th June and 19th June 2007 with all accessions of a similar type being harvested together across all replicates.

Figure 3.1. Lettuce diversity set trial at Warwick HRI, UK in 2007.

Heads were harvested and processed as described (section 2.2.2.) and additional processing data including untrimmed weight and head diameter were recorded. Approximately ~75g unwashed mixed material was sealed per bag with material

from one head filling two bags. Bags were hung on a racking system at 5° for storage (see figure 2.3). Bags were then phenotypically assessed for post harvest discolouration as described (section 2.2.3.) on days 1, 3, 6, 9 and 13.

3.2.3. Statistical analysis

As the data exhibited a variable mean relationship it was transformed before analysis. For the intensity mean score browning, pinking and visible scores the data was multiplied by 50 and for mean score overall discolouration scores it was multiplied by 2. This would allow data to be comparable between intensity as they would be on a scale of 100. The percentage scores of extent of pinking, browning, visible and overall discolouration were transformed to angles (by multiplying by 90°) prior to analysis. As the field trial produced an unbalanced data set, it was analysed by REML with the fixed treatment rep/block/plot/head/bag for days 1, 3, 6, 9 and 13. Scores were adjusted through the REML analysis to adjust for block effects which may have resulted in negative values for the estimated means (an equivalent could also occur with missing values).

T-tests were carried out for each individual transformed day data for the different discolouration measurements between the F_7 mapping population parents Saladin and Iceberg (as section 2.4.2.).

Correlation analyses were conducted between all discolouration measures and morphological traits (correlations between browning, pinking and overall discolouration and between them and processing data (untrimmed/trimmed weight

and trimmed diameters)) (as section 2.4.4.). R values were generated which were then compared to the associated *p* values (see table 3.2.).

Table 3.2. Correlation analysis parameters. Where *df* (degrees of freedom).

		Probability (p value)		
Population	**df**	**0.05**	**0.01**	**0.001**
Lettuce diversity set	25	0.381	0.487	0.597

3.3. Results

Spectrophotomic readings were taken from lettuce leaf and midrib tissue for pinking and browning values as a confirmatory measure. Data suggested all tissue was scored consistently and correctly; pink was always scored as pink whilst brown was always scored as brown.

3.3.1. Diversity for post harvest discolouration within the WHRI lettuce diversity set

REML analysis of the data sets for each day showed that accessions were significantly different (****P* <0.001) for all measures of discolouration.

All post harvest discolouration measures of colour intensity (mean score) demonstrated a comparable linear trend over time when meaned across all accessions. Pinking, browning and overall discolouration increased at a similar rate over the 5 time points with a crossover of the rate of browning and pinking intensity occurring around day 3 (see figure 3.2.a). Between days 6 and 9 the rates of all 3 measures plateau but begin to increase again by day 13.

The extent of post harvest discolouration (%) gave a curved response over time (see figure 3.2.b), with an increase in the extent of discolouration up to day 6 followed by a levelling off to day 13. As with colour intensity, browning appears to be more prevalent than pinking in the early stages post harvest.

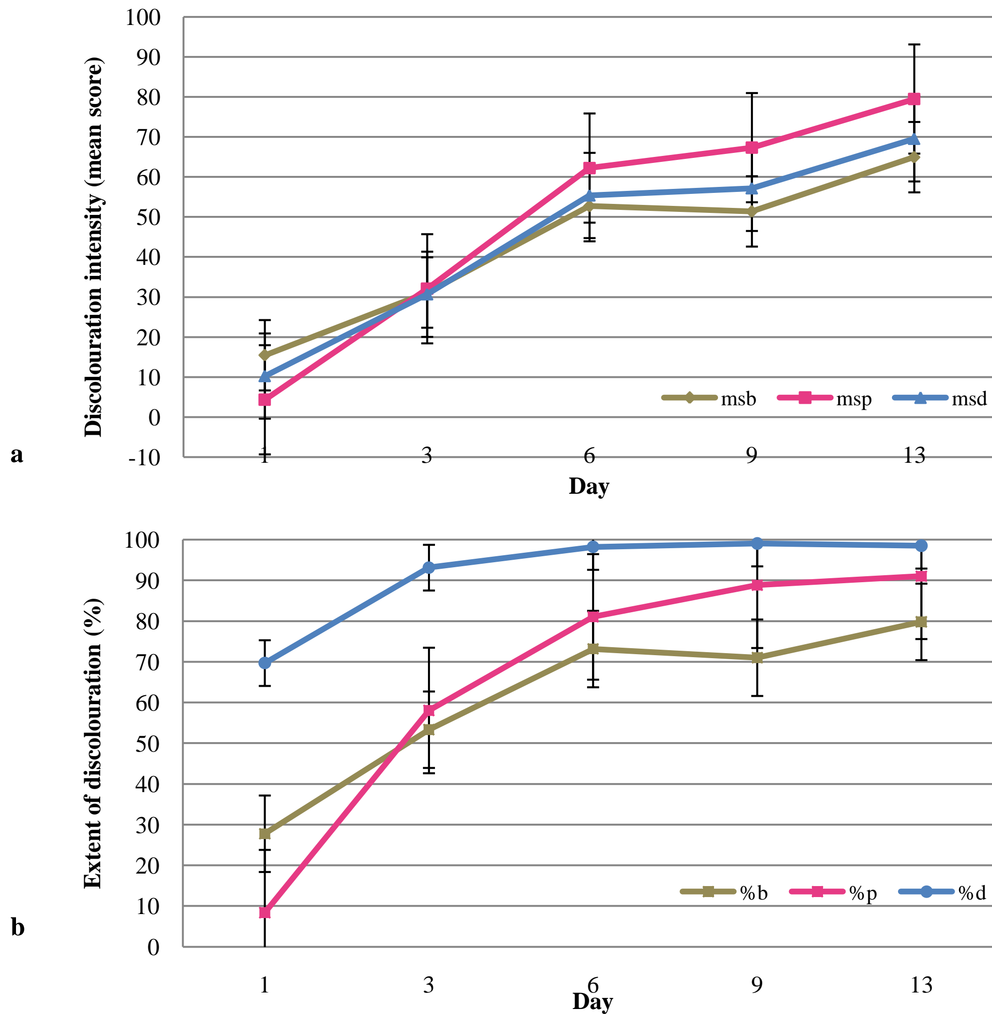


Figure 3.2. Means for a) intensity and b) extent (%) of post harvest pinking, browning and overall discolouration over 13 days across all accessions of the WHRI lettuce diversity set. Error bars represent se (standard error) from means. Where *msb* (mean score browning); *msp* (mean score pinking); *msd* (mean score discolouration); %*b* (percentage browning); *%p* (percentage pinking); *%d* (percentage overall discolouration).

Differences early on are potentially of greater importance to the food processor and retailers due to the products limited time on the shelf before the ‘best before’ time expires. Day 3 is especially important as that is the current threshold level for the major retailers; therefore it is appropriate to focus on post harvest performance at

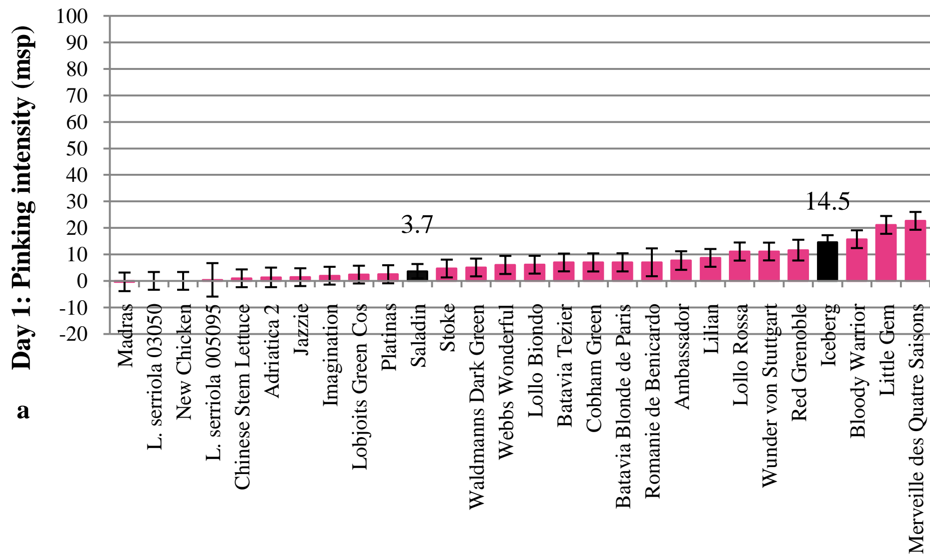
days 1 and 3 (DAC Pink personal communication). From day 6 onwards accessions were reaching maximum discolouration scores; the majority of accessions displayed maximum tissue coverage by some type of discolouration on day 6 for the extent of overall discolouration. The minimum value was 59.3% for Lollo Rossa and this would still be classed as unmarketable by retailers (graphs of discolouration on day 6, 9 and 13 in Appendix A).

Post harvest pinking of prepacked leaf tissue

The lettuce diversity set exhibited significant differences (***P <0.001) between accessions for pinking intensity for days 1 (Wald $_{[56]}$ = 130.41) and 3 (Wald $_{[58]}$ = 223.42), indicating genetic variation for this trait (see figure 3.3a and b). There were a lot of changes in ranking of accessions between days 1 and 3 for pinking intensity. Some accessions remained in similar rank positions on day 1 and day 3 while others changed considerably. Madras (-0.4) had the lowest level of pinking intensity on day 1, although at day 3 it was no longer the best performing accession as it had moved within the accession distribution (30.4). *L. serriola* 005095, New Chicken and *L. serriola* 03050 were also accessions with low pinking intensity on day 1 and on day 3, they were therefore ‘good’ accessions in respect to this post harvest quality. Little Gem and Bloody Warrior were poor performing accessions on day 1 with high pinking intensity; they also remained poor performing accessions on day 3. Interestingly, Waldmanns Dark Green (5.1) was a mid performing accession for pinking intensity on day 1, although by day 3 it had the third highest intensity value (47.5).

The lettuce diversity set also showed significant differences (***P <0.001) between accessions for the extent of pinking for days 1 (Wald $_{[56]}$ = 133.09) and 3 (Wald $_{[56]}$ =

190.53), signifying genetic variation for this trait (see figure 3.4a and b). As for intensity there were changes in ranking of accessions between days 1 and 3 for the extent of pinking. In a comparable trend to pinking intensity, some accessions remained in similar rank positions on day 1 and 3 while others changed considerably. Madras, *L. serriola* 03050 and New Chicken showed no signs of pinking on day 1, whilst *L. serriola* 005095 also revealed low levels of the extent of pinking (0.8%). *L. serriola* 03050, New Chicken and *L. serriola* 005095 were good accessions in respect to post harvest quality for the extent of pinking, as they were also the best performing accessions on day 3. However, as for intensity Madras was no longer a good performing accession for the extent of pinking by day 3 as its score had increased significantly it had moved within the distribution of accessions (from -0.5% to 47.3%). Little Gem and Bloody Warrior were poor performing accessions on both day 1 and day 3, while Merveille des Quatre Saisons was the worst performing accession on day 1 (33.5) but by day 3 it was not such a poor performing accession.



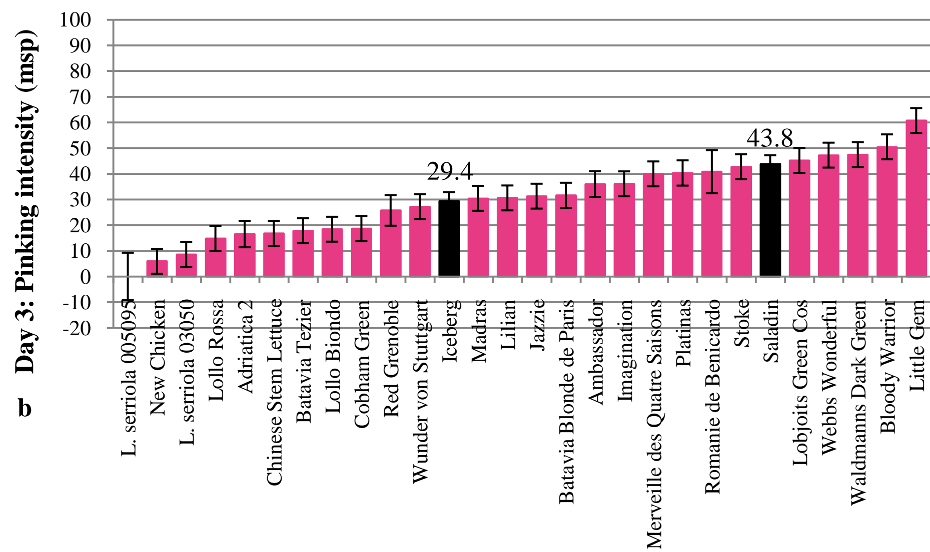


Figure 3.3. Transformed adjusted means (from REML) for lettuce post harvest pinking intensity on a) day 1 and b) day 3 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML).

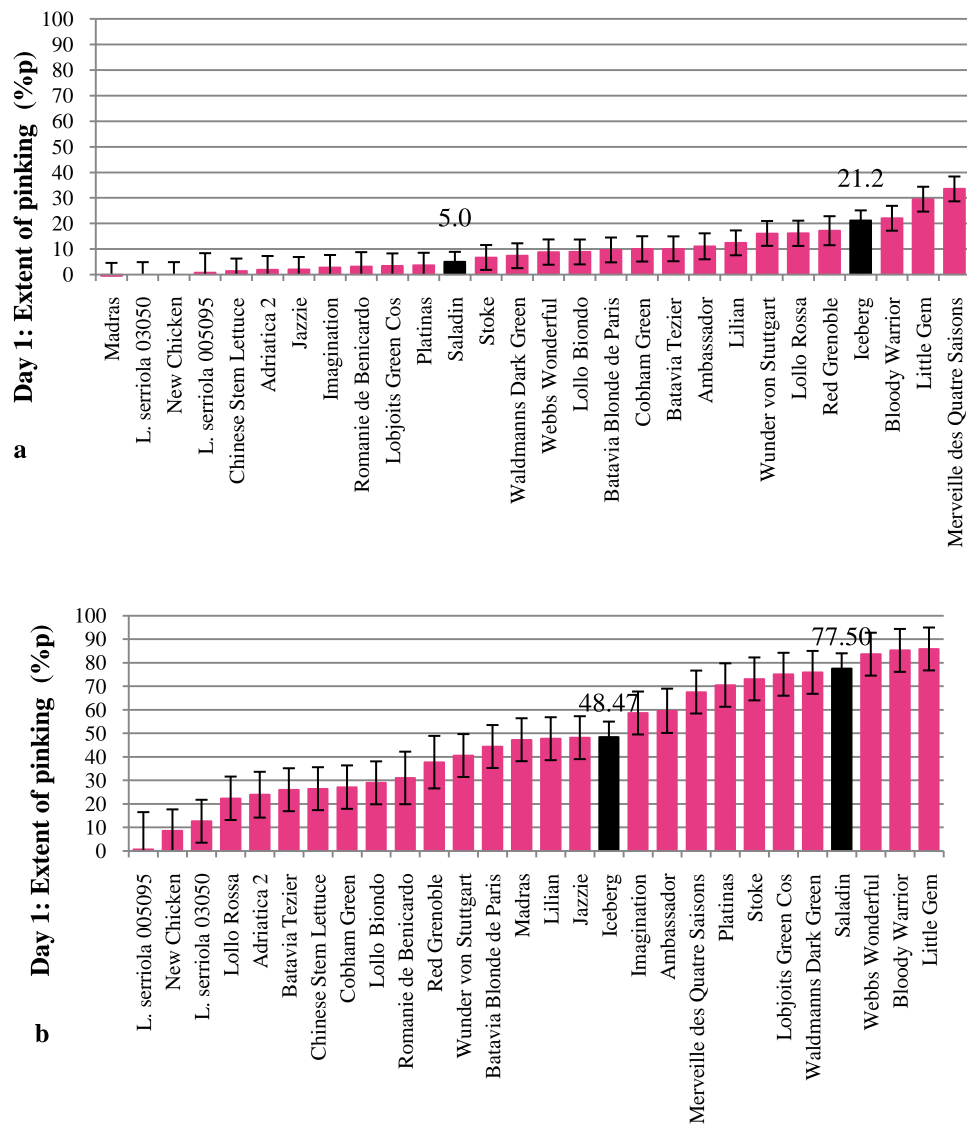


Figure 3.4. Transformed adjusted means (from REML) for lettuce post harvest extent of pinking on a) day 1 and b) day 3 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F7 WHRI mapping population parents are highlighted with respective adjusted means (from REML).

Post harvest browning of prepacked leaf tissue

The lettuce diversity set exhibited significant differences (****P* <0.001) between accessions for browning intensity for days 1 (Wald $_{[59]}$ = 719.55) and 3 (Wald $_{[48]}$ = 198.96), indicating genetic variation for this trait (see figure 3.5. a and b). There was a change in ranking of accessions between days 1 and 3 for browning intensity as for

all measures of pinking. The majority of accessions changed their ranking position for browning intensity, with only a minority remaining in similar rank positions on day 1 and day 3. Madras, Saladin and Lobjoits Green Cos remained good performing accessions on days 1 and 3. Although *L. serriola* 005095 showed no signs of browning on day 1, by day 3 it had moved ranks within the distribution to become a 'poor' accession for browning intensity (39.2). Adriatica 2 and Ambassador remained poor performing accessions on days 1 and 3. New Chicken was also a poor performing accession for browning intensity on day 1 (41.9), although as no change in its score had occurred between days 1 to 3 it moved to the middle of the distribution.

The lettuce diversity set also showed significant differences (***P <0.001) between accessions for extent of browning for days 1 ($\text{Wald}_{[58]} = 671.9$) and 3 ($\text{Wald}_{[48]} = 256.49$), indicating genetic variation for this trait (see figure 3.6. a and b). As for browning intensity, there were many changes in ranks of accessions between days 1 and 3 for the extent of browning intensity. The majority of accessions at the extremes of the distribution for the extent of browning stayed in similar rank positions on day 1 and 3, whilst the remaining accessions moved considerably. *L. serriola* 005095, Madras and Romanie de Benicardo were good performing accessions on days 1 and 3, and therefore were good accessions in respect to this post harvest trait. Additionally, Madras was constantly a good performing accession for all measures of browning on day 1 and day 3, suggesting that it is a good accession for post harvest browning. *L. serriola* 03050 had low levels for the extent of browning on day 1 (6.2%), which had increased by day 3 to be become a poor performing accession (59%). Adriatica 2, Ambassador, Cobham Green and Lilian remained poor performing accessions for the extent of browning on days 1 and 3.

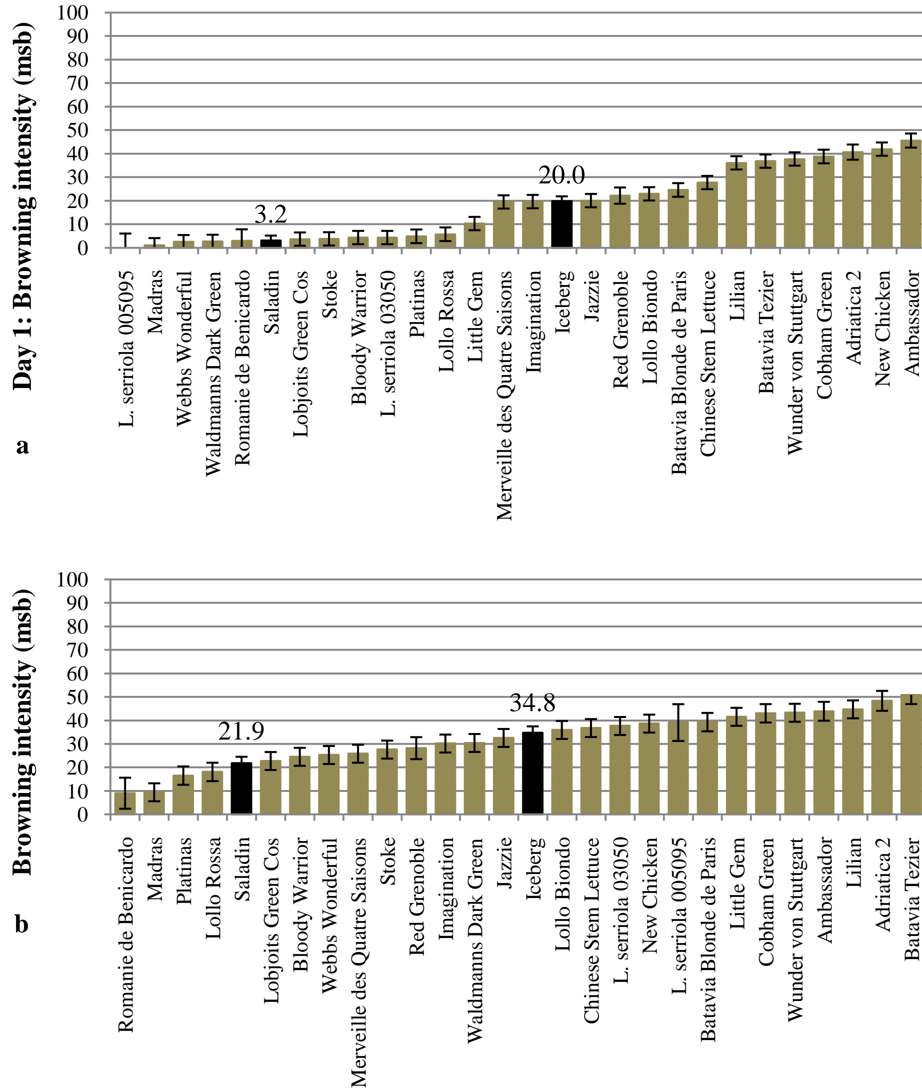


Figure 3.5. Transformed adjusted means (from REML) for lettuce post harvest browning intensity on a) day 1 and b) day 3 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML).

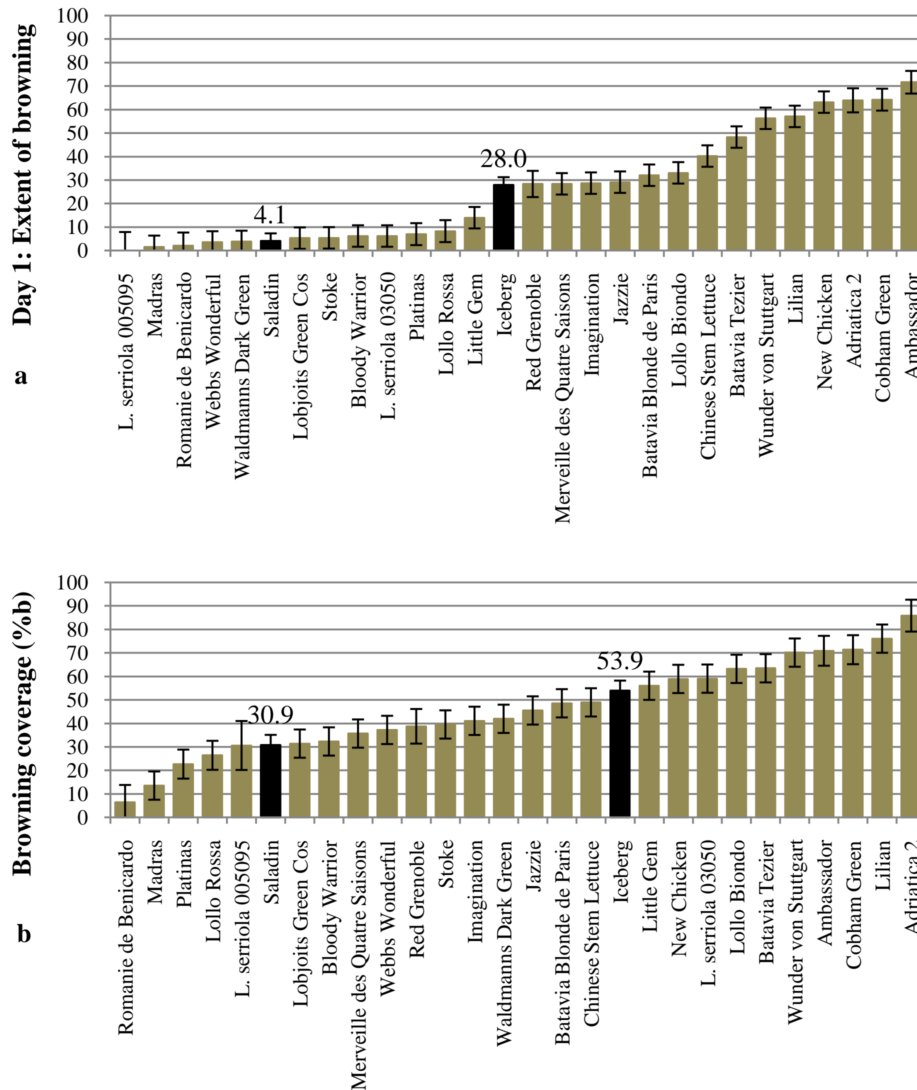


Figure 3.6. Transformed adjusted means (from REML) for lettuce post harvest extent of browning on a) day 1 and b) day 3 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML).

Post harvest overall discolouration of prepacked leaf tissue

Where discolouration could not be classified as a ‘pinking’ or ‘browning’ response it was scored simply as ‘visible’ discolouration so that its affect would not be lost. Consumers are likely to be indifferent as to whether post harvest discolouration is due to pinking or browning, as any type of visible discolouration would make the

product less acceptable. Therefore it is relevant to combine data for all types of discolouration including the 'unclassified' scores to give an overall discolouration score.

There were significant differences between accessions (****P* <0.001) for discolouration intensity on day 1 ($Wald_{[56]} = 442.34$) and day 3 ($Wald_{[58]} = 123.73$), indicating genetic variation for this trait (see figure 3.7. a and b). As for pinking and browning measures, ranking positions of accessions changed between day 1 and day 3 for overall discolouration. All accessions were showing overall discolouration intensity on day 1 and day 3 which would suggest that each accession is exhibiting pinking, browning or a combination of both types of post harvest discolouration. Madras, *L. serriola* 005095 and *L. serriola* 03050 were the best performing accessions on day 1 and day 3 for overall discolouration intensity, suggesting that they were good accessions in respect to this post harvest trait. Webbs Wonderful was also a good performing accession on day 1 (8.2) for overall discolouration intensity, however by day 3 it had become a poor accession (36.6). Ambassador, Batavia Tezier and Lilian remained poor performing accessions on day 1 and day 3. However, on day 1 New Chicken was also a poor performing accession (27.9) but on day 3 it had changed rank to become a best performing accession (27.1), suggesting that no additional discolouration had occurred.

The lettuce diversity set also showed significant differences (****P*<0.001) between accessions for the extent of overall discolouration for day 1 ($Wald_{[56]} = 287$) and day 3 ($Wald_{[58]} = 149.91$), suggesting genetic variation for this trait (see figure 3.8. a and b). As expected, ranks of accessions changed between day 1 and day 3 for the extent of overall discolouration. All accessions were showing overall discolouration on day 1 and day 3, with 6 accessions having reached the possible maximum level for the

extent of overall discolouration on day 1 and the majority of accessions having done so by day 3. Madras and Lollo Rossa were the best performing accessions on day 1 and day 3 for the extent of overall discolouration, therefore suggesting that they were good accessions in respect to this post harvest quality. Interestingly Lollo Rossa was the only accession to have <50% of the available extent of overall discolouration (47%). Red Grenoble and Chinese Stem Lettuce were mid performing accessions on day 1, although on day 3 they were good performing accessions within the diversity set. Wunder von Stuttgart, New Chicken, Ambassador, Adriatica 2, Lilian and Cobham Green were all at or extremely near the possible maximum level for the extent of overall discolouration on day 1.

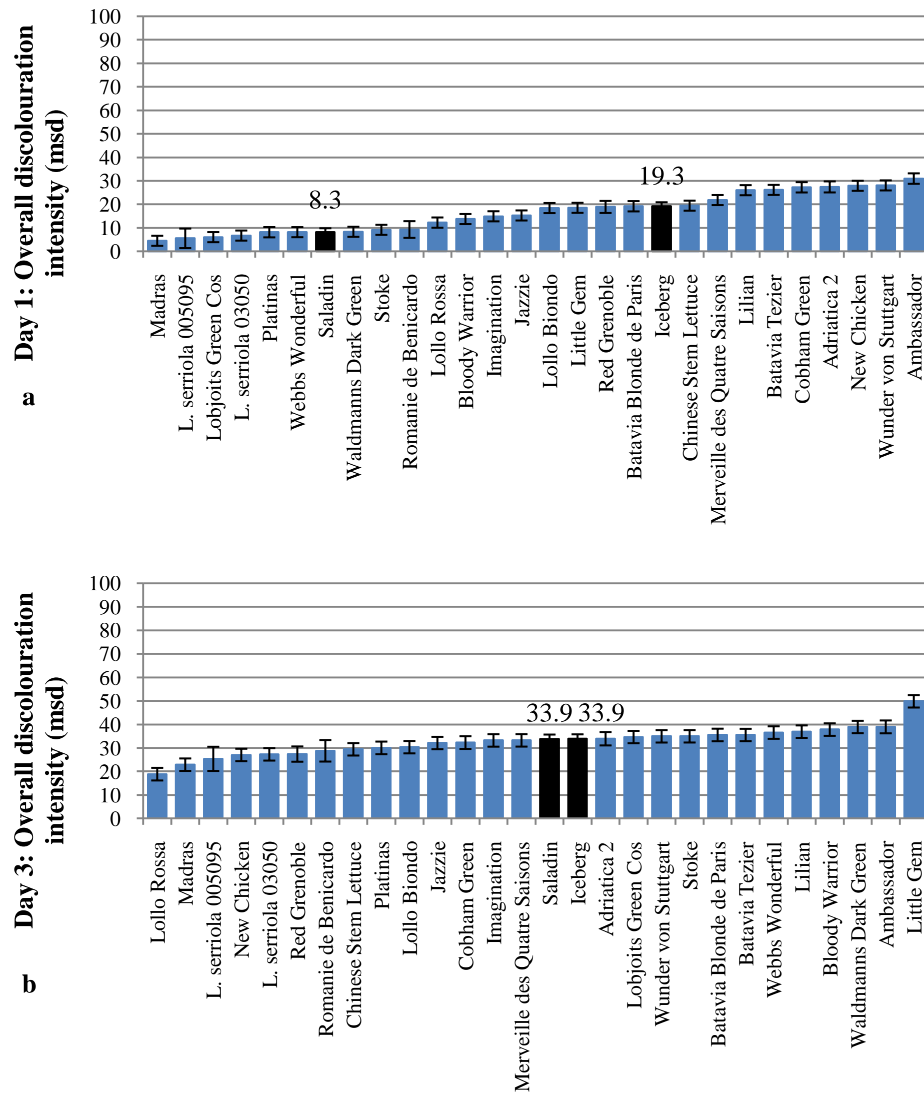


Figure 3.7. Transformed adjusted means (from REML) for lettuce post harvest overall discolouration intensity on a) day 1 and b) day 3 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML).

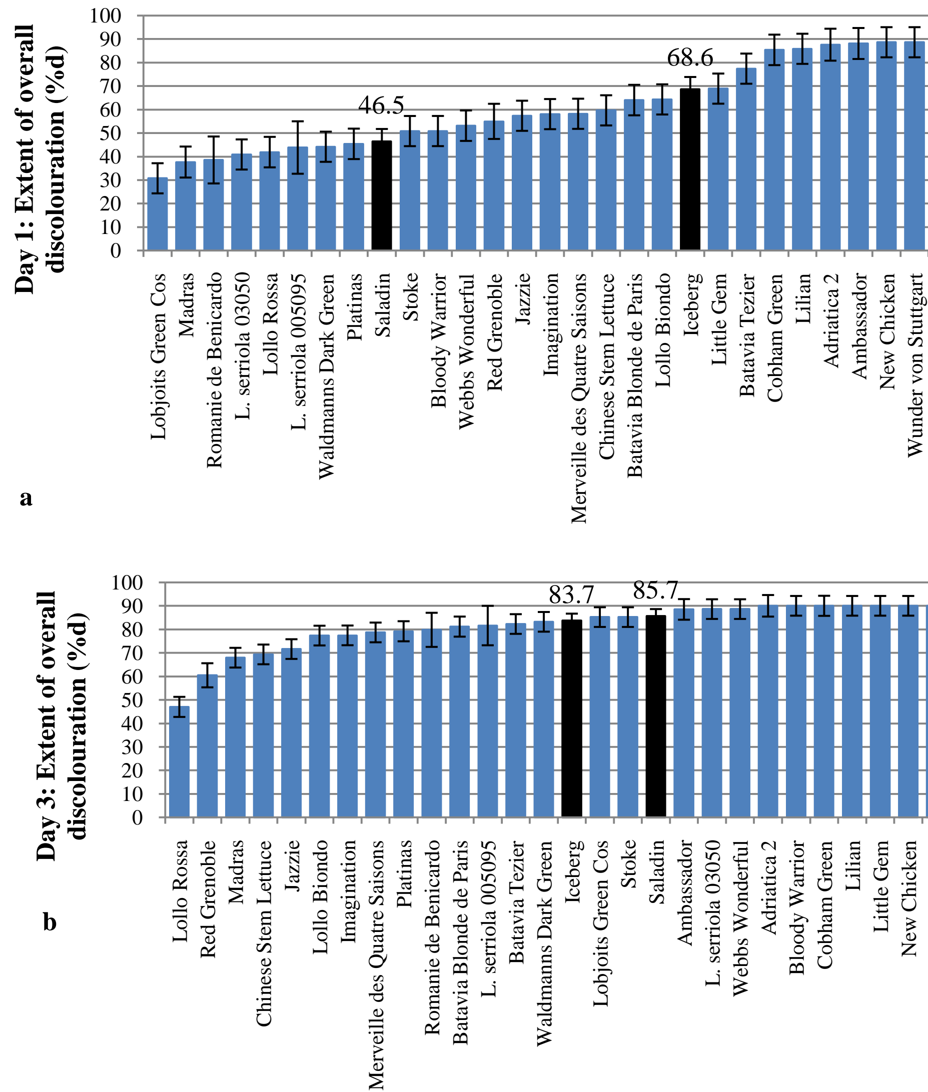


Figure 3.8. Transformed adjusted means (from REML) for lettuce post harvest extent of overall discolouration on a) day 1 and b) day 3 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML).

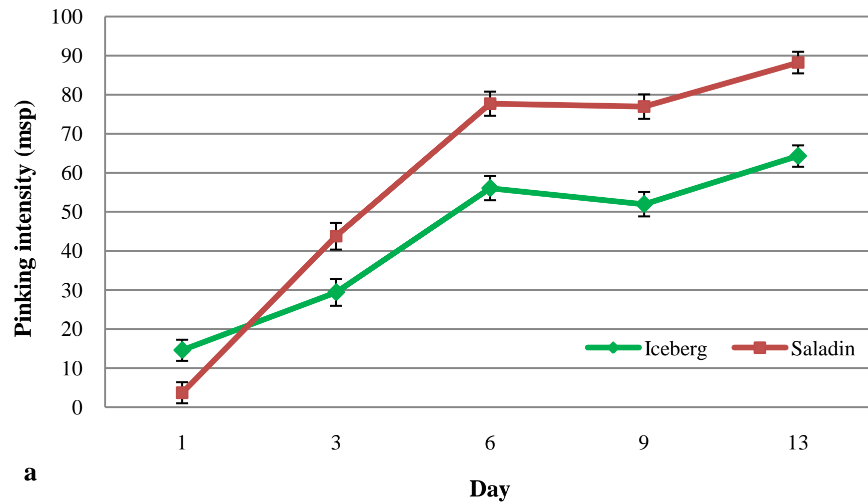
3.3.2. Performance of the mapping population parents

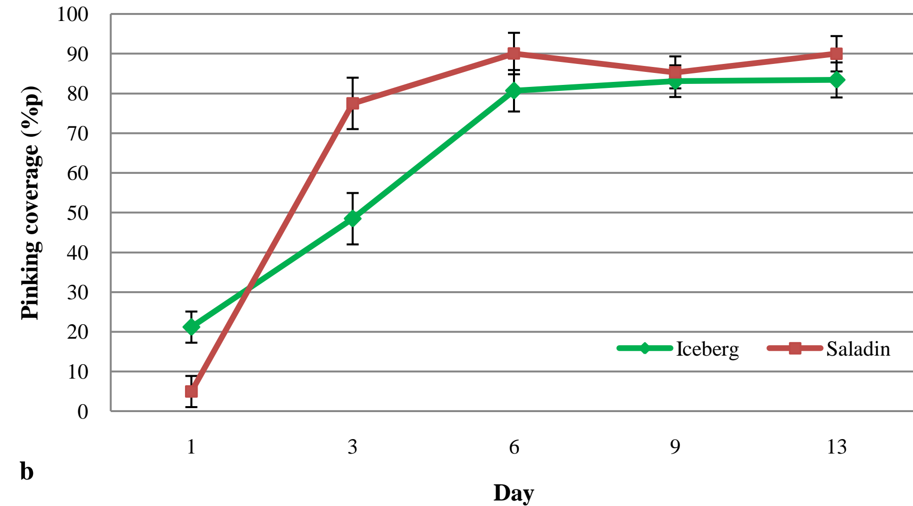
The mapping parent's cvs Saladin and Iceberg were compared for each measurement of discolouration on each day using T-tests. Different rates of discolouration were observed resulting from significant genetic variation between the parents.

Pinking

The parental accessions were significantly different (***P <0.001) for pinking intensity on all days (day 1, 3, 6, 9 and 13), however their ranking changed (see figure 3.9.a). The mapping parents also exhibited a linear response to pinking intensity. Saladin was the more resistant parental accession for pink intensity on day 1, but by day 3 Saladin and Iceberg had reversed rank positions. A difference in the rate of pinking intensity caused Iceberg to become the more resistant parental accession to pinking intensity on day 3 and it remained so through to day 13.

Saladin and Iceberg were also significantly different (***P <0.001) for extent of pinking on days 1, 3, 6 and 13 with the exception of day 9 (see figure 3.9.b). As for pinking intensity the ranking changed, however the mapping parents revealed a curved response to the extent of pinking. Saladin had the lowest values for the extent of pinking on day 1, but by day 3 Saladin and Iceberg had reversed rank positions. Differences in the rate of the extent of pinking resulted in Iceberg being the more resistant parental accession on day 3, 6 and 13. On day 6 Saladin had reached the possible maximum level for the extent of pinking, while Iceberg was near causing the curved response.





	Significance level					
	2 mapping parent accessions			Lettuce diversity set accessions		
Pinking intensity (msp)	**T-test Statistic**	**df**	**T-test P value**	**REML Wald Statistic**	**ddf**	**REML P value**
Day 1	3.97	33	***<0.001	130.41	56	***<0.001
Day 3	-3.59	35	***<0.001	223.42	58	***<0.001
Day 6	-6.96	46	***<0.001	785.05	47	***<0.001
Day 9	-8.19	45	***<0.001	314.05	57	***<0.001
Day 13	-8.25	29	***<0.001	556.67	117	***<0.001

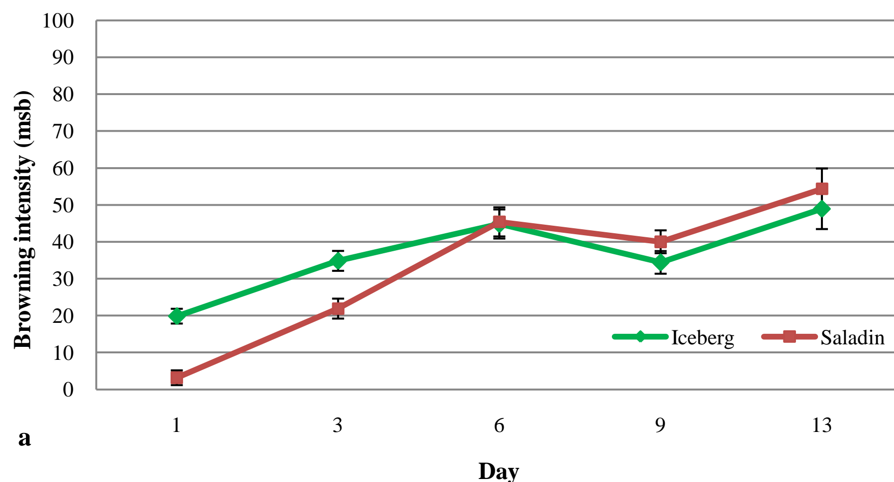
	Significance level					
	2 mapping parent accessions			Lettuce diversity set accessions		
Extent of pinking (%p)	**T-test Statistic**	**df**	**T-test P value**	**REML Wald Statistic**	**ddf**	**REML P value**
Day 1	4.51	39	***<0.001	133.09	56	***<0.001
Day 3	-3.86	31	***<0.001	190.53	56	***<0.001
Day 6	-3.80	45	***<0.001	240.53	46	***<0.001
Day 9	-0.96	46	0.344	193.58	58	***<0.001
Day 13	-3.59	45	***0.001	153.41	56	***<0.001

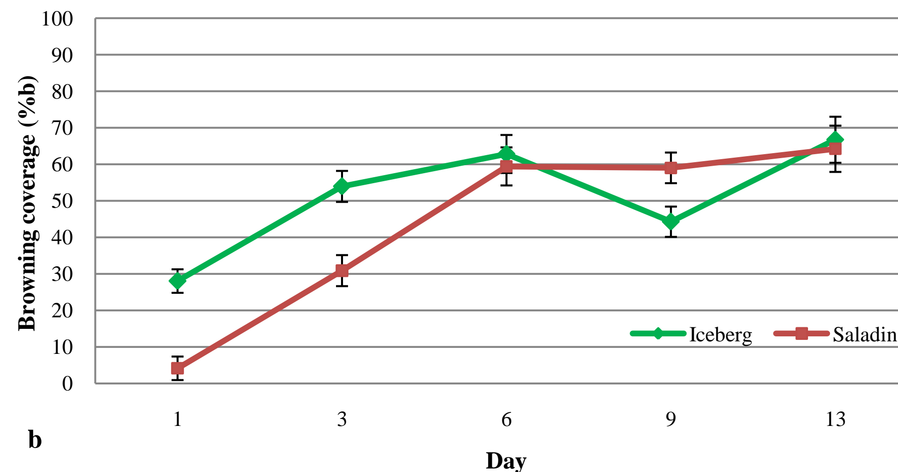
Figure 3.9. Transformed adjusted means (from REML) for lettuce post harvest a) pinking intensity and b) extent of pinking over 13 days for the WHRI F_7 mapping population parents Saladin and Iceberg. Error bars represent sems from REML. REML ndf = 27. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *msp* (mean score pinking); *%p* (percentage pinking); *df* (degrees of freedom); *ddf* (denominator degrees of freedom); *ndf* (numerator degrees of freedom).

Browning

As for the pinking response, the mapping parents showed significant differences (***P <0.001) for day 1 and day 3 for browning intensity (see figure 3.10a). Saladin was the more resistant parental accession for brown intensity on day 1 and day 3 with Iceberg the most susceptible. Due to lower and higher browning intensity rates for Iceberg and Saladin respectively, the accessions had converged by day 6. The mapping parents had similar values for browning intensity day 9 and day 13, therefore suggesting similar rates of reaction.

The mapping parents also showed significant differences (***P <0.001) for day 1 and day 3 for extent of browning (see figure 3.10.b). As for browning intensity, Iceberg was more susceptible to extent of browning on day 1 with an extremely high discolouration score in comparison to Saladin. Saladin was also more resistant to extent of browning on day 3. As observed for intensity, the accessions converged by day 6 due to differences in rates of the extent of browning and remained similar to day 13 resulting in a curved response. Interestingly, there was a significant decrease of Iceberg extent of browning on day 9, which may be due to sampling error or a change of tissue distribution within the bags.





	Significance level					
	2 mapping parent accessions			**Lettuce diversity set accessions**		
Browning intensity (msb)	**T-test Statistic**	**df**	**T-test P value**	**REML Wald Statistic**	**ddf**	**REML P value**
Day 1	5.63	30	***<0.001	719.55	59	***<0.001
Day 3	3.61	46	***<0.001	198.96	48	***<0.001
Day 6	-0.18	39	0.857	131.95	48	***<0.001
Day 9	-1.74	45	0.090	245.91	57	***<0.001
Day 13	-0.75	45	0.459	107.61	55	***<0.001

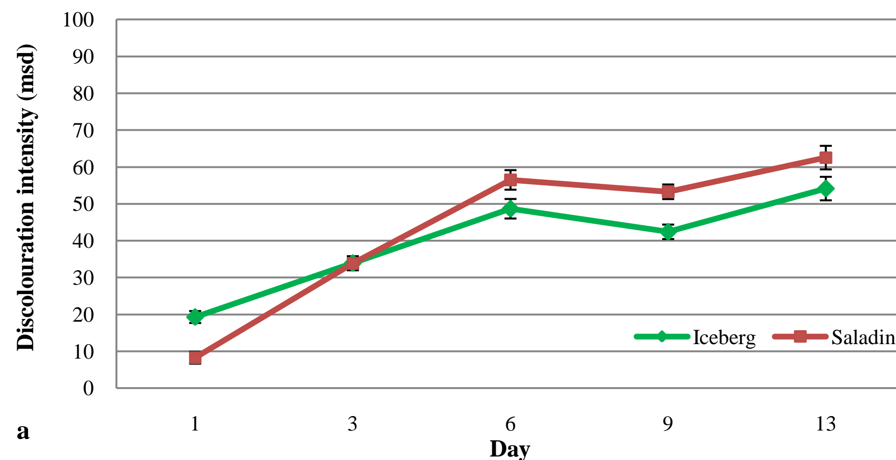
	Significance level					
	2 mapping parent accessions			**Lettuce diversity set accessions**		
Extent of browning (%b)	**T-test Statistic**	**df**	**T-test P value**	**REML Wald Statistic**	**ddf**	**REML P value**
Day 1	7.51	39	***<0.001	671.9	58	***<0.001
Day 3	3.82	45	***<0.001	256.49	48	***<0.001
Day 6	0.48	35	0.631	165.69	48	***<0.001
Day 9	-3.61	46	***<0.001	264.83	57	***<0.001
Day 13	0.47	45	0.640	131.23	56	***<0.001

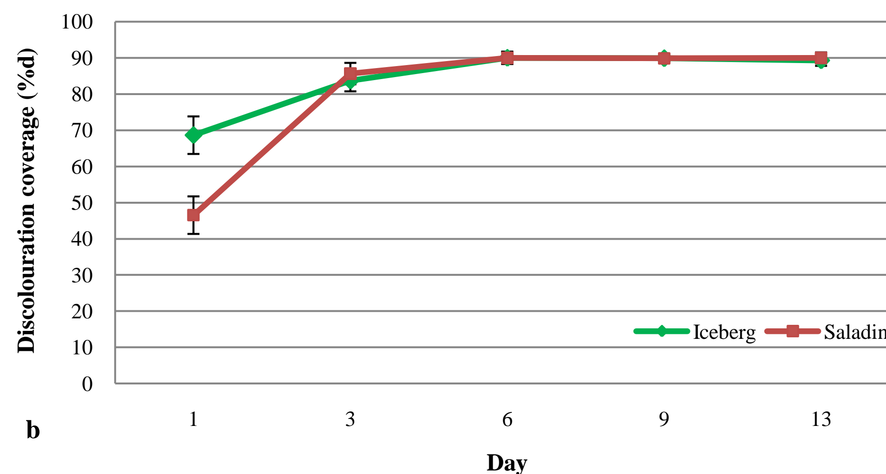
Figure 3.10. Transformed adjusted means (from REML) for lettuce post harvest a) browning intensity and b) extent of browning over 13 days for the WHRI F_7 mapping population parents Saladin and Iceberg. Error bars represent sems from REML. REML ndf = 27. Significant effects shown as **P* <0.05, ***P* <0.01 and ****P* <0.001. Where *msb* (mean score browning); *%b* (percentage browning); *df* (degrees of freedom); *ddf* (denominator degrees of freedom); *ndf* (numerator degrees of freedom).

Overall discolouration

The mapping parents showed significant differences for overall discolouration intensity on days 1 and 9 (***$P < 0.001$) and days 6 and 13 (**$P < 0.005$) but not on day 3 (see figure 3.11.a). As for all measures of discolouration, Saladin was the most resistant mapping parent to overall discolouration intensity on day 1. The mapping parents were not significantly different on day 3, suggesting that the reversal in the pinking response counterbalanced the better browning response of Saladin. Due to a difference in rates for overall discolouration intensity, Saladin and Iceberg had reversed rank positions by day 6 (which was probably due to differences in pinking). Iceberg had the lowest values for intensity which remained on day 13.

Due to differences in pinking as seen for overall discolouration intensity, Saladin and Iceberg were only significantly different (***$P < 0.001$) for extent of overall discolouration on day 1 (see figure 3.11.b).





	Significance level					
	2 mapping parent accessions			Lettuce diversity set accessions		
Overall discolouration intensity (msd)	**T-test Statistic**	**df**	**T-test P value**	**REML Wald Statistic**	**ddf**	**REML P value**
Day 1	6.23	32	***<0.001	442.34	56	***<0.001
Day 3	0.06	46	0.950	123.73	58	***<0.001
Day 6	-3.01	36	**0.005	217.02	45	***<0.001
Day 9	-6.16	45	***<0.001	249.91	56	***<0.001
Day 13	-3.02	45	**0.004	175.05	56	***<0.001

	Significance level					
	2 mapping parent accessions			Lettuce diversity set accessions		
Extent of overall discolouration (%d)	**T-test Statistic**	**df**	**T-test P value**	**REML Wald Statistic**	**ddf**	**REML P value**
Day 1	5.78	46	***<0.001	287	56	***<0.001
Day 3	-0.38	45	0.706	149.91	58	***<0.001
Day 6	NA	45	NA	182.3	55	***<0.001
Day 9	NA	46	NA	184.94	227	***<0.001
Day 13	-0.98	45	0.333	138.42	55	***<0.001

Figure 3.11. Transformed adjusted means (from REML) for lettuce post harvest a) overall discolouration intensity and b) extent of overall discolouration over 13 days for the WHRI F_7 mapping population parents Saladin and Iceberg. Error bars represent sems from REML. REML ndf = 27. Significant effects shown as **P* <0.05, ***P* <0.01 and ****P* <0.001. Where *msd* (mean score discolouration); *%d* (percentage discolouration); *df* (degrees of freedom); *ddf* (denominator degrees of freedom); *ndf* (numerator degrees of freedom); *NA* (not applicable).

3.3.3. Correlations amongst traits

All discolouration measures and morphological traits were assessed for potential relationships (correlations between browning and pinking and between them and morphological traits, (full correlation matrix in Appendix A)). Only the highly significant correlations (****P* <0.001) are described below (see table 3.3.).

The two measures of pinking (intensity and extent) were positively correlated with each other over all days ($R_{[25]} \geq 0.94$). Similarly measures of browning positively correlated with each other, with mean browning intensity and mean extent of browning highly correlated ($R_{[25]} \geq 0.97$). As for pinking and browning, measures of overall discolouration were generally positively correlated with one another.

While measures of pinking were generally positively correlated with measures of browning, negative correlations were also recorded. Day 1 values for browning intensity and extent of browning were both negatively correlated with pinking intensity and extent of pinking on day 3 and day 9 (also day 6 for pinking intensity). They also demonstrated negative correlations with mean score pinking (across days) and mean extent of pinking (across days).

For morphological traits; weight was negatively correlated with browning intensity and extent of browning on days 1 and 3. However, it was positively correlated with pinking intensity on day 6, 9 and 13, in addition to extent of pinking day 3 and day 6. Additionally weight was negatively correlated with measures of overall discolouration on day 1; however this may be consequential of the strong browning relationships recorded on day 1. Diameter of head was also negatively correlated with nearly all measures of pinking, while positively correlating with measures of browning.

Table 3.3. Correlation matrix from the WHRI lettuce diversity for post harvest discolouration and morphological parameters scored in 2007 experimental trial. Read across then down. Only significant effects are shown and highly significant effects ***P <0.001 are shown bold. Where *unt wt* (untrimmed weight, g); *tr wt* (trimmed weight, g); *tr dia 1* (trimmed diameter 1, mm); *tr diam 2* (trimmed diameter 2), *msb* (mean score browning); *msp* (mean score pinking); *%b* (percentage browning); *%p* (percentage pinking). Numerical value before discolouration measurement is day. Degree of freedom is 25.

	unt wt	tr wt	tr dia 1	tr dia 2	1msb	3msb	msb	1%b	3%b	msp
1msb	**-0.75**	**-0.72**	**0.84**	**0.86**						
3msb	**-0.75**	**-0.68**	**0.63**	**0.61**	**0.83**					
msb		-0.49		0.49	**0.70**	**0.77**				
1%b	**-0.75**	**-0.73**	**0.84**	**0.86**	**0.99**	**0.83**	**0.73**			
3%b	**-0.79**	**-0.76**	**0.66**	**0.65**	**0.86**	**0.96**	**0.83**	**0.87**		
6%b			0.40	0.45	**0.63**	**0.70**	**0.94**	**0.66**	**0.78**	
%b	-0.57	**-0.60**	0.55	**0.61**	**0.83**	**0.85**	**0.97**	**0.85**	**0.92**	
1msp										
3msp	**0.59**	0.55	**-0.81**	**-0.59**	**-0.67**			**-0.65**		
6msp	**0.80**	**0.76**	**-0.88**	**-0.75**	**-0.74**	-0.50		**-0.73**	-0.53	
9msp	**0.75**	**0.65**	**-0.84**	**-0.65**	**-0.69**	-0.50		**-0.67**	-0.50	
13msp	**0.67**	0.54	**-0.65**	-0.52						
msp	**0.75**	**0.66**	**-0.85**	**-0.66**	**-0.64**			**-0.63**		
1%p										
3%p	**0.63**	**0.59**	**-0.81**	-0.58	**-0.69**	-0.52		**-0.67**	-0.51	**0.89**
6%p	**0.72**	**0.70**	**-0.74**	**-0.59**	**-0.59**			-0.58		**0.92**
9%p		0.49	**-0.63**	**-0.56**	**-0.61**			**-0.61**		**0.65**
13%p										**0.71**
%p	**0.66**	**0.62**	**-0.78**	**-0.59**	**-0.62**			**-0.61**		**0.94**

3.4. Discussion

A retailer and consumer survey conducted through MINTeL (2007) suggested that issues with post harvest quality impact on both whole head and processed lettuce products. This would therefore signify that research related to post harvest discolouration is required for the current and future salads market supported by a continuously increasing demand for fresh salad products (Soliva-Fortuny and Martin-

Belloso 2003; Schene 2007). The current study was an initial study into genetic variation for post harvest discolouration in lettuce.

Post harvest discolouration in lettuce accessions was quantified as pinking or browning using an adapted visual scoring system developed by Hilton *et al.* (2009). It was also possible to calculate the mean score and percentage of each type of discolouration for each accession. The two measures represented different aspects of the discolouration response: with mean score (ms) measuring the production of compounds as intensity of colour, whilst percentage (%) related to diffusion of the coloured pigments throughout the tissue as the extent of discolouration; both effects make the produce unacceptable to the consumer (Tomás-Barberán and Espín 2001).

Whilst consumer reaction varies for discolouration type neither is commercially acceptable as any type of visible discolouration makes the product less desirable. Measurements of an overall discolouration response are acceptable for retailers as it combines scores for pinking and browning, however it also incorporates data that could not be classified (as either pink or brown) so all affects on discolouration are included. The accepted commercial viewpoint is that extent of overall discolouration is the best reflection of consumer product acceptance as it reflects presence or absence of pigments (Clifford *et al.* 2001). However, for genetic studies it is important to distinguish between different discolouration responses to determine whether they have a different genetic basis.

Phenotypic variation for all discolouration responses (pinking, browning and overall discolouration) was observed in the diversity set representing the primary lettuce gene pool. This was shown to be due to significant genetic variation as shown by significant Wald statistics for line effects in the REML analysis. When meaned

across all accessions, the lettuce diversity set were generally more susceptible to 'browning' in the early stages post harvest and 'pinking' in the later stages post harvest. Accessions were subsequently assessed individually per scoring day which revealed that they changed ranks within the distribution. These changes in rank positions across days were indicative of differing rates of discolouration. There were many changes in ranking positions of accessions between days 1 and 3 for all measures of discolouration; this would suggest differences between intrinsic rates of discolouration. More changes of rank positions occurred for the browning response in comparison to the pinking response which suggests more variation between accessions for rate of browning. For overall discolouration the majority of accessions had reached the possible maximum score for the extent of discolouration by day 3.

Intensity and extent of discolouration were positively correlated for all measures of pinking, browning and overall discolouration across days ($R \geq 0.9$) which suggests that although measured as separate traits, intensity and extent may have the same genetic basis (see table 3.3.).

Interestingly browning on day 1 was negatively correlated with pinking on day 3, 6 and 9 and mean pinking across days (see table 3.3.). The negative correlation implies that that pinking and browning are associated in an antagonistic way. Higher levels of browning on day 1 resulted in lower levels of pinking from day 3 onwards. This might be explained if the browning mechanism is a pre-determined protective mechanism preventing any further damage to the tissue while the pinking response is an alternative response to a stress producing compounds for repair of tissue damage.

This agrees with the finding that overall accessions within the lettuce diversity set were more susceptible to browning in the early stages post harvest and pinking in the later stages. It is important for breeders to understand the relationship between pinking and browning as 'tradeoffs' between the two responses may be needed in order find the best response for maximum shelf life. Similarly it is important for breeders to know whether reduced pinking and/or browning is associated with changes in other traits of interest as linkage or pleiotropic effects.

Potentially important correlations for breeders were observed between morphological traits and post harvest discolouration for accessions of the diversity set. Weight was negatively correlated with browning on day 1 and day 3 but positively correlated with pinking from day 3 onwards and diameter was positively correlated with browning on all days and negatively correlated with pinking on all days (see table 3.3).

There is a possible explanation of these correlations. Heavier heads would have grown at a quicker rate (as accessions were harvested within a similar time period) and therefore would have higher turgor pressure. Turgor pressure is required for all cell expansion and consequently plant growth. Head diameter is also indirectly consequential of stiffness as turgor pressure is required for plants to maintain their shape (Haman and Izuno 1993). Diameter decreases when heads are tighter whereas tissue with naturally less stiff leaves or those which have wilted would loosen and therefore we would expect to see a larger diameter. Negative correlations observed between weight and diameter measurements of accessions from the diversity set would support this idea. More rigid tissue with higher turgor pressure is more susceptible to cracking whilst in the field and/or during processing and storage (Newman *et al.* 2009). This additional accidental damage would increase the

wounding response and thus induce the phenylpropanoid pathway resulting in post harvest discolouration.

The most rigid tissue would potentially have a heavier weight and smaller diameter, which correlated with low levels of browning and high levels of pinking for accessions within the diversity set. Cell expansion, plant growth and volume maintenance would require a higher rate of secondary metabolism (Lynn *et al.* 1987; Haman and Izuno 1993). Polyphenol production would be increased for PPO oxidation where tannins are being used for growth rather than undergoing non enzymatic browning, while the pinking response could be initiated due to a weight stress for repair.

There was significant genetic variation for post harvest discolouration (established via T-tests) between mapping parents' cvs Saladin and Iceberg. Saladin displayed less pinking on day 1, however by day 3 the parents had reversed positions and Iceberg showed significantly less pinking than Saladin (see figure 3.9. a and b). The crossover suggests that Saladin may have beneficial alleles for the early stages post harvest while Iceberg alleles affect later stages for pinking; therefore both parents may potentially have useful alleles operating at different stages post harvest. Saladin was also the most resistant parental accession to both browning responses on day 1 and day 3 (see figure 3.10. a and b), so may have beneficial alleles post harvest for browning.

In addition to Saladin and Iceberg exhibiting significant genetic variation for post harvest discolouration, they were also distributed within the range seen in the diversity set (see figures 3.3. to 3.8.). Therefore the differences observed between Saladin and Iceberg is representative of the genetic variation observed in the lettuce

diversity set, and a ‘Saladin x Iceberg’ population would be suitable for genetic analysis of post harvest pinking and browning. The suggestion that both parents may possess beneficial alleles for the control of pinking may result in transgressive segregation in any subsequent mapping population (Grant 1975; de Vicente and Tanksley 1993; Rieseberg *et al.* 2003). Furthermore as Saladin and Iceberg are not at the extremes of the lettuce diversity set distribution, there may be additional genetic variation which could be exploited.

3.5. Conclusions

- There is significant genetic variation for post harvest discolouration between accessions of the lettuce diversity set.
- There is significant genetic variation between the parent’s of the Warwick HRI RIL mapping population, cvs Saladin and Iceberg, for post harvest discolouration.
- The differences for post harvest discolouration between Saladin and Iceberg are representative of the genetic variation between accessions of the lettuce diversity set.
- The Warwick HRI Saladin x Iceberg mapping population is suitable for studying the genetic basis of post harvest discolouration in lettuce.

CHAPTER 4

Generation of a *Lactuca sativa* linkage map as a tool for the genetic analysis of reduced post harvest discolouration in a lettuce RIL population

4.1. Introduction

The intra-specific cross between Saladin and Iceberg is potentially of high importance for crop breeding research as it is derived from two lettuce cultivars. The majority of current published linkage maps are based upon populations derived from inter-specific crosses between *Lactuca sativa* and a wild species relative (Johnson *et al.* 2000; Syed *et al.* 2006; Truco *et al.* 2007). Although this means there is a higher level of polymorphism between the parents which facilitates construction of a linkage map; the majority of polymorphisms observed in such populations have generally been ‘bred out’ of the cultivated crop. Although the use of two cultivars in crossing results in reduced levels of polymorphism the resultant linkage map is of more direct application in lettuce breeding. The F_7 Saladin x Iceberg RIL mapping population was produced from 130 of the most informative RILs that represented the major recombination events found within the population (Pink 2004; Pink 2009). The construction of a genetic linkage map for this population would provide a valuable resource, allowing the genetic characterisation of economically important traits and the development of markers for use in conventional marker assisted breeding programmes.

The aim of this study was to

- Generate a high density linkage map based on the F_7 RIL Saladin x Iceberg mapping population.

4.2. Material and methods

4.2.1. Genetic mapping

A genetic linkage map was produced based on 125 (of the 130) of the F_7 Saladin x Iceberg RILs using a variety of molecular markers.

DNA extraction

Genomic DNA of the mapping population parents and 125 F_7 RILs were extracted using the QIAGEN DNeasy 96 Plant Kit (QIAGEN Ltd., West Sussex, UK; Catalog No. 69181). A disc (10 mm) of leaf material was collected from young leaf from each mapping population parent and RILs and frozen in liquid nitrogen. The extraction was conducted according to the manufacturers' instructions from the QIAGEN DNeasy 96 Plant Handbook. DNA samples were suspended in 1 x TE (10 x TE: 100 mM Tris HCL pH8.0, 10 mM EDTA).

Fluorescent label amplified fragment length polymorphisms (AFLPs)

The AFLP procedure was performed according to the method described by Vos *et al.* 1995 using *Eco*RI/*Mse*I simultaneously (Analysis System I AFLP Starter Primer Kit, Catalog No. 10544-103 and 10483-014; Life Technologies, Gibro-BRL, Rockville, Md) and according to the manufacturers' instructions. A total of 46 primer combinations in lettuce were employed (with 3-base selectivity) and all combinations applied to every individual (see table 4.1.). The subsequent samples were run on an ABI DNA Sequencer 3100 in the WHRI Genome Centre.

Table 4.1. List of primer combinations used for AFLP analysis. The name and last three selective nucleotides of the primers are shown. Nomanclature from Vuylsteke *et al.* 1999.

Primer		**M47**	**M48**	**M49**	**M50**	**M54**	**M59**	**M60**	**M61**	**M62**
		CAA	**CAC**	**CAG**	**CAT**	**CCT**	**CTA**	**CTC**	**CTG**	**CTT**
E33	**AAG**		X	X		X	X	X	X	X
E35	**ACA**	X	X	X		X	X	X	X	X
E36	**ACC**		X							
E37	**ACG**								X	
E38	**ACT**		X	X		X	X	X		X
E41	**AGG**		X	X		X	X	X		X
E44	**ATC**		X	X	X	X	X	X	X	X
E45	**ATG**	X	X	X	X	X	X	X	X	X

The AFLP genotype data was analysed using SoftGenetics GeneMarker: Version 1.6 software and analysed as described in the software's 'Quick Start Guide'. Polymorphic alleles were scored by the presence/absence of peaks in the electropherogram in comparison to the parental genotypes. AFLP markers were designated with the name of the two primers (E#M#) used for amplification, followed by the product size in bp from the electropherogram and reference to the parent (i is Iceberg and s is Saladin).

Conserved ortholog set markers (COSs)

Conserved ortholog set (COS) markers (Fulton *et al.* 2002) developed through the Compositdb project at UCDavis

(http://cgpdb.ucdavis.edu/database/genome_viewer/viewer/) were used for PCR based genotyping (see table 4.2.).

Table 4.2. COS primer combinations for PCR analysis. Where *Chr* (Marker chromosome location on Map2 JMR3 (http://cgpdb.ucdavis.edu/database/genome_viewer/viewer/)

Marker	Chr	Forward primer	Reverse primer
LE9008	1	CGAGAAGCTGCTGAAGTTGATGAG	TGGGCCACATATTGTCAATACACG
LE9212	1	GCCTCTATCAGATGTATGCCAGATG	GGAGTGTCTTGCAGACAAATCATC
LE3023	2	CGACCCTACATGGCTGAACT	TGGTACACCTTCCCATTCGT
LE9016	2	CCGATGTCAATGATGTGCTAGATG	AGCATTTCCTCCTCTTTCTTCAGC
LE9037	2	TGGTCTATCTCTACCTAAGGATGC	CTGAACTCACAATGTTGAGACTAG
LE9050	2	ACTACTTCTTCTGCTATAGAGTCC	CTTCTACACCCTCAAACTCCTTATC
LE3007	3	TCCGTCTCTATTGGGTTTCCT	CTCTGGCCTTGCTTGTGATAG
LE3014	3	GAAAATCGACCAGACCCGTA	ACGGGTTCAAAGAATCCACA
LE9003	4	CAGGAACCGCTGAAGTTGAGGTTG	CACAACTGCATTAACAGTATTGAG
LE9006	4	GTGAGCTTGATCATGCATTCCTGC	CCTTGATTGATAGTTTCATTGCCAC
LE9033	4	TCAATTGTGAAGTTAGATTCACCAG	CACTAAGGATTGTTAAACCATCTAG
LE9039	4	GTTCAGACGATTATTCACCAGATG	TTTGGTGATTGCATAACCCCATCC
LE9015	5	AACTCTTGTGCTTCTACTTGCAGA	TCCTTGAGAGAGGTAACTAATCTC
LE9023	5	GCAGCTTTGCTTACCTCGATTTCC	TGTTCTTTAGCTTTCTCAAGCCTC
LE9048	5	TCAATGCAAGTACATGGTTACGTC	TTCTCCAATCTCAACTCTGTATGG
LE9251	5	CATAAGAGCCTTTAAGTTTGACAT	GTTGATGTATGTACGGTAGATGTCG
LE9030	6	AAGGAGAAGGTGAACCATGGATAG	TCTAAACGAGAATCTTCTTGAACC
LE9018	7	TCTTGCAAAATCTAATGTCACAAG	CTGCAACAAGTTCCTTCATTATCC
LE9019	7	GGCTCAGAAGCGTTGGATTGATTG	CTCACCAGAATCAACAGCAGCAAG
LE9022	7	CTGTAAGGAATAAGAAACGAGTTG	GAAAACTCTGCTATATCCAAATTGC
LE1164	8	AGATCCTTCCATCTTTGCCA	AACCAAGGGTGGCTTCAAA
LE9041	8	CATCGTCTGTAGGAATACTTGGATC	GACATATTCCCGATCAGAGATGTTG
LE9052	8	ACTACTTCTTCTGCTATAGAGTCC	CTTCTACACCCTCAAACTCCTTATC
LE9214	8	CACTGACAGTATTACATTGCAAC	CTTCCAAGACTTATGTGAAATTCC
LE9013	9	AAGTTGGGTGGAGAATCACATTGG	GATGCATAGCTCTCCAGGTTGTTC
LE9038	9	GATGGAGCGTCCGATCAGTGTCTG	GGATCACCATCATAGTCAGCTTGT

Polymerase chain reaction (PCR)

The 10 µl reaction mixture consisted of: 1 µl 10 x PCR reaction buffer, 1 µl forward primer (5 mM); 1 µl reverse primer (5 mM); 1 µl template DNA (~10 ng/µl); 4.6 µl dH_20 and 0.1 µl DNA polymerase (Invitrogen™ *Taq* DNA polymerase, Invitrogen ™, UK). PCR was performed using a thermal cycler, (Applied Biosystems Gene Amp® PCR system 9700, Applied Biosystems™, Singapore). The PCR reaction program was: 95°C for 5 min, 34 cycles of (94°C (30 s); 55°C (30 s); 72°C (30 s)), extended at 72°C for 10 min. The PCR products were determined visually by agarose gel electrophoresis (1% gel).

Expressed sequence tags (ESTs)

Expressed sequence tag (EST) markers were developed and genotyped by Rijk Zwaan based on the Serriola x Salinas integrated map, which is the current 'framework' lettuce map (Truco *et al.* 2007) (see table 4.3.). Twenty-six EST markers were run at Rijk Zwaan Breeding Station, Fijnaart and genotype data scored and provided for incorporation into the linkage map.

Table 4.3. EST marker id's from Rijk Zwaan. The primer sequences remain confidential although they provide anchor points to the integrated map. Where *Chr* (Marker chromosome location on Map2 JMR3 (http://cgpdb.ucdavis.edu/database/genome_viewer/viewer/)

Marker	Chr	Marker	Chr	Marker	Chr	Marker	Chr	Marker	Chr
RZ-A	8	RZ-G	4	RZ-M	4	RZ-S	2	RZ-Y	4
RZ-B	8	RZ-H	3	RZ-N	8	RZ-T	2	RZ-Z	4
RZ-C	4	RZ-I	4	RZ-O	2	RZ-U	2		
RZ-D	4	RZ-J	2	RZ-P	2	RZ-V	4		
RZ-E	1	RZ-K	5	RZ-Q	1	RZ-W	8		
RZ-F	3	RZ-L	5	RZ-R	1	RZ-X	4		

SNP mapping

Illumina Golden gate assays OPA3 and 4

Genomic DNA from the F_7 Saladin x Iceberg mapping population was supplied at ~150 ng/µl to the DNA Technologies Core, UCDavis Genome Center USA, for SNP genotyping. The DNA Technologies Core provided custom high throughput SNP genotyping using the Illumina Golden Gate Assay to generate SNP specific PCR products. The third and fourth generation OPA Illumina assay (OPA3/OPA4) each consisted of 384 SNPs (768 in total) which were originally developed for the Salinas x Serriola mapping population (Truco *et al.* 2007). Fluorescent labelled products representing the different SNPs were combined with Illumina beads (VeraCode bar-

coded beads) in solution for the BeadXpress platform. Each fluorescent signal was associated with a particular address, which consequently translated to a particular locus where the presence of the signals indicated genotype. The DNA Technologies Core additionally conducted replicates to serve as internal controls for assay reproducibility requiring >99.5% homology.

BeadStudio software (version 3.1.3.0, Illumina Inc.) was used to analyse the output genotype data. Genotypes segregated into clusters representing homozygotes and heterozygotes (where applicable), which could be manually altered through the SNP graphs (see figure 4.1.). Maria Truco at UCDavis provided the initial genotype scores for OPA4.

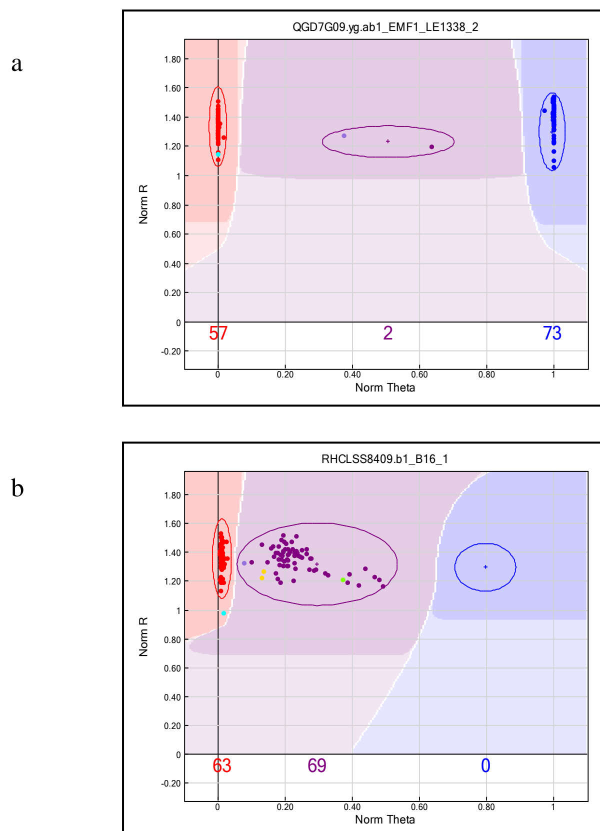


Figure 4.1. SNP graphs from Beadstudio 3.1.3.0 scored a) co-dominantly and b) dominantly. AA genotype information (red); BB genotype information (dark blue); AB genotype information (dark purple). Saladin genotype (lime green); Iceberg genotype (turquoise); Salinas genotype (yellow); artificial heterozygote (light purple).

Single position polymorphism (SPP) genotyping

The Saladin x Iceberg F_7 population mapping output was compared to the MCB19 10NR map under construction in Prof Richard Michelmores' lab, at the Genome Center, UCDavis. The MCB19 10NR map is based on the Salinas x Serriola mapping population (Truco *et al.* 2007) and was produced using the Affymetrix high density GeneChip® microarray designed to detect single feature polymorphisms (SFPs) in more than 35,000 lettuce genes. Primers were derived for every 100 bins per chromosome where gaps were present in the current map version based on polymorphisms between Saladin and Iceberg. The primers were specifically designed around single position polymorphism (SPP) sites of the associate EST/contig which were detected by hybridisation intensity differences from the Affymetrix high density GeneChip® microarray data output allowing identification of position (see table 4.4.). Primers were developed using the primer design software Primer3 (v. 0.4.0) (Rozen and Skaletsky 2000). The relevant EST/contig sequence was submitted with parameters of product size 200-250 bp and targets as SPP site.

Single sequence conformation polymorphism (SSCP) analysis

The mapping parents and the entire F_7 population were amplified using the associated primers and analysed on SSCP gels. The notched glass plate of the SSCP tank was sequentially cleaned with H_2O, EtOH, RAIN-X and H_2O. The un-notched glass plate was cleaned with H_2O, EtOH, 1 ml binding solution (500 µl ethanol, 500 µl 10% glacial acetic acid, 50 µl 5% binding silane) and EtOH. The glass plates were clamped together and 80 ml MDE gel (55.2 ml dH_2O, 20 ml 2x MDE solution, 4.8 ml 10x TBE, 400 µl 10% ammonium persulfate, 80 µl temed) poured and allowed to polymerize > 1 hr.

Table 4.4. SPP markers from map MCB10_10NR_SxI (RW Michelmore personal communication) used to develop markers covering every 100 bins of the lettuce genome in order to enable coalescence in the F_7 Saladin x Iceberg linkage map. Where *Marker Code* (code on original map); *Sequence ID* (EST/Contig data retrieved from http://cgpdb.ucdavis.edu/cgpdb2/CGP_ContigViewer/); *Chr* (chromosome present on); *SPP Position* (single positional polymorphism from EST/Contig sequence).

Marker Code	Sequence ID	Chr	SPP Position	Marker Code	Sequence ID	Chr	SPP Position	Marker Code	Sequence ID	Chr	SPP Position
AZTD	CLS_S3_Contig2493	1	531_550	AZAO	CLS_S3_Contig2055	4	99_104	AWFJ	CLS_S3_Contig10615	8	921_926
AJDS	CLSM16652.b1_H11.ab1	1	127_134	BCHA	CLS_S3_Contig4064	4	513_516	AMQR	CLSM8885.b1_I13.ab1	8	403_422
AIMC	CLSM13691.b1_E16.ab1	1	493_516	AUIH	CLSY8890.b1_C15.ab1	4	241_244	ATJI	CLSY3988.b1_H13.ab1	8	513_518
BGGF	CLS_S3_Contig6529	1	113_126	AXJO	CLS_S3_Contig11326	4	383_402	BTEH	QGB28E23.yg.ab1	8	459_464
AIKO	CLSM13565.b1_J07.ab1	1	67_72	ATRK	CLSY4971.b1_F20.ab1	5	289_292	BTUF	QGC10O20.yg.ab1	8	219_234
AYEW	CLS_S3_Contig1528	1	349_358	BKJN	CLS_S3_Contig9062	5	119_130	ASRF	CLSY1344.b1_P23.ab1	8	91_102
AVGS	CLS_S3_Contig10032	2	445_448	BEWJ	CLS_S3_Contig5657	5	67_86	AMGU	CLSM7725.b1_I11.ab1	8	599_616
BSOW	QGB20I09.yg.ab1	2	287_290	ARFM	CLSX2754.b1_C17.ab1	5	351_354	ASUI	CLSY1763.b1_E10.ab1	8	105_118
AZKZ	CLS_S3_Contig230	2	357_362	BRZR	QGB13D02.yg.ab1	5	85_100	BHCQ	CLS_S3_Contig7056	8	791_796
BKUD	CLS_S3_Contig9313	2	339_352	BHAY	CLS_S3_Contig7016	5	153_156	AZOT	CLS_S3_Contig239	9	75_100
AREY	CLSX2692.b1_G01.ab1	2	253_256	BEVL	CLS_S3_Contig5635	5	327_338	BUGI	QGC15F13.yg.ab1	9	267_272
AZVA	CLS_S3_Contig2539	2	425_430	AHTB	CLSM11856.b1_P12.ab1	5	479_492				
BHKL	CLS_S3_Contig7239	2	875_878	AOVB	CLSS2687.b1_N24.ab1	5	597_602				
ASZZ	CLSY2574.b1_K20.ab1	2	95_100	BUZC	QGC24H06.yg.ab1	6	111_114				
AXWL	CLS_S3_Contig1329	2	561_564	AWNO	CLS_S3_Contig10808	6	201_220				
BFPJ	CLS_S3_Contig6129	2	403_418	BTJM	QGB6H05.yg.ab1	6	147_150				
BCOF	CLS_S3_Contig4235	2	277_282	ALGQ	CLSM4746.b1_D11.ab1	6	107_124				
AKLQ	CLSM20182.b1_K06.ab1	2	783_788	AIED	CLSM1289.b1_B11.ab1	6	49_58				
BIKC	CLS_S3_Contig7846	3	615_636	ALJA	CLSM4981.b1_J22.ab1	7	315_320				
BVZI	QGD11G21.yg.ab1	3	269_272	BAIJ	CLS_S3_Contig2862	7	129_140				
BLNH	CLS_S3_Contig9764	3	281_286	AOIW	CLSS13293.b1_I12.ab1	7	217_226				
ARTT	CLSX5061.b1_I17.ab1	3	227_234	BBRG	CLS_S3_Contig3686	7	165_170				
BLII	CLS_S3_Contig9647	4	287_306	BLAG	CLS_S3_Contig9458	7	351_364				
BLTJ	CLS_S3_Contig9909	4	87_90	AHEI	CLSL2531.b1_E10.ab1	8	223_228				

Samples were loaded onto the gel with 0.5x formamide loading dye (950 µl formamide, 10 µl 1N NaOH, 40 µl 1% dyes) after an initial denature at 98°C for 5 mins and direct cooling on ice. Gels were ran in 0.6x TBE at 1 W for ~16 hr. The notched glass was removed and the un-notched glass with the adhered gel was placed in a shaker for 8 min with fixing solution (10 ml glacial acetic acid, 210 ml 95% ethanol, 780 ml H_2O, 1.5 g silver nitrate) and rinsed twice with H_2O. The developing solution (30 g NaOH, 1 L H_2O, 1 ml 37% formaldehyde) was added and shaken for 8-10 min or until bands appeared which were then scored with the corresponding parent.

All genotype data was scored co-dominantly (A (positive parent 1), B (positive parent 2), H (heterozygous) and U (unknown data point)).

Joinmap®4 analysis

Linkage analysis for genotype scores for all markers from the mapping population was performed using Joinmap® 4 software with default parameters, except for those listed below (Stam 1995; Van Ooijen 2006). The calculations of the linkage maps used all pair wise recombination estimates smaller than 0.49, LOD scores higher than 0.01, a jump of 5 and the regression mapping algorithm. A RIx (where x=generation; 7 in this study) population was used. Markers were assigned to linkage groups by increasing the LOD score for grouping with steps of one LOD unit. Recombination frequencies were converted to map distance in centimorgans using Kosambi's mapping function (Kosambi 1944). After an initial generation of a 'best fit' linkage map with all markers (ignoring anchoring points), markers were consequently assigned to a chromosome based on their linkage with anchor markers. The map was regenerated as individual linkage groups independently and marker

order was forced (as appropriate) during linkage analysis. The linkage map was drawn using the MapChart programme (Voorrips 2002).

4.3. Results

The newly generated F_7 Saladin x Iceberg mapping population was screened to create a new linkage map for a presumed genetically fixed population. Genotypic data was obtained using 674 polymorphic markers including AFLPs, COSs, ESTs OPAs and SPPs (see table 4.5.). The genetic map was generated using 425 markers mapping to 18 linkage groups covering all 9 chromosomes (see figure 4.2. and table 4.5. and 4.6.). The map length covered 1039.7 cM, which was 139.7 cM longer than the estimated map length of 900 cM. The smallest and largest linkage groups were 3.3 cM (LG 6c) and 167.4 cM (LG 2). The average distance between markers over the map is 2.4 cM. The length of the linkage groups did not correlate with the numbers of mapped markers on them. The largest linkage group (LG 5) had only 41 markers, while the smallest linkage group (LG 6) had 22 markers. LG 4 had the most markers (86), while LG 9 had the least markers (17) (see table 4.6.). Inconsistent marker coverage was also observed on LG 2 which had an average inter-locus interval of 2.2 cM but an interval of 37.2 cM was observed between 109.4-146.6 cM for markers BVZJ-OP4 and BDAY-OP4/BHOQ-OP4.

Table 4.5. Marker characteristics of the Saladin x Iceberg genetic map. Where *Sal* (Saladin); *Ice* (Iceberg); *No* (number).

Marker type	Map anchor	No. polymorphic markers for Sal x Ice	No. markers mapped for Sal x Ice
AFLP	-	335	163
COS	Map2 JMR3	3	2
EST	Map2 JMR3	21	18
OPA	MCB19 JNR3	305	237
SPP	MCB19 JNR3	9	4
Morphological	-	1	1
Total	-	674	425

Clustering of markers (where markers mapped to the same position) was observed on all linkage groups. Each of the 9 linkage groups had a minimum of 2 clustering markers which were either 2 AFLPs or 2 OPAs. LG 1 had a clustering of 3 and 4 OPA markers, LG 2 had a cluster of 8 OPA markers, LG 6 had a cluster of 4 OPA markers and LG 8 had a cluster of 5 OPA markers. LG 7 had a clustering of 3 OPA markers and 2 additional clusters of 4 markers which included 3 OPA markers and a morphological locus for seed colour and 6 markers which included 5 OPA markers and 1 SPP marker.

All linkage groups with the exception of LG 6 contained loci displaying segregation distortion (see table 4.7. and Appendix B). Six of the linkage groups (2, 3, 4, 5, 7 and 8) had loci showing segregation distortion towards both the Saladin and the Iceberg allele. For LG 3 and LG 4 proportion of loci showing segregation distortion towards the Saladin or Iceberg allele were approximately equal; segregation distortion at loci on LG 2, 7 and 8 favoured the Iceberg allele, while LG 5 favoured the Saladin allele. For LG1 all loci showed segregation distortion for the Saladin allele 1 and for LG9 all segregation distortion was for the Iceberg allele. Linkage groups 8 and 9 showed distorted segregation for approximately 50% of loci, both in favour of the Iceberg allele.

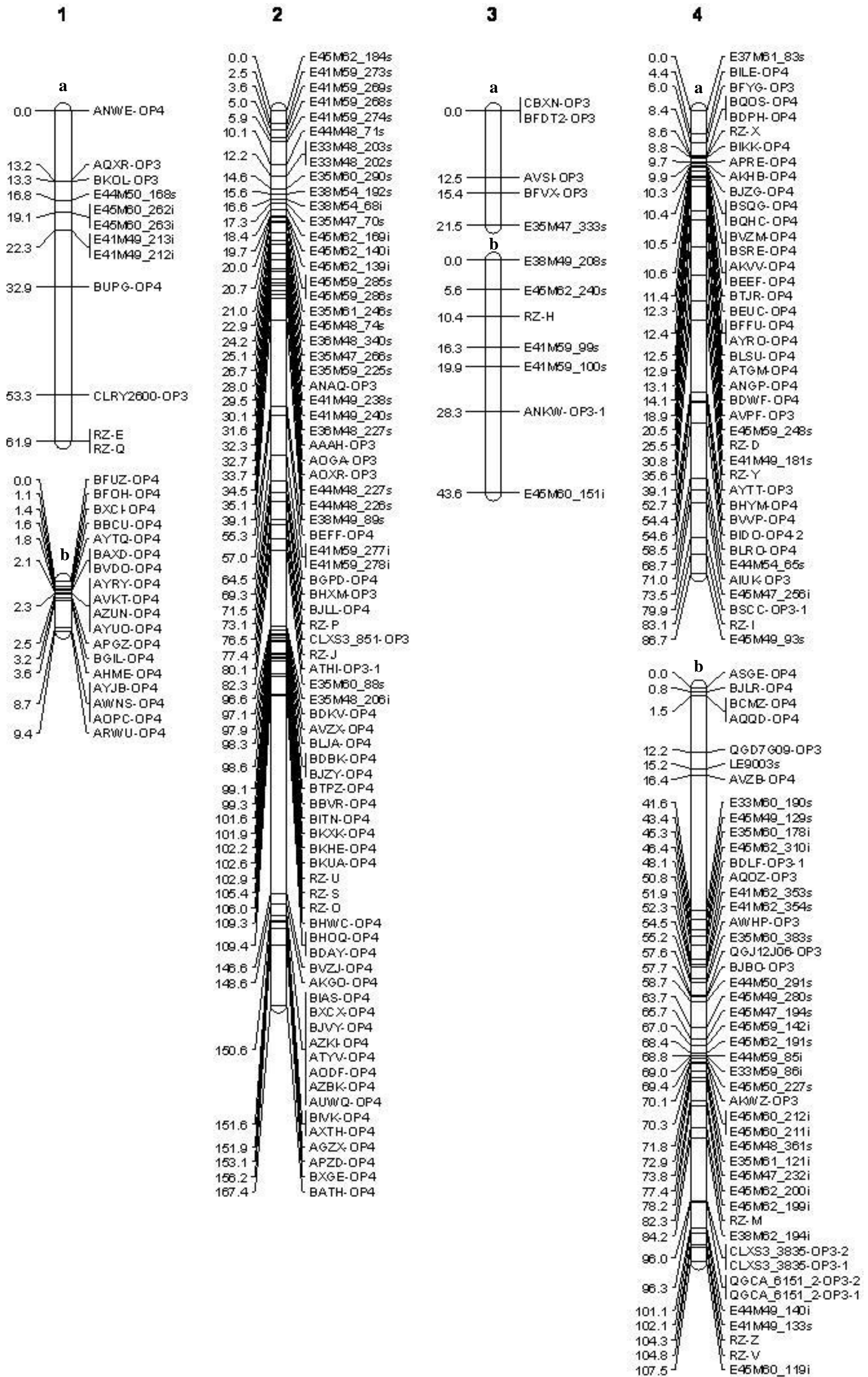


Figure 4.2. Linkage map of *Lactuca sativa* based on the F_7 Saladin x Iceberg RIL mapping population. Recombination distances are in Kosambi's cM. Numerical value refers to chromosome number, letter refers to linkage group for each chromosome.

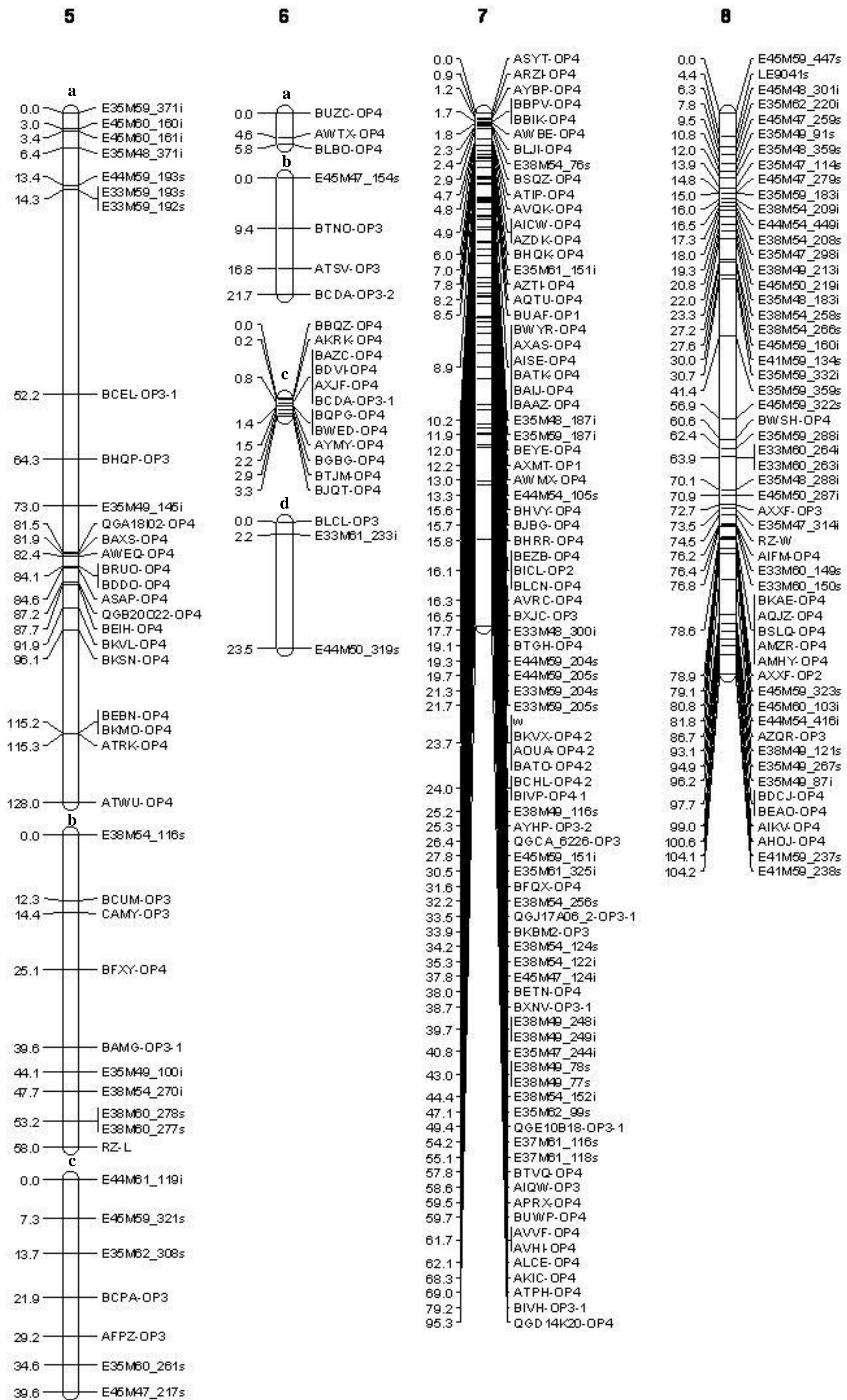


Figure 4.2. Continued.

9

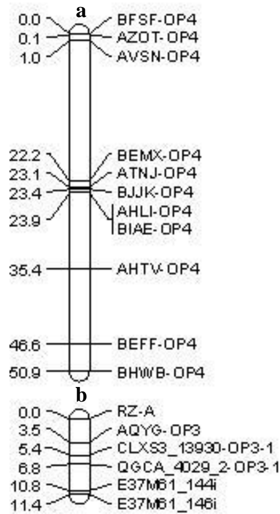


Figure 4.2. continued.

The Saladin x Iceberg map was generated with the marker order and placement on linkage group based on the MCB10_10NR map currently under construction in Prof Richard Michelmores' lab at The University of California, Davis, (see Appendix B). The component linkage groups for LGs 1, 5 and 7 of the Saladin x Iceberg map are positioned in the correct orientation with anchor markers in the right order based on their original bin position in the MCB10_10NR map. Anchor markers for LG 8 are also in the correct order and orientation, but there is a single outlier marker AHOJ-OP4 which is not locating at its estimated position in the MCB10_10NR map. Component LGs corresponding to LG 4 are again in the correct orientation, anchor markers for LG 4b are in the right order and the majority of anchor markers for LG 4a are also in the correct order but there are two major outliers not locating at the correct estimated positions; BIDO-OP4-2 and BLRO-OP4.

Table 4.6. Characteristics of linkage groups of Saladin x Iceberg mapping population. Where *LG* (linkage group); c*LGs* (number of component linkage groups); *cM* (centimorgans); *Av* (average); *No* (number); *Morph* (morphological).

LGs	cLGs	Total length (cM)	Markers	Av. loci interval (cM)	No of markers					
					AFLP	COS	EST	OPA	SPP	Morph
1	2	71.3	30	2.4	5	0	2	23	0	0
2	1	167.4	77	2.2	32	0	5	40	0	0
3	2	65.1	12	5.4	6	0	1	5	0	0
4	2	194.2	86	2.3	32	1	7	46	0	0
5	3	225.6	41	5.5	18	0	1	21	1	0
6	4	54.3	22	2.5	3	0	0	17	2	0
7	1	95.3	85	1.1	26	0	0	57	1	1
8	1	104.2	55	1.9	39	1	1	14	0	0
9	2	62.3	17	3.7	2	0	1	13	1	0
Total	18	1039.7	425	2.4	163	2	18	236	5	1

Table 4.7. Summary of markers showing segregation distortion for each parental genotype for each chromosome and the number of markers showing significant segregation distortions (**P* **< 0.05).** Where *LG* (linkage group); *No* (number); *Sal* (Saladin); *Ice* (Iceberg); *H* (heterozygote); *U* (unkown).

LG	% Sal allele	% Ice allele	% H	% U	% Non-distorted markers	No of distorted markers					
						AFLP	COS	EST	OPA	SPP	Gene
1	13.3	0	6.6	0	80.1	0	0	0	6	0	0
2	7.1	16.2	1.3	1.3	74.1	9	0	1	10	0	0
3	8.3	8.3	0	0	83.4	1	0	0	1	0	0
4	12.8	10.5	2.3	0	74.4	7	1	2	12	0	0
5	22	4.9	2.4	0	70.7	3	0	0	9	0	0
6	0	0	0	0	100	0	0	0	0	0	0
7	2.4	16.5	1.2	0	79.9	10	0	0	6	0	1
8	12.7	34.5	1.8	0	51	24	1	0	2	0	0
9	0	47.1	5.9	0	47	1	0	0	8	0	0
Total	9.3	15.4	2.1	0.2	73	55	2	3	54	0	1

Most of the component linkage groups for LG 3 (LG 3a), LG 6 (LG 6a, 6b and 6c) and LG 9 (LG 9a) are positioned in the correct orientation with anchor markers in the right order, however the orientation of a single component LG for each linkage group (LG 3b, LG 6d and LG 9b) is unknown as each only has a single anchor point (ANKW-OP3-1, BLCL-OP3 and AQYG-OP3 respectively). LG 2 appears in the correct orientation with anchor markers in the right order, however a small section (encompassing markers BIAS-OP4, AKGO-OP4 and BVZJ-OP4) appears to have been 'inverted' and should be positioned within the estimated region of 40 cM (see figure 4.3.).

The SPP markers were specifically designed to provide genome wide coverage for *Lactuca satvia*, as they were derived for every 100 bins per chromosome based on the MCB10_10NR map. However, due to equipment and time constraints the mapping population RILs were only genotyped for 9 of the 59 SPP markers (see table 4.4.). Of these 4 were mapped onto LG 5a, LG 6a and c, and LG 9a. The addition of the remaining SPP markers (particularly in conjunction with the 249 unmapped markers) would be beneficial due to the estimated genome coverage which could allow coalescence into 9 linkage groups (see table 4.8.).

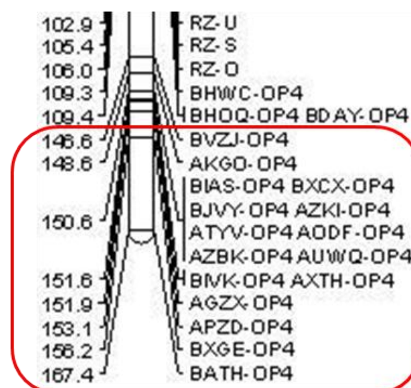


Figure 4.3. Inverted marker order observed in LG 2.

4.4. Discussion

Genetic maps based on crosses between lettuce cultivars have previously been reported to have higher numbers of linkage groups than those derived from interspecific crosses between lettuce and related wild *Lactuca* sp. The high number of linkage groups in the Sal x Ice map could be due to low intraspecific polymorphism and a high frequency of monomorphic regions between the parental lines Saladin and Iceberg (Truco *et al.* 2007).

Many lettuce linkage maps often exceed the theoretical estimated length as in this study (Kesseli *et al.* 1994; Syed *et al.* 2006; Truco *et al.* 2007). Error in scoring can increase the number of apparent recombinants and thus dramatically inflate map distances (Kesseli *et al.* 1994; Jeuken *et al.* 2001). Inflation has been recorded with error rates below 2% (Jeuken *et al.* 2001). If a locus locates internally it may be placed in the correct position however it will cause length expansion as seen in LG 2 (although this could also be the result of translocation) (Kesseli *et al.* 1994).

The majority of the initial mapping studies in lettuce were based on populations segregating for disease resistance (Landry *et al.* 1987; Kesseli *et al.* 1994; Syed *et al.* 2006) and the Sal x Ice RIL population was produced initially to study resistance to pests and disease (*Myzus persicae* and *Bremia lactucae*) (DAC Pink personal communication). The opportunity to determine the genetic control of discolouration in lettuce provided by the Sal x Ice RIL population allows possible interactions between disease and agronomic traits to be established, thus allowing breeding to combine improved shelf life with already established traits for growers.

Kesseli *et al.* (1994) and Waycott *et al.* (1999) each constructed linkage maps based on populations derived from crosses between cultivars of *Lactuca sativa.* Like the Sal x Ice map constructed in this study, these maps were fragmented with many linkage groups containing many marker types exceeding the chromosomal number of lettuce. Johnson *et al.* (2000) reviewed an AFLP marker based framework map for lettuce QTL analysis of a *L. sativa* x *L. serriola* map. The most recent genetic map for a single lettuce population was produced by Syed *et al.* (2006), which was also based on a cross derived from *L. sativa* and *L. serriola.* Both these interspecific maps consisted of only 10 linkage groups, including 9 major and 1 minor group.

More recently integrated maps based on different types of crosses have been aligned to produce a more informative map with greater utility. Jeuken *et al.* (2001) constructed the first integrated map of lettuce. The map was based on 488 markers (using 124 markers for alignment) from 2 populations derived from crosses between *L. saligna* and *L. sativa*. Markers coalesced into 9 linkage groups; however they only spanned 854 cM with a mean interval of 1.8 cM. Most recently Truco *et al.* (2007) constructed a high density integrated map of lettuce (*Lactuca* spp.). The map was based on 2,744 markers (using 560 markers for alignment) from 7 intra and inter specific mapping populations. Markers were assigned to 9 chromosomal linkage groups covering a genetic distance of 1505 cM and had a mean interval of 0.7 cM.

The use of AFLP markers in linkage analysis has been reported as a reliable and reproducible procedure for numerous crops as it can cover the entire genome when sufficient markers are obtained (Nikaido *et al.* 1999; Duran *et al.* 2004). From the markers used for map construction in this study the AFLP markers were more informative as 95% of those assigned to a chromosome mapped compared to 77.6% for OPA markers. For the remaining marker sets >55% of assigned markers mapped

(see table 4.9.). However approximately 33% of the AFLP markers showed significant segregation distortion, compared to 23% of OPA markers (see table 4.7.).

The haploid genome of lettuce consists of 9 linkage groups; as the Sal x Ice lettuce map consists of 18 linkage groups some of the linkage groups will join once additional markers are added, thus forming 9 linkage groups each representing a single chromosome. The addition of new markers (not limited to the un-genotyped SPP markers and the current unmapped markers) could aid in coalescence of the linkage groups.

Two-hundred and forty nine markers remained unmapped; the majority of these were AFLP markers which could not be assigned to groups (see table 4.8.). Many formed small tight linkage groups within marker type, however as they did not contain anchor markers from previous maps their position remains unknown at present so they were excluded from the map construction. Most of the COS, EST, OPA and SPP markers were assigned to a LG but a minority did not map. This could be due to high recombination frequencies ('low linkage') between markers, causing excessive and un-mappable genetic distances.

Table 4.8. Mapping of assigned and unassigned markers. Where *No* (number).

Marker	No. not assigned to chromosome	No. assigned to chromosome	No. assigned mapped
AFLP	162	173	163
COS	0	3	2
EST	0	21	18
OPA	1	304	236
SPP	0	9	5
Morphological	0	1	1
Total	163	511	425

The addition of the SPP markers would be beneficial due to the estimated genome coverage which could allow coalescence into 9 linkage groups (see table 4.9.). The

un-genotyped SPP markers for LG 1 could allow length extension of the group at both ends in addition to a possible joining of LG 1a and 1b via markers BBGF and AIKO. The 2 genotyped SPP markers (BIKC and BLNH) and the un-genotyped marker ARTT would also allow extension in length at both ends of LG 3, while BVZI could assist in the coalescence of LG 3a and 3b. The genotyped SPP marker (AXJO) and the un-genotyped markers BLII, BLTJ and AUIH could aid in length extension at both ends of LG 4, whilst AZAO and BCHA could aid in linking LG 4a and 4b. For LG 5, the addition of the numerous un-genotyped SPP markers could possibly allow coalescence into 1 group via the joining of LG 5a and 5b (BKJN, BEWJ, ARFM, BRZR and BHAY) and of LG 5b and 5c (BEVL, AHTB and AOVB). The single SPP marker (BUGI) for LG 9 could also help with the linking of LG 9a and 9b. For LG 6 the addition of the currently un-genotyped marker AWNO could link LG 6a and 6b, while ALGO and AIED could not only link LG 6c and 6d but aid in the determination of the orientation of LG 6d and extension of the linkage group as a whole. For both LG 7 and LG 8 the addition of genotyped markers (BAIJ and AMGU respectively) and numerous un-genotyped SPP markers would increase the density of each linkage group, assist in confirming the order of markers and allow length extension at the end of the groups (and start for chromosome 7). Finally, the addition of un-genotyped SPP marker AKLQ, could possibly assist in the split of the inverted marker order observed in LG 2 (see figure 4.2.), while markers AVGS, BSOW, AZKZ, BKUD and AREY could allow their correct positioning. The remaining markers would increase the density of the linkage group and improve the correct marker order.

Markers were generally evenly distributed across linkage groups although a few distances exceeded 20 cM (LG 1a, 2, 4b, 5a, 6d and 9a), with the largest distance of

37.3 cM recorded on LG 2 (see figure 4.2. and 4.3.). The maximum number of markers mapped at any locus was 8 on LG 2 which were all OPA markers. There were many small clusters of OPA markers distributed across the map, which could suggest the markers had been developed from the same/similar sequence IDs. High numbers of double AFLP clusters were also observed, however the majority of these appear to be the product of stutter peaks as there is a single base pair difference; E41M49_212i and E41M49_213i on LG 1a. Clustering of AFLP markers has also been recorded by Truco *et al.* (2007) and van Os *et al.* (2006) in ultra dense maps of lettuce and potato respectively.

Different markers types target different genomic regions and thus reveal dissimilar distribution patterns. Therefore in order to generate a versatile molecular map it is beneficial to use a variety of markers during construction (Klein *et al.* 2000; Sebastian *et al.* 2000; Mei *et al.* 2004; van der Linden *et al.* 2004; Syed *et al.* 2006). Accurate high resolution maps act as extremely important tools to detect/locate genes/QTL encoding desirable agronomic traits, and versatile markers can be used to hasten the improvement of plants (Jeuken *et al.* 2001; Syed *et al.* 2006).

Table 4.9. Chromosome position of SPP markers (yet to be genotyped) on map MCB10_10NR. Where *Marker Code* (code on original map); *Sequence ID* (EST/Contig data retrieved from http://cgpdb.ucdavis.edu/cgpdb2/CGP_ContigViewer/); *Chr* (chromosome present on); *x: x* (chromosome: position).

Marker Code	Sequence ID	Chr : position (bin)	Marker Code	Sequence ID	Chr : position (bin)	Marker Code	Sequence ID	Chr : position (bin)
AZTD	CLS_S3_Contig2493	1 : 214	AKLQ	CLSM20182.b1_K06.ab1	2 : 2482	ALGQ	CLSM4746.b1_D11.ab1	6 : 1688
AJDS	CLSM16652.b1_H11.ab1	1 : 333	BVZI	QGD11G21.yg.ab1	3 : 625	AIED	CLSM1289.b1_B11.ab1	6 : 1725
AIMC	CLSM13691.b1_E16.ab1	1 : 404	ARTT	CLSX5061.b1_I17.ab1	3 : 1424	ALJA	CLSM4981.b1_J22.ab1	7 : 27
BGGF	CLS_S3_Contig6529	1 : 1588	BLII	CLS_S3_Contig9647	4 : 268	AOIW	CLSS13293.b1_I12.ab1	7 : 755
AIKO	CLSM13565.b1_J07.ab1	1 : 1657	BLTJ	CLS_S3_Contig9909	4 : 322	BBRG	CLS_S3_Contig3686	7 : 861
AYEW	CLS_S3_Contig1528	1 : 2286	AZAO	CLS_S3_Contig2055	4 : 550	BLAG	CLS_S3_Contig9458	7 : 1637
AVGS	CLS_S3_Contig10032	2 : 409	BCHA	CLS_S3_Contig4064	4 : 1242	AHEI	CLSL2531.b1_E10.ab1	8 : 379
BSOW	QGB20I09.yg.ab1	2 : 518	AUIH	CLSY8890.b1_C15.ab1	4 : 1913	AWFJ	CLS_S3_Contig10615	8 : 438
AZKZ	CLS_S3_Contig230	2 : 734	BKJN	CLS_S3_Contig9062	5 : 314	AMQR	CLSM8885.b1_I13.ab1	8 : 526
BKUD	CLS_S3_Contig9313	2 : 852	BEWJ	CLS_S3_Contig5657	5 : 691	ATJI	CLSY3988.b1_H13.ab1	8 : 1008
AREY	CLSX2692.b1_G01.ab1	2 : 946	ARFM	CLSX2754.b1_C17.ab1	5 : 915	BTEH	QGB28E23.yg.ab1	8 : 1074
AZVA	CLS_S3_Contig2539	2 : 1136	BRZR	QGB13D02.yg.ab1	5 : 1570	BTUF	QGC10O20.yg.ab1	8 : 1410
BHKL	CLS_S3_Contig7239	2 : 1667	BHAY	CLS_S3_Contig7016	5 : 1893	ASRF	CLSY1344.b1_P23.ab1	8 : 1680
ASZZ	CLSY2574.b1_K20.ab1	2 : 1708	BEVL	CLS_S3_Contig5635	5 : 2125	ASUI	CLSY1763.b1_E10.ab1	8 : 2505
AXWL	CLS_S3_Contig1329	2 : 1818	AHTB	CLSM11856.b1_P12.ab1	5 : 2899	BHCQ	CLS_S3_Contig7056	8 : 2775
BFPJ	CLS_S3_Contig6129	2 : 1952	AOVB	CLSS2687.b1_N24.ab1	5 : 2911	BUGI	QGC15F13.yg.ab1	9 : 459
BCOF	CLS_S3_Contig4235	2 : 2350	AWNO	CLS_S3_Contig10808	6 : 696			

Comparison of the Sal x Ice linkage map to other lettuce maps, to determine the linkage group orientation and marker order, suggests it is a good quality map. All the linkage groups with the exception of those with a single anchor marker (LG 3b, 6d and 9b) are known to be in the correct orientation. This is specifically due to 237 OPA and 4 SPP markers that mapped to the same linkage groups (and generally in the same order) as for the MCB19 JNR3 map under construction at The University of California, Davis. Additionally 2 Cos and 18 EST markers were mapped to the same linkage groups as for Map2 JMR3 (Truco *et al.* 2007).

The Sal x Ice map also has 57 and 22 common AFLP markers (within 5 bp) respectively with lettuce linkage maps published by Jeuken *et al.* (2001) and Syed *et al.* (2006) (by implication these will also be 'anchors' with the integrated map of Truco *et al.* 2007). Common markers for both published maps were present on linkage groups 2, 4, 5, 7 and 8 on the Saladin x Iceberg map (see Appendix B).

The morphological marker for seed colour ' w' on the Sal x Ice map correlates with the mapped position for brown seed 'br' on linkage group 7 as published by Waycott *et al.* (1999). OPA marker BKVX is of high importance as it co-locates with the marker 'w'. Interestingly Waycott *et al.* (1999) also recorded co-segregation of the loci determining plump involucres 'pl' with that for brown seed 'br'.

The large number of common markers with other maps allows accurate cross referencing between maps and thus provides the opportunity to utilise larger number of assigned markers and integration between maps of different populations. This map contains a variety of molecular markers having anchor points with many other maps; it is therefore a valuable resource for lettuce crop improvement.

The map produced in this study is based on an intraspecific cross between lettuce cultivars. This can result in the reduction of polymorphism as the genotypic variation within the progeny has been established in a narrow genetic background. Thus most linkage maps are generally based on interspecific crosses as they contain a wide range of genetic variation; however linkage maps based on these types of crosses may have limitations for breeders.

In contrast, maps generated from intraspecific crosses have greater utility for lettuce breeders and can be used directly for breeding purposes as they contain informative markers within the cultivated gene pool (Kesseli *et al.* 1994; Truco *at el.,* 2007). Self pollinated species such as lettuce have limited polymorphism within the cultivated species (Truco *et al.* 2007); therefore the development of comprehensive maps from intraspecific crosses is frequently limited. Although, interspecific crosses exhibit higher levels of polymorphism, segregation distortion is often more apparent. Moreover lower recombination frequencies or segregation distortion towards the cultivated allele could limit the access to the wild allele in the genome in interspecific crosses (Truco *et al.* 2007).

Genetic maps developed using a single population can be of limited use for other populations if the markers are not polymorphic in those populations. Integrated maps combining information from several populations can increase the amount of markers located within a genomic region and thus increase the chance of obtaining polymorphic markers in a desired population (Truco *at el.,* 2007). Due to the limitations observed with intraspecific crosses the most suitable and informative alternative could be the production of integrated maps of many populations with both common and specific markers (Jeuken *et al.* 2001; Truco *at el.,* 2007). Integrated

maps have been developed for several crop species in addition to lettuce including melon (Périn *et al.* 2002) and tomato (Haanstra *et al.* 1999).

The Sal x Ice map has common markers for each linkage group with 'Map2 JMR3' and/or map 'MCB19 10NR', both developed at The University of California, Davis and this allows integration with maps based on different populations. A linkage map based on the F_2 and F_5 Sal x Ice RIL population was produced previously at Warwick HRI, The University of Warwick, and by rescoring of common AFLP primer combinations with common panels this could be integrated with the F_7 map to provide more information on the mapping population as a whole.

Some segregation distortion from the Mendelian ratio was expected as although individuals for the RIL population were not consciously selected for any particular traits, there could have been inadvertent selection for traits associated with seed production due to the single seed descent (SSD) protocol used to generate the RILs. Some RILs were naturally lost while others were excluded due to inadequate seed production through re-generation from an F_6 to an F_7 population. The linkage maps based on the earlier F_2 and F_5 Saladin x Iceberg generations could be compared with the F_7 map to test whether segregation distorted has occurred in this population during SSD.

In this study all linkage groups with the exception of LG 6, exhibited segregation distortion, with 27% of the loci skewed. Of that, distortion favouring the Saladin alleles accounted for ~34% compared to 57% for the Iceberg allele. The distorted loci favoured one parental allele in 6 out of 8 linkage groups (LGs 1, 5, 7, 8 and 9). While 2 linkage groups (LG 1 and LG 9) were completely skewed to a single parental allele. The clustering of distorted loci suggested that the distortions were

genuine and unlikely to be due to miss scoring of loci which would be expected to be more randomly distributed.

Segregation distortion at 19% of loci was observed in the genetic map constructed by Syed *et al.* (2006). Distorted loci were observed on all linkage groups with the exception of LG 6 as also recorded in this study. Unlike the current study the segregation distortion was evenly distributed between the 2 parental genotypes. However similarly to the Sal x Ice F_7 map, the map generated by Syed *et al.* (2006) revealed a high number of distorted loci in the lower part of LG 4.

For the high density integrated lettuce map (Truco *et al.* 2007), the majority of markers segregated close to the Mendelian ratio for intraspecific crosses but a high proportion of distorted loci were recorded in the interspecific crosses. Two types of distortion were also observed; distortion of multiple linkage groups within a specific population and the distortion of specific regions in multiple populations (Truco *et al.* 2007). Genes of interest can be located in significantly distorted genomic regions which has been observed in this study on LG 7 around the markers for seed colour 'w' which is located within a significantly distorted region spanning 4.4 cM (Truco *et al.* 2007).

It has been reported that intraspecific crosses have a higher level of distortion in comparison to interspecific crosses, which was observed during production of the high density lettuce map and for other crops including *Brassica rapa* (Jenczewski *et al.* 1997; Suwabe *et al.* 2002; Truco *et al.* 2007). It has been postulated that the degree of distortion observed within a mapping population should correlate with the degree of genomic divergence observed within the parents (Kesseli *et al.* 1994; Taylor and Ingvarsson 2003).

The genetic linkage map constructed in this study is of good quality and can be cross referenced with many published maps (Waycott *et al.* 1999; Jeuken *et al.* 2001; Syed *et al.* 2006; Truco *et al.* 2007). However map coverage could be improved by the additional screening of the mapping population with more markers and/or by filling the 'unknown' data points within the current marker set. Individually or in conjunction these actions may 'pull in' all the unmapped assigned loci and may additionally allow the assignment of the remaining AFLP markers. The advantage of the map presented here is that it is based on a cross from within a crop. Any polymorphic markers identified as being linked to agronomically important traits are likely to be of direct value to lettuce breeders and could be used for marker assisted selection/plant breeding and further genetic studies.

4.5. Conclusions

- A good quality linkage map covering all 9 lettuce linkage groups has been generated based on the cross Saladin x Iceberg; the quality of the map makes it suitable for QTL analysis.

CHAPTER 5

Understanding genetic variation for post harvest discolouration in a selected RIL population

5.1. Introduction

From the preliminary experiments involving the parents of the Warwick HRI mapping population (Chapter 3) it was shown that Saladin and Iceberg were significantly different for post harvest discolouration traits. QTL analysis been used to understand the genetic variation and control of such complex traits via linkage analysis between markers and phenotypic observations (Cichy *et al.* 2009). Marker assisted selection based on QTL analysis can increase the heritability of a desired trait. Although advances have been made using QTL mapping to improve several important crop agronomic traits (Xing *et al.* 2002; Kelly *et al.* 2003), QTL studies into lettuce and in particular post harvest traits has been minimal (Zhang *et al.* 2007).

The aims of this experiment were to

- Demonstrate genetic variation for post harvest discolouration in the F_7 Saladin x Iceberg mapping population.
- Identification of significant QTL for post harvest discolouration traits.
- Demonstrate stability over environments of extreme phenotypes for post harvest discolouration in the F_7 Saladin x Iceberg mapping population.

5.2. Materials and methods

5.2.1. Saladin x Iceberg RIL population

Ninety-four highly informative lines (based on recombination events) from the Saladin x Iceberg F_7 population of recombinant inbred lines (RILs) were selected for the 2008 field trials (see section 2.1.2.).

5.2.2. Extreme discolouration RIL subset

Eleven RILs were selected from the 94 F7 Sal x Ice RILs based on their extreme phenotypes (observed in the UK and NL for the field trial of selected mapping population) for extreme post harvest discolouration (see table 5.1.) and on their genotypes at the QTL identified as determining post harvest discolouration in the initial study (see section 5.3.3). These traits were distinct in lines or present in combinations. The selected lines displayed a variety of morphologies (see figure 5.1.).

Table 5.1. Saladin x Iceberg F_7 RILs with extreme phenotypic post harvest discolouration selected for 2009 experimental trial. Numbers refer to seed numbers as stored in the Warwick HRI Genetic Resource Unit (all seed numbers have the prefix LJ). Where *No* (number).

Line No	Extreme phenotype	Line No	Extreme phenotype
5023	Low pinking	5043	High browning
5051	Low pinking	5053	High browning
5045	High pinking	5002	Low discolouration
5075	High pinking	5042	Low discolouration
5022	Low browning	5066	High discolouration
5055	Low browning		

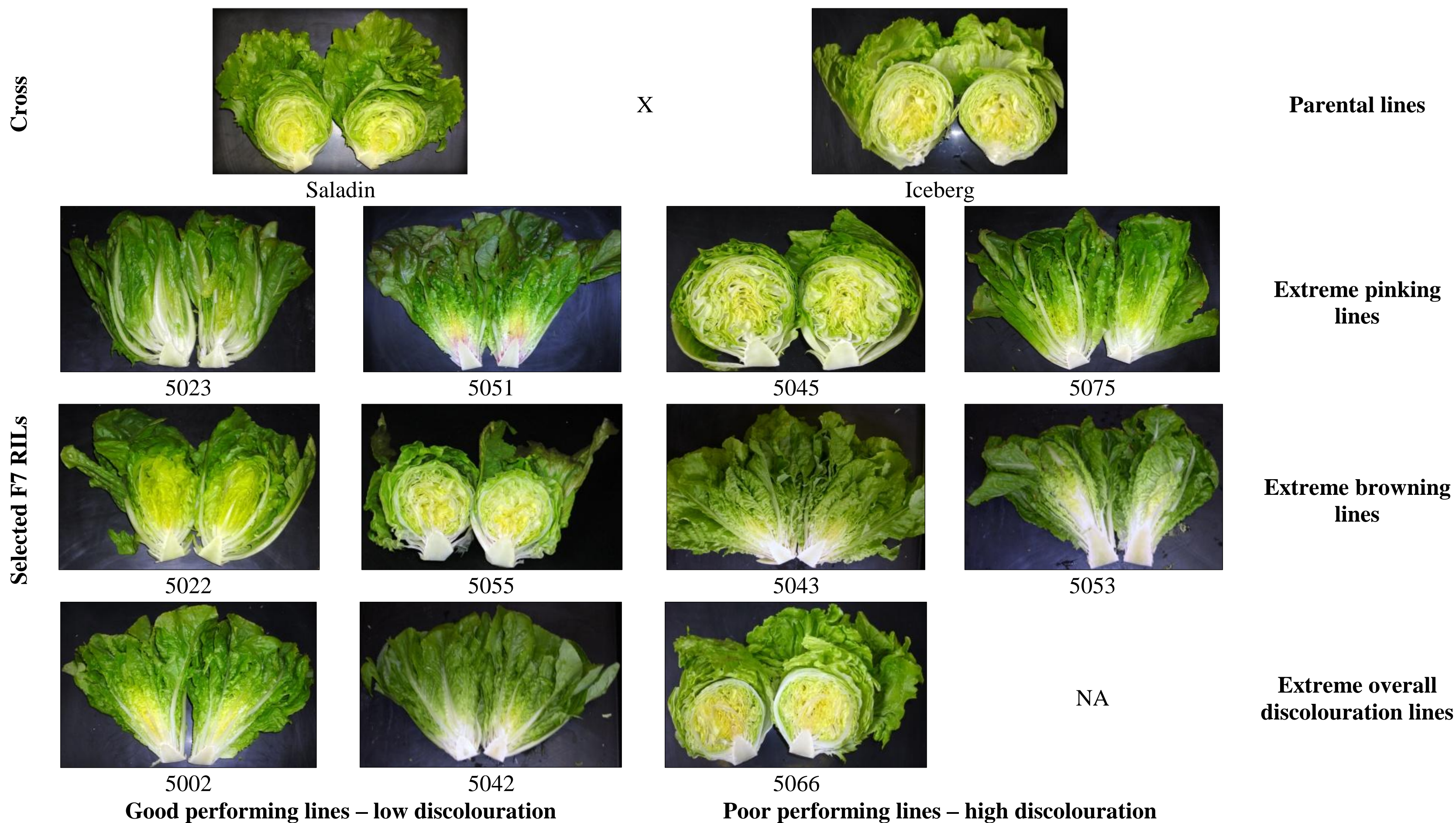


Figure 5.1. Saladin x Iceberg F_7 RILs with both extreme genotypic and phenotypic post harvest discolouration harvested during the 2009 experimental trial. Numbers refer to seed numbers as stored in the Warwick HRI Genetic Resource Unit (all seed numbers have the prefix LJ).

5.2.3. Field trial and assessment of post harvest discolouration of the F_7 Saladin x Iceberg mapping population

The 94 most genetically informative RILs from the F_7 mapping population and the mapping parents were grown in the UK and The Netherlands in 2008. The UK site was the Big Cherry experimental field at Warwick HRI, Wellesbourne (Latitude: 52.183. Longitude: 1.583) while the Netherlands site was the experimental fields at Rijk Zwaan breeding station, Fijnaart (Latitude: 51.633. Longitude: 4.467), (see figure 5.2.).

Figure 5.2. Map displaying field trial sites for 2007, 2008 and 2009. Topography map created using Google maps (http://maps.google.com/). Warwick 2007/8/9 and Fijnaart 2008.

Plants for the UK assessment of post harvest discolouration were grown in a replicated field trial, with the two replicates separated with 6 weeks between planting dates. Plants were raised and maintained as previously described (see section 2.2.1.), with the trial planted on 28th April and 9th June 2009. The trial replicates were designed in 4 blocks, with each containing a single plot (of 12 plants) for each of the

96 lines (94 F_7 RILs and the 2 mapping parents), randomised in a column/row design so all treatments occurred equally in each row and column (see appendix C for field plan and randomisation). Crop protection was as previously described, with the additional use of Quavor flo' and 'Roviral' (1.5 L/ha) and slug pellets according to good agricultural practice. Fencing and netting also surrounded and covered the trial plots to provide protection from the local fauna (see figure 5.3.a.). Harvests occurred on 24th June, 26th June, 1st July, 8th July, 15th July, 5th August, 12th August and 20th August 2008 when >50% of heads reached maturity level for harvest.

Plants for the NL assessment of post harvest discolouration were grown in a replicated field trial, with the two replicates run concurrently. The trial was designed in 2 blocks, with each containing a single plot of each of the 96 lines (94 F_7 RILs and the 2 mapping parents), again randomised in a column/row design (see appendix C for field plan and randomisation). Plants grown in The Netherlands were grown under Rijk Zwaan growing, transplanting and maintenance procedures (see figure 5.3.b.). The plant arrangement was as for the associated UK trial (see section 2.2.1.), with 30 x 36 cm spacing between plants. Harvests occurred on 15th September, 17th September, 22nd September and 24th September 2008 in numerical plot order following the standard procedure for Rijk Zwaan breeding trials.

Figure 5.3. Field sites a) UK and b) NL of the selected mapping population trials in 2008.

Heads were harvested and processed as previously described (see section 2.2.2.). Approximately ~100g unwashed mixed material was sealed per bag with material from one head filling two bags. Bags were stored vertically at 5° for storage (see figure 2.3.). Bags were then phenotypically assessed for post harvest discolouration as described (see section 2.2.3.) on days 1 and 3. The 'visible' score classification was removed from the scoring protocol, with tissue discolouration classified as either clean, pink or brown. In the event of no classification the tissue was kept and discolouration back dated.

5.2.4. Field trial of extreme discolouration lines

Plants of the subset of RILs for assessment of post harvest discolouration were grown in a replicated field trial during the 2009 growing season on the experimental site Pump Ground at Warwick HRI, UK (Latitude: 52.183. Longitude: 1.583). After germination plants were raised and maintained as previously described (see section 2.2.1.), with the trial planted on 22nd April 2009. The trial was designed in 3 blocks each containing 2 plots of each of the extreme RILs and Saladin and Iceberg, randomised in a column/row design so all treatments occurred equally in each row and column (see appendix C for field plan and randomisation). Crop protection was as described, with the additional use of Quavor flo' and 'Roviral' (1.5 L/ha). Fencing and flappers also surrounded the land to provide protection from the local fauna (see figure 5.4.). Harvests occurred on 27th and 28th July 2009 with RILs being harvested in numerical plot order.

Figure 5.4. Lettuce RILs with extreme discolouration genotypes at Warwick HRI, UK in 2009.

Heads were harvested and processed as described (see section 2.2.2.). Approximately ~100g unwashed mixed material was sealed per bag with material from one head filling two bags. Bags were stored vertically at 5° for storage (see figure 2.3.). Bagged leaves were then phenotypically assessed for post harvest discolouration as previously described (see section 2.2.3.) with the amendments as for the 94 RILs described above on days 1, 2, 3 and 4.

5.2.5. QTL analysis

The phenotypic data from the 94 most informative RILs were combined with their genotype data and used for QTL analysis as previously described (see section 2.2.3.). All quantitative traits measured used adjusted means from REML.

5.2.6. Statistical analysis

REML analysis

Trial data for the 94 RILs were analysed for each site singly and across sites. As the data exhibited a variable mean relationship it was transformed before analysis. For

the intensity mean score browning and pinking scores the data were multiplied by 50 and for mean score overall discolouration scores they were multiplied by 25. This allowed data to be compared more easily between traits as they were all transformed to a 100 point scale. The percentage scores of extent of pinking, browning and overall discolouration were transformed to angles (by multiplying by 90°) prior to analysis. As the field trial produced an unbalanced data set (due to missing values) it was analysed by REML. For the UK trial data the fixed treatment was block/rep/plot/head/bag for days 1 and 3, and day/plot/treat/rep/head/bag for across days. For the NL trial data the fixed treatment was rep/block/plot/head/bag for days 1 and 3, and day/treat/rep/head/bag for across days. For the trial data across sites the fixed treatment were weighted for each site for days 1 and 3. Scores were adjusted through the REML analysis to take account of possible block effects; in some cases this resulted in negative values for the estimated means (a similar situation occurred with some missing values).

The data from the trial re-assessing the extreme discolouration lines also exhibited a variable mean relationship; it was therefore transformed before analysis. Angular transformation was used for percentage scores, while for mean score browning and pinking scores the data were transformed to a 100 point scale by multiplication by 50 or 25 as described above. As the field trial produced an unbalanced data set, it was also analysed by REML with the fixed treatment rep/bed/plot/head/bag for days 1, 2, 3 and 4. Scores were adjusted through the REML analysis to adjust for block effects again this resulted in negative values for some of the estimated means or missing values.

Correlation analysis

Correlation analyses were conducted between all measures of post harvest discolouration for 94 RILs (as section 2.4.4.). R values were generated which were then compared to the R values expected for different levels of probability to assess their significance (see table 5.2.).

Table 5.2. Correlation analysis parameters. Where *df* (degrees of freedom).

		Probability (p value)		
Population	**df**	**0.05**	**0.01**	**0.001**
F_7 Saladin x Iceberg RIL mapping population	70	0.205	0.267	0.381

5.3. Results

5.3.1. Variation for discolouration within the RIL mapping population

REML analysis of the data sets for each day showed that RILs from the mapping population were significantly different (*$P \leq 0.05$) for all measures of discolouration, however some measures were dependent on site.

When meaned across all the RILs; the levels of pinking and browning measured by ‘intensity and extent’ were similar on day 1. However, by day 3 there was significantly less browning in the population than pinking.

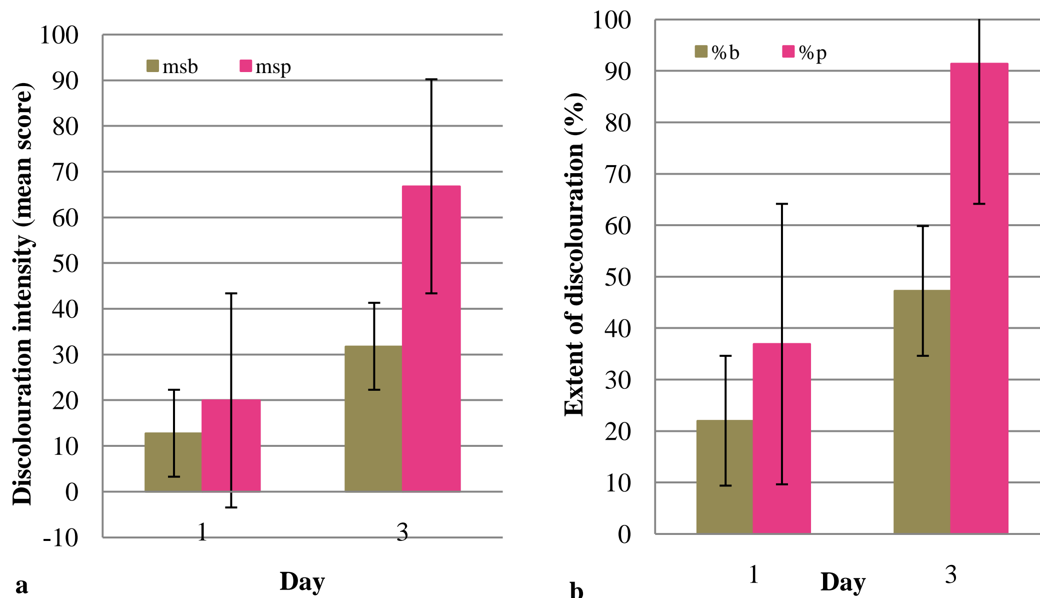


Figure 5.5. Means for a) intensity and b) extent of post harvest pinking and browning over 3 days across all RILs from the mapping population. Error bars represent se (standard error) from means. Where *msb* (mean score browning); *msp* (mean score pinking); %*b* (percentage browning); *%p* (percentage pinking).

Post harvest pinking of prepacked leaf tissue

Although the trials carried out in the UK and NL showed different levels of pinking (both intensity and extent) there were consistent effects over both trials. Generally on day 1 the parents were significantly different from each other (see table 5.3.) and distributed towards the extremes of the distribution of the RILs. But by day 3 they had converged towards the middle of the distribution and were generally not significantly different. However, even when the differences between the parents were not significant Iceberg was always the poorer performing parent. Generally lower scores were recorded for intensity than extent in both trials for each day.

The REML analysis showed significant differences (**P <0.01) between the 94 RILs for intensity for day 1 in both the UK and NL trials and for day 3 in the NL trial and when analysed across both sites. Significant differences (**P <0.01) were also

recorded between RILs for extent for both days in both trials. The REML analyses therefore indicated the existence of genetic variation for pinking.

Table 5.3. Differences between parents Saladin and Iceberg for post harvest pinking. Where *S* (significant differences); *NS* (not significant differences).

	Pinking intensity		**Extent of pinking**	
Site	Day 1	Day 3	Day 1	Day 3
UK	S	NS	S	NS
NL	NS	S	NS	NS
Across sites	S	S	S	NS

There was also a highly significant site effect for both intensity and extent on day 1 indicating that the plants' growing environment significantly influences their post harvest performance (see table 5.4. a and b). Day was also a highly significant (***P <0.001) factor for both measures of pinking in both trials this was due to a significant increase in mean levels of pinking in the RILs over time (see figure 5.6. (a-d) and figure 5.7. (a-d)). There was also a significant interaction between day and genotype for intensity and extent indicating differences between the RILs in the rate of increase in pinking over time (see Appendix C).

All the RILs showed pink discolouration by day 1 in both trials as measured by intensity and extent (see figure 5.6. (a-d) and figure 5.7. (a-d)). As stated above pinking increased at different rates for different RILs and rank orders changed over days; the changes in rank orders also differed between trials again indicating that the growing conditions of the plants influences their post harvest performance.

Table 5.4. REML significance level of pinking a) intensity and b) extent for the RIL mapping population in both the UK and NL. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *ndf* (numerator degrees of freedom); *ddf* (denominator degrees of freedom): *F pr* (probability); *Line* (genotype/RIL); *x* (interaction).

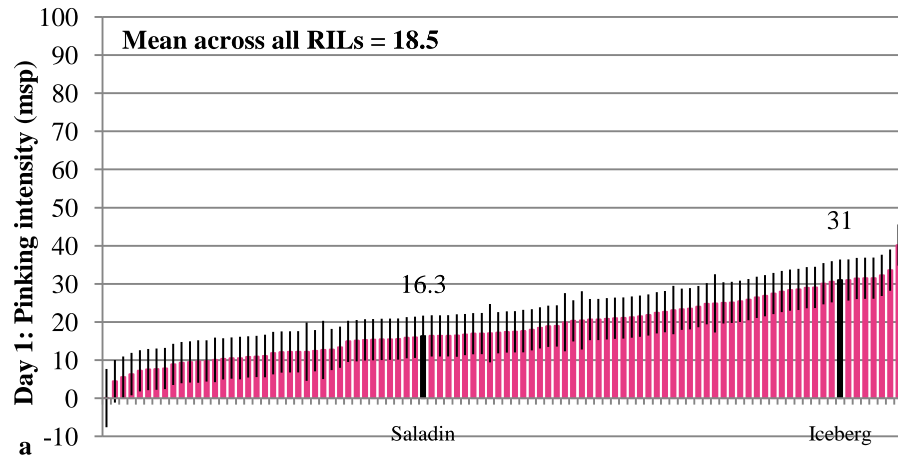
a

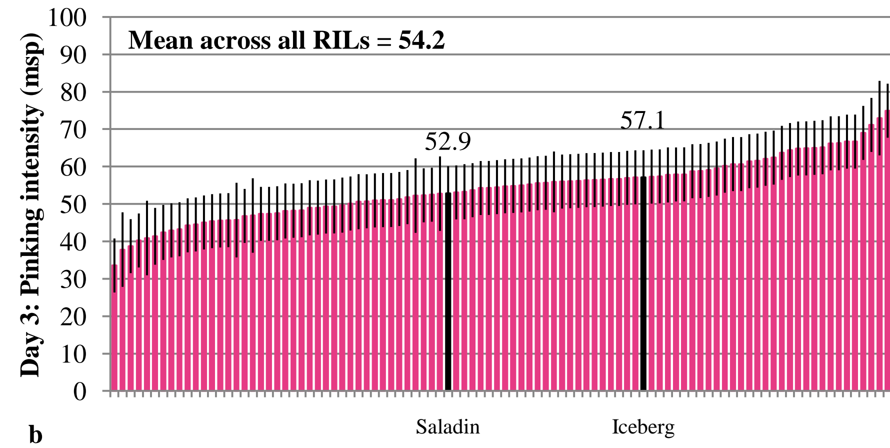
Site	Day	Fixed term	Wald statistic	ndf	F statistic	ddf	F pr
UK	1	Line	198.19	95	2.11	34.5	**0.007
	3	Line	121.82	95	1.29	37.2	0.194
NL	1	Line	209.04	95	2.2	66.9	***<0.001
	3	Line	179.94	95	1.91	53.5	**0.005
Across sites	1	Site	29.43	1	29.43	22.5	***<0.001
	1	Line	294.91	95	3.1	107.1	***<0.001
	1	Site x Line	112.32	95	1.18	112.3	0.199
	3	Site	1.59	1	1.59	1.4	0.378
	3	Line	201.1	95	2.11	117.5	***<0.001
	3	Site x Line	100.65	95	1.05	112	0.395

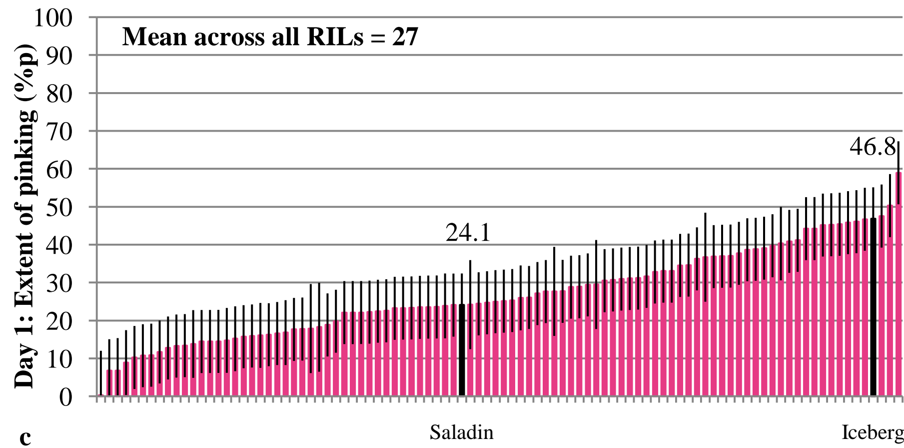
b

Site	Day	Fixed term	Wald statistic	ndf	F statistic	ddf	F pr
UK	1	Line	186.29	95	2.11	34.9	**0.01
	3	Line	132.4	95	1.29	1.39	**0.007
NL	1	Line	212.13	95	2.2	67.3	***<0.001
	3	Line	153.54	95	1.91	94.8	**0.01
Across sites	1	Site	31.2	1	29.43	22.1	***<0.001
	1	Line	288.47	95	3.1	107.1	***<0.001
	1	Site x Line	109.95	95	1.18	112.9	0.23
	3	Site	17.68	1	1.59	1.4	0.094
	3	Line	185.24	95	2.11	163	***<0.001
	3	Site x Line	100.7	95	1.05	153.6	0.37

The range of variation seen in the RILs for intensity in both trials on each day was similar (~40). Similarities were also observed for the extreme values of the distribution. The range of variation for extent in the RILs on both trials for day 1 was also similar (~55). However by day 3 the range had decreased (particularly in the NL trial) as the majority of the RILs were reaching the possible maximum score for discolouration (see figure 5.6. b and d and figure 5.7. b and d). Some RILs significantly outperformed the better parent (Saladin) for pinking although no one RIL consistently outperformed Saladin on all days and at both sites (see table 5.5.).







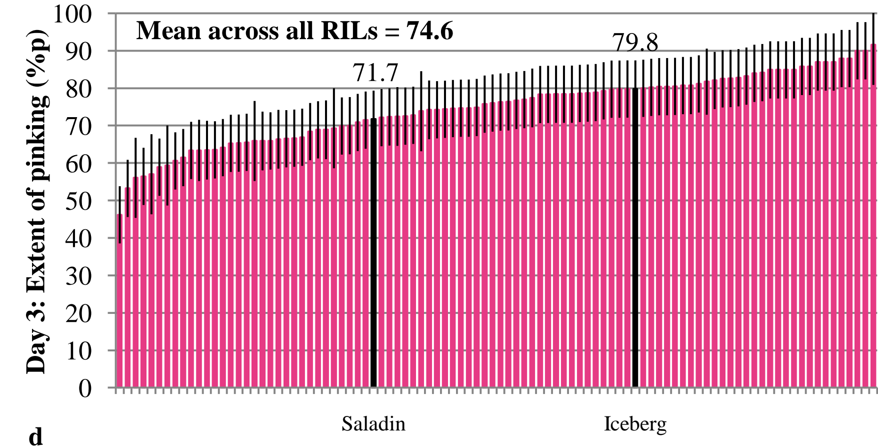
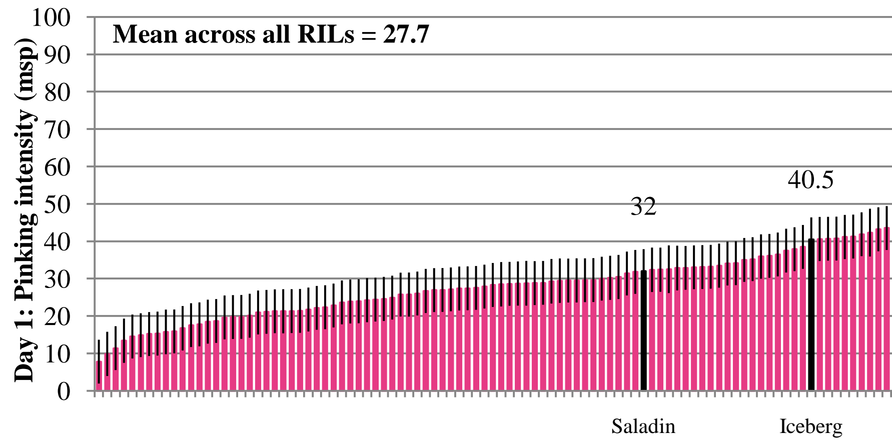
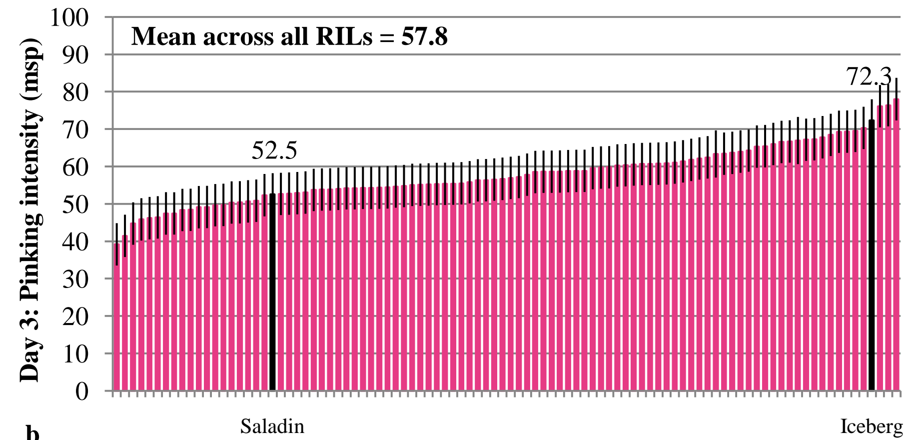
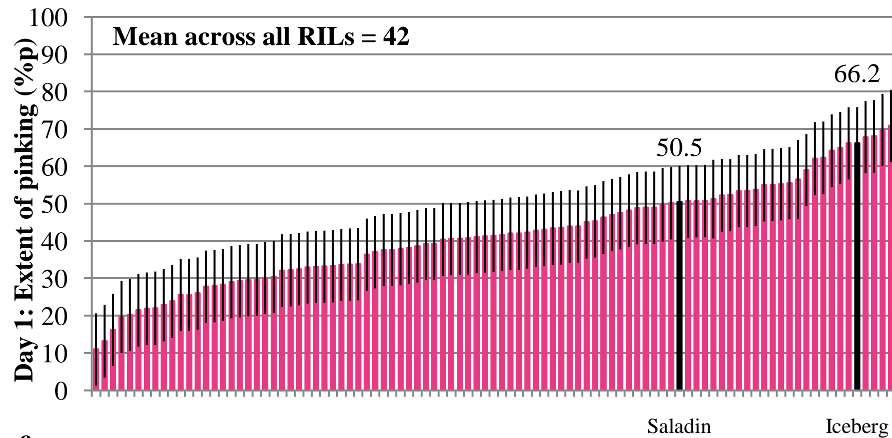


Figure 5.6. Transformed adjusted means (from REML) for lettuce post harvest pinking intensity from the UK trial on a) day 1 and b) day 3 and extent of pinking on c) day 1 and d) day 3 for the F_7 mapping population. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI population parents are highlighted with respective adjusted means (from REML).







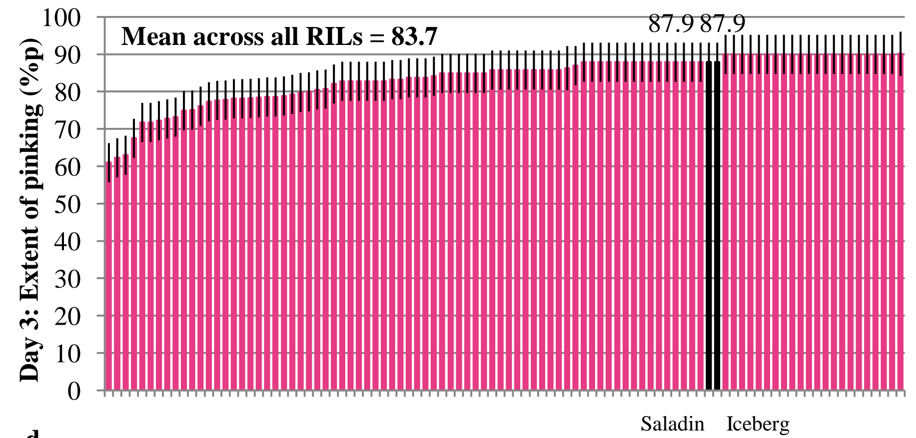


Figure 5.7. Transformed adjusted means (from REML) for lettuce post harvest pinking intensity from the NL trial on a) day 1 and b) day 3 and extent of pinking on c) day 1 and d) day 3 for the F_7 mapping population. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI population parents are highlighted with respective adjusted means (from REML).

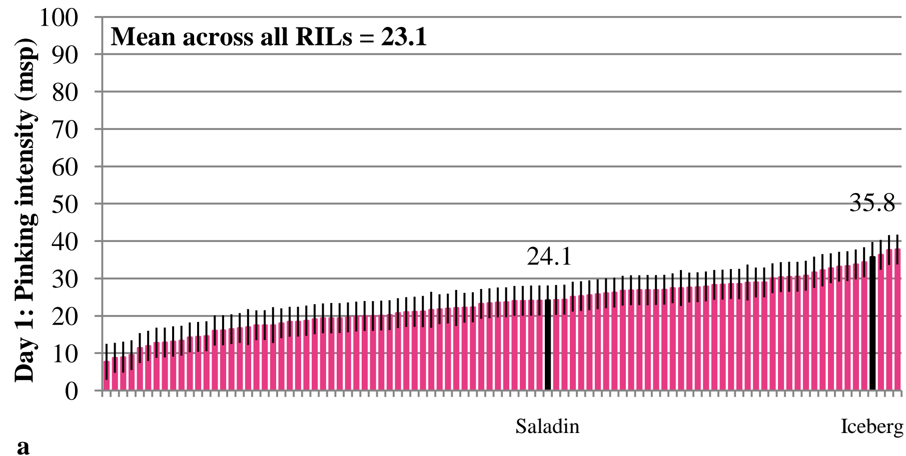
Table 5.5. RILs performing significantly better than the best parent Saladin for post harvest pinking. Where ✓ (the line is performing significantly better than Saladin); × (the line is not performing significantly better than Saladin).

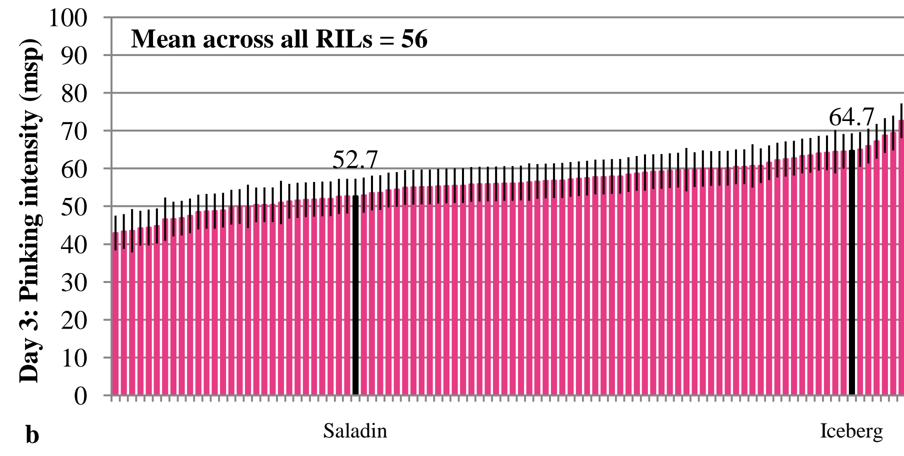
	UK trial				NL trial				Across site analysis			
	Pinking intensity		Extent of pinking		Pinking intensity		Extent of pinking		Pinking intensity		Extent of pinking	
RIL \ Day	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**
5002	×	×	×	×	×	×	×	✓	×	×	×	✓
5010	×	×	×	×	✓	×	✓	×	×	×	×	×
5017	×	×	×	×	✓	×	✓	×	✓	×	✓	×
5019	×	×	×	×	×	×	×	✓	×	×	×	×
5022	×	×	×	×	×	×	×	×	×	×	✓	×
5023	✓	×	×	×	×	×	×	✓	✓	✓	✓	✓
5024	×	×	×	×	✓	✓	✓	✓	✓	×	✓	✓
5031	×	×	×	×	×	×	✓	×	✓	×	✓	×
5032	×	×	×	×	×	×	✓	×	×	×	×	×
5035	×	×	×	×	✓	×	✓	✓	✓	×	✓	✓
5042	×	×	×	×	✓	×	✓	✓	×	×	×	✓
5051	×	×	×	×	×	✓	✓	✓	×	×	✓	✓
5056	✓	×	×	×	×	×	×	×	×	×	×	×
5057	×	×	×	×	✓	×	✓	×	×	×	×	×
5059	×	×	×	×	✓	×	✓	×	✓	×	✓	×
5063	×	✓	✓	✓	✓	×	✓	×	✓	✓	✓	✓
5066	×	×	×	×	×	×	×	✓	×	×	×	×
5068	×	×	×	×	×	×	×	✓	×	×	×	×
5071	×	×	×	×	✓	×	✓	✓	✓	×	✓	×
5072	×	×	×	×	✓	×	✓	×	✓	×	✓	×
5074	×	×	✓	×	×	×	×	×	×	×	×	×
5075	×	×	×	×	×	×	×	×	×	×	✓	×
5081	×	×	×	×	×	×	✓	✓	×	×	×	×
5082	×	×	×	×	×	×	✓	×	×	×	×	×
5085	×	×	×	×	✓	×	✓	×	✓	×	✓	×
5086	×	×	×	×	✓	×	✓	✓	✓	×	✓	✓
5095	×	×	×	×	×	×	✓	×	×	×	×	×
5098	×	×	×	×	×	×	×	×	×	×	×	✓
5106	×	×	×	×	✓	×	×	×	✓	×	✓	×
5110	×	×	×	×	×	×	✓	×	×	×	×	×
5121	×	×	×	×	✓	×	✓	×	✓	×	✓	×
5123	×	×	✓	✓	×	×	×	×	✓	×	✓	✓

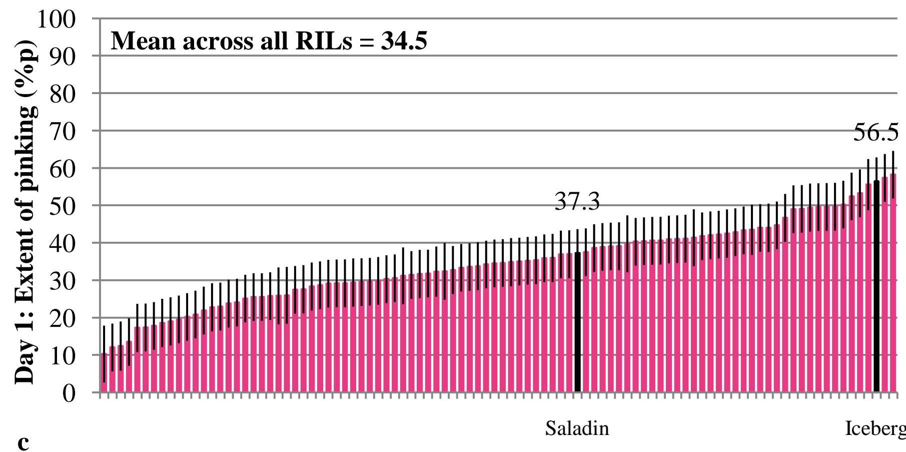
When the data from the two sites were combined in a weighted analysis the mapping parents were shown to be significantly different for intensity on both days but only on day 1 for extent (see table 5.3.). For intensity the lowest extreme scores were always higher than for either trial individually while the highest extreme scores were always lower than that recorded for the individual trials for each day. The range for

intensity of the RILs was therefore slightly lower than for the individual trials for each day. The parental scores on day 1 for intensity were an average across the two sites. However on day 3, while the score for Saladin was similar to that for both trials, the Iceberg score was an average across sites (see figure 5.8. a and b). For extent the lowest extreme scores were similar to that for the NL trial on day 1 but higher than both trials on day 3. The highest extreme scores were an average of both trials for day 1 but were equal to both trials on day 3. The range for extent of the RILs was an average for both trials for day 1 but it was less than both trials for day 3 as RILs were reaching the possible maximum score for discolouration. The parental scores on both days were an average across the two sites (see figure 5.8. c and d). The means of all RILs for pinking across sites were averages of both trials. As recorded for both trials, some RILs had significantly outperformed the better parent (Saladin) for pinking (see table 5.5.), although no one RIL consistently outperformed Saladin.

RILs 5002, 5022, 5023, 5042, 5043, 5051, 5066 and 5075 were also selected for inclusion in the 'extreme discolouration subset' for later experiments to examine stability of phenotypes over environments/years (as they showed consistent transgressive segregation) (see section 5.3.4.).







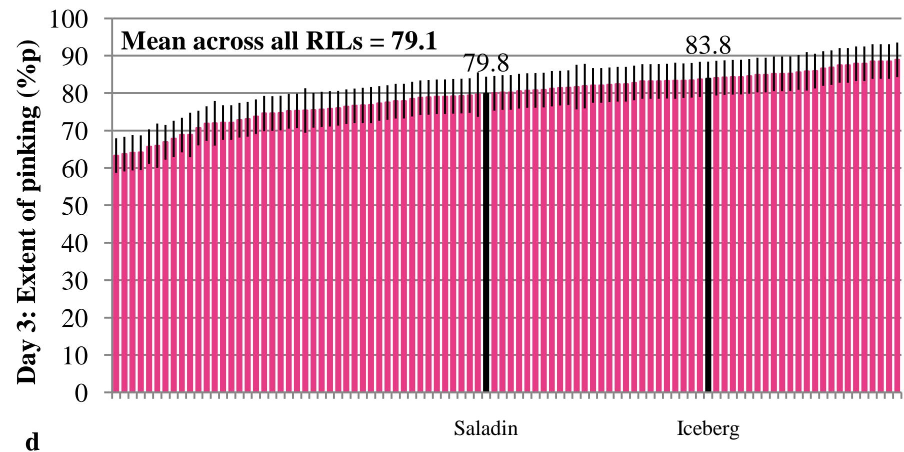


Figure 5.8. Transformed adjusted means (from REML) for lettuce post harvest pinking intensity across sties on a) day 1 and b) day 3 and extent of pinking on c) day 1 and d) day 3 for the F_7 mapping population. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI population parents are highlighted with respective adjusted means (from REML).

Post harvest browning of prepacked leaf tissue

The trials carried out in the UK and NL showed different levels of both intensity and extent of browning but as with pinking some effects were consistent over both trials. Generally for the UK trial the parents were not significantly different (see table 5.6.); and were both positioned at the middle of the distribution of the scores for the RILS on day 1. For the NL trial the parents were generally significantly different on day 1 (see table 5.6.); distributed more towards the extremes of the RIL distribution. Although the differences between parents were not always significant; as with pinking Iceberg was the poorer performing parent at all times. The scores for browning intensity were generally lower than the scores for extent of browning in both trials for each day.

Table 5.6. Differences between parents Saladin and Iceberg for post harvest browning. Where *S* (significant differences); *NS* (not significant differences).

	Browning intensity		**Extent of browning**	
Site	Day 1	Day 3	Day 1	Day 3
UK	NS	NS	NS	NS
NL	S	NS	S	NS
Across sites	NS	NS	NS	NS

The REML analysis did not show significant differences between RILs for intensity, however significant differences were recorded for extent for day 3 in the NL trial and when analysed across both sites. Therefore, although there was less conclusive evidence for genetic variation for browning than for pinking nevertheless the REML analyses did indicate genetic variation for browning.

There was also a highly significant site effect for browning (extent only) on day 3, again indicating that the growing environment of the plants influences their post

harvest performance (see table 5.7. a and b). Day was also a highly significant (***P <0.001) factor for browning (both intensity and extent) in both trials again due to an increase in levels across the RILs over time (see figure 5.9. (a-d) and figure 5.10. (a-d)). As with pinking a significant interaction was observed between day and genotype for both measures of browning indicating differences between the RILs in the rate of increase of browning over time (see Appendix C).

Table 5.7. REML significance level of browning a) intensity and b) extent for the RIL mapping population in both the UK and NL. Significant effects shown as **P* <0.05, ***P* <0.01 and ****P* <0.001. Where *ndf* (numerator degrees of freedom); *ddf* (denominator degrees of freedom): *F pr* (probability); *Line* (genotype/RIL) ; *x* (interaction).

a

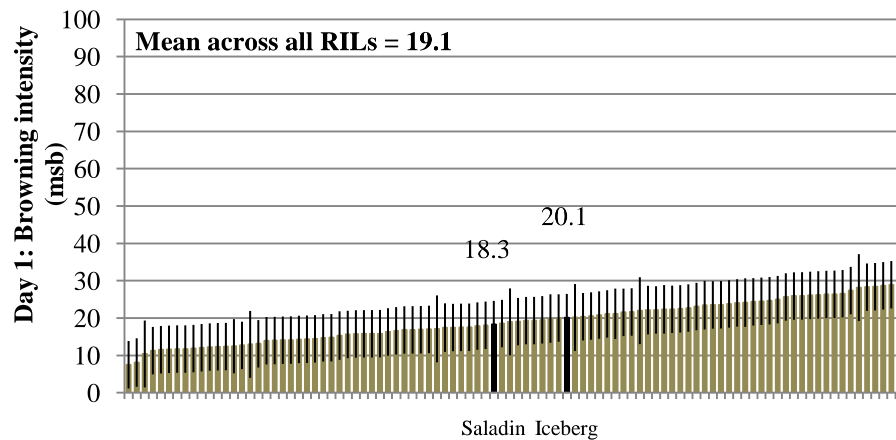
Site	Day	Fixed term	Wald statistic	ndf	F statistic	ddf	F pr
UK	1	Line	67.8	95	chi	0.71	0.984
	3	Line	100.39	95	1.06	87.6	0.397
NL	1	Line	119.23	95	1.26	60.4	0.166
	3	Line	132.53	95	1.4	58.7	0.081
Across sites	1	Site	2.66	1	2.66	1.9	0.249
	1	Line	105.28	95	1.11	119.9	0.295
	1	Site x Line	81.76	95	0.86	140.8	0.782
	3	Site	13.86	1	13.86	2	0.066
	3	Line	139.57	95	1.47	140	*0.019
	3	Site x Line	93.36	95	0.98	152.1	0.531

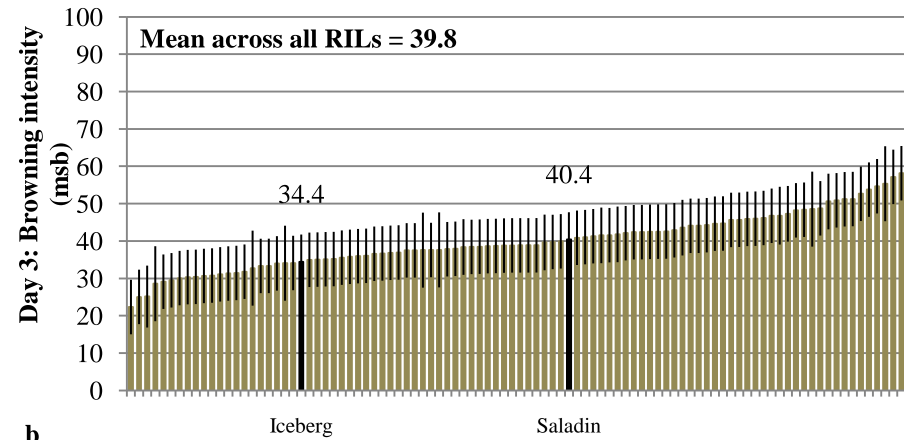
b

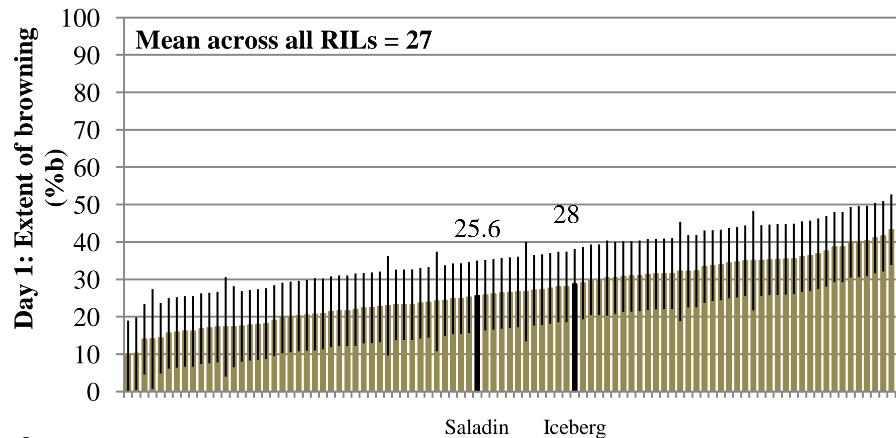
Site	Day	Fixed term	Wald statistic	ndf	F statistic	ddf	F pr
UK	1	Line	69.47	95	chi	0.73	0.977
	3	Line	86.13	95	0.91	87.6	0.681
NL	1	Line	116.22	95	1.23	59.8	0.195
	3	Line	139.96	95	1.48	58.5	*0.05
Across sites	1	Site	6.31	1	6.31	1.5	0.164
	1	Line	103.17	95	1.09	107	0.336
	1	Site x Line	82.51	95	0.87	130.8	0.765
	3	Site	20.79	1	20.79	2	*0.047
	3	Line	132.81	95	1.4	119.3	*0.041
	3	Site x Line	93.28	95	0.98	140.2	0.533

All of the RILs showed brown discolouration by day 1 for both trials as measured by intensity and extent, however, lower scores were recorded in the NL trial (see figure

5.9. (a-d) and figure 5.10. (a-d)). As indicated by the REML analysis, scores for browning increased at different rates for different RILs and by day 3 the rank order had changed from day 1. The changes in rank order differed between the trials agreeing with the finding from the REML analysis that growing environment significantly influences post harvest performance. The range of variation for all RILs for intensity in both trials for each day was similar (~23 for day 1 and ~33 for day 3). Some RILs performed significantly worse than the worse parent (Iceberg) for browning and although no one RIL consistently performed poorer than Iceberg (see table 5.8.), this did provide some evidence for transgressive segregation.







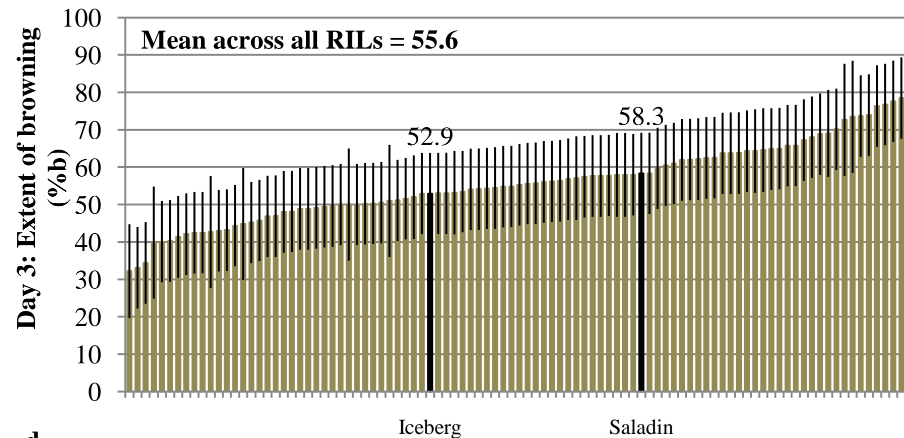
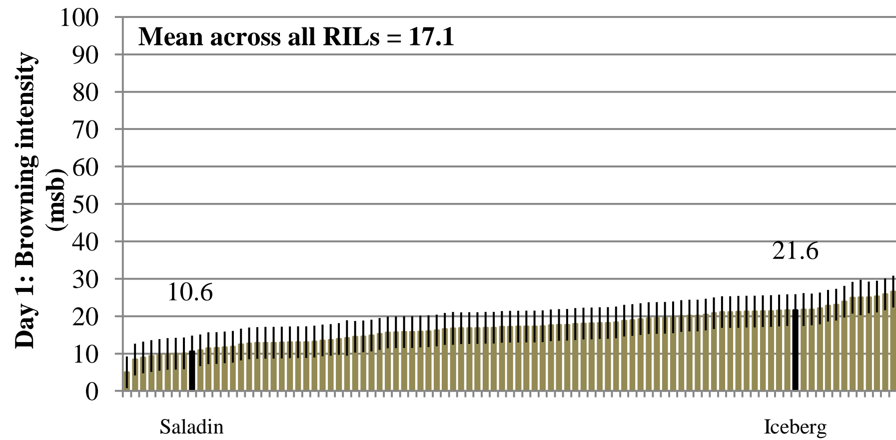
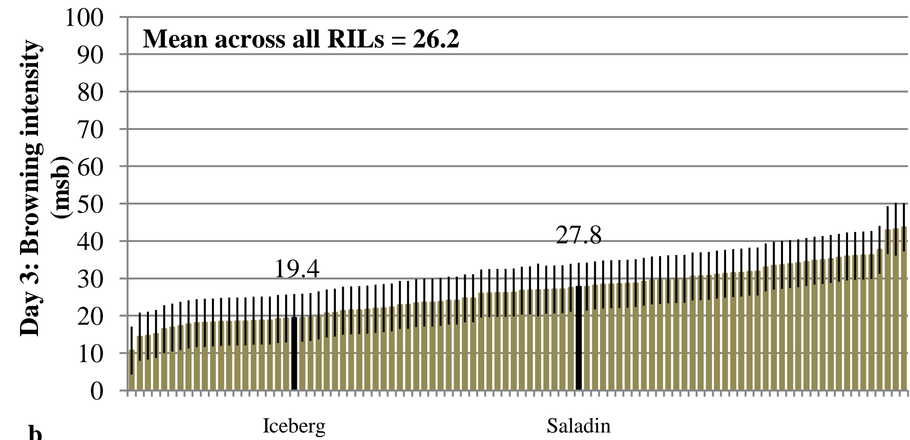
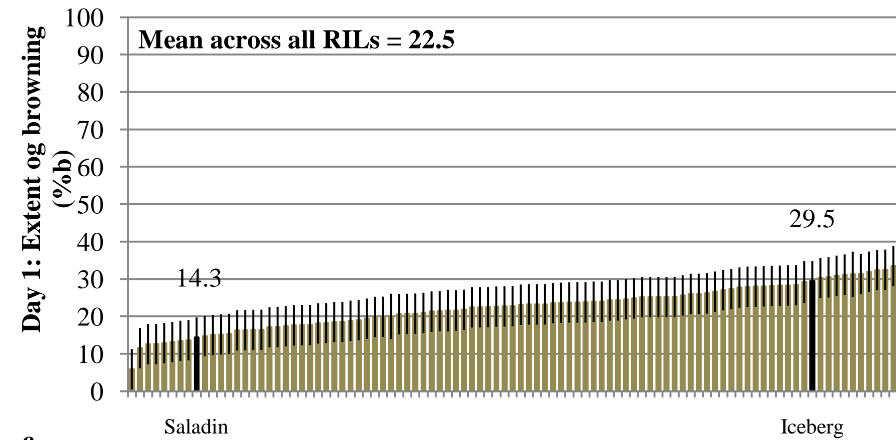


Figure 5.9. Transformed adjusted means (from REML) for lettuce post harvest browning intensity from the UK trial on a) day 1 and b) day 3 and extent of browning on c) day 1 and d) day 3 for the F_7 mapping population. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI population parents are highlighted with respective adjusted means (from REML).







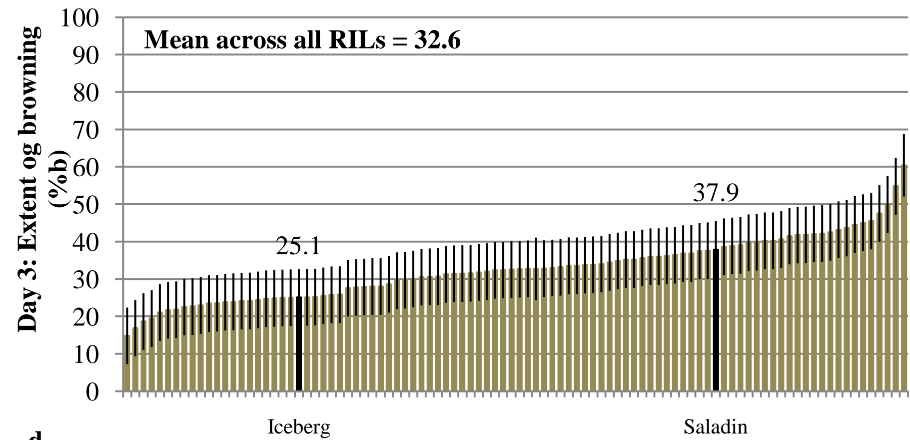


Figure 5.10. Transformed adjusted means (from REML) for lettuce post harvest browning intensity from the NL trial on a) day 1 and b) day 3 and extent of browning on c) day 1 and d) day 3 for the F_7 mapping population. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI population parents are highlighted with respective adjusted means (from REML).

Table 5.8. RILs performing significantly worse than the poor parent Iceberg for post harvest browning. Where ✓ (the line is performing significantly worse than Iceberg); × (the line is not performing significantly worse than Iceberg).

	UK				**NL**				**Across site**			
	Browning intensity		**Extent of browning**		**Browning intensity**		**Extent of browning**		**Browning intensity**		**Extent of browning**	
RIL \ Day	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**
5043	✓	×	×	×	×	×	×	×	×	×	×	×
5053	×	✓	×	×	×	✓	×	×	×	✓	×	✓
5096	×	✓	✓	×	×	×	×	×	×	×	×	×
5101	×	×	×	×	×	✓	×	✓	×	×	×	×
5102	×	×	×	×	×	✓	×	✓	×	×	×	×

When the data from the two sites were combined in a weighted analysis the mapping parents were not significantly different for browning (see table 5.6.). The range of browning for the RILs was lower than for either trial for each day. The lowest extreme scores for browning (both intensity and extent) were always higher than for each trial for day 1, but were similar to the UK trial for day 3. For intensity the highest extreme scores were similar to that for the UK trial on day 1 and were an average across sites for day 3. For extent the highest extreme scores were similar to that for the NL trial on both days. The parental scores for browning were averages of both trials (see figure 5.11. a - d). Again there was some evidence of transgressive segregation with some RILs having significantly worse scores compared to the worse parent (Iceberg) for browning (see table 5.8.). Although no one RIL always performed poorer than Iceberg, RIL 5053 performed the worst for most measures of browning for both trials and across sites.

RILs 5043 and 5053 were also selected for inclusion in the 'extreme discolouration subset' for later experiments (as they showed consistent transgressive segregation) (see section 5.3.4.).

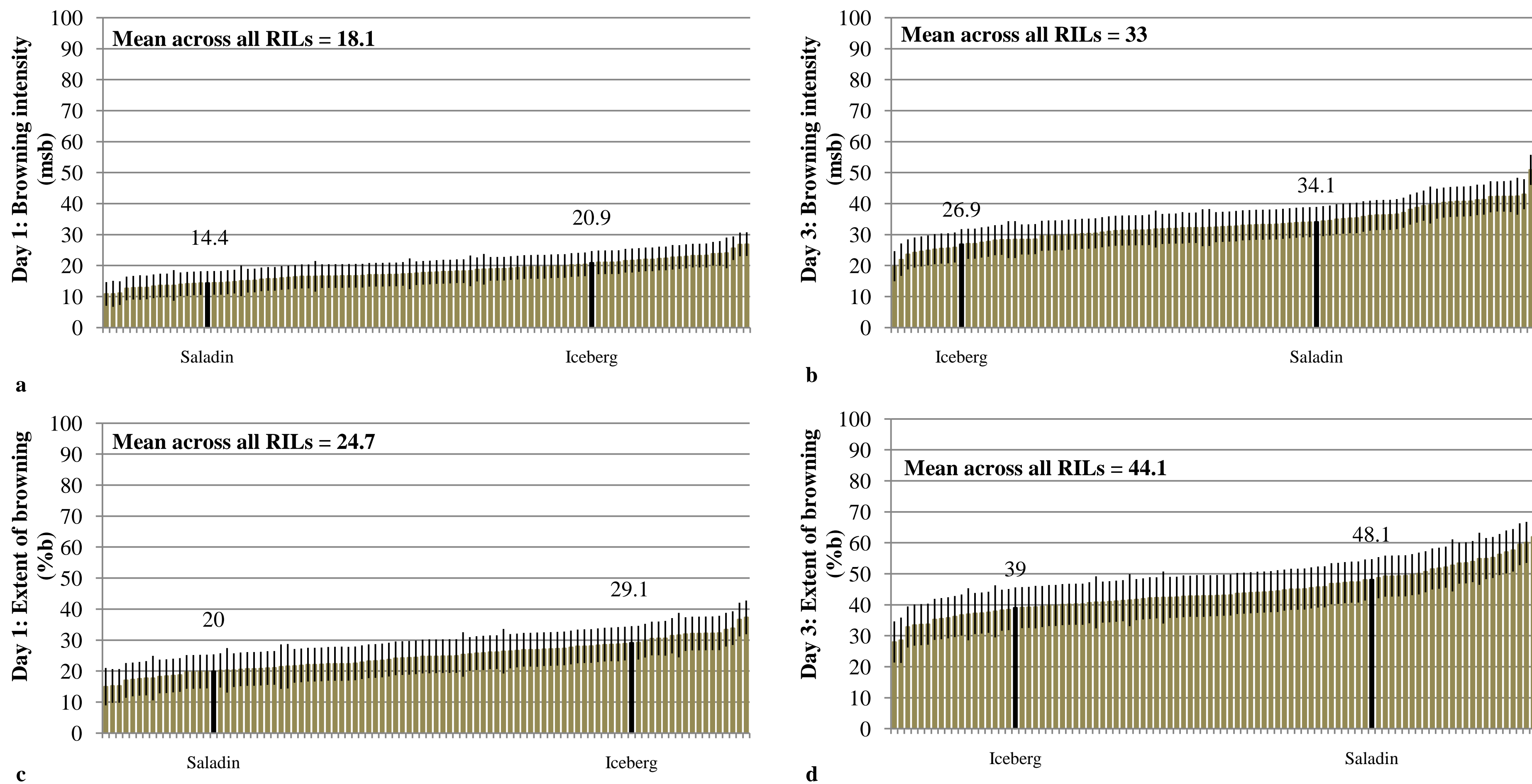


Figure 5.11. Transformed adjusted means (from REML) for lettuce post harvest browning intensity across sties on a) day 1 and b) day 3 and extent of browning on c) day 1 and d) day 3 for the F_7 mapping population. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI population parents are highlighted with respective adjusted means (from REML).

Post harvest overall discolouration of prepacked leaf tissue

Trials carried out in the UK and NL showed different levels of overall discolouration (both intensity and extent) but again some effects were consistent over both trials. Generally the mapping parents were not significantly different on either day (table 5.9.). Although on day 1 they were distributed towards the extreme of the distribution of the RILs (towards the high discolouration scores). Although the differences between parents were not always significant at all times, as for pinking and browning Iceberg was the poorer performing parent. Scores for overall discolouration intensity were generally lower than scores for extent of overall discolouration in both trials for each day.

Table 5.9. Differences between parents Saladin and Iceberg for post harvest overall discolouration. Where *S* (significant differences); *NS* (not significant differences).

	Overall discolour intensity		**Extent of overall discolour**	
Site	Day 1	Day 3	Day 1	Day 3
UK	NS	NS	NS	NS
NL	S	NS	NS	NS
Across sites	S	NS	S	NS

The REML analysis showed significant differences (**P* <0.05) between RILs for intensity and extent on day 1 and day 3 in the NL trial and also when the data from UK and NL were combined for analysis across sites indicating genetic variation for overall discolouration. There was also a highly significant site effect for overall discolouration (both for intensity and extent) on day 1 again providing evidence that the growing environment of the plant influences post harvest performance (see table 5.10. a and b). Day was also a highly significant (****P* <0.001) factor for both measures of overall discolouration in both trials due to an increase in levels of discolouration across the RILs over time (see figure 5.12. (a-d) and figure 5.13. (a-

d)). Again a significant interaction was observed between day and genotype indicating differences between the RILs in the rate of increase of overall discolouration over time (see Appendix C).

Table 5.10. REML significance level of overall discolouration a) intensity and b) extent for the RIL mapping population in both the UK and NL. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *ndf* (numerator degrees of freedom); *ddf* (denominator degrees of freedom): *F pr* (probability); *Line* (genotype/RIL) ; *x* (interaction).

a

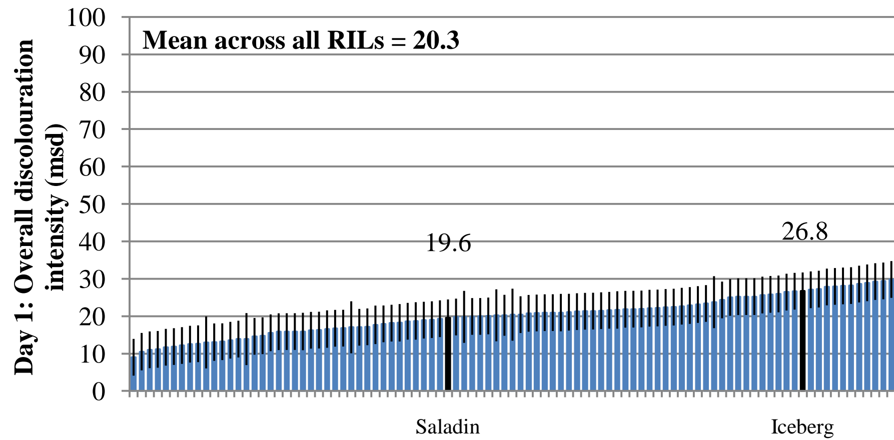
Site	Day	Fixed term	Wald statistic	ndf	F statistic	ddf	F pr
UK	1	Line	103.79	95	1.1	48	0.363
	3	Line	96.46	95	1.02	87.3	0.472
NL	1	Line	170.17	95	1.79	67.1	**0.006
	3	Line	152.79	95	1.62	61.7	*0.022
Across sites	1	Site	13.37	1	13.37	15.9	**0.002
	1	Line	174.99	95	1.84	111.4	***<0.001
	1	Site x Line	98.97	95	1.04	109.3	0.419
	3	Site	2.88	1	2.88	1.7	0.25
	3	Line	145.97	95	1.54	113.8	**0.01
	3	Site x Line	103.28	95	1.09	137.8	0.324

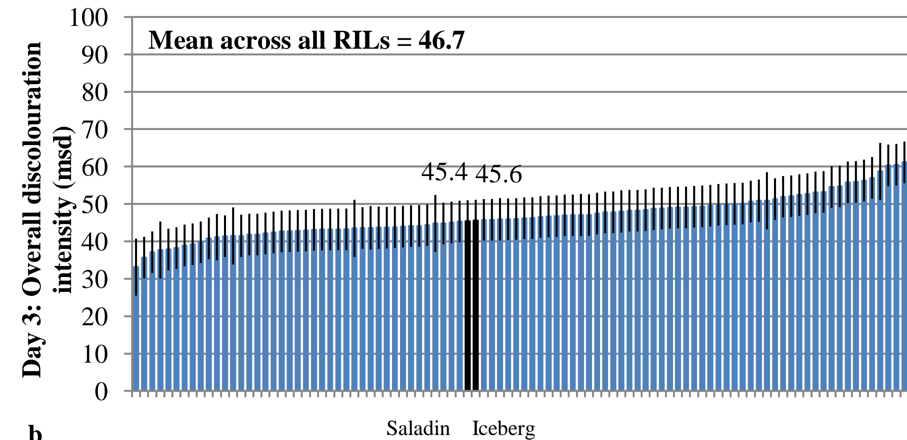
b

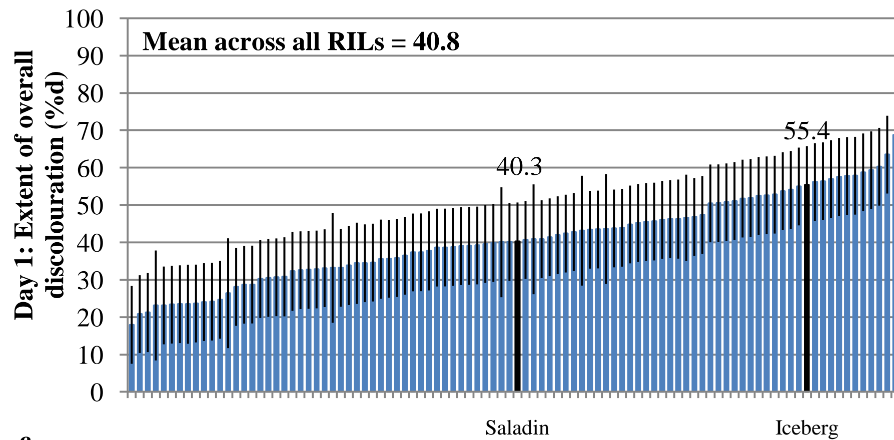
Site	Day	Fixed term	Wald statistic	ndf	F statistic	ddf	F pr
UK	1	Line	107.69	95	1.14	56.4	0.298
	3	Line	98.32	95	chi	1.03	0.387
NL	1	Line	196.44	95	2.07	68.4	***<0.001
	3	Line	205.9	95	2.17	95.8	***<0.001
Across sites	1	Site	11.68	1	11.68	17.3	***0.003
	1	Line	207.23	95	2.18	123.7	***<0.001
	1	Site x Line	96.89	95	1.02	123.8	0.456
	3	Site	8.42	1	8.42	1.1	0.193
	3	Line	201.56	95	2.12	144.2	***<0.001
	3	Site x Line	102.66	95	1.08	133.8	0.337

All RILs showed overall discolouration by day 1 in both trials as measured by intensity and extent (see figure 5.12. (a-d) and figure 5.13. (a-d)). As indicated in the REML analysis, Scores for overall discolouration increased at different rates for different RILs by day 3 and the rank order of lines changed over time; the changes in rank orders also differed per trial as found for both pinking and browning. The range

of variation observed in the RILs for intensity in both trials for each day was 20-30. Similarities were also observed for the extreme values of the distribution. The range of variation observed in the RILs for extent in both trials for day 1 was also similar (~50). However by day 3 the range had decreased in both trials as the majority of the RILs were reaching the possible maximum score for discolouration. There was some evidence of transgressive segregation as some RILs significantly outperformed the better parent (Saladin) and others were significantly worse than the worse parent (Iceberg) for overall discolouration, (see table 5.11. and table 5.12.).







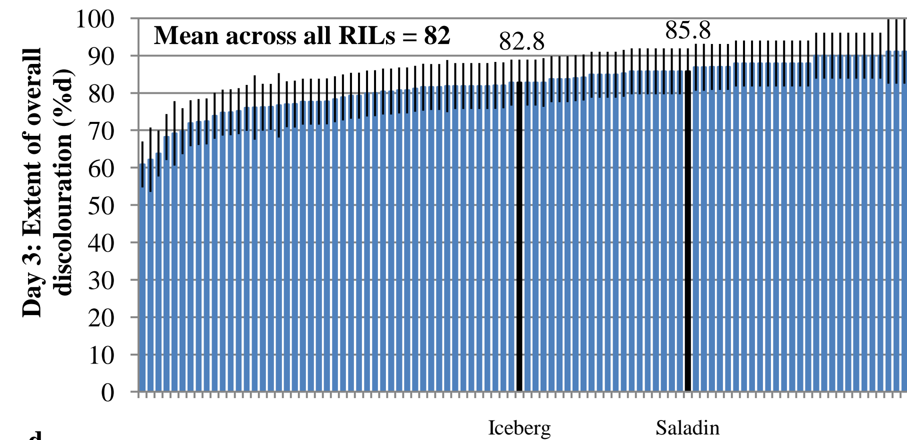
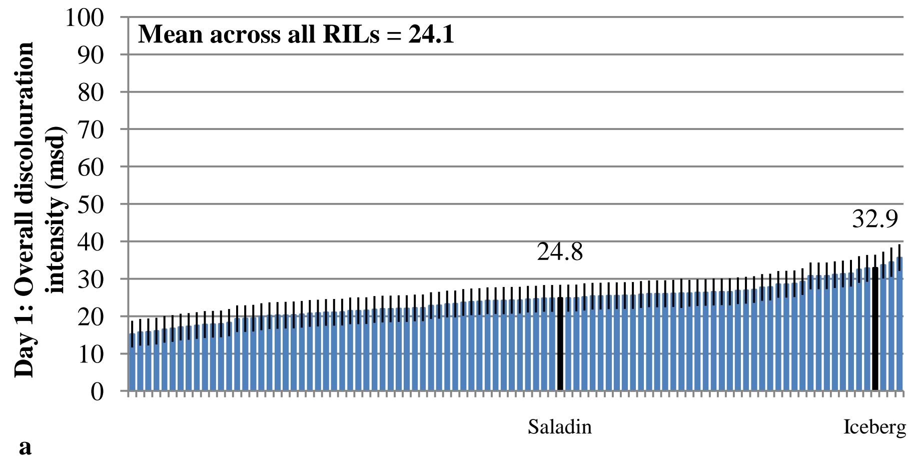
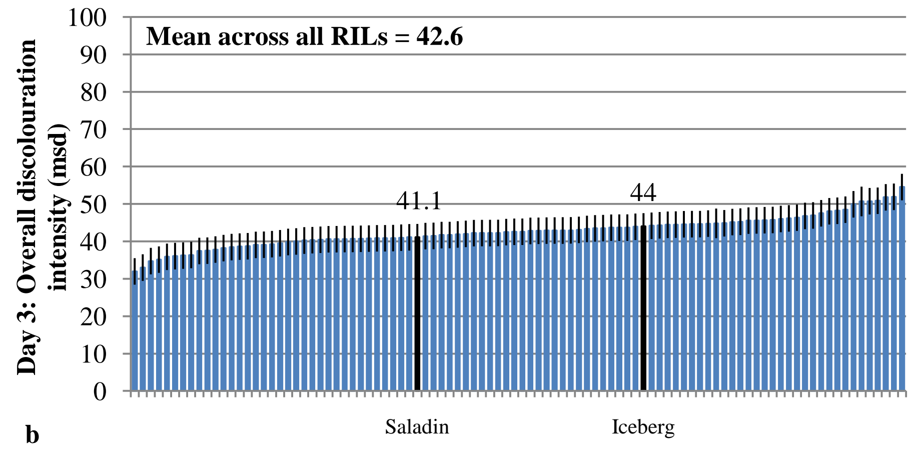
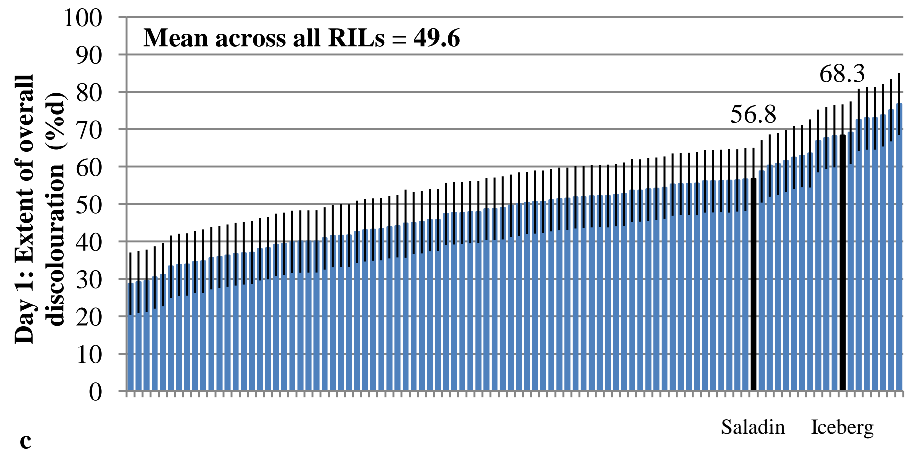


Figure 5.12. Transformed adjusted means (from REML) for lettuce post harvest overall discolouration intensity from the UK trial on a) day 1 and b) day 3 and extent of pinking on c) day 1 and d) day 3 for the F_7 mapping population. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI population parents are highlighted with respective adjusted means (from REML).







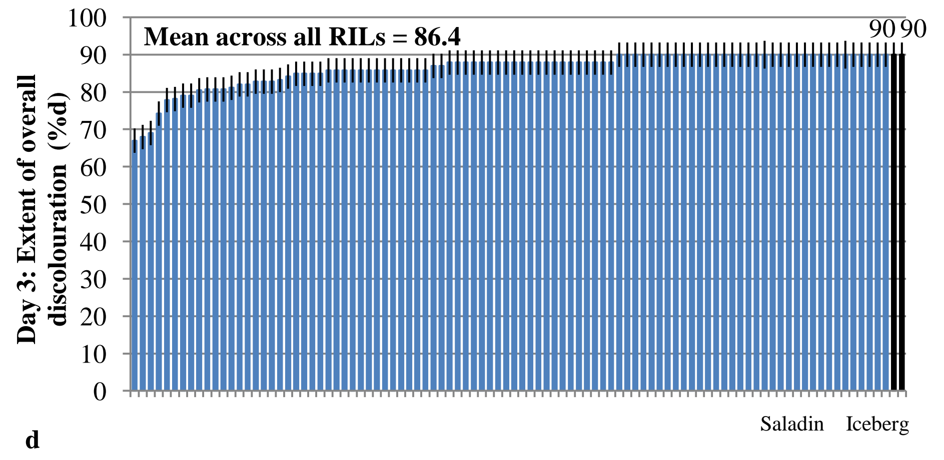


Figure 5.13. Transformed adjusted means (from REML) for lettuce post harvest overall discolouration intensity from the NL trial on a) day 1 and b) day 3 and extent of pinking on c) day 1 and d) day 3 for the F_7 mapping population. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI population parents are highlighted with respective adjusted means (from REML).

Table 5.11. RILs performing significantly better than the best parent Saladin for post harvest overall discolouration. Where ✓ (the line is performing significantly better than Saladin); × (the line is not performing significantly better than Saladin).

	UK				NL				Across site			
	Overall discolour intensity		Extent of overall discolour		Overall discolour intensity		Extent of overall discolour		Overall discolour intensity		Extent of overall discolour	
RIL \ Day	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**
5002	×	×	×	×	×	×	×	✓	×	×	✓	×
5008	×	×	×	×	×	×	×	×	×	×	✓	×
5014	×	×	×	×	×	×	×	✓	×	×	×	×
5017	×	×	×	×	×	×	✓	✓	×	×	✓	×
5018	×	×	×	×	×	×	✓	×	×	×	✓	×
5019	×	×	×	×	×	×	×	✓	×	×	×	×
5022	×	×	×	×	×	×	✓	×	×	×	✓	×
5023	×	×	×	✓	×	×	✓	×	✓	×	✓	×
5024	×	×	×	×	×	✓	×	✓	×	×	✓	×
5027	×	×	×	✓	×	×	×	×	×	×	✓	×
5031	×	×	×	×	×	×	✓	✓	×	×	✓	×
5032	×	×	×	×	×	×	✓	×	×	×	✓	×
5035	×	×	×	×	×	×	✓	✓	×	×	✓	×
5042	×	×	×	×	×	×	✓	✓	×	×	✓	×
5051	×	×	×	×	×	×	✓	✓	×	×	✓	×
5056	×	×	×	×	×	×	×	×	×	×	✓	×
5057	×	×	×	×	×	×	✓	×	×	×	✓	×
5059	×	×	×	×	×	×	✓	✓	×	×	✓	×
5062	×	×	×	×	×	×	×	×	×	×	✓	×
5063	×	×	×	✓	×	×	✓	✓	×	×	✓	×
5066	×	×	×	×	×	✓	×	×	×	×	×	×
5071	×	×	×	×	×	×	×	✓	×	×	✓	×
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5082	×	×	×	×	×	×	✓	×	×	×	×	×
5085	×	×	×	×	×	×	×	×	×	×	✓	×
5086	×	×	×	×	×	×	✓	✓	✓	×	✓	✓
5091	×	×	×	×	×	×	×	✓	×	×	×	×
5095	×	×	×	×	×	×	✓	✓	×	×	×	×
5098	×	×	×	×	×	×	×	✓	×	×	×	×
5103	×	×	×	×	×	×	✓	×	×	×	✓	×
5106	×	×	×	✓	×	×	✓	×	✓	×	✓	×
5107	×	×	×	×	×	×	×	×	×	×	✓	×
5121	×	×	×	×	×	×	✓	×	×	×	✓	×
5123	✓	×	✓	×	×	×	×	×	✓	×	✓	×

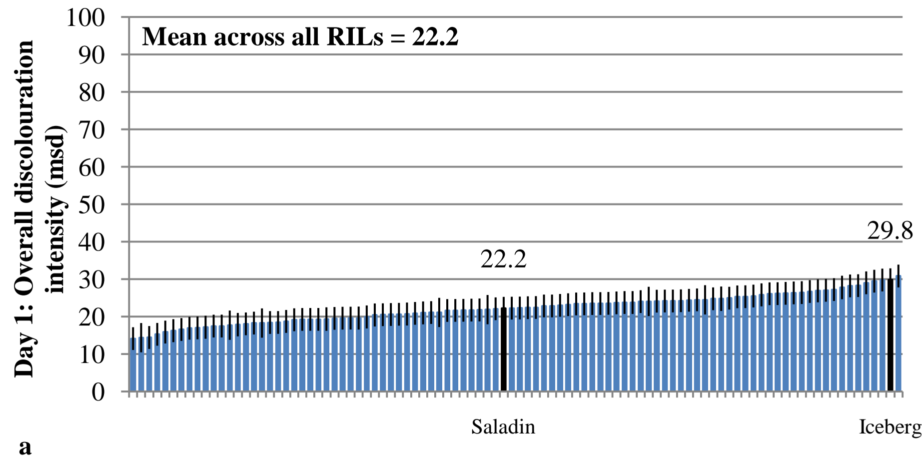
Table 5.12. RILs performing significantly worse than the poor parent Iceberg for post harvest overall discolouration. Where ✓ (the line is performing significantly worse than Iceberg); × (the line is not performing significantly worse than Iceberg).

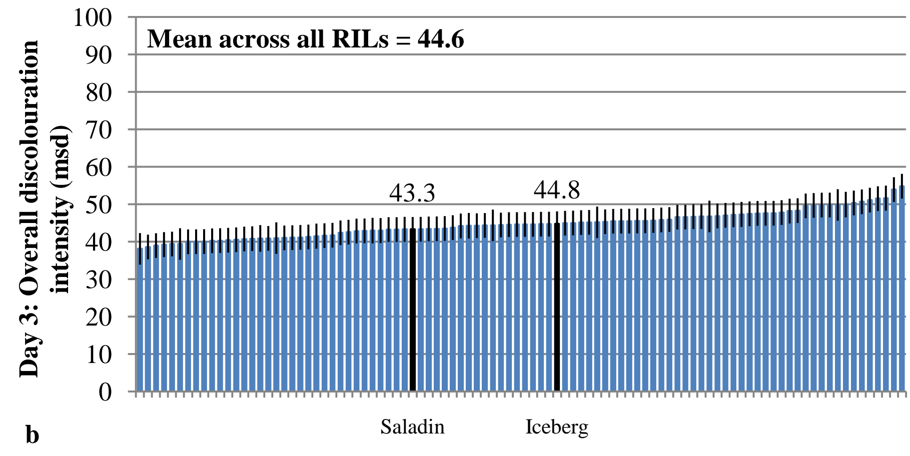
	UK				**NL**				**Across site**			
	Overall discolour intensity		**Extent of overall discolour**		**Overall discolour intensity**		**Extent of overall discolour**		**Overall discolour intensity**		**Extent of overall discolour**	
RIL \ Day	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**
5019	×	✓	×	×	×	×	×	×	×	×	×	×
5053	×	×	×	×	×	✓	×	×	×	✓	×	×
5068	×	✓	×	×	×	×	×	×	×	×	×	×
5095	×	✓	×	×	×	×	×	×	×	×	×	×
5096	×	✓	×	×	×	×	×	×	×	✓	×	×
5102	×	×	×	×	×	✓	×	×	×	✓	×	×
5104	×	×	×	×	×	×	×	×	×	×	×	×
5120	×	✓	×	×	×	×	×	×	×	✓	×	×

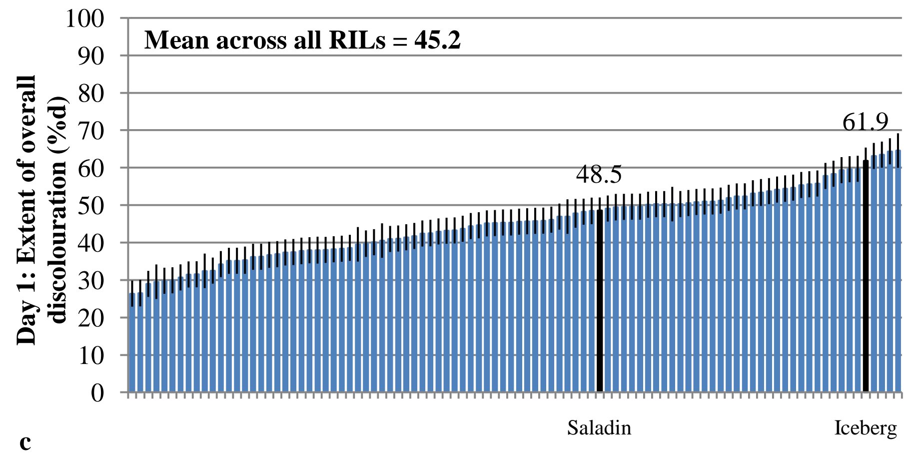
When the data from the two sites were combined in a weighted analysis the mapping parents were significantly different for intensity on day 1 (see table 5.9.). For overall discolouration intensity the extreme scores for the RILs on day 1 were similar to those in the individual trials, however on day 3 the lowest extreme score was higher than for both trials and the highest extreme score was similar to the NL trial. For extent the extreme scores for day 1 were similar to that for the NL trial, but higher than that of either trial for day 3. The range for extent was similar to that observed in the individual trials for each day. The parental scores for overall discolouration (both intensity and extent) on both days were an average across the two sites (see figure 5.14. a-d). The means of all RILs for overall discolouration across sites were averages of both trials. Again some RILs significantly outperformed the better parent (Saladin) for overall discolouration (see table 5.11.). RILs 5023 and 5063 performed significantly better than the best parent (Saladin) both trials. RILs 5081 and 5082 performed significantly better than Saladin only in the NL trial reflecting the influence of growing conditions on the trait. RILs 5072, 5074, 5075 and 5076

only out performed Saladin when the data were analysed across sites. No one RIL consistently performed poorer than Iceberg. Several RILs scored significantly lower than Iceberg for overall discolouration (see table 5.12.). RILs 5019, 5068 and 5095 only performed significantly poorer than Iceberg in the UK trial.

RILs 5002, 5022, 5023, 5042, 5051, 5053, 5066 and 5075 were also selected for inclusion in the 'extreme discolouration subset' for later experiments (as they showed consistent transgressive segregation) (see section 5.3.4.).







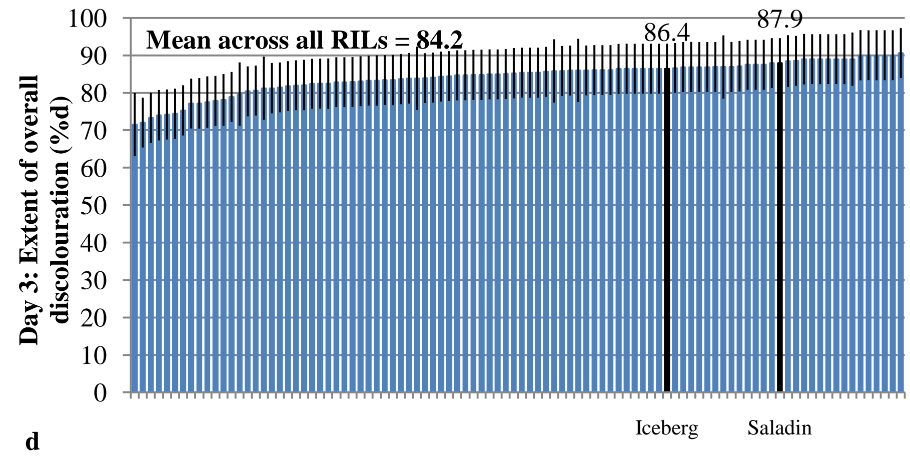


Figure 5.14. Transformed adjusted means (from REML) for lettuce post harvest overall discolouration intensity across sties on a) day 1 and b) day 3 and extent of overall discolouration on c) day 1 and d) day 3 for the F_7 mapping population. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI population parents are highlighted with respective adjusted means (from REML).

5.3.2. Correlations amongst traits assessed on the 94 RILs

The post harvest discolouration traits scored on the 94 RILs were analysed for potential relationships by testing for correlations between browning and pinking measurements within and across sites, (full correlation matrix in Appendix C). Only the highly significant correlations (***P <0.001) are described below (see table 5.13.).

The two measures of pinking (intensity and extent) were highly positively correlated with each other both within and across days (mean UK site $R_{[90]} \geq 0.95$, mean NL site $R_{[90]} \geq 0.94$ and mean across sites $R_{[90]} \geq 0.95$). Similarly the measures of browning were highly positively correlated with each other (mean UK site $R_{[90]} \geq 0.96$, mean NL site $R_{[90]} \geq 0.96$ and mean across sites $R_{[90]} \geq 0.95$). However, only data collected in the NL trial showed a positive correlation between the measures of overall discolouration within and across days (mean NL site $R_{[90]} \geq 0.85$).

Post harvest discolouration scores form the UK and NL trials were not correlated; however positive correlations were recorded between specific sites and across site data. Across site pinking was generally positively correlated for all days and across days with both UK and NL pinking and UK and NL overall discolouration measures, but not browning. Whilst across site browning was generally positively correlated for all days and across days with both UK and NL browning. The intensity of overall discolouration across sites did not reveal any correlations with any other measures of discolouration. Although the across site extent of overall discolouration was positively correlated with pinking measures in both the UK and NL, but only UK browning measures.

Table 5.13. Correlation matrix from the WHRI Saladin x Iceberg mapping population for post harvest discolouration and morphological parameters scored in 2008 experimental trial. Read across then down. Only significant effects are shown and highly significant effects ****P* <0.001 are shown bold. Where *msb* (browning intensity); *msp* (pinking intensity); *msd* (overall discolouration intensity); *%b* (extent of browning); *%p* (extent of pinking); *%d* (extent of overall discolouration); *UK* (UK site); *NL* (Netherlands site). Degree of freedom is 90.

	msp	msb	%p	%b	%d	UK msp	UK msb	UK %p	UK %b	NL msp	NL msb	NL %p
%p	**0.95**											
%b		**0.95**										
%d	**0.89**		**0.92**	0.23								
UK msp	**0.73**		**0.67**	0.27	**0.68**							
UK msb		**0.83**		**0.82**	0.27	**0.38**						
UK %p	**0.76**		**0.77**		**0.75**	**0.95**	0.29					
UK %b		**0.74**		**0.81**	**0.37**	**0.48**	**0.96**	**0.40**				
NL msp	**0.82**	-0.25	**0.80**		**0.71**	0.23		0.30				
NL msb	-0.32	**0.67**	-0.32	**0.57**		-0.28		-0.31		-0.21		
NL msd	**0.62**		**0.59**		**0.58**					**0.85**	**0.35**	
NL %p	**0.76**	-0.26	**0.82**		**0.72**			0.28		**0.94**		
NL %b	-0.26	**0.61**	-0.25	**0.56**		-0.23		-0.26			**0.96**	
NL %d	**0.71**		**0.77**		**0.73**			0.23		**0.90**		**0.95**

5.3.3. QTL analysis

The REML analyses demonstrated significant variation between the RILs for post harvest discolouration traits (section 5.3.1.) indicating genetic variation for these traits. The adjusted means from the REML analyses were combined with the linkage map (Chapter 4) to carry out QTL analysis on all traits in order to investigate the genetic control of this variation further.

The QTL analysis was performed separately on the data for each of the phenotypic post harvest discolouration traits: pinking (intensity and extent), browning (intensity and extent) and overall discolouration (intensity and extent) for day 1 and day 3 in addition to an analysis carried out using data summarised across days. For each assessment day the data were analysed for the UK and NL trial separately and a weighted across site analysis, to identify if putative QTL were environmentally stable or environment specific.

Interval mapping using MapQTL ®4.0 software identified 80 putative QTL for post harvest discolouration traits (see appendix C), while the subsequent MQM increased robustness of the identification of putative QTL (by taking into account the effect of other QTL) and reduced the number of putative QTL to 56 (see table 5.15. and figure 5.15.).

The 56 putative QTL (*P <0.05) were detected with LOD significance threshold greater than 1.95; the LOD significance threshold of 1.95 was based on the simulation study based on an F_7 RIL experimental population (Van Ooijen 1999) (see table 5.15). Individual putative QTL accounted for between 6.1-22.6% of the phenotypic variation in this population and QTL were identified on all the linkage groups except LG 1, with the highest number of QTL being mapped on LG 7 (23). The highest number of putative QTL (20) were detected for browning traits (10 for intensity and 10 for extent), followed by 19 putative QTL for pinking traits (11 for intensity and 8 for extent) and then 17 for overall discolouration traits (10 for intensity and 7 for extent) (see table 5.14. table 5.15.). Thirty-one putative QTL related to assessment of discolouration on day 1 which was expected as a result of greater variation observed between the parents on day 1 in the preliminary trial (see Chapter 3). Eleven putative QTL were identified for day 3 and a similar number (14) from the analysis across days. The number of putative QTL identified for both trials and across sites (20 for UK trial; 17 for NL trial; 19 for across site) was similar. However there were different numbers of putative QTL per trait between sites (see table 5.14.).

Table 5.14. Summary table for putative QTL for QTL impacting on post harvest discolouration traits in a Saladin x Iceberg RIL population. Where *UK* (United Kingdom); *NL* (The Netherlands).

Trait	Data set			Total	
	UK	NL	Across sites		
Pinking intensity	7	0	4	11	19
Extent of pinking	3	3	2	8	
Browning intensity	2	3	5	10	20
Extent of browning	3	3	4	10	
Overall discolouration intensity	4	5	1	10	17
Extent of overall discolouration	1	3	3	7	
Total	20	17	19	56	56

Many of the putative QTL co-located to the same position and are therefore most likely to be identifying the same underlying genetic factor (i.e. QTL). It was therefore possible to consolidate the large number of putative QTL down to a smaller number of QTL for post harvest discolouration. The 56 putative QTL were then reduced to 21 QTL based on their co-location on the map (see figure 5.15.).

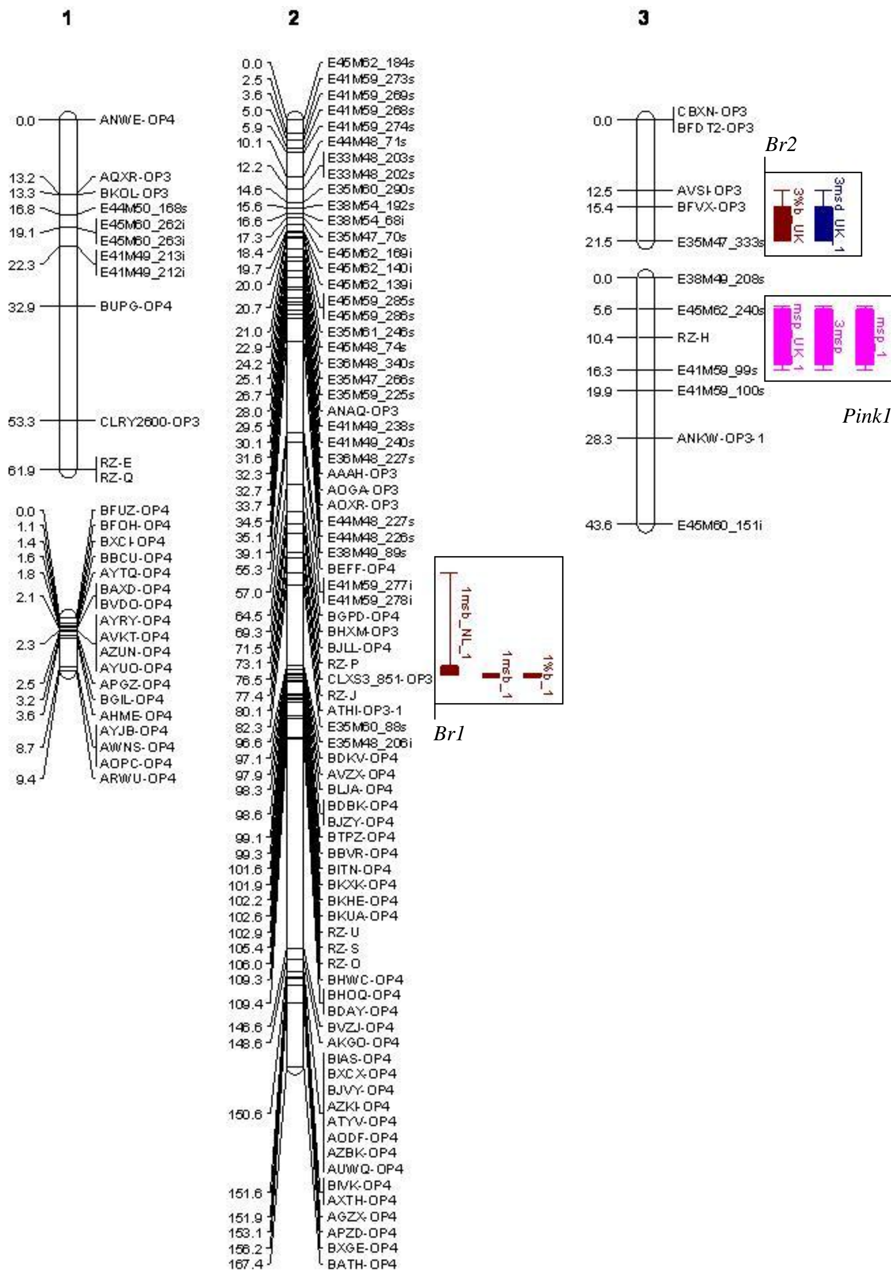


Figure 5.15. Putative QTL impacting on post harvest discolouration of lettuce tissue from the Saladin x Iceberg RIL population grown in 2 sites: UK and NL. Where *UK* (United Kingdom); *NL* (The Netherlands), *msb* (browning intensity); *%b* (extent of browning); *msp* (pinking intensity); *%p* (extent of pinking); *msd* (overall discolouration intensity); *%d* (extent of overall discolouration) ; *Br* (browning); *Pink* (pinking); *Dis* (overall discolouration). Number before discolouration parameter refers to day. Box encloses putative QTL forming significant QTL. Number after discolouration parameter refers to QTL number.

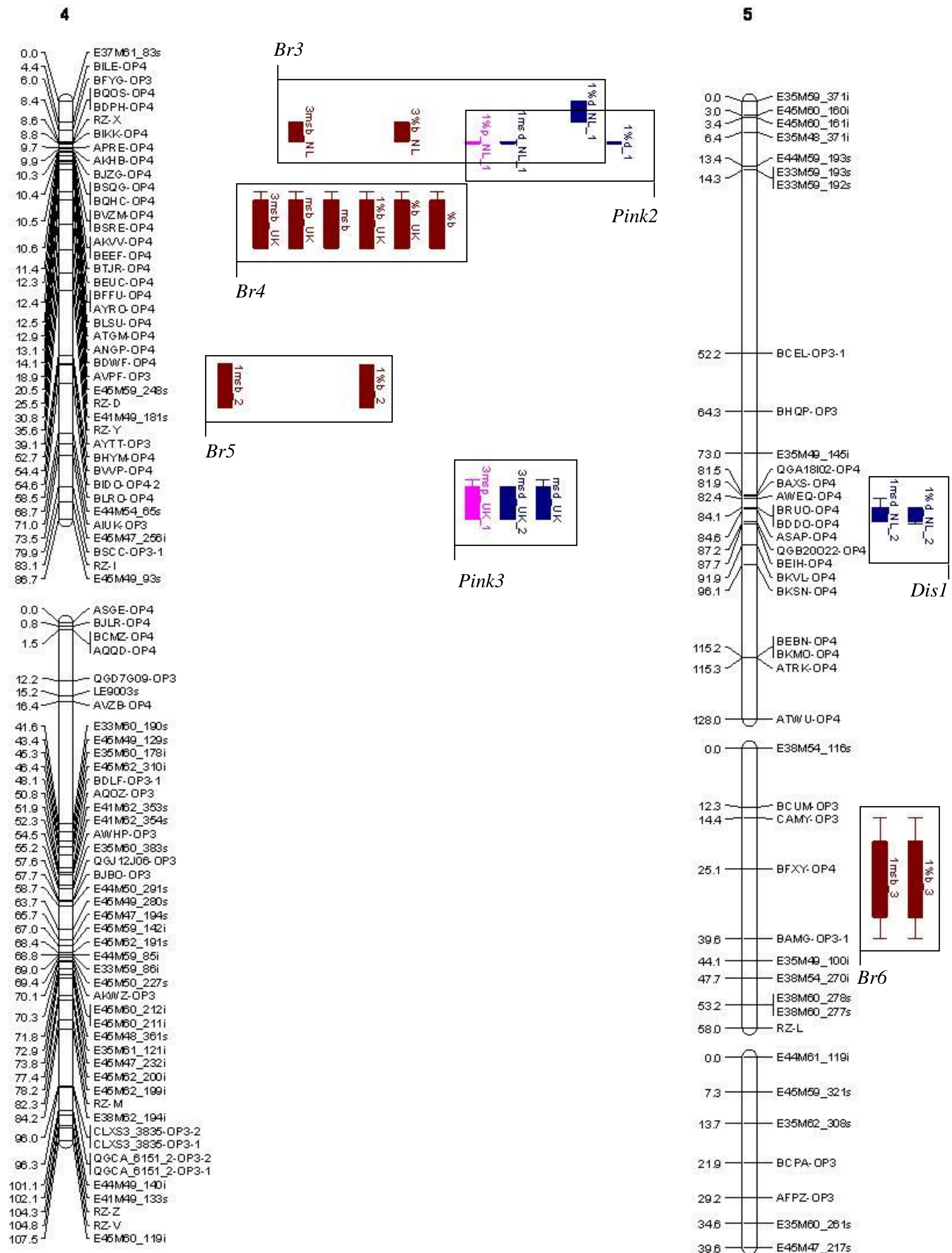


Figure 5.15. continued.

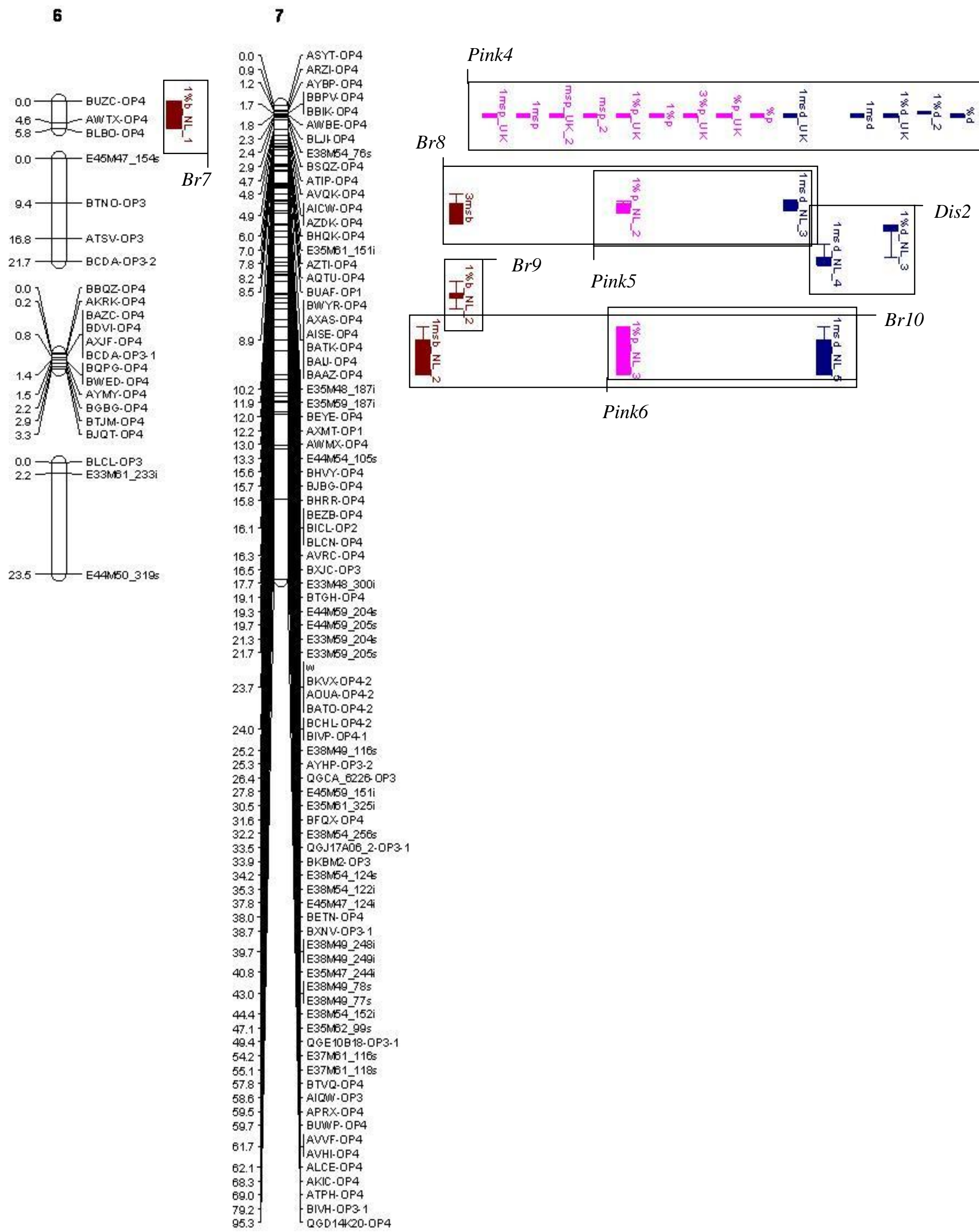


Figure 5.15. continued.

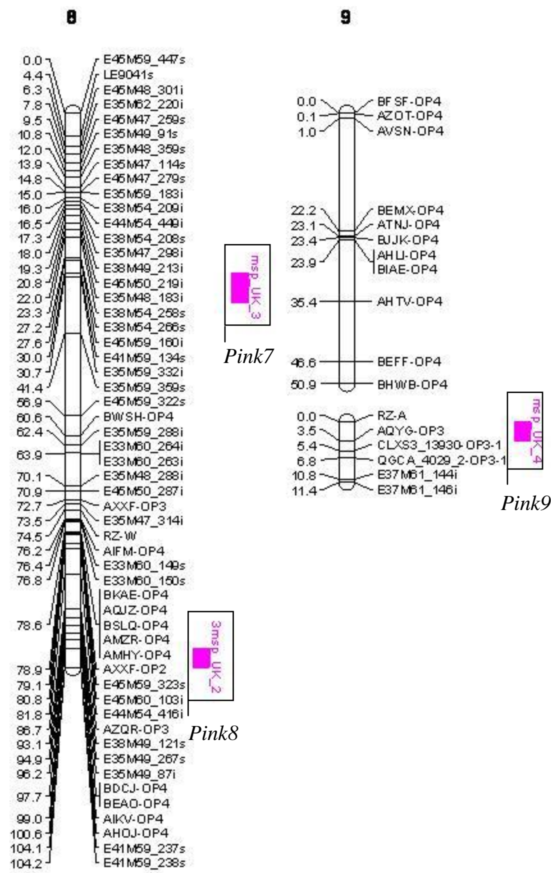


Figure 5.15. continued.

Table 5.15. Putative MQM QTL impacting on post harvest discolouration of lettuce tissue from the Saladin x Iceberg RIL population grown in 2 sites: UK and NL. Additive effect equals half the difference between homozygous alleles at the QTL: positive number indicates an additive allelic effect of Saladin; negative number indicates a negative allelic effect of Saladin. Confidence interval was based on a 2 LOD support interval with a significant LOD value of 1.95. Where *UK* (United Kingdom); *NL* (The Netherlands), *LG* (linkage group from F_7 Saladin x Iceberg linkage map (see Chapter 4)); *LOD* (logarithm of odds), *cM* (centimorgans); *msb* (browning intensity); *%b* (extent of browning); *msp* (pinking intensity); *%p* (extent of pinking); *msd* (overall discolouration intensity); *%d* (extent of overall discolouration). Number before discolouration parameter refers to day.

Trait	LG	Location (cM)	LOD peak	Confidence interval (cM)	Nearest Locus	Additive effect	Genetic variation explained by QTL (%)	Allelic contribution
3msb_UK	4a	25.5	2.26	11.6	RZ-D	2.50	10.5	Saladin
msb_UK	4a	25.5	2.77	11.6	RZ-D	2.06	12.8	Saladin
1%b_UK	4a	25.5	2.29	11.6	RZ-D	2.06	10.7	Saladin
3%b_UK	3a	21.5	1.98	9	E35M47_333s	-3.27	9.8	Iceberg
%b_UK	4a	25.5	2.85	11.6	RZ-D	3.00	13.2	Saladin
1msp_UK	7	2.3	3.63	0.7	BLJI-OP4	3.18	16.3	Saladin
3msp_UK_1	4a	83.1	2.28	8.2	RZ-I	2.57	9.6	Saladin
3msp_UK_2	8	104.2	2.24	4.2	E41M59_238s	-2.66	9.7	Iceberg
msp_UK_1	3b	10.4	2.05	11.3	RZ-H	2.10	7.8	Saladin
msp_UK_2	7	2.3	2.48	0.7	BLJI-OP4	2.29	9.5	Saladin
msp_UK_3	8	30.7	2.19	5.7	E35M59_332i	-2.53	8.4	Iceberg
msp_UK_4	9b	0	3.16	3.5	RZ-A	2.52	11.3	Saladin
1%p_UK	7	2.3	3.8	0.7	BLJI-OP4	4.86	17	Saladin
3%p_UK	7	2.3	2.08	0.7	BLJI-OP4	2.92	9.7	Saladin
%p_UK	7	2.3	2.08	0.7	BLJI-OP4	8.78	8.6	Saladin
1msd_UK	7	2.3	2.73	0.7	BLJI-OP4	1.87	12.6	Saladin
3msd_UK_1	3a	21.5	2.05	9	E35M47_333s	-1.62	8.3	Iceberg
3msd_UK_2	4a	83.1	4.07	6.8	RZ-I	2.54	17	Saladin
msd_UK	4a	83.1	2.48	8.2	RZ-I	1.67	11.7	Saladin
1%d_UK	7	2.3	2.51	0.7	BLJI-OP4	3.86	11.6	Saladin

Table 5.15. continued.

Trait	LG	Location (cM)	LOD peak	Confidence interval (cM)	Nearest Locus	Additive effect	Genetic variation explained by QTL (%)	Allelic contribution
1msb_NL_1	2	97.9	1.97	18.2	AVZX-OP4	-1.37	8.2	Iceberg
1msb_NL_2	7	49.4	3.41	9.8	QGE10B18-OP3-1	1.85	14.8	Saladin
3msb_NL	4a	6	2.35	4	BFYG-OP3	-2.57	10.9	Iceberg
1%b_NL_1	6a	4.6	2.04	5.8	AWTX-OP4	-1.66	7.9	Iceberg
1%b_NL_2	7	38	2.33	5.5	BETN-OP4	5.48	8.1	Saladin
3%b_NL	4a	6	2.35	4	BFYG-OP3	-3.08	10.9	Iceberg
1%p_NL_1	4a	8.6	2.3	0.4	RZ-X	4.47	9.3	Saladin
1%p_NL_2	7	21.3	4.66	2.4	E33M59_204s	-8.07	22.6	Iceberg
1%p_NL_3	7	47.1	2.53	9.8	E35M62_99s	4.96	12	Saladin
1msd_NL_1	4a	8.6	4.77	0.4	RZ-X	1.96	15.4	Saladin
1msd_NL_2	5a	84.6	2.63	4.8	ASAP-OP4	1.29	7.7	Saladin
1msd_NL_3	7	19.7	4	2.2	E44M59_205s	-1.93	13.3	Iceberg
1msd_NL_4	7	31.6	2.11	4.4	BFQX-OP4	-1.49	6.1	Iceberg
1msd_NL_5	7	49.4	5.19	9.8	QGE10B18-OP3-1	2.30	17.4	Saladin
1%d_NL_1	4a	0	3.21	4.4	E37M61_83s	4.39	13.1	Saladin
1%d_NL_2	5a	84.6	2.35	3.6	ASAP-OP4	3.49	9.2	Saladin
1%d_NL_3	7	25.2	2.01	6.5	E38M49_116s	-3.19	7.8	Iceberg
1msb_1	2	98.6	2.71	0.7	BLJA-OP4 / BDBK-OP4	-1.20	10.8	Iceberg
1msb_2	4a	58.5	2.67	9.1	BLRO-OP4	-2.30	10.1	Iceberg
1msb_3	5b	25.1	2.49	25.2	BFXY-OP4	1.65	9.6	Saladin
3msb	7	21.7	1.97	6	E33M59_205s	1.98	11	Saladin
msb	4a	25.5	2.12	11.6	RZ-D	1.29	9.9	Saladin
1%b_1	2	98.6	2.51	0.7	BLJA-OP4 / BDBK-OP4	-1.61	10.2	Iceberg
1%b_2	4a	58.5	2.22	8.9	BLRO-OP4	-2.94	8.1	Iceberg
1%b_3	5b	25.1	2.79	25.2	BFXY-OP4	2.54	11.8	Saladin

Table 5.15. continued.

Trait	LG	Location (cM)	LOD peak	Confidence interval (cM)	Nearest Locus	Additive effect	Genetic variation explained by QTL (%)	Allelic contribution
%b	4a	25.5	2.18	11.6	RZ-D	1.67	10.2	Saladin
1msp	7	2.3	3.53	0.7	BLJI-OP4	2.74	15.9	Saladin
3msp	3b	10.4	2.55	11.3	RZ-H	2.14	11.8	Saladin
msp_1	3b	10.4	2.16	11.3	RZ-H	1.81	8.9	Saladin
msp_2	7	2.3	2.28	0.7	BLJI-OP4	1.88	9.5	Saladin
1%p	7	2.3	3.6	0.7	BLJI-OP4	4.37	16.2	Saladin
%p	7	2.3	3.37	0.7	BLJI-OP4	3.14	15.2	Saladin
1msd	7	2.3	4.25	0.7	BLJI-OP4	1.63	16.9	Saladin
1%d_1	4a	8.6	2.13	0.4	RZ-X	2.97	8.8	Saladin
1%d_2	7	1.7	2.84	0.5	BBPV-OP4	3.16	12	Saladin
%d	7	2.3	2.33	0.7	BLJI-OP4	2.05	10.8	Saladin

Significant QTL for post harvest pinking traits

The 19 putative QTL (**P* <0.05) for post harvest pinking traits could be reduced to 9 QTL positions based on their co-location on the map; they were identified on 5 linkage groups (LGs 3b (*Pink1*), 4a (*Pink2* and *Pink3*), 7 (*Pink4*, *Pink5* and *Pink6*), 8 (*Pink7* and *Pink8*) and 9b (*Pink9*)) (see figure 5.15, table 5.15. and table 5.16.). The total phenotypic variation explained by all putative QTL for pinking traits was between 7.8-22.6%, with 9.3-22.6% explaining phenotypic variation on day 1 and 9.6-11.8% explaining phenotypic variation on day 3. The largest amount of genetic variation explained by a putative QTL was for '1%p_NL_2' at 22.6%. Ten putative QTL were identified for pinking traits measured in the UK trial, 3 were identified for pinking traits measured in the NL trial and 6 were identified through a weighted analysis across sites (see table 5.14.). Twelve putative QTL located to 2 overlapping regions on separate linkage groups forming 2 significant QTL. Of these, 3 putative QTL co-localised on LG 3b (5-16.3 cM) for pinking intensity from the UK trial and across site analysis for day 3 and across day data forming QTL *Pink1,* while 9 putative QTL co-localised on LG 7 (1.7-2.4 cM) for pinking (both intensity and extent) for the UK trial and across site for both days and across days forming QTL *Pink4*. The remaining QTL were identified by a single locating putative QTL for pinking (*Pink2, Pink3, Pink5, and Pink6, Pink7, Pink8* and *Pink9*). The Saladin marker allele resulted in 16 putative QTL for pinking traits while the Iceberg marker allele resulted in 3; this translated into 7 QTL linked to the Saladin marker allele and 3 QTL with the Iceberg marker allele.

Significant QTL for post harvest browning traits

The 20 putative QTL (**P* <0.05) for post harvest browning traits could be reduced to 10 QTL positions based on their co-location on the map; they were identified on 6

linkage groups (LGs 2 (*Br1*), 3 (*Br2*), 4a (*Br3*, *Br4* and *Br5*), 5b (*Br6*), 6a (*Br7*) and 7 (*Br8*, *Br9* and *Br10*)) (see figure 5.15., table 5.15. and table 5.16.). The total phenotypic variation explained by all putative QTL for browning traits was between 7.9-14.8%, with 8.1-10.8% explaining phenotypic variation on day 1 and 10.9-12.8% explaining phenotypic variation on day 3. The largest amount of genetic variation explained by a putative QTL was for '1msb_NL_2' at 14.8%. Five putative QTL were identified for browning traits measured in the UK trial, 6 were identified for browning traits measured in the NL trial and 9 were identified through a weighted analysis across sites (see table 5.14.). Fifteen putative QTL located to 5 overlapping regions on different linkage groups forming 5 significant QTL. Three putative QTL co-localised on LG 2 (80.1-98.3 cM) for all measurements of browning from the NL trial and across sites for day 1 forming QTL *Br1*. Three clusters of peaks were present on LG 4a for browning traits. Two putative QTL co-localised (4.4-8.4 cM) for browning (both intensity and extent) from the NL trial for day 3 forming QTL *Br3*. Six putative QTL co-localised (18.9-30.5 cM) for browning (both intensity and extent) for the UK trial and across site and for both days and across days forming QTL *Br4*. Two putative QTL co-localised (54.6-63.5 cM) for browning (both intensity and extent) across sites for day 1 forming *Br5*. Two putative QTL co-localised on LG 5b (14.4-39.6 cM) for browning (both intensity and extent) across sites for day 1 forming *Br6*. The remaining QTL were identified by a single locating putative QTL for browning (*Br7, Br8, Br9* and *Br10*). The Saladin marker allele resulted in 11 putative QTL for browning traits while the Iceberg marker allele resulted in 9; this translated into 5 QTL linked to the Saladin marker allele and 5 QTL linked to the Iceberg marker allele.

Significant QTL for post harvest overall discolouration traits

The 17 putative QTL (**P* <0.05) for post harvest overall discolouration traits could be reduced to 2 QTL positions based on their co-location on the map; they were identified on 2 linkage groups (including LGs 5a (*Dis1*) and 7 (*Dis2*)) (see figure 5.15., table 5.15. and table 5.16.). The total phenotypic variation explained by all identified peaks for overall discolouration traits was between 6.1-17.4%, with 7.7-15.4% explaining phenotypic variation on day 1 and 8.3-17% explaining phenotypic variation on day 3. The largest amount of genetic variation explained by a putative QTL was for '1msd_NL_'5 at 17.4%. Five putative QTL were identified for overall discolouration traits measured in the UK trial, 8 were identified for overall discolouration traits measured in the NL trial and 4 were identified through a weighted analysis across sites (see table 5.14.). Four putative QTL located to 2 overlapping regions on different linkage groups forming 2 significant QTL. Two putative QTL co-localised on LG 5a (82.4-87.7 cM) for overall discolouration (both intensity and extent) from the NL trial for day 1 forming QTL *Dis1*. Two putative QTL also co-localised on LG 7 (24-32.2 cM) for overall discolouration (both intensity and extent) for the NL trial for day 1 forming QTL *Dis2*. The remaining putative QTL for overall discolouration measurements localised with either pink or brown measurements and were thus treated as a specific colour; putative QTL were included as pink on LGs 4a and 7, as brown on LG 3a and as both pink and brown on LG 7. The Saladin marker allele resulted in 13 putative QTL for overall discolouration traits while the Iceberg marker allele resulted in 5; this translated into 1 QTL linked to the Saladin marker allele and 1 QTL linked to the Iceberg marker allele.

Table 5.16. Significant QTL summary based on MQM mapping impacting on post harvest discolouration of lettuce tissue from the Saladin x Iceberg RIL population Confidence interval was based on a 2 LOD support interval with a significant LOD value of 1.95. Where *LG* (linkage group from F_7 Saladin x Iceberg linkage map (see Chapter 4)); *cM* (centimorgans); *Br* (post harvest browning); *Pink* (post harvest pinking); *Dis* (post harvest overall discolouration).

QTL	LG	Confidence Interval (cM)	Underlying locus	Allelic contribution
Br1	2	18.2	ATHI-OP3-1 / E35M60_88s / E35M48_206i / BDKV-OP4 / AVZX-OP4 / BLJA-OP4	Iceberg
Br2	3a	9	AVSI-OP3 / BFVX-OP3 / E35M47_333s	Iceberg
Br3	4a	4	BILE-OP4 / BFYG-OP3 / BQOS-OP4 / BDPH-OP4	Iceberg
Br4	4a	11.6	BQOS-OP4 / BDPH-OP4 / RZ-X / BIKK-OP4	Saladin
Br5	4a	8.9	BIDO-OP4-2 / BLRO-OP4	Iceberg
Br6	5b	25.2	CAMY-OP3 / BFXY-OP4 / BAMG-OP3-1	Saladin
Br7	6a	5.8	BUZC-OP4 / AWTX-OP4 / BLBO-OP4	Iceberg
Br8	7	6	E33M48_300i / BTGH-OP4 / E44M59_204s / E44M59_205s / E33M59_204s / E33M59_205s / w / BKVX-OP4-2 / AOUA-OP4-2 / BATO-OP4-2	Saladin
Br9	7	5.5	E38M54_122i / E45M47_124i / BETN-OP4 / BXNV-OP3-1 / E38M49_248i / E38M49_249i / E35M47_244i	Saladin
Br10	7	9.8	E38M54_152i / E35M62_99s / QGE10B18-OP3-1 / E37M61_116s	Saladin
Pink1	3b	11.3	E45M62_240s / RZ-H / E41M59_99s	Saladin
Pink2	4a	0.4	BQOS-OP4 / BDPH-OP4 / RZ-X / BIKK-OP4	Saladin
Pink3	4a	8.2	BSCC-OP3-1 / RZ-I / E45M49_93s	Saladin
Pink4	7	0.7	BBPV-OP4 / BBIK-OP4 / AWBE-OP4 / BLJI-OP4 / E38M54_76s	Saladin
Pink5	7	2.4	E44M59_204s / E44M59_205s / E33M59_204s / E33M59_205s	Iceberg
Pink6	7	9.8	E38M54_152i / E35M62_99s / QGE10B18-OP3-1 / E37M61_116s	Saladin
Pink7	8	5.7	E41M59_134s / E35M59_332i	Iceberg
Pink8	8	4.2	AHOJ-OP4 / E41M59_237s / E41M59_238s	Iceberg
Pink9	9b	3.5	RZ-A / AQYG-OP3	Saladin
Dis1	5a	5.3	AWEQ-OP4 / BRUO-OP4 / BDDO-OP4 / ASAP-OP4 / QGB20O22-OP4 / BEIH-OP4	Saladin
Dis2	7	8.2	BCHL-OP4-2 / BIVP-OP4-1 / E38M49_116s / AYHP-OP3-2 / QGCA_6226-OP3 / E45M59_151i / E35M61_325i / BFQX-OP4 / E38M54_256s	Iceberg

5.3.4. Stability of discolouration phenotype for the extreme phenotype RILs

From the REML analyses (section 5.3.1.) it was found that the plant's growing environment has a significant effect on the post harvest discolouration traits. This has implications for lettuce breeders as if the phenotype is largely determined by environmental factors selection in the field will be difficult. Marker assisted selection of genotypes offers a way of overcoming this. In order to show whether the post harvest traits were sufficiently heritable to select, RILs were selected from the population that had the beneficial marker alleles from an initial QTL analysis and that were showing significant transgressive segregation for at least one trait (pinking, browning or overall discolouration).

Post harvest pinking of prepacked leaf tissue

There were significant differences (***P <0.001) between the selected RILs for pinking (both intensity and extent) for days 1, 2 and 3 confirming genetic variation for this trait (see table 5.17. a and b, figure 5.16. (a-d) and figure 5.17. (a-d)). However by day 4 RILs were only significantly different for pinking intensity, but not for extent of pinking as the majority had reached the possible maximum score for the extent of pinking. There was little movement in ranking of RILs between days for pinking (both intensity and extent), which would be expected if the phenotypes were largely determined by genotype and not by environment.

Table 5.17. REML significance level of a) pinking intensity and b) extent of pinking over 4 days for the extreme RIL set. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *ddf* (denominator degrees of freedom): P (probability).

		Significance level		
a	**Pinking intensity**	**REML Wald Statistic**	**ddf**	**REML P value**
	Day 1	77.61	23.2	***<0.001
	Day 2	62.56	20.7	***<0.001
	Day 3	82.6	21.5	***<0.001
	Day 4	85.81	22.3	***<0.001

		Significance level		
b	**Extent of pinking**	**REML Wald Statistic**	**ddf**	**REML P value**
	Day 1	80.12	22.3	***<0.001
	Day 2	54.89	21.2	***0.001
	Day 3	60	20.3	***<0.001
	Day 4	18.24	23.2	0.189

The mapping parents Saladin and Iceberg were significantly different for pinking (both intensity and extent) on day 1 and day 3. Saladin was the best performing parent for pinking. Low pinking (LP) RILs 5023 and 5051 (initially selected for low pinking scores) performed significantly better than Saladin on all days (except RIL 5023 on day 4 for extent of pinking). RILs 5002 and 5042 also performed significantly better than Saladin on all days but these were selected for low browning and low overall discolouration respectively. Generally RILs 5055 and 5066 selected for low browning and high overall discolouration respectively, had significantly higher scores of pinking (both intensity and extent) than the high pinking (HP) RILs (selected for high pinking scores) on all days. The most extreme ‘extreme pinking’ RILs, LP RIL 5051 and HP RIL 5045 also revealed significant differences for pinking (both intensity and extent) on all days. The extreme RIL set demonstrated a pattern for rate of pinking; with those exhibiting a high pinking score early on revealing lower rates of discolouration to those with an initially low pinking score.

Post harvest browning of prepacked leaf tissue

There were significant differences (*P <0.05) between the selected RILs for browning intensity for days 1, 2 and 3 and for extent of browning for days 1 and 3, confirming genetic variation for this trait (see table 5.18. a and b, figure 5.18. (a-d) and figure 5.19. (a-d)). As for measurements of pinking, there was little movement in ranking of RILs between days for browning.

Table 5.18. REML significance level of a) browning intensity and b) extent of browning over 4 days for the extreme RIL set. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *ddf* (denominator degrees of freedom): P (probability).

a

	Significance level		
Browning intensity	**REML Wald Statistic**	**ddf**	**REML P value**
Day 1	30.37	26.6	*0.023
Day 2	29.03	26.2	*0.029
Day 3	42.19	26.9	**0.003
Day 4	21.51	30.8	0.095

b

	Significance level		
Extent of browning	**REML Wald Statistic**	**ddf**	**REML P value**
Day 1	29.92	26.4	*0.024
Day 2	23.17	26.5	0.077
Day 3	33.3	26.9	*0.014
Day 4	18.82	31	0.154

The mapping parents Saladin and Iceberg were significantly different for browning (both intensity and extent) on day 3 and day 4. Iceberg was the best performing parent for browning. No RILs including low browning (LB) RILs 5022 and 5055 (selected for low browning scores) performed significantly better than Iceberg. Generally high browning (HB) RILs 5043 and 5053 (selected for high browning scores) performed worse than poorest performing parent Saladin. Significant variation was observed between LB and HB RILs for browning on all days (except day 4 for intensity). In addition there were no RILs which showed browning scores

above 60% of possible maximum scores. In contrast to the pinking response, the two browning scores were at similar levels and also showed similar rates of increase.

Post harvest overall discolouration of prepacked leaf tissue

There were significant differences (**P* <0.05 and ***P* <0.01) between the selected RILs for overall discolouration (for intensity and extent) for days 1, 2 and 3 confirming genetic variation for this trait (see table 5.19. a and b, figure 5.20. (a-d) and figure 5.21. (a-d)). By day 2 approximately half of the RILs were nearing the possible maximum score for the extent of overall discolouration.

Table 5.19. REML significance level of a) overall discolouration intensity and b) extent of overall discolouration over 4 days for the extreme RIL set. Significant effects shown as **P* <0.05, ***P* <0.01 and ****P* <0.001. Where *ddf* (denominator degrees of freedom): *P* (probability).

a

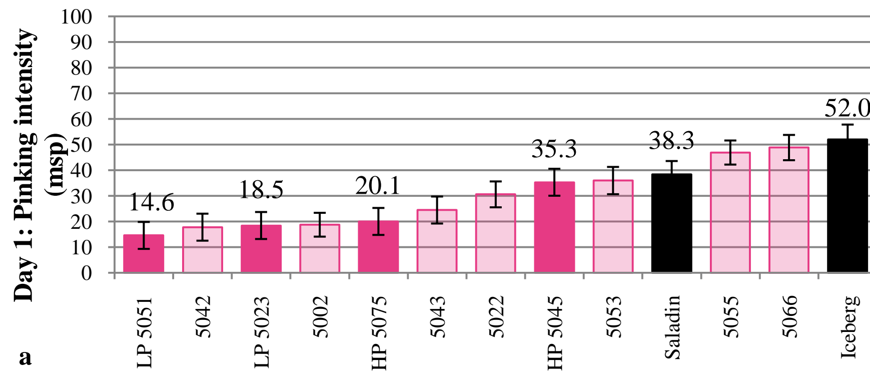
	Significance level		
Overall discolouration intensity	**REML Wald Statistic**	**ddf**	**REML P value**
Day 1	54.52	28	***<0.001
Day 2	32.23	27.4	*0.019
Day 3	43.87	26.4	**0.003
Day 4	17.85	28	0.188

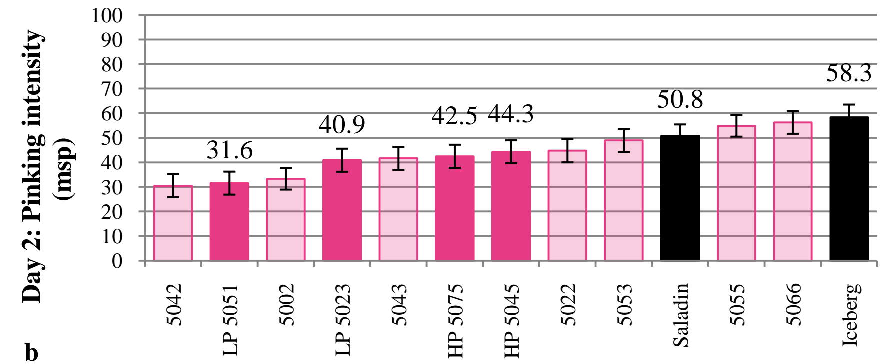
b

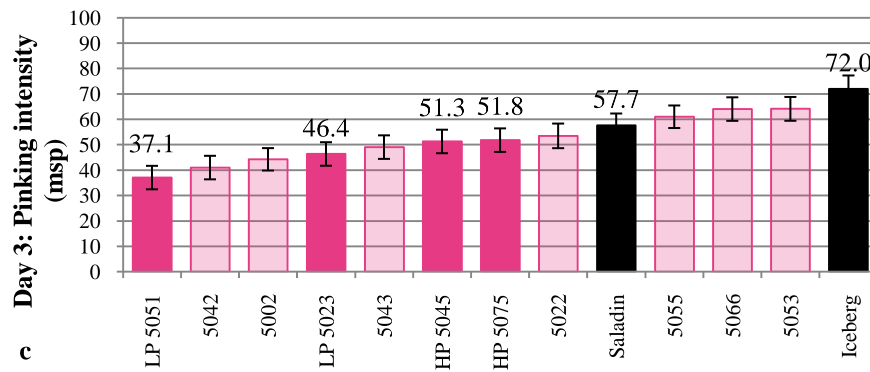
	Significance level		
Extent of overall discolouration	**REML Wald Statistic**	**ddf**	**REML P value**
Day 1	64.97	29.6	***<0.001
Day 2	60.98	25.8	***<0.001
Day 3	44.56	20.9	**0.005
Day 4	17	26.4	0.22

The mapping parents Saladin and Iceberg were not significantly different for overall discolouration (both intensity and extent) until day 4. Iceberg was the best performing parent for overall discolouration intensity while Saladin was the best performing parent for extent of overall discolouration. Low overall discolouration (LD) RILs 5002 and 5042 (selected for low overall discolouration scores) showed similar scores of overall discolouration intensity on all days and they performed

significantly better than both parents on day 1 for overall discolouration intensity and on all days for the extent of overall discolouration. A range of RILs from the extreme set selected for other phenotypes also performed significantly better than both parents (and LD RILs for overall discolouration intensity) until day 3 (including RILs 5023, 5051 and 5075). Although it was not the most susceptible RIL to overall discolouration and did not perform significantly worse than the poorest parent the RIL 5066 selected for high overall discolouration scores, was amongst the poorest performing RILs from within the extreme RIL set. Significant variation was observed between LD and HD RILs for overall discolouration (both intensity and extent) on days 1 and 2, and for extent of overall discolouration on days 3 and 4. A similar pattern for rate of overall discolouration intensity was recorded as for pinking intensity; with those initially displaying low levels of overall discolouration intensity demonstrating higher rates of reaction to those with a primarily high overall discolouration intensity score (with RILs not reaching the possible maximum score for overall discolouration intensity).







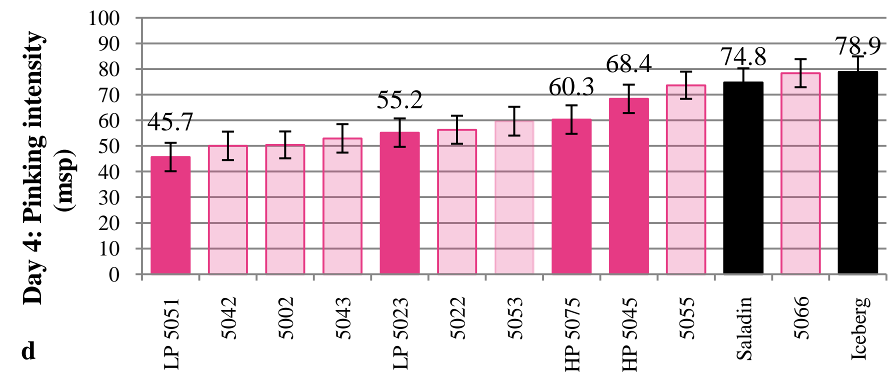
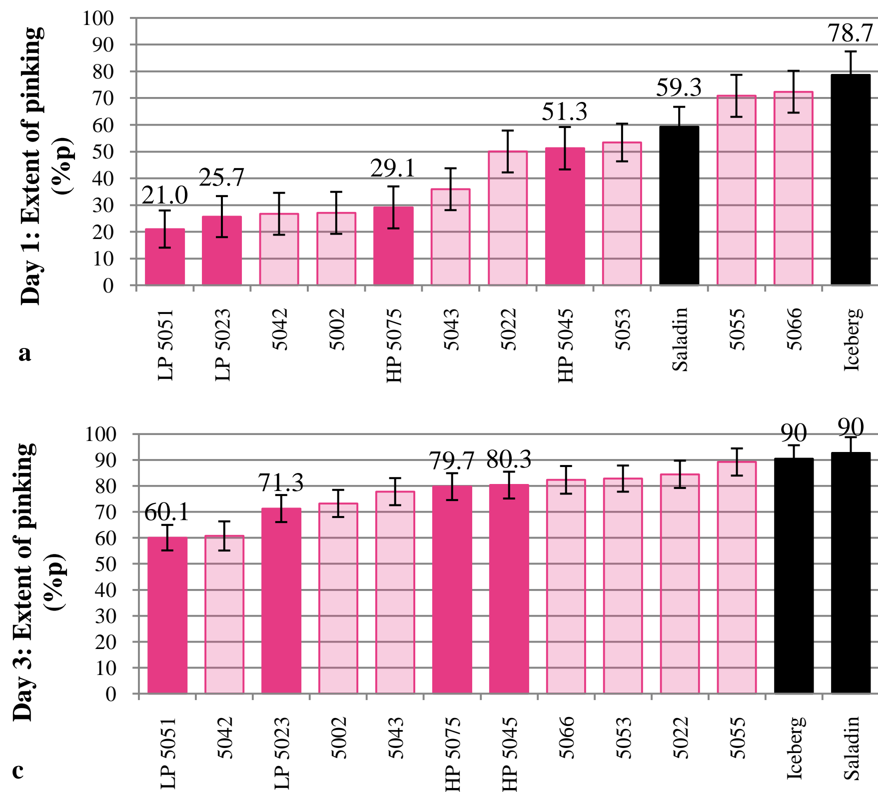


Fig.5.16. Transformed adjusted means (from REML) for lettuce post harvest pinking intensity on a) day 1, b) day 2, c) day 3 and d) day 4 for the F_7 mapping population discolouration extreme RILs. Error bars represent sems (standard error of the mean) from REML. The extreme RILs for pinking intensity are highlighted with respective adjusted means (from REML). Where *LP* (low pinking RIL); *HP* (high pinking RIL).



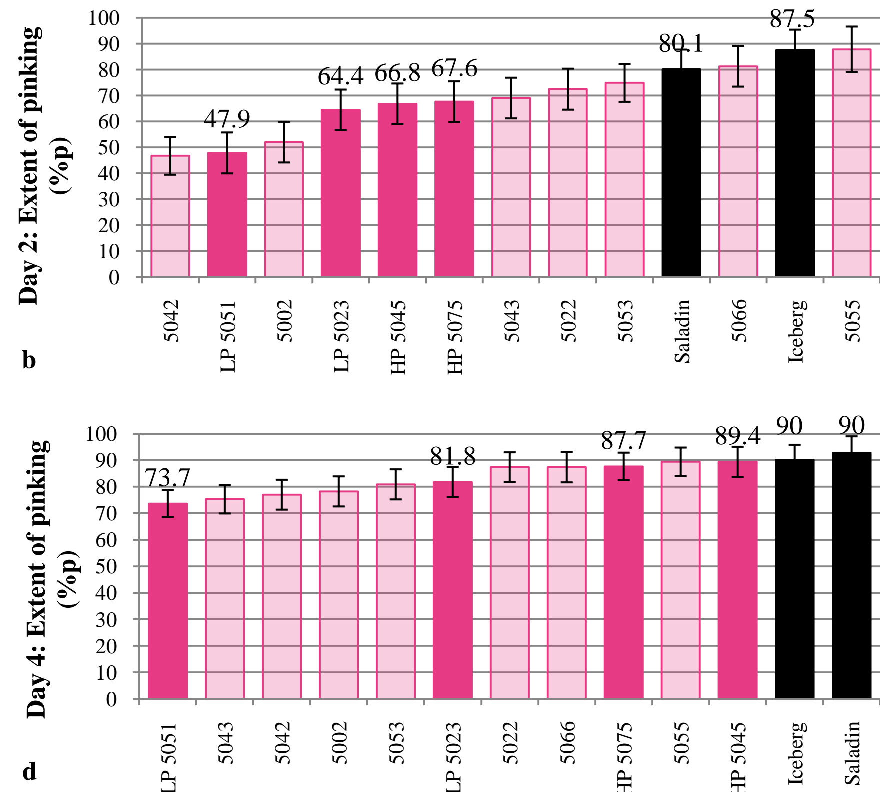
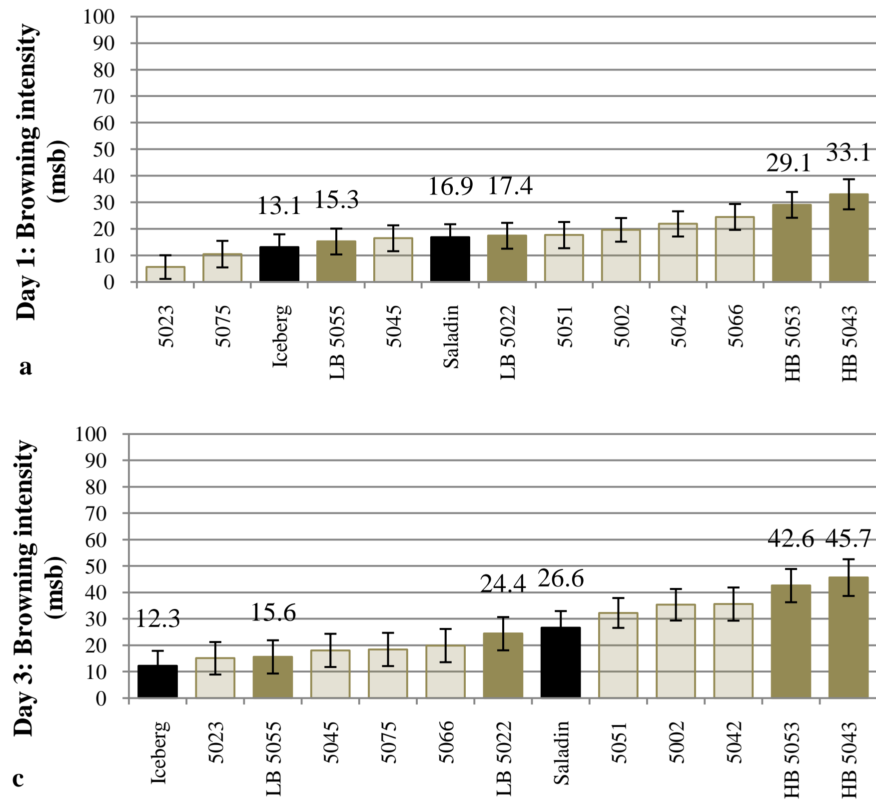
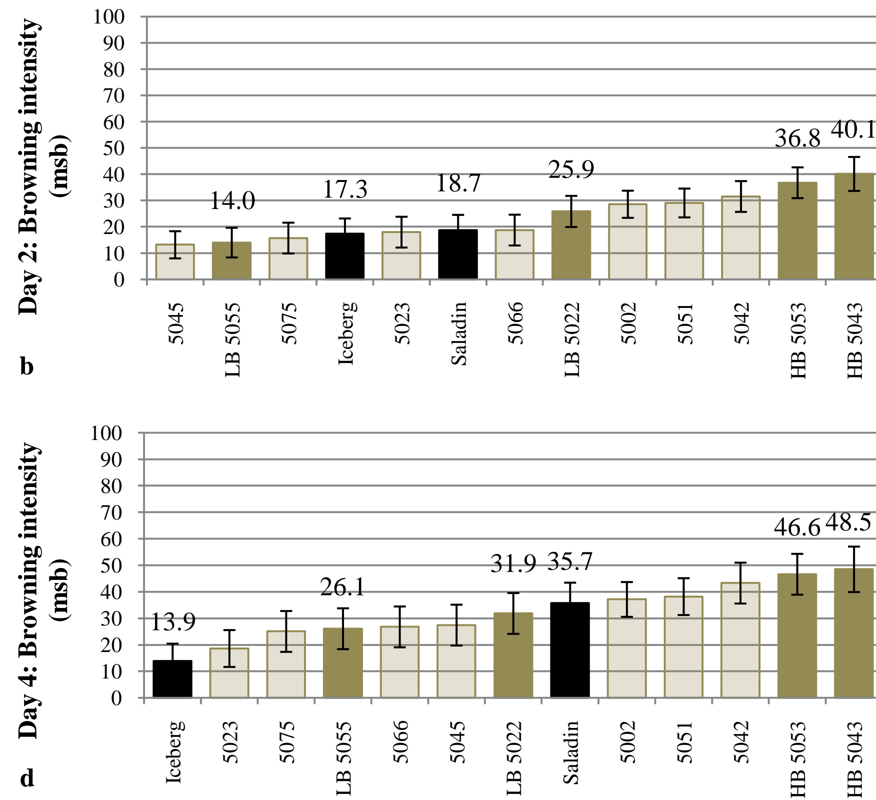
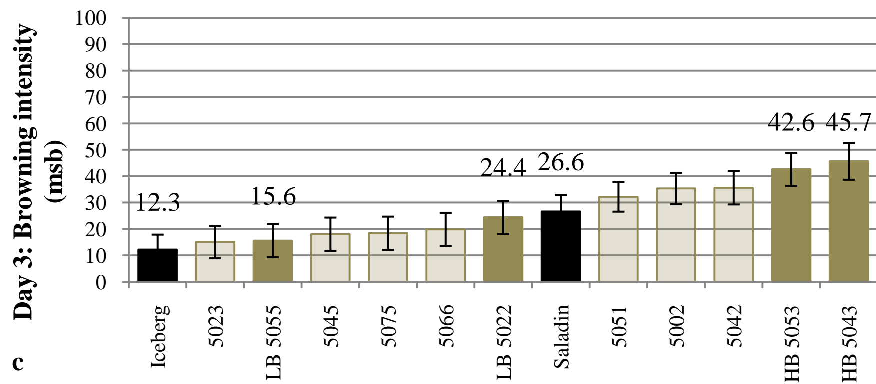


Fig.5.17. Transformed adjusted means (from REML) for extent of lettuce post harvest pinking on a) day 1, b) day 2, c) day 3 and d) day 4 for the F_7 mapping population discolouration extreme RILs. Error bars represent sems (standard error of the mean) from REML. The extreme RILs for pinking intensity are highlighted with respective adjusted means (from REML). Where *LP* (low pinking RIL); *HP* (high pinking RIL).







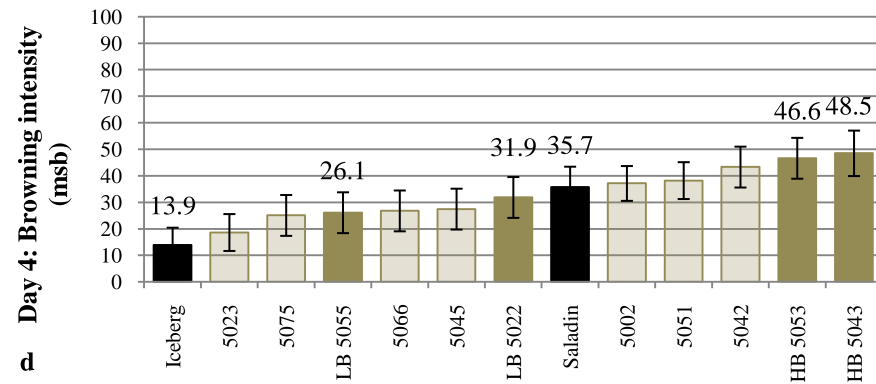
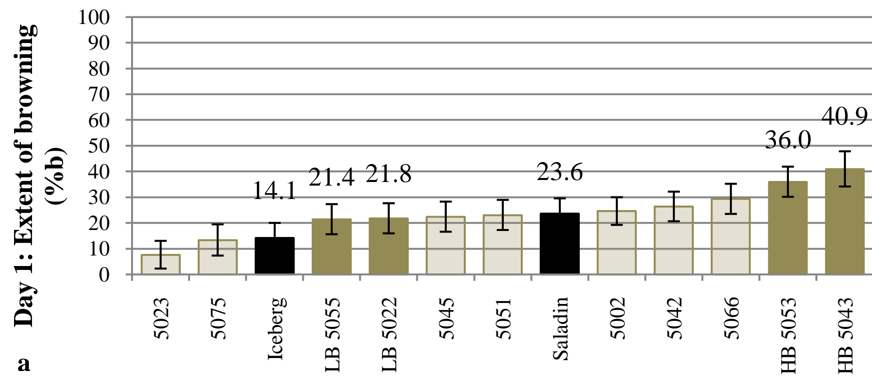
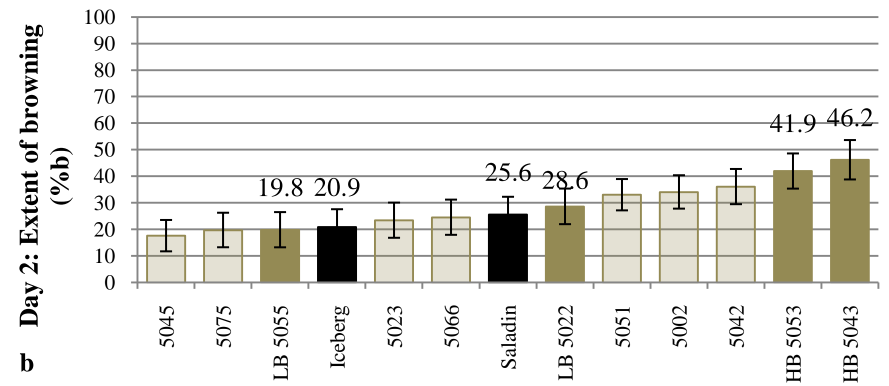
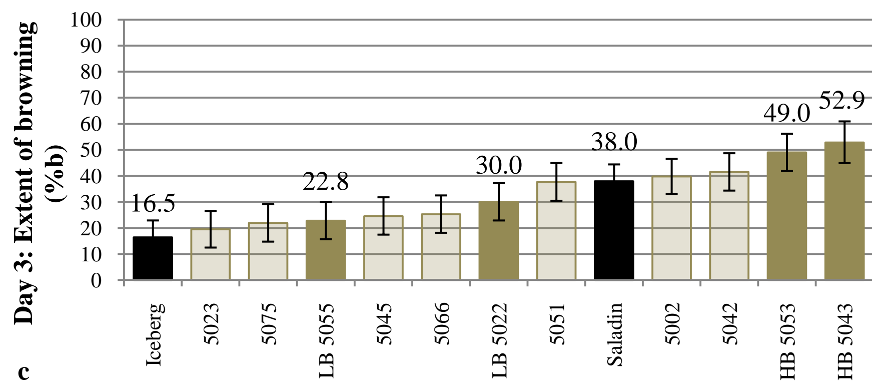


Figure 5.18. Transformed adjusted means (from REML) for lettuce post harvest browning intensity on a) day 1, b) day 2, c) day 3 and d) day 4 for the F_7 mapping population discolouration extreme RILs. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML). Where *LH* (low browning RIL); *HB* (high browning RIL).







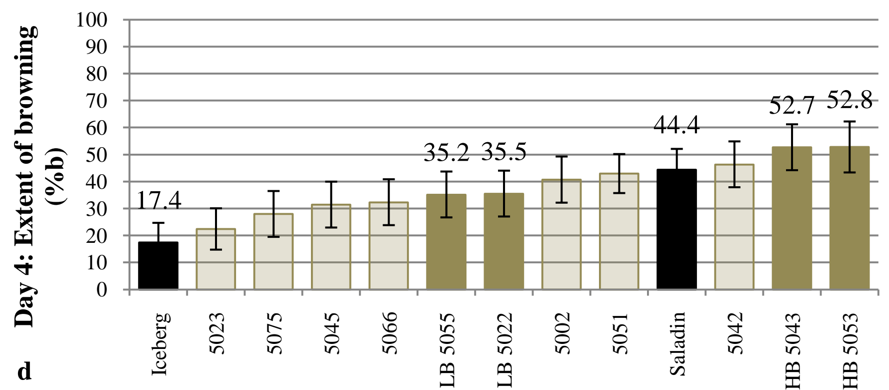


Figure 5.19. Transformed adjusted means (from REML) for extent of lettuce post harvest browning on a) day 1, b) day 2, c) day 3 and d) day 4 for the F_7 mapping population discolouration extreme RILs. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML). Where *LH* (low browning RIL); *HB* (high browning RIL).

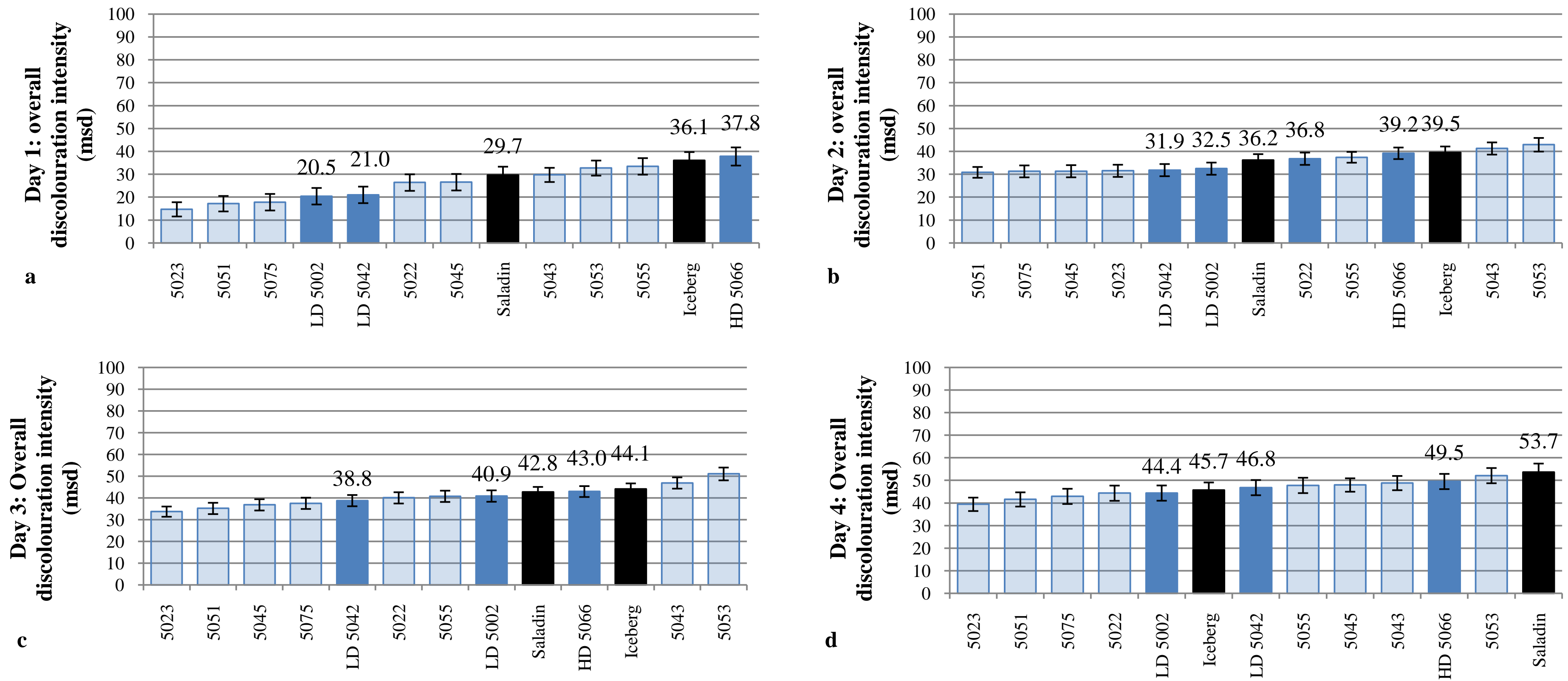
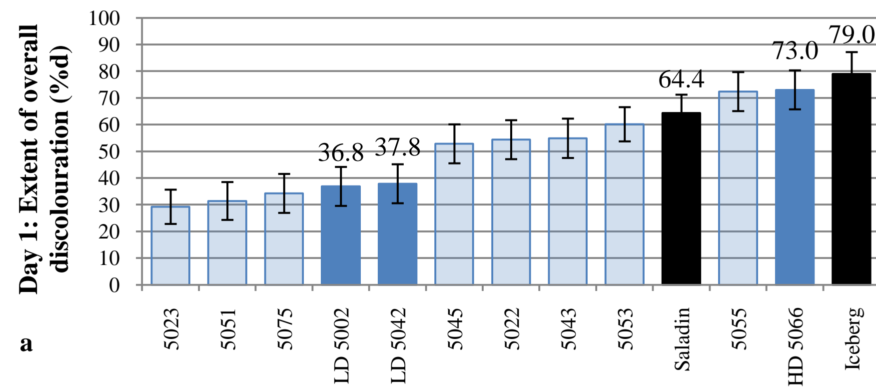
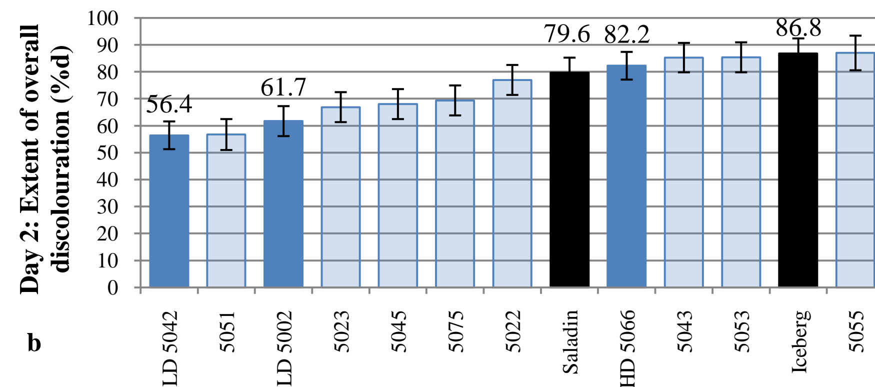
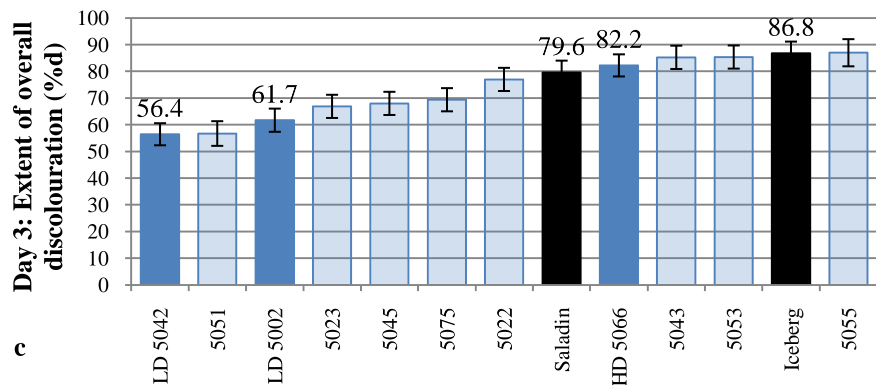


Figure 5.20. Transformed adjusted means (from REML) for lettuce post harvest overall discolouration intensity on a) day 1, b) day 2, c) day 3 and d) day 4 for the F_7 mapping population discolouration extreme RILs. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML). Where *LD* (low overall discolouration RIL); *HD* (high overall discolouration RIL).







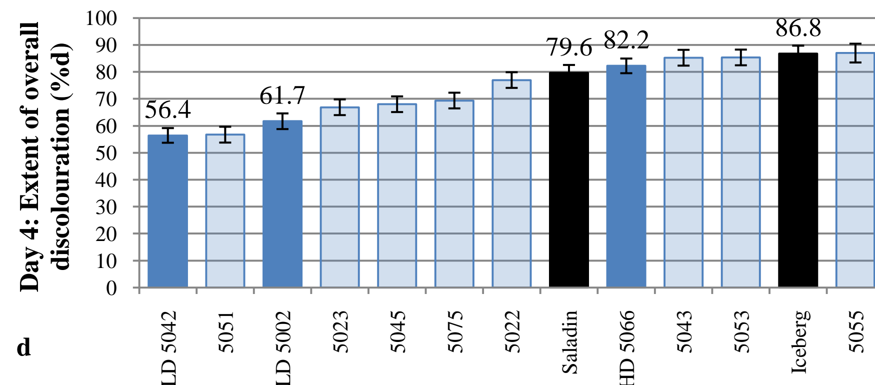


Figure 5.21. Transformed adjusted means (from REML) for extent of lettuce post harvest overall discolour on a) day 1, b) day 2, c) day 3 and d) day 4 for the F_7 mapping population discolouration extreme RILs. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML). Where *LH* (low browning RIL); *HB* (high browning RIL).

5.4. Discussion

Significant phenotypic variation for all measures of post harvest discolouration was observed in the Saladin x Iceberg RIL population, with a wide range of phenotypes recorded when assessed in different environments (UK and NL). When meaned across all RILs, the level of pinking and browning was similar on day 1 but in the later stages post harvest 'pinking' generally became the more important form of discolouration. This agrees with the findings for the lettuce diversity set reported in Chapter 3.

The parents of the RIL population, Saladin and Iceberg were generally significantly different for all measures of discolouration on day 1 and pinking on day 3. Although the parents were not always significantly different, Saladin was always the best performing parent. Saladin and Iceberg are both cultivars of lettuce and therefore an inbred population resulting from a cross between them might be expected to show high levels of transgressive segregation (Rieseberg *et al.* 1999, 2003). Transgressive segregation was observed in the RIL population for each discolouration response. For pinking, there were RILs which performed better than the best parent Saladin. In contrast, for browning, transgressive segregation was recorded for RILs which performed worse than the poorest parent Iceberg. For overall discolouration there were RILs which performed either better or worse than the best or poorest parent which is not unexpected given the results for the individual discolouration responses. However, no one RIL consistently showed transgressive segregation. Quantitative genetic studies suggest that the accumulation of complementary genes is the principal cause of transgression, although epistasis, overdominance and a high

mutation rate have also been reported as having a possible role (Rieseberg *et al.* 1999, 2003).

The partitioning of the phenotypic variation by REML analysis showed that the effect of line (i.e. genotype) was consistent across sites for pinking and overall discolouration. However, significant variation between RILs for browning was site specific and only recorded in the NL trial. There were also significant site effects: for pinking for day 1 and for browning for day 3, indicating that the plants' growing environment significantly influences their post harvest performance. This is in agreement with Hilton *et al.* (2009) who showed that post harvest discolouration of lettuce could be manipulated by changes in growing conditions. The trials in the UK and NL were designed for genetic analysis with the aim to provide robust phenotypes for QTL analysis and to determine whether there was any phenotypic plasticity (Gurganus *et al.* 1999) over environments, which was achieved.

Post harvest discolouration is a complex trait and therefore each type of discolouration is likely to be controlled by many QTL with small effect (Zhang *et al.* 2007). The QTL analysis carried out in this study identified 21 QTL for post harvest discolouration traits; 10 QTL for browning, 9 QTL for pinking and 2 QTL which were identified as influencing overall discolouration. These were identified by the co-location of many putative QTL for the same response (see figure 5.15.). The putative QTL all had relatively low effects and 'explained' low levels of the observed genetic variation (for pinking the highest was 22.6% and the lowest was 7.8%, for browning the highest was 14.8% and the lowest was 7.9%, and for overall discolouration the highest was 17.4% and the lowest was 6.1%) and much of the observed variation was left unexplained by the QTL analysis suggesting that there are probably other QTL for each trait that were not detected in this experiment

(Zhang *et al.* 2007). False negative QTL errors (Type II QTL errors) are determined by the experimental set-up and the size of the genetic effect of QTL (Van Ooijen 1999). More replicates of discolouration data (including plot, rep and site) may have allowed more QTL to be identified. A few significant QTL were based on single putative QTL (*Br7, Br9, Pink7, Pink8* and P*ink9*) which could be an example of Type I errors (false positives), which are determined by the significance threshold (Van Ooijen 1999). However the possibilities of these errors have been minimised by using the MQM approach.

The marker alleles for significant QTL were from both mapping parents, Saladin and Iceberg; this can be explained as the parents were not significantly different for all measures of discolouration. Of the 21 QTL, 12 QTL were linked to the best performing parental marker allele, Saladin (6 for pinking, 5 for browning, and 1 for overall discolouration) and 9 QTL were linked to the poorest performing parental marker allele, Iceberg (3 for pinking, 5 for browning, and 1 for overall discolouration). Thus some beneficial alleles were derived from the poorer performing parent Iceberg. This provides an explanation of the transgressive segregation observed in the RILs (see tables 5.5., 5.8., 5.11. and 5.12.) as beneficial alleles from the two parents can accumulate in RILs to give genotypes with better/worse phenotypes than the parents. Some of the RILs which had significantly improved phenotypes in comparison to the best performing parent Saladin possessed beneficial alleles from Iceberg.

Three clusters of QTL determining post harvest discolouration were recorded on LG 4 and LG 7 (see figure 5.22.). Three QTL for browning were loosely clustered on LG 4 (*Br3, Br4, Br5*; 4.4-63.5 cM) and 3 QTL for browning were also loosely clustered on LG 7 (*Br8, Br9, Br10*; 17.7-54.2 cM). Three QTL for pinking were

loosely clustered on LG 7 (*Pink4, Pink5* and *Pink6*; 1.7-54.2 cM). These three regions could in fact be the manifestation of 3 larger single QTL, or they could be linked. *Br3* and *Pink2* are closely clustered on LG 4 (4.4-8.8 cM), while *Br8* and *Pink5* (19.3-21.7 cM), and *Br10* and *Pink6* (44.4-54.2 cM) were co located separately on LG 7. This would suggest that although there were no highly significant phenotypic correlations (see table 5.13.) between pinking and browning, the two responses do have some common underlying genetic basis which may impact on the efficiency of a purely phenotypic selection process in a breeding programme.

The nearest markers associated with the QTL could be exploited by breeders using marker assisted selection (MAS) to breed for reduced post harvest discolouration (by selecting for beneficial alleles) and therefore extend the shelf life of salad products (see table 5.16.). However before going to the expense of applying MAS, it would be useful to gain an understanding of the stability of phenotype over environment and years; this was tested through the field trial of RILs with extreme phenotypes.

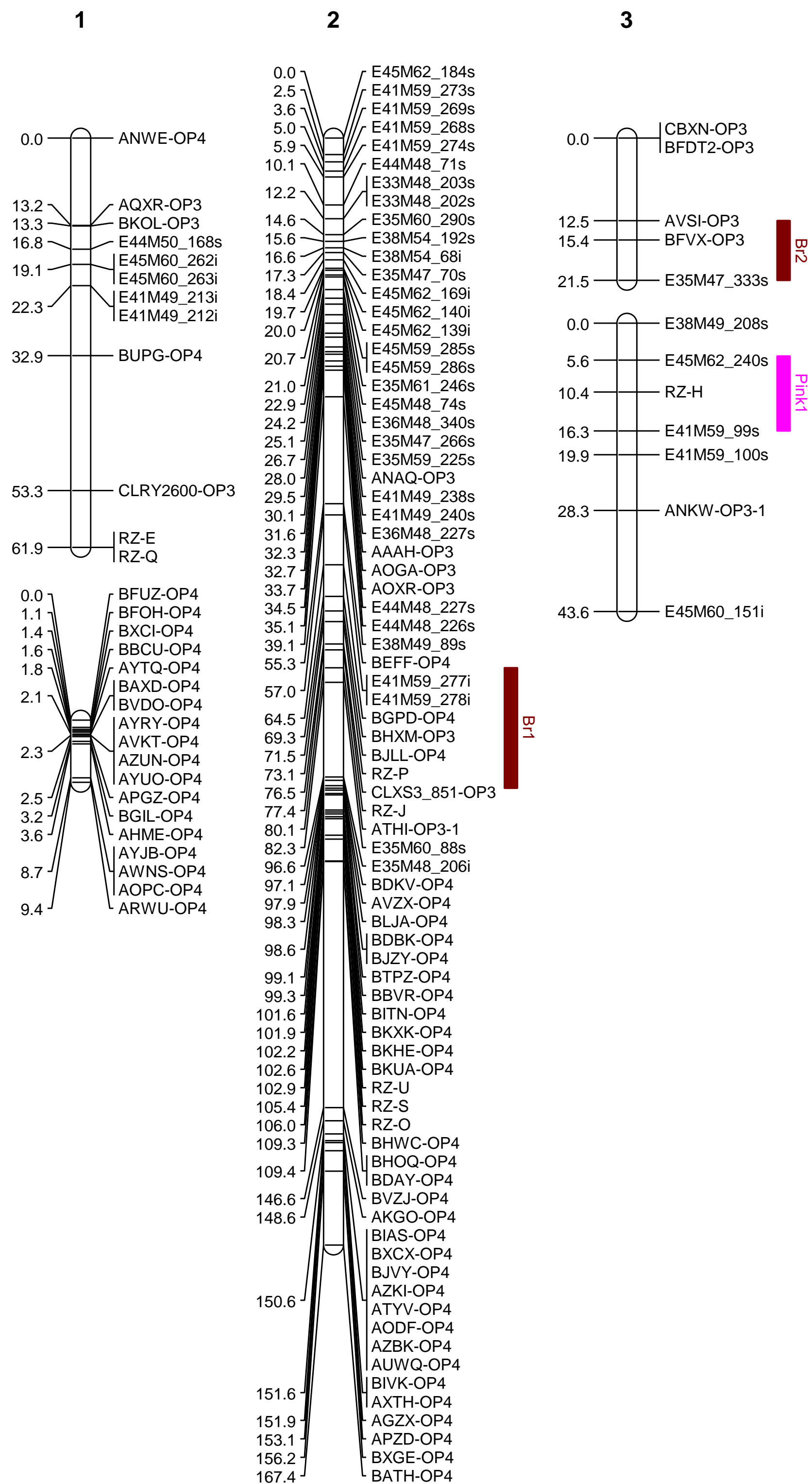


Figure 5.22. QTL impacting on post harvest discolouration of lettuce tissue from the Saladin x Iceberg RIL population. Where *Br* (browning); *Pink* (pinking); *Dis* (overall discolouration). Number after discolouration parameter refers to QTL number for that specific trait.

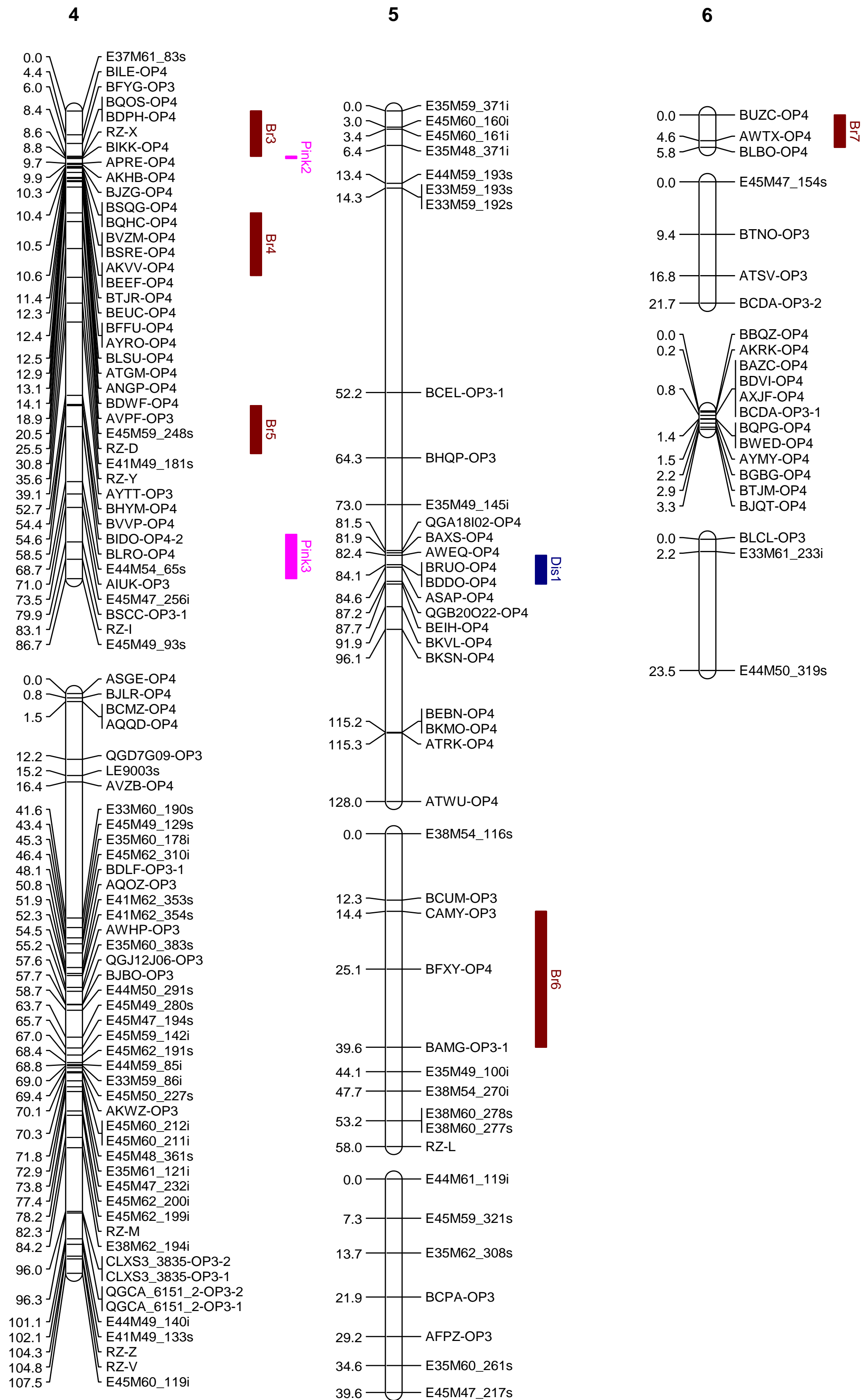


Figure 5.22. continued

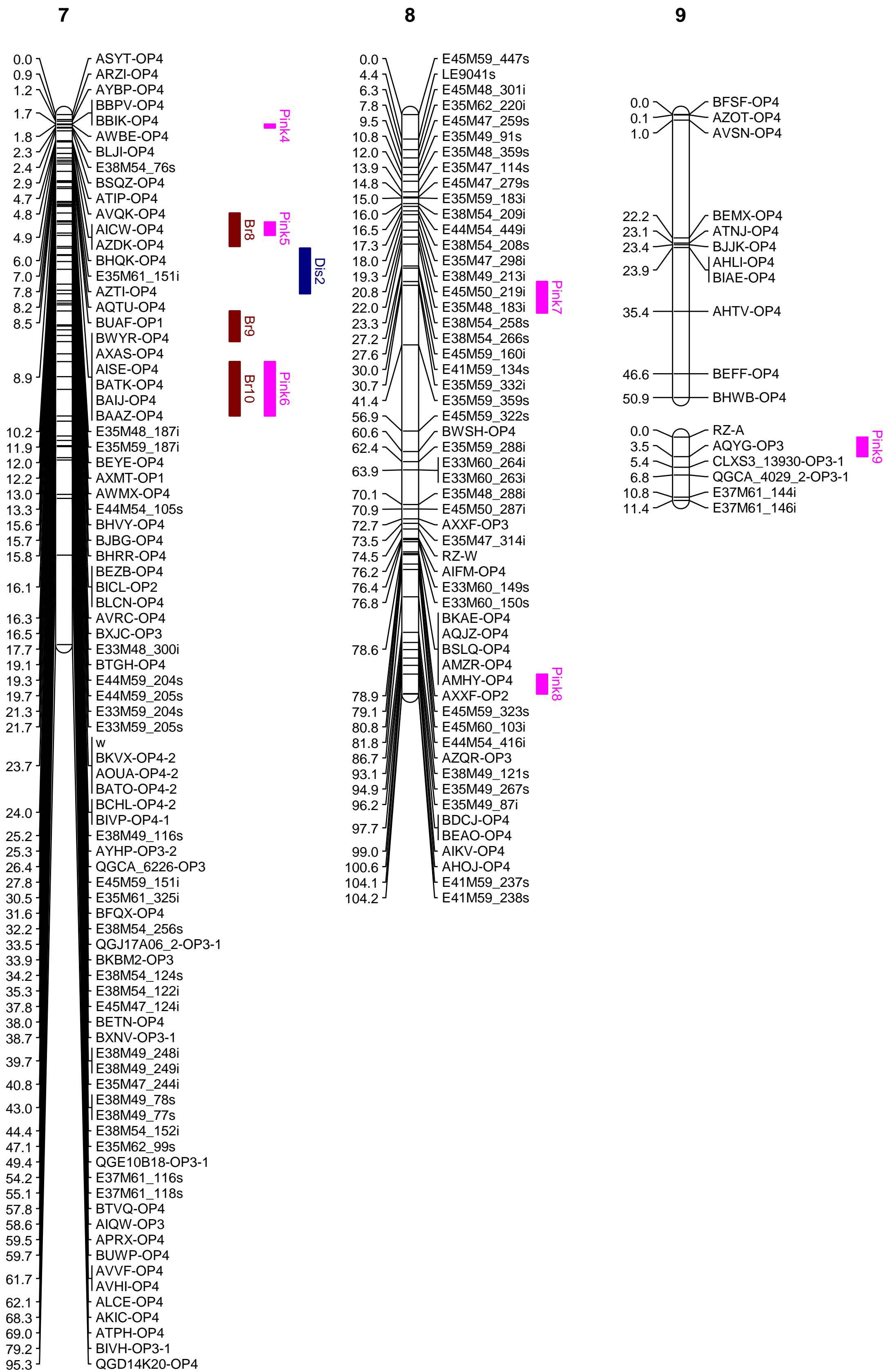


Figure 5.22. continued

Although some RILs performed significantly better or worse than the RILs selected for a specific type of discolouration, RILs generally performed as they did in the 2008 trial; those selected for an extreme discolouration type (i.e. pink, brown or overall discolouration) showed the same phenotype in the 2009 trial. This would suggest that although a site effect was observed in the 2008 trial and the extreme discolouration subset RILs were only tested in a UK trial in 2009 (see table 5.4.) the phenotypes of these RILs were largely determined by genotype and not environment.

Certain RILs from the extreme subset showed significantly lower levels of post harvest discolouration in comparison to the best parent Saladin. Saladin is an iceberg type lettuce; it is accepted by consumers as a high quality lettuce and has been widely used in breeding of modern cultivars. The results therefore indicate that it would be possible to extend the shelf life of lettuce as a component of bagged salads by accumulating naturally occurring alleles that give a good phenotype. An increase of the shelf life would have great financial benefit to the cut salad industry and it therefore may be beneficial to exploit the commercial potential of RILs showing reduced discolouration through breeding. However, before selecting for significantly reduced post harvest discolouration it would be useful for lettuce breeders to have some understanding of any possible relationship between post harvest discolouration and agronomic traits, in order to be able to optimise shelf life while still being able to select for different quality traits (this is addressed in Chapter 6).

5.5. Conclusions

- There was significant genetic variation for post harvest discolouration in the F_7 Saladin x Iceberg RIL mapping population.
- Twenty-one significant QTL for post harvest discolouration traits have been identified.
    - 9 QTL for pinking.
    - 10 QTL for browning.
    - 2 QTL for overall discolouration.
- Post harvest discolouration quality traits can be selected for by lettuce breeders; markers for use in MAS have been identified for each QTL.
- Phenotypes for post harvest discolouration in the F_7 Saladin x Iceberg RILs demonstrated stability over environments.

CHAPTER 6

Assessing agronomic traits and their potential influence on post harvest discolouration in lettuce

6.1. Introduction

Agronomic traits determine the acceptance of the product in the market; however there have been limited QTL studies into agronomic traits of lettuce. For breeders it is important to understand the relationship between agronomic traits and post harvest discolouration as changes in other traits of interest may have pleiotropic effects. In Chapter 3 significant correlations were found between head diameter or weight and post harvest discolouration which were hypothesised to be due to water relations in the leaf.

Breeders need to understand the relationship between agronomic traits and post harvest discolouration in order to optimise shelf life while still selecting for different quality traits. QTL analysis can allow the assessment of linkage (i.e. co location) of QTL determining different traits. It can also provide DNA markers linked to agronomic traits for marker assisted selection and also to select for recombinants to break any undesirable linkages between traits.

The aims of this experiment were to

- Identify significant QTL for a range of important agronomic traits.
- Determine whether QTL for agronomic traits were co-located with any post harvest QTL identified in Chapter 5.

6.2. Materials and methods

6.2.1. Field trial of pre harvest agronomic traits of the F_7 Saladin x Iceberg mapping population

The F_7 Saladin x Iceberg mapping population (125 lines) were grown for observational purposes in a replicated field trial during the 2007 growing season on the experimental site Sheep Pens (west) at Warwick HRI, UK (Latitude: 52.183. Longitude: 1.583). Plants were raised and maintained as previously described (section 2.2.1.), with the trial planted on 1st May 2007. The trial was designed in 4 blocks, containing a single plot (of 12 plants) of the 125 lines in numerical order (see appendix D for field plan). Crop protection was as previously described (see section 2.2.1.) with the additional use of 'Greencrop Saffron FL' propyzamide herbicide (3.5 L/ha) according to good agricultural practice. Fencing and flappers also surrounded the experimental plot to provide protection from the local fauna (figure 3.1.).

6.2.2. Assessment of pre harvest agronomic traits

The F_7 RILs and the mapping population parents were scored prior to sowing on a two-point scale for seed colour (brown/white). Morphological traits were then assessed in the field on an ordinal scale when >50% of heads of each line reached maturity level. The RILs were scored on a two-point scale for whether they were heading or non-heading (yes/no). They were also scored on a three-point scale per trait for green pigmentation, anthocyanin pigmentation, indented leaf edges, savoy/blistering of leaves and plant diameter (small/medium/large) (see figure 6.1.).

Green pigmentation (1/2/3)

Anthocyanin pigmentation (1/2/3)

1

No anthocyanin pigmentation, therefore leaf as green pigmentation (1/2/3) from above.

Indented leaf edges (0/1/2)

Savoy/blistering of leaves (0/1/2)

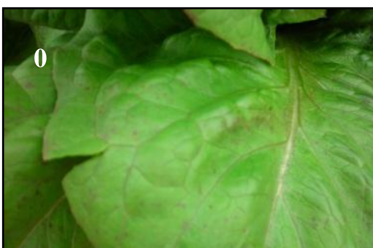


Figure 6.1. Ordinal scale for morphological trait scoring for the F_7 Saladin x Iceberg mapping population. Where *number* (trait score).

When the line appeared to be segregating all phenotypes were noted and the genotype was excluded from the analysis. RILs were scored for days to harvest and days to bolting and were subsequently also classified on a three point scale as early/mid/late.

6.2.3. Statistical analysis

Histograms were produced for all agronomic traits scored on 2 or 3 point scales (see section 6.2.2.) and for the different measures relating to head weight of plants using data gathered from the field trials of the RIL population described in Chapter 5. The histograms (see figure 6.2. a-n) were used to assess the distribution of the trait data. For traits measured on a two point scale chi square tests were carried out to determine if the numbers in each class were equal.

Chi Square analyses

Chi square tests were conducted to see if the distribution for traits with 2 categories were significantly different from 1:1, with 1 degree of freedom.

Correlation analysis

Correlation analyses were conducted between measures of agronomic traits and all measures of post harvest discolouration for RILs (see section 2.4.4.). R values were generated which were then compared to the R values expected for different levels of probability to assess their significance (see table 5.2.).

6.2.4. QTL analysis

One hundred and twenty-five RILs used to develop the genetic linkage map (see Chapter 4) were used for QTL analysis as previously described (see section 2.2.3.) using phenotype data scored on each RIL as described above.

6.3. Results

6.3.1. Variation for morphological traits

For the trait of heading and non-heading of the plant, the observed numbers of RILs for each category are not significantly different from that expected for equal frequencies ($X^2_{[1]} = 0.17$), while for head diameter the middle category of 'medium size' was dominant (see figure 6.2. a and b). For both untrimmed and trimmed head weights recorded in the UK trial, frequencies were skewed towards lower weights, while the frequency distribution for trimmed weight in the NL trial and for trimmed weight across sites formed bell shaped normal distributions (see figure 6.2. k-n). For leaf morphology, the frequency distribution of savoy/blistering of leaves was skewed towards leaves with sever blistering, while the central category of slight indentation was dominant for leaf indented edges (see figure 6.2. c and d). For leaf pigmentation, the central category of medium greenness was dominant for greenness of leaf; while the frequency distribution of red anthocyanin pigmentation was severely skewed to no pigmentation (see figure 6.2. e and f). For seed colour (brown and white) the observed numbers of RILs for each category are not significantly different from that expected for equal frequencies ($X^2_{[1]} = 2.72$).

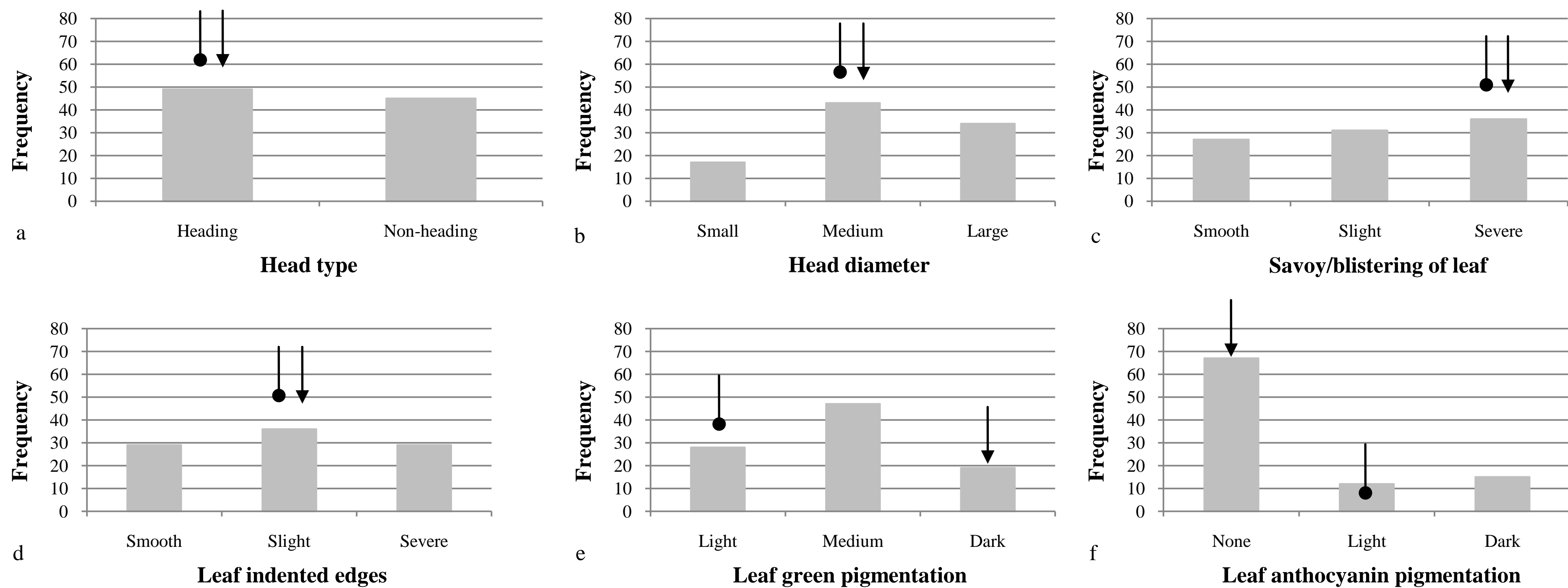
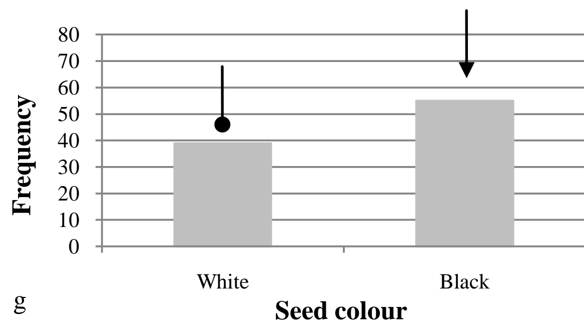
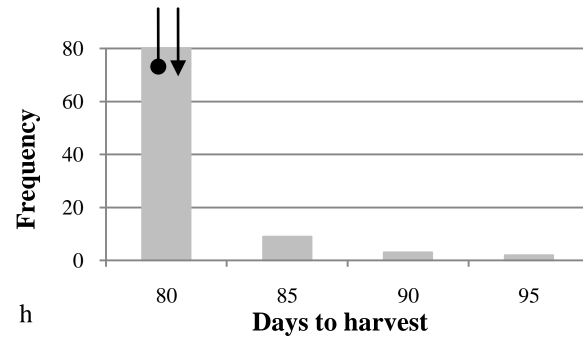
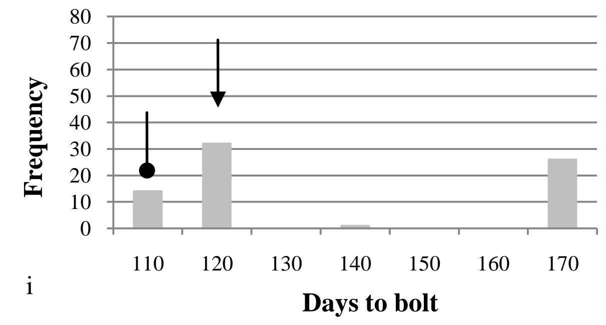
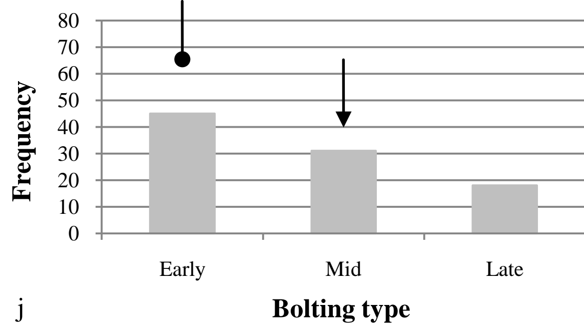


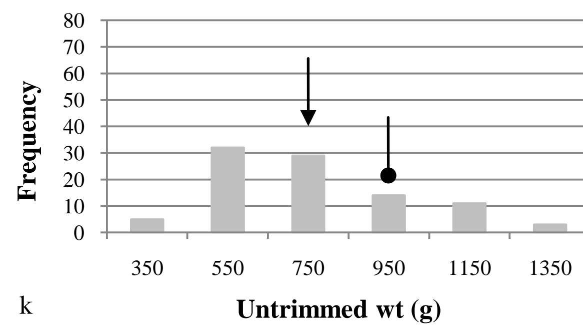
Figure 6.2. Distributions of selected traits in the RIL population for a) production of a head, b) head diameter, c) savoy/blistering of the leaf, d) indentation of leaf edges, e) green pigmentation of leaf, f) anthocyanin pigmentation of leaf, g) seed colour before sowing, h) days to ready for harvest, i) days to plant has bolted, j) bolting type, k) untrimmed weight of head in UK trial, l) trimmed weight of plant head in UK trial, m) trimmed weight of plant head in NL trial, n) trimmed weight of plant head across sites. Saladin is ⟶ and Iceberg is —●. Where *wt* (weight); *g* (grams).

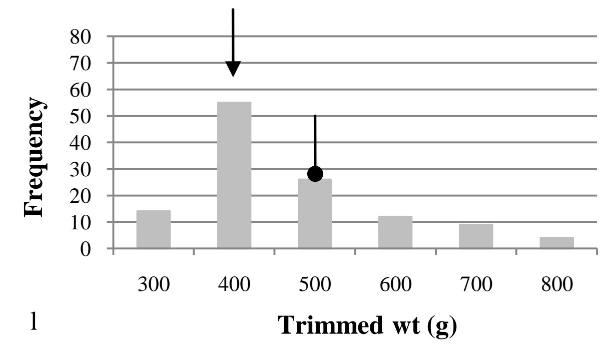


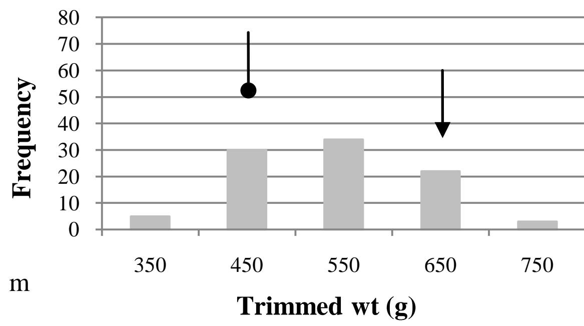












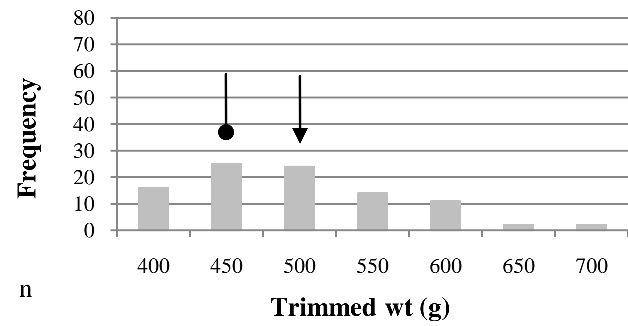


Figure 6.2. continued.

For life cycle related traits, the distribution frequencies of the RIL population were severely skewed. The frequency distribution for days to harvest was skewed to fewer days until harvest, while for days to bolting the distribution frequency was bi-modal but for general bolting time the distribution was skewed to early bolters (see figure 6.2. h-j).

6.3.2. Correlations amongst traits

Morphological traits (from the observational trial above) and all measures of post harvest discolouration between and across sites (recorded in Chapter 5) were assessed for potential relationships (correlations between morphological traits and between them and browning and pinking for each trial and across sites (the full correlation matrix is given in Appendix D)). Only the highly significant correlations (***P <0.001) are described below (see table 6.1.). Although there were significant correlations, many of them had relatively low values for the coefficient of correlation ‘r; rather than give all values of r for the different data sets (i.e within site and across sites) only the highest value is given to illustrate the degree of correlation (all of the coefficients of correlation are given in Appendix D).

Plant weights (trimmed and untrimmed) were positively correlated with each for the UK trial and across site data. The highest correlation was plant trimmed and untrimmed weight in the UK ($R_{[90]} \geq 0.72$). However plant weight (trimmed) recorded in the NL trial did not correlate with measures from the UK trial or the across site data. This is likely to be due to the fact that plants grown in the NL trial were more severely trimmed following Rijk Zwaan standard procedures, than the plants grown at Wellesbourne. Measures of plant weight (trimmed and untrimmed in

both trials) were positively correlated with diameter (the highest correlation was with untrimmed weight ($R_{[90]} \geq 0.41$)) but negatively correlated with head production (the highest correlation was with untrimmed weight ($R_{[90]} \geq -0.38$)). While diameter was negatively correlated with head production ($R_{[90]} \geq -0.58$). Head production was highly correlated with indented leaf edges ($R_{[90]} \geq 0.72$), while both were positively correlated with days to bolting ($R_{[90]} \geq 0.36$ and $R_{[90]} \geq 0.53$). Savoy/blistering of the leaves was also positively correlated with both production of a head ($R_{[90]} \geq 0.39$) and indented leaf edges ($R_{[90]} \geq 0.34$). Greenness of leaf and leaf anthocyanin pigmentation were also positively correlated ($R_{[90]} \geq 0.35$).

Plant weight measurements (trimmed and untrimmed) were positively correlated with browning on day 3 in the UK trial (the highest correlation was between trimmed weight and extent of browning ($R_{[90]} \geq 0.37$)) and positively correlated with pinking on day 3 in the NL trial (the highest correlation was between trimmed weight and extent of pinking ($R_{[90]} \geq 0.35$)). Head diameter was generally negatively correlated with pinking in both trials and across sites (the highest correlation was with pinking intensity on day 1 in the UK trial ($R_{[90]} \geq -0.31$)) and was positively correlated with browning on day 3 in both trials and across sites (the highest correlation was with browning intensity in the UK trial ($R_{[90]} \geq 0.35$)). Head production was positively correlated with pinking in both trials and across sites (the highest correlation was with extent of pinking on day 3 across sites ($R_{[90]} \geq 0.55$)) and was generally negatively correlated with browning in both trials and across sites (the highest correlation was with browning intensity on day 3 across sites ($R_{[90]} \geq -0.45$)).

Table 6.1. Correlation matrix from the WHRI Saladin x Iceberg mapping population for post harvest discolouration and morphological parameters scored in 2008 experimental trials. Read across then down. Only significant effects are shown and highly significant effects ***P <0.001 are shown bold. Where *unt wt* (untrimmed weight, g); *tr wt* (trimmed weight, g); *dia* (diameter); *head* (production of head); *indnt* (indented leaf edges); *sav* (savoy leaves); *grn* (green); *anth* (anthocyanin pigmentation); *dharv* (days to harvest); *dbolt* (days to bolt); *msb* (mean score browning); *msp* (mean score pinking); *msd* (mean score overall discolouration); *%b* (percentage browning); *%p* (percentage pinking); *%d* (percentage overall discolouration); *UK* (UK site); *NL* (Netherlands site). Degree of freedom is 90.

	msp	**msb**	**%p**	**%b**	**%d**	**UK msp**	**UK msb**	**UK %p**	**UK %b**	**NL msp**	**NL msb**	**NL %p**	**UK unt wt**	**dia**	**head**	**indnt**	**grn**
UK tr wt		0.30		0.28			**0.34**		0.33								
UK unt wt		**0.42**		**0.39**			0.34		0.31	-0.23	0.24						
dia	-0.28	0.31	-0.30	0.23	-0.25	-0.21	0.30	-0.30	0.23				**0.41**				
head	**0.58**	**-0.44**	**0.59**	-0.32	**0.45**	**0.40**	-0.33	**0.47**	-0.25	**0.49**	**-0.34**	**0.46**	**-0.38**	**-0.58**			
indnt	**0.36**	-0.21	**0.36**		0.28	0.27	-0.22	0.30		0.29		0.27		-0.32	**0.72**		
sav	0.30	-0.21	0.32		0.32		-0.25		-0.22	0.31		0.31	-0.30	-0.28	**0.39**	**0.34**	
grn	-0.22		-0.25		-0.29	-0.25		-0.27			0.21					-0.23	
anth																	**0.35**
dharv					-0.21				-0.21								
dbolt	0.24		0.21			0.23		0.24							**0.36**	**0.53**	
seed	0.32	-0.21	**0.34**		0.29	0.26		0.31		0.22	-0.23	0.22		-0.27	0.28	0.24	

Leaf indentation and savoy/blistering of leaves both showed positive correlations with pinking. Savoy/blistering of leaves was generally positively correlated with pinking for both trials and across sites trial (the highest correlation was with extent of pinking on day 1 across sites ($R_{[90]} \geq 0.38$)). Leaf indentation was positively correlated with pinking for both trials and across sites (the highest correlation was with extent of pinking on day 1 across sites ($R_{[90]} \geq 0.34$) and pinking intensity on day 1 across sites ($R_{[90]} \geq 0.34$)). Seed colour was generally positively correlated with pinking from both trials and across site (the highest correlation was with extent of pinking on day 1 across sites ($R_{[90]} \geq 0.31$)).

6.3.3. QTL mapping for agronomic traits

Correlation analysis demonstrated there were significant relationships between the different agronomic traits and between agronomic traits and post harvest discolouration traits (see section 6.3.1 and Chapter 5). Histograms also showed bell shaped normal distributions for most traits, relating to normal distributions. Scores (see figure 6.1.) and means were subsequently combined with the linkage map (see Chapter 4) to carry out a QTL analysis for all agronomic traits to investigate the genetics of each trait.

QTL analysis was performed separately on agronomic trait data and weight-based data from both trials in addition to data summarised across sites. Interval mapping using MapQTL ®4.0 software identified a total of 48 putative QTL for agronomic traits (see appendix D), while the subsequent multiple QTL model mapping (MQM) increased accuracy of the identification of QTL (by taking into account the effect of other QTL) and reduced the number of QTL to 20 (see figure 6.3. and table 6.2.).

The 20 QTL (*P <0.05) were detected with LOD significance threshold greater than 1.95 which was based on the simulation study based on an F_7 RIL experimental population (Van Ooijen 1999) (see table 6.2.). Individual putative QTL accounted for between 6.2-98.6% of the recorded variation for individual traits scored in this population and were located on 6 linkage groups (LGs 2, 4a and b, 5a, b and c, 7, 8 and 9) with highest number being mapped on LG 7 (see figure 6.3. and table 6.2.). Fourteen QTL were detected for morphological traits (2 for savoying, 3 for indentation, 4 for heading, 1 for diameter and 4 for plant weight), 5 QTL were detected for leaf pigmentation traits (4 for anthocyanin and 1 for greenness) and 1 for bolting) (see figure 6.3. and table 6.2.). A QTL was identified for seed colour; this was subsequently mapped onto LG 7 at 23.7 cM corresponding to the previously identified major gene for seed colour (Waycott *et al.* 1999). The Saladin marker allele was linked to 11 QTL for agronomic traits while the Iceberg marker allele was linked to 9 QTL for agronomic traits.

QTL clustering

Eight QTL for agronomic traits located to 4 overlapping regions on 2 separate linkage groups; other QTL although not overlapping were clustered close together with these co-localising QTL (figure 6.3.). Two QTL co-localised on LG 7 (2.4-4.7 cM) for head diameter (*Diameter*) and untrimmed weight from the UK trial (*Untr_UK_wt*); a QTL for leaf indentation (*Indentation_2*) was also tightly clustered with the co-localised QTL (spanning 1.7-2.4 cM). Two QTL for production of a head (*Heading_1*) and trimmed weight from the UK trial (*Tr_UK_wt*) co-localised on LG 7 (19.7-23.4 cM). Two QTL also co-localised on LG 7 (29-79 cM) for production of a head (*Heading_3*) and savoy/blistering of leaves (*Savoy_2*). There was also a tight cluster of 3 weight related QTL on LG 7 (19.7-33.9 cM; *Tr-UK_wt,*

Tr_wt and *Tr_NL_wt*), while the remaining weight related QTL (*Untr_UK_wt*) was located in the same region (15 cM distance). Three QTL for production of a head loosely clustered on LG 7 (spanning 16.1-79 cM; *Heading_1, Heading_2* and *Heading_3*). Two QTL co-localised on LG 8 (30-40.7 cM) for production of a head (*Heading_4*) and indented leaf edges (*Indentation_3*).

Table 6.2. MQM QTL impacting on agricultural traits of individuals from the Saladin x Iceberg RIL population. Additive effect equals half the difference between homozygous alleles at the QTL: positive number indicates an additive allelic effect of Saladin; negative number indicates a negative allelic effect of Saladin. Confidence interval was based on a 2 LOD support interval with a significant LOD value of 1.95. Where *LG* (linkage group from F_7 Saladin x Iceberg linkage map (see Chapter 4)); *LOD* (logarithm of odds), *cM* (centimorgans); *heading* (production of an enclosed head); *diameter* (head diameter (cm)); *green* (of leaf); *anthocyanin* (pigmentation on leaf); *indentation* (on leaf edges); *savoy* (blistering of leaf) *Untr* (untrimmed head); *Tr* (trimmed head); *wt* (weight (g)); *UK* (United kingdom); *NL* (the Netherlands).

Trait	LG	Location (cM)	LOD peak	Confidence interval (cM)	Nearest Locus	Additive effect	Genetic variation explained by QTL (%)	Allelic contribution
Heading_1	7	23.7	2.05	9.1	BKVX-OP4-2 / AOUA-OP4-2 / BATO-OP4-2 / BCHL-OP4-2	-0.16	6.5	Iceberg
Heading_2	7	49.4	2.25	17.1	QGE10B18-OP3-1	0.16	6.8	Saladin
Heading_3	7	74	13.25	10	ATPH-OP4 / BIVH-OP3-1	0.01	63.8	Saladin
Heading_4	8	30.7	2.43	10.7	E35M59_332i	-0.19	9.6	Iceberg
Diameter	7	2.9	3.79	2.3	BSQZ-OP4	-0.30	17	Iceberg
Green	4a	6	6.52	4	BFYG-OP3	-0.41	20.5	Iceberg
Anthocyanin_1	2	82.3	3.66	12.2	E35M60_88s	0.20	6.2	Saladin
Anthocyanin_2	4a	73.5	5.68	7.5	E45M47_256i	-0.25	10	Iceberg
Anthocyanin_3	5c	26.9	18.8	12.3	AFPZ-OP3	0.56	45.2	Saladin
Anthocyanin_4	9a	28.9	15.5	10	BIAE-OP4	-0.66	44.1	Iceberg
Indentation_1	5b	47.7	3.53	9.1	E38M54_270i	0.28	12.4	Saladin
Indentation_2	7	2.3	4.56	0.7	BLJI-OP4	0.32	15.4	Saladin
Indentation_3	8	30.7	2.59	10.7	E35M59_332i	-0.29	9.1	Iceberg
Savoy_1	5a	78	47.48	8.5	E35M49_145i / QGA18I02-OP4	1.00	98.5	Saladin
Savoy_2	7	74	1378.7	10.2	ATPH-OP4 / BIVH-OP3-1	1.00	88.3	Saladin
Bolting	4b	94.2	2.16	11.8	CLXS3_3835-OP3-2	-12.57	11.6	Iceberg
Untr UK wt	7	2.9	2.21	2.3	BSQZ-OP4	-68.13	8.4	Iceberg
Tr UK wt	7	21.7	2.25	4	E33M59_205s	55.64	11.6	Saladin
Tr NL wt	7	33.5	2.15	3.4	QGJ17A06_2-OP3-1	30.10	10	Saladin
Tr wt	7	26.4	2.99	5.3	QGCA_6226-OP3	28.23	13.7	Saladin

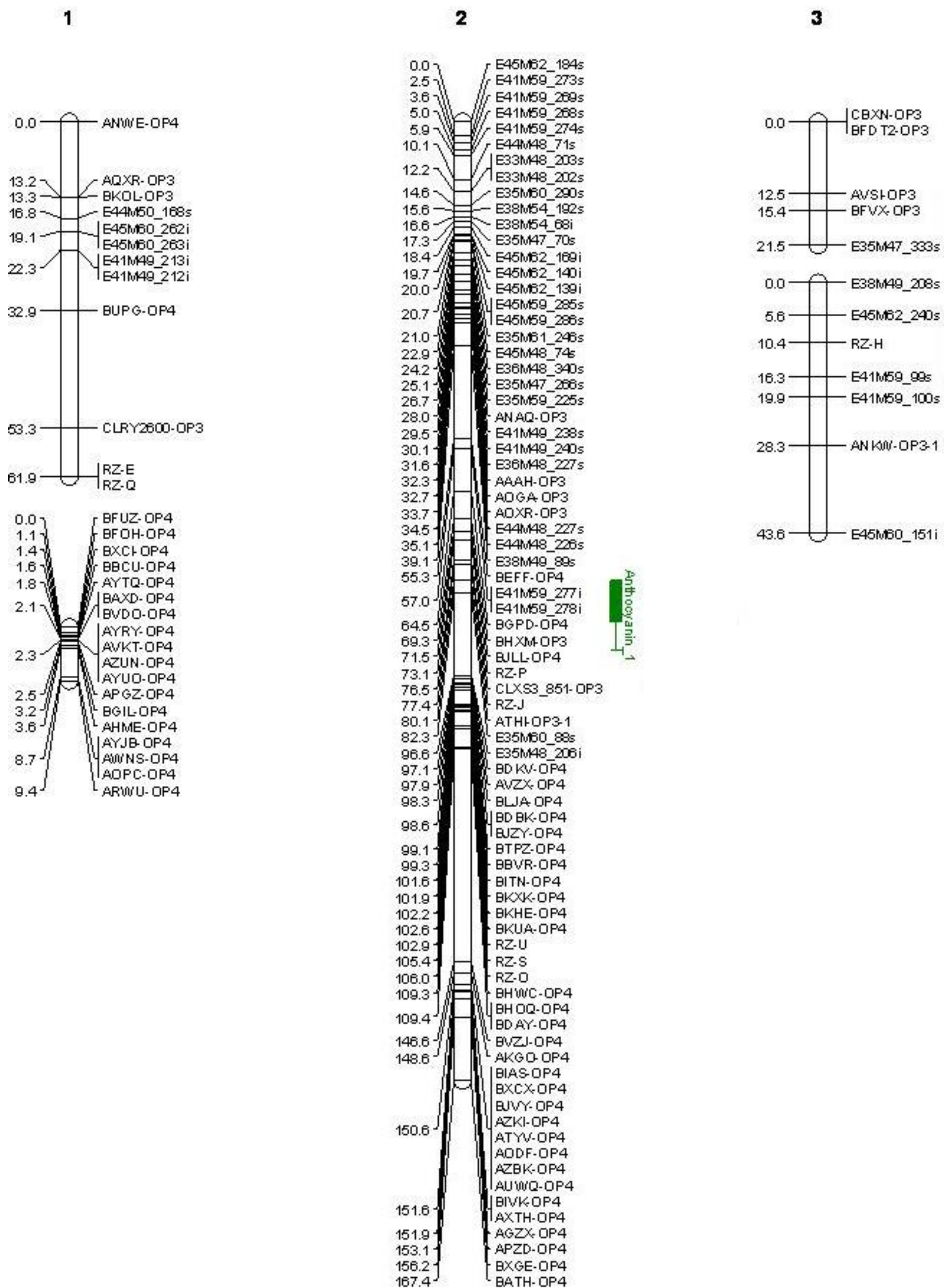


Figure 6.3. MQM QTL impacting on agricultural traits of lettuce tissue of individuals from the Saladin x Iceberg RIL population. Where *heading* (production of an enclosed head); *diameter* (of head (cm)); *green* (of leaf); *anthocyanin* (pigmentation on leaf); *indentation* (on leaf edges); *savoy* (blistering of leaf) *Untr* (untrimmed head); *Tr* (trimmed head); *wt* (weight (g)); Red are morphological QTL; black are life cycle QTL; green are pigmentation QTL.

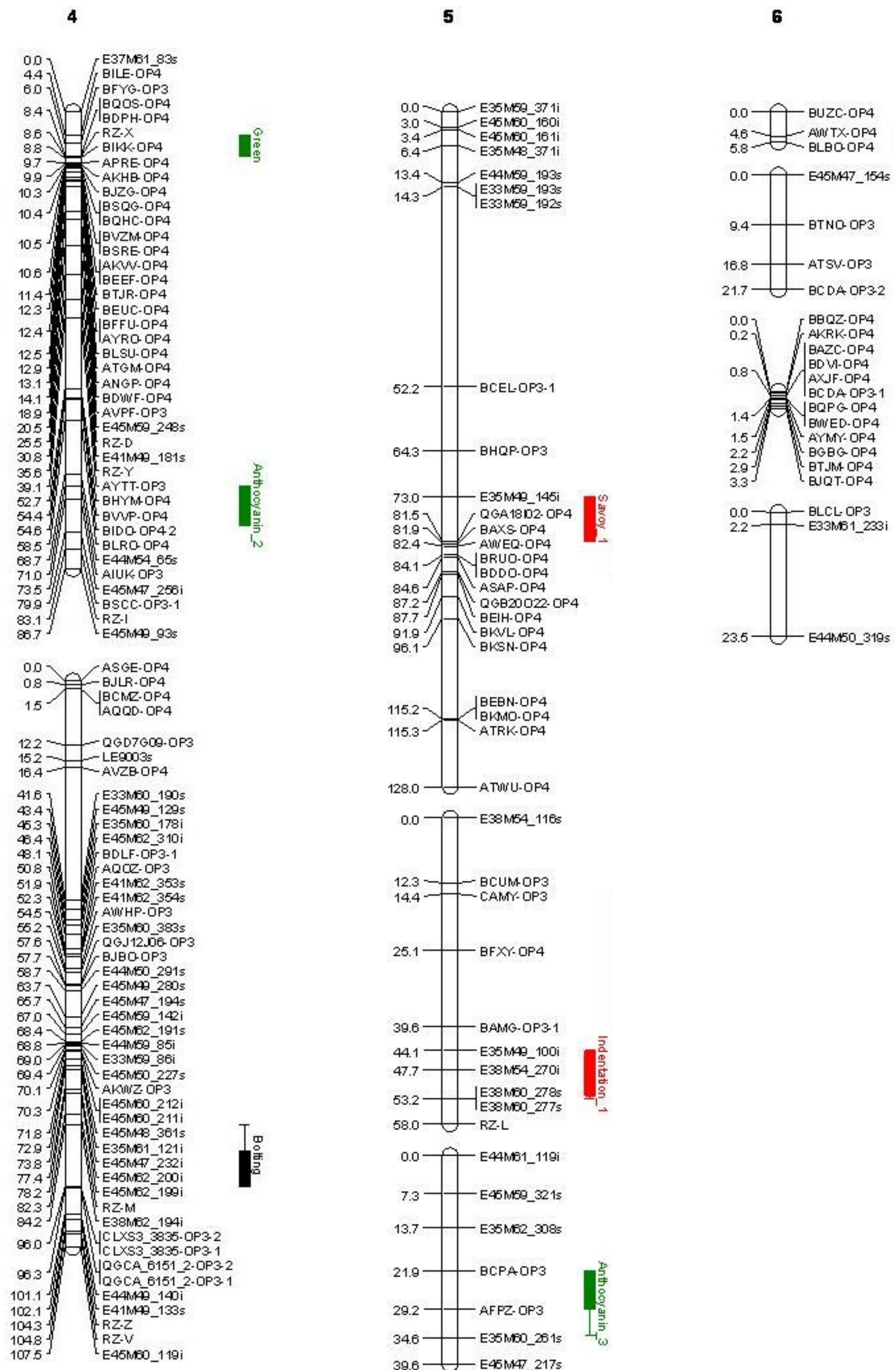


Figure 6.3. continued.

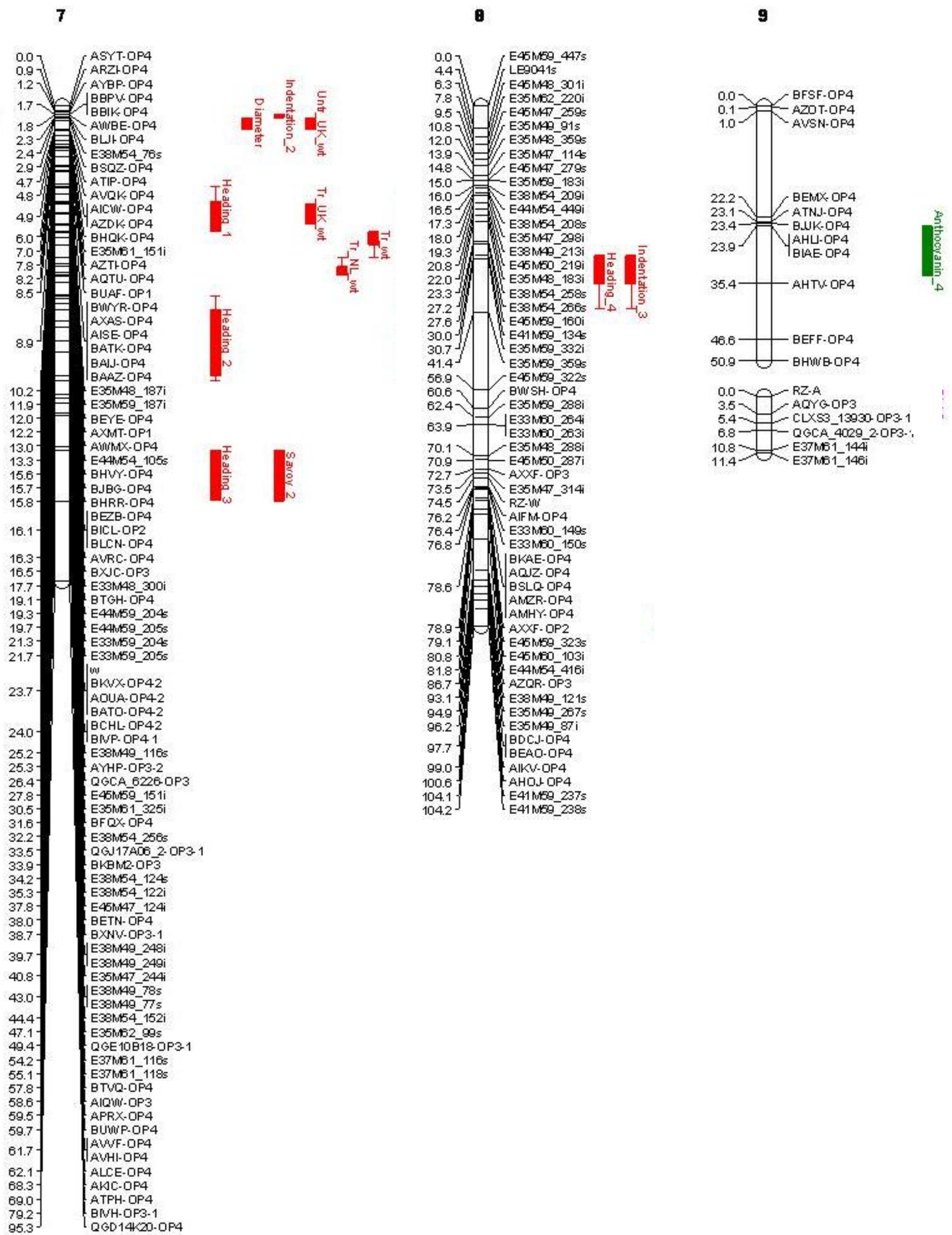


Figure 6.3. continued.

Clustering of agronomic and post harvest discolouration QTL

Ten QTL for agronomic traits and 10 QTL for post harvest discolouration traits (identified in Chapter 5) (located to 6 overlapping regions on 4 separate linkage groups (see figure 6.4.), other QTL for both types of traits were also clustered independently and with these co-locating QTL (see figure 6.4.). Two QTL co-localised on LG 2 (80.1-92.3 cM) for anthocyanin pigmentation of leaf (*Anthocyanin_1*) and post harvest browning (*Br1*). Two QTL co-localised on LG 4a (4.4-8.4 cM) for greenness of leaf (*Green*) and post harvest browning (*Br3*), a QTL for post harvest pinking (*Pink2*) was also tightly clustered with these co-localised QTL (spanning 8.4-8.8 cM). Two QTL for anthocyanin pigmentation of leaf (*Anthocyanin_2*) and post harvest pinking (*Pink3*) loosely clustered on LG 4a (spanning 71-86.7 cM). Two QTL for savoy/blistering of leaf (*Savoy_1*) and post harvest overall discolouration (*Dis1*) loosely clustered on LG 5a (spanning 73-87.7 cM). Two QTL for indentation of leaf edges (*Indentation_2*) and post harvest pinking (*Pink4*) co-localised on LG 7 (1.7-2.4 cM); 2 QTL for agronomic traits (*Diameter* and *Untri_UK_w*t) were also tightly clustered with the co-localised QTL (as above). Seven QTL were co-located on LG 7 within the region of 16.1-33.9 cM; 1 QTL for production of a head (*Heading_1*), 3 QTL for weight based traits (*Tr_UK_wt, Tr_wt* and *Tr_NL_wt*) and 3 QTL for post harvest discolouration (*Br8*, *Pink5* and *Dis2*). Four QTL for production of a head (*Heading_2*), post harvest browning (*Br9* and *Br10*) and pinking (*Pink6*) co-located and are tightly clustered to this large set of co-localised QTL within the region of 35.3-55.1 cM. Three QTL, 1 for indentation of leaf edges (*Indentation_3*), 1 for production of a head (*Heading_4*) and 1 for post harvest pinking (*Pink7*) co-located on LG 8 (30-40.7 cM).

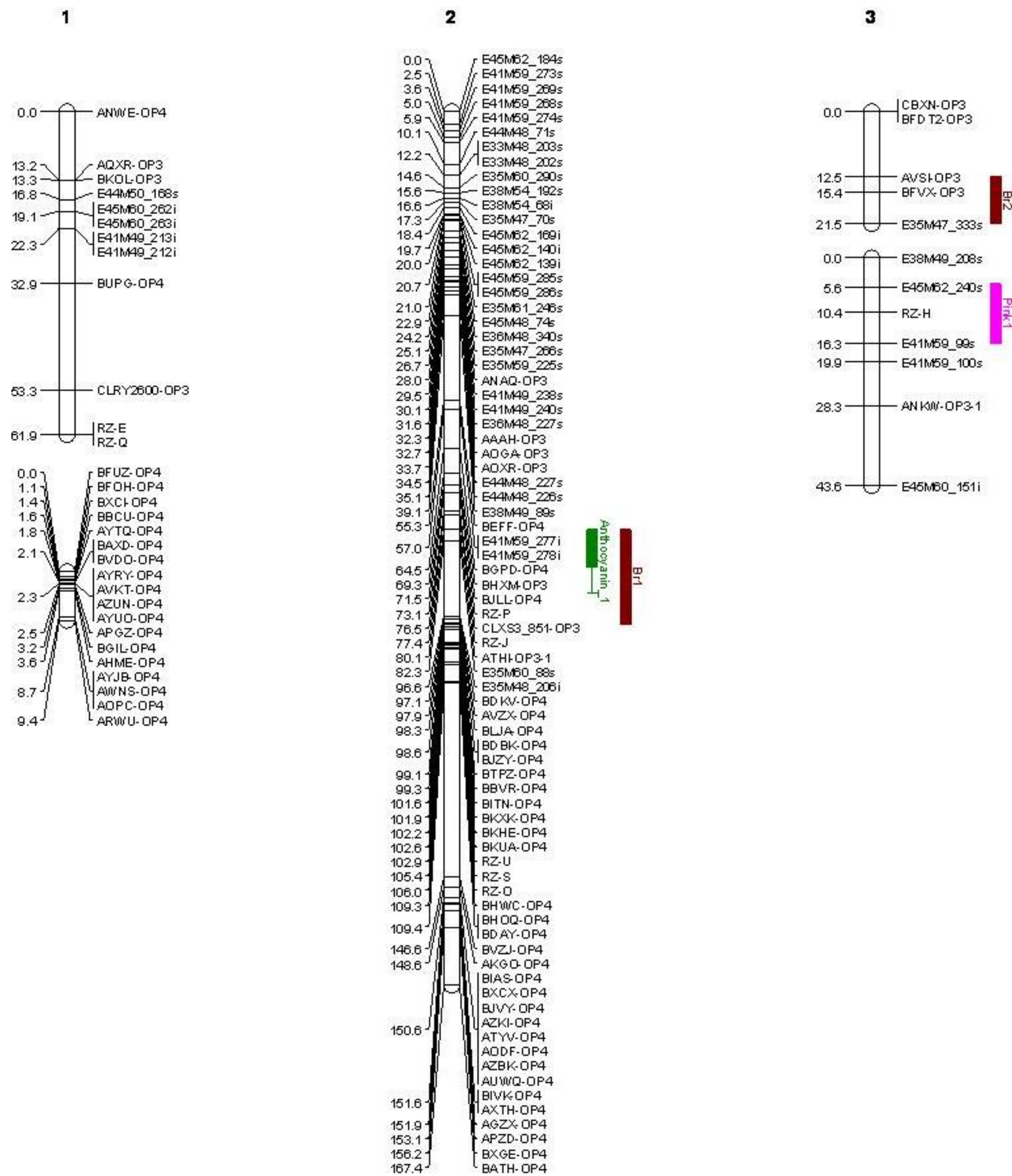


Figure 6.4. MQM QTL impacting on agricultural traits or on post harvest discolouration of lettuce tissue of individuals from the Saladin x Iceberg RIL population. Where *heading* (production of an enclosed head); *diameter* (of head (cm)); *green* (of leaf); *anthocyanin* (pigmentation on leaf); *indentation* (on leaf edges); *savoy* (blistering of leaf) *Untr* (untrimmed head); *Tr* (trimmed head); *wt* (weight (g)); *Browning* (post harvest browning); *pinking* (post harvest pinking); *discolouration* (post harvest overall discolouration); Red are morphological QTL; black are life cycle QTL; green are pigmentation QTL; pink are pinking QTL; brown are browning QTL; blue are overall discolouration QTL.

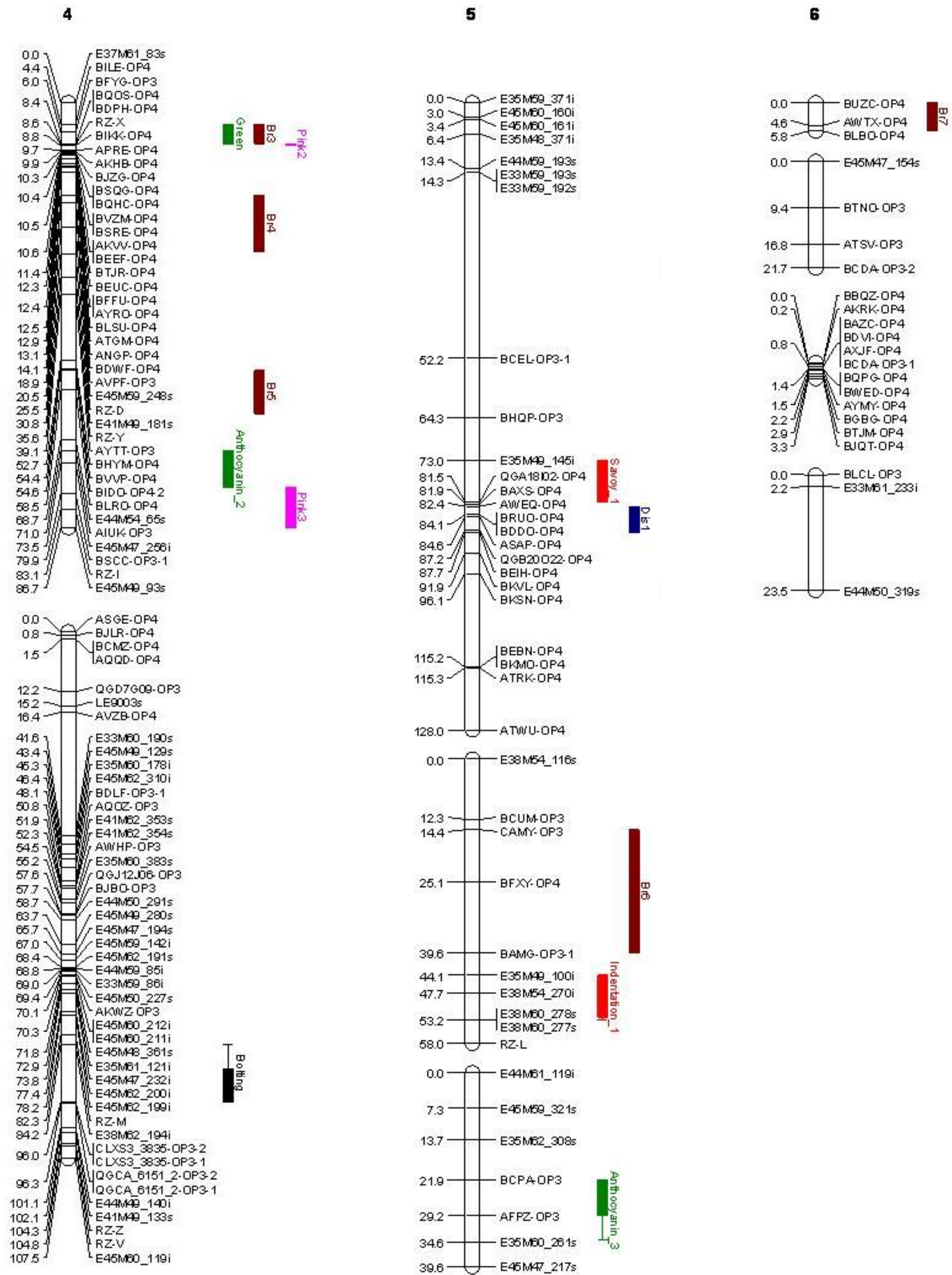


Figure 6.4. continued.

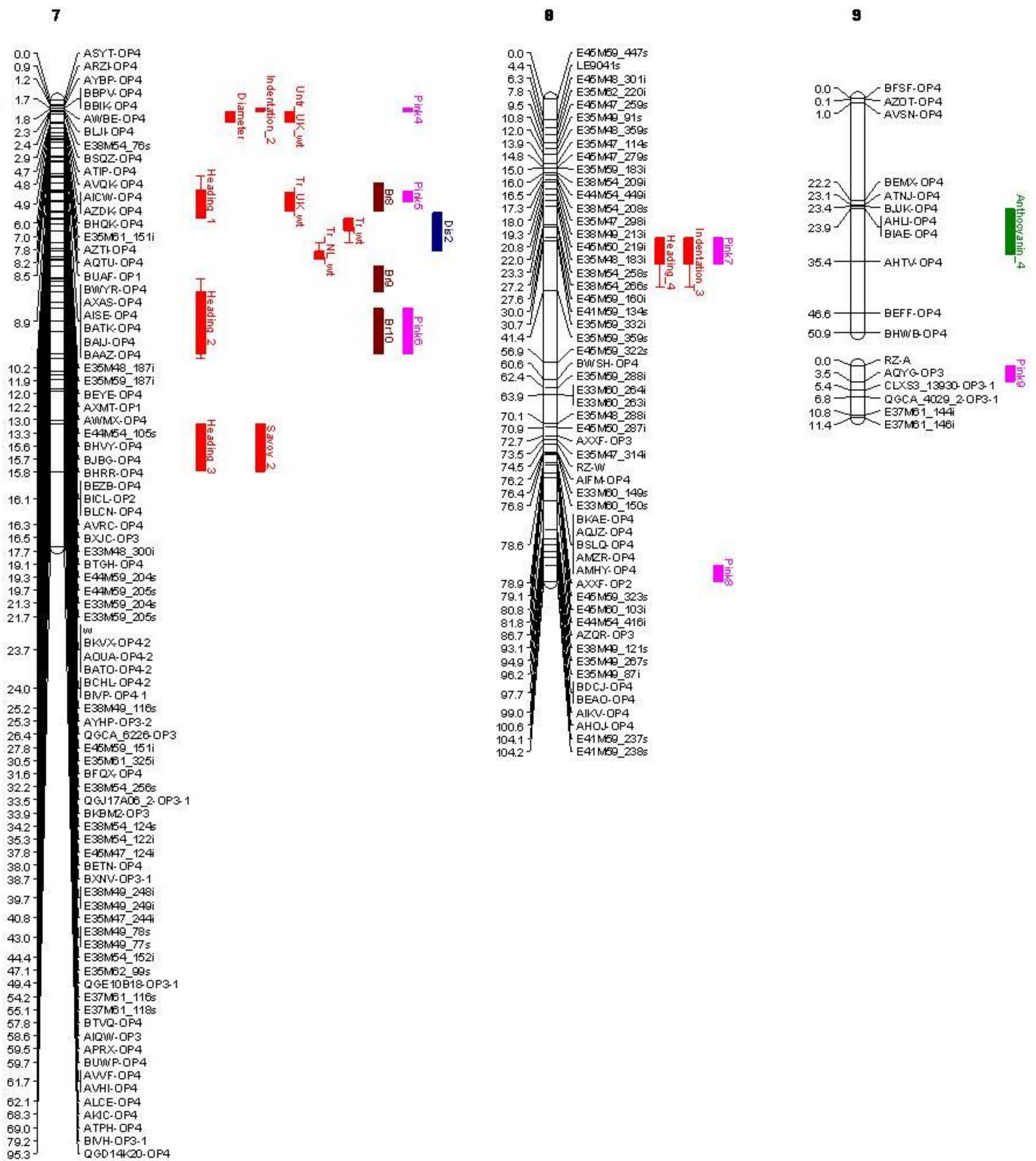


Figure 6.4. continued.

6.4. Discussion

Phenotypic variation for all discolouration traits was observed in the Saladin x Iceberg population (see Chapter 5). Additionally the parents of this population are known to vary for a number of other traits; they have different morphologies and have been shown to have significantly different levels of resistance to downy mildew (*Bremia lactucae*) and *Myzus persicae* (DAC Pink personal communication). A wide range of phenotypes were recorded in the RIL population, with most agronomic traits showing a normal probability distribution. Correlations between some agronomic traits and post harvest quality had previously been recorded for the lettuce diversity set representing the primary lettuce genepool (see Chapter 3). Correlations between morphological and post harvest discolouration traits are potentially of high importance for breeders as they may be due to pleiotropic effects or tightly linked loci. Therefore information about the associations between traits and any possible genetic linkages between them is important to lettuce breeders as they need to know if selection for one trait is affecting other economically important traits.

Although a large number of significant correlations were found (table 6.1.) many of these had low values for the coefficient of correlation ‘r’. The r value for a correlation can be used to calculate the variation in common between traits which is given as r^2. Thus for higher r values, the greater the variation in common between the traits and greater the potential problem might be for breeders. The highest r value found was for the correlation between an agronomic trait and a post harvest discolouration trait was between head production and extent of pinking (on day 3 across sites) with a value for $R_{[90]} \geq 0.55$; this gives an r^2 value of 0.30 i.e. only 30% of the variation is in common. The highest r value recorded between agronomic

traits was between head production and indented leaf edges ($R_{[90]} \geq 0.72$); giving an r^2 value of 0.52 i.e. 52% of the variation in common. In general, the r^2 values for the significant correlations are low indicating that the variation in common between traits is low (~<20%) and in general it is not anticipated that the correlated effects would cause any major problems for breeders. However, there are significant correlations and these may still limit the progress breeders can make by phenotypic selection; information on the genetic basis of the correlations would be of value.

This study reports QTL for agronomic traits and links between certain pre harvest traits to post harvest spoilage due to discolouration. As discussed in Chapter 3, some 'head' traits appear to influence the susceptibility of lettuce tissue to post harvest discolouration possibly through alterations in the water relationships within the plant tissue. The correlations observed between head type, diameter and weight for the RIL population also supports this idea.

Leaves with severe savoying/blistering are more three-dimensional and less compact, and are therefore more susceptible to damage during handling. This could explain why this trait showed a highly significant positive correlation with post harvest pinking (the highest correlation was with extent of pinking on day 1 across sites ($R_{[90]} \geq 0.38$)) (see table 6.1.).

Indentation of leaf edges was also correlated with pinking. It is difficult to see a physiological reason for this relationship, and the significant correlation could be due to linkage.

In *Arabidopsis,* seed coat pigments are predominantly flavonoids (specifically condensed tannins of the cyanidin type and flavonols of the quercetin type) which are an end product of phenylpropanoid metabolism (Chapple *et al.* 1994). Condensed

tannins undergo oxidative reactions which give seeds their brown pigmentation (Debeaujon *et al.* 2000); this reaction also results in post harvest browning in vegetative tissue (Lopez-Galvez *et al.* 1996). RILs with brown seeds generally showed increased scores for post harvest discolouration which could be explained by these lines having higher levels of polyphenols.

In addition to affecting post harvest discolouration traits, selection based on a particular morphological trait may have indirect effects on other morphological traits. Head type has been shown to significantly influence post harvest quality and significant correlations were also found with days to bolting; a possible explanation is that heads which are more tightly closed take longer for the lettuce flowering stalk to break through the head tissue. This is an important trait for growers as it reflects 'holding ability' in the field and thus saleability.

Leaf indentation is also positively correlated to days to bolting; however it is also correlated to head production so it may be due to linkage. The same may be suggested for savoy/blistering of leaves as it is positively correlated with indented leaves and head production, although blistering of the leaves can reduce compactness of the head making it appear larger and looser.

Twenty QTL were identified for agronomic traits and 21 QTL were identified for post harvest discolouration. The correlations between the phenotypic scores for the traits can be explained by the co-location of QTL for the different traits on the map (see figure 6.4.).

Clusters of QTL determining both agronomic (head and leaf morphology based) and post harvest discolouration traits were recorded on LG 7 (1.7-55.1 cM) and LG 8 (30-40.7 cM) (see figure 6.4.). This could explain the correlations observed between

the traits of a head production, head diameter, plant weight (trimmed and untrimmed) and leaf indentation with all 3 measures of post harvest discolouration. QTL for head production, diameter and weight were co-located on LG 7 in two regions (2.4-2.7 cM and 19.7-23.4 cM). These data along with the consistent phenotypic correlations suggest that the QTL in these two regions have a 'general' effect on head development. The QTL for post harvest discolouration in this region may also have the same genetic basis and be a reflection of the susceptibility of the head to damage which then induces a discolouration response.

The clustering of the 4 QTL related to head weight on LG 7 (2.4-33.9 cM) would be expected as the phenotypic data that they are based on are directly and indirectly measuring the same trait either in different environments or in some cases were measurements made on the same head (see figure 6.3.). The 2 QTL for head production (*Heading_2* and *Heading_3*) which loosely clustered with the above two regions (including QTL *Heading_1* (spanning 16.1-79 cM)) could also be the manifestation of 1 larger single QTL. A larger data set would increase the LOD accuracy provided for each marker under the QTL potentially refining QTL length.

QTL for leaf indentation (*Indentation_2*; 1.7-2.4 cM) and savoy/blistering of leaves (*Savoy_2*; 29-79 cM) also clustered with the above regions and go some way to explaining the observed phenotypic correlations (see figure 6.3.). The co-location of these two QTL indicate that this is a region which influences leaf morphology QTL for head production and indented leaves also co-localised on LG 8 (30-40.7 cM) again suggesting possible linkage of genes influencing these 2 traits.

There were also overlapping QTL regions for traits which did not show a significant phenotypic correlation. QTL for anthocyanin pigmentation co-localised and

clustered with both QTL for post harvest browning (on LG 2; 80.1-92.3 cM) and post harvest pinking (on LG 4a; 71-86.7 cM) (see figure 6.4.). Anthocyanins and the metabolites involved in post harvest discolouration are both produced via the phenylpropanoid pathway and are initially regulated by a common enzyme PAL. If polyphenols are being sequestered to the anthocyanin pathway this might result in there being less available for the oxidative reactions causing post harvest discolouration upon wounding.

QTL for greenness leaf, post harvest browning and pinking co-localised and clustered within LG 4a (4.4-8.8 cM) (see figure 6.4.). It is difficult to see a physiological basis for this but it could simply be due to the darkness of the leaf and how this influences how discolouration is perceived by eye, although it may be due to linkage of genes influencing these two traits.

The agronomic traits investigated in this trial are generally complex traits likely to be controlled by many QTL with small effect. For some traits such as head diameter, greenness of leaf, leaf indentation and days to bolting, much of the observed variation was left unexplained by the QTL analysis suggesting that there are probably QTL with smaller effects that could not be detected (Zhang *et al.* 2007). Type II QTL errors (false negative) are determined by the experimental set-up and the size of the genetic effect of QTL (Van Ooijen 1999). This trial was designed for observational purposes, so with an improved design and more a quantitative scoring method for some traits additional QTL may have been identified.

Where QTL for different traits are co-located or clustered and the traits they determine are positively correlated, selection of the ideal genotype could improve several traits at the same time. However when QTL for desirable and undesirable traits map together, it is necessary to determine whether there are in fact multiple

QTL or a single QTL with pleiotropic effects. This can be determined via fine mapping and analysis of near-isogenic substitution lines. If QTL for both desirable and undesirable traits are actually a single QTL with pleiotropic effects, selecting for an improved genotype would be extremely difficult (Zhang *et al.* 2007) and would require fine mapping to identify whether the pleiotropic effect is due to closely linked genes, markers could then be used to select for recombination to break the linkage.

In this study it is apparent that although QTL for some agronomic traits occur independently of QTL for post harvest discolouration, some co-localise and could result in the need for 'trade offs' by breeders during selection. By combining information on QTL for agronomic traits with the information on QTL for post harvest discolouration it may be possible to identify the degree of linkage between desirable traits in different lettuce types and undesirable post harvest discolouration, therefore adding value to the salads marketing by increasing the resources available for breeding.

This study has provided the basis for breeders to understand the relationships between agronomic traits and post harvest discolouration responses so that they can now carry out selection in a more informed manner. If there are still genetic linkages between important QTL then this genetic analysis is also the first step in trying to fine map the QTL and to identify markers to allow selection for recombination to break the linkage or to show that the correlated effects are due to pleiotropic effects (i.e. the same gene, which means they are stuck with it).

6.5. Conclusions

- Twenty significant QTL for a range of important agronomic traits have been identified, providing markers for MAS of these traits
- There were significant correlations between agronomic traits and post harvest discolouration traits, however, traits were generally not highly correlated.
- Some QTL for agronomic traits were linked to QTL for post harvest discolouration explaining the associations between traits.
- Post harvest discolouration and agronomic traits can generally be independently selected for by breeders, however in some cases pleiotropic effects may occur.

CHAPTER 7

Explaining the genetic variation causing post harvest discolouration

7.1. Introduction

QTL for post harvest discolouration in lettuce were identified in Chapter 5. The full determination of the heritability of post harvest discolouration would allow development of improved varieties for trait combinations (including organoleptic and metabolomic characteristics) by breeders. Therefore, it is important to attempt to link gene function and gene regulation to phenotype. Hypothetically, it should be possible to link metabolomic changes in biochemical pathways to the enzymes involved and consequently the underlying genetic adjustment that lead to a specific phenotype (Fiehn 2002). By comparing the post harvest discolouration phenotype with the levels of key metabolites in the phenylpropanoid pathway, it may be possible to identify which genes are controlling the biosynthesis of these metabolites. Mapping of ESTs derived from genes known to be involved in the phenylpropanoid pathway is a complementary approach to attempt to identify the genetic basis of any co locating QTL (Zhang *et al.* 2005) and provide further information about the genes underlying the post harvest discolouration phenotype.

The aims of this experiment were to

- Demonstrate variation in levels of metabolites related to post harvest discolouration in the F_7 Saladin x Iceberg mapping population.
- Identification of putative candidate genes involved in post harvest discolouration for SNP detection.
- Detect SNPs for candidate genes and map on the Sal x Ice linkage map.

7.2. Material and methods

7.2.1. Saladin x Iceberg RIL population

All 125 RILs from the Saladin x Iceberg F_7 population were used for candidate gene mapping (see section 2.1.2.).

7.2.2. Extreme discolouration RIL subset

All 11 RILs from the extreme discolouration RIL subset (see section 5.2.2.) were used for metabolite analysis.

7.2.3. Metabolite analysis

When material was harvested from the first rep of the field trial of extreme discolouration lines for bagged phenotypic assessment (see section 5.2.4.), tissue was also harvested for metabolite analysis (see section 2.3.1.). Extraction and identification for PAL activity, PPO activity and total phenolic content was as stated (see sections 2.3.1. and 2.3.2.).

7.2.4. Statistical analysis

ANOVA

Data from each metabolite analysis were analysed using general ANOVA with comparison contrasts (see table 7.1.). Metabolite activity was analysed by day and for differences across days for all lines and between comparison contrasts to identify main effects due to genotype.

Table 7.1. Comparison contrasts of RILs for ANOVA. For RIL number relating to extreme discolouration phenotype see sections 5.2.2. and 5.3.4. Where *v* (comparison); *L* (low); *H* (high); *P* (pinking); *B* (browning); *D* (overall discolouration).

Groupings of RILs	Comparison against Saladin	Comparison against Iceberg
Saladin v Iceberg	Saladin v LP RILs	Iceberg v LP RILs
LP RILs v HP RILs	Saladin v HP RILs	Iceberg v HP RILs
LB RILs v HB RILs	Saladin v LB RILs	Iceberg v LB RILs
LD RILs v HD RILs	Saladin v HB RILs	Iceberg v HB RILs
	Saladin v LD RILs	Iceberg v LDRILs
	Saladin v HD RILs	Iceberg v HD RILs

Correlation analysis

Correlation analyses were conducted between all discolouration measures and metabolite activity (correlations between browning, pinking and overall discolouration and between them and PAL activity, PPO activity, TPC and protein levels (as section 2.4.4.)). R values were generated which were then compared to the associated *p* values (see table 7.2.).

Table 7.2. Correlation analysis parameters. Where *df* (degrees of freedom).

Population	df	Probability (p value)		
		0.05	**0.01**	**0.001**
F_7 extreme RILs trial	11	0.553	0.684	0.801

7.2.5. EST data mining

Candidate genes were selected from the phenylpropanoid pathway for potential SNP mapping (see table 7.3.). The TAIR database was searched by gene product name (i.e. CHS/chalcone synthase) for locus number and protein sequence in *Arabidopsis* (http://www.arabidopsis.org/).

The searched *Arabidopsis* gene protein sequences were 'BLAST'ed against lettuce ESTs (http://cgpdb.ucdavis.edu/cgpdb2/blast_search/) using the CLS_S3_Sat.assembly database (L.sativa | CAP3: 100/95) and tblastn (protein vs DNA) programme. The contig/EST with the highest identity to the candidate gene was selected and the sequence retrieved from the CLS_S3_ESTs_Sat.assembly file (http://cgpdb.ucdavis.edu/cgpdb2/est_info_assembly.php).

Table 7.3. Candidate gene information relating to the phenylpropanoid pathway. Information from candidate genes in *Arabidopsis thaliana* from the TAIR database (www.arabidopsis.org). More gene information in appendix.

Gene Name	Annotation	TAIR Accession	Locus	Other names
Acetyl CoA carboxylase	ACCase	/	/	/
Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	ACCD	504954673	ATCG00500	/
Anthocyanidin synthase	ANS	2127218	AT4G22880	F7H19.60, LDOX, LEUCOANTHOCYANIDIN DIOXYGENASE, TANNIN DEFICIENT SEED 4, TT18
Anthocyanin reductase	ANR	2195733	AT1G61720	BAN, BANYULS, T13M11.8, T13M11_8
Anthocyaninless 2	ANL2	2127008	AT4G00730	AHDP, ARABIDOPSIS THALIANA HOMEODOMAIN PROTEIN, F6N23.10
Arabidopsis reductase 1	AR1	2121894	AT4G24520	ARABIDOPSIS CYTOCHROME REDUCTASE, ARABIDOPSIS P450 REDUCTASE 1,, F22K18.280
Arabidopsis reductase 2	AR2	2128951	AT4G30210	ARABIDOPSIS P450 REDUCTASE 2, F9N11.60
Aureusidin synthase	AS	/	/	/
Chalcone flavanone isomerise	CFI	2097228	AT3G55120	A11, T15C9.120, TT5
Chalcone isomerise	CHI	2156957	AT5G66220	K2A18.30
Chalcone reductase	CHR	/	/	/
Chalcone synthase	CHS	2159098	AT5G13930	MAC12.28, TT4
Cytochrome P450	CYP98A3	2058440	AT2G40890	FAMILY 98, SUBFAMILY A, POLYPEPTIDE 3, T20B5.9
Dihydroflavonal 4-reductase	DFR	2165427	AT5G42800	M318, MJB21.18, TT3
Enhancer of glabra 3	EGL3	2026629	AT1G63650	ATMYC-2, EGL1, F24D7.16
Flavanone 3'5' hydroxylase	F3'5'H	/	/	/
Flavanone 3 5 hydroxylase	F35H	/	/	/
Flavanone 3'-hydroxylase	F3'H	2142878	AT5G07990	CYP75B1, CYTOCHROME P450 75B1, D501, F13G24.190, TT7
Flavanone 3-hydroxylase	F3H	2081008	AT3G51240	F24M12.280, F3'H, TT6
Flavone synthase	FSI/ FS2	/	/	/

Table 7.3. continued.

Gene Name	Annotation	TAIR Accession	Locus	Other names
Flavonol 7-O-rhamnosyl transferase	F7ORT	2198791	AT1G06000	T21E18.5
Flavonol synthase 1	FLS1	2159542	AT5G08640.	T2K12.5
Flavonol synthase 2	FLS2	2160564	AT5G63580	MBK5.4
Flavonol synthase 3	FLS3	2160589	AT5G63590	MBK5.5
Flavonol synthase 4	FLS4	504954954	AT5G63595	/
Flavonol synthase 5	FLS5	2160594	AT5G63600	MBK5.7
Flavonol synthase 6	FLS6	504954874	AT5G43935	/
Glutathione S-transferase	GST	2167215	AT5G17220	GLUTATHIONE S-TRANSFERASE PHI 12, GLUTATHIONE S-TRANSFERASE 26, MKP11.22, TT19
Hydroxycinnamoyl-coa shikimate transferase	COA	2154334	AT5G48930	HCT, HYDROXYCINNAMOYL-COA QUINATE HYDROXYCINNAMOYL TRANSFERASE, K19E20.4
Isoflavone 2-hydroxylase	I2_H	/	/	/
Isoflavone reductase	IFR	2025192	AT1G75280	F22H5.17
Isoflavone synthase	IFS	/	/	/
Leucoanthocyanidin dioxygenase / Anthocyanidin synthase	LDOX/ANS	2127218	AT4G22880	F7H19.60, TANNIN DEFICIENT SEED 4, TT18
Leucoanthocyanidin reductase	LCR/ LAR	/	/	/
Malonyl-CoA:anthocyanidin 5-O-glucoside-6"-O-malonyltransferase	5MAT	2093620	AT3G29590	MTO24.5
MYB domain protein 3	MYB3	2009452	AT1G22640	T22J18.19
MYB domain protein 4	MYB4	2121259	AT4G38620	T9A14.11
O-methyltransferase	OMT1	2153423	AT5G54160	K18G13.3
Phenylammonia lyase 1	PAL1	2057981	AT2G37040	CI0004, T1J8.22
Phenylammonia lyase 2	PAL2	2101958	AT3G53260	T4D2.190
Phenylammonia lyase 3	PAL3	2146708	AT5G04230	F21E1.150

Table 7.3. continued.

Gene Name	Annotation	TAIR Accession	Locus	Other names
Phenylammonia lyase 4	PAL4	2076244	AT3G10340	F14P13.6
Polyphenol oxidase	PPO	2162677	AT5G48100	LAC15, LACCASE-LIKE 15, MDN11.18, TT10
Production of anthocyanin 1	PAP1	2027523	AT1G56650	ATMYB75, F25P12.92, MYB DOMAIN PROTEIN 75, MYELOBLASTOSIS PROTEIN 75, SIAA1,
Production of anthocyanin 2	PAP2	2201532	AT1G66390	ATMYB90, MYB DOMAIN PROTEIN 90, MYB90, T27F4.14
Transparent testa 1	TT1	2008386	AT1G34790	F11O6.15
Transparent testa 12	TT12	2077725	AT3G59030	F17J16.80
Transparent testa 15	TT15	/	/	/
Transparent testa 16	TT16	2166766	AT5G23260	ABS, AGAMOUS-LIKE 32, AGL32, ARABIDOPSIS BSISTER, AT5G23260.1, MKD15.12
Transparent testa 19	TT19	2167215	AT5G17220	ARABIDOPSIS THALIANA GLUTATHIONE S-TRANSFERASE PHI 12, GLUTATHIONE S-TRANSFERASE 26, GST26, MKP11.22
Transparent testa 2	TT2	2169538	AT5G35550	ATMYB123, MOK9.18, MYB DOMAIN PROTEIN 123,
Transparent testa 8	TT8	2118524	AT4G09820	F17A8.170
Transparent testa glabra 1	TTG1	2153914	AT5G24520	K18P6.4, , UNARMED 23
Transparent testa glabra 2	TTG2	2049852	AT2G37260	ATWRKY44, DR. STRANGELOVE 1, DSL1, F3G5.5, WRKY44
UDP flavonoid glucosyl transferase	UF3GT/3GT	2166552	AT5G54060	MJP23.2
Vestitone reductase	VR	/	/	/
72_-dihydroxy 4_-methoxyisoflavanol dehydratase	DMID	/	/	/
4-coumarate:CoA ligase 1	4CL1	2017602	AT1G51680	F19C24.11
4-coumarate:CoA ligase 2	4CL2	2094716	AT3G21240	MXL8.10
4-coumarate:CoA ligase 3	4CL3	2015003	AT1G65060	F16G16.6
4-coumarate:CoA ligase 5	4CL5	2094771	AT3G21230	MXL8.9

A reciprocal blast of the lettuce EST/contig sequence was also conducted against the NCBI *Arabidopsis thaliana* sequences database, using the RefSeq protein database and BLASTX (nucleotide vs protein) programme to ensure the correct candidate gene would be amplified (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=3702). If the highest hit was the correct *Arabidopsis* candidate gene primers were designed for the lettuce sequence. Using the contig viewer, information regarding intron structure for each candidate gene was utilised to design primers to regions of the lettuce sequence avoiding possible conserved splice sites (http://cgpdb.ucdavis.edu/cgpdb2/CGP_ContigViewer/).

7.2.6. SNP detection

Parental screen

The parents of the WHRI mapping population, Saladin and Iceberg were screened for polymorphism for the candidate genes.

Primer design

Primers for the candidate genes were developed using the primer design software Primer3 (v. 0.4.0) (Rozen and Skaletsky 2000). The relevant EST/contig sequence was submitted with parameters of product size 100-300 bp and targets as intron site plus 12 bp (the aim was to include a single intron within a small product). Where no intron site was present, the whole sequence was amplified.

Polymerase chain reaction (PCR)

The 20 µl reaction mixture consisted of: 4 µl 5 x iProof HF buffer; 0.4 µl forward primer (10 mM); 0.4 µl reverse primer (10 mM); 2 µl template DNA (~10 ng/µl); 12.4 µl dH_20 and 0.2 µl iProof DNA polymerase. PCR was performed using a PTC-225 Peltier thermal cycler, DNA engine tetrad (MJ Research, USA). The PCR reaction program was: 98°C for 30 s, 40 cycles of (98°C (5 s); 57°C (10 s); 72°C (25 s)), extended at 72°C for 5 min. The PCR products were determined visually by agarose gel electrophoresis (1% gel).

Single sequence conformation polymorphism (SSCP) analysis

Parental samples for each candidate gene amplification were run on SSCP gels to see if any SNPs were present between DNA sequences of Saladin and Iceberg (as for Chapter 4). When a polymorphism for a candidate gene was recorded between the parents, the entire F_7 population was amplified using the associated primers and analysed on SSCP gels as the parents (see figure 7.1.). If no polymorphism was observed then the PCR products for the parental DNA were sequenced.

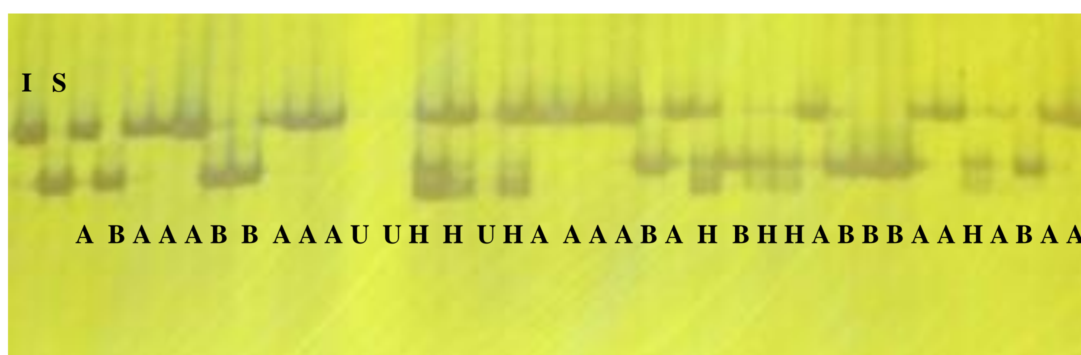


Figure 7.1. SSCP gel showing polymorphic candidate gene PPO PCR products. Where *S* (Saladin); *I* (Iceberg); *H* (heterozygote); *A* (A/Iceberg genotype); *B* (B/Saladin genotype); *U* (unknown genotype).

DNA purification and sequencing

The PCR products were purified to remove free primers and nucleotides before sequencing. For 20 µl PCR product, 0.5 µl of usb exonuclease 1 10 un/µl and 0.5 µl usb shrimp alkaline phosphatase (SAP) 1 un/µl was added. Samples were incubated at 37°C for 30 min, then 80°C for 15 min. DNA was diluted to 2ng/µl per 100 bases of PCR product length and the associated forward primers were diluted to 3 µM. Eight µl of each DNA sample and associated primers were sent for sequencing at Davis Sequencing, USA.

Joinmap®4 analysis

Linkage analysis for genotype scores for putative gene specific markers from the mapping population was performed using Joinmap®4 software as described (section 4.2.1.).

7.3. Results

7.3.1. Metabolite profiling

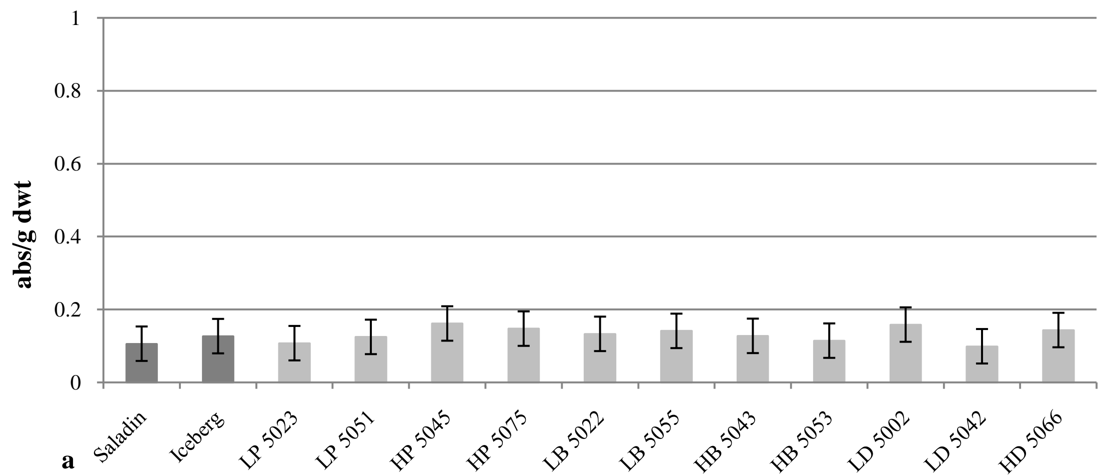
PAL activity

ANOVA revealed significant variation (***P* <0.004) between lines for PAL activity when expressed as absorbance per gram of dry weight of lettuce (abs/g dwt) for day 4 post storage and for the differences of PAL activity across days (****P* <0.001) (see table 7.4.).

PAL activity of the 13 lines varied from 0.099 abs/g dwt to 0.161 abs/g dwt for harvest day and from 0.089 abs/g dwt to 0.579 abs/g dwt on day 4 (see figure 7.2. (a-c)). All RILs had similar levels of PAL activity on harvest day. Generally PAL activity increased from harvest day to day 4. Mean PAL activity was 0.13 abs/g dwt and 0.32 abs/g dwt respectively for harvest day and day 4. Saladin had one of the lowest PAL activities on both days. Significant differences were seen between the mapping parents Saladin and Iceberg (**P <0.029) for PAL activity on day 4. Low discolouration RILs generally had lower PAL activity than high discolouration RILs. LB 5055 had significantly lower PAL activity than both HBs 5043 and 5053, while LD 5022 had significantly lower PAL activity than HD 5066. No RIL had significantly lower PAL activity than Saladin or significantly higher PAL activity than Iceberg. However, LB 5055 and LD 5002 showed similar PAL activity to Saladin and all three lines had significantly lower PAL activity than Iceberg. HP 5045, HB 5043, HB 5053 and LB 5022 all showed similar PAL activity to Iceberg; all had significantly higher PAL activity than Saladin. Saladin PAL activity remained constant post harvest while PAL activity decreased for LB 5055 (-0.04 abs/g dwt) and LD 5002 (-0.08 abs/g dwt). Transgressive segregation was observed with HB 5043 PAL activity increasing at a higher rate across days than Iceberg and all RILs (0.645 abs/g dwt).

Table 7.4. PAL activity for RILs from the extreme discolouration subset over 4 days. ANOVA GT df = 12. ANOVA comparison df = 1. Significant effects shown as **P* <0.05, ***P* <0.01 and ****P* <0.001. Where *PAL* (phenylalanine ammonia lyase); *GT* (genotype/accession); *RIL;* (recombinant inbred line); *v* (comparison); *vr* (variance ratio); *prob* (ANOVA probability).

	PAL activity					
	Day harvest		Day 4		Across days	
Variation	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**
GT	0.35	0.974	2.85	**0.004	4.47	***<0.001
Saladin v Iceberg	0.19	0.662	5	*0.029	2.67	0.109
Low pinking RILs v High pinking RILs	1.32	0.255	0.74	0.394	0.01	0.919
Low browning RILs v High browning RILs	0.23	0.63	9.15	**0.004	10.92	**0.002
Low overall discolouration RILs v High overall discolouration RILs	0.13	0.72	6.38	*0.014	5.73	*0.021
Saladin v Low pinking RILs	0.06	0.807	1.59	0.212	0.68	0.415
Saladin v High pinking RILs	1.4	0.241	3.85	*0.05	0.82	0.37
Saladin v Low browning RILs	0.58	0.451	1.97	0.166	1.42	0.239
Saladin v High browning RILs	0.13	0.717	14.99	***<0.001	15.14	***<0.001
Saladin v Low overall discolouration RILs	0.31	0.58	0.8	0.374	0.05	0.824
Saladin v High overall discolouration RILs	0.63	0.431	8.79	**0.004	5.14	*0.028
Iceberg v Low pinking RILs	0.33	0.568	1.11	0.297	0.35	0.558
Iceberg v High pinking RILs	0.46	0.501	0.38	0.538	0.96	0.333
Iceberg v Low browning RILs	0.06	0.802	1.39	0.243	0.48	0.492
Iceberg v High browning RILs	0.02	0.887	1.66	0.202	4.02	*0.051
Iceberg v Low overall discolouration RILs	0	0.961	2.84	0.097	2.76	0.103
Iceberg v High overall discolouration RILs	0.13	0.724	0.53	0.47	0.4	0.529



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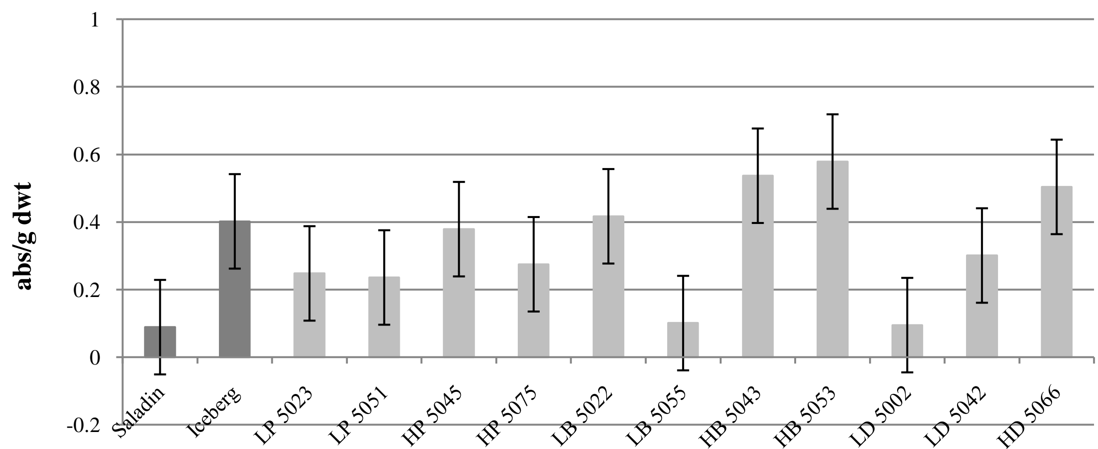

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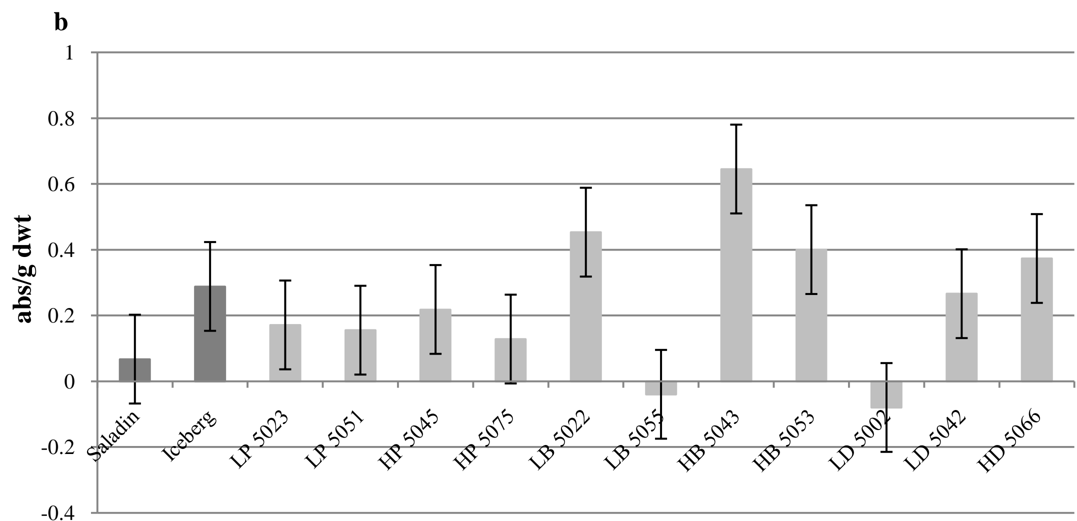


c

Figure 7.2. Mean PAL (phenylalanine ammonia lyase) activity for RILs from the extreme discolouration subset on a) harvest day, b) 4 days after storage and c) the difference between PAL activity levels across the 4 days. PAL activity measured as absorbance per gram of dry weight of lettuce (abs/g dwt). Error bar represents seds (standard error of differences of the mean) from ANOVA. Where *dwt* (dry weight); *g* (gram).

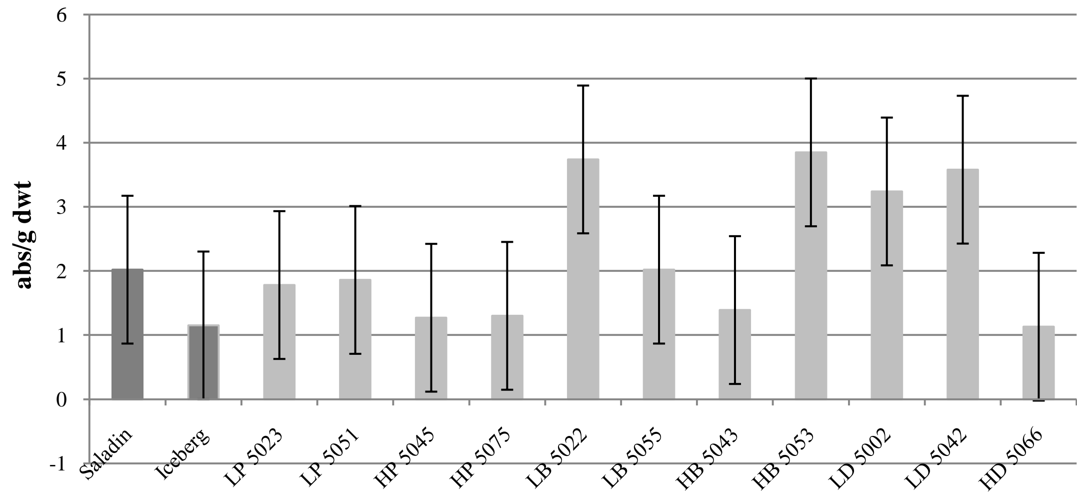
PPO activity

ANOVA revealed significant variation (***P* <0.003) between lines for PPO activity at 4 days post storage (see table 7.5.).

PPO activity of the 13 lines varied from 1.13 abs/g dwt to 3.85 abs/g dwt for harvest day and from 0.94 abs/g dwt to 4.7 abs/g dwt on day 4 (see figure 7.3. (a-c)). Generally, low discolouration RILs had higher PPO activity than high discolouration RILs on harvest day. LB 5022 had significantly higher PPO activity than HB 5043, while LDs 5022 and 5042 had significantly higher PPO activity than HD 5066 (**P* <0.026). Generally, PPO activity increased from harvest day to day 4. Mean PPO activity was 2.18 abs/g dwt and 2.508 abs/g dwt respectively for harvest day and day 4. LB 5055 had significantly higher PPO activity than HB 5043 and Saladin. Saladin and Iceberg PPO activity increased at similar rates post harvest. Generally PPO for low discolouration RILs increased at higher rates across days than for high discolouration RILs.

Table 7.5. PPO activity for RILs from the extreme discolouration subset over 4 days. ANOVA GT df = 12. ANOVA comparison df = 1. Significant effects shown as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Where *PPO* (polyphenol oxidase); *GT* (genotype/accession); *RIL;* (recombinant inbred line); *v* (comparison); *vr* (variance ratio); *prob* (ANOVA probability).

	PPO activity					
	Day harvest		Day 4		Across days	
Variation	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**
GT	1.63	0.111	3.11	**0.003	1.69	0.11
Saladin v Iceberg	0.58	0.45	0.73	0.399	0	0.995
Low pinking RILs v High pinking RILs	0.44	0.511	1.05	0.311	0.34	0.564
Low browning RILs v High browning RILs	0.1	0.757	1.01	0.32	2.39	0.131
Low overall discolouration RILs v High overall discolouration RILs	5.21	*0.026	0.32	0.575	0.62	0.437
Saladin v Low pinking RILs	0.04	0.841	0.14	0.708	0.66	0.422
Saladin v High pinking RILs	0.55	0.462	0.21	0.648	1.66	0.206
Saladin v Low browning RILs	0.73	0.397	5.27	*0.026	1.58	0.217
Saladin v High browning RILs	0.36	0.551	2.17	0.147	0	0.996
Saladin v Low overall discolouration RILs	1.92	0.172	0.13	0.719	1.24	0.272
Saladin v High overall discolouration RILs	0.61	0.44	0.64	0.426	2.71	0.108
Iceberg v Low pinking RILs	0.46	0.501	0.37	0.547	0.65	0.426
Iceberg v High pinking RILs	0.02	0.892	2.08	0.156	1.64	0.209
Iceberg v Low browning RILs	3	0.089	1.72	0.196	1.6	0.214
Iceberg v High browning RILs	2.19	0.145	0.24	0.627	0	0.998
Iceberg v Low overall discolouration RILs	5.12	*0.028	1.81	0.185	1.22	0.276
Iceberg v High overall discolouration RILs	0	0.986	2.74	0.105	2.69	0.11



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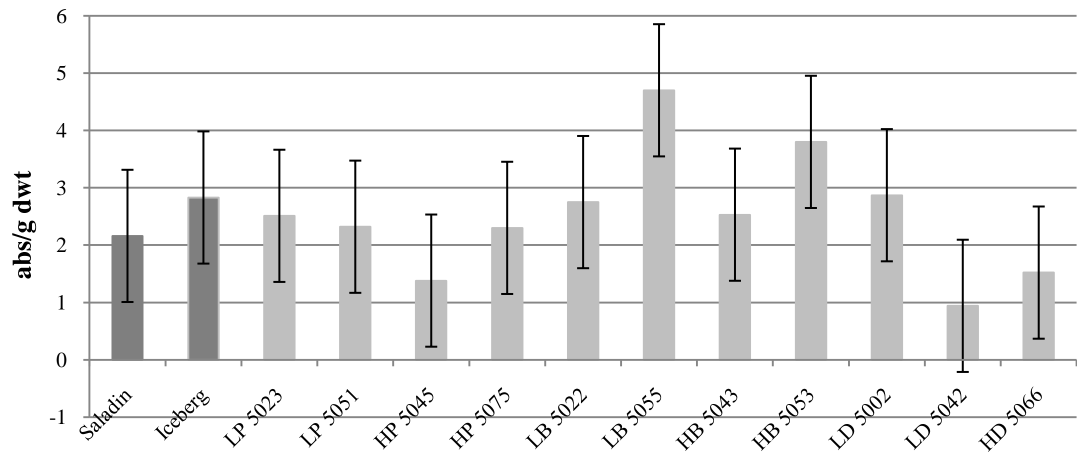

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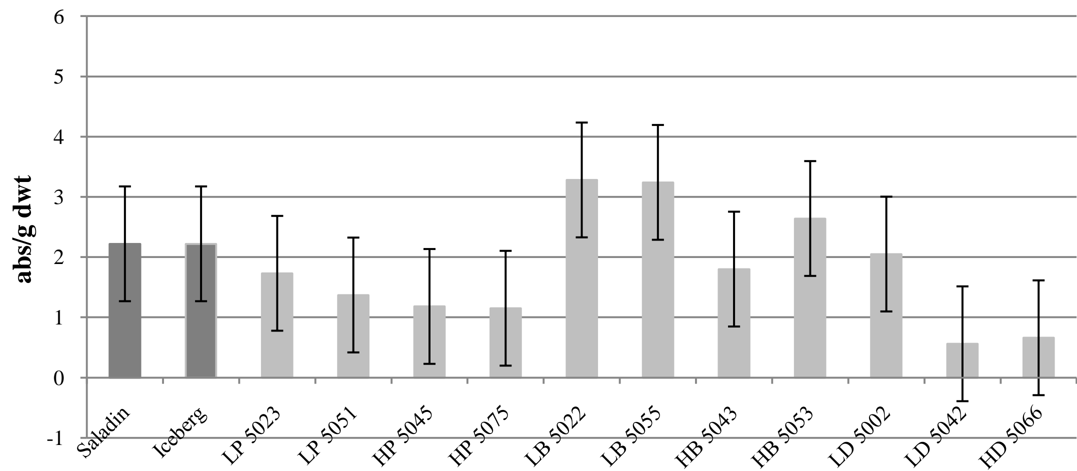

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Figure 7.3. Mean PPO (polyphenol oxidase) activity for RILs from the extreme discolouration subset on a) harvest day, b) 4 days after storage and c) the difference between PPO activity levels across the 4 days. PPO activity measured as absorbance per gram of dry weight abs/g dwt. Error bar represents seds (standard error of differences of the mean) from ANOVA. Where *dwt* (dry weight); *g* (gram).

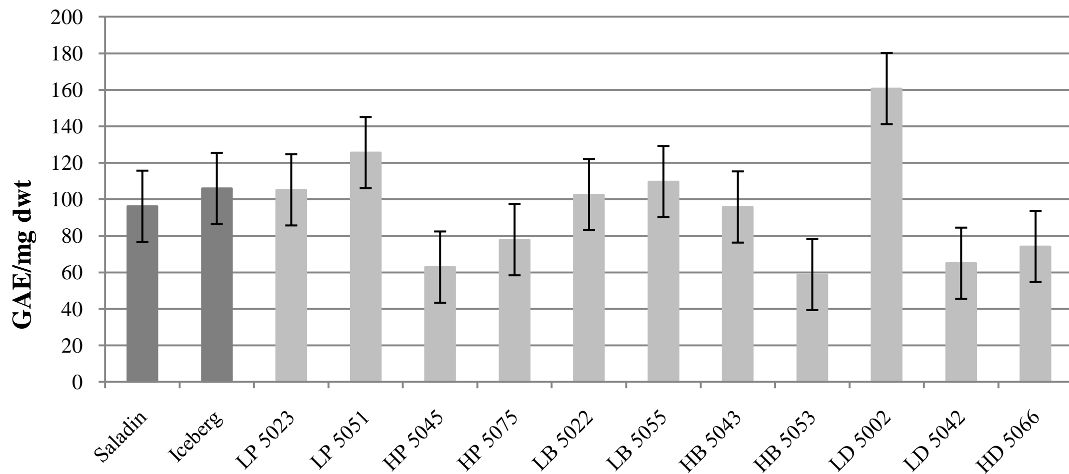
Total phenolic content

ANOVA revealed significant variation between lines for total phenolic content (TPC) when expressed as gallic acid equivalents per gram of dry mass of lettuce (GAE/g dwt) for harvest day (***P <0.001), day 4 post storage (**P <0.003) and for the difference of TPC across days (**P <0.002) (see table 7.6.).

TPC of the 13 lines varied from 58.8 mg GAE/g dwt to 150.7 mg GAE/g dwt for harvest day and from 77.8 mg GAE/g dwt to 170.3 mg GAE/g dwt on day 4 (see figure 7.4. (a-c)). Generally, low discolouration RILs had higher TPCs than high discolouration RILs on harvest day; the mean TPC of LP RILs was significantly higher than for HP RILs (**P < 0.002), the mean TPC of LB RILs was significantly higher than for HB RILs (*P < 0.05) and the mean TPC of LD RILs was significantly lower than for HD RILs (*P < 0.038). Generally, TPC increased from harvest day to day 4. Mean TPC was 95.4 mg GAE/g dwt and 106.8 mg GAE/g dwt respectively for harvest day and day 4. As for harvest day, low discolouration RILs generally had higher TPCs than high discolouration RILs. LP 5051 had significantly higher TPC than both HPs 5045 and 5075, while LD 5022 had significantly higher TPC than HD 5066. HB 5053 had significantly higher TPC than both LBs 5022 and 5055. Generally, TPC for low discolouration RILs decreased across days, while it increased across days for high discolouration RILs. Saladin and Iceberg showed an increase in TPC across days. Transgressive segregation was recorded; HB 5053 TPC increased at a higher rate across days than Iceberg and all RILs (87.2 mg GAE/g dwt) and LP 5023 TPC increased at a lower rate across days than Saladin and all RILs (87.2 mg GAE/g dwt).

Table 7.6. Total phenolic content (TPC) for RILs from the extreme discolouration subset over 4 days. ANOVA GT df = 12. ANOVA comparison df = 1. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *TPC* (total phenolic content); *GT* (genotype/accession); *RIL;* (recombinant inbred line); *v* (comparison); *vr* (variance ratio); *prob* (ANOVA probability).

	TPC					
	Day harvest		Day 4		Across days	
Variation	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**
GT	4.52	***<0.001	2.93	**0.003	3.22	**0.002
Saladin v Iceberg	0.2	0.656	2.01	0.161	0.04	0.836
Low pinking RILs v High pinking RILs	10.99	**0.002	1.04	0.313	5.03	*0.03
Low browning RILs v High browning RILs	4.01	*0.05	6.46	**0.01	7.37	**0.009
Low overall discolouration RILs v High overall discolouration RILs	4.55	*0.038	3.29	0.075	0.11	0.745
Saladin v Low pinking RILs	1.15	0.288	0	0.957	3.48	0.068
Saladin v High pinking RILs	1.96	0.168	0.48	0.49	0.02	0.879
Saladin v Low browning RILs	0.28	0.602	0.5	0.48	1.2	0.278
Saladin v High browning RILs	1.1	0.299	1.65	0.203	1.04	0.314
Saladin v Low overall discolouration RILs	0.84	0.364	0.43	0.515	0.01	0.926
Saladin v High overall discolouration RILs	1.02	0.317	0.93	0.34	0.04	0.847
Iceberg v Low pinking RILs	0.28	0.601	2.82	0.098	4.5	*0.039
Iceberg v High pinking RILs	3.71	0.059	5.61	*0.021	0.16	0.691
Iceberg v Low browning RILs	0	0.994	5.52	*0.022	1.79	0.187
Iceberg v High browning RILs	2.53	0.118	0.18	0.672	0.59	0.447
Iceberg v Low overall discolouration RILs	0.14	0.708	1.11	0.297	0.02	0.875
Iceberg v High overall discolouration RILs	2.13	0.151	5.67	*0.02	0.16	0.689



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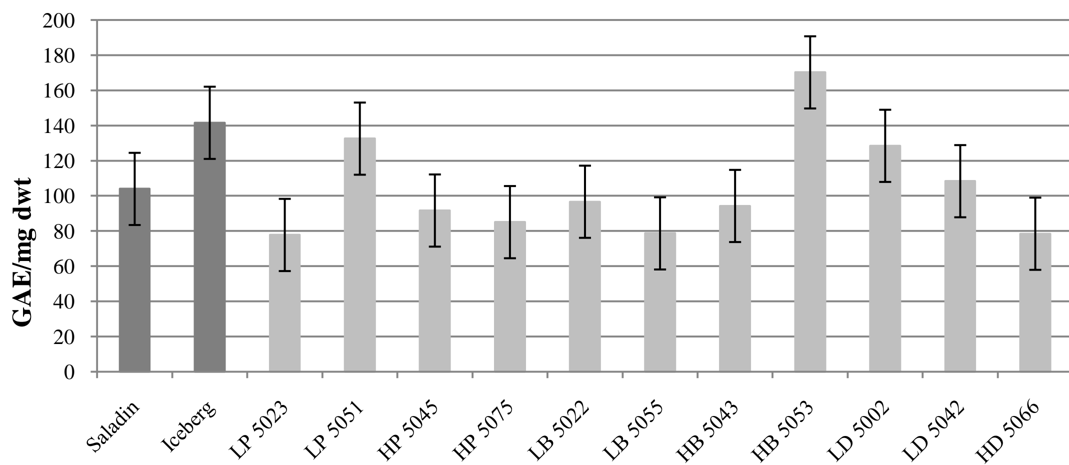
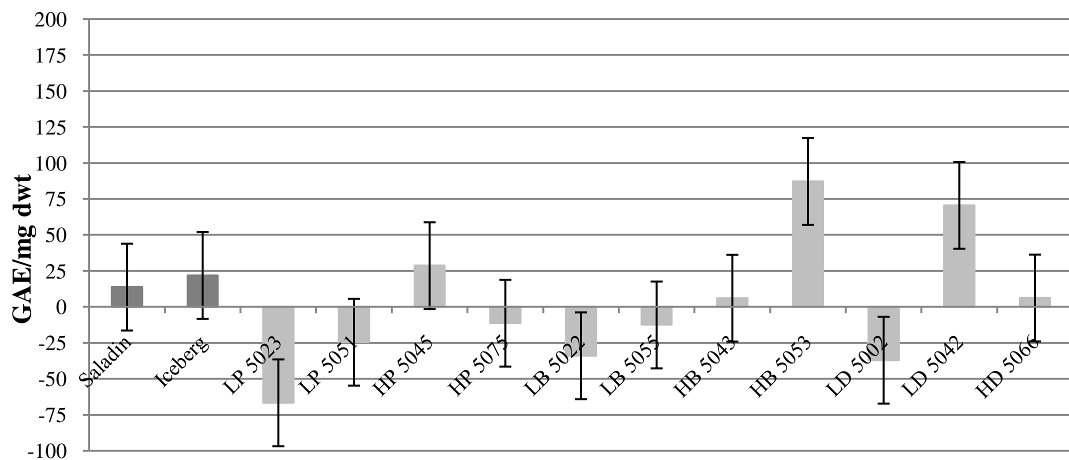


b



c

Figure 7.4. Mean TPC (total phenolic content) for RILs from the extreme discolouration subset on a) harvest day, b) 4 days after storage and c) the difference between TPC levels across the 4 days. TPC expressed as gallic acid equivalents (GAE) per gram of dry mass of lettuce. Error bar represents seds (standard error of differences of the mean) from ANOVA. Where *dwt* (dry weight); *g* (gram).

7.3.2. Correlation amongst traits

All discolouration measures (browning and pinking), morphological traits (plant weight) and metabolite activity were assessed for potential relationships (full correlation matrix in Appendix E) (see table 7.7.). The relationships recorded between discolouration parameters and between them and any morphological traits has been discussed in Chapter 5.

PAL activity on day 4 and PAL differences in activity across days were positively correlated ($R_{[11]} \geq 0.91$). Both of these parameters were generally positively correlated with browning (intensity and extent) and overall discolouration (intensity) on days 1 and 2 post harvest. The highest correlation for PAL activity on day 4 was with overall discolouration intensity on day 2 ($R_{[11]} \geq 0.66$) and the highest correlation for the difference between PAL activity across days was with browning intensity on day 1($R_{[11]} \geq 0.66$).

PPO activity on day 4 and PPO differences in activity across days were positively correlated ($R_{[11]} \geq 0.84$). The differences between PPO activity across days was positively correlated with extent of overall discolouration on day 3 ($R_{[11]} \geq 0.6$).

TPC on harvest day and differences in TPCs across days were negatively correlated ($R_{[11]} \geq -0.7$). The difference between TPC across days was generally positively correlated with overall discolouration (intensity) on days 3 and 4 post harvest (the highest correlation was with overall discolouration intensity on day 4 ($R_{[11]} \geq 0.69$)). TPC on harvest day was negatively correlated with PAL activity on day 4 ($R_{[11]}$ ≥ 0.59).

Protein level differences across days were negatively correlated with protein levels on harvest day ($R_{[11]} \geq -0.67$) and positively correlated with protein levels on day 4

($R_{[11]}$ ≥0.89). Protein levels on harvest day generally negatively correlated with browning post harvest (the highest correlation was with browning intensity on day 2 ($R_{[11]}$ ≥0.59)). It was also positively correlated with pinking (intensity and extent) on day 4 ($R_{[11]}$ ≥0.62 and $R_{[11]}$ ≥0.74 respectively).

Table 7.7. Correlation matrix from the RILs from the extreme discolouration RIL subset for post harvest discolouration and metabolite activity. Read across then down. Only significant effects are shown and highly significant effects ****P* <0.001 are shown bold, ***P* <0.01 are shown italics. Where *msb* (mean score browning); *msp* (mean score pinking); *msd* (mean score overall discolouration); *%b* (percentage browning); *%p* (percentage pinking); *%d* (percentage overall discolouration); *H* (harvest day); *Ac* (across days). Numerical value before measurement is day. Degree of freedom is 11.

	1msb	**2msb**	**1%b**	**1%b**	**4msp**	**5%p**	**4msd**	**msd**	**3%d**	**4 PAL**	**4 PPO**	**H TPC**	**H Pro**	**4 Pro**
4 PAL	0.61							0.56						
Ac PAL	*0.70*		0.63							**0.91**				
Ac PPO									0.60		**0.84**			
H TPC										-0.59				
Ac TPC							*0.69*	0.56		0.46		*-0.70*		
H Pro		-0.59		-0.58	0.62	*0.74*								
Ac Pro													-0.67	**0.89**

7.3.3. Candidate gene mapping

EST data mining for putative candidate genes

For each candidate gene, the locus number and protein sequence in *Arabidopsis* (see table 7.3.) was retrieved from the TAIR database (see table 7.8). Of the 61 potential candidate genes there were 13 for which a locus could not be identified from the database (Acetyl CoA carboxylase (ACCase), Aureusidin synthase (AS), Chalcone reductase (CHR), 72_-dihydroxy 4_-methoxyisoflavanol dehydratase (DMID), Flavanone 35 hydroxylase (F35H), Flavanone 3'5' hydroxylase (F3’5’H), Flavone synthase (FS1/FS2), Isoflavone 2-hydroxylase (I2_H), Isoflavone synthase (IFS), Leucoanthocyanidin reductase (LAR/ LCR), Transparent testa 15 (TT15) and Vestitone reductase (VR)).

The contig/EST with the highest identity to each of the remaining 48 candidate gene protein sequence was selected (see table 7.8). One gene (Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (ACCD)) did not generate any hits against the associated contig/EST database. To ensure that the contig/EST had its highest identity to the associated gene a reciprocal blast was conducted against an *Arabidopsis* database. Of the 4 copies of the 4-coumarate:CoA ligase (4CL) gene, the sequences were associated with 2 contigs, however only one of these was for 4CL. Of the 6 copies of the Flavonol synthase (FLS) gene, the sequences were all associated with 1 contig that was for FLS. Of the 4 copies of the Phenylammonia lyase (PAL) gene, the sequences were all associated with 1 contig that was for PAL2. Of the 2 copies of the Production of anthocyanin (PAP) gene, the sequences were all associated with 1 contig that was for PAP2. Of the 2 copies of the Arabidopsis reductase (AR) gene, the sequences were associated with 2 contigs, however only

one of these was for AR2. When there were a number of gene copies in *Arabidopsis,* which all aligned to the same lettuce contig/EST, then all copies may not have not have been found in lettuce or the extra copies may not exist in lettuce. If there were 1 or 2 copies such as for AR and PAP then both reasons are possible, however with increased copies such as for 4CL, FLS and PAL the latter is more possible. This resulted in 17 unique potential candidate genes suitable for analysis.

Table 7.8. EST data mining results for candidate genes relating to the phenylpropanoid pathway. Information from candidate genes in *Arabidopsis thaliana* from the TAIR database (www.arabidopsis.org). Where ✓ (suitable); × (not suitable); *NA* (not applicable).

Gene annotation	Locus	Contig from assembly	Reciprocal blast protein	Suitable for candidate gene	No. introns
4CL1	AT1G51680	CLS_S3_Contig7103	4CL1	✓	0
4CL2	AT3G21240	CLS_S3_Contig2246	ACYL-COA SYNTHETASE 5	×	NA
4CL3	AT1G65060	CLS_S3_Contig2246	ACYL-COA SYNTHETASE 5	×	NA
4CL5	AT3G21230	CLS_S3_Contig2246	ACYL-COA SYNTHETASE 5	×	NA
5MAT	AT3G29590	CLS_S3_Contig8439	transferase family protein	×	NA
ACCase	/	/	/	×	NA
ACCD	ATCG00500	/	/	×	NA
ANL2	AT4G00730	CLSM11952.b1_O11.ab1	ATML1	×	NA
ANR	AT1G61720	CLS_S3_Contig4844	dihydroflavonol 4-reductase family / dihydrokaempferol 4-reductase family	×	NA
ANS	AT4G22880	CLS_S3_Contig4920	FLS	×	NA
AR1	AT4G24520	CLS_S3_Contig7982	AR2	×	NA
AR2	AT4G30210	CLS_S3_Contig7982	AR2	✓	15
AS	/	/	/	×	NA
CFI/CHI	AT3G55120	CLS_S3_Contig3861	CFI/CHI	✓	3
CHR	/	/	/	×	NA
CHS	AT5G13930	CLSM3570.b1_D05.ab1	CHS	✓	1
COA	AT5G48930	CLS_S3_Contig8834	COA	✓	1
CYP98A3	AT2G40890	CLS_S3_Contig1544	CYP98A3	✓	2
DFR	AT5G42800	CLS_S3_Contig9432	cinnamoyl-CoA reductase 1	×	NA
DMID	/	/	/	×	NA

Table 7.8. continued.

Gene annotation	Locus	Contig from assembly	Reciprocal blast protein	Suitable for candidate gene	No. introns
EGL3	AT1G63650	CLS_S3_Contig2106	MYC2	×	NA
F35H	/	/	/	×	NA
F3'5'H	/	/	/	×	NA
F3H	AT3G51240	CLS_S3_Contig3385	F3H	✓	4
F3'H	AT5G07990	CLS_S3_Contig9541	F3'H	✓	3
F7ORT	AT1G06000	CLS_S3_Contig7415	UDP-glucoronosyl/UDP-glucosyl transferase family protein	×	NA
FLS1	AT5G08640	CLS_S3_Contig4920	FLS	✓	2
FLS2	AT5G63580	CLS_S3_Contig4920	FLS	✓	2
FLS3	AT5G63590	CLS_S3_Contig4920	FLS	✓	2
FLS4	AT5G63595	CLS_S3_Contig4920	FLS	✓	2
FLS5	AT5G63600	CLS_S3_Contig4920	FLS	✓	2
FLS6	AT5G43935	CLS_S3_Contig4920	FLS	✓	2
FSI/FS2	/	/	/	×	NA
GST	AT5G17220	CLS_S3_Contig2862	GST	✓	2
I2_H	/	/	/	×	NA
IFR	AT1G75280	CLS_S3_Contig11204	IFR	✓	4
IFS	/	/	/	×	NA
LAR	/	/	/	×	NA
LCR/LAR	/	/	/	×	NA
LDOX/ANS	AT4G22880	CLS_S3_Contig4920	FLS	×	NA
MYB3	AT1G22640	CLS_S3_Contig10237	MYB4	×	NA

Table 7.8. continued.

Gene annotation	Locus	Contig from assembly	Reciprocal blast protein	Suitable for candidate gene	No. introns
MYB4	AT4G38620	CLS_S3_Contig10237	MYB4	✓	1
OMT1	AT5G54160	CLS_S3_Contig7929	OMT1	✓	3
PAL1	AT2G37040	CLS_S3_Contig2068	PAL2	✓	1
PAL2	AT3G53260	CLS_S3_Contig2068	PAL2	✓	1
PAL3	AT5G04230	CLS_S3_Contig2068	PAL2	✓	1
PAL4	AT3G10340	CLS_S3_Contig2068	PAL2	✓	1
PAP1	AT1G56650	CLSS2363.b1_E15.ab1	PAP2	×	NA
PAP2	AT1G66390	CLSS2363.b1_E15.ab1	PAP2	✓	2
PPO	AT5G48100	CLS_S3_Contig4395	PPO	✓	4
TT1	AT1G34790	CLSM13222.b1_L17.ab1	JKD	×	NA
TT12	AT3G59030	CLS_S3_Contig7142	MATE efflux family protein	×	NA
TT14/TT19	AT5G17220	CLS_S3_Contig2862	GST	×	NA
TT15	/	/	/	×	NA
TT16	AT5G23260	CLS_S3_Contig4782	AP1	×	NA
TT2	AT5G35550	CLS_S3_Contig1853	MYB111	×	NA
TT8	AT4G09820	CLS_S3_Contig2106	MYC2	×	NA
TTG1	AT5G24520	CLS_S3_Contig4839	TTG1	✓	0
TTG2	AT2G37260	CLS_S3_Contig4165	WRKY33	×	NA
UF3GT/3GT	AT5G54060	CLSM17476.b1_G02.ab1	glycosyltransferase family protein	×	NA
VR	/	/	/	×	NA

SNP detection

Of the 17 candidate genes, 2 of these had no introns (4CL1 and Transparent testa glabra 1 (TTG1)) so primers were designed for the entire sequence (see table 7.9). For the remaining candidate genes, primers were designed around each intron. Most primers were designed to include a single intron within a small product (250-300 bp), however when they were close together they were included within a primer pair (see table 7.8 and 7.6). Of the 40 primer pairs, 8 did not produce PCR products for Saladin and Iceberg. However, there were still alternative primers for each gene (see table 7.9). All PCR products were then run on an SSCP gel. Of the PCR products for 32 primer pairs, 13 produced polymorphic bands between Saladin and Iceberg. Some of these were for the same gene which resulted in 10 candidate genes for analysis (AR2), Chalcone isomerise (CHI), Chalcone synthase (CHS), Hydroxycinnamoyl-coa shikimate transferase (COA), Flavanone 3-hydroxylase (F3H), Flavanone 3'-hydroxylase (F3'H), Glutathione S-transferase (GST), Isoflavone reductase (IFR), Polyphenol oxidase (PPO) and 4CL1.

For the primers that produced a PCR product that was not polymorphic between Saladin and Iceberg on an SSCP gel, products were sequenced. Polymorphisms were found for Cytochrome P450 (CYP98A3) and PAL2 (see table 7.10.). Monomorphic sequence was confirmed between the parental lines for O-methyltransferase (OMT) (primer pairs 1 and 2) and PAP2 (primer pair 2). The other sequences were not reproducible.

Table 7.9. Candidate gene primers and PCR product information for agarose and SSCP gel.

Forward primer	Sequence	Reverse primer	Sequence	Estimated product size	Introns included	PCR product	SSCP polymorphism
AR2_f1	TTTTCTTCGGAACGCAGACT	**AR2_r1**	AGTTGGCTCACCATCTCCAT	203	521, 582, 659	✓	×
AR2_f2	TACGCTGTTGATGACGAGGA	**AR2_r2**	CCTGATCTGCAAGACCATCA	247	659, 717, 801	×	×
AR2_f3	TGATGGTCTTGCAGATCAGG	**AR2_r3**	ATCTGCATGGATGTTGAGCA	278	838, 908	✓	✓
AR2_f4	TGCTCAACATCCATGCAGAT	**AR2_r4**	AATGAGGATCCAGCATTTGG	261	110, 1193	✓	×
AR2_f5	ACACCAAATGCTGGATCCTC	**AR2_r5**	AAGTGGAGGTTTGGCTGATG	267	1416, 1501	✓	✓
AR2_f6	CATCAGCCAAACCTCCACTT	**AR2_r6**	CAGGCCCAATCATGATAACC	285	1652, 1749	✓	×
AR2_f7	GTCCCGGTTATCATGATTGG	**AR2_r7**	ATACGCTCCCTCAGAAAGCA	300	1977, 2100	✓	×
CHI_f1	AATCCGTCGTATTTCCACCA	**CHI_r1**	CAGCAGCAGTTTTTCCCTTC	186	166	✓	✓
CHI_f2	AAGGGAAAAACTGCTGCTGA	**CHI_r2**	CAAACACCAACGCACATTTC	155	323	×	×
CHI_f3	GAAATGTGCGTTGGTGTTTG	**CHI_r3**	TGCCCCAATTTCTCATTCTC	221	546	✓	✓
CHS_f	AGAGAAGTTCCAGCGCATGT	**CHS_r**	GGGGACTTCGACAACTACCA	151	265	✓	✓
COA_f	CCCTTTGCTGGTCGATTAAG	**COA_r**	AATCCATCGGCGAGAGTATG	272	524	✓	✓
CYP98A3_f1	AAGGATTTGATCTGGGCTGA	**CYP98A3_r1**	CGCTTCCCAAATGTCAATCT	248	1107	✓	×
CYP98A3_f2	CCTTAGCGACGACACAATCA	**CYP98A3_r2**	GTTTGCATTGGCTTTGTGTG	277	1503	✓	×
F3H_f1	ATCTCGTAGGGCGGAGATTT	**F3H_r1**	TATCGGGCCACCTTGAGTAG	296	416	×	×
F3H_f2	GATGGTGGCAAGAGTTGGA	**F3H_r2**	ATGGATAGCCGACTGGTTGT	148	844	✓	×
F3H_f3	AAATCTCGTAGGGCGGAGAT	**F3H_r3**	TATCGGGCCACCTTGAGTAG	298	416	×	×
F3H_f4	CCTCAACCCGATCTCACATT	**F3H_r4**	ATGGATAGCCGACTGGTTGT	247	844	✓	✓
F3'H_f1	TGCTCAGTGCACCTGTTTTC	**F3'H_r1**	GAATTCTCCGGCTAACACCA	267	508	✓	×
F3'H_f2	AGAGGGAGGGAAGCTTTCAG	**F3'H_r2**	CCATTTCTTGTTGGGCTTGT	152	950	✓	✓
F3'H_f3	ACAAGCCCAACAAGAAATGG	**F3'H_r3**	AAGGAGAGTGGTGTGGATGG	135	1120	×	×

Table 7.9. continued.

Forward primer	Sequence	Reverse primer	Sequence	Product size	Introns included	PCR product	SSCP polymorphism
FLS_f1	ATAGGGTTTGGCCACCTTCT	**FLS_r1**	GAGGTACAACCCCAAGAGCA	258	478	×	×
FLS_f2	TGCTCTTGGGGTTGTACCTC	**FLS_r2**	TTGGTCCGACCTCAAACTCT	272	807	✓	×
GST_f	TCCCGAGTTCCTGAAGTTACA	**GST_r**	GCCTCGACTTCTAGCCATTG	260	292/340	✓	✓
IFR_f1/2	AAGTCCGGCCATCCTACTTT	**IFR_r1**	TGTGTGACCTACCGTGGAGA	186	284	✓	✓
		IFR_r2	GATCCACATCGTTTCCGAAC	280	417	✓	×
IFR_f3	CTGGCTATTTCCTCCCAACA	**IFR_r3**	CCGATCTTCTTCTCCCACAA	238	654	✓	×
IFR_f4	GACGGAAATGCAAAAGTGGT	**IFR_r4**	CGTTCAACGGAACTGGAGAT	241	855	×	×
OMT_f1	CGCCTCTCCTGCTTATGAAC	**OMT_r1**	GGCCATTGAACCCATCATAC	228	492	✓	×
OMT_f2	ATTTTGATTTGCCCCATGTC	**OMT_r2**	ATTCGGCCACAATCACTTTC	207	803	✓	×
OMT_f3	ATGTTGGTGGGGACATGTTT	**OMT_r3**	ATTCGGCCACAATCACTTTC	153	869	✓	×
PAL2_f	CTCCACCCCTCAAGATTCTG	**PAL2_r**	GCGATTCTTACACCCTCGAC	254	1402	✓	×
PAP2_f1/2	GCATGGACTGCTGATGAAGA	**PAP2_r1**	TTCTCCCCGCTATCAATGAC	241	249	×	×
		PAP2_r2	CATTGGCAGTTCTTCCAGGT	262	379	✓	×
PPO_f1	TCCATTCACAAGCTGCTGTC	**PPO_r1**	ATCGGAAAGCAAAACCCTTT	299	243	✓	✓
PPO_f2	TCCATTCACAAGCTGCTGTC	**PPO_r2**	ATCGGAAAGCAAAACCCTTT	299	393	✓	✓
PPO_f3	ATGAAGAGGGGACATTGTGG	**PPO_r3**	ACCAGGCTGACCGTTGATAG	242	635	✓	×
PPO_f4	CTATCAACGGTCAGCCTGGT	**PPO_r4**	GGAGGAGGTCTAGGGTTTGG	237	762	✓	×
TTG1_f	TTCTACCACCACCGTCTTCC	**TTG1_r**	AAAGCAATGGCAATCCAATC	1034	/	✓	×
4CL1_f	CCGACGCCTATTTCTCTCTC	**4CL1_r**	CCACCAATTCCTCGTTGTTC	912	/	✓	✓

Table 7.10. SNPs in candidate genes for the phenylpropanoid pathway. Where number following _ refers to primer pair numbers.

Gene	SNP position
CYP98A3_1	291, 386
CYP98A3_2	283
PAL2	50-51, 93, 115

SNP mapping

The Saladin x Iceberg RILs were screened with primers for candidate genes GST and PPO, and the markers were mapped as described in Chapter 4. GST mapped on LG 7 as on the MCB10_10NR map and to its estimated position at 8.9 cM. GST also co-located with markers BAIJ-OP4 and BAAZ-OP4 (see figure 7.4). The marker for GST mapped in a 'hot spot' for discolouration QTL (1.7 – 54.2 cM) (identified in Chapter 5). It did not extend the length of the group and all other characteristics of the LG and map remained constant; the average loci interval of LG 7 and the map respectively was still 1.1 cM and 2.4 cM. The marker did not show segregation distortion ($X^2_{[2]}$=2.07.) PPO did not map on LG 9b as expected, as there was not enough linkage between the few markers on that LG. The marker for PPO showed highly significant (***P <0.001) segregation distortion ($X^2_{[2]}$=38.83).

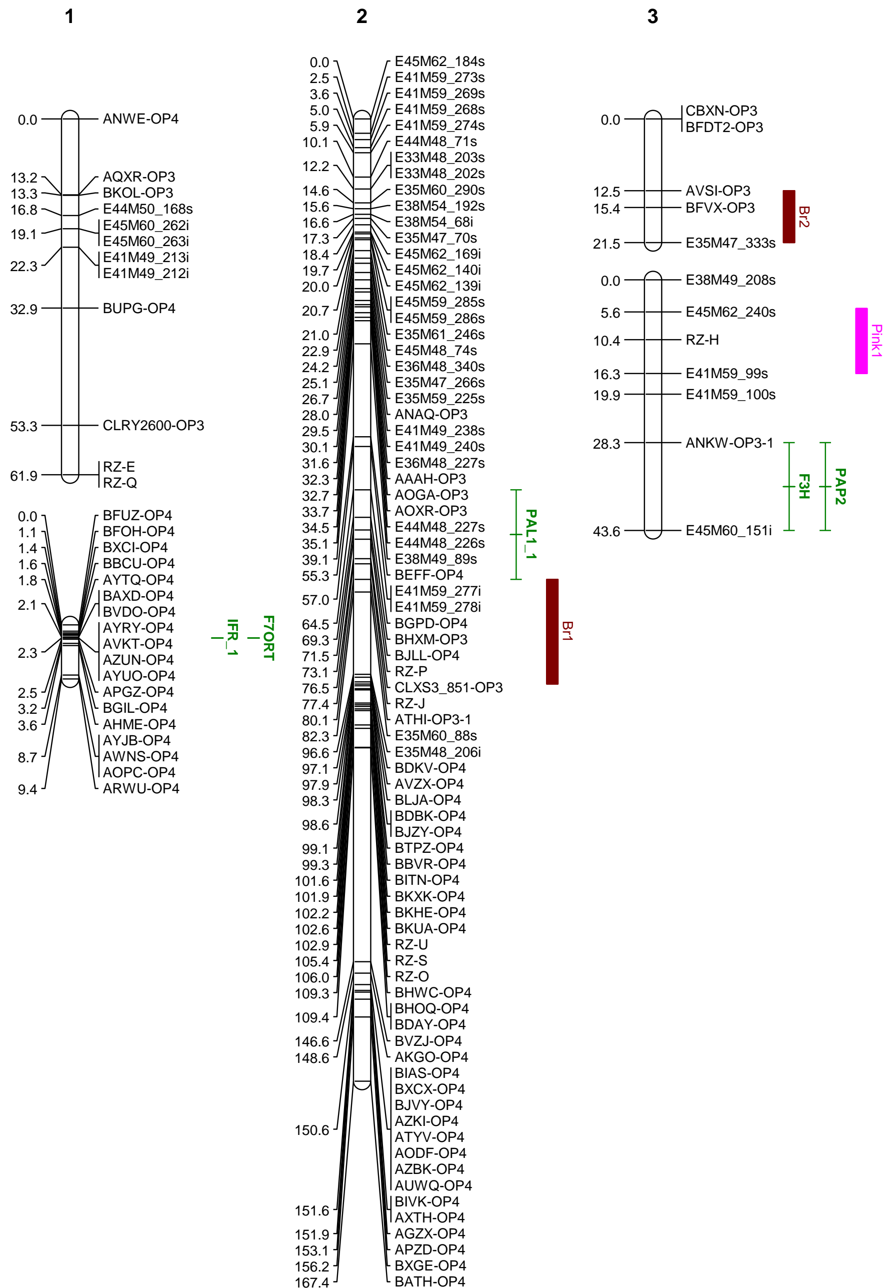


Figure 7.5. Candidate genes and QTL impacting on post harvest discolouration of lettuce tissue from the Saladin x Iceberg RIL population. Green bars represent candidate gene placed positions. Red marker and arrow represents mapped marker. Number after _ refers to number of gene positions for a candidate gene. Where *a* and *b* represent the potential area for candidate gene to map which is split across component linkage groups. Full colour blocks represent QTL. Where *Br* (browning); *Pink* (pinking); *Dis* (overall discolouration). Number after discolouration parameter refers to QTL number for that specific trait.

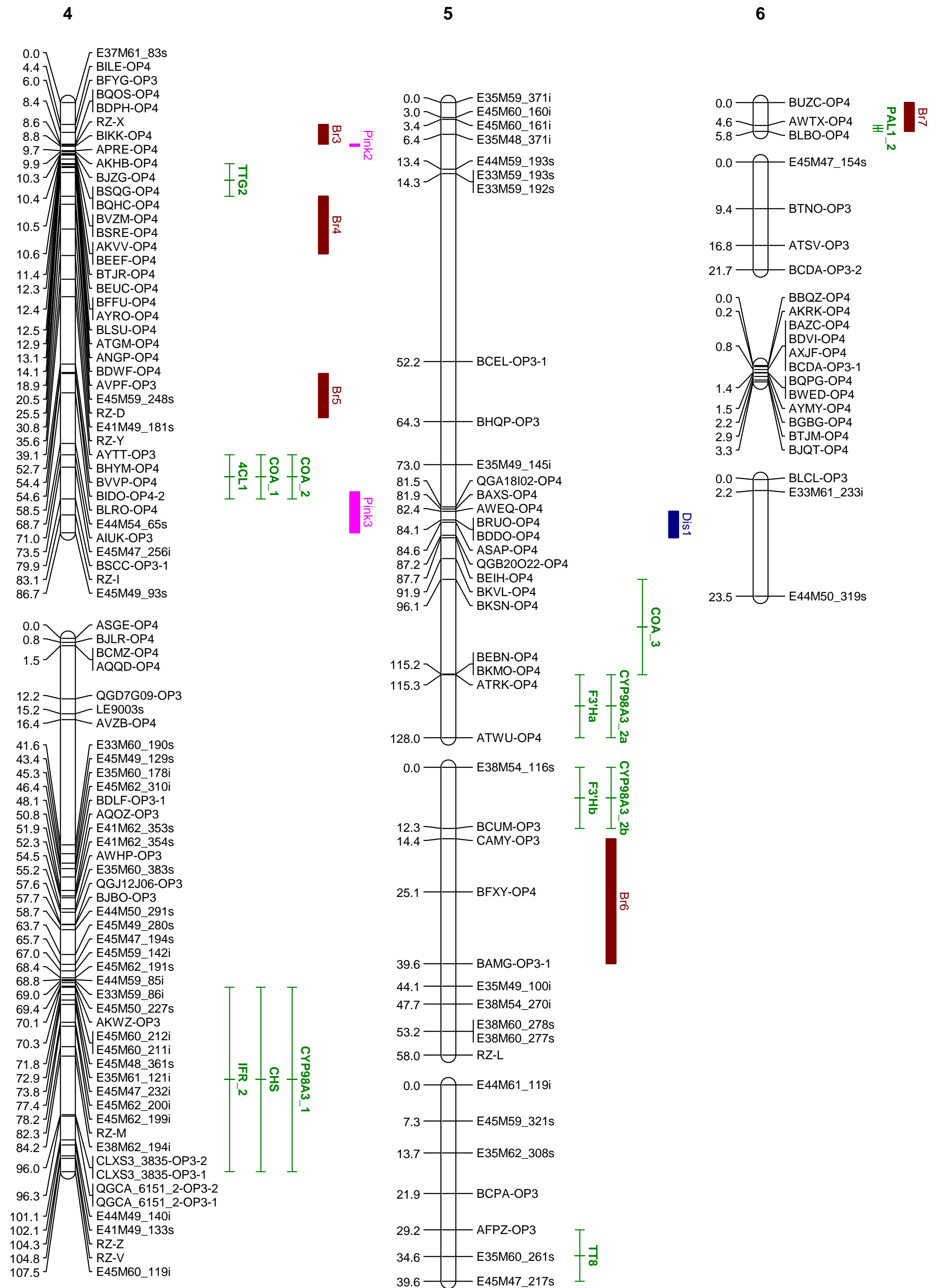


Figure 7.5. continued.

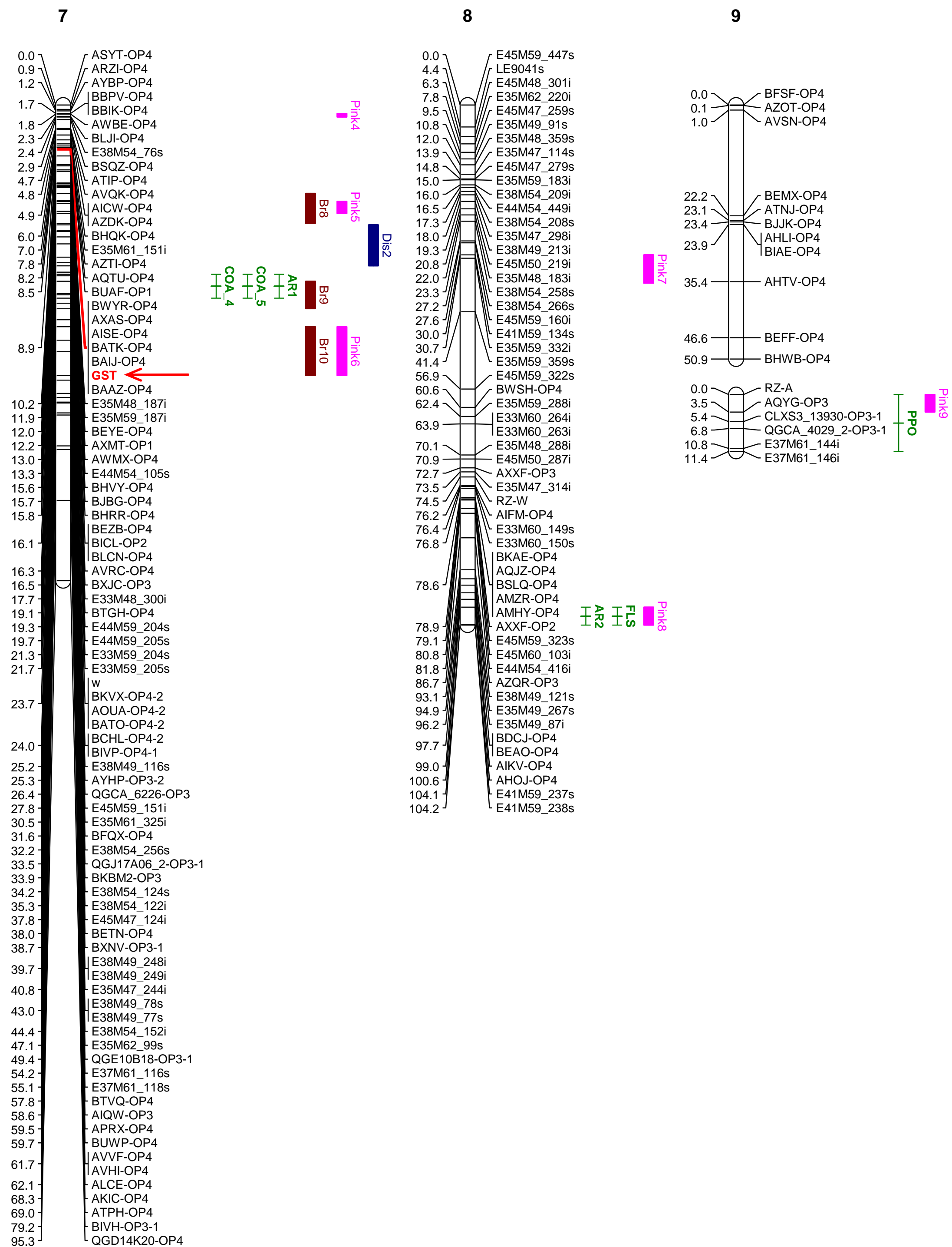


Figure 7.5. continued.

Comparative studies of the MCB19_10NR_map with the Sal x Ice map allowed map positions of candidate genes to be estimated (see table 7.11. and 7.9. and figure 7.5.). The MCB19_10NR_map was searched for the contig/EST from the assembly for the

associated candidate gene (see table 7.11.) and the *Arabidopsis* locus (see table 7.12.). Contig/EST data was then cross-referenced to identify markers and their positions on the MCB19_10NR_map, and if any of these were present in the Sal x Ice map. Some candidate genes were placed to an exact position on the Sal x Ice map, while for other genes only a position within an area on the map could be identified (see figure 7.5.).

Table 7.11. Candidate gene map positions for the phenylpropanoid pathway on map MCB19_10NR_map based on contig. Where *LG* (linkage group).

Gene annotation	Contig from assembly	LG	Order	Origin marker code	Gene for contig from map
4CL1	CLS_S3_Contig7103	4	1064, 1065, 1083	BHER	4CL
AR2	CLS_S3_Contig7982	8	1739, 1740	BIPX	AR2
CYP98A3	CLS_S3_Contig1544	4	2604	AYFM	CYP98A3
F3H	CLS_S3_Contig3385	3	1135, 1136	BBEO	F3H
FLS	CLS_S3_Contig4920	8	887, 916	BDRC	FLS
PPO	CLS_S3_Contig4395	9	1015	BCUX	PPO

Table 7.12. Candidate gene map positions for the phenylpropanoid pathway on map MCB19_10NR_map based on locus. Where *LG* (linkage group); *order* (marker order number on map).

Gene annotation	Locus	Contig from map	LG	Order	Origin marker code	Gene for contig from map
AR1	AT4G24520	CLSZ1307.b1_F15.ab1	7	813, 815, 819, 821, 822, 823	AUOP	NADPH-ferrihemoprotein reductase (NADPH-cytochrome p450 reductase)
CHS	AT5G13930	CLSM403.b2_E05.ab1	4	2124, 2125, 2136, 2139, 2140, 2147, 2157, 2158, 2159	ALBH	CHS
COA	AT5G48930	QGC3a03.yg.ab1	4	1090	BVIT	anthranilate N-hydroxycinnamoyl/benzoyltransferase family
		QGA10I08.yg.ab1	4	1123	BQHX	anthranilate N-hydroxycinnamoyl/benzoyltransferase family
		CLS_S3_Contig8847	5	137	BKAJ	anthranilate N-hydroxycinnamoyl/benzoyltransferase family
		CLS_S3_Contig6733	7	732	BGOW	anthranilate N-hydroxycinnamoyl/benzoyltransferase family
		CLSZ1823.b1_M24.ab1	7	738	AURU	anthranilate N-hydroxycinnamoyl/benzoyltransferase family
CYP98A3	AT2G40890	CLS_S3_Contig186	5	963	AYSM	CYP98A3
F3'H	AT5G07990	QGC7D22.yg.ab1	5	257, 258	BVNU	F3'H and F3'5'H
F7ORT	AT1G06000	QGB12K08.yg.ab1	1	2028	BRYX	glycosyltransferase family
IFR	AT1G75280	CLSY3307.b1_E12.ab1	1	1950, 1952, 1959, 1960	ATEW	IFR
		CLSM11379.b2_E14.ab1	4	2027, 2048, 2078, 2086, 2094, 2095	AHOL	IFR
PAL	AT2G37040	CLSL2456.b1_P13.ab1	2	2243, 2250, 2255	AHDU	PAL1
		CLS_S3_Contig2329	6	402, 405, 406, 407, 409	AZME	PAL1
PAP2	AT1G66390	CLSS6254.b1_L03.ab1	3	1270, 1271, 1272, 1273	APZN	myb family transcription factor
TT8	AT4G09820	QGG26L04.yg.ab1	5	2974	BZKG	bHLH protein
TTG2	AT2G37260	CLLX3544.b1_O21.ab1	4	633	AALQ	WRKY family transcription factor

7.4. Discussion

Metabolite levels in food are becoming increasingly important in today's market of health and 'superfoods'. Natural antioxidants, principally phenolic substances, are thought to have both nutraceutical and health benefits (Fan *et al.* 2007; Siddhuraju and Becker 2007). Epidemiological studies have also revealed an inverse relationship between flavonoid dietary intake (resulting from the phenylpropanoid pathway) and the risk of some cancers and coronary heart disease (Duthie and Crozier 2000; Puupponen-Pimia *et al.* 2001; Hung *et al.* 2004; Tripoli *et al.* 2007). In addition, changes in these compounds may alter the plant's defence against pest and diseases. It is therefore important to monitor any affect on the metabolome that may occur when breeding for new varieties with improved phenotypes.

All lines showed similar PAL activity on harvest day; however activity generally increased across days at different rates (rank positions within the distribution of lines across days were reflected to differing rates of discolouration), (with the exception of LB 5055 and LD 5002 which showed a decrease in PAL activity across days). PAL activity on day 4 was positively correlated with browning (intensity and extent) and overall discolouration (intensity) on days 1 and 2 post harvest (see table 7.12.). An increase in post harvest discolouration would be expected for RILs with higher PAL activity as PAL is the rate limiting enzyme of the pathway controlling the flux of substrates into the pathway, thus affecting the degree of post harvest discolouration (Wanner *et al.* 1995). PAL activity on day 4 was also negatively correlated with TPC on harvest day (see table 7.12) which would confirm that as PAL activity increases total phenolics decrease, as the plant contains a finite amount of substrate

for the biosynthesis of polyphenols which are metabolised through the phenylpropanoid pathway. PAL activity has previously been shown to affect post harvest browning of cut salads (Clifford *et al.* 2001). The level of browning and overall discolouration observed in the field trials was related to an increased PAL activity, which would suggest that the level of discolouration of a line is under genetic control and is influenced by the availability of phenolic substrates for the phenylpropanoid pathway following tissue wounding. The relationship between discolouration and PAL activity, coupled with increased PAL transcripts observed in *Nicotiana* following wounding, supports the idea that an effective control of post harvest discolouration could be manipulated or controlled for reduced polyphenol biosynthesis (Fukasawa-Akada *et al.* 1996; Hisaminato *et al.* 2001). The first gene copy of PAL1 (PAL_1) was placed to an estimated position (using comparative genomics) on LG 2 (64.5-80.1 cM) of the Sal x Ice map, which was close to *Br1* (see figure 7.5.). The second gene copy of PAL1 (PAL_2) was placed on LG 6a (4.6-5.8 cM), which co-located with *Br7*, providing initial evidence that these genes may underlie the two QTL.

All lines of the subset showed similar PPO activity on harvest day; however, activity generally increased across days at different rates. PPO activity on day 4 was positively correlated with overall discolouration (intensity) on day 3 (see table 8.10). PPO oxidises phenolic substrates from the phenylpropanoid pathway, which leads to the development of coloured pigments causing discolouration spoilage (Joslin and Pointing 1951; Zawistowski *et al.* 1991; Martinez and Whitaker 1995; Solomon *et al.* 1996; Lopez-Galvez *et al.* 1996; Toivonen and Brummell 2008; Van Vliet *et al.* 2009). PPO is reported to be positioned on LG 9b (RW Michelmore personal communication) which also contains a QTL for pinking (*Pink9*), however as there is

only a small number of markers on this group it was hard to define its position in the Sal x Ice map (see figure 7.5.). PPO is compartmentalised and thus always present in cells at supraoptimal levels and released upon cell damage (Degl'Innocenti *et al.* 2005; Toivonen and Brummell 2008). It has been suggested that the degree of discolouration observed is due to substrate availability (driven by PAL activity) rather than PPO activity (Vaughn *et al.* 1988; Clifford *et al.* 2001). The absence of correlations between discolouration phenotypes and PPO activity and the presence of correlations with PAL activity supports this.

Other candidate genes thought to be involved in the discolouration were also investigated for polymorphisms between the mapping parents, with the aim to see if they could explain the genetic basis of the discolouration QTL.

GST was mapped on the Sal x Ice linkage map to LG 7 at 8.86 cM. This was in a hot spot region for 7 discolouration QTL (*Pink4, Pink5, Pink6, Br8, Br9, Br10* and *Dis2*). GST has been shown to be involved in the accumulation of both anthocyanins and proanthocyanidins. *Arabidopsis* mutants lacking GST (TT19) have displayed no pigments on leaves and stems (Kitamura *et al.* 2004). Potential map positions for genes AR1, COA_4 and COA_5 were also identified in this region co locating with *Br9* (35.3–38.7 cM). AR controls the conversion of 3-OH-anthocyanins to epi-flavan-3-ols that upon condensation become condensed tanins, which are the precursors to post harvest browning (see figure 1.2.) (Lepiniec *et al.* 2008). COA synthesises substrates for the phenylpropanoid pathway that influences the accumulation of flavonoids (Li *et al.* 2010). The potential co-location of these many candidate genes with *Br9* and close proximity to other discolouration QTL suggests that the candidate genes could be the genetic basis controlling the QTL and hence the phenotype.

Seven candidate genes were identified as being positioned on LG 4 (4 on LG 4a and 3 on LG 4b). The potential map position of TTG2 was located on LG 4a (12.3-18.9 cM) which was loosely clustered with *Pink2, Br3* and *Br4.* TTG2 has been shown to be involved in proanthocyanidin synthesis. *Arabidopsis* mutants have displayed loss of seed pigmentation due to loss of tannin accumulation (Smyth *et al.* 2002). This reaction also results in post harvest browning in vegetative tissue (Lopez-Galvez *et al.* 1996). COA_1, COA_2 and 4CL1 were positioned on LG 4a (71-79.9 cM), co-locating with *Pink3* (78.5-79.9 cM) and close to *Br5.* COA has been shown to synthesise substrates for the pathway (as above (Li *et al.* 2010)) as does 4CL1, which both indirectly regulate production of post harvest discolouration pigments (see figure 1.2. and appendix E). The positions of IFR_2, CHS and CYP98A3_1 were co-located on LG 4b (70.3-107.4 cM). IFR is involved in isoflavonoid production while CYP98A3 is involved in flavonoid biosynthesis; both occur via a side branch of the main pathway (see figure 1.2.). CHS is one of the first enzymes involved in the pathway and therefore could indirectly affect the activity of IFR and CYP98A3.

Four candidate genes (COA_3, F3'H, CYP98A3 and TT8) were placed on LG 5. F3'H and CYP98A3 could occur somewhere over two component linkage groups (5 a and b). F3'H, CYP98A3 and COA_3 are tightly clustered between 2 discolouration QTL (*Dis1* and *Br6*). COA, F3'H and CYP98A3 function at the same level of the pathway but on different branches; all regulating flavonoid biosynthesis (see above and figure 1.2.).

PAP2 and F3H were placed on LG 3b (28.3-43.6 cM) and were loosely clustered with *Pink1*. PAP2 is involved in the production of anthocyanin pigments while F3H converts flavanones to 3'-OH-flavanones; both genes function at the start of the phenylpropanoid pathway and indirectly affect the level of discolouration.

FLS and AR2 were positioned on LG 8 (100.6-104.2 cM) which co-locates with *Pink8.* FLS controls the conversion of dihydroflavonols to flavonols that occurs upstream of AR on the same branch of the pathway leading to condensed tannins, which eventually become the discolouration pigments (see figure 1.2.) (Lepiniec *et al.* 2008).

The candidate genes IFR and F7ORT were both placed on the map by a comparative approach on LG 1 at 2.33 cM and are thought to respectively affect isoflavonoid and flavonol production. These genes could not be placed in the region of any discolouration QTL, however LG 1b is small (9.4 cM) and with extension of group length QTL could be discovered; as QTL identified only accounts for a proportion of genetic variation and polymorphism may not translate to a phenotypic effect.

By using direct genetic analysis and comparative genomics this study has gone some way to explain the genetic basis of QTL causing the discolouration phenotypes observed in the Sal x Ice RIL population. However further research is needed to investigate the roles of candidate genes (particularly PAL and PPO) and their relationship to the QTL identified in chapter 5 and the desired phenotype for new lettuce varieties. Markers could then be developed to select for optimal PAL and/or PPO activities to give the desired phenotype.

7.5. Conclusions

- Significant variation in levels of metabolites related to post harvest discolouration has been observed in RILs from the extreme discolouration RIL subset.
    - Significant variation was recorded for PAL on day 4 post harvest and for the differences in PAL activities across days.
    - Significant variation was recorded for PPO on day 4 post harvest.
    - Significant variation was recorded for TPC on harvest day, day 4 post harvest and for the differences in TPCs across days.
- Polymorphisms between the mapping parents Saladin and Iceberg have been identified for 12 candidate genes involved in post harvest discolouration including, AR2, CHI, CHS, COA, CYP98A3, F3H, F3'H, GST, IFR, PAL, PPO and 4CL1.
- Three candidate genes (GST, IFR and F7ORT) have been mapped on the Sal x Ice linkage map.
- Potential map positions of 20 candidate genes and/or gene copies have been identified on the Sal x Ice linkage map by comparative genomics for AR1, AR2, CHS, COA, CYP98A3, F3H, F3'H, FLS, PAL, PAP2, PPO, TT8, TTG2 and 4CL.
- Several candidate genes are positioned in the same map area as QTL for post harvest discolouration identified in Chapter 5, providing initial evidence that they may be the underlying genetic basis for the QTL.

CHAPTER 8

Assessing induced genetic variation for post harvest discolouration

8.1. Introduction

The forward genetics approach embraces various means of identifying a single or sets of genes which are responsible for a particular phenotype. Rijk Zwaan, a world leading company in breeding and supplying vegetable seed for commercial cultivation, has developed mutants from various lettuce cultivars (via random mutagenesis using EMS (ethylmethane sulphonate)) which have produced offspring displaying reduced post harvest discolouration (JW Schut personal communication). However the genetic basis of these interesting and potentially beneficial phenotypes is unknown. A first step in gaining information about the genetic changes brought in these mutant lines is to analyse the post harvest discolouration phenotype and the level of key metabolites in the phenylpropanoid pathway associated with the discolouration response, in order to attempt to identify lesions in the pathway which may indicate mutations in genes controlling the biosynthesis of these metabolites.

The aims of this experiment were to

- Demonstrate phenotypic variation for post harvest discolouration in mutant lines.
- Associate variation in metabolite levels to post harvest discolouration in the mutant lines.

8.2. Material and methods

8.2.1. Rijk Zwaan discolouration mutants and wild type

Dr Johan Schut, from the Rijk Zwaan breeding station in Fijnaart, Netherlands supplied two 'discolouration mutants'. Mutant genotype 'TroubaLessOxida' has reportedly shown resistance to pinking post processing and was developed from wild type variety Troubadour 4250, which was also provided as a control. Mutant 09R.9511 has reportedly shown resistance to browning post processing, however as the original parent was an individual F_2 plant with parents Silvinas and Bedford (common outdoor iceberg lettuce varieties) which were not available for these experiments; the iceberg variety Saladin was used for comparisons as this is the pedigree of both of these lines.

The cv. Iceberg and the gene bank accession *L. serriola* 03050 were also included in some experiments. These are parents with Saladin of the WHRI and the UC Davis mapping population respectively. However the aim of these experiments was to investigate the mutants by comparing them with wild type controls and this is the focus of this chapter; discussion of the natural variation between Iceberg and Saladin was covered in Chapter 7.

8.2.2. Leaf disc trial

The 6 lines were assessed during autumn 2008 and 2009. Three seeds of each line were planted in 5 inch pots into Levington M2 compost. Germination occurred under cool glasshouse conditions at ambient temperature in a genotype grouped

arrangement. Seedlings were subjected to glasshouse raising conditions as described (see section 2.2.1.). The 5th, 10th, 15th and 20th leaf (in emergence order) was removed from each plant when fully expanded to provide 8 x 5mm leaf disc samples (avoiding all major veins and leaf tip).

Leaf discs were cut from each leaf with a sterile cork borer avoiding all major veins and the leaf tip/edges. Four leaf discs were placed between 90 mm filter papers (Whatman® Schleicher & Schuell qualitative circles, catalogue number 1001 090, Whatman International Ltd) saturated in 50 mM MES (2-(N-morpholino) ethanesulfonic acid) buffer solution at pH 5.8 in parafilm sealed or unsealed Petri dishes (see figure 8.1.). The leaf orientation remained constant with the upper leaf surface facing down. Samples were stored at 4°C with the discolouration at wound surface position on the underside of the filter paper scored daily until day 10.

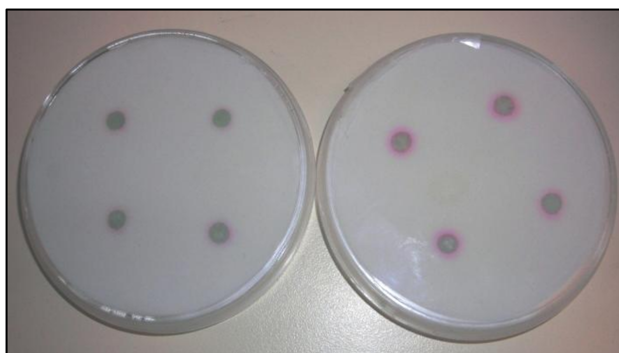


Figure 8.1. Example of lettuce leaf discs between 50 mM mes buffer soaked filter paper in sealed Petri dishes.

On each assessment date (days 1 through to 10) Petri dishes were removed from storage and arranged under a halogen light source for assessment. The intensity of pinking was scored as 3 categories including visible, slight and severe (see figure 8.2.). When no discolouration was recorded, data was classified as clean. The spread of discolouration onto the filter paper was split into 5 categories spanning 0.5

mm until 2.5 mm. There was a small amount of browning however it was not possible to score it in a meaningful way.

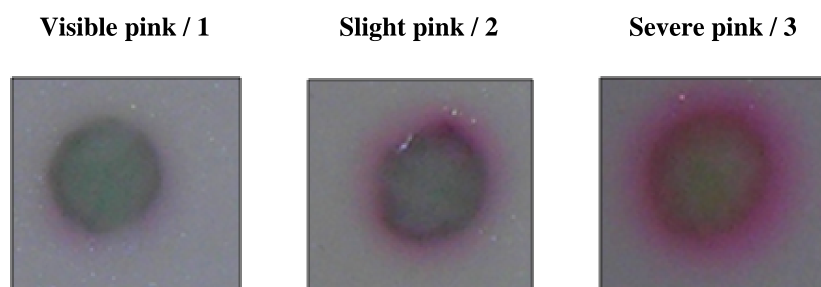


Figure 8.2. Discolouration scale for leaf disc pinking.

Each leaf disc was given a single score as a discolouration coordinate of its most intense representation and spread, resulting in 4 tallies per score sheet per Petri dish on each assessment day (see table 8.1.).

Table 8.1. Sample score grid for a single Petri dish representing the discolouration. Where / represents discs impossible to score. Italicised text is output score.

		Discolouration Intensity (PINK)			
		Clean	**Visible**	**Slight**	**Severe**
Spread		**0**	**1**	**2**	**3**
0 ≤ 0.5 mm	**0**	0	*10*	*20*	*30*
0.5 ≤ 1 mm	**1**	/	*11*	*21*	*31*
1 ≤ 1.5 mm	**2**	/	*12*	*22*	*32*
1.5 ≤ 2 mm	**3**	/	*13*	*23*	*33*
2 ≤ 2.5 mm	**4**	/	*14*	*24*	*34*

8.2.3. Field trial

The mutant TroubaLessOxida and wild type Troubadour 4250 were grown in the replicated field trial with the F_7 RIL mapping population (09R.9511 was not available at this time) for the use in the assessment of post harvest discolouration during the 2008 growing season on the UK experimental site Big Cherry, Warwick HRI, Wellesbourne (Latitude: 52.183. Longitude: 1.583) and the Netherlands

experimental site at Rijk Zwaan breeding station, Fijnaart (Latitude: 51.633. Longitude: 4.467). Transplants were raised, maintained, harvested, processed and scored as previously described (as section 5.2.1.).

8.2.4. Glasshouse trial

The 6 lines were grown for phenotypic post harvest discolouration assessment (see figure 8.3.). Plants were grown during the 2009 autumn to 2010 spring period in glasshouse 16 compartment 46 at Warwick HRI, UK (Latitude: 52.183. Longitude: 1.583). The trial was designed in a Latin square with a 6 x 6 single plant randomisation (with 2 replicates separated by 3 months), ensuring that each accession occurred equally in each row and in each column (see appendix F for field plan and randomisation). The reps were planted on 1st September 2009 and 14th January 2010. Seeds were sown in 5 inch pots into Levington M2 compost with 40 mm spacers between plots. Germination and plant raising took place in the glasshouse compartment with plants arranged according to the randomisation. Plants were subjected to the 'natural' day cycle, temperature and light threshold of the compartment. Plants were predominantly watered with tap water at the base by hand when needed (following an initial watering upon sowing with liquid feed Vitax 2:1:4 on tap (where the concentrated feed was diluted to 1:200)). Harvests occurred on 14th December 2009, 4th January, 11th January and 19th April 2010 with all accessions of a similar type being harvested together across replicates upon maturity.

Figure 8.3. Lettuce wild types and discolouration mutant lines at Warwick HRI, UK in 2009.

Heads were harvested and processed as described (section 2.2.2.). Approximately 50g unwashed mixed material was sealed per bag with material from one head filling one bag. Bags were stored vertically at 5°C (see figure 2.3.). Bags were then phenotypically assessed for post harvest discolouration as previously described (section 2.2.3. with amendments as described in section 5.2.2.) on days 1, 2, 3 and 4.

8.2.5. Metabolite analysis

When material was harvested from the first rep of the glasshouse trial for bagged phenotypic assessment, tissue was also harvested for metabolite analysis (see section 2.3.1.). Extraction and identification for PAL activity, PPO activity and total phenolic content was as previously described (see sections 2.3.1. and 2.3.2.).

8.2.6. Statistical analysis

ANOVA analysis

The leaf disc data was transformed to counts for each eventuality given in table 8.1. based on the restrictions within the scoring system. Data were analysed using

general ANOVA for severity and spread for main effects (line, leaf number, day and condition) and all possible interactions.

Data from each metabolite analysis were analysed using general ANOVA with comparison contrasts (Troubadour 4250 v TroubaLessOxida and Saladin v 09R.0511). Metabolite activity was analysed by day and for differences across days for 5 lines (*L. serriola* 03050 was excluded due to limited amounts of leaf material) and between comparison contrasts to identify main effects due to genotype.

REML analysis

The data from the field trial of the pinking mutant and wild type exhibited a variable mean relationship so it was transformed before analysis as described previously (section 5.2.5.). As the field trial produced an unbalanced data set, it was analysed by REML. The REML fixed treatment was day*GT for site with the random variables browning intensity, pinking intensity, overall discolouration intensity, extent of browning, extent of pinking, extent of overall discolouration for days 1 and 3. Scores were adjusted through the REML analysis to adjust for block effects which in some cases could result in negative values for the estimated means (this could also occur with estimates for missing values). Standard errors of differences of means (sems) were also calculated.

Data for the glasshouse trial of the mutant subset also revealed a variable mean relationship and was transformed before analysis as above. The REML fixed treatment was rep/bed/plot/head/bag, with the random variables browning intensity, pinking intensity, overall discolouration intensity, extent of browning, extent of pinking, extent of overall discolouration for days 1, 2, 3 and 4. As above, scores were adjusted through the REML analysis.

Correlation analysis

Correlation coefficients were calculated for all pair wise combinations of discolouration measures, morphological traits and metabolite levels (browning, pinking and overall discolouration, PAL activity, PPO activity and total phenolic content and between them and morphological traits (trimmed weight)). R values were generated which were then compared to the associated *p* values (see table 8.2.).

Table 8.2. Correlation analysis parameters. Where *df* (degrees of freedom).

		Probability (p value)		
Population	**df**	**0.05**	**0.01**	**0.001**
Wild type and mutant lines	4	0.811	0.917	0.974

8.3. Results

8.3.1. Phenotypic variation for post harvest discolouration in mutant lines.

Leaf disc assessment

The ANOVA showed significant variation (***P <0.001) between lines, leaf number and days for pinking (for both severity and spread). A significant interaction (***P <0.001) was also recorded between leaf number, line and day for pinking (severity and spread) confirming genetic variation for this trait (see table 8.3. a, figure 8.4. (a-f) and figure 8.5. (a-f)). There were also significant interactions (**P <0.005) between line and leaf number with condition across days for pinking spread.

Table 8.3. Mean pinking (severity and spread) of lettuce lines for the 5th, 10th, 15th and 20th leaf over 10 days. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *no* (number), *condition* (sealed or non sealed samples); *x* (interaction); *df* (degrees of freedom); *vr* (variance ratio); *prob* (probability).

	Pinking severity			Pinking spread		
Variation	**df**	**Vr**	**ANOVA F prob**	**df**	**vr**	**ANOVA F prob**
Line	5	130.02	***<.001	5	170.63	***<.001
Leaf no	3	9.54	***<.001	3	7.91	***<.001
Line x Leaf no	15	3.16	**0.003	15	7.45	***<.001
Condition	1	1.39	0.245	1	3.65	0.063
Line x Condition	5	0.26	0.931	5	0.46	0.803
Leaf no x Condition	3	1.48	0.232	3	1.85	0.152
Line x Leaf no x Condition	14	0.92	0.541	14	0.55	0.887
Day no	9	407.08	***<.001	9	591.43	***<.001
Line x Day no	45	39.5	***<.001	45	57.73	***<.001
Leaf no x Day no	27	5.44	***<.001	27	3.89	***<.001
Condition x Day no	9	0.44	0.915	9	1.5	0.144
Line x Leaf no x Day no	133	2.84	***<.001	133	3.01	***<.001
Line x Condition x Day no	45	1.14	0.252	45	1.67	**0.005
Leaf no x Condition x Day no	27	1.04	0.406	27	2.44	***<.001
Line x Leaf no x Condition x Day no	124	0.92	0.707	124	0.98	0.533

'Browning mutant' 09R.9511 showed similar pinking severity to Saladin for leaf 5 and leaf 20 (see figure 8.4. c and d). Pinking severity of leaf 10 remained extremely similar to leaf 5 for 09R.9511; there was then a significant drop in the scores for pinking severity for leaf 15 followed by a significant rise in pinking severity for leaf 20. 'Pinking mutant' TroubaLessOxida showed extremely low levels of pinking severity for each leaf (see figure 8.4. b). TroubaLessOxida showed significantly lower levels of pinking severity than the wild type Troubadour 4250 for leaf 5 and leaf 10 (see figure 8.4. a and b).

'Browning mutant' 09R.9511 showed a similar response in terms of development of both pinking severity and spread (with a significant decrease in scores for pinking spread for leaf 15 followed by a significant rise for leaf 20). This resulted in significant differences between 09R.9511 and Saladin for pinking spread for leaves 5, 10 and 15 (see figure 8.5. c and d). 'Pinking mutant' TroubaLessOxida showed

extremely low levels of pinking spread for each leaf (see figure 8.5. b). TroubaLessOxida showed significantly lower levels of pinking severity than the wild type Troubadour 4250 for each leaf (see figure 8.5. a and b).

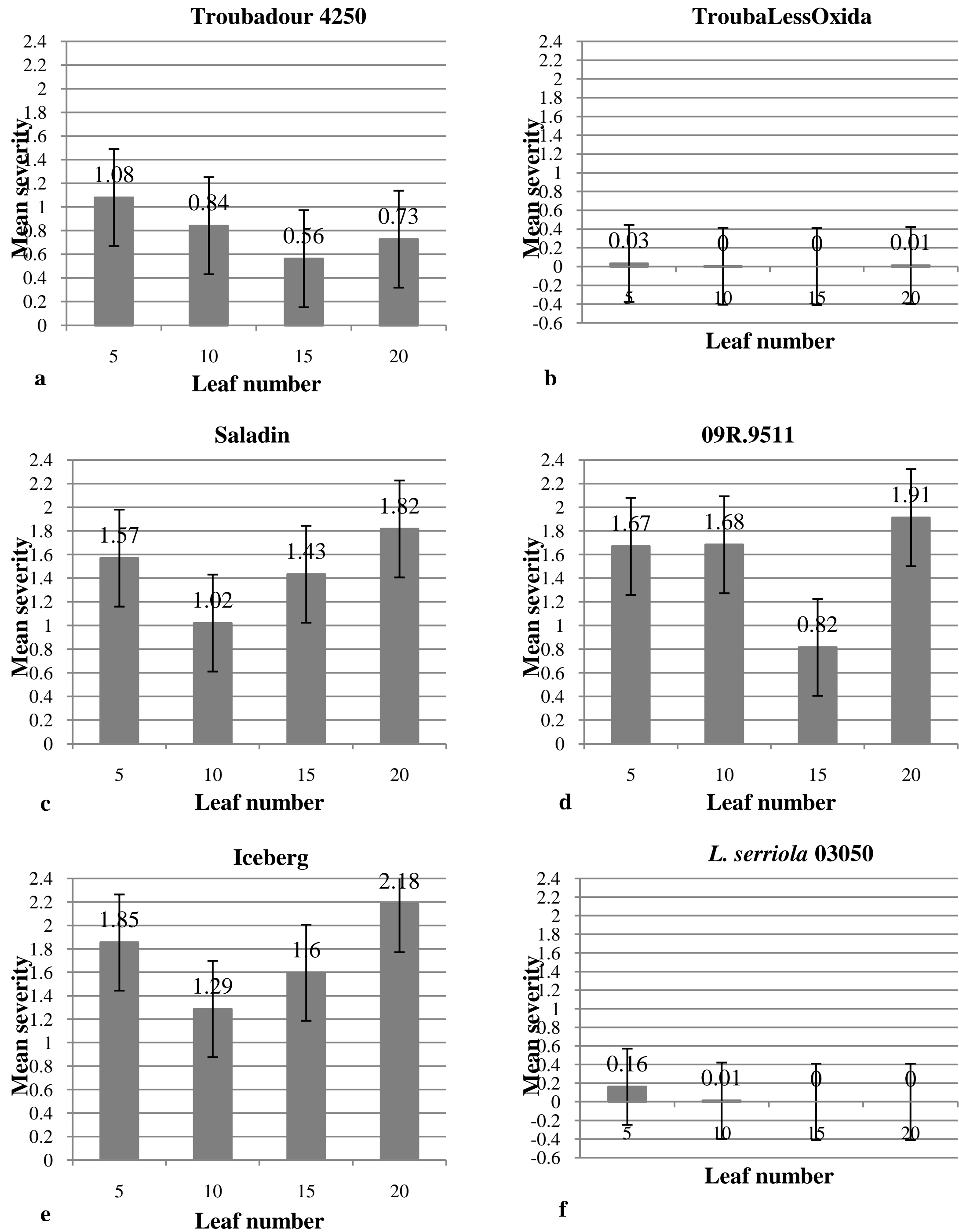


Figure 8.4. Mean pinking severity of lettuce lines for the 5th, 10th, 15th and 20th leaf (in emergence order) for a) Troubadour 4250, b) TroubaLessOxida, c) Saladin, d) 09R.9511, e) Iceberg and f) *L. serriola* 03050. Error bars represent sems from ANOVA.

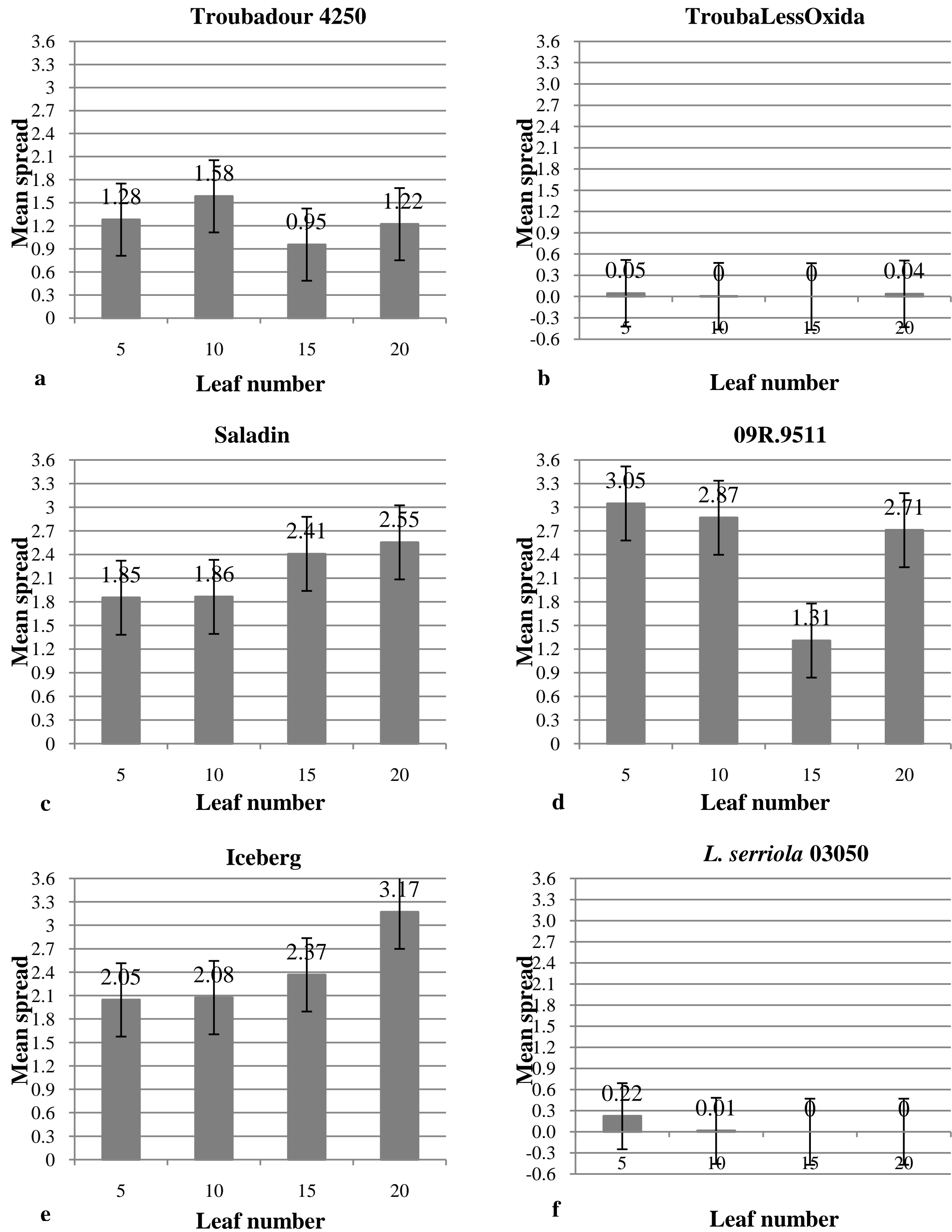


Figure 8.5. Mean pinking spread of lettuce lines for the 5th, 10th, 15th and 20th leaf (in emergence order) for a) Troubadour 4250, b) TroubaLessOxida, c) Saladin, d) 09R.9511, e) Iceberg and f) *L. serriola* 03050. Error bars represent sems from ANOVA.

Field trial of pinking mutant and wild type

Pinking

REML analysis of the 'Pinking mutant' TroubaLessOxida and wild type variety Troubadour 4250 showed significant differences (****P* <0.001) between lines for all measures of pinking on day 1 and day 3. The pinking responses demonstrated similar trends. However, extent of pinking always had higher scores than pinking intensity for each line per day.

TroubaLessOxida and Troubadour 4250 were significantly different for pinking (both intensity ($Wald_{[33.9]} = 200.65$) and extent ($Wald_{[32.9]} = 136.14$)) and for pinking across days (intensity ($Wald_{[34.7]} = 26.8$) and extent ($Wald_{[34.4]} = 11.7$)), indicating genetic variation for this trait (see figure 8.6. a and b). TroubaLessOxida had significantly lower levels of pinking than Troubadour 4250 on day 1 and 3. TroubaLessOxida pinking on day 3 (intensity (12.2) and extent (24.3)) was lower than pinking for Troubadour 4250 on day 1 (intensity (43.7) and extent (69.4)).

Browning

REML analysis of the 'Pinking mutant' TroubaLessOxida and wild type variety Troubadour 4250 showed significant differences between lines for all measures of browning on day 1. The browning responses demonstrated similar trends. However, the extent of browning was always relatively higher than browning intensity for each accession per day.

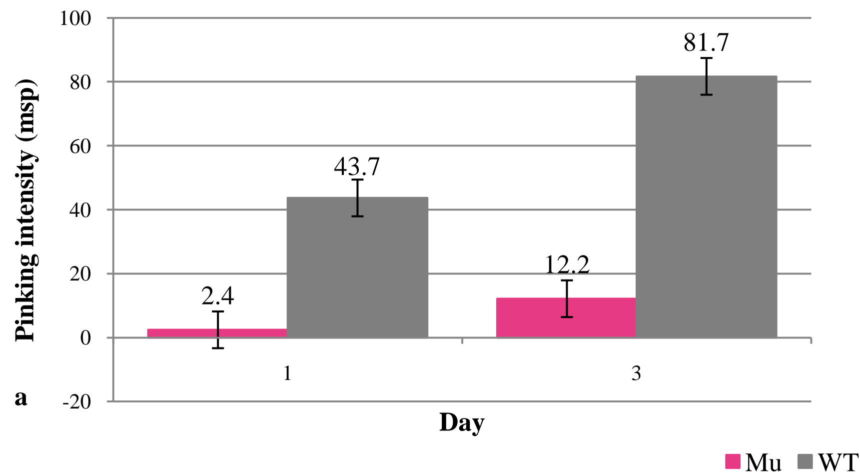
TroubaLessOxida and Troubadour 4250 were significantly different for browning intensity ($Wald_{[34.9]} = 10.56$) and browning across days (both intensity ($Wald_{[35.9]} = 5.38$) and extent ($Wald_{[34.4]} = 10.45$), indicating genetic variation for this trait (see figure 8.7. a and b). TroubaLessOxida had significantly lower levels of browning

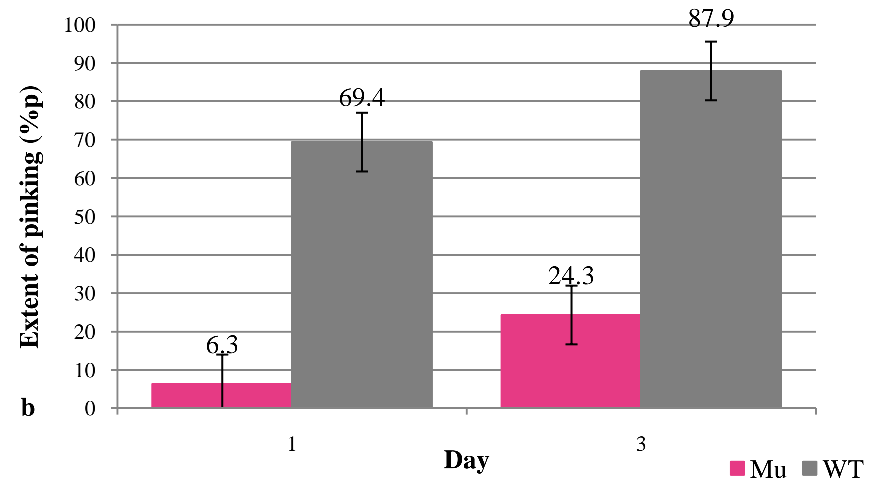
than Troubadour 4250 on day 1. However due to varying rates of browning the lines revealed similar scores for browning on day 3.

Overall discolouration

REML analysis of the 'Pinking mutant' TroubaLessOxida and wild type variety Troubadour 4250 revealed significant differences (***P <0.001) between lines for all measures of overall discolouration on day 1 and day 3. The overall discolouration responses demonstrated similar trends however the extent of overall discolouration was always higher than overall discolouration intensity for each accession per day (as recorded for both pinking and browning).

TroubaLessOxida and Troubadour 4250 were significantly different for overall discolouration (both intensity (Wald $_{[82.83]}$ = 82.83) and extent (Wald $_{[36]}$ = 70.53)) and overall discolouration across days (intensity (Wald $_{[28.14]}$ = 28.14) and extent (Wald $_{[36]}$ = 17.88), indicating genetic variation for this trait (see figure 8.8. a and b). TroubaLessOxida had significantly lower levels of overall discolouration than Troubadour 4250 on days 1 and 3. TroubaLessOxida showed approximately a quarter of the amount of overall discolouration intensity showed by Troubadour 4250 on day 1, and approximately half of that observed on day 3. The extent of overall discolouration for TroubaLessOxida approximately increased twofold from day 1 to day 3, however for Troubadour 4250 it increased by ~10 as it was near the possible maximum level for the extent of overall discolouration score possible on day 1 which it reached by day 3. TroubaLessOxida overall discolouration intensity on day 3 (intensity (26.7) and extent (58.8)) was lower than that observed for Troubadour 4250 on day 1 (intensity (39.8) and extent (80.2)).

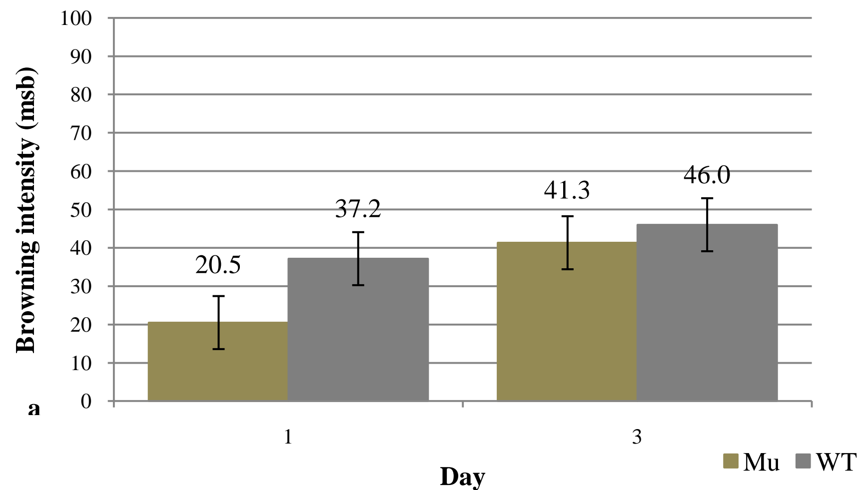


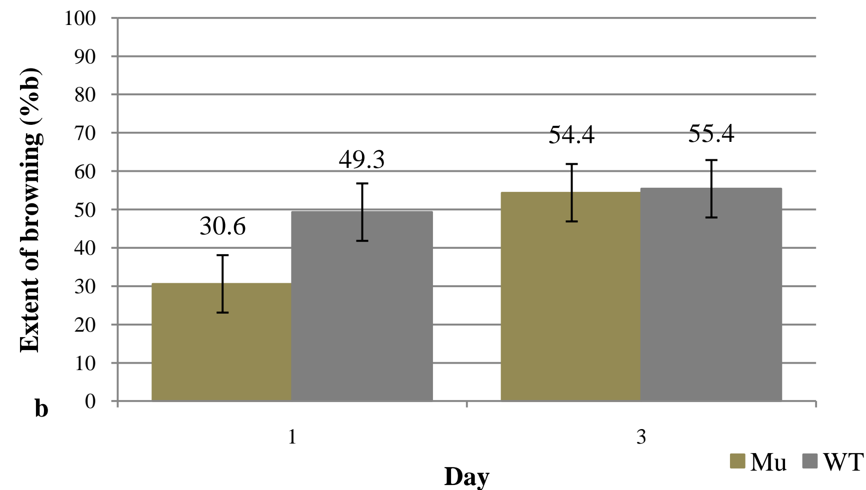


	Significance level of Troubadour wild type and pinking mutant		
Pinking intensity (msp)	**REML Wald Statistic**	**ddf**	**REML P value**
Day	26.8	34.7	***<0.001
GT	200.65	33.9	***<0.001

	Significance level of Troubadour wild type and pinking mutant		
Extent of pinking (%p)	**REML Wald Statistic**	**ddf**	**REML P value**
Day	11.7	34.4	**0.002
GT	136.14	32.9	***<0.001

Figure 8.6. Transformed adjusted means (from REML) for lettuce post harvest a) pinking intensity and b) extent of pinking on days 1 and 3 for the Rijk Zwaan wild type breeding line Troubadour 4250 and respective 'pinking mutant' TroubaLessOxida. Error bars represent seds from REML. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. REML ndf = 1. Where *msp* (mean score pinking); *%p* (percentage pinking); *ddf* (denominator degrees of freedom); *ndf* (numerator degrees of freedom); *GT* (genotype); *x* (interaction); *Mu* (mutant: TroubaLessOxida); *WT* (wild type: Troubadour 4250).

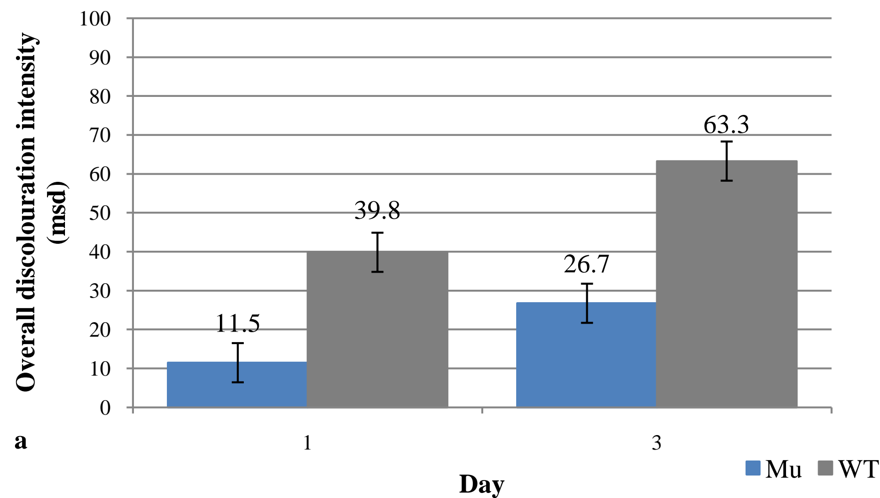


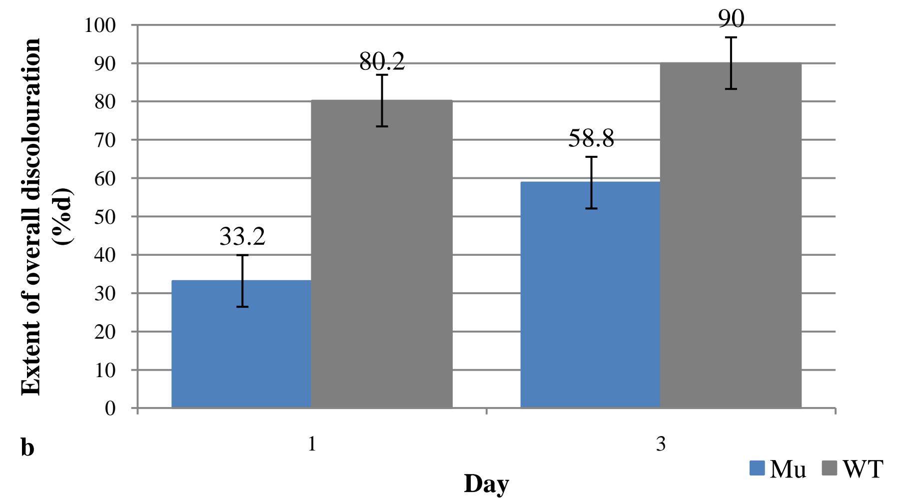


	Significance level of Troubadour wild type and pinking mutant		
Browning intensity (msb)	**REML Wald Statistic**	**ddf**	**REML P value**
Day	10.56	34.9	**0.003
GT	5.38	35.9	*0.026

	Significance level of Troubadour wild type and pinking mutant		
Extent of browning (%b)	**REML Wald Statistic**	**ddf**	**REML P value**
Day	10.45	34.4	**0.003
GT	3.39	33.9	0.074

Figure 8.7. Transformed adjusted means (from REML) for lettuce post harvest a) browning intensity and b) extent of browning on 3 days for the Rijk Zwaan wild type breeding line Troubadour 4250 and respective 'pinking mutant' TroubaLessOxida. Error bars represent seds from REML. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. REML ndf = 1. Where *msp* (mean score pinking); *%p* (percentage pinking); *ddf* (denominator degrees of freedom); *ndf* (numerator degrees of freedom); *GT* (genotype); *x* (interaction); *Mu* (mutant: TroubaLessOxida); *WT* (wild type: Troubadour 4250).





	Significance level of Troubadour wild type and pinking mutant		
Overall discolouration intensity (msd)	**REML Wald Statistic**	**ddf**	**REML P value**
Day	28.14	28.14	***<0.001
GT	82.83	82.83	***<0.001

	Significance level of Troubadour wild type and pinking mutant		
Extent of overall discolouration (%d)	**REML Wald Statistic**	**ddf**	**REML P value**
Day	17.88	36	***<0.001
GT	70.53	36	***<0.001

Figure 8.8. Transformed adjusted means (from REML) for lettuce post harvest a) overall discolouration intensity and b) extent of overall discolouration on 3 days for the Rijk Zwaan wild type breeding line Troubadour 4250 and respective 'pinking mutant' TroubaLessOxida. Error bars represent seds from REML. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. REML ndf = 1. Where *msp* (mean score pinking); *%p* (percentage pinking); *ddf* (denominator degrees of freedom); *ndf* (numerator degrees of freedom); *GT* (genotype); *x* (interaction); *Mu* (mutant: TroubaLessOxida); *WT* (wild type: Troubadour 4250).

Glasshouse trial

Pinking

REML analysis revealed there was significant variation (***P <0.001) between lines for pinking (both intensity and extent) and for day (see table 8.4. a and b). There was also a significant interaction between line and day indicating differences in rate of development of pinking intensity (see table 8.4. a). The rates for pinking of lines remained constant across the 4 days as there was minimal changing in the ranking of the lines (see figure 8.9. (a-d) and figure 8.10. (a-d)).

Table 8.4. Lettuce post harvest pinking a) intensity and b) extent of lettuce lines from REML. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *GT* (genotype); *x* (interaction); *df* (degrees of freedom); *Ch* (Chi-squared).

a

	Significance level of lettuce lines			
Pinking intensity (msp)	**REML Wald Statistic**	**df**	**Wald df**	**Chi P value**
GT	379.23	5	75.85	***<0.001
Day	149.6	3	49.87	***<0.001
GT x Day	30.1	15	2.01	**0.01

b

	Significance level of lettuce lines			
Extent of pinking (%p)	**REML Wald Statistic**	**df**	**Wald df**	**Chi P value**
GT	300.81	5	60.16	***<0.001
Day	70.04	3	23.35	***<0.001
GT x Day	15.33	15	1.02	0.428

The pinking mutant TroubaLessOxida and its wild type Troubadour 4250 were significantly different for pinking on each day, indicating genetic variation for this trait. Troubadour 4250 had low levels of pinking intensity and median levels of extent of pinking in comparison to the other lines. The level of pinking for TroubaLessOxida was constant on day 1 and day 2, after which it increased 2 fold by day 3 and remained constant on day 4. Scores for pinking for Troubadour 4250 were

high on day 1 and then increased steadily to day 4. Saladin and 09R.9511 were not significantly different for any type of pinking.

Browning

REML analysis revealed significant variation (***P <0.001) between lines for browning and day (see table 8.5. a and b). A significant interaction was recorded between line and day, indicating differences in rate of development of browning intensity (see table 8.5. a). There were varying intrinsic rates for browning (both intensity and extent) for lines as rank orders changed across days (see figure 8.11. and figure 8.12.). The browning mutant 09R.9511 was always the best performing line for browning, followed by Saladin. Although 09R.9511 was the most resistant line, it still displayed browning on each day. Various lines performed the worst depending on day post harvest.

Table 8.5. Lettuce post harvest browning a) intensity and b) extent of lettuce lines from REML. Significant effects shown as **P* <0.05, ***P* <0.01 and ****P* <0.001. Where *GT* (genotype); *x* (interaction); *df* (degrees of freedom); *Ch* (Chi-squared).

a	**Significance level of lettuce lines**			
Browning intensity (msb)	**REML Wald Statistic**	**df**	**Wald df**	**Chi P value**
GT	182.26	5	36.45	***<0.001
Day	49.77	3	16.59	***<0.001
GT x Day	29.09	15	1.94	*0.016

b	**Significance level of lettuce lines**			
Extent of browning (%b)	**REML Wald Statistic**	**df**	**Wald df**	**Chi P value**
GT	157.57	5	31.51	***<0.001
Day	14.77	3	4.92	**0.002
GT x Day	20.74	15	1.38	0.145

The browning mutant 09R.9511 and Saladin were significantly different for browning (intensity on days 3 and 4 and extent on day 1, 3 and 4) indicating genetic variation for this trait. Mutant 09R.9511 had increased 2 fold by day 2, and after which browning intensity remained constant. Troubadour 4250 and TroubaLessOxida were not significantly different for any type of browning.

Overall discolouration

REML analysis revealed there was significant variation between lines for overall discolouration (both intensity and extent) and for day (see table 8.6. a and b). There was also a significant interaction between line and day indicating differences in rate of development of extent of overall discolouration (see table 8.6. b). There were varying intrinsic rates for overall discolouration for lines as rank orders changed across days (see figure 8.11. (a-d) and figure 8.12. (a-d)). Various lines performed the best for overall discolouration depending on day post harvest; this included the two mutant lines.

Table 8.6. Lettuce post harvest overall discolouration a) intensity and b) extent of lettuce lines from REML. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *GT* (genotype); *x* (interaction); *df* (degrees of freedom); *Ch* (Chi-squared).

a

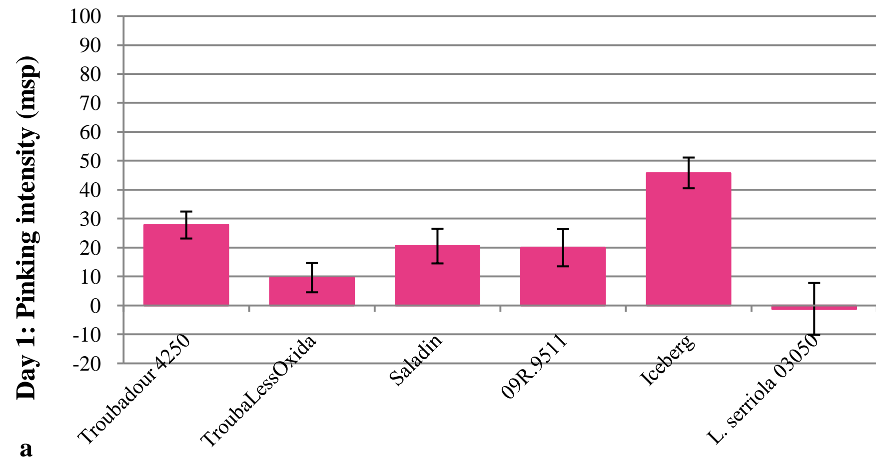
	Significance level of lettuce lines			
Overall discolouration intensity (msd)	**REML Wald Statistic**	**df**	**Wald df**	**Chi P value**
GT	238.93	5	47.79	***<0.001
Day	183.51	3	61.17	***<0.001
GT x Day	20.11	15	1.34	0.168

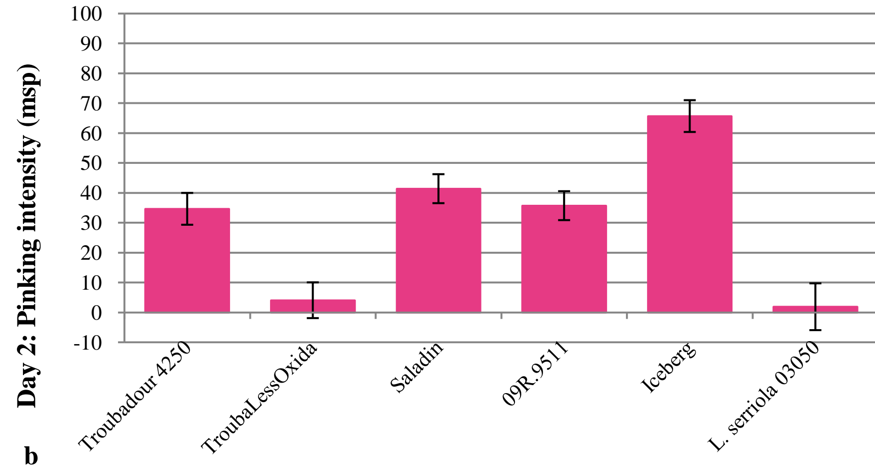
b

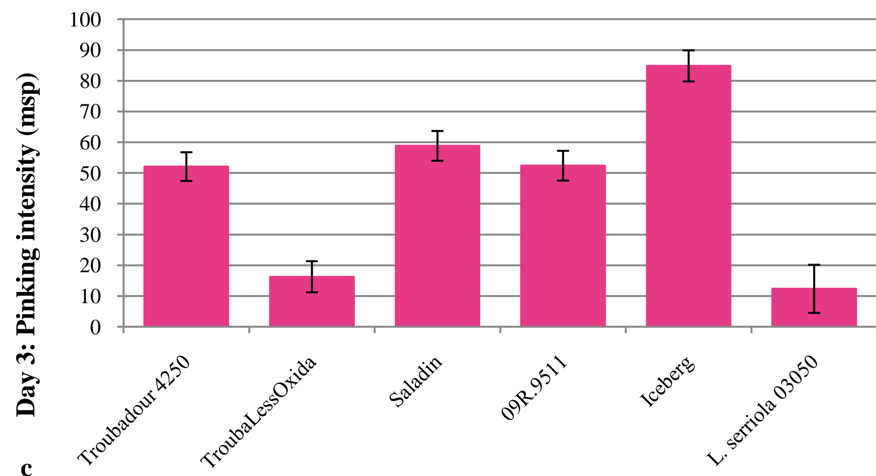
	Significance level of lettuce lines			
Extent of overall discolouration (%d)	**REML Wald Statistic**	**df**	**Wald df**	**Chi P value**
GT	107.86	5	21.57	***<0.001
Day	106.97	3	35.66	***<0.001
GT x Day	38.1	15	2.54	***<0.001

The pinking mutant TroubaLessOxida and its wild type Troubadour 4250 were significantly different for overall discolouration (for intensity on all days and extent on days 2, 3 and 4) indicating genetic variation for this trait. The level of overall discolouration for both TroubaLessOxida and Troubadour 4250 increased steadily across all days; however this occurred for the wild type at a quicker rate. The level of overall discolouration intensity recorded for TroubaLessOxida and Troubadour 4250 on day 1 was relatively high in comparison to the other accessions; although by day 3 they were low and high respectively.

The browning mutant 09R.9511 and Saladin were only significantly different for overall discolouration for intensity on days 3 and 4 and for extent on day 2, indicating genetic variation for this trait later in the post harvest period. The level of overall discolouration for 09R.9511 increased at a high rate between day 1 and day 2 and then slowed to become steady. The level of overall discolouration intensity for Saladin steadily increased across all days.







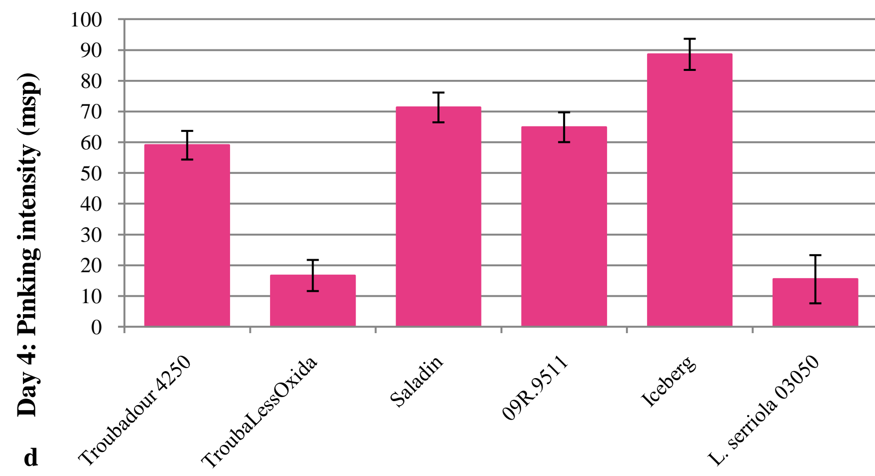


Figure 8.9. Transformed adjusted means (from REML) for lettuce post harvest pinking intensity on a) day 1, b) day 2, c) day 3 and d) day 4 for lettuce lines. Error bars represent sems from REML.

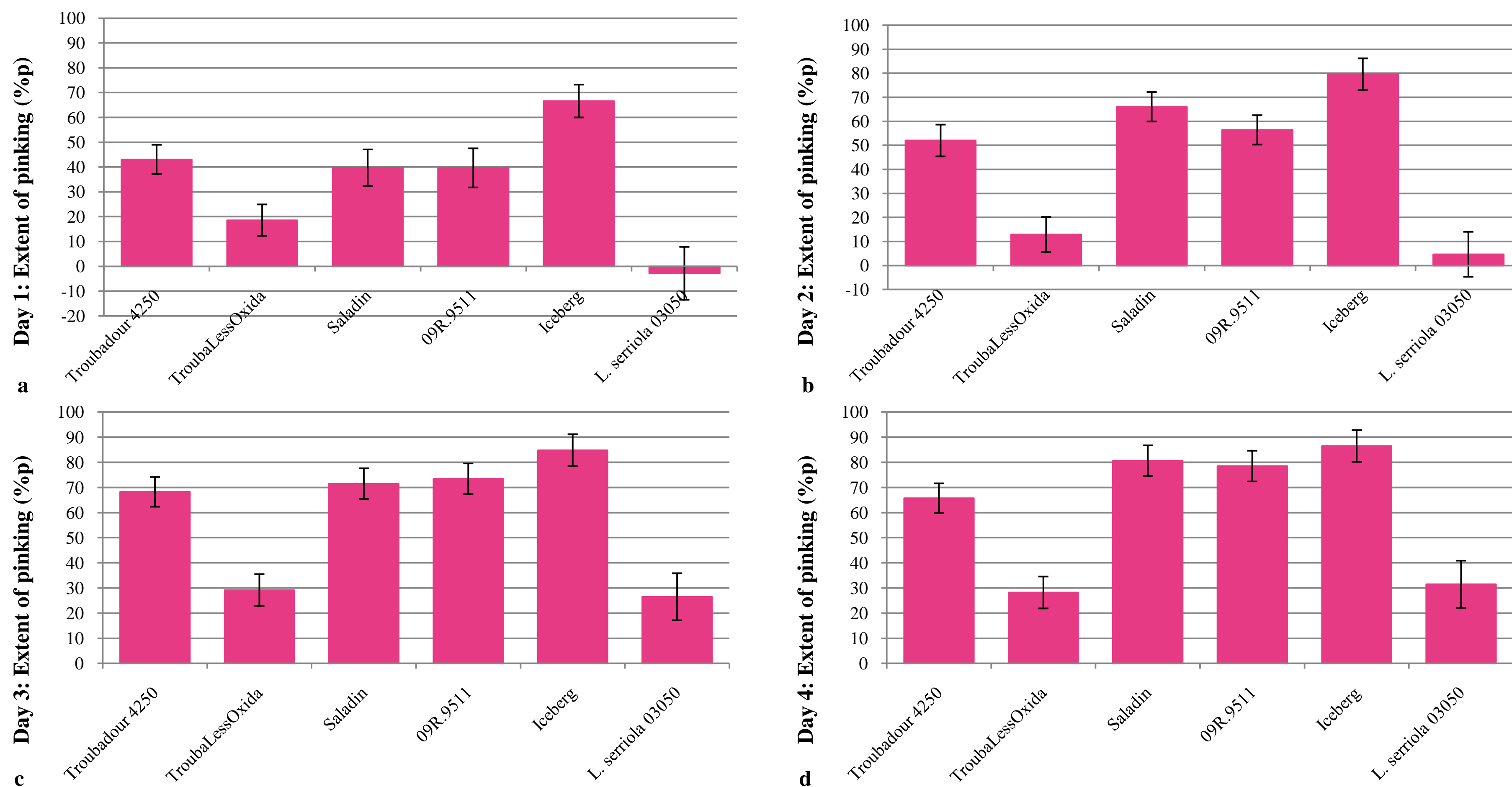
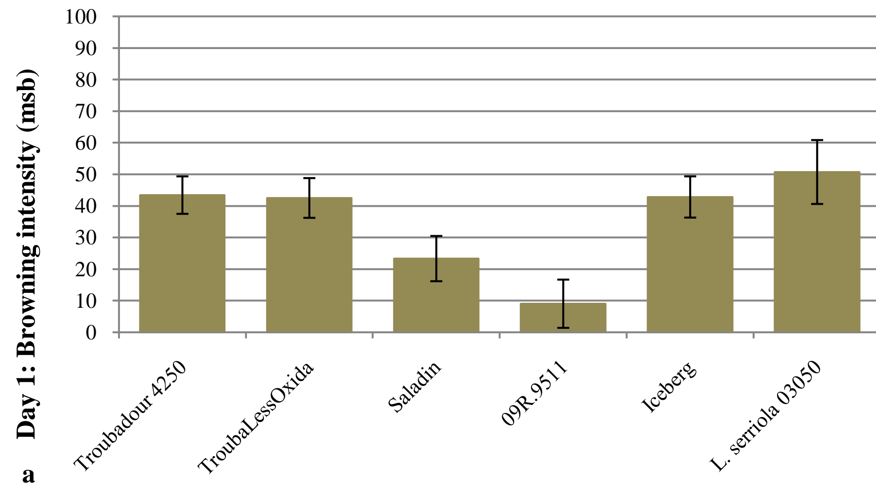
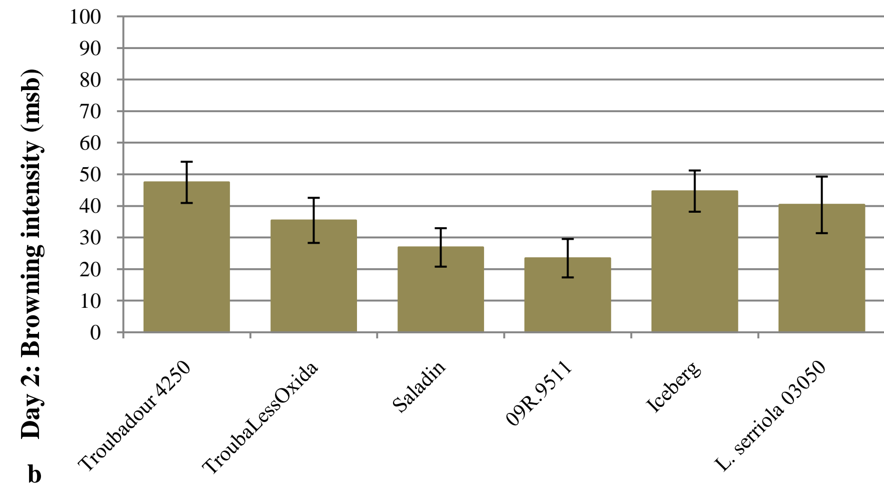
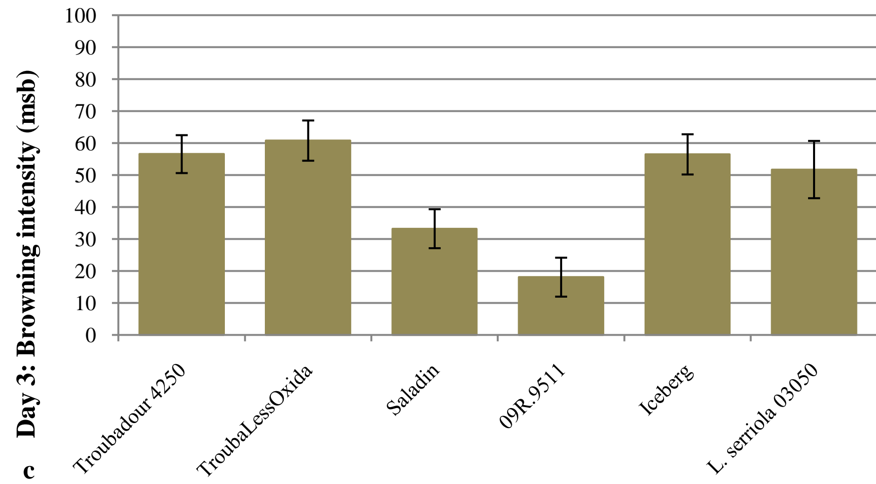


Figure 8.10. Transformed adjusted means (from REML) for lettuce post harvest extent of pinking on a) day 1, b) day 2, c) day 3 and d) day 4 for lettuce lines. Error bars represent sems from REML.







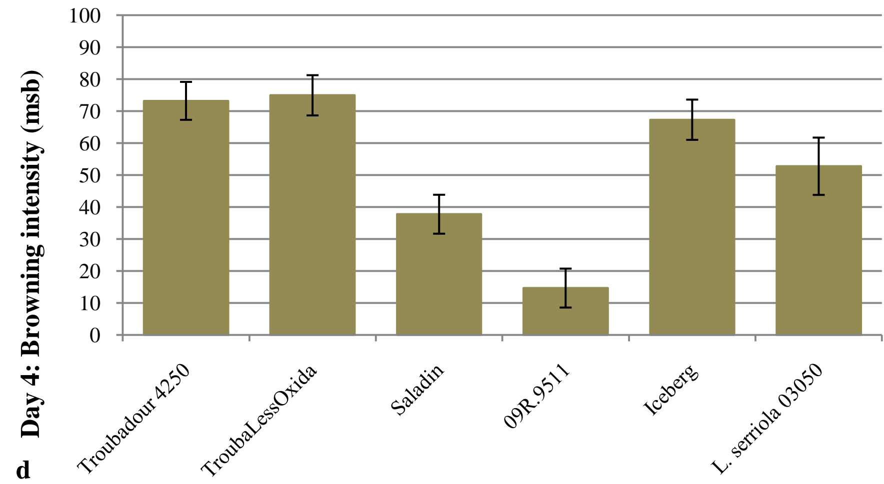
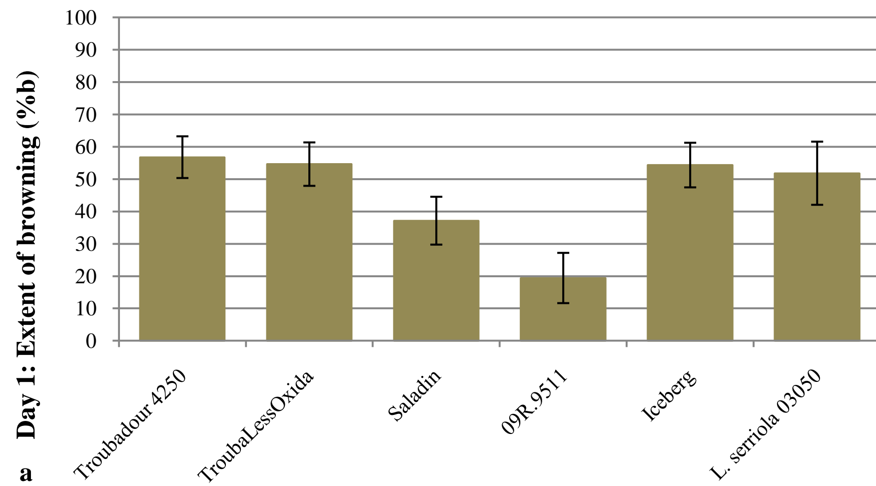
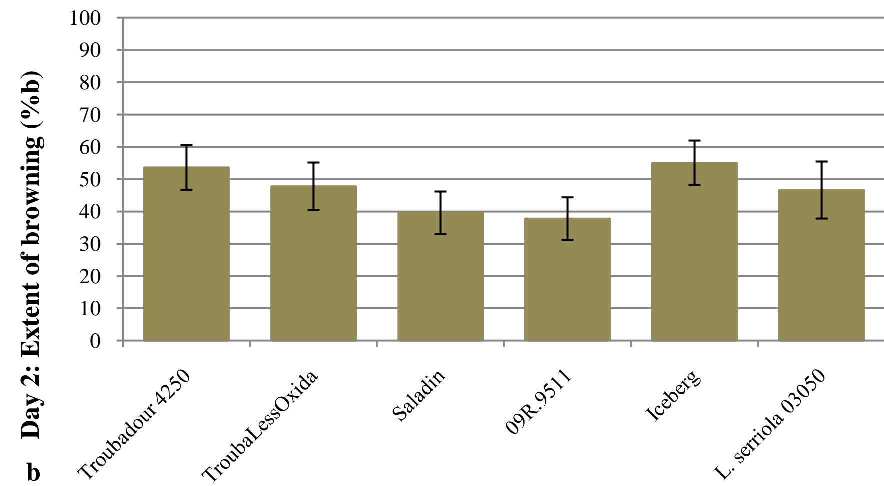
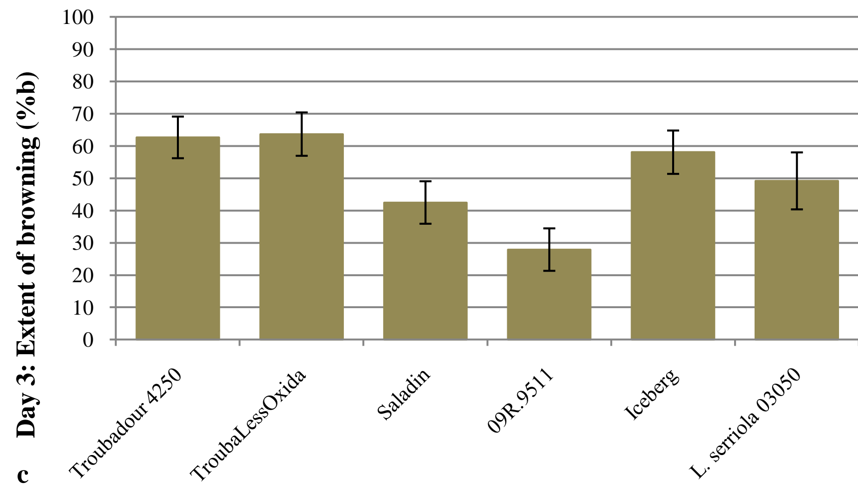


Figure 8.11. Transformed adjusted means (from REML) for lettuce post harvest browning intensity on a) day 1, b) day 2, c) day 3 and d) day 4 for lettuce lines. Error bars represent sems from REML.







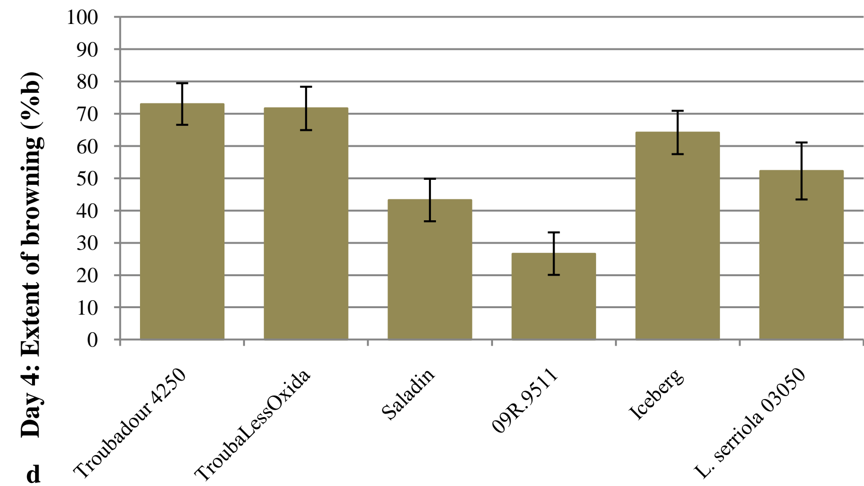
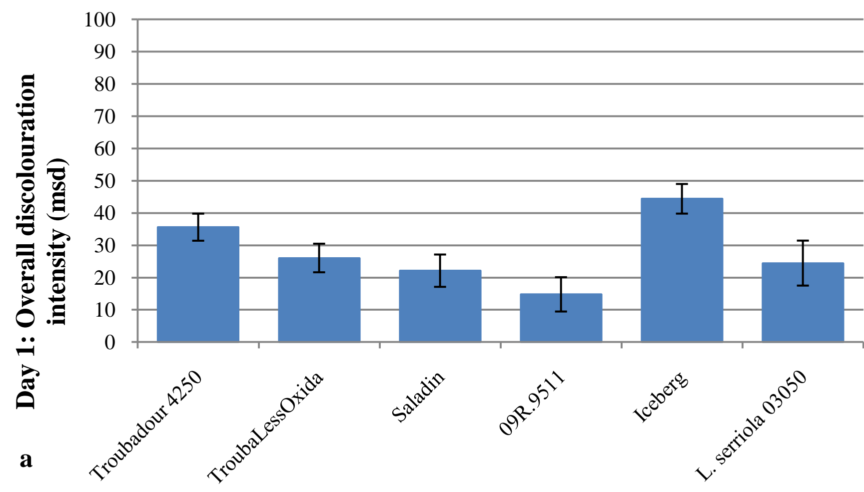
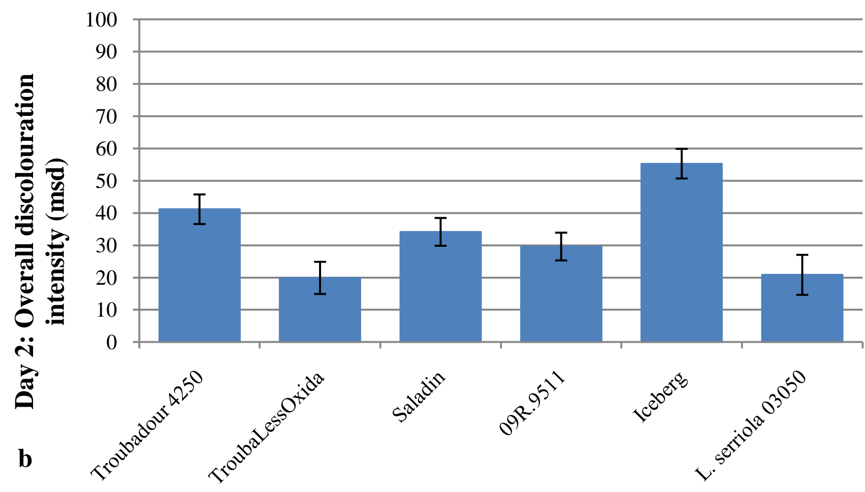
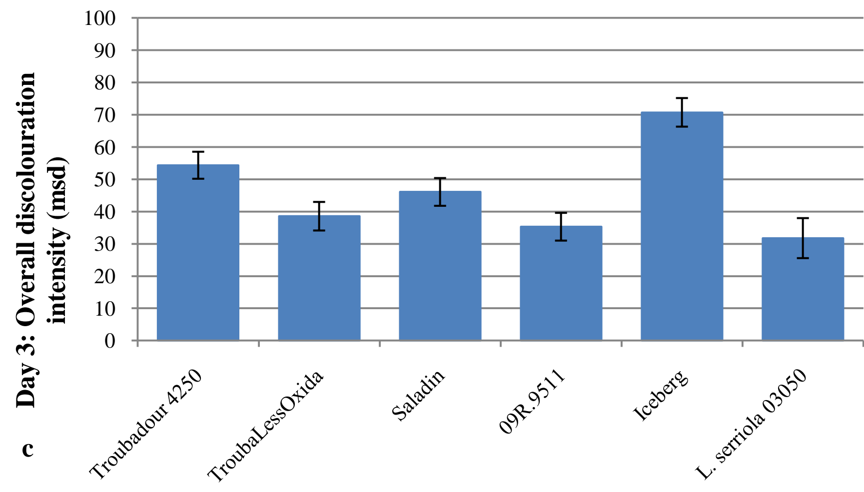


Figure 8.12. Transformed adjusted means (from REML) for lettuce post harvest extent of browning on a) day 1, b) day 2, c) day 3 and d) day 4 for lettuce lines.

Error bars represent sems from REML.







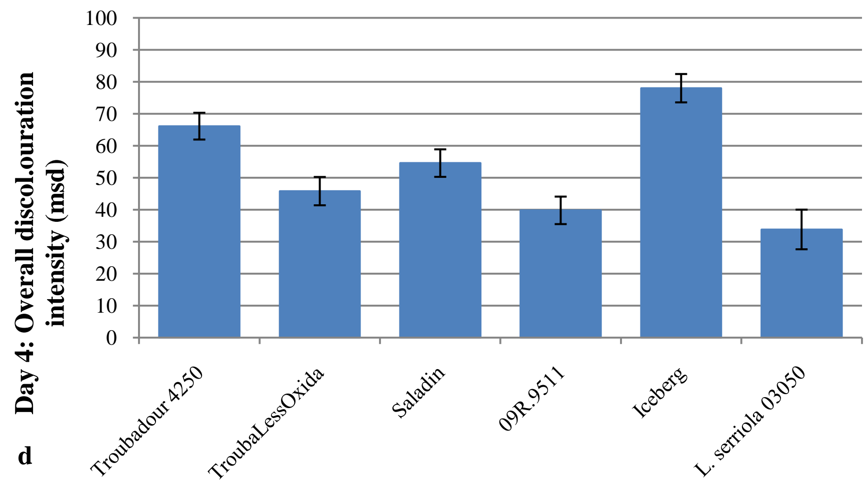
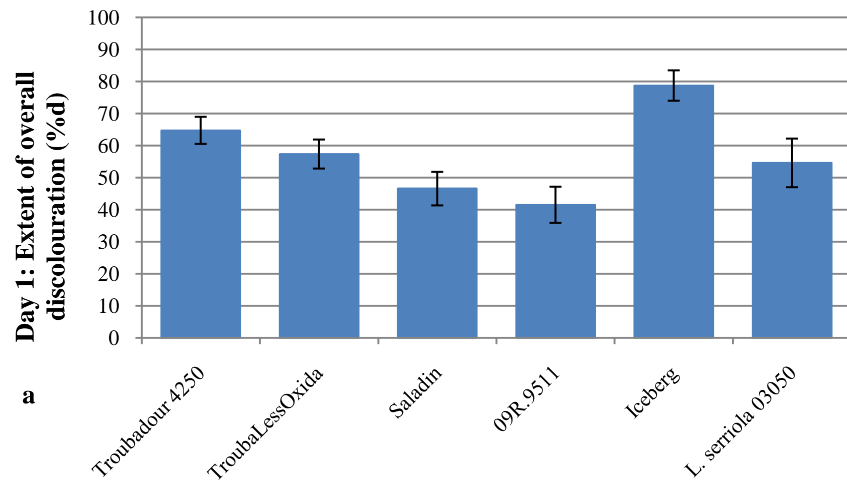
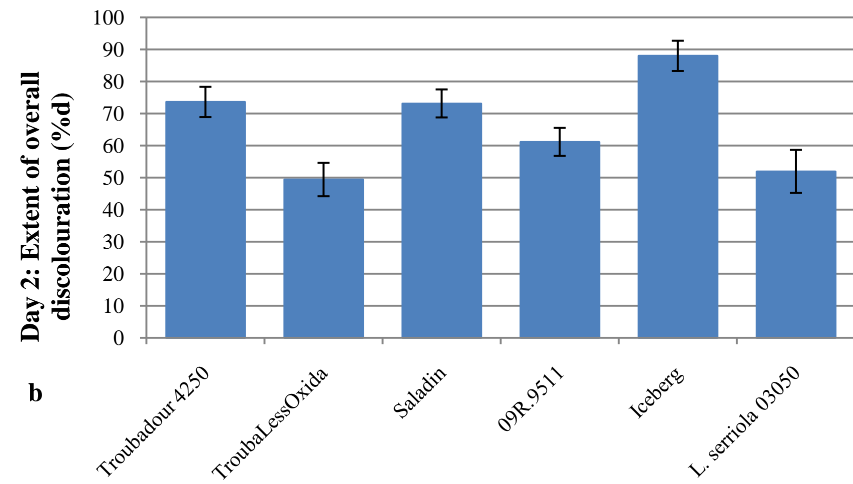
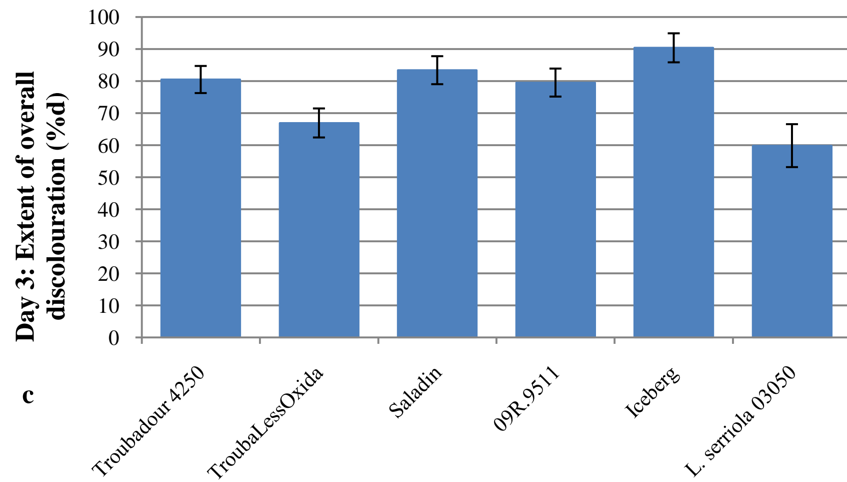


Figure 8.13. Transformed adjusted means (from REML) for lettuce post harvest overall discolouration intensity on a) day 1, b) day 2, c) day 3 and d) day 4 for lettuce lines. Error bars represent sems from REML.







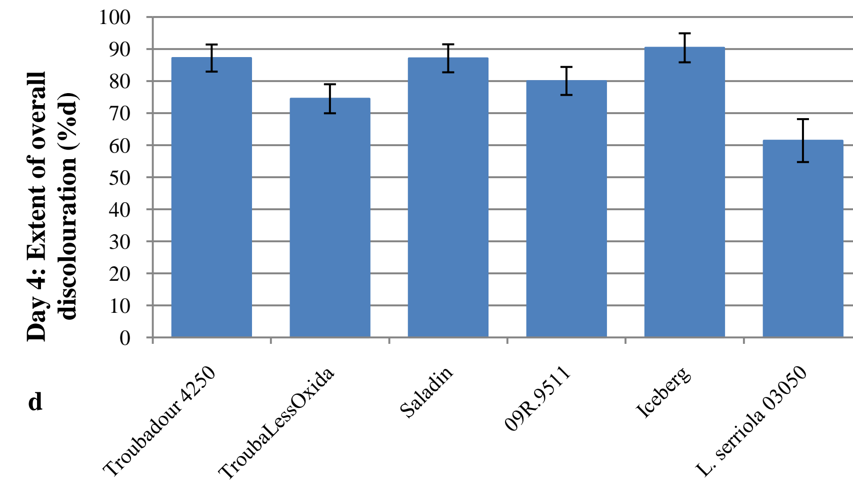


Figure 8.14. Transformed adjusted means (from REML) for lettuce post harvest extent of overall discolouration on a) day 1, b) day 2, c) day 3 and d) day 4 for lettuce lines. Error bars represent sems from REML.

8.3.2. Metabolite profiling

PAL activity

ANOVA revealed significant variation (***P <0.001) between lines for PAL activity when expressed as absorbance per gram of dry weight of lettuce (abs/g dwt), for day 4 post storage and for the differences of PAL activity across days (*P <0.017) (see table 8.7.).

Table 8.7. PAL activity for lettuce lines over 4 days. ANOVA GT df = 4. ANOVA comparison df = 1. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *PAL* (phenylalanine ammonia lease); *GT* (genotype/accession); *v* (comparison); *vr* (variance ratio); *prob* (ANOVA probability).

	PAL Activity					
	Harvest day		**Day 4**		**Difference across days**	
Variation	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**
GT	2.41	0.074	11.92	***<0.001	5.04	*0.017
TroubaLessOxida v Troubadour 4250	3.57	0.07	35.6	***<0.001	5.4	*0.042
09R.9511 v Saladin	0.06	0.805	3.47	0.082	0.06	0.807

PAL activity of the 5 lines varied from 0.113 abs/g dwt to 0.434 abs/g dwt for harvest day and from 0.159 abs/g dwt to 0.466 abs/g dwt on day 4 (see figure 8.15. (a-c)). TroubaLessOxida always had the highest levels of PAL activity on both days. Generally PAL activity increased from harvest day to day 4. Significant differences were seen between 'pinking mutant' TroubaLessOxida and wild type Troubadour 4250 (***P <0.001) for PAL activity on day 4. Mean PAL activity was 0.2393 abs/g dwt on day 4. However Troubadour 4250 PAL activity decreased across days (-0.073 abs/g dwt). Significant differences were recorded between TroubaLessOxida and Troubadour 4250 (*P <0.042) for the difference in PAL activity across days.

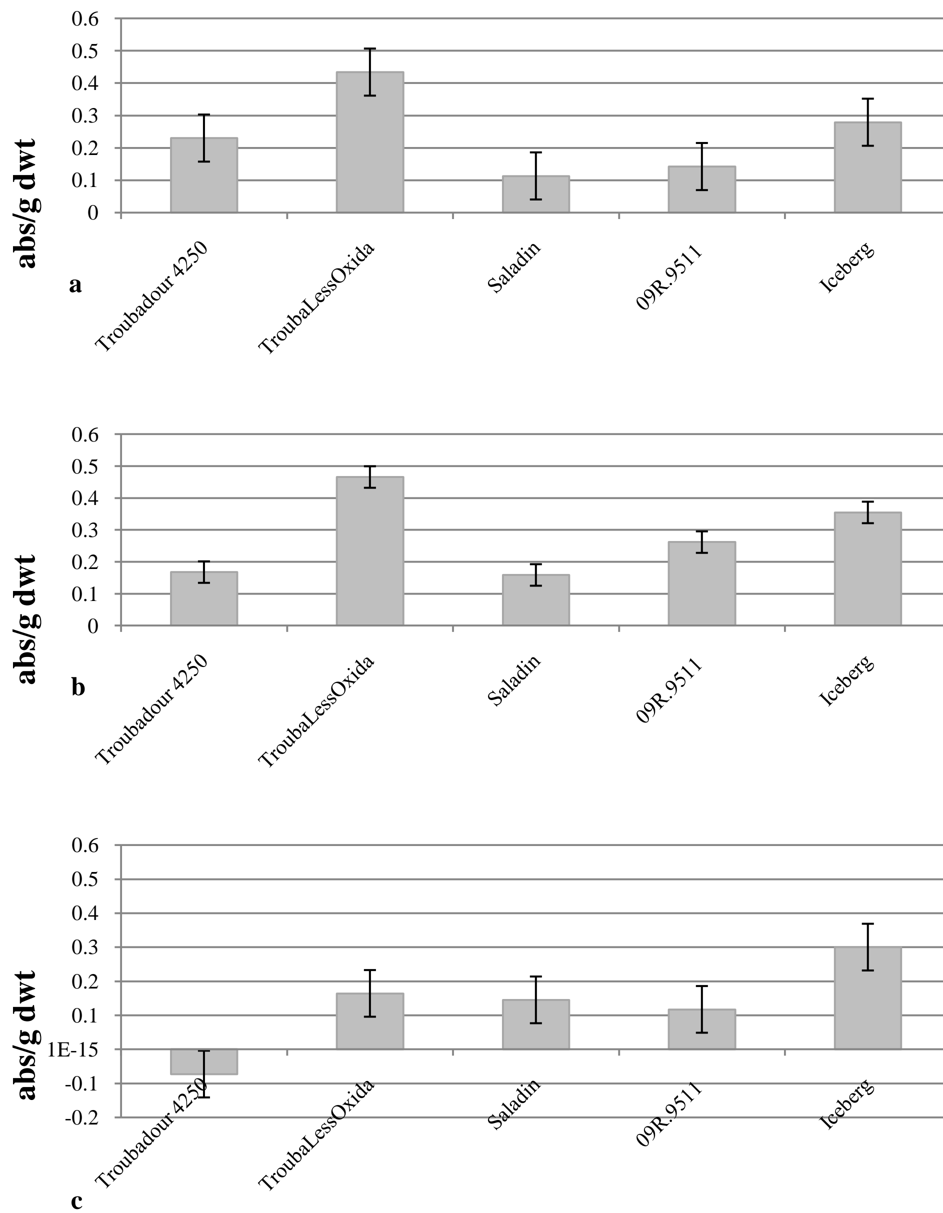


Figure 8.15. Mean PAL (phenylalanine ammonia lyase) activity for lettuce lines on a) harvest day, b) 4 days after storage and c) the difference between PAL activity levels across the 4 days. PAL activity measured as absorbance per gram of dry lettuce weight (abs/g dwt). Error bar represents seds (standard error of differences of the mean) from ANOVA. Where *dwt* (dry weight); *g* (gram).

PPO activity

ANOVA revealed significant variation (***P <0.001) between lines for PPO activity on harvest day and day 4, and for the differences between PPO activities across days (**P <0.009) (see table 8.8.).

Table 8.8. PPO activity for lettuce lines over 4 days. ANOVA GT df = 4. ANOVA comparison df = 1. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *PPO* (polyphenol oxidase); *GT* (genotype/accession); *v* (comparison); *vr* (variance ratio); *prob* (ANOVA probability).

	PPO activity					
	Harvest day		Day 4		Difference across days	
Variation	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**
GT	6.21	***<0.001	18.79	***<0.001	4.26	**0.009
TroubaLessOxida v Troubadour 4250	0.05	0.817	0.06	0.809	0.09	0.768
09R.9511 v Saladin	2.26	0.142	2.05	0.163	2.06	0.163

PPO activity of the 5 lines varied from 0.78 abs/g dwt to 2.04 absorbance abs/g dwt for harvest day, and from 0.92 abs/g dwt to 3.6 abs/g dwt on day 4 (see figure 8.16. (a-c)). Troubadour 4250 always had the lowest levels of PPO activity on both days while Saladin and 09R.9511 respectively had the highest levels of PPO activity on harvest day and day 4. PPO activity increased from harvest day to day 4 for all lines. Mean PPO activity was 1.36 abs/g dwt and 2.07 abs/g dwt respectively for harvest day and day 4. Troubadour 4250 and TroubaLessOxida showed similar levels of PPO activity for all days as did Saladin and 09R.9511. However Troubadour 4250 and TroubaLessOxida had significantly lower levels of PPO activity on both days than Saladin and 09R.9511. All lines showed an increase in PPO activity across days; 09R.9511 recorded the highest increase in PPO activity across days (2.05 abs/g dwt).

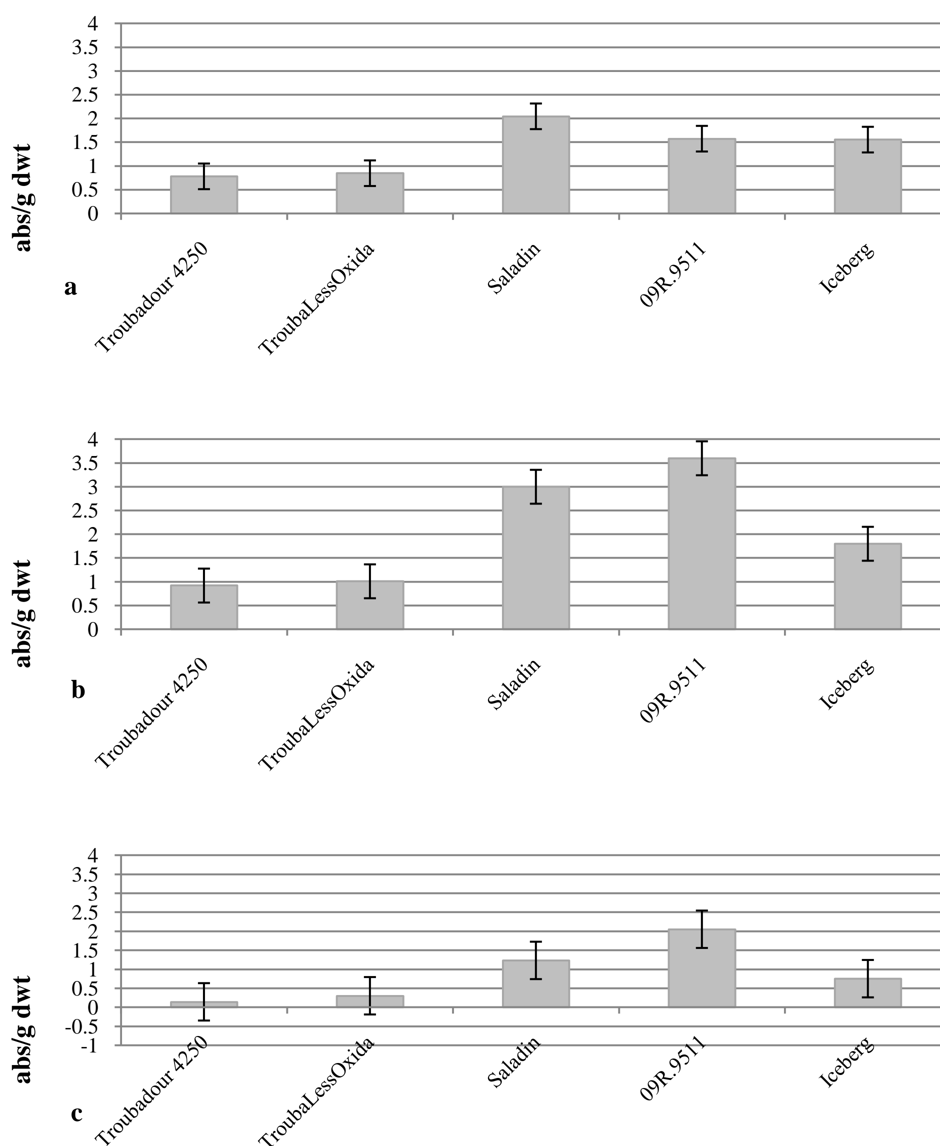


Figure 8.16. Mean PPO (polyphenol oxidase) activity for lettuce lines on a) harvest day, b) 4 days after storage and c) the difference between PPO activity levels across the 4 days. PPO activity measured as absorbance per gram of dry weight (abs/g dwt). Error bar represents seds (standard error of differences of the mean) from ANOVA. Where *dwt* (dry weight); *g* (gram).

Total phenolic content

ANOVA revealed significant variation (**P <0.009) between lines for total phenolic content (TPC) when expressed as gallic acid equivalents per gram of dry weight of lettuce (GAE/g dwt) for the differences of TPC across days (see table 8.9.).

Table 8.9. Mean total phenolic content (TPC) of lettuce lines over 4 days. ANOVA GT df = 4. ANOVA comparison df = 1. Significant effects shown as *P <0.05, **P <0.01 and ***P <0.001. Where *TPC* (total phenolic content); *GT* (genotype/accession); *v* (comparison); *vr* (variance ratio); *prob* (ANOVA probability).

	TPC					
	Harvest day		Day 4		Difference across days	
Variation	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**	**vr**	**ANOVA F prob**
GT	0.8	0.532	1.65	0.18	3.9	**0.009
TroubaLessOxida v Troubadour 4250	2.9	0.096	0.57	0.457	1.46	0.234
09R.9511 v Saladin	0.13	0.722	2.73	0.106	7.92	**0.007

TPCs in the 5 lines varied from 48.7 mg GAE/g dwt to 69.9 mg GAE/g dwt for harvest day, and from 43.9 mg GAE/g dwt to 78.6 mg GAE/g dwt on day 4 (see figure 8.17. (a-c)). Generally TPC decreases across days; mean TPC was 60.98 mg GAE/g dwt on harvest day and 57.58 mg GAE/g dwt on day 4. However Troubadour 4250 TPC remained constant and 'browning mutant' 09R.9511 TPC increased (19.2 mg GAE/g dwt) across days. Significant differences were recorded between 09R.9511 and Saladin for the difference in TPC across days (**P <0.007).

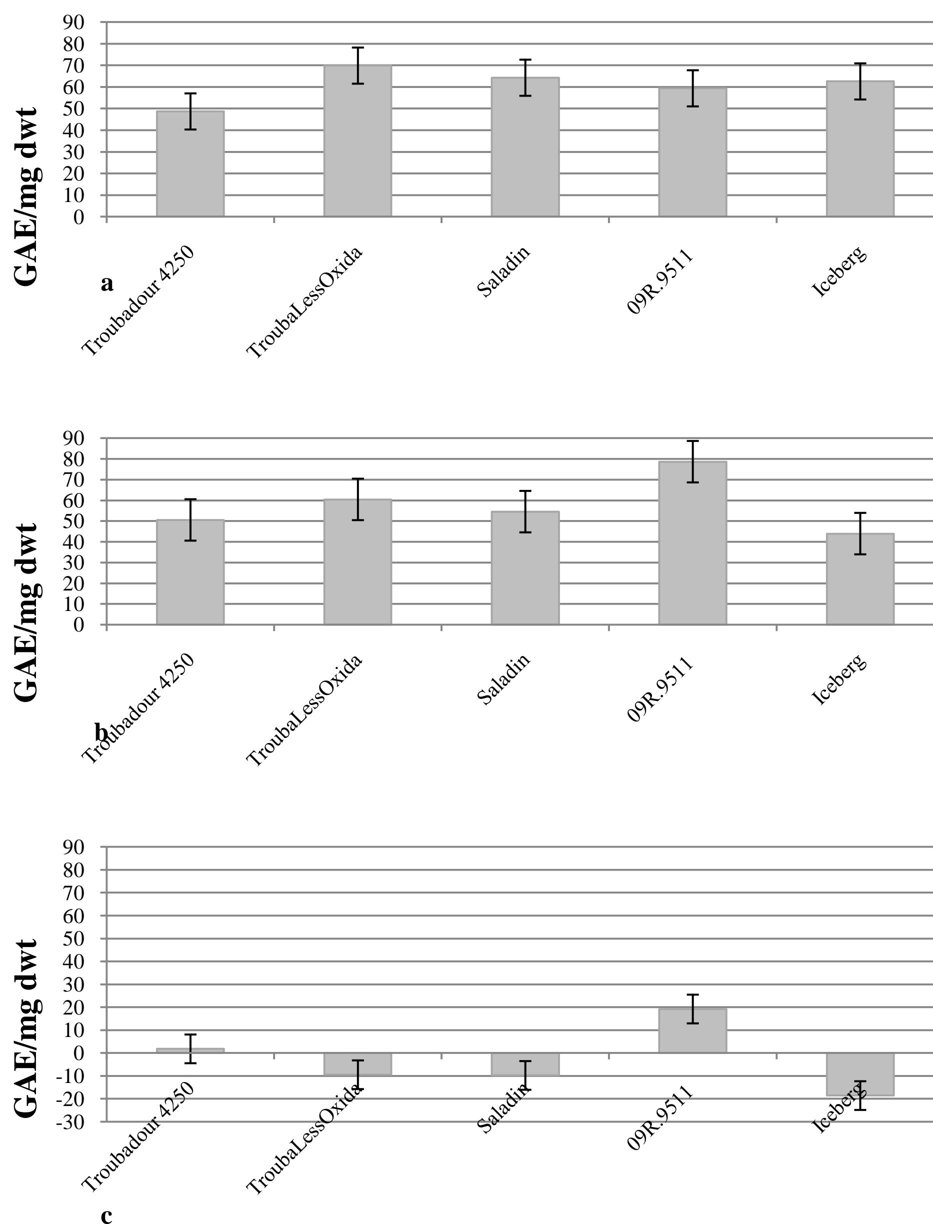


Figure 8.17. Mean TPC (total phenolic content) of lettuce lines on a) harvest day, b) 4 days after storage and c) the difference between TPC levels across the 4 days. TPC expressed as gallic acid equivalents (GAE) per gram of dry mass of lettuce. Error bar represents seds (standard error of differences of the mean) from ANOVA. Where *dwt* (dry weight); *mg* (milligram); *g* (gram).

8.3.3. Correlations amongst traits

All discolouration measures (browning and pinking), morphological traits (plant weight) and metabolite activity were assessed for potential relationships (full correlation matrix in Appendix F) (see table 8.10.).

The two measures of pinking (intensity and extent) were positively correlated with each other over all days, with mean pinking intensity and mean extent of pinking highly correlated ($R_{[3]} \geq 0.98$). Similarly measures of browning positively correlated with each other, with mean browning intensity and mean extent of browning perfectly correlated ($R_{[3]} = 1$). As for pinking and browning, measures of overall discolouration were generally positively correlated with one another. No highly significant correlations were recorded between pinking and browning.

For metabolite related traits; PPO activity on day 4 and PPO differences in activity across days were positively correlated ($R_{[3]} \geq 0.98$). PPO activity on day 4 was negatively correlated with browning (intensity and extent) for all days (the highest correlation was with extent of browning on day 4 ($R_{[3]} \geq -0.98$)). PPO activity on harvest day was positively correlated with trimmed weight ($R_{[3]} \geq 0.95$). TPC was negatively correlated with overall discolouration intensity on day 1 and day 4 ($R_{[3]} \geq -0.89$ and $R_{[3]} \geq -0.91$ respectively).

Table 8.10. Correlation matrix from the mutant lines for post harvest discolouration, plant weight and metabolite activity. Read across then down. Only significant effects are shown and highly significant effects ***P <0.001 are shown bold, **P <0.01 are shown italics. Where *tr wt* (trimmed weight, g); *msb* (mean score browning); *msd* (mean score overall discolouration); *%b* (percentage browning); *H* (harvest day); *Ac* (across days). Numerical value before measurement is day. Degree of freedom is 3.

	msb	**1msb**	**4msb**	**%b**	**1%b**	**3%b**	**4%b**	**1msd**	**4msd**	**tr wt**	**4 PPO**
H PPO										*0.95*	
4 PPO	**-0.97**	*-0.96*	**-0.98**	**-0.98**	**-0.97**	**-0.97**	**-0.98**			*0.95*	
Ac PPO	**-0.97**	*-0.96*	**-0.98**	**-0.98**	**-0.97**	**-0.98**	**-0.98**				**0.98**
H TPC											
4 TPC								-0.89	-0.91		

8.4. Discussion

Mutations can be induced by methods such as using EMS or RNAi gene silencing (e.g. Hammond *et al.* 2000; Lu *et al.* 2003). In lettuce, chemical mutagenesis has been conducted in both the scientific community and industry showing the high potential of this method for breeding (JW Schut personal communication; A Abbott personal communication; Robinson 1986). However the technology has limitations as it generates mutations in many genes and may result in only moderate or weak phenotypes (Lee *et al.* 2010).

Chemical mutagenesis using EMS has been used at Rijk Zwaan to generate two mutant lines, TroubaLessOxida and 09R.9511, which have previously been recorded to display lower levels of post harvest discolouration (pinking and browning respectively). Pinking mutant TroubaLessOxida has shown significantly lower scores for pinking than wild type Troubadour 4250 for day’s 1-4 post harvest across different environments and years, suggesting genetic stability for this trait. It has

also shown significantly lower scores for browning than Troubadour 4250, for browning for day 1, although this variation was specific to NL suggesting phenotypic plasticity (Gurganus *et al.* 1999) for this trait. Significant differences were also recorded between the mutant and wild type for overall discolouration for day's 1-4 post harvest but this difference can be explained by the significant differences in pinking. Browning mutant 09R.9511 showed significantly lower scores for browning than Saladin for day's 1-4 post harvest across different environments and years, suggesting genetic stability for this trait. Lines were not significantly different for pinking and were generally not significantly different for overall discolouration.

Intensity and extent of discolouration were generally positively correlated for all measures of pinking, browning and overall discolouration across days (see table 8.10.) which suggest that although measured as separate traits, intensity and extent may have the same genetic basis as discussed in Chapter 3.

The mutants, Troubadour 4250 and Saladin, were generally situated between *L. serriola* 03050 and Iceberg for each discolouration trait for each day. Iceberg showed high scores for all measures of discolouration in comparison to all other lines. *L. serriola* 03050 showed extremely low levels of post harvest discolouration similar to that of TroubaLessOxida for pinking and 09R.9511 for browning, which were all generally significantly lower than recorded for all commercial breeding lines (including Saladin, Troubadour 4250 and Iceberg). Iceberg and *L. serriola* 03050 are lines with naturally occurring alleles showing extreme phenotypes compared to the other lines tested. *L. serriola* 03050 contains alleles giving as good a phenotype as the mutated lines (for the respective type of discolouration), this provides the option of alternative routes to achieve the same phenotype via exploitation of naturally occurring alleles through breeding.

The mutant lines had significantly higher levels of PAL activity than their comparative wild type control. Troubadour 4250 and TroubaLessOxida were significantly different for PAL activity on harvest day and day 4 post harvest, but also for the difference between activity levels across days. As the lines were significantly different on harvest day it would suggest that the higher levels of PAL activity in the mutant are largely predetermined. Saladin and 09R.9511 were only significantly different for PAL activity on day 4 post harvest, suggesting that increased PAL activity was induced at higher levels in the mutant upon wounding. Higher levels of PAL activity was recorded in the mutant lines which also demonstrated reduced discolouration phenotypes. However lines with increased PAL activity would be expected to show higher levels of post harvest discolouration as biosynthesis of polyphenols for oxidation would be expected to increase leading to the formation of coloured pigments (Joslin and Pointing 1951; Zawistowski *et al.* 1991; Martinez and Whitaker 1995; Lopez-Galvez *et al.* 1996; Solomon *et al.* 1996; Peiser *et al.* 1998; Hisaminato *et al.* 2001; Toivonen and Brummell 2008; Van Vliet *et al.* 2009).

All lines showed similar levels for total phenolic content (TPC) on harvest day; however levels generally decreased across days at different rates (rank positions within the distribution of lines across days were reflected to differing rates of discolouration), (with the exception of 09R.9511 which showed an increase in TPC across days). A decrease in TPC would be expected as once the plant has been removed from its nutrient and water supply it contains a finite amount of substrate for the biosynthesis of polyphenols. TPCs of lettuce have also been known to be altered by cultivar, type and colour, in addition to growing environment (Liu *et al.* 2007).

PPO activity on day 4 was negatively correlated with browning (intensity and extent) for all days (see table 8.10). The negative correlation implies that PPO activity on day 4 and browning are associated in an antagonistic way. Higher levels of PPO on day 4 resulted in lower levels of browning. TPC was negatively correlated with overall discolouration intensity on day 1 and day 4 (see table 8.10.). Higher TPCs resulted in lower levels of overall discolouration. However the mutant lines have strong phenotypes giving extreme scores which would have affected the correlation analysis.

The results obtained in this study suggest that the mutations are not directly affecting PAL or PPO levels (as lower enzyme activity would be expected for phenotypes with reduced discolouration). As the mutants have higher levels of PAL, PPO and TPC a worse phenotype than the wild type would be expected. The fact that better phenotypes have been observed, means that mutations must have occurred somewhere within the phenylpropanoid pathway downstream from PPO to stop the phenotype developing (see figure 1.2. and 8.18.). The mapping parents Saladin and Iceberg did follow the predicted pattern (see Chapter 7). The mutation may be novel and therefore if combined with naturally occurring beneficial alleles could produce an even better phenotype. However, although this mutation cannot be explained simply in terms of the biosynthetic pathway, the phenotypes have been confirmed in this study.

PPO activity on day 4 and PPO differences in activity across days were positively correlated ($R_{[3]} \geq 0.98$). PPO activity increased across days from time of wounding which occurred at different rates depending on the line. PPO activity for Troubadour 4250, TroubaLessOxida and Iceberg remained unchanged however there was a ~50% increase in activity for Saladin and >100% increase in activity for 09R.9511.

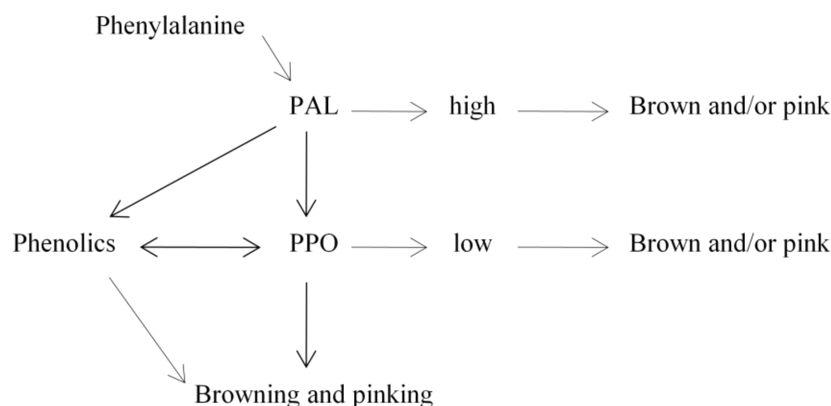


Figure 8.18. Section of the phenylpropanoid pathway and affect of enzyme regulation.

PPO activity on harvest day was also positively correlated with trimmed weight ($R_{[3]}$ $\geq$0.95). Greater plant weight has been associated with higher enzyme activities in central and secondary metabolism in *Arabidopsis* (Mitchell-Olds and Pedersen 1998). It has been reported that plant growth is not directly related to absolute levels of amino acids, sugars and starch, but is in fact related to flux which is indicated by the capacity of enzymes to use resources (Cross *et al.* 2006).

This study indicates that a desired phenotype with reduced levels of post harvest discolouration can be achieved by two approaches to breeding. The induced variation observed in TroubaLessOxida and 09R.9511 has shown phenotypes with reduced pinking and browning respectively. By a balanced amount of backcrossing rounds of the mutants, the many randomly induced point mutations in the genome induced by EMS can be removed so they do not affect any other properties of the line. However mutation breeding is still not acceptable for some markets such as for ‘organic products’. As current public opinion in the EU opposes any type of genetic engineering in food products, the use of natural alleles could be beneficial. *L. serriola* 03050 contains natural alleles which have given as good a phenotype as TroubaLessOxida and 09R.9511 for post harvest discolouration which could used in

classical breeding. *L. serriola* has been used extensively in lettuce breeding as a source of several disease resistance genes (Crute 1988). However, *L. serriola* 03050 will have many 'background' deleterious alleles which will also require a backcross programme to remove. MAS using markers identified by approaches described in Chapter 5 would be helpful in this.

8.5. Conclusions

- Mutation using EMS produced a line 'TroubaLessOxida' with an improved phenotype for pinking and significantly increased PAL activities.
- Mutation using EMS produced a line '09R.9511' with an improved phenotype for browning with significantly increased TPC in the later stages post harvest.
- *L. serriola* 03050 contains naturally occurring alleles giving as good a phenotype as the mutated lines (for the respective type of discolouration), which gives an alternative route to achieve the same phenotype.

CHAPTER 9

General Discussion

In today's increasing market of "food perfection", any detrimental change to the visual characteristics of a product is likely to incite an unfavourable consumer response. A prime example of this is pre-packed cut salads. There is an escalating demand for this product due to the increasingly busy lifestyles of (time poor/cash rich) consumers, however, the limited shelf life of fresh processed lettuce primarily due to post harvest spoilage is a major food industry concern with losses entering the £millions per annum (Martinez and Whitaker 1995; Soliva-Fortuny and Martin-Belloso 2003).

This study has significantly improved the understanding of post harvest discolouration in lettuce. The emphasis has been to investigate the genetic control of post harvest discolouration and to provide the tools and knowledge for breeding: however, work to understand the metabolic causes of post harvest discolouration in lettuce has also been in initiated.

Genetic variation in the degree of post harvest discolouration was observed in a lettuce diversity set (of 28 lines) that represents the wider plant genetic resource collection of the lettuce genepool held in the Warwick genetic resources unit (see section 3.3.1.). Diversity sets which are chosen to represent the primary and secondary crop genepool provide a tool to assess whether significant genetic variation exists for a given trait. The lettuce diversity set is available to study other traits and has now been extended to 96 accessions to provide an increased representation of the crop diversity.

The parents of the WHRI lettuce mapping population, Saladin and Iceberg were included in the diversity set. They showed significantly different responses for post harvest discolouration and the difference between them was representative of a major

part of the variation seen in the diversity set. Saladin had significantly lower levels of post harvest discolouration (pinking, browning and overall discolouration) compared to Iceberg (see sections 3.3.2.). This meant that the existing population of F_7 recombinant inbred lines (RILs) derived from a cross between Saladin and Iceberg was suitable for genetic analysis of post harvest discolouration traits.

As a precursor to the genetic analysis, a good quality linkage map was generated based on the F_7 Saladin x Iceberg population (Sal x Ice map). It covers all 9 LGs with most component LGs in the correct orientation and marker order. It was of sufficient quality to use for QTL analyses in the present study (see Chapter 5 and 6), and is a valuable resource for future genetic studies in lettuce. As it was derived from a lettuce x lettuce cross it has direct application to the cultivated genepool (compared to intraspecific crosses between lettuce and wild species relatives) and is therefore valuable resource for lettuce breeding. The map has been integrated with the genechip map MCB10_10NR map created by Michelmore's group in UC Davis, based on marker positions, and can therefore be used for comparative genomic approaches (as in section 7.3.3.), increasing the value of the map for genetic studies. Due to common markers, the Sal x Ice map was also anchored to other published lettuce linkage maps published by Jeuken *et al.* (2001) and Syed *et al.* (2006). However, although the Sal x Ice map is of good quality it could still be improved. Future work to add new markers would help coalesce the map into 9 linkage groups and fill gaps; the addition of markers in key positions may also allow the inclusion of the markers which have been scored on the population during this study (249 un-mapped markers) but which currently do not map to any linkage group.

Significant genetic variation in the post harvest discolouration response was demonstrated for the RILs from the mapping population. In addition environmental

variation was found in the form of a site effect for browning, although none were recorded for pinking or overall discolouration. Hilton *et al.* (2009) found that the majority of agronomic factors did not have a major impact on post harvest discolouration on lettuce. However, preliminary investigations of meteorological data suggested that rainfall could affect post harvest discolouration (Hilton *et al.* 2009). Both irrigation methods and rainfall have been shown to affect general lettuce post harvest visual quality (Fonseca 2006). No effect of weather was recorded for any trials in this study. Although lines for each trial were transplanted on the same day and harvested within a restricted period, time of transplanting, timing of harvest and crop maturity has also been shown to have an effect on post harvest discolouration (Beverley *et al.* 1993; Bergquist *et al.* 2005; Hilton *et al.* 2009). As the environment cannot be controlled in the field in the UK, it would be beneficial to attempt to produce genotypes with stable phenotypes over environments. RILs with extreme phenotypes demonstrated stability over environments, therefore showing that it was possible to select lines showing phenotypic stability opening up the possibility of breeding varieties which do not develop post harvest discolouration.

QTL analysis is the first step in providing tools for efficient breeding of quantitative traits which are influenced by the environment. Although the QTL identified in this study accounted for a proportion of the genetic variation observed in the RILs, the remaining variation is unexplained. The genetic basis of discolouration can be begun to be understood based on phenotypic correlations between discolouration responses. The markers linked to the 21 significant QTL identified for discolouration can be used for MAS (section 5.3.3.), therefore making it possible to select for genotypes showing less post harvest discolouration, because this selection can be carried out on

genotypes it is independent of the environment. A possible strategy for MAS would be to first select for beneficial alleles at QTL found in all environments. Secondly, deleterious alleles at QTL would be selected against or this could also be achieved by trialling lines in different environments to give as much environmental stability of the trait as possible.

Although, this study has provided breeders with the tools to breed for reduced discolouration in lettuce, it is also important for them to understand what effect this may have on other important agronomic traits. Correlations between important agronomic traits and post harvest discolouration traits were recorded, but generally these were not highly correlated (see section 6.3.2.). Twenty significant QTL were identified for a range of important agronomic traits, and markers linked to them can be also be used for MAS (see section 6.3.3.). Some QTL for agronomic traits were linked to QTL for post harvest discolouration which explains the weak correlations between the traits at the phenotypic level (see section 6.3.3.). However, because the correlations are weak, post harvest discolouration and agronomic traits can generally be independently selected for by breeders without having to compromise on traits. However, linkage between QTL means that there is a chance pleiotrophic effects may occur, therefore future work to fine map of the QTL would provide for more informed breeding.

Another approach to provide more information is to identify the metabolic effect of QTL. This research has also initiated studies to understand the metabolic changes underlying the phenotype change. Significant variation in levels of metabolites related to post harvest discolouration, including PAL, PPO and TPC, was observed in the extreme RILs (see section 7.3.1.). The differences in metabolite levels were correlated with the discolouration phenotype (see section 7.3.2.). Therefore, it would

be beneficial in future work to assess the entire RIL population for these metabolites and conduct a QTL analysis on the data to see if QTL for metabolites co-locate with discolouration QTL.

Another approach to providing more genetic information is to attempt to identify the genes underlying QTL. This study has initiated work to identify associated candidate genes. Twenty-three genes associated with the biosynthetic pathway responsible for discolouration (the phenylpropanoid pathway) have been placed on the Sal x Ice map using comparative genomic approaches (see section 7.3.3.). Some of these co-locate within the region of a discolouration QTL and are therefore candidate genes for the QTL effect. There are a number of future studies that could be carried out to further the understanding of the genetics of the discolouration response. It would be valuable to accurately map all candidate genes on the Sal x Ice map by directly mapping the genes in the Sal x Ice RILs. It would also be useful to determine the expression of candidate genes associated with discolouration QTL firstly in the parental lines and then if significant differences are found in the RILs to map expression QTL and see if they co locate to the QTL identified in this study. Natural allelic variation for candidate genes could be assessed in a wider lettuce diversity set (the diversity set at WHRI has now been increased to 96) and induced variation could be searched for in a Saladin TILLING population which has been produced at Warwick HRI (at WHRI).

Rijk Zwaan have induced phenotypic variation via mutagenesis leading to mutants with reduced post harvest browning and pinking. The phenotype was confirmed for both mutants in this study; although the point of mutation could not be identified the data indicated it to be downstream of PPO for both lines (see section 8.4.). Another approach to help characterise the mutants would be to cross the mutants and look at

complementation. It would be beneficial to look at the offspring's post harvest discolouration phenotype and see if it is reflected in the metabolome. The mutants could also be crossed with the best performing RILs or RILs with beneficial alleles at all identified QTL to combine the QTL and mutation/s for breeding.

However, the lettuce primary and secondary genepool contains naturally occurring alleles, which give as good a phenotype (e.g. the phenotype of *L. serriola* 03050) as the mutated lines (see section 8.3.1.). This gives an alternative route to producing a desired phenotype using natural allelic variation.

However, it is necessary to approach breeding for reduced post harvest discolouration with a note of caution. During this study, there was insufficient time to look at the potential impact reducing post harvest discolouration may have on other abiotic and biotic stress responses, particularly pest and disease resistance. Browning is thought to be a wound response which deters pests, if lines were bred for reduced discolouration it is important to ensure that resistance to pests and/or disease is not compromised. These factors must be looked into so that breeders can take a balanced approach.

Nevertheless, this study indicates that a desired phenotype with reduced levels of post harvest discolouration can be achieved and has provided the tools and knowledge to do this.

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Appendix A

Additional information from Chapter 3

Field trial plan and randomisation

Plants were transplanted to the field into 3 x 1.83 m wide beds with a plot length of 1.05 and spacing distance of 35 cm (see figure A.1.a). Beds were marked using a tractor with a wheel base of 1.83 m and plots were then individually marked using a 'Wolf Garten' row marker. A plot configuration of 12 plants per plot and rep (4 rows x 3 plant arrangement) of the same accession was used, creating a formation of two central heads surrounded by guard plants. The plant material was randomised within a rep by an alpha design (see figure A.1.b).

Rep

Plot number 1-30

1	2	3
30	30	30
29	29	29
28	28	28
27	27	27
26	26	26
25	25	25
'	'	'
5	5	5
4	4	4
3	3	3
2	2	2
1	1	1

31.5m long

Bed

1 2 3

5.49 m wide

Overall area 172.94 m^2

a

```
rep 1 ------------------------
   plot   1   2   3   4   5   6
block+-----------------------
  1   |  28  13   9  14   1  30
  2   |  27  29  21   8  15  19
  3   |  12  20   4  11   6  24
  4   |  18  17  22   7  10   2
  5   |  23  25   5   3  26  16
rep 2 ------------------------
   plot   1   2   3   4   5   6
block+-----------------------
  1   |  11  22  25  16  18  29
  2   |  20   3  10  24   2  14
  3   |   5  19  17  13  21  26
  4   |  27  28  12  15  23   9
  5   |   1  30   8   7   4   6
rep 3 ------------------------
   plot   1   2   3   4   5   6
block+-----------------------
  1   |  23   5  20   6  21   7
  2   |   9  12   1  18  17  16
  3   |   3  10  15   8  28  22
  4   |  14  29  25   4  30  26
  5   |  11  27   2  13  19  24
```

b

Figure A.1. Field trial plan (a) and randomised alpha design for field plan (b).

Post harvest discolouration of prepacked leaf tissue on days 6, 9 and 13.

REML analysis was conducted for each measure of pinking, browning and overall discolouration on days 6, 9 and 13 (see figures A.3. - A.8.).

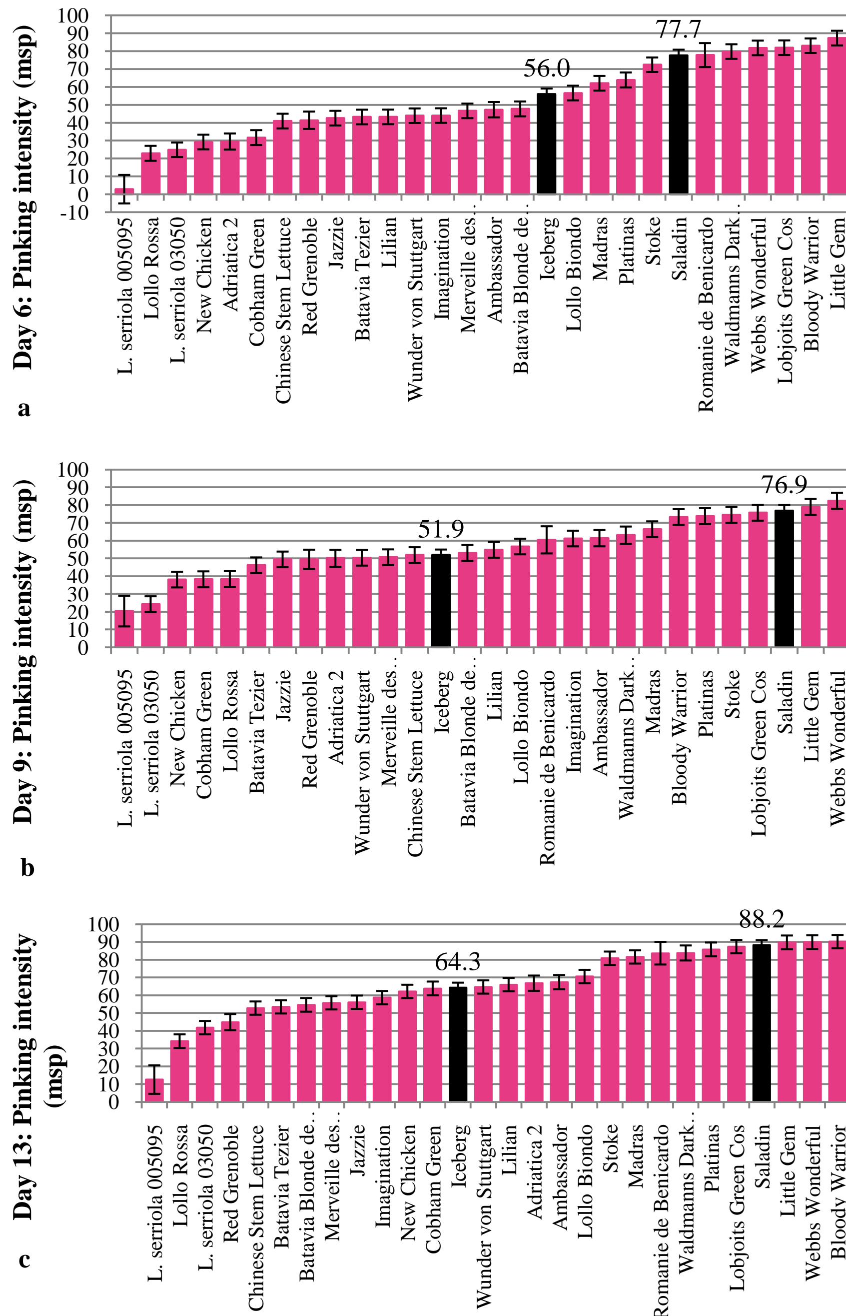

Figure A.2. Transformed adjusted means (from REML) for lettuce post harvest pinking intensity on a) day 6, b) day 9 and c) day 13 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML). Where *Batavia Blonde de..* (Batavia Blonde de Paris); *Merveille des..* (Merveille des Quatre Saisons); *Waldmans Dark..* (Waldmans Dark Green).

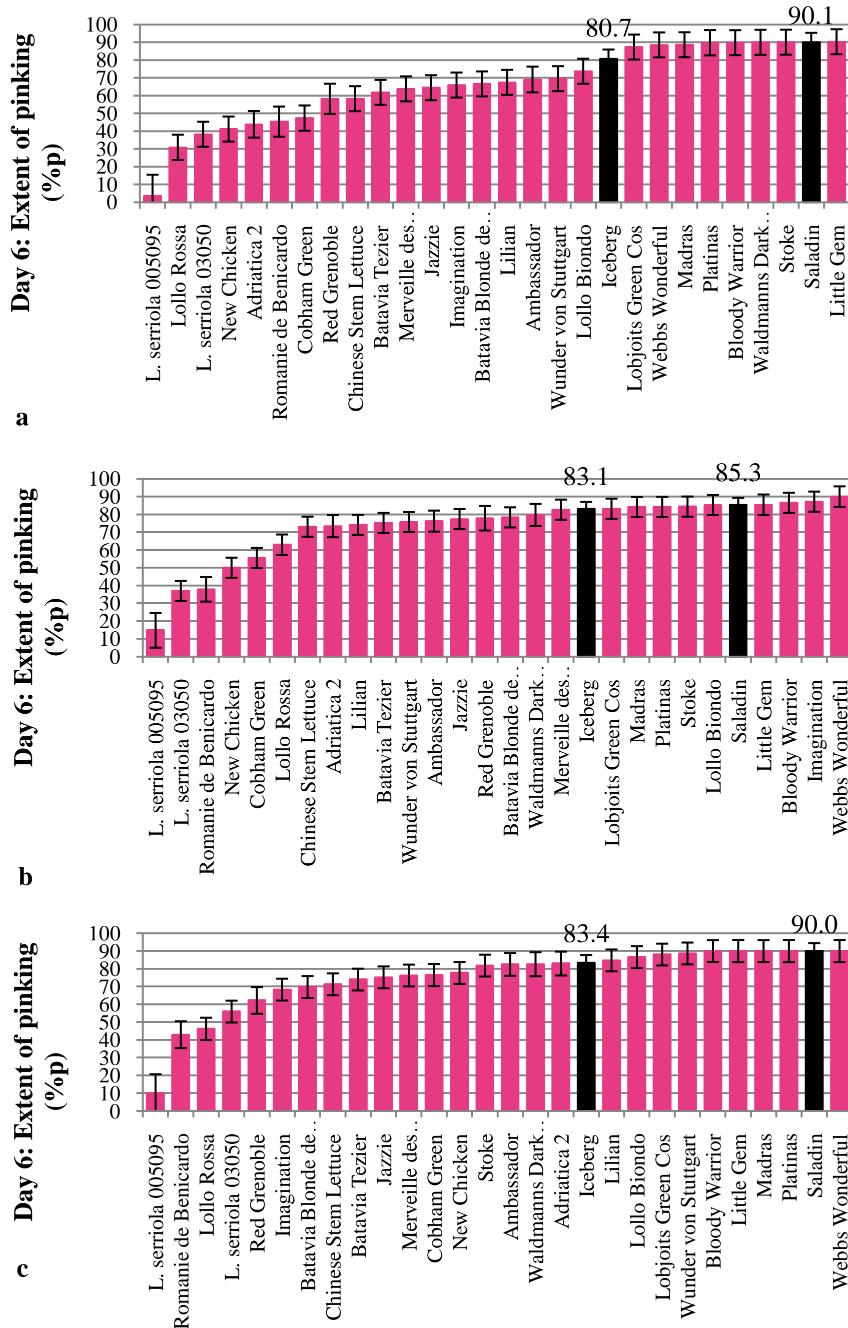

Figure A.3. Transformed adjusted means (from REML) for lettuce post harvest extent of pinking on a) day 6, b) day 9 and c) day 13 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML). Where *Batavia Blonde de..* (Batavia Blonde de Paris); *Merveille des..* (Merveille des Quatre Saisons); *Waldmans Dark..* (Waldmans Dark Green).

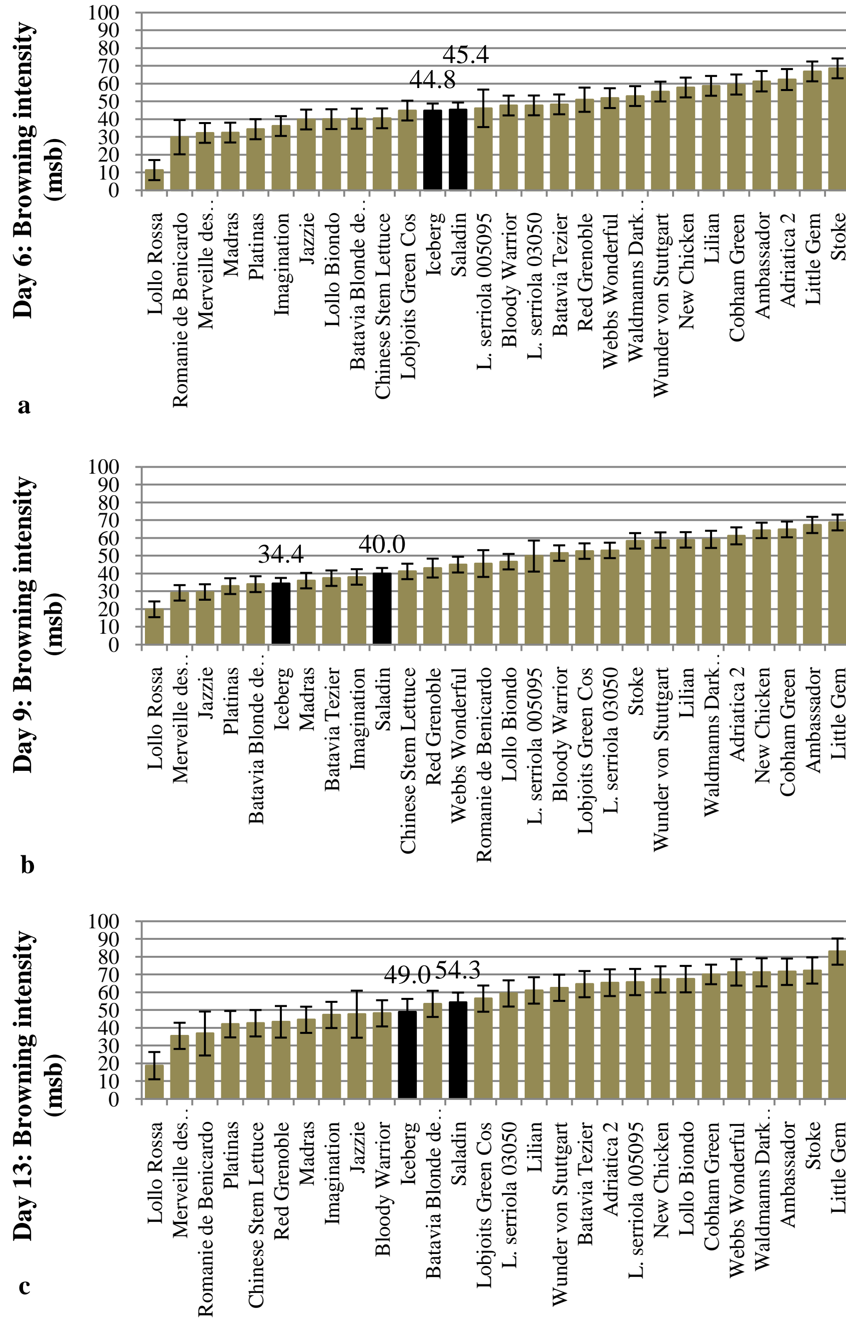

Figure A.4. Transformed adjusted means (from REML) for lettuce post harvest browning intensity on a) day 6, b) day 9 and c) day 13 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML). Where *Batavia Blonde de..* (Batavia Blonde de Paris); *Merveille des..* (Merveille des Quatre Saisons); *Waldmans Dark..* (Waldmans Dark Green).

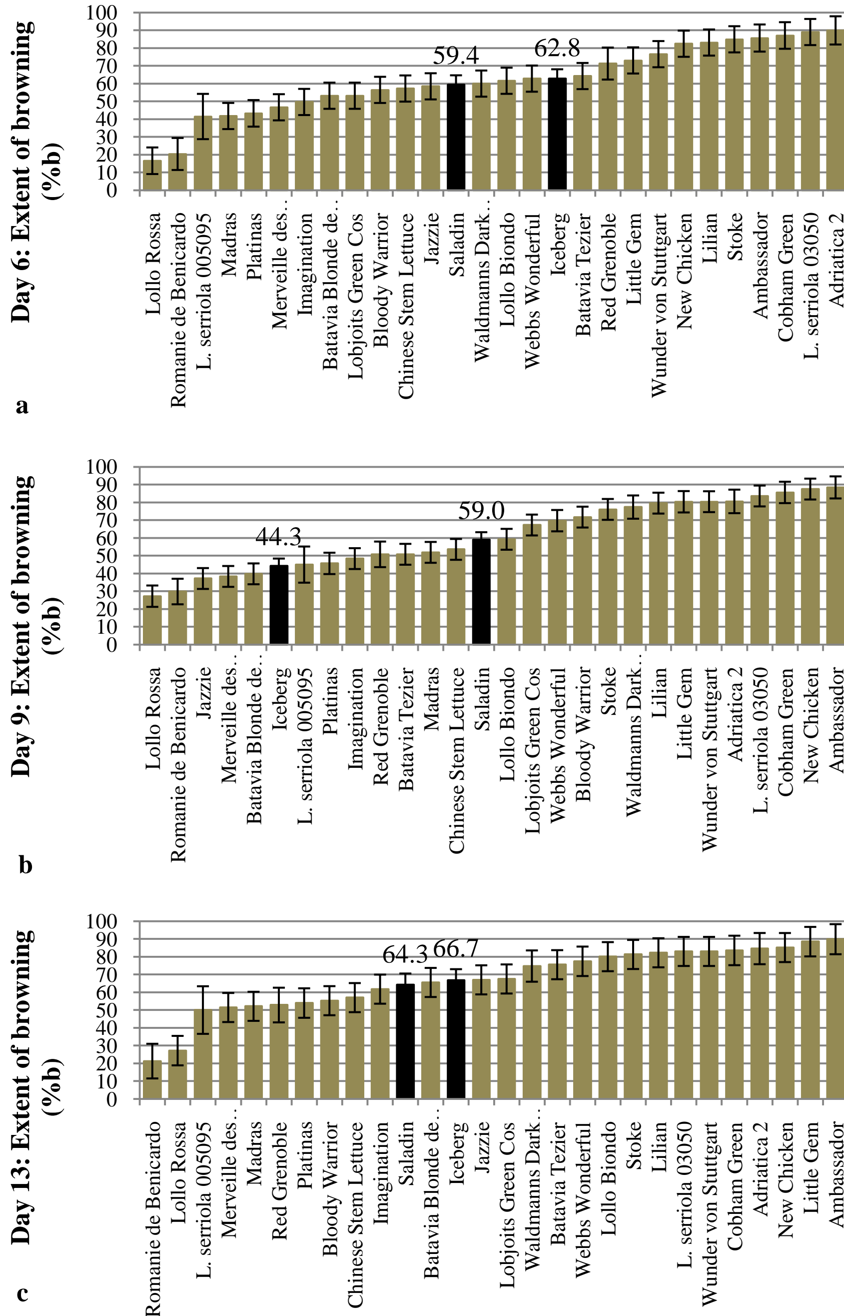

Figure A.5. Transformed adjusted means (from REML) for lettuce post harvest extent of browning on a) day 1 and b) day 3 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML). Where *Batavia Blonde de..* (Batavia Blonde de Paris); *Merveille des..* (Merveille des Quatre Saisons); *Waldmans Dark..* (Waldmans Dark Green).

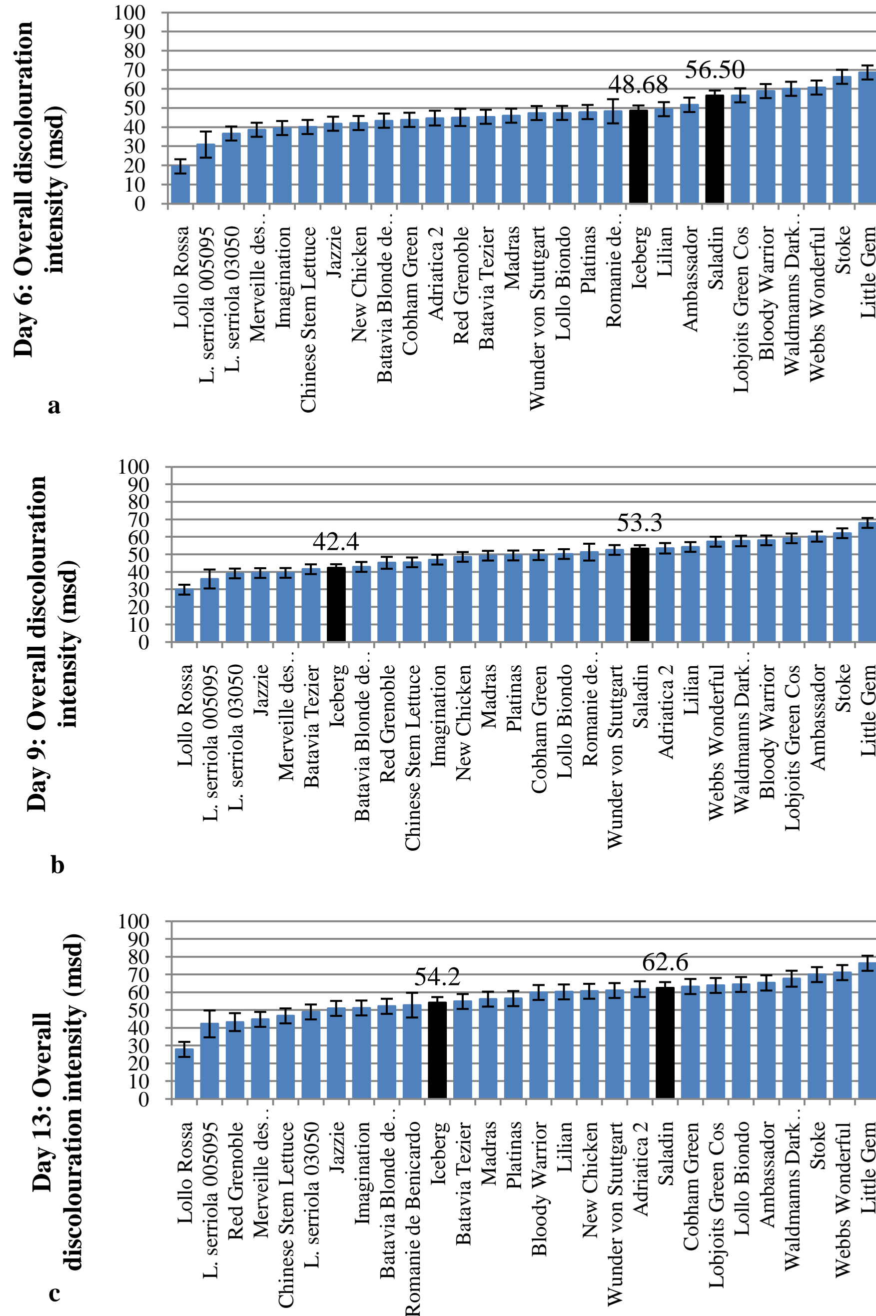

Figure A.6. Transformed adjusted means (from REML) for lettuce post harvest overall discolouration intensity on a) day 6, b) day 9 and c) day 13 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML). Where *Batavia Blonde de..* (Batavia Blonde de Paris); *Merveille des..* (Merveille des Quatre Saisons); *Waldmans Dark.* (Waldmans Dark Green).

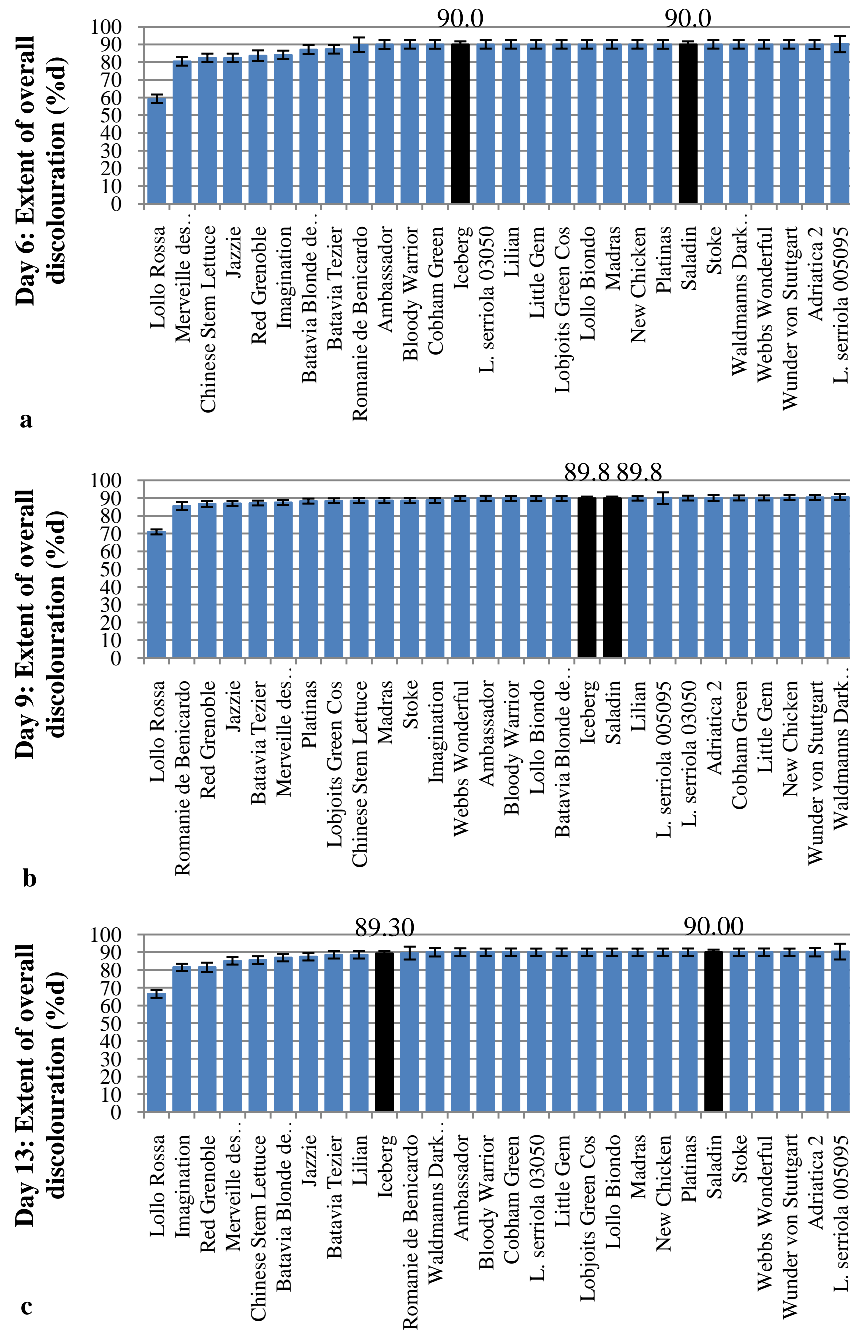

Figure A.7. Transformed adjusted means (from REML) for lettuce post harvest overall discolouration intensity on a) day 6, b) day 9 and c) day 13 for the WHRI lettuce diversity set. Error bars represent sems (standard error of the mean) from REML. The F_7 WHRI mapping population parents are highlighted with respective adjusted means (from REML). Where *Batavia Blonde de..* (Batavia Blonde de Paris); *Merveille des..* (Merveille des Quatre Saisons); *Waldmans Dark..* (Waldmans Dark Green).

Correlations amongst traits

Table A.1. Correlation matrix for the WHRI lettuce diversity between post harvest discolouration measurements and morphological parameters. Read across then down. Only significant effects shown with ****P* <0.001 in bold, ***P*<0.01 in italics and **P*<0.05 in regular font. Where *unt wt* (untrimmed weight, g); *tr wt* (trimmed weight, g); *tr dia1* (trimmed diameter 1, mm); *tr diam2* (trimmed diameter 2), *msb* (mean score browning); *msp* (mean score pinking); *msd* (mean score overall discolouration); *msv* (mean score visible discolouration); *%b* (percentage browning); *%p* (percentage pinking); *%d* (percentage overall discolouration); *%v* (percentage visible discolouration). Numerical value before discolouration measurement is day.

	unt wt	**tr wt**	**tr dia1**	**tr dia2**	**1msb**	**3msb**	**6msb**	**9msb**	**13msb**	**msb**	**1%b**	**3%b**	**6%b**	**9%b**	**13%b**	**%b**	**1msp**	**3msp**	**6msp**	**9msp**
unt wt																				
tr wt	**0.95**																			
tr dia1	**-0.76**	**-0.66**																		
tr dia2	**-0.72**	**-0.67**	**0.82**																	
1msb	**-0.75**	**-0.72**	**0.84**	**0.86**																
3msb	**-0.75**	**-0.68**	**0.63**	**0.61**	**0.83**															
6msb					0.46	*0.59*														
9msb					0.45	*0.50*	**0.85**													
13msb					0.37	*0.58*	**0.87**	**0.83**												
msb	-0.44	*-0.49*	0.38	*0.49*	**0.70**	**0.77**	**0.92**	**0.89**	**0.89**											
1%b	**-0.75**	**-0.73**	**0.84**	**0.86**	**0.99**	**0.83**	0.47	0.48	0.40	**0.73**										
3%b	**-0.79**	**-0.76**	**0.66**	**0.65**	**0.86**	**0.96**	**0.64**	*0.57*	**0.63**	**0.83**	**0.87**									
6%b	-0.40	-0.41	0.40	0.45	**0.63**	**0.70**	**0.93**	**0.77**	**0.83**	**0.94**	**0.66**	**0.78**								
9%b					0.38	0.40	**0.83**	**0.97**	**0.82**	**0.85**	0.41	0.48	**0.75**							
13%b				0.30	*0.53*	**0.70**	**0.86**	**0.77**	**0.95**	**0.92**	*0.56*	**0.77**	**0.90**	**0.74**						
%b	*-0.57*	*-0.60*	*0.55*	**0.61**	**0.83**	**0.85**	**0.82**	**0.79**	**0.79**	**0.97**	**0.85**	**0.92**	**0.93**	**0.74**	**0.89**					
1msp																				
3msp	*0.59*	*0.55*	**-0.81**	*-0.59*	**-0.67**	-0.43					**-0.65**	-0.45					0.34			
6msp	**0.80**	**0.76**	**-0.88**	**-0.75**	**-0.74**	*-0.50*					**-0.73**	*-0.53*						**0.87**		
9msp	**0.75**	**0.65**	**-0.84**	**-0.65**	**-0.69**	*-0.50*					**-0.67**	*-0.50*						**0.85**	**0.92**	

Table A.1. continued.

	unt wt	tr wt	tr dia1	tr dia2	1msb	3msb	6msb	9msb	13msb	msb	1%b	3%b	6%b	9%b	13%b	%b	1msp	3msp	6msp	9msp	13msp	msp	1%p	3%p
13msp	**0.67**	*0.54*	**-0.65**	*-0.52*			0.29	0.41	0.44					*0.54*	0.32			**0.66**	**0.80**	**0.78**				
msp	**0.75**	**0.66**	**-0.85**	**-0.66**	**-0.64**	-0.45					**-0.63**	-0.44		0.30				**0.90**	**0.97**	**0.94**	**0.87**			
1%p																	**0.95**	0.34						
3%p	**0.63**	*0.59*	**-0.81**	*-0.58*	**-0.69**	*-0.52*					**-0.67**	*-0.51*						**0.97**	**0.86**	**0.87**	**0.65**	**0.89**		
6%p	**0.72**	**0.70**	**0.74**	**0.59**	**-0.59**	**-0.39**				**-0.58**	**-0.40**							**0.77**	**0.92**	**0.87**	**0.77**	**0.92**		**0.82**
9%p	**0.46**	**0.49**	**-0.63**	**-0.56**	**-0.61**					**-0.61**						**-0.39**		**0.61**	**0.68**	**0.74**	**0.36**	**0.65**		**0.67**
13%p	0.44	0.36					0.41	0.43	*0.55*	0.36			0.39	*0.55*	*0.54*	0.31		0.46	**0.62**	*0.59*	**0.88**	**0.71**		0.48
%p	**0.66**	**0.62**	**-0.78**	*-0.59*	**-0.62**	-0.42					**-0.61**	-0.42					0.28	**0.89**	**0.91**	**0.90**	**0.73**	**0.94**	0.28	**0.93**
1msd	**-0.78**	**-0.73**	**0.79**	**0.83**	**0.97**	**0.84**	0.48	0.47	0.38	**0.71**	**0.96**	**0.87**	**0.64**	0.39	*0.52*	**0.82**		*-0.55*	**-0.68**	**-0.66**		*-0.58*		*-0.59*
3msd						0.42	**0.62**	*0.53*	**0.66**	*0.53*		0.38	0.47	*0.50*	**0.62**	0.41	0.43	**0.63**	0.45	0.44	0.43	*0.52*	0.36	*0.53*
6msd	*0.49*	0.42	**-0.61**	-0.44			**0.61**	*0.54*	**0.66**	0.39			0.42	**0.62**	*0.52*			**0.75**	**0.81**	**0.77**	**0.82**	**0.85**		**0.70**
9msd	0.35		-0.44				**0.68**	**0.78**	**0.73**	*0.57*			*0.50*	**0.84**	*0.58*	0.39		**0.61**	**0.62**	**0.68**	**0.80**	**0.73**		*0.54*
13msd							**0.72**	**0.76**	**0.88**	**0.67**		0.30	**0.63**	**0.82**	**0.79**	*0.55*		0.45	*0.53*	*0.52*	**0.81**	**0.64**		0.40
msd							**0.79**	**0.78**	**0.86**	**0.70**		0.32	**0.66**	**0.83**	**0.76**	*0.56*		*0.56*	*0.56*	*0.57*	**0.75**	**0.67**		0.48
1%d	**-0.78**	**-0.73**	**0.73**	**0.72**	**0.90**	**0.89**	*0.58*	*0.50*	*0.50*	**0.76**	**0.90**	**0.92**	**0.75**	0.41	**0.65**	**0.87**		-0.46	*-0.58*	*-0.55*		-0.47		*-0.53*
3%d						0.35	**0.64**	**0.65**	**0.74**	**0.66**	0.29	0.41	**0.64**	**0.72**	**0.75**	**0.61**		0.34	0.34	0.31	**0.68**	0.46		0.32
6%d	0.30	0.30					*0.56*	*0.52*	**0.64**	*0.53*			**0.61**	**0.61**	**0.65**	0.49		0.28	0.44	0.41	**0.70**	*0.51*		
9%d						0.30	*0.55*	*0.50*	**0.63**	*0.55*		0.33	**0.62**	*0.57*	**0.69**	*0.53*			0.36	0.37	**0.62**	0.44		
13%d	0.31	0.30					*0.55*	*0.51*	**0.63**	*0.52*			*0.58*	**0.60**	**0.65**	0.48		0.29	0.45	0.40	**0.72**	*0.52*		0.28
%d			0.37	0.41	**0.66**	**0.70**	**0.73**	**0.66**	**0.74**	**0.83**	**0.68**	**0.75**	**0.85**	**0.66**	**0.84**	**0.87**					0.30			
1msv			-0.41	-0.47														0.43	0.46	*0.50*	0.36	0.48		0.39
3msv			*0.52*	0.29	0.34						0.32						-0.38	**-0.77**	*-0.53*	*-0.55*	-0.43	*-0.59*	-0.41	**-0.75**
6msv	*-0.54*	-0.42	0.42	0.33			*-0.51*	*-0.55*	*-0.55*				-0.39	**-0.67**	-0.46			*-0.54*	**-0.64**	**-0.65**	**-0.90**	**-0.74**		*-0.53*
9msv	**0.76**	**0.68**	*-0.55*	*-0.54*	*-0.59*	**-0.65**	*-0.53*	-0.47	-0.39	**-0.60**	**-0.60**	**-0.68**	*-0.59*		-0.48	**-0.62**			0.48	0.45	0.43	0.41		
13msv			0.29				*-0.55*	**-0.67**	**-0.68**	*-0.55*			-0.47	**-0.78**	**-0.60**	-0.45			-0.46	-0.45	**-0.84**	*-0.57*	0.33	
msv							**-0.73**	**-0.77**	**-0.71**	**-0.68**		-0.38	**-0.62**	**-0.78**	**-0.66**	*-0.58*					*-0.53*	-0.40		
1%v			-0.43	*-0.53*	-0.39				0.29		-0.38							0.46	*0.49*	0.48	0.35	*0.50*		0.40
3%v			*0.57*	0.35	0.39						0.38						-0.44	**-0.77**	*-0.57*	*-0.53*	-0.40	*-0.59*	-0.46	**-0.76**
6%v	*-0.55*	-0.43	0.39				*-0.53*	*-0.57*	*-0.56*	-0.40			-0.43	**-0.69**	-0.48			*-0.53*	**-0.64**	**-0.63**	**-0.88**	**-0.73**		*-0.51*
9%v	**0.75**	**0.64**	*-0.59*	*-0.57*	**-0.64**	**-0.73**	*-0.54*	*-0.51*	-0.43	**-0.65**	**-0.65**	**-0.75**	**-0.64**		*-0.53*	**-0.69**			0.45	0.48	0.38	0.40		0.32
13%v	-0.38		0.32				*-0.54*	**-0.66**	**-0.69**	*-0.53*			-0.47	**-0.77**	**-0.60**	-0.43			*-0.50*	*-0.50*	**-0.86**	**-0.60**	0.34	
%v							**-0.67**	**-0.78**	**-0.69**	**-0.63**			*-0.57*	**-0.86**	**-0.61**	*-0.52*		-0.42	-0.43	-0.41	**-0.73**	*-0.54*		-0.40

Table A.1. continued.

	6%p	9%p	13%p	%p	1msd	3msd	6msd	9msd	13msd	msd	1%d	3%d	6%d	9%d	13%d	%d	1msv	3msv	6msv	9msv	13msv	msv	1%v
13%p	**0.73**	0.35																					
%p	**0.94**	**0.79**	**0.67**																				
1msd	*-0.54*	*-0.55*		*-0.52*																			
3msd	0.45	0.37	*0.50*	*0.56*																			
6msd	**0.78**	0.46	**0.76**	**0.77**		**0.71**																	
9msd	*0.57*		**0.70**	*0.57*		**0.65**	**0.89**																
13msd	*0.53*		**0.82**	*0.49*		**0.66**	**0.86**	**0.89**															
msd	*0.56*		**0.78**	*0.56*		**0.80**	**0.91**	**0.94**	**0.95**														
1%d	-0.43	-0.43		-0.42	**0.93**	0.29																	
3%d	0.43		**0.83**	0.42	0.29	**0.65**	**0.67**	**0.68**	**0.84**	**0.82**	0.39												
6%d	*0.59*		**0.87**	0.48		0.46	**0.69**	**0.66**	**0.78**	**0.75**		**0.84**											
9%d	*0.52*		**0.83**	0.44		*0.52*	**0.62**	**0.60**	**0.74**	**0.72**	0.32	**0.86**	**0.96**										
13%d	*0.59*		**0.92**	*0.49*		0.46	**0.70**	**0.64**	**0.79**	**0.76**		**0.85**	**0.98**	**0.94**									
%d			**0.61**		**0.69**	*0.51*	0.37	0.44	**0.64**	**0.65**	**0.80**	**0.83**	**0.76**	**0.80**	**0.76**								
1msv	*0.55*	**0.62**	0.37	*0.52*		0.42	0.45		0.35	0.34			0.30	0.29									
3msv	-0.48	-0.42		**-0.65**		**-0.66**	**-0.60**	*-0.51*	-0.38	*-0.51*													
6msv	**-0.69**		**-0.86**	**-0.63**		-0.44	**-0.82**	**-0.82**	**-0.82**	**-0.80**		**-0.73**	**-0.79**	**-0.67**	**-0.78**	-0.46		0.43					
9msv	0.44	0.30		0.34	**-0.66**						**-0.66**					-0.41							
13msv	-0.47		**-0.84**			-0.40	**-0.70**	**-0.79**	**-0.88**	**-0.80**		**-0.77**	**-0.73**	**-0.63**	**-0.76**	*-0.56*			**0.89**				
msv			*-0.58*			**-0.64**	**-0.64**	**-0.76**	**-0.74**	**-0.79**		**-0.70**	*-0.56*	*-0.52*	*-0.56*	*-0.56*		*0.56*	**0.68**	0.38	**0.68**		
1%v	*0.55*	*0.59*	0.37	*0.53*		0.45	*0.51*		0.37	0.37			0.33	0.31	0.31		**0.95**						
3%v	-0.48	-0.43		**-0.65**		**-0.61**	**-0.60**	-0.46		-0.47								**0.97**	0.39			*0.50*	
6%v	**-0.69**		**-0.85**	**-0.60**		-0.42	**-0.82**	**-0.83**	**-0.82**	**-0.80**		**-0.71**	**-0.82**	**-0.70**	**-0.81**	-0.47		0.38	**0.98**		**0.87**	**0.64**	
9%v	0.40	0.30		0.33	**-0.72**						**-0.71**					*-0.51*				**0.95**		0.39	
13%v	*-0.52*		**-0.86**	-0.42		-0.41	**-0.73**	**-0.81**	**-0.89**	**-0.81**		**-0.78**	**-0.76**	**-0.67**	**-0.79**	*-0.56*			**0.90**		**0.99**	**0.68**	
%v	-0.43		**-0.73**	-0.42		*-0.52*	**-0.74**	**-0.85**	**-0.83**	**-0.84**		**-0.78**	**-0.69**	**-0.61**	**-0.70**	*-0.56*		*0.49*	**0.84**		**0.86**	**0.86**	

	3%v	6%v	9%v	13%v	%v
3%v					
6%v	0.33				
9%v					
13%v		**0.90**			
%v	0.46	**0.85**		**0.86**	

Appendix B

Additional information from Chapter 4

Table B.1. Summary of markers showing significant (*$P < 0.05$) segregation distortion for each parental genotype. Where *Chr* (chromosome); *a* (Saladin allele); *b* (Iceberg allele); *h* (heterozygote); *u* (unkown); *P* (probability).
Table B.1. Where df=2

Chr	Locus	Marker	a	h	b	u	X^2 value	P value
1	ANWE-OP4	OPA	0	67	58	0	2228.05	***$P < 0.001$
1	BKOL-OP3	OPA	60	8	57	0	19.09	***$P < 0.001$
1	BUPG-OP4	OPA	0	90	28	7	4288.72	***$P < 0.001$
1	CLRY2600-OP3	OPA	62	8	55	0	19.42	***$P < 0.001$
1	AQXR-OP3	OPA	62	6	57	0	8.72	*$P < 0.05$
1	BFUZ-OP4	OPA	62	6	55	2	9.2	*$P < 0.05$
2	BEFF-OP4	OPA	24	101	0	0	5107.27	***$P < 0.001$
2	BJLL-OP4	OPA	39	7	48	31	22.04	***$P < 0.001$
2	E41M49_238s	AFLP	17	0	69	39	33.31	***$P < 0.001$
2	E41M49_240s	AFLP	21	0	65	39	24.23	***$P < 0.001$
2	E41M59_277i	AFLP	25	0	65	35	19.49	***$P < 0.001$
2	E41M59_278i	AFLP	25	0	65	35	19.49	***$P < 0.001$
2	BKUA-OP4	OPA	64	7	54	0	14.06	***$P < 0.001$
2	ATHI-OP3-1	OPA	60	7	58	0	13.28	**$P < 0.01$
2	CLXS3_851-OP3	OPA	58	7	58	2	13.63	**$P < 0.01$
2	BDAY-OP4	OPA	64	6	54	1	9.47	**$P < 0.01$
2	BHOQ-OP4	OPA	64	6	53	2	9.79	**$P < 0.01$
2	BHWC-OP4	OPA	64	6	52	3	10.13	**$P < 0.01$
2	E35M60_88s	AFLP	32	0	60	33	10.12	**$P < 0.01$
2	E41M59_273s	AFLP	58	0	30	37	10.45	**$P < 0.01$
2	RZ-J	EST	53	6	61	5	9.76	**$P < 0.01$
2	AVZX-OP4	OPA	60	6	57	2	8.87	*$P < 0.05$
2	BIAS-OP4	OPA	45	1	23	56	7.13	*$P < 0.05$
2	E35M47_70s	AFLP	35	0	58	32	7.25	*$P < 0.05$
2	E38M49_89s	AFLP	33	0	57	35	7.93	*$P < 0.05$
2	E41M59_268s	AFLP	55	0	34	36	6.45	*$P < 0.05$
3	AVSI-OP3	OPA	65	6	54	0	9.5	**$P < 0.01$
3	E41M59_99s	AFLP	31	0	52	42	6.72	*$P < 0.05$
4	AKHB-OP4	OPA	3	0	59	63	52.37	***$P < 0.001$
4	BFYG-OP3	OPA	33	2	90	0	26.41	***$P < 0.001$
4	BHYM-OP4	OPA	50	71	0	4	2587.29	***$P < 0.001$
4	BIDO-OP4-2	OPA	0	61	63	1	1861.55	***$P < 0.001$
4	BLRO-OP4	OPA	0	76	49	0	2871.34	***$P < 0.001$
4	BVVP-OP4	OPA	0	60	63	2	1815.73	***$P < 0.001$
4	E45M60_119i	AFLP	67	0	20	38	27.17	***$P < 0.001$
4	LE9003s	COS	26	0	68	31	20.56	***$P < 0.001$
4	RZ-X	EST	32	1	87	5	26.02	***$P < 0.001$
4	CLXS3_3835-OP3-1	OPA	83	1	41	0	14.81	***$P < 0.001$
4	CLXS3_3835-OP3-2	OPA	83	1	41	0	14.81	***$P < 0.001$
4	QGCA_6151_2-OP3-1	OPA	83	2	40	0	15.03	***$P < 0.001$
4	QGCA_6151_2-OP3-2	OPA	83	2	40	0	15.03	***$P < 0.001$
4	AVPF-OP3	OPA	42	4	79	0	13.31	**$P < 0.01$
4	E37M61_83s	AFLP	31	0	61	33	11.4	**$P < 0.01$
4	E41M49_133s	AFLP	57	0	28	40	11.4	**$P < 0.01$
4	E41M49_133s	AFLP	57	0	28	40	11.4	**$P < 0.01$
4	RZ-M	EST	72	5	43	5	12.41	**$P < 0.01$
4	E44M49_140i	AFLP	61	0	33	31	9.96	**P < 0.01
4	E35M60_383s	AFLP	58	0	34	33	7.82	*P < 0.05
4	E41M62_353s	AFLP	57	0	35	33	6.8	*P < 0.05
4	E45M49_280s	AFLP	56	0	33	36	7.45	*$P < 0.05$
4	RZ-D	EST	45	2	72	6	6.23	*$P < 0.05$
5	BFXY-OP4	OPA	63	47	0	15	1248.55	***$P < 0.001$

Table B.1. continued.

Chr	Locus	Marker	a	h	b	u	X^2 value	P value
5	E35M49_145i	AFLP	26	0	66	33	19.13	***$P < 0.001$
5	BCPA-OP3	OPA	60	7	55	3	14.04	***$P < 0.001$
5	E35M62_308s	AFLP	62	0	31	32	11.97	**$P < 0.01$
5	ASAP-OP4	OPA	77	0	48	0	8.82	*$P < 0.05$
5	AWEQ-OP4	OPA	74	0	48	3	7.57	*$P < 0.05$
5	BAXS-OP4	OPA	77	0	48	0	8.82	*$P < 0.05$
5	BDDO-OP4	OPA	75	0	49	1	7.51	*$P < 0.05$
5	BHQP-OP3	OPA	52	5	68	0	6.91	*$P < 0.05$
5	BRUO-OP4	OPA	74	0	49	2	7.11	*$P < 0.05$
5	E35M60_261s	AFLP	56	0	34	35	6.89	*$P < 0.05$
5	QGA18I02-OP4	OPA	75	0	49	1	7.51	*$P < 0.05$
7	AWMX-OP4	OPA	0	18	69	38	262.53	***$P < 0.001$
7	BETN-OP4	OPA	0	87	38	0	3773.8	***$P < 0.001$
7	BFQX-OP4	OPA	4	0	57	64	47.75	***$P < 0.001$
7	QGD14K20-OP4	OPA	66	59	0	0	1728.07	***$P < 0.001$
7	E33M59_205s	AFLP	26	0	62	37	16.36	***$P < 0.001$
7	E33M59_204s	AFLP	27	0	61	37	14.74	***$P < 0.001$
7	E38M54_76s	AFLP	30	0	59	36	11.01	**$P < 0.01$
7	QGE10B18-OP3-1	OPA	60	7	57	1	13.51	**$P < 0.01$
7	E44M59_205s	AFLP	31	0	59	35	10.28	**$P < 0.01$
7	w	Gene	47	0	78	0	9.79	**$P < 0.01$
7	AIQW-OP3	OPA	59	6	60	0	8.53	*$P < 0.05$
7	E33M48_300i	AFLP	33	0	56	36	7.45	*$P < 0.05$
7	E35M59_187i	AFLP	34	0	58	33	7.82	*$P < 0.05$
7	E35M61_325i	AFLP	36	0	58	31	6.72	*$P < 0.05$
7	E44M54_105s	AFLP	35	0	57	33	6.8	*$P < 0.05$
7	E44M59_204s	AFLP	32	0	57	36	8.55	*$P < 0.05$
7	E45M59_151i	AFLP	34	0	57	34	7.35	*$P < 0.05$
8	BWSH-OP4	OPA	0	62	61	2	1938.59	***$P < 0.001$
8	E35M47_114s	AFLP	23	0	70	32	25.61	***$P < 0.001$
8	E35M47_298i	AFLP	16	0	77	32	42.12	***$P < 0.001$
8	E35M48_183i	AFLP	24	0	70	31	24.36	***$P < 0.001$
8	E35M48_359s	AFLP	25	0	69	31	22.41	***$P < 0.001$
8	E35M59_183i	AFLP	21	0	71	33	29.07	***$P < 0.001$
8	E35M59_332i	AFLP	74	0	18	33	36.09	***$P < 0.001$
8	E38M49_213i	AFLP	21	0	66	38	25.03	***$P < 0.001$
8	E38M54_208s	AFLP	14	0	75	36	43.89	***$P < 0.001$
8	E38M54_209i	AFLP	13	0	75	37	45.77	***$P < 0.001$
8	E44M54_449i	AFLP	18	0	74	33	36.09	***$P < 0.001$
8	E45M47_279s	AFLP	25	0	67	33	20.94	***$P < 0.001$
8	E45M59_160i	AFLP	65	0	26	34	18.42	***$P < 0.001$
8	LE9041s	COS	12	0	80	33	52.52	***$P < 0.001$
8	E35M62_220i	AFLP	28	0	65	32	16.43	***$P < 0.001$
8	E45M48_301i	AFLP	27	0	66	32	18.09	***$P < 0.001$
8	E45M47_259s	AFLP	29	0	63	33	14.22	***$P < 0.001$
8	E45M50_219i	AFLP	41	0	80	4	14.69	***$P < 0.001$
8	E35M49_267s	AFLP	61	0	31	33	11.4	**$P < 0.01$
8	E41M59_237s	AFLP	59	0	29	37	11.79	**$P < 0.01$
8	E41M59_238s	AFLP	60	0	29	36	12.38	**$P < 0.01$
8	E33M60_150s	AFLP	58	0	31	36	9.73	**$P < 0.01$
8	E38M54_258s	AFLP	31	0	58	36	9.73	**$P < 0.01$
8	AZQR-OP3	OPA	59	6	60	0	8.53	*$P < 0.05$
9	AHTV-OP4	OPA	0	52	72	1	1356.55	***$P < 0.001$
9	BEFF-OP4	OPA	24	101	0	0	5107.27	***$P < 0.001$
9	BHWB-OP4	OPA	0	61	64	0	1846.73	***$P < 0.001$
9	BJJK-OP4	OPA	46	6	73	0	14.44	***$P < 0.001$
8	AZQR-OP3	OPA	59	6	60	0	8.53	*$P < 0.05$
8	E33M60_149s	AFLP	57	0	32	36	8.55	*$P < 0.05$
8	E35M49_91s	AFLP	34	0	58	33	7.82	*$P < 0.05$
8	E41M59_134s	AFLP	33	0	57	35	7.93	*$P < 0.05$
9	AHLI-OP4	OPA	47	6	71	1	13.37	**$P < 0.01$
9	BIAE-OP4	OPA	46	6	67	6	13.13	**$P < 0.01$
9	ATNJ-OP4	OPA	47	5	73	0	10.32	**$P < 0.01$
9	BEMX-OP4	OPA	48	4	73	0	7.26	*$P < 0.05$

Table B.1. continued.

Chr	Locus	Marker	a	h	b	u	X^2 value	P value
9	E37M61_144i	AFLP	35	0	57	33	6.8	$*P < 0.05$

Table B.2. Map alignment of the F_7 Sal x Ice map and the MCB19 10NR map. Where *LG* (linkage group); *cM* (centimorgan).

Marker order	LG	Marker	Map position (cM)	Chromosome position on MCB19 10NR map (bin)
1	1a	ANWE-OP4	0	917
2	1a	AQXR-OP3	13.246	942
3	1a	BKOL-OP3	13.322	947
14	1b	BFOH-OP4	1.128	1590
16	1b	BBCU-OP4	1.61	1630
19	1b	BVDO-OP4	2.068	1632
20	1b	AYRY-OP4	2.331	1682
21	1b	AVKT-OP4	2.332	1633
22	1b	AZUN-OP4	2.332	2320
23	1b	AYUO-OP4	2.332	1669
53	2	ANAQ-OP3	28.035	889
57	2	AAAH-OP3	32.331	810
58	2	AOGA-OP3	32.689	778
59	2	AOXR-OP3	33.679	862
66	2	BGPD-OP4	64.513	1864
67	2	BHXM-OP3	69.295	357
68	2	BJLL-OP4	71.496	2461
72	2	ATHI-OP3-1	80.138	2314
75	2	BDKV-OP4	97.064	2385
79	2	BJZY-OP4	98.577	2388
84	2	BKHE-OP4	102.151	2403
85	2	BKUA-OP4	102.592	2394
89	2	BHWC-OP4	109.271	2485
90	2	BHOQ-OP4	109.393	2483
92	2	BVZJ-OP4	146.635	964
93	2	AKGO-OP4	148.603	954
94	2	BIAS-OP4	150.588	913
108	3a	CBXN-OP3	0	485
109	3a	BFDT2-OP3	0	601
110	3a	AVSI-OP3	12.467	648
118	3b	ANKW-OP3-1	28.326	725
123	4a	BQOS-OP4	8.446	394
124	4a	BDPH-OP4	8.446	418
126	4a	BIKK-OP4	8.789	421
132	4a	BVZM-OP4	10.467	423
134	4a	AKVV-OP4	10.617	427
135	4a	BEEF-OP4	10.617	440
136	4a	BTJR-OP4	11.425	461
137	4a	BEUC-OP4	12.285	563
143	4a	BDWF-OP4	14.06	418
144	4a	AVPF-OP3	18.881	762
150	4a	BHYM-OP4	52.721	720
152	4a	BIDO-OP4-2	54.554	2471
153	4a	BLRO-OP4	58.532	369
155	4a	AIUK-OP3	70.985	451
157	4a	BSCC-OP3-1	79.909	1175

Table B.2. continued.

Marker order	LG	Marker	Map position (cM)	Chromosome position on MCB19 10NR map (bin)
158	4a	RZ-I	83.066	1199
162	4b	BCMZ-OP4	1.528	1314
163	4b	AQQD-OP4	1.528	1199
166	4b	AVZB-OP4	16.39	1314
171	4b	BDLF-OP3-1	48.091	1833
172	4b	AQOZ-OP3	50.792	1488
175	4b	AWHP-OP3	54.519	1498
178	4b	BJBO-OP3	57.656	1538
187	4b	AKWZ-OP3	70.057	1638
213	5a	BCEL-OP3-1	52.212	127
214	5a	BHQP-OP3	64.289	39
223	5a	BEIH-OP4	87.696	96
225	5a	BKSN-OP4	96.14	130
226	5a	BEBN-OP4	115.249	245
227	5a	BKMO-OP4	115.249	244
228	5a	ATRK-OP4	115.255	250
231	5b	BCUM-OP3	12.319	1603
232	5b	CAMY-OP3	14.358	1612
233	5b	BFXY-OP4	25.138	1918
234	5b	BAMG-OP3-1	39.592	1955
243	5c	BCPA-OP3	21.942	2812
244	5c	AFPZ-OP3	29.197	2841
247	6a	BUZC-OP4	0	258
248	6a	AWTX-OP4	4.623	338
251	6b	BTNO-OP3	9.414	740
252	6b	ATSV-OP3	16.847	832
253	6b	BCDA-OP3-2	21.726	918
254	6c	BBQZ-OP4	0	938
258	6c	AXJF-OP4	0.825	899
259	6c	BCDA-OP3-1	0.827	918
263	6c	BGBG-OP4	2.195	909
264	6c	BTJM-OP4	2.938	940
265	6c	BJQT-OP4	3.299	943
266	6d	BLCL-OP3	0	1454
286	7	BUAF-OP1	8.488	388
288	7	AXAS-OP4	8.859	394
291	7	BAIJ-OP4	8.868	411
292	7	BAAZ-OP4	8.868	412
295	7	BEYE-OP4	11.958	478
297	7	AWMX-OP4	13	444
299	7	BHVY-OP4	15.622	533
300	7	BJBG-OP4	15.715	511
301	7	BHRR-OP4	15.806	521
302	7	BEZB-OP4	16.131	529
303	7	BICL-OP2	16.131	531
304	7	BLCN-OP4	16.131	517
305	7	AVRC-OP4	16.296	518
315	7	AOUA-OP4-2	23.7	645
316	7	BATO-OP4-2	23.719	647
319	7	E38M49_116s	25.229	677

Table B.2. continued.

Marker order	LG	Marker	Map position (cM)	Chromosome position on MCB19 10NR map (bin)
320	7	AYHP-OP3-2	25.328	649
327	7	BKBM2-OP3	33.901	636
332	7	BXNV-OP3-1	38.707	971
343	7	BTVQ-OP4	57.823	1023
344	7	AIQW-OP3	58.56	905
348	7	AVHI-OP4	61.677	1071
349	7	ALCE-OP4	62.055	1070
351	7	ATPH-OP4	68.982	1111
352	7	BIVH-OP3-1	79.174	1131
378	8	BWSH-OP4	60.608	318
384	8	AXXF-OP3	72.686	281
393	8	AMZR-OP4	78.603	273
404	8	BEAO-OP4	97.721	559
406	8	AHOJ-OP4	100.57	591
409	9a	BFSF-OP4	0	231
410	9a	AZOT-OP4	0.119	223
411	9a	AVSN-OP4	1.027	231
412	9a	BEMX-OP4	22.209	458
413	9a	ATNJ-OP4	23.091	474
415	9a	AHLI-OP4	23.919	483
416	9a	BIAE-OP4	23.934	484
417	9a	AHTV-OP4	35.447	466
419	9a	BHWB-OP4	50.872	217
421	9b	AQYG-OP3	3.495	1011

Table B.3. Common markers (+/- 5bp) between the Saladin x Iceberg genetic map and the lettuce map constructed by Syed *et al.,* (2006). Where *LG* (linkage group); *cM* (centimorgan).

Marker from Syed *et al.,* (2006).	LG present on from Syed *et al.,* (2006).	Marker from Saladin x Iceberg F7 map	LG present on from Saladin x Iceberg F7 map
E35/M59-F-190-SER	1	E35M59_187i	7
E45/M48-F-070-SAT	1	E45M48_47s	2
E45/M48-F-297-SAT	1	E45M48_301i	8
E35/M48-F-293-SER	2	E35M48_288i	8
E35/M48-F-364-SAT	3	E35M48_371i	5a
E35/M49-F0096-SAT	3	E35M49_100i	5b
E35/M59-F-187-SER	3	E35M59_187i	7
E35/M59-F-220-SAT	4	E35M59_225s	2
E35/M49-F-268-SAT	4	E35M49_267s	8
E44/M48-F-223-SER	5	E44M48_266s	2
E35/M59-F-361-SER	5	E35M59_359s	8
E45/M48-F-075-SER	5	E45M48_74s	2
E45/M48-F-365-SAT	5	E45M48_361s	4b
E35/M48-F-376-SAT	5	E35M48_371i	5a
E35/M48-F-288-SER	5	E35M48_288i	8
E35/M59-F-182-SAT	5	E35M59_183i	8
E35/M48-F-204-SAT	7	E35M48_206i	2
E35/M48-F-188-SAT	7	E35M48_187i	7
E35/M49-F-146-SER	7	E35M49_145i	5a
E35/M48-F-290-SAT	8	E35M48_288i	8
E35/M59-F-183-SAT	9	E35M59_183i	8
E45/M48-F-079-SER	10	E45M48_74s	2

Table B.4. Common markers (+/- 5bp) between the Saladin x Iceberg genetic map and the lettuce map constructed by Jeuken *et al.,* (2001). Where *LG* (linkage group); *cM* (centimorgan).

Marker from Jeuken *et al.,* (2001)	LG present on from Jeuken *et al.,* (2001)	Marker from Saladin x Iceberg F7 map	LG present on from Saladin x Iceberg F7 map
E35M48-288sal	1	E35M48_288i	8
E38M54-189!	1	E38M54_192s	2
E35M48-206	1	E35M48_206i	2
E38M54-123	1	E38M54_122i	7
E35M59-354	2	E35M59_359s	8
E45M49-128	2	E45M49_129s	4b
E35M59-328	2	E35M59_332i	8
E44M49-144sal	2	E44M49_140i	4b
E45M48-72sal	3	E45M48_74s	2
E44M49-136	3	E44M49_140i	4b
E38M54-149!	3	E38M54_152i	7
E35M60-180	3	E35M60_178i	4b
E35M48-182	3	E35M48_183i	8
E35M60-85	4	E35M60_88s	2
E35M60-84	4	E35M60_88s	2
E45M49-92sal	4	E45M49_93s	4a
E45M49-282	4	E45M49_280s	4b
E38M54-157sal	4	E38M54_152i	7
E45M49-276salA	4	E45M49_280s	4b
E45M49-97sal	4	E45M49_93s	4a
E35M60-259	4	E35M60_261s	5c
E45M49-278salB	4	E45M49_280s	4b
E35M49-143	4	E35M49_145i	5a
E35M60-90sal	5	E35M60_88s	2
E44M49-145sal	5	E44M49_140i	4b
E35M48-374salA	5	E35M48_371i	5a
E35M48-376	5	E35M48_371i	5a
E38M54-253sal	5	E38M54_256s	7
E35M59-333sal	5	E35M59_332i	8
E35M49-89!	5	E35M49_87i	8
E45M48-70sal	6	E45M48_74s	2
E38M54-269satA	6	E38M54_266s	8
E35M48-354sal	6	E35M48_359s	8
E35M60-288sal	6	E35M60_290s	2
E35M49-87sal	6	E35M49_87i	8
E35M49-84	6	E35M49_87i	8
E35M48-187sal	6	E35M48_187i	7
E35M59-369sal	6	E35M59_371i	5a
E35M60-92	6	E35M60_88s	2
E45M48-300	6	E45M48_301i	8
E35M49-140	7	E35M49_145i	5a
E35M49-91!	7	E35M49_91s	8
E38M54-187salB	8	E38M54_192s	2
E38M54-208	8	E38M54_208s	8
E35M49-96!	8	E35M49_91s	8
E38M54-258	8	E38M54_258s	8
E38M54-210	8	E38M54_209i	8
E35M48-364	8	E35M48_359s	8
E35M49-142	8	E35M49_145i	5a
E45M48-301sal	8	E45M48_301i	8
E45M48-77sal	9	E45M48_74s	2
E35M59-361sal	9	E35M59_359s	8
E35M48-192	9	E35M48_187i	7
E38M54-127	9	E38M54_122i	7
E38M54-127	9	E38M54_124s	7
E35M48-371	9	E35M48_371i	5a
E35M59-227sal	9	E35M59_225s	2

Appendix C

Additional information from Chapter 5

Field trial plan and randomisation for UK site for selected F_7 Saladin x Iceberg mapping population

Plants were transplanted to the field into 8 x 1.83 m wide beds with a plot length of 1.05 and spacing distance of 35 cm (see figure C.1.).

Guard	Guard	Guard	Guard		Guard	Guard	Guard	Guard	
24	48	72	96		24	48	72	96	
23	47	71	95		23	47	71	95	
22	46	70	94		22	46	70	94	
21	45	69	93		21	45	69	93	
20	44	68	92		20	44	68	92	
19	43	67	91		19	43	67	91	
18	42	66	90		18	42	66	90	
'	'	'	'		'	'	'	'	27.3 m long
10	34	58	82		10	34	58	82	
9	33	57	81		9	33	57	81	
8	32	56	80		8	32	56	80	
7	31	55	79		7	31	55	79	
6	30	54	78		6	30	54	78	
5	29	53	77		5	29	53	77	
4	28	52	76		4	28	52	76	
3	27	51	75		3	27	51	75	
2	26	50	74		2	26	50	74	
1	25	49	73		1	25	49	73	
Guard	Guard	Guard	Guard		Guard	Guard	Guard	Guard	Baseline
Bed 1	Bed 2	Bed 3	Bed 4		Bed 5	Bed 6	Bed 7	Bed 8	
7.32 m				5.49 m Tractor path	7.32 m				
Rep 1					Rep 2				

Overall width 20.3 m and overall area 549.6 m^2

Figure C.1. Field trial plan for UK site.

Beds were marked using a tractor with a wheel base of 1.83 m and plots were then individually marked using a ‘Wolf Garten’ row marker. A plot configuration of 12 plants per plot and rep (4 rows x 3 plant arrangement) of the same accession was

used, creating a formation of two central heads surrounded by guard plants. The plant material was randomised within a rep by a column/row design (see figure C.2.).

rep 1

block \ plot	1	2	3	4	5	6
1	12	90	58	8	24	67
2	39	71	62	19	18	23
3	84	78	61	56	60	73
4	6	87	70	64	15	96
5	92	14	81	21	29	49
6	83	68	26	57	31	89
7	74	2	38	86	3	34
8	51	36	55	10	20	77
9	44	94	85	80	45	27
10	22	75	32	48	40	88
11	5	11	25	4	13	9
12	37	95	33	35	91	54
13	76	16	53	43	17	30
14	69	65	66	63	41	1
15	72	79	46	59	7	42
16	47	52	93	82	28	50

rep 2

block \ plot	1	2	3	4	5	6
1	28	5	66	72	22	17
2	76	55	69	21	37	78
3	93	20	24	96	56	25
4	44	81	53	4	2	70
5	91	67	75	7	51	16
6	92	88	95	38	73	64
7	94	65	87	10	32	19
8	54	68	52	6	30	18
9	29	11	23	59	36	31
10	46	62	33	74	85	13
11	12	40	86	41	26	9
12	27	48	60	71	83	43
13	45	50	57	35	8	49
14	58	84	42	63	80	15
1	39	90	1	34	14	47
16	82	79	89	77	3	61

Figure C.2. Randomised column/row design for field plan for UK site.

Field trial plan and randomisation for NL site for selected F_7 Saladin x Iceberg mapping population

Plants were transplanted to the field into 2 x 1.5 m wide beds with a plot length of 1.09 and spacing distance of 36 cm (see figure C.3.a.). Beds and plots were marked according to Rijk Zwaan procedures. The plot configuration and field plan design type used were as the UK site (see figure C.3.b.).

Plot number 1-96

Rep 1	Rep 2	
Guard	Guard	
1	96	
2	95	
3	94	
4	93	
5	92	
6	91	
7	90	
8	89	
9	88	105.8 m long
10	87	
11	86	
'	'	
86	11	
87	10	
88	9	
89	8	
90	7	
91	6	
92	5	
93	4	
94	3	
95	2	
96	1	
Guard	Guard	Baseline
Bed 1	Bed 2	

3 m wide
Overall area 317.4 m^2

a

Rep 1	Rep 1 cont	Rep 2	Rep 2 cont
69	44	31	89
74	73	18	76
56	52	83	56
10	62	34	8
93	59	96	86
61	1	95	24
85	30	84	92
65	63	58	48
31	92	63	35
76	66	42	72
4	88	29	23
51	33	17	79
47	7	44	81
5	34	68	71
43	2	69	90
16	36	94	67
89	68	22	27
58	25	37	45
54	86	88	43
75	81	21	80
53	49	46	54
8	84	74	87
22	79	9	52
39	17	82	15
40	13	25	40
29	90	13	12
23	78	55	38
38	57	19	4
72	35	6	77
94	9	47	73
55	14	10	57
95	45	41	60
24	3	49	26
26	37	70	61
64	18	93	16
11	27	7	53
80	28	30	33
32	15	75	3
41	91	14	64
82	70	78	62
42	67	20	51
46	87	85	91
48	60	32	39
77	50	11	5
21	6	50	65
83	71	36	1
19	96	59	66
12	20	28	2

b

Figure C.3. **Field trial plan for NL site (a) and randomised column/row design for field plan for NL site (b).** Where *cont* is rep continued.

Field trial plan and randomisation for extreme discolouration RIL set

Plants were transplanted to the field into 4 x 1.83 m wide beds with a plot length of 1.05 and spacing distance of 35 cm (see figure C.4.a.). Beds were marked using a tractor with a wheel base of 1.83 m and plots were then individually marked using a 'Wolf Garten' row marker. The plot configuration and field plan design type used were as the UK site (see figure C.4.b.).

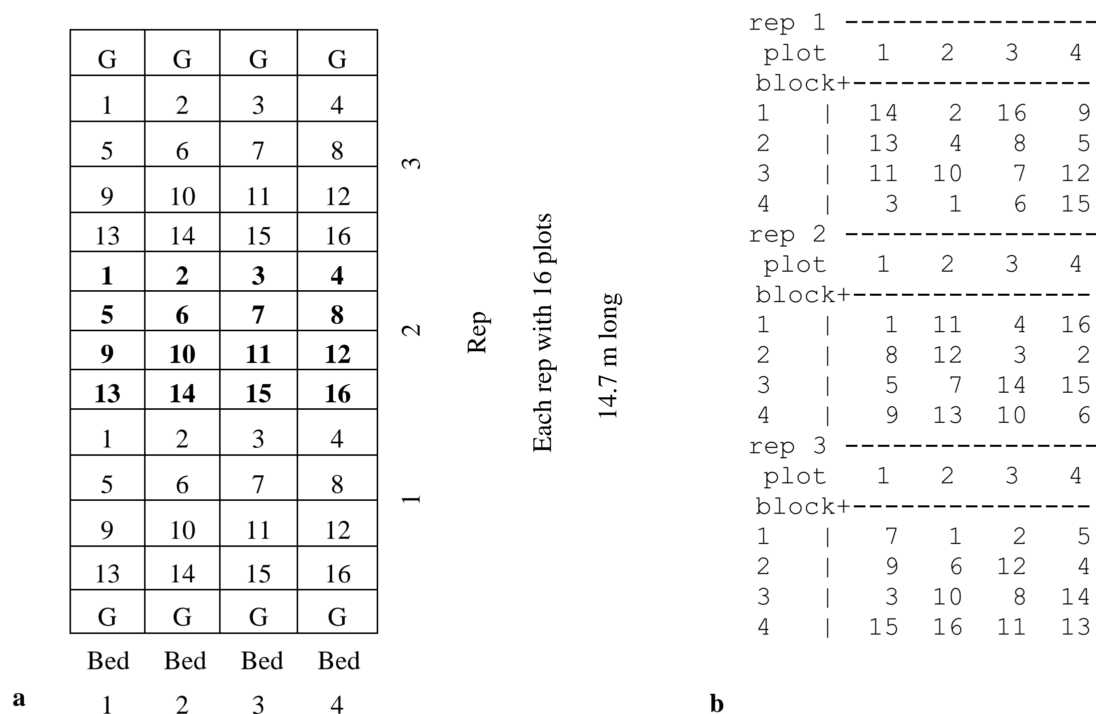

Figure C.4. Field trial plan for extreme discolouration RIL set (a) and Randomised column/row design for field plan (b). Where *G* (guard plant; either Saladin or Iceberg).

REML analysis output across days data for the selected RILs.

Table C.1. REML analysis of discolouration across days within the selected RIL mapping population. Where *df* (degrees of freedom); *chi* (chi squared probability); *Line* (genotype/RIL).

Site	Discolouration measure	Fixed term	Wald statistic	df	Wald/df	chi pr
UK	Browning intensity (msb)	Line	79.27	95	0.83	0.877
		Day	1585.54	1	1585.54	***<0.001
		Line x Day	168.14	95	1.77	***<0.001
	Pinking intensity (msp)	Line	154.81	95	1.63	***<0.001
		Day	4715.69	1	4715.69	***<0.001
		Line x Day	148.87	95	1.57	***<0.001
	Overall discolouration intensity (msd)	Line	94.41	95	0.99	0.498
		Day	5564.58	1	5564.58	***<0.001
		Line x Day	186.66	95	1.96	***<0.001
	Extent of browning (%b)	Line	73.35	95	0.77	0.952
		Day	1346.53	1	1346.53	***<0.001
		Line x Day	153.92	95	1.62	***<0.001
	Extent of pinking (%p)	Line	183.28	95	1.93	***<0.001
		Day	4916.7	1	4916.7	***<0.001
		Line x Day	153.63	95	1.62	***<0.001
	Extent of overall discolouration (%d)	Line	111.57	95	1.17	0.118
		Day	4403.34	1	4403.34	***<0.001
		Line x Day	211.83	95	2.23	***<0.001
NL	Browning intensity (msb)	Line	139.06	95	1.46	**0.002
		Day	356.74	1	356.74	***<0.001
		Line x Day	154.23	95	1.62	***<0.001
	Pinking intensity (msp)	Line	218.32	95	2.3	***<0.001
		Day	5148.7	1	5148.7	***<0.001
		Line x Day	309.82	95	3.26	***<0.001
	Overall discolouration intensity (msd)	Line	174.55	95	1.84	***<0.001
		Day	4863.58	1	4863.58	***<0.001
		Line x Day	293.73	95	3.09	***<0.001
	Extent of browning (%b)	Line	139.74	95	1.47	**0.002
		Day	285.66	1	285.66	***<0.001
		Line x Day	165.42	95	1.74	***<0.001
	Extent of pinking (%p)	Line	232.91	95	2.45	***<0.001
		Day	4266.27	1	4266.27	***<0.001
		Line x Day	407.52	95	4.29	***<0.001
	Extent of overall discolouration (%d)	Line	237.73	95	2.5	***<0.001
		Day	4411.12	1	4411.12	***<0.001
		Line x Day	353.46	95	3.72	***<0.001

Quantitative trait loci from interval mapping

Table C.2. QTL impacting on post harvest discolouration of lettuce tissue from the Saladin x Iceberg RIL population grown in 2 sites: UK and NL. Additive effect equals half the difference between homozygous alleles at the QTL: positive number indicates an additive allelic effect of Saladin; negative number indicates a negative allelic effect of Saladin. Confidence interval was based on a 2 LOD support interval with a significant LOD value of 1.95. Where *UK* (United Kingdom); *NL* (The Netherlands), *LG* (linkage group from F_7 Saladin x Iceberg linkage map (see Chapter 4)); *LOD* (logarithm of odds), *cM* (centimorgans); *msb* (browning intensity); *%b* (extent of browning); *msp* (pinking intensity); *%p* (extent of pinking); *msd* (overall discolouration intensity); *%d* (extent of overall discolouration). Number before discolouration parameter refers to day.

Trait	LG	Location (cM)	LOD peak	Confidence interval (cM)	Nearest Locus	Additive effect	Genetic variation explained by QTL (%)	Allelic contribution
3msb_UK	4a	25.5	2.25	16.4	RZ-D	2.51	10.6	Saladin
msb_UK	4a	25.5	2.76	11.6	RZ-D	2.07	12.9	Saladin
1%b_UK	4a	25.5	2.29	12.6	RZ-D	2.84	10.9	Saladin
3%b_UK	3a	21.5	2.14	21.5	E35M47_333s	-3.55	12	Iceberg
%b_UK_1	4a	25.5	2.85	11.6	RZ-D	3.01	13.3	Saladin
%b_UK_2	4a	58.5	2.14	8.9	BLRO-OP4	-5.04	9.9	Iceberg
1msp_UK_1	7	2.3	3.62	2.4	BLJI-OP4	3.18	16.3	Saladin
1msp_UK_2	7	2.9	2.48	4.6	BSQZ-OP4	2.65	11.5	Saladin
1msp_UK_3	7	7.8	2.26	3.2	AZTI-OP4	2.53	10.5	Saladin
1msp_UK_4	7	16.5	2.47	3.5	BXJC-OP3	-2.65	11.4	Iceberg
3msp_UK_1	4a	83.1	2.14	13.2	RZ-I	2.61	10.1	Saladin
3msp_UK_2	8	104.2	2.11	3.6	E41M59_238s	-2.8	11	Iceberg
msp_UK	7	2.3	2.09	2.4	BLJI-OP4	2.3	9.7	Saladin
1%p_UK_1	7	2.3	3.79	2.4	BLJI-OP4	4.85	17	Saladin
1%p_UK_2	7	2.9	2.61	4.6	BSQZ-OP4	4.05	12	Saladin
1%p_UK_3	7	7.8	2.51	3.2	AZTI-OP4	3.96	11.6	Saladin
1%p_UK_4	7	16.5	2.72	7.2	BXJC-OP3	-4.14	12.5	Iceberg
1%p_UK_5	7	23.7	2.11	4.7	w	-3.67	9.8	Iceberg
3%p_UK	7	2.3	2.07	2.4	BLJI-OP4	2.92	9.7	Saladin

Table C.2. continued.

Trait	LG	Location (cM)	LOD peak	Confidence interval (cM)	Nearest Locus	Additive effect	Genetic variation explained by QTL (%)	Allelic contribution
%p_UK_1	7	2.3	3.5	2.4	BLJI-OP4	3.88	15.8	Saladin
%p_UK_2	7	2.9	2.25	4.6	BSQZ-OP4	3.14	10.5	Saladin
%p_UK_3	7	4.9	2.22	4.6	AICW-OP4 / AZDK-OP4	3.12	10.3	Saladin
%p_UK_4	7	8.2	2.11	3.2	AQTU-OP4	3.06	9.8	Saladin
%p_UK_5	7	12	1.96	7.2	BEYE-OP4	2.92	9.2	Saladin
%p_UK_6	7	16.5	2.19	7.4	BXJC-OP3	-3.1	10.2	Iceberg
%p_UK_7	7	23.7	2.18	5.9	w	-3.1	10.1	Iceberg
1msd_UK	7	2.3	2.73	2.4	BLJI-OP4	1.87	12.6	Saladin
3msd_UK	4a	83.1	2.33	13.2	RZ-I	1.87	11.1	Saladin
msd_UK	4a	83.1	2.5	13.2	RZ-I	1.69	12	Saladin
1%d_UK	7	2.3	2.51	2.4	BLJI-OP4	3.86	11.6	Saladin
1msb_NL_1	7	0.9	2.55	2.4	ARZI-OP4	-1.56	11.8	Iceberg
1msb_NL_2	7	4.8	2.68	4.6	AVQK-OP4	-1.6	12.3	Iceberg
1msb_NL_3	7	49.4	2.24	15.5	QGE10B18-OP3-1	1.5	10.4	Saladin
3msb_NL	4a	6	2.35	4	BFRY-OP3	-2.57	10.9	Iceberg
1%b_NL_1	7	0.9	2.37	2.4	ARZI-OP4	-1.9	11	Iceberg
1%b_NL_2	7	4.8	2.42	4.6	AVQK-OP4	-1.92	11.2	Iceberg
1%b_NL_3	7	49.4	2.25	16.2	QGE10B18-OP3-1	1.89	10.4	Saladin
3%b_NL	4a	6	2.35	4	BFRY-OP3	-3.08	10.9	Iceberg
1%p_NL	7	21.3	1.96	23.9	E33M59_204s	-4.86	11.5	Iceberg
%p_NL	7	13	2.01	3.4	AWMX-OP4	-4.39	12.7	Iceberg
1msd_NL_1	5a	57.2	2.06	86.8	BCEL-OP3-1 / BHQP-OP3	-1.63	12.6	Iceberg
1msd_NL_2	5a	84.6	2.18	38.1	ASAP-OP4	1.46	10.1	Saladin
1%d_NL	4a	0	1.98	8.4	E37M61_83s	3.8	10.3	Saladin
1msb_1	4a	25.5	2.91	11.6	E45M59_248s / RZ-D	1.36	13.4	Saladin
1msb_2	4a	52.7	2.75	10.3	BHYM-OP4	2.31	12.7	Saladin
1msb_3	4a	58.5	3.01	8.9	BLRO-OP4	-2.68	13.7	Iceberg
3msb	7	21.7	2.08	6.2	E33M59_205s	2.04	13.2	Saladin
msb	4a	25.5	2.1	16.4	RZ-D	1.28	9.8	Saladin
1%b_1	4a	25.5	3.14	11.6	E45M59_248s / RZ-D	1.95	14.4	Saladin

Table C.2. continued.

Trait	LG	Location (cM)	LOD peak	Confidence interval (cM)	Nearest Locus	Additive effect	Genetic variation explained by QTL (%)	Allelic contribution
1%b_2	4a	52.7	2.87	10.3	BHYM-OP4	3.29	13.5	Saladin
1%b_3	4a	58.5	3.28	8.9	BLRO-OP4	-3.84	14.8	Iceberg
%b_1	4a	25.5	2.16	11.6	RZ-D	1.66	10.1	Saladin
%b_2	4a	58.5	2	14.1	BLRO-OP4	-3.1	9.3	Iceberg
1msp_1	7	2.3	3.54	2.4	BLJI-OP4	2.74	16	Saladin
1msp_2	7	4.9	2.96	4.6	AICW-OP4 / AZDK-OP4	2.51	13.5	Saladin
1msp_3	7	7.8	2.43	5	AZTI-OP4	2.28	11.2	Saladin
1msp_4	7	8.9	2.33	3	BWYR-OP4 / AXAS-OP4 / AISE-OP4 / BATK-OP4 / BAIJ-OP4 / BAAZ-OP4	2.23	10.8	Saladin
1msp_5	7	13	2.36	6	AWMX-OP4	-3.28	13.2	Iceberg
1msp_6	7	16.5	2.57	3	BXJC-OP3	-2.35	11.8	Iceberg
1msp_7	7	21.7	2.05	5	E33M59_205s	-2.81	11.6	Iceberg
3msp	1	10.4	2.56	16.3	RZ-H	2.14	11.8	Saladin
msp_1	3b	10.4	2.31	16	RZ-H	1.98	10.8	Saladin
msp_2	7	2.3	2.43	2.4	BLJI-OP4	2.03	11.3	Saladin
msp_3	7	4.9	2.01	4.6	AICW-OP4 / AZDK-OP4	1.85	9.4	Saladin
1%p_1	7	1.75	3.29	2.4	BBPV-OP4 / BBIK-OP4 / AWBE-OP4	4.15	14.9	Saladin
1%p_2	7	4.9	3.05	4.6	AICW-OP4 / AZDK-OP4	4.02	13.9	Saladin
1%p_3	7	7.8	2.71	3	AZTI-OP4	3.78	12.4	Saladin
1%p_4	7	13	2.49	4	AWMX-OP4	-5.31	13.9	Iceberg
1%p_5	7	16.5	2.85	3	BXJC-OP3	-3.9	13	Iceberg
1%p_6	7	21.3	2.23	5	E33M59_205s	-4	12.6	Iceberg
%p_1	7	2.3	3.37	2.4	BLJI-OP4	3.14	15.3	Saladin
%p_2	7	4.9	2.47	4.6	AICW-OP4 / AZDK-OP4	2.7	11.4	Saladin
%p_3	7	8.9	2.07	5	BAIJ-OP4 / BAAZ-OP4	2.45	9.6	Saladin
%p_4	7	13	2.31	4	AWMX-OP4	-3.84	13.2	Iceberg
%p_5	7	16.5	2.18	3	BXJC-OP3	-2.55	10.1	Iceberg
%p_6	7	21.7	2.09	5	E33M59_205s	-2.85	11.8	Iceberg
1msd	7	2.3	2.39	2.4	BLJI-OP4	1.27	11.2	Saladin
1%d_1	7	1.75	2.39	2.4	BBPV-OP4 / BBIK-OP4 / AWBE-OP4	3.03	11.1	Saladin
1%d_2	7	4.9	2.12	4.6	AICW-OP4 / AZDK-OP4	2.87	9.9	Saladin
%d	7	2.3	2.33	2.4	BLJI-OP4	2.06	10.9	Saladin

Correlations amongst traits

All discolouration measures and morphological traits were assessed for potential relationships (correlations between browning, pinking and overall discolouration traits per and across trial sites (see table C.3.).

Table C.3. Correlation matrix for the Saladin x Iceberg lettuce RIL population between post harvest discolouration measurements and morphological parameters within and across sites. Read across then down. Only significant effects shown with ******P* <0.001 in bold, ***P*<0.01 in italics and **P*<0.05 in regular font. Where *UK* (UK trial site); *NL* (NL trial site); *msb* (browning intensity); *msp* (pinking intensity); *msd* (overall discolouration intensity); *%b* (extent of browning); *%p* (extent of pinking); *%d* (extent of overall discolouration). Numerical value before discolouration measurement is day.

	1msp	3msp	msp	1msb	3msb	msb	1msd	3msd	msd	1%p	3%p	%p	1%b	3%b	%b	1%d	3%d	%d	UK 1msp
1msp																			
3msp	**0.682**																		
msp	**0.900**	**0.931**																	
1msb																			
3msb	-0.242			**0.542**															
msb				**0.782**	**0.948**														
1msd	**0.889**	**0.648**	**0.825**	**0.458**															
3msd	0.219		0.209																
msd	0.224		0.213					**1.000**											
1%p	**0.995**	**0.673**	**0.892**		-0.242		**0.882**	0.221	0.225										
3%p	**0.631**	**0.864**	**0.830**				**0.605**			**0.636**									
%p	**0.955**	**0.802**	**0.951**		-0.225		**0.861**	0.206	0.210	**0.960**	**0.825**								
1%b				**0.963**	**0.497**	**0.735**	**0.514**				0.209								
3%b				**0.518**	**0.939**	**0.893**							**0.490**						
%b				**0.820**	**0.864**	**0.952**	*0.317*						**0.820**	**0.901**					
1%d	**0.898**	**0.678**	**0.848**	**0.359**			**0.963**			**0.905**	**0.647**	**0.894**	**0.448**		0.221				
3%d	**0.563**	**0.789**	**0.747**				**0.566**			**0.568**	**0.879**	**0.730**	0.232			**0.606**			
%d	**0.884**	**0.761**	**0.890**	*0.332*			**0.937**			**0.890**	**0.760**	**0.924**	**0.428**		0.230	**0.979**	**0.755**		
UK 1msp	**0.654**	**0.605**	**0.681**	0.251			**0.695**	0.215	0.218	**0.644**	**0.618**	**0.692**	**0.349**		0.205	**0.682**	**0.492**	**0.688**	
UK 3msp				**0.489**	**0.822**	**0.795**							**0.498**	**0.762**	**0.747**		0.246		
UK msp	**0.560**	**0.767**	**0.733**	*0.285*			**0.628**			**0.552**	**0.755**	**0.674**	**0.384**		*0.274*	**0.629**	**0.627**	**0.679**	**0.859**
UK 1msb		*0.297*	0.247	**0.817**	**0.405**	**0.610**	**0.501**				**0.347**	0.221	**0.865**	**0.412**	**0.700**	**0.468**	**0.362**	**0.478**	**0.455**
UK 3msb				**0.489**	**0.822**	**0.795**							**0.498**	**0.762**	**0.747**		0.246		
UK msb				**0.680**	**0.765**	**0.825**	*0.279*						**0.705**	**0.722**	**0.825**	0.223	*0.319*	0.266	0.256
UK 1msd	**0.499**	**0.547**	**0.571**	**0.588**		**0.368**	**0.713**			**0.488**	**0.582**	**0.564**	**0.677**	0.248	**0.498**	**0.687**	**0.508**	**0.696**	**0.886**
UK 3msd	0.218		0.207					**1.000**	**1.000**	0.219									0.214
UK msd	0.220		0.209					**1.000**	**1.000**	0.221		0.206							0.217
UK 1%p	**0.666**	**0.610**	**0.690**	0.239			**0.701**	0.225	0.228	**0.661**	**0.634**	**0.711**	**0.339**			**0.696**	**0.502**	**0.702**	**0.993**
UK 3%p	**0.502**	**0.758**	**0.700**	0.224			**0.548**			**0.506**	**0.866**	**0.680**	*0.322*			**0.578**	**0.713**	**0.660**	**0.646**

Table C.3. continued.

	1msp	3msp	msp	1msb	3msb	msb	1msd	3msd	msd	1%p	3%p	%p	1%b	3%b	%b	1%d	3%d	%d	UK 1msp
UK %p	**0.651**	**0.738**	**0.760**	0.254			**0.694**			**0.651**	**0.806**	**0.765**	**0.363**			**0.707**	**0.651**	**0.750**	**0.925**
UK 1%b		*0.330*	*0.284*	**0.778**	**0.367**	**0.567**	**0.512**				**0.398**	0.264	**0.865**	**0.377**	**0.677**	**0.493**	**0.390**	**0.506**	**0.498**
UK 3%b				**0.452**	**0.730**	**0.712**							**0.468**	**0.770**	**0.739**		*0.317*		
UK %b		0.264		**0.690**	**0.642**	**0.738**	**0.369**				*0.280*		**0.745**	**0.674**	**0.814**	*0.327*	**0.398**	**0.373**	**0.345**
UK 1%d	**0.518**	**0.577**	**0.599**	**0.548**		*0.282*	**0.711**			**0.516**	**0.618**	**0.598**	**0.657**		**0.421**	**0.736**	**0.544**	**0.746**	**0.861**
UK 3%d	**0.395**	**0.626**	**0.565**	*0.279*		0.225	**0.479**			**0.395**	**0.673**	**0.529**	**0.341**	0.222	*0.312*	**0.503**	**0.801**	**0.618**	**0.488**
UK %d	**0.525**	**0.641**	**0.640**	**0.515**		*0.289*	**0.702**			**0.524**	**0.689**	**0.630**	**0.619**		**0.425**	**0.730**	**0.669**	**0.773**	**0.824**
NL 1msp	**0.869**	**0.492**	**0.724**		*-0.280*	*-0.271*	**0.700**			**0.869**	**0.414**	**0.785**		-0.212		**0.716**	**0.404**	**0.693**	0.207
NL 3msp	**0.621**	**0.772**	**0.767**				**0.498**			**0.616**	**0.590**	**0.663**				**0.534**	**0.579**	**0.589**	0.257
NL msp	**0.824**	**0.692**	**0.821**		-0.254	-0.250	**0.660**			**0.821**	**0.552**	**0.800**		-0.215		**0.689**	**0.539**	**0.706**	0.252
NL 1msb		-0.253	-0.249	**0.633**	**0.398**	**0.536**					*-0.314*	-0.259	**0.501**	**0.345**	**0.478**		-0.243		
NL 3msb	-0.254	*-0.285*	*-0.297*	*0.320*	**0.684**	**0.629**				-0.252	*-0.314*	*-0.300*	0.223	**0.636**	**0.528**				
NL msb	-0.265	*-0.309*	*-0.315*	**0.479**	**0.655**	**0.668**				-0.262	**-0.353**	*-0.322*	**0.356**	**0.600**	**0.572**		-0.240		-0.218
NL 1msd	**0.772**	**0.384**	**0.612**				**0.724**			**0.773**	*0.285*	**0.668**				**0.692**	*0.303*	**0.647**	
NL 3msd	**0.362**	**0.470**	**0.458**		*0.306*	*0.267*	**0.372**			**0.359**	*0.295*	**0.367**		*0.273*	0.225	**0.339**	**0.364**	**0.371**	
NL msd	**0.646**	**0.496**	**0.615**				**0.624**			**0.644**		**0.592**				**0.586**	**0.386**	**0.581**	
NL 1%p	**0.860**	**0.481**	**0.713**		-0.264	-0.260	**0.690**			**0.870**	**0.411**	**0.785**				**0.719**	**0.406**	**0.696**	
NL 3%p	**0.498**	**0.578**	**0.596**				**0.377**			**0.503**	**0.674**	**0.613**				**0.414**	**0.658**	**0.513**	0.248
NL %p	**0.854**	**0.558**	**0.757**		-0.262	-0.263	**0.678**			**0.864**	**0.524**	**0.822**				**0.712**	**0.514**	**0.720**	0.231
NL 1%b		-0.212		**0.574**	**0.357**	**0.484**					*-0.282*		**0.496**	*0.322*	**0.460**		-0.215		
NL 3%b		**-0.258**	**-0.248**	**0.273**	**0.612**	**0.558**					**-0.248**	**-0.231**		**0.633**	**0.514**				
NL %b		**-0.276**	**-0.262**	**0.441**	**0.589**	**0.605**					**-0.299**	**-0.252**	**0.355**	**0.589**	**0.564**		**-0.227**		
NL 1%d	**0.816**	**0.437**	**0.665**				**0.724**			**0.827**	**0.348**	**0.731**				**0.752**	**0.359**	**0.711**	
NL 3%d	**0.457**	**0.531**	**0.545**				**0.359**			**0.465**	**0.597**	**0.557**				**0.395**	**0.650**	**0.494**	**0.205**
NL %d	**0.816**	**0.504**	**0.706**				**0.713**			**0.828**	**0.440**	**0.766**				**0.745**	**0.460**	**0.733**	
NL 3%b		**-0.258**	**-0.248**	**0.273**	**0.612**	**0.558**					**-0.248**	**-0.231**		**0.633**	**0.514**				
NL %b		**-0.276**	**-0.262**	**0.441**	**0.589**	**0.605**					**-0.299**	**-0.252**	**0.355**	**0.589**	**0.564**		**-0.227**		
NL 3%b		**-0.258**	**-0.248**	**0.273**	**0.612**	**0.558**					**-0.248**	**-0.231**		**0.633**	**0.514**				
NL %b		**-0.276**	**-0.262**	**0.441**	**0.589**	**0.605**					**-0.299**	**-0.252**	**0.355**	**0.589**	**0.564**		**-0.227**		
NL 1%d	**0.816**	**0.437**	**0.665**				**0.724**			**0.827**	**0.348**	**0.731**				**0.752**	**0.359**	**0.711**	
NL 3%b		**-0.258**	**-0.248**	**0.273**	**0.612**	**0.558**					**-0.248**	**-0.231**		**0.633**	**0.514**				
NL %b		**-0.276**	**-0.262**	**0.441**	**0.589**	**0.605**					**-0.299**	**-0.252**	**0.355**	**0.589**	**0.564**		**-0.227**		
NL 1%d	**0.816**	**0.437**	**0.665**				**0.724**			**0.827**	**0.348**	**0.731**				**0.752**	**0.359**	**0.711**	
NL 3%d	**0.457**	**0.531**	**0.545**				**0.359**			**0.465**	**0.597**	**0.557**				**0.395**	**0.650**	**0.494**	**0.205**
NL %d	**0.816**	**0.504**	**0.706**				**0.713**			**0.828**	**0.440**	**0.766**				**0.745**	**0.460**	**0.733**	

Table C.3. continued.

	UK 3msb	UK msp	UK 1msb	UK 3msb	UK msb	UK 1msd	UK 3msd	UK msd	UK 1%p	UK 3%p	UK %p	UK 1%b	UK 3%b	UK %b	UK 1%d	UK 3%d
UK msp	0.243															
UK 1msb	**0.530**	**0.529**														
UK 3msb	**1.000**	0.243	**0.530**													
UK msb	**0.942**	**0.382**	**0.782**	**0.942**												
UK 1msd	**0.349**	**0.833**	**0.816**	**0.349**	**0.573**											
UK 3msd																
UK msd							**1.000**									
UK 1%p		**0.852**	**0.431**		0.227	**0.869**	0.224	0.227								
UK 3%p		**0.875**	**0.488**		*0.308*	**0.674**			**0.655**							
UK %p		**0.945**	**0.500**		*0.289*	**0.861**			**0.933**	**0.882**						
UK 1%b	**0.527**	**0.559**	**0.978**	**0.527**	**0.772**	**0.832**			**0.478**	**0.523**	**0.546**					
UK 3%b	**0.938**	*0.303*	**0.520**	**0.938**	**0.891**	**0.361**				0.247		**0.521**				
UK %b	**0.857**	**0.481**	**0.836**	**0.857**	**0.957**	**0.659**			*0.319*	**0.427**	**0.404**	**0.848**	**0.893**			
UK 1%d	*0.289*	**0.817**	**0.783**	*0.289*	**0.518**	**0.966**			**0.860**	**0.688**	**0.862**	**0.820**	*0.305*	**0.618**		
UK 3%d	**0.425**	**0.689**	**0.495**	**0.425**	**0.503**	**0.575**			**0.495**	**0.771**	**0.674**	**0.514**	**0.508**	**0.581**	**0.610**	
UK %d	**0.355**	**0.851**	**0.766**	**0.355**	**0.559**	**0.934**			**0.826**	**0.774**	**0.882**	**0.801**	**0.394**	**0.663**	**0.972**	**0.780**
NL 1msp	-0.221			-0.221	-0.211				0.224	0.236	0.250					
NL 3msp		0.240							*0.270*	*0.284*	*0.300*					0.237
NL msp		**0.229**							**0.269**	**0.285**	**0.301**					**0.235**
NL 1msb		**-0.226**								**-0.269**	**-0.250**					
NL 3msb		**-0.257**							**-0.207**	**-0.332**	**-0.289**					**-0.230**
NL msb		**-0.278**							**-0.228**	**-0.349**	**-0.310**					**-0.243**
NL 1msd																
NL 3msd																
NL msd																
NL 1%p	**-0.212**			**-0.212**					**0.217**	**0.236**	**0.246**					
NL 3%p		**0.205**							**0.264**	**0.237**	**0.278**					
NL %p	**-0.214**			**-0.214**					**0.250**	**0.260**	**0.278**					**0.207**
NL 1%b		**-0.218**								**-0.269**	**-0.242**					**-0.212**
NL 3%b										**-0.267**	**-0.212**					**-0.234**
NL %b		**-0.235**								**-0.307**	**-0.257**					**-0.260**
NL 1%d											**0.207**					
NL 3%d									**0.214**		**0.228**					
NL %d									**0.216**	**0.206**	**0.230**					

Table C.3. continued.

	UK %d	NL 1msp	NL 3msp	NL msp	NL 1msb	NL 3msb
NL 3msp	0.221	**0.641**				
NL msp		**0.910**	**0.901**			
NL 1msb						
NL 3msb	-0.230				**0.548**	
NL msb	-0.231			-0.205	**0.790**	**0.946**
NL 1msd		**0.928**	**0.562**	**0.827**	0.253	

	UK %d	NL 1msp	NL 3msp	NL msp	NL 1msb	NL 3msb	NL msb	NL 1msd	NL 3msd	NL msd	NL 1%p	NL 3%p	NL %p	NL 1%b	NL 3%b	NL %b
NL 3msd		**0.415**	**0.755**	**0.640**	0.222	**0.536**	**0.479**	**0.488**								
NL msd		**0.766**	**0.769**	**0.847**	*0.276*	*0.327*	**0.347**	**0.851**	**0.873**							
NL 1%p		**0.994**	**0.631**	**0.901**				**0.921**	**0.416**	**0.762**						
NL 3%p		**0.490**	**0.754**	**0.686**				**0.402**	**0.574**	**0.574**	**0.492**					
NL %p		**0.967**	**0.732**	**0.942**				**0.882**	**0.500**	**0.794**	**0.972**	**0.680**				
NL 1%b					**0.950**	**0.557**	**0.778**	*0.304*	*0.291*	**0.346**						
NL 3%b					**0.460**	**0.947**	**0.873**		**0.494**	*0.301*				**0.498**		
NL %b	-0.210				**0.738**	**0.914**	**0.957**		**0.477**	**0.364**				**0.787**	**0.927**	
NL 1%d		**0.950**	**0.600**	**0.859**				**0.963**	**0.476**	**0.822**	**0.957**	**0.442**	**0.923**			
NL 3%d		**0.463**	**0.702**	**0.641**				**0.404**	**0.589**	**0.583**	**0.469**	**0.904**	**0.635**			
NL %d		**0.936**	**0.687**	**0.900**				**0.935**	**0.550**	**0.853**	**0.943**	**0.596**	**0.953**			
tr wt																

Appendix D

Additional information from Chapter 6

Field trial plan and randomisation

Plants were transplanted to the field into 4 x 1.83 m wide beds with a plot length of 1.05 and spacing distance of 35 cm (see figure D.1.). Beds were marked using a tractor with a wheel base of 1.83 m and plots were then individually marked using a 'Wolf Garten' row marker. Plots were marked in numerical order. The trial randomisation was also in numerical order (see figure D.2.).

Guard	Guard	Guard	Guard
32	64	96	128
31	63	95	127
30	62	94	126
29	61	93	125
28	60	92	124
27	59	91	123
26	58	90	122
25	57	89	121
'	'	'	'
8	40	72	104
7	39	71	103
6	38	70	102
5	37	69	101
4	36	68	100
3	35	67	99
2	34	66	98
1	33	65	97
Guard	Guard	Guard	Guard

Plot number

35.7 m long

Bed

1 2 3 4

7.32 m wide

Overall area 261.3 m^2

Figure D.1. Field trial plan.

Guard	Guard	Guard	Guard
5033	5064	5095	1003
5032	5063	5094	1004
5031	5062	5093	5125
5030	1004	5092	5124
5029	5061	5091	5123
5028	5060	5090	5122
6027	5059	5089	5121
5026	5058	5088	5120
5025	5057	5087	5119
5024	5056	5086	5118
5023	5055	5085	5117
5022	5054	5084	5116
5021	5053	5083	5115
5020	5052	5082	5114
5019	5051	5081	5113
5018	5050	5080	5112
5017	5049	5079	5111
5016	5048	5078	5110
5015	5047	5077	5109
5014	5046	5076	5108
5013	5045	5075	5107
5012	5044	5074	5106
5011	5043	5073	5105
5010	5042	5072	5104
5009	5041	5071	5103
5008	5040	1003	5102
5007	5039	5070	5101
5006	5038	5069	5100
5005	5037	5068	5099
5004	5036	5067	5097
5003	5035	5066	5097
5002	5034	5065	5096
Guard	Guard	Guard	Guard

Bed

1 2 3 4

Figure D.2. Randomised design for field plan. Number refers to LJ seed number from the WHRI GRU for plot. Guard plants were either Saladin or Iceberg.

Correlations amongst traits

All discolouration measures and morphological traits were assessed for potential relationships (correlations between browning, pinking and overall discolouration and between them and morphological traits per and across trial sites (see table D.1.).

Table D.1. Correlation matrix for the Saladin x Iceberg lettuce RIL population between post harvest discolouration measurements and morphological parameters within and across sites. Read across then down. Only significant effects shown with ******P* <0.001 in bold, *****P*<0.01 in italics and ****P*<0.05 in regular font. Where *UK* (UK trial site); *NL* (NL trial site); *msb* (browning intensity); *msp* (pinking intensity); *msd* (overall discolouration intensity); *%b* (extent of browning); *%p* (extent of pinking); *%d* (extent of overall discolouration) *unt wt* (untrimmed weight, g); *tr wt* (trimmed weight, g); *dia* (head diameter); *head* (heading); *indt* (indented leaf edges); *sav* (savoy/blistering of leaves); *grn* (leaf green colour); *anth* (leaf anthocyanins); *dharv* (days to harvest); *dbolt* (days to bolting); *seed* (seed colour). Numerical value before discolouration measurement is day.

	1msp	3msp	msp	1msb	3msb	msb	1msd	3msd	msd	1%p	3%p	%p	1%b	3%b	%b	1%d	3%d	%d	UK 1msp
1msp																			
3msp	**0.682**																		
msp	**0.900**	**0.931**																	
1msb																			
3msb	-0.242			**0.542**															
msb				**0.782**	**0.948**														
1msd	**0.889**	**0.648**	**0.825**	**0.458**															
3msd	0.219		0.209																
msd	0.224		0.213					**1.000**											
1%p	**0.995**	**0.673**	**0.892**		-0.242		**0.882**	0.221	0.225										
3%p	**0.631**	**0.864**	**0.830**				**0.605**			**0.636**									
%p	**0.955**	**0.802**	**0.951**		-0.225		**0.861**	0.206	0.210	**0.960**	**0.825**								
1%b				**0.963**	**0.497**	**0.735**	**0.514**				0.209								
3%b				**0.518**	**0.939**	**0.893**							**0.490**						
%b				**0.820**	**0.864**	**0.952**	*0.317*						**0.820**	**0.901**					
1%d	**0.898**	**0.678**	**0.848**	**0.359**			**0.963**			**0.905**	**0.647**	**0.894**	**0.448**		0.221				
3%d	**0.563**	**0.789**	**0.747**				**0.566**			**0.568**	**0.879**	**0.730**	0.232			**0.606**			
%d	**0.884**	**0.761**	**0.890**	*0.332*			**0.937**			**0.890**	**0.760**	**0.924**	**0.428**		0.230	**0.979**	**0.755**		
UK 1msp	**0.654**	**0.605**	**0.681**	0.251			**0.695**	0.215	0.218	**0.644**	**0.618**	**0.692**	**0.349**		0.205	**0.682**	**0.492**	**0.688**	
UK 3msb				**0.489**	**0.822**	**0.795**							**0.498**	**0.762**	**0.747**		0.246		
UK msp	**0.560**	**0.767**	**0.733**	*0.285*			**0.628**			**0.552**	**0.755**	**0.674**	**0.384**		*0.274*	**0.629**	**0.627**	**0.679**	**0.859**
UK 1msb		*0.297*	0.247	**0.817**	**0.405**	**0.610**	**0.501**				**0.347**	0.221	**0.865**	**0.412**	**0.700**	**0.468**	**0.362**	**0.478**	**0.455**
UK 3msb				**0.489**	**0.822**	**0.795**							**0.498**	**0.762**	**0.747**		0.246		
UK msb				**0.680**	**0.765**	**0.825**	*0.279*						**0.705**	**0.722**	**0.825**	0.223	*0.319*	0.266	0.256
UK 1msd	**0.499**	**0.547**	**0.571**	**0.588**		**0.368**	**0.713**			**0.488**	**0.582**	**0.564**	**0.677**	0.248	**0.498**	**0.687**	**0.508**	**0.696**	**0.886**
UK 3msd	0.218		0.207					**1.000**	**1.000**	0.219									0.214
UK msd	0.220		0.209					**1.000**	**1.000**	0.221		0.206							0.217
UK 1%p	**0.666**	**0.610**	**0.690**	0.239			**0.701**	0.225	0.228	**0.661**	**0.634**	**0.711**	**0.339**			**0.696**	**0.502**	**0.702**	**0.993**
UK 3%p	**0.502**	**0.758**	**0.700**	0.224			**0.548**			**0.506**	**0.866**	**0.680**	*0.322*			**0.578**	**0.713**	**0.660**	**0.646**

	sav	grn	anth	dharv	dbolt	seed
anth		**0.355**				
dharv						
dbolt						
seed	*0.273*					

Table D.1. continued.

	1msp	3msp	msp	1msb	3msb	msb	1msd	3msd	msd	1%p	3%p	%p	1%b	3%b	%b	1%d	3%d	%d	UK 1msp
UK %p	0.651	0.738	0.760	0.254			0.694			0.651	0.806	0.765	0.363			0.707	0.651	0.750	0.925
UK 1%b		0.330	0.284	0.778	0.367	0.567	0.512				0.398	0.264	0.865	0.377	0.677	0.493	0.390	0.506	0.498
UK 3%b				0.452	0.730	0.712							0.468	0.770	0.739		0.317		
UK %b		0.264		0.690	0.642	0.738	0.369				0.280		0.745	0.674	0.814	0.327	0.398	0.373	0.345
UK 1%d	0.518	0.577	0.599	0.548		0.282	0.711			0.516	0.618	0.598	0.657		0.421	0.736	0.544	0.746	0.861
UK 3%d	0.395	0.626	0.565	0.279		0.225	0.479			0.395	0.673	0.529	0.341	0.222	0.312	0.503	0.801	0.618	0.488
UK %d	0.525	0.641	0.640	0.515		0.289	0.702			0.524	0.689	0.630	0.619		0.425	0.730	0.669	0.773	0.824
NL 1msp	0.869	0.492	0.724		-0.280	-0.271	0.700			0.869	0.414	0.785		-0.212		0.716	0.404	0.693	0.207
NL 3msp	0.621	0.772	0.767				0.498			0.616	0.590	0.663				0.534	0.579	0.589	0.257
NL msp	0.824	0.692	0.821		-0.254	-0.250	0.660			0.821	0.552	0.800		-0.215		0.689	0.539	0.706	0.252
NL 1msb		-0.253	-0.249	0.633	0.398	0.536					-0.314	-0.259	0.501	0.345	0.478		-0.243		
NL 3msb	-0.254	-0.285	-0.297	0.320	0.684	0.629				-0.252	-0.314	-0.300	0.223	0.636	0.528				
NL msb	-0.265	-0.309	-0.315	0.479	0.655	0.668				-0.262	-0.353	-0.322	0.356	0.600	0.572		-0.240		-0.218
NL 1msd	0.772	0.384	0.612				0.724			0.773	0.285	0.668				0.692	0.303	0.647	
NL 3msd	0.362	0.470	0.458		0.306	0.267	0.372			0.359	0.295	0.367		0.273	0.225	0.339	0.364	0.371	
NL msd	0.646	0.496	0.615				0.624			0.644		0.592				0.586	0.386	0.581	
NL 1%p	0.860	0.481	0.713		-0.264	-0.260	0.690			0.870	0.411	0.785				0.719	0.406	0.696	
NL 3%p	0.498	0.578	0.596				0.377			0.503	0.674	0.613				0.414	0.658	0.513	0.248
NL %p	0.854	0.558	0.757		-0.262	-0.263	0.678			0.864	0.524	0.822				0.712	0.514	0.720	0.231
NL 1%b		-0.212		0.574	0.357	0.484					-0.282		0.496	0.322	0.460		-0.215		

	1msp	3msp	msp	1msb	3msb	msb	1msd	3msd	msd	1%p	3%p	%p	1%b	3%b	%b	1%d	3%d	%d	UK 1msp
NL 3%b		-0.258	-0.248	0.273	0.612	0.558					-0.248	-0.231		0.633	0.514				
NL %b		-0.276	-0.262	0.441	0.589	0.605					-0.299	-0.252	0.355	0.589	0.564		-0.227		
NL 1%d	0.816	0.437	0.665				0.724			0.827	0.348	0.731				0.752	0.359	0.711	
NL 3%d	0.457	0.531	0.545				0.359			0.465	0.597	0.557				0.395	0.650	0.494	0.205
NL %d	0.816	0.504	0.706				0.713			0.828	0.440	0.766				0.745	0.460	0.733	
trim_wt					0.236														
NL tr wt																			
UK tr wt					0.337	0.303								0.314	0.280				
UK unt wt	-0.236			0.226	0.448	0.416				-0.239			0.222	0.433	0.394				
dia	-0.290	-0.232	-0.282		0.339	0.309				-0.291	-0.238	-0.297		0.292	0.232	-0.261		-0.252	-0.314
head	0.541	0.530	0.583	-0.261	-0.453	-0.437	0.361			0.538	0.550	0.589		-0.341	-0.316	0.419	0.415	0.448	0.443
indt	0.340	0.326	0.361		-0.250	-0.213	0.271			0.341	0.312	0.359				0.287		0.284	0.305
sav	0.375		0.296		-0.335	-0.208	0.383			0.377		0.322		-0.349		0.381		0.323	
grn		-0.213	-0.223				-0.249				-0.280	-0.246	-0.212			-0.295		-0.292	-0.245
anth																			
dharv				-0.243									-0.223			-0.236		-0.209	
dbolt		0.245	0.236								0.240	0.208							
seed	0.300	0.295	0.324		-0.259	-0.215	0.238			0.317	0.303	0.343				0.287	0.205	0.291	0.236

	UK 3msb	UK msp	UK 1msb	UK 3msb
UK msp	0.243			
UK 1msb	0.530	0.529		
UK 3msb	1.000	0.243	0.530	

Table D.1. continued.

	UK 3msb	UK msp	UK 1msb	UK 3msb	UK msb	UK 1msd	UK 3msd	UK msd	UK 1%p	UK 3%p	UK %p	UK 1%b	UK 3%b	UK %b	UK 1%d	UK 3%d
UK msb	**0.942**	**0.382**	**0.782**	**0.942**												
UK 1msd	**0.349**	**0.833**	**0.816**	**0.349**	**0.573**											
UK 3msd																
UK msd							**1.000**									
UK 1%p		**0.852**	**0.431**		0.227	**0.869**	0.224	0.227								
UK 3%p		**0.875**	**0.488**		*0.308*	**0.674**			**0.655**							
UK %p		**0.945**	**0.500**		*0.289*	**0.861**			**0.933**	**0.882**						
UK 1%b	**0.527**	**0.559**	**0.978**	**0.527**	**0.772**	**0.832**			**0.478**	**0.523**	**0.546**					
UK 3%b	**0.938**	*0.303*	**0.520**	**0.938**	**0.891**	**0.361**				0.247		**0.521**				
UK %b	**0.857**	**0.481**	**0.836**	**0.857**	**0.957**	**0.659**			*0.319*	**0.427**	**0.404**	**0.848**	**0.893**			
UK 1%d	*0.289*	**0.817**	**0.783**	*0.289*	**0.518**	**0.966**			**0.860**	**0.688**	**0.862**	**0.820**	*0.305*	**0.618**		
UK 3%d	**0.425**	**0.689**	**0.495**	**0.425**	**0.503**	**0.575**			**0.495**	**0.771**	**0.674**	**0.514**	**0.508**	**0.581**	**0.610**	
UK %d	**0.355**	**0.851**	**0.766**	**0.355**	**0.559**	**0.934**			**0.826**	**0.774**	**0.882**	**0.801**	**0.394**	**0.663**	**0.972**	**0.780**
NL 1msp	-0.221			-0.221	-0.211				0.224	0.236	0.250					
NL 3msp		0.240							*0.270*	*0.284*	*0.300*					0.237
NL msp		**0.229**							**0.269**	**0.285**	**0.301**					**0.235**
NL 1msb		**-0.226**								**-0.269**	**-0.250**					
NL 3msb		**-0.257**							**-0.207**	**-0.332**	**-0.289**					**-0.230**
NL msb		**-0.278**							**-0.228**	**-0.349**	**-0.310**					**-0.243**
NL 1msd																
NL 3msd																
NL msd																
NL 1%p	**-0.212**			**-0.212**					**0.217**	**0.236**	**0.246**					
NL 3%p		**0.205**							**0.264**	**0.237**	**0.278**					
NL %p	**-0.214**			**-0.214**					**0.250**	**0.260**	**0.278**					**0.207**
NL 1%b		**-0.218**								**-0.269**	**-0.242**					**-0.212**
NL 3%b										**-0.267**	**-0.212**					**-0.234**
NL %b		**-0.235**								**-0.307**	**-0.257**					**-0.260**
NL 1%d											**0.207**					
NL 3%d									**0.214**		**0.228**					
NL %d									**0.216**	**0.206**	**0.230**					
trim_wt	**0.305**			**0.305**	**0.237**								**0.264**			
NL tr wt																
UK tr wt	**0.361**			**0.361**	**0.339**								**0.373**	**0.327**		
UK unt wt	**0.363**			**0.363**	**0.337**								**0.360**	**0.309**		
dia	**0.348**	**-0.211**		**0.348**	**0.299**				**-0.344**		**-0.297**		**0.325**	**0.232**	**-0.234**	
head	**-0.404**	**0.395**		**-0.404**	**-0.330**	**0.246**			**0.461**	**0.388**	**0.469**		**-0.355**	**-0.246**	**0.304**	**0.238**
indt	**-0.311**	**0.274**		**-0.311**	**-0.224**				**0.311**	**0.230**	**0.303**		**-0.244**		**0.209**	
sav					**-0.246**				**0.208**				**-0.343**	**-0.216**		
grn		**-0.254**	**-0.253**			**-0.291**			**-0.248**	**-0.246**	**-0.274**	**-0.287**			**-0.353**	
anth																
dharv			**-0.244**			**-0.224**						**-0.235**		**-0.206**	**-0.240**	
dbolt		**0.234**								**0.245**	**0.239**					
seed		**0.264**							**0.258**	**0.301**	**0.305**				**0.227**	

Table D.1. continued.

	UK %d	NL 1msp	NL 3msp	NL msp	NL 1msb	NL 3msb	NL msb	NL 1msd	NL 3msd	NL msd	NL 1%p	NL 3%p	NL %p	NL 1%b	NL 3%b	NL %b
NL 3msp	0.221	**0.641**														
NL msp		**0.910**	**0.901**													
NL 1msb																
NL 3msb	-0.230				**0.548**											
NL msb	-0.231			-0.205	**0.790**	**0.946**										
NL 1msd		**0.928**	**0.562**	**0.827**	0.253											
NL 3msd		**0.415**	**0.755**	**0.640**	0.222	**0.536**	**0.479**	**0.488**								
NL msd		**0.766**	**0.769**	**0.847**	*0.276*	*0.327*	**0.347**	**0.851**	**0.873**							
NL 1%p		**0.994**	**0.631**	**0.901**				**0.921**	**0.416**	**0.762**						
NL 3%p		**0.490**	**0.754**	**0.686**				**0.402**	**0.574**	**0.574**	**0.492**					
NL %p		**0.967**	**0.732**	**0.942**				**0.882**	**0.500**	**0.794**	**0.972**	**0.680**				
NL 1%b					**0.950**	**0.557**	**0.778**	*0.304*	*0.291*	**0.346**						
NL 3%b					**0.460**	**0.947**	**0.873**		**0.494**	*0.301*				**0.498**		
NL %b	-0.210				**0.738**	**0.914**	**0.957**		**0.477**	**0.364**				**0.787**	**0.927**	
NL 1%d		**0.950**	**0.600**	**0.859**				**0.963**	**0.476**	**0.822**	**0.957**	**0.442**	**0.923**			
NL 3%d		**0.463**	**0.702**	**0.641**				**0.404**	**0.589**	**0.583**	**0.469**	**0.904**	**0.635**			
NL %d		**0.936**	**0.687**	**0.900**				**0.935**	**0.550**	**0.853**	**0.943**	**0.596**	**0.953**			
tr wt																
NL tr wt			**0.343**	0.252					0.214			**0.348**				
UK tr wt																
UK unt wt		-0.209		-0.228		0.241	0.235									
dia	-0.207															
head	*0.309*	**0.409**	**0.489**	**0.491**	**-0.355**	*-0.280*	**-0.344**	0.265	0.232	*0.284*	**0.397**	**0.493**	**0.460**	*-0.281*		-0.206
indt		0.242	*0.285*	*0.285*					0.215	0.228	0.241	*0.284*	*0.272*			
sav		**0.344**	0.215	*0.305*				**0.406**		*0.289*	*0.329*		*0.310*	0.227		
grn	*-0.324*					0.257	0.206								0.245	
anth																
dharv	-0.226															
dbolt																
seed	0.235	0.214		0.222		-0.222	-0.228				0.220		0.218			

	NL %d	tr wt	NL tr wt	UK tr wt	UK unt wt	dia	head	indt
NL tr wt		**0.666**						
UK tr wt		**0.765**						
UK unt wt		**0.615**		**0.778**				
dia					**0.413**			
head	**0.357**				**-0.381**	**-0.583**		
indt	0.229					*-0.322*	**0.716**	
sav	*0.314*	-0.232		*-0.299*	*-0.303*	*-0.284*	**0.385**	**0.342**
anth			-0.211					
dharv								
dbolt							**0.362**	**0.530**
seed		*-0.333*	*-0.281*			*-0.270*	*0.283*	0.240
grn								-0.234

Quantitative trait loci from interval mapping

Table D.2. MQM QTL impacting on agricultural traits of individuals from the Saladin x Iceberg RIL population. Additive effect equals half the difference between homozygous alleles at the QTL: positive number indicates an additive allelic effect of Saladin; negative number indicates a negative allelic effect of Saladin. Confidence interval was based on a 2 LOD support interval with a significant LOD value of 1.95. Where *LG* (linkage group from F_7 Saladin x Iceberg linkage map (see Chapter 4)); *LOD* (logarithm of odds), *cM* (centimorgans); *heading* (production of an enclosed head); *diameter* (head diameter (cm)); *green* (of leaf); *anthocyanin* (pigmentation on leaf); *indentation* (on leaf edges); *savoy* (blistering of leaf) *Untr* (untrimmed head); *Tr* (trimmed head); *wt* (weight (g)); *UK* (United kingdom); *NL* (the Netherlands).

Trait	LG	Location (cM)	LOD peak	Confidence interval (cM)	Nearest Locus	Additive effect	Genetic variation explained by QTL (%)	Allelic contribution
Heading_1	7	1.2	5.47	2.4	AYBP-OP4	0.24	23.5	Saladin
Heading_2	7	2.9	4.43	3.6	BSQZ-OP4	0.22	19.6	Saladin
Heading_3	7	7.8	2.77	4.9	AZTI-OP4	0.18	12.7	Saladin
Heading_4	7	12	2.09	7.2	BEYE-OP4	0.16	9.7	Saladin
Heading_5	7	17.7	2.87	7.2	E33M48_300i	-0.22	18.1	Iceberg
Heading_6	7	23.7	2.38	4	w	-0.17	11	Iceberg
Heading_7	7	74	perfect fit	10.2	ATPH-OP4 / BIVH-OP3-1	0.50	100	Saladin
Heading_8	8	35.7	perfect fit	10.7	E35M59_332i / E35M59_359s	-0.50	100	Iceberg
Diameter_1	7	1.2	3.07	2.4	AYBP-OP4	-0.27	14	Iceberg
Diameter_2	7	2.9	3.8	4.6	BSQZ-OP4	-0.30	17.1	Iceberg
Diameter_3	7	7.8	3.07	3.2	AZTI-OP4	-0.27	14	Iceberg
Diameter_4	7	16.5	3.16	5.8	BXJC-OP3	0.27	14.3	Saladin
Green_1	4a	6	9.94	4	BFYG-OP3	-0.49	38.6	Iceberg
Green_2	4a	18.9	4.5	7.4	AVPF-OP3	-0.33	19.8	Iceberg
Green_3	4a	44.1	5.21	17.1	AYTT-OP3 / BHYM-OP4	-0.48	39.9	Iceberg
Green_4	4a	58.5	2.21	63.5	BLRO-OP4	0.46	10.3	Saladin
Anthocyanin_1	5c	29.2	6.67	12.7	AFPZ-OP3	0.40	27.9	Saladin
Anthocyanin_2	9a	35.4	4.61	35.6	AHTV-OP4	-0.69	20.2	Iceberg
Indentation_1	5b	47.7	2.35	58	E38M54_270i	0.28	12.9	Saladin
Indentation_2	7	2.3	3.77	2.4	BLJI-OP4	0.33	17	Saladin
Indentation_3	7	2.9	2.69	4.6	BSQZ-OP4	0.28	12.5	Saladin

Table D.2. continued.

Trait	LG	Location (cM)	LOD peak	Confidence interval (cM)	Nearest Locus	Additive effect	Genetic variation explained by QTL (%)	Allelic contribution
Indentation_4	8	35.7	2.76	10.7	E35M59_332i	-0.58	48.4	Iceberg
Savoy_1	5a	78	5.8	8.5	E35M49_145i / QGA18I02-OP4	0.67	67.5	Saladin
Savoy_2	7	74	3.19	10.2	ATPH-OP4 / BIVH-OP3-1	0.67	65.4	Saladin
Bolting_1	4b	94.2	3.42	11.9	CLXS3_3835-OP3-2 / CLXS3_3835-OP3-1	-16.58	26.2	Iceberg
Bolting_2	4b	107.5	2.73	2.7	E45M60_119i	-13.76	16.3	Iceberg
Bolting_3	5a	78	47.58	8.5	E35M49_145i / QGA18I02-OP4	-30.93	99	Iceberg
Bolting_4	7	74	51.21	10.2	ATPH-OP4 / BIVH-OP3-1	-30.93	99	Iceberg
Bolting_5	8	35.7	45.72	10.7	E35M59_332i / E35M59_359s	-30.93	99	Iceberg
Untr UK wt_1	4a	58.5	2.09	13.9	BLRO-OP4	-144.15	9.7	Iceberg
Untr UK wt_2	5b	39.6	2.52	33.3	BAMG-OP3-1	78.12	11.6	Saladin
Untr UK wt_3	7	0.9	2.58	2.4	ARZI-OP4	-78.70	11.9	Iceberg
Untr UK wt_4	7	2.9	2.99	4.6	BSQZ-OP4	-84.66	13.7	Iceberg
Untr UK wt_5	7	7.8	2.11	19.3	AZTI-OP4	-71.47	9.8	Iceberg
Untr UK wt_6	7	8.9	2.02	19.3	BWYR-OP4 / AXAS-OP4 / AISE-OP4 / BATK-OP4 / BAIJ-OP4 / BAAZ-OP4	-70.04	9.4	Iceberg
Untr UK wt_7	7	23.7	2.24	5.6	w	74.07	10.4	Saladin
Tr UK wt_1	4a	58.5	2.38	8.9	BLRO-OP4	-99.49	11	Iceberg
Tr UK wt_2	7	21.7	2.18	24	E33M59_205s	53.78	12.2	Saladin
Tr NL wt_1	7	19.1	2.02	1.6	BTGH-OP4	-29.32	9.4	Iceberg
Tr NL wt_2	7	23.7	1.96	12.3	BKVX-OP4-2 / AOUA-OP4-2 / BATO-OP4-2 / BCHL-OP4-2 / BIVP-OP4-1	-28.64	9.2	Iceberg
Tr NL wt_3	7	33.5	2.15	26.2	QGJ17A06_2-OP3-1 / BKBM2_OP3	30.10	10	Saladin
Tr wt_1	7	8.2	2.17	3.2	AQTU-OP4	-23.87	10.1	Iceberg
Tr wt_2	7	12.2	2.26	1.4	AXMT-OP1	-24.16	10.7	Iceberg
Tr wt_3	7	15.6	3.16	4.4	BHVY-OP4	-28.86	14.4	Iceberg
Tr wt_4	7	19.1	2.37	19.3	BTGH-OP4	-24.96	11	Iceberg
Tr wt_5	7	23.7	2.38	5.5	w	24.91	11	Saladin
Tr wt_6	7	26.4	3	5.3	QGCA_6226-OP3	28.23	13.7	Saladin
Tr wt_7	7	38.7	2.25	14.9	BXNV-OP3-1	24.36	10.4	Saladin

Appendix E

Additional information from Chapter 7

Table E.1. Candidate gene information relating to the phenylpropanoid pathway. Information from candidate genes in *Arabidopsis thaliana* from the TAIR database (www.arabidopsis.org).

Gene Name	Biological process	Cellular component	Molecular function
ACCase	/	/	/
ACCD	Fatty acid biosynthetic process	Chloroplast, membrane, chloroplast envelope	Acetyl-CoA carboxylase activity
ANS	Response to wounding, vacuole organization, anthocyanin biosynthetic process, response to jasmonic acid stimulus, proanthocyanidin biosynthetic process	Unknown	Leucocyanidin oxygenase activity
ANR	Negative regulation of flavonoid biosynthetic process	/	Oxidoreductase activity
ANL2	Root development, anthocyanin accumulation in tissues in response to UV light	Nucleus	Transcription factor activity, transcription regulator activity
AR1	Response to oxidative stress, phenylpropanoid metabolic process	Endoplasmic reticulum	NADPH-hemoprotein reductase activity
AR2	Phenylpropanoid metabolic process	Chloroplast, endoplasmic reticulum	NADPH-hemoprotein reductase activity
AS	/	/	/
CFI	Response to UV and UV-B, flavonoid biosynthetic process	Endoplasmic reticulum, nucleus, plant-type vacuole membrane	Chalcone isomerase activity
CHI	Flavonoid biosynthetic process	/	Chalcone isomerase activity
CHR	/	/	/
CHS	Flavonoid biosynthetic process, response to; oxidative stress, gravity and wounding, chalcone biosynthetic process, response to jasmonic acid stimulus, auxin polar transport, response to UV-B, regulation of anthocyanin biosynthetic process	Endoplasmic reticulum, nucleus, plant-type vacuole membrane	Naringenin-chalcone synthase activity
CYP98A3	Lignin biosynthetic process, positive regulation of flavonoid biosynthetic process, auxin homeostasis	Mitochrondrian, plasma membrane, endoplasmic reticulum, microsome	Monooxygenase activity, p-coumarate 3-hydroxylase activity

Table E.1. continued.

DFR	Anthocyanin biosynthetic process	Endoplasmic reticulum membrane	Dihydrokaempferol 4-reductase activity
EGL3	Regulation of transcription, epidermal cell fate specification	Nucleus	DNA binding
F3'5'H	/	/	/
F35H	/	/	/
F3'H	Response to UV, flavonoid biosynthetic process	/	Functions in oxygen binding, flavonoid 3'-monooxygenase activity
F3H	Flavonoid biosynthetic process, response to UV-B	Unknown	Naringenin 3-dioxygenase activity
FSI/ FS2	/	/	/
F7ORT	Flavonol biosynthetic process	Unknown	UDP-glycosyltransferase activity, transferase activity, transferring glycosyl groups
FLS1	Flavonoid biosynthetic process	Unknown	Flavonol synthase activity
FLS2	Flavonoid biosynthetic process	Unknown	Flavonol synthase activity
FLS3	Flavonoid biosynthetic process	Unknown	Flavonol synthase activity
FLS4	Flavonoid biosynthetic process	Unknown	Flavonol synthase activity
FLS5	Flavonoid biosynthetic process	Unknown	Flavonol synthase activity
FLS6	Unknown	Unknown	Flavonol synthase activity
GST	Toxin catabolic process	Cytoplasm	Glutathione transferase activity
COA	Lignin biosynthetic process, positive regulation of flavonoid biosynthetic process, auxin homeostasis	Unknown	Transferase activity, quinate O-hydroxycinnamoyltransferase activity, shikimate O-hydroxycinnamoyltransferase activity
I2_H	/	/	/
IFR	Response to oxidative stress and cadmium ion	Plasma membrane	Catalytic activity, binding, transcription repressor activity
IFS	/	/	/
LDOX/ANS	Response to wounding, vacuole organization, anthocyanin biosynthetic process, response to jasmonic acid stimulus, proanthocyanidin biosynthetic process	Unknown	Leucocyanidin oxygenase activity
LCR/ LAR	/	/	/
5MAT	Anthocyanin biosynthetic process	Unknown	Transferase activity, O-malonyltransferase activity

Table E.1. continued.

MYB3	Cinnamic acid biosynthetic process, response to wounding, response to salt stress, regulation of transcription, DNA-dependent, response to abscisic acid stimulus, response to auxin stimulus, response to ethylene stimulus, response to gibberellin stimulus, response to jasmonic acid stimulus, response to salicylic acid stimulus, negative regulation of metabolic process, response to cadmium ion	/	DNA binding, has transcription factor activity
MYB4	Negative regulation of transcription, response to salt stress, response to abscisic acid stimulus, response to auxin stimulus, response to ethylene stimulus, response to gibberellin stimulus, response to jasmonic acid stimulus, response to salicylic acid stimulus, response to cadmium ion, response to UV-B	/	DNA binding, has transcription factor activity
OMT1	Lignin biosynthetic process, flavonol biosynthetic process	Cytoplasm, cytosol, nucleus, plasma membrane	O-methyltransferase activity, caffeate O-methyltransferase activity
PAL1	Defense response, response to oxidative stress, phenylpropanoid biosynthetic process, response to wounding	Cytoplasm	Phenylalanine ammonia-lyase activity
PAL2	Defense response, response to oxidative stress, phenylpropanoid biosynthetic process, response to wounding	Cytoplasm	Phenylalanine ammonia-lyase activity
PAL3	Defense response, response to wounding	Cytoplasm	Phenylalanine ammonia-lyase activity
PAL4	Biosynthetic process, L-phenylalanine catabolic process	Cytoplasm	Ammonia ligase activity, ammonia-lyase activity, catalytic activity
PPO	Lignin biosynthetic process , related to flavonoid biosynthetic process	Endomembrane system	Functions in copper ion binding, laccase activity
PAP1	Response to salt stress, regulation of transcription, DNA-dependent, removal of superoxide radicals, anthocyanin biosynthetic process, sucrose mediated signaling, response to jasmonic acid stimulus, anthocyanin metabolic process, regulation of anthocyanin biosynthetic process	/	DNA binding
PAP2	Regulation of transcription	/	DNA binding, has transcription factor activity
TT1	Flavonoid biosynthetic process	Nucleus	Transcription factor activity
TT12	Proanthocyanidin biosynthetic process, maintenance of seed dormancy	Membrane, plant-type vacuole membrane	Antiporter activity, solute:hydrogen antiporter activity, transporter activity, transmembrane transporter activity

Table E.1. continued.

TT16	Regulation of cell shape, seed development, ovule development, regulates ptoanthocyanidin biosynthetic process	Nucleus	Transcription factor activity
TT19	Toxin catabolic process	Cytoplasm	Glutathione transferase activity
TT2	Regulation of transcription, DNA-dependent, proanthocyanidin biosynthetic process	Nucleus	DNA binding
TT8	Trichome differentiation, regulates flavonoid biosynthetic process, proanthocyanidin biosynthetic process	Nucleus	DNA binding, has transcription factor activity
TTG1	Cell fate commitment, epidermal cell fate specification, trichome differentiation, regulation of protein localization	Cytoplasm, nucleus	DNA binding, nucleotide binding, protein binding
TTG2	Regulation of transcription, DNA-dependent, epidermal cell fate specification, seed coat development	Nucleus	Transcription factor activity
UF3GT/3GT	N-terminal protein myristoylation	Unknown	Transferase activity, transferring glycosyl groups
VR	/	/	/
4CL1	Response to UV, phenylpropanoid metabolic process, response to fungus, response to wounding	Unknown	4-coumarate-CoA ligase activity
4CL2	Response to UV, phenylpropanoid metabolic process, response to fungus, response to wounding	Unknown	4-coumarate-CoA ligase activity
4CL3	Response to UV, phenylpropanoid metabolic process, pollen exine formation, not involved with response to wounding, defense response to fungus	Unknown	4-coumarate-CoA ligase activity
4CL5	Phenylpropanoid biosynthetic process	Unknown	4-coumarate-CoA ligase activity
ACCase	/	/	/

Correlations amongst traits

All discolouration measures, morphological traits and metabolite profiles were assessed for potential relationships (correlations between browning, pinking and overall discolouration and between them and morphological traits and PPO activity, PAL activity and TPC per and across trial sites (see table E.2.).

Table E.2. Correlation matrix for the Saladin x Iceberg lettuce RIL population between post harvest discolouration measurements, morphological traits and metabolite activity within and across sites. Read across then down. Only significant effects shown with ****P* <0.001 in bold, ***P*<0.01 in italics and **P*<0.05 in regular font. Where *msb* (browning intensity); *msp* (pinking intensity); *msd* (overall discolouration intensity); *%b* (extent of browning); *%p* (extent of pinking); *%d* (extent of overall discolouration)); *tr wt* (trimmed weight, g); *Acr* (across days); *DH* (harvest day); *D4* (day 4); *PPO* (polyphenols oxidase activity); *PAL* (phenylalanine ammonia lyase activity); *TPC* (total phenolic content);. Numerical value before discolouration measurement is day.

	msb1	msb2	msb3	msb4	msb	b%1	b%2	b%3	b%4	b%	msp1	msp2	msp3	msp4	msp	p%1	p%2	p%3	p%4	p%
PAL_DH																				
PAL_D4	0.610																			
PAL_AD	*0.702*					0.630														
PPO_DH																				
PPO_D4																				
PPO_AD																				
TPC_DH																				
TPC_D4																				
TPC_AD																				
Pro_DH		-0.592	-0.567		-0.557		-0.576							0.615					*0.743*	
Pro_D4																				
Pro_AD																				
tr_wt																				

Table E.2. continued.

	msd1	msd2	msd3	msd4	msd	d%1	d%2	d%3	d%4	d%	PAL_DH	PAL_D4	PAL_AD	PPO_DH	PPO_D4	PPO_AD
PAL_DH																
PAL_D4		0.661			0.558											
PAL_AD		0.642										**0.906**				
PPO_DH																
PPO_D4																
PPO_AD								0.596							**0.837**	
TPC_DH												-0.588				
TPC_D4																
TPC_AD			0.581	*0.688*	0.560											
Pro_DH																
Pro_D4																
Pro_AD																
tr_wt																

	TPC_DH	TPC_D4	TPC_AD	Pro_DH	Pro_D4	Pro_AD	tr_wt
PAL_DH							
PAL_D4							
PAL_AD							
PPO_DH							
PPO_D4							
PPO_AD							
TPC_DH							
TPC_D4							
TPC_AD	*-0.700*						
Pro_DH							
Pro_D4							
Pro_AD				-0.667	**0.894**		
tr_wt							

Appendix F

Additional information from Chapter 8

Field trial plan and randomisation

Plants were grown in 5 inch pots into with 40 mm spacers between plots. The trial randomisation was designed in a 6x6 Latin square (see figure F.1.).

row	column	Plant	row	column	Plant
1	1	6	4	1	4
1	2	3	4	2	1
1	3	4	4	3	2
1	4	1	4	4	5
1	5	5	4	5	3
1	6	2	4	6	6
2	1	5	5	1	2
2	2	2	5	2	5
2	3	3	5	3	6
2	4	6	5	4	3
2	5	4	5	5	1
2	6	1	5	6	4
3	1	3	6	1	1
3	2	6	6	2	4
3	3	1	6	3	5
3	4	4	6	4	2
3	5	2	6	5	6
3	6	5	6	6	3

Figure D.2. Latin square design for glasshouse plan. Plant refers to line id, where Saladin is 1, Iceberg is 2, Troubadour 4250 is 3, TroubaLessOxida is 4, 09R.9511 is 5 and *L. serriola* 03050 is 6.

Correlations amongst traits

All discolouration measures, morphological traits and metabolite profiles were assessed for potential relationships (correlations between browning, pinking and overall discolouration and between them and morphological traits and PPO activity, PAL activity and TPC per and across trial sites (see table F.1.).

Table F.1. Correlation matrix for the Saladin x Iceberg lettuce RIL population between post harvest discolouration measurements, morphological traits and metabolite activity within and across sites. Read across then down. Only significant effects shown with ****P* <0.001 in bold, ***P*<0.01 in italics and **P*<0.05 in regular font. Where *msb* (browning intensity); *msp* (pinking intensity); *msd* (overall discolouration intensity); *%b* (extent of browning); *%p* (extent of pinking); *%d* (extent of overall discolouration)); *tr wt* (trimmed weight, g); *Acr* (across days); *DH* (harvest day); *D4* (day 4); *PPO* (polyphenols oxidase activity); *PAL* (phenylalanine ammonia lyase activity); *TPC* (total phenolic content);. Numerical value before discolouration measurement is day.

	msb	**msb1**	**msb2**	**msb3**	**msb4**	**b%**	**b%1**	**b%2**	**b%3**	**b%4**	**msp**	**msp1**	**msp2**	**msp3**	**msp4**	**p%**	**p%1**	**p%2**
msb																		
msb1	**0.999**																	
msb2	0.89	0.88																
msb3	**0.995**	**0.994**																
msb4	**0.997**	**0.996**		**0.997**														
b%	**0.999**	**0.997**		**0.996**	**1**													
b%1	**0.999**	**0.999**		**0.996**	**0.999**	**0.999**												
b%2	0.906	0.906	**0.992**			0.886	0.889											
b%3		*0.99*		**0.995**	**0.998**	**0.996**	**0.995**											
b%4	**0.993**	**0.991**		**0.994**	**0.999**	**0.998**	**0.997**		**0.999**									
msp																		
msp1											0.879							
msp2											**0.998**	0.899						
msp3											**0.999**	0.898	**1**					
msp4											*0.989*		*0.979*	*0.982*				
p%											*0.978*		*0.964*	*0.968*	**0.997**			
p%1											0.921	**0.995**	0.938	0.937				
p%2											*0.988*		*0.971*	*0.973*	**0.998**	**0.996**		
p%3											0.944		0.922	0.93	*0.976*	*0.989*		*0.973*
p%4											0.927		0.904	0.91	*0.972*	*0.985*		*0.977*
msd												0.941					0.912	

	msd2	**msd3**	**msd4**	**d%**	**d%1**	**d%2**	**d%3**	**d%4**	**tr wt**
msd3	0.929								
msd4	0.899	*0.988*							
d%	*0.979*	*0.96*	0.949						
d%1									
d%2	0.948			*0.961*					
d%3	0.904					*0.967*			
d%4	0.881			0.924		*0.975*	0.917		
tr wt									

Table F.1. continued.

	msb	msb1	msb2	msb3	msb4	b%	b%1	b%2	b%3	b%4	msp	msp1	msp2	msp3	msp4	p%	p%1	p%2	p%3	p%4	msd	msd1
msd1			0.940																		0.912	
msd2											0.915	*0.986*	0.928	0.931			*0.986*				0.937	
msd3												0.9399					0.911				**0.998**	0.912
msd4												0.893									**0.992**	0.93
d%												0.952	0.883	0.886			0.947				**0.965**	
d%1		0.879	0.922					0.9575														0.987
d%2											0.929	0.885	0.933	0.939	0.909	0.89	0.903	0.905				
d%3											0.978		0.97	0.976	0.982	0.976		0.981	0.957	0.945		
PPO_D4	*-0.97*	-0.958		*-0.964*	*-0.976*	*-0.976*	*-0.969*		*-0.971*	*-0.979*												
PPO_Acr	*-0.967*	*-0.964*			*-0.978*	*-0.976*	*-0.975*		*-0.978*	*-0.984*												
TPC_DH																						
TPC_D4																						-0.889
TPC_Acr																						

	msd2	msd3	msd4	d%	d%1	d%2	d%3	d%4	tr wt	PAL_DH	PAL_D4	PAL_Acr	PPO_DH	PPO_D4	PPO_Acr	TPC_DH	TPC_D4	TPC_Acr
PAL_D4																		
PAL_Acr																		
PPO_DH									0.951									
PPO_D4									0.948									
PPO_Acr														*0.978*				
TPC_DH																		
TPC_D4			-0.909															
TPC_Acr																		