Investigating the Genetic Control of Postharvest Shelf Life and Vitamin C content in Broccoli (*Brassica oleracea* var. *italica*)

by

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DECLARATION

I declare that the material contained in this thesis is obtained from my own work and that none of the work has been published or submitted to another university for another degree. Any data, material, methods and information obtained from external sources (published or unpublished) are referenced accordingly.

SUMMARY

Broccoli (*Brassica oleracea* var. *italica*) is a popular vegetable, known for its nutritional benefits. However, the marketability of broccoli is limited by a short shelf life. Broccoli is susceptible to rapid postharvest senescence, which causes visible head yellowing and wilting from dehydration. Visible quality loss is also accompanied by a decline in nutrients, resulting in a product with reduced postharvest nutritional value. These factors combined cause broccoli to become unmarketable, leading to severe wastage in the retail chain. Postharvest yellowing in broccoli has been shown to be controlled by genotype, as a doubled haploid population (MGDH) created from an F_1 cross between GD33, a poor performing DH line (yellow in 2 days) and Mar34, a good performing DH line (staying green > 4 days) exhibited natural variation for shelf life. Therefore, to investigate the genetic control of quality in broccoli, the fixed mapping population was assessed for shelf life, morphological traits and vitamin C content and stability in replicated field trials. Visual inspections identified head yellowing, stem turgor and bud compactness as the main traits affecting the marketability of broccoli. Two methods to quantify head yellowing were also evaluated. Spectrophotometer readings were found to be more sensitive than Image J in detecting colour change, but Image J data was more reproducible. Vitamin C quantification using HPLC, confirmed that natural variation was present in the MGDH population at harvest. Vitamin C content during postharvest storage, detected by plate assays, found vitamin C to be unstable, degrading quickly after harvest. A unique broccoli x broccoli linkage map, covering ~72.9% of the *B.oleracea* genome, was also constructed by genotyping the MGDH population with SSR and AFLP markers. QTL analysis of the trait data positioned 48 significant QTL in the linkage map for head yellowing (4), colour co-ordinates (17), morphological traits (17), bud quality (2) and postharvest vitamin C content (3) and stability (5). The identification of QTLs associated with the above traits has provided useful information for breeders to breed for improved nutritional and quality in broccoli using marker-assisted selection (MAS). The location of QTLs has also provided targets for fine-scale mapping and for the identification of candidate genes underlying traits.

CHAPTER 1:
INTRODUCTION AND LITERATURE REVIEW

1.1 The *Brassica* genus

The *Brassica* genus belongs to the *Brassicaceae* family, which consists of ~340 genera and ~3350 species (Schmidt *et al.*, 2001). Members of the *Brassica* genus are of agricultural importance as they are a rich source of oil seeds and include a variety of vegetable and fodder crops summarized in Table 1.1 (Ostergaard and King, 2008; Schmidt *et al.*, 2001). The six ancestral and most widely cultivated *Brassica* species include three diploids: *B. rapa*, *B. nigra* and *B. oleracea*, the genomes of which have been denoted A, B and C respectively, and three amphidiploids derived from the diploid species (*B. juncea* (AB), *B. napus* (AC) and *B. carinata* (BC)) which make up the 'triangle of U' proposed by U (1935) (Figure 1.1). These species are key to describing the evolution and relationships within the *Brassica* genus.

Table 1.1: A summary of the crop plants associated with *Brassica* species and their common names.

Species	Common name
B. rapa	Turnip, Chinese cabbage.
B. nigra	Black mustard.
B. oleracea	Cabbage, Kale, Broccoli, Cauliflower
B. juncea	Indian mustard.
B. napus	Oilseed rape, rutabaga.
B. carinata	Ethiopian mustard.

Brassicas are closely related to the model plant organism *Arabidopsis thaliana* whose genome has been sequenced and annotated. Lineages between the two species are of particular importance as it allows for the translation of information by exploiting conserved sequences and gene order through comparative genomics (Li *et al.*, 2003). Comparative mapping studies have revealed collinear regions between the genomes of *Brassica* and *A.thaliana* in chromosomal segments (Lukens *et al.*,

2003; Li *et al*., 2003; Babula *et al*., 2003) through genome alignment. This allows for the potential identification of gene orthologs for the improvement of traits in closely related crop plants like *Brassica* (Schmidt *et al*., 2001).

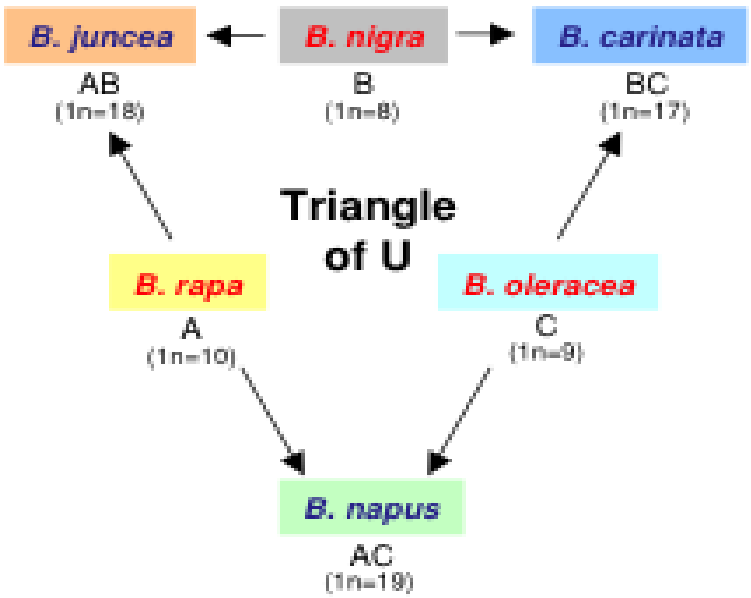


Figure 1.1: The triangle of U for the *Brassica* species which consists of three diploids (*B. rapa, B. nigra and B. oleracea*) and three amphidiploids (*B. juncea, B. carinata and B. napus*) reproduced from Ostergaard and King (2008). Genome type and haploid chromosome number is indicated for each species.

1.1.2 Broccoli (Brassica oleracea var. *italica).*

Broccoli (*Brassica oleracea* var. *italica*) and other important vegetable crops such as kale, Kohlrabi, cabbage, cauliflower and Brussels sprouts all have 'C' genomes (n=9) but are all morphologically different (Bohuon *et al*., 1996). The main identifying characteristic of broccoli is its enlarged inflorescence, which is the part that is eaten (Lan and Paterson, 2000). Broccoli is believed to have originated in the eastern Mediterranean region from the cultivation of Kale, with Italy being the centre of diversification (Kalloo and Bergh, 1993). The edible parts of broccoli are the florets consisting of unopened flower buds and branch tissue (Figure 1.2). Demand for fresh

broccoli in the UK has increased making broccoli a crop of commercial importance; in 2009 a total area of 8,950 hectares of broccoli and cauliflower was planted in the UK, which was estimated to have a market value of £46,325,000 (DEFRA, 2009).

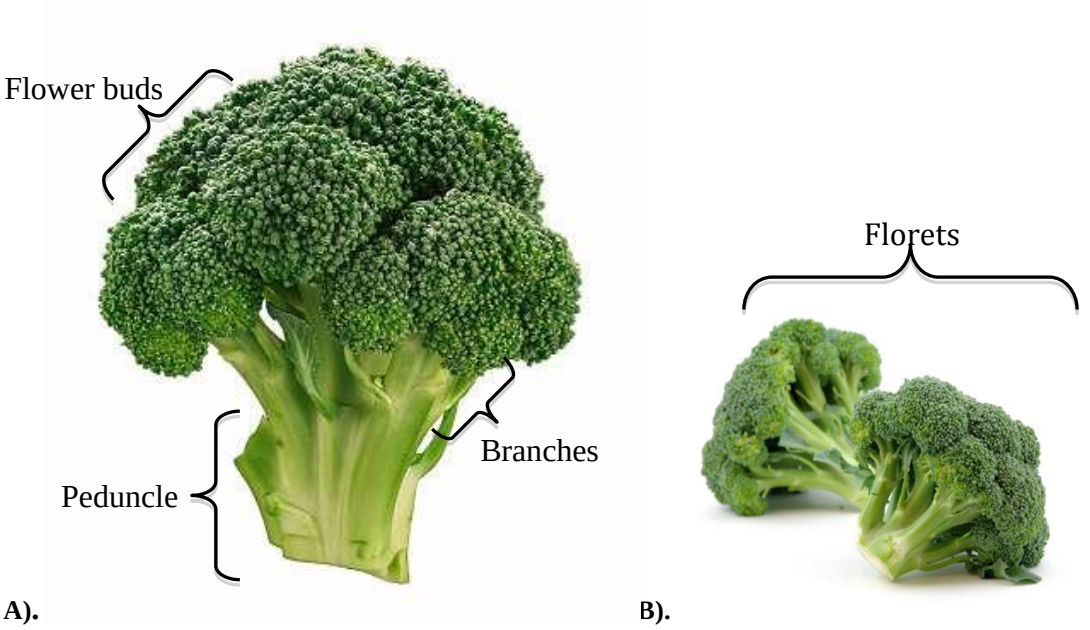


Figure 1.2: A photographic illustration of the edible parts of broccoli A). The whole broccoli head including the peduncle, branches and flowers buds and B). Prepared florets.

1.2 Evaluating shelf life and postharvest quality and in broccoli.

Shelf life can be defined as the period of time that fresh produce retains desirable attributes such as appearance, colour, texture and nutritional status. The main aspects that affect postharvest quality in fresh produce, leading to a reduction in shelf life include: water loss (dehydration), respiration rate, storage temperature, mechanical damage, chlorophyll loss, nutrient recycling, microbial growth and sensitivity to ethylene. In broccoli, factors that particularly affect postharvest quality include head yellowing, wilting and tissue softening caused by postharvest senescence, which can incite an unfavourable consumer response as consumers obviously buy fresh produce based on its appearance. Consumers perceive head yellowing in broccoli as poor

quality, which leads to rejection of produce. Currently, methods to evaluate colour and colour change in broccoli rely on visual inspection, which is subjective. A challenge of this project was to find a suitable quantitative method for assessing head yellowing in broccoli. Therefore, the next section reviews the theories behind colour vision and quantitative methods of measuring colour change in broccoli as an alternative to visual assessments.

1.2.1 Colour perception

To perceive colour, a light source, an observer and an object are required. The human eye can detect colours as wavelengths in the visible spectrum (~400-700nm), which consists of red, orange, yellow, green, blue, indigo and violet wavelengths of light. Colours are classified in terms of their hue (colour), lightness (brightness) and saturation/chroma (vividness). However, colour is a matter of perception and subjective interpretation, as two people looking at the same object might perceive the colour of the object differently. The basis of colour vision stems from two colour vision theories.

1.2.2 Theories of colour vision

The Trichromatic theory

Thomas Young and Herman Helmoltz proposed the trichromatic colour vision in the early 1800s; they stated that colour vision in humans is meditated by the stimulation of three types of cone photoreceptors, found in the retina (Shaaban & Deeb, 1998). Each cone is sensitive to different wavelengths in the visible spectrum (Figure 1.3): Long (L, ~560 nm), Medium (M, ~530 nm) and short (S, ~430 nm). To perceive

colour the activity in the three different photoreceptors is compared (Soloman & Lennie, 2007).

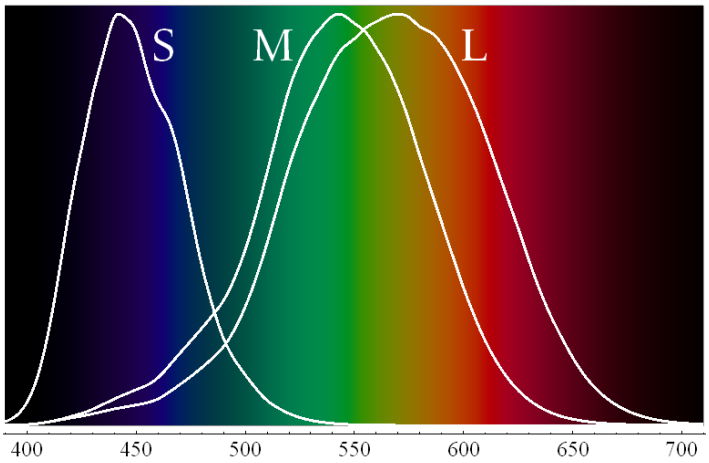


Figure 1.3: Long, medium and short wavelengths (nm) in the visible light spectrum detected by cone photoreceptors in the human eye.

The Opponent process theory of vision.

Hering proposed the opponent process theory in the late 1800s. The theory assumes that the receptors in the human eye perceive colour in pairs of opposites; light-dark, red-green and yellow-blue. Object recognition in the opponent theory is based on luminosity or hue gradients (Mureika, 2005). Therefore, the colors of an object are mainly encoded in the opponent color signals whilst the spatial patterns or textures are mostly encoded in the light–dark component (Qui *et al.*, 2007).

1.2.3 Quantitative approaches to assess head yellowing:

The RGB colour model

The RGB colour model (Figure 1.4) is an additive colour model consisting of the three primary colours of red (R), green (G) and blue (B). The primary colours can be combined together in various ways to produce a broad array of other colours.

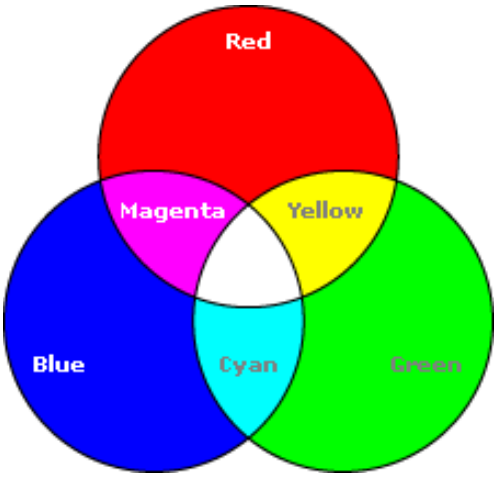


Figure 1.4: The primary (red, green and blue) and secondary colours (magenta, cyan and yellow) of the additive RGB colour model.

The secondary colours of the RGB colour model are cyan, magenta and yellow, which are formed by the mixing of two primary colours i.e. red and green combine to make yellow. The RGB colour model relates very closely to the way humans perceive colour with the cone receptors in the eye. It also provides a quantitative approach to measuring head discolouration and colour change in broccoli. Image J is a public domain Java imaging processing software programme (Rasband, W.S., 1997). It can be used to determine the RGB values from photographic images.

The Hunter Lab colour space

The Hunter *Lab* colour space (Figure 1.5) is based on the opponent colour theory and measures colours using 3 dimensions: *L*, *a* and *b*. The value *L* is regarded as the degree of lightness or darkness of a colour where as the co-ordinates *a* and *b* represents the chromatic portion of the colour space. On the red-green colour scale (*a*), red is represented by +*a* and green by –*a*. Similarly on the blue-yellow colour scale (*b*), yellow is represented by +*b* and blue by -*b*.

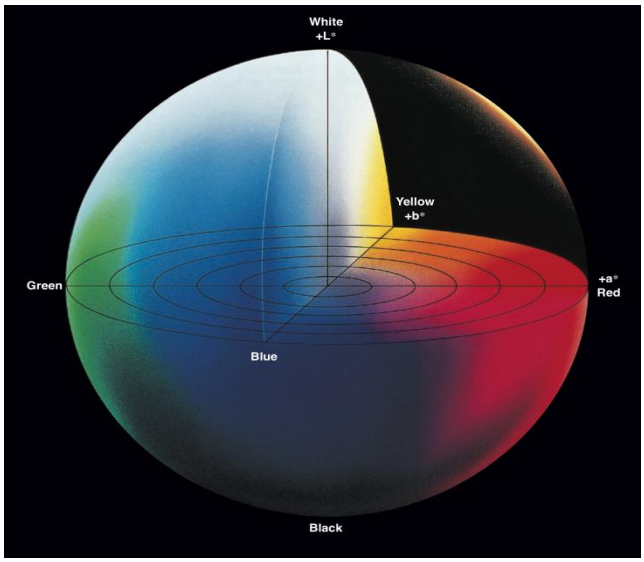


Figure 1.5: A 3-D representation of the Hunter *Lab* colour space taken from the Konica colour communication booklet. 'L' represents the lightness of an object (0 =black, 100 = white), 'a' represents colour on a red (+) to green (-) colour scale and 'b' represents colour on a yellow (+) to blue (-) colour scale. .

The L-value ranges from 0 (black) to 100 (white) whereas *a*-value ranges from -60 (green) to +60 (red) and the *b*-value from -60 (blue) to +60 (yellow). The co-ordinates in the Hunter *Lab* colour scale can also be used to calculate the hue angle (colour) and chroma (vividness) of a sample.

1.3 Natural and postharvest senescence.

Colour change in broccoli occurs as a result of postharvest senescence, the processes of which is very similar to natural leaf senescence. Natural senescence is a highly regulated process, resulting in developmental change and alterations in gene expression, that lead to the mobilisation of cellular components and death of individual cells, organs or whole organisms (Hodges and Forney, 2000; King and O'Donoghue, 1995; King *et al.*,1995). In plants, senescence can occur naturally as seen in autumnal leaves, fruit ripening and the abscission of flower petals (King and O'Donoghue, 1995) or it can be induced by abiotic and biotic stresses such as

nutrient or water deficiency, wounding, extremes of temperature, attack from pathogens and exogenous compounds, for example, ethylene (Buchanan-Wollaston *et al.*, 2003; Hodges and Forney, 2000; King and O'Donoghue, 1995). Although mechanisms for triggering senescence can be different, similarities have been observed between natural and artificially induced plant senescence, these include: a reduction in photosynthetic capacity, the breakdown of chloroplasts (Ghosh *et al.*, 2001), loss of chlorophyll (Hortenstiener, 2006; Yamauchi *et al.*, 1997), the degradation of macromolecules such as proteins and lipids (Palma *et al.*, 2002; Zhuang *et al.*, 1997; Coupe *et al.*, 2003) and the recycling and remobilisation of nutrients to developing parts of the plant (Coupe *et al.*, 2003). The model plant species *Arabidopsis* has been paramount in the study of mechanisms described above that regulate and occur during leaf senescence (Buchanan-Wollaston *et al.*, 2003; Lim *et al.*, 2003; Yoshida, 2003)

1.3.1 Senescence in broccoli.

Broccoli heads are composed of immature florets arrange in whorls on a fleshy stem (Buchanan-Wollaston *et al.*, 2001; Nishikawa *et al.*, 2005). Each floret contains hundreds of immature flower buds; the sepals that protect the flowers are rich in chlorophyll, which gives broccoli its characteristic green appearance (Buchanan-Wollaston *et al.*, 2001; Nishikawa *et al.*, 2005). However, during storage the sepals become susceptible to rapid yellowing due to chlorophyll degradation (Buchanan-Wollaston *et al.*, 2001: Nishikawa *et al.*, 2005). In broccoli, senescence in induced prematurely as a result of harvesting and post-harvest stresses (Coupe *et al.*, 2003; Gapper *et al.*, 2005). This initiates rapid deterioration and quality loss such as senescing of buds, opening of buds to expose yellow petals, softening of tissue and

wilting, all of which causes broccoli to become unmarketable (Wurr *et al.*, 2002; Serrano *et al.*, 2006). Post-harvest senescence is also observed in other perishable crops such as asparagus, lettuce and cauliflower (King and O'Donoghue, 1995; Downs *et al.*, 1997). As with broccoli, these crops are all harvested before reaching maturity and are still undergoing rapid pre-harvest growth (King and O'Donoghue, 1995; Downs *et al.*, 1997). Deterioration in young plant organs such as those described above is associated with the inability to maintain metabolic homeostasis (King and O'Donoghue, 1995), as losses due to respiration and transpiration can no longer be replaced (Brosnan and Sun, 2001). Decapitation of the crop removes the supply of energy, water, nutrients and hormones and therefore triggers the onset of postharvest senescence (Brosnan and Sun, 2001). Other factors that promote post-harvest senescence are respiration rate, storage temperature and sensitivity to ethylene (Brash *et al.*, 1994; Albanese *et al.*, 2007). As broccoli is harvested when it is immature it has a high respiration rate, which has been shown to increase with temperature (Brash *et al.*, 1995). Respiration rate and storage temperature are limiting factors of shelf life, as broccoli heads stored at 10^{o}C have a respiration rate of 38-43 ml CO_2/kg.hr rising to 80-90 ml CO_2/kg.hr when stored at 15^{o}C (Cantwell and Suslow, recommendations for maintaining postharvest quality: Broccoli factsheet, UC Davis). Broccoli stored at 10^{o}C has a shelf life of ~5 days. However, most retailers issue shelf life guidelines of only 4 days from delivery to consumption (Wurr *et al.*, 2002). To determine the genetic control of head yellowing in broccoli the underlying processes leading to loss of green colour such as the dismantling of chloroplasts preceding chlorophyll degradation need to be understood. The next section summarises these processes.

1.4 Changes in the chloroplast and chlorophyll degradation.

1.4.1 Dismantling of chloroplasts during senescence.

During senescence in higher plants photosynthetic capability is reduced due to the dismantling of chloroplasts and the transition of chloroplasts to gerontoplasts (Ghosh *et al.*, 2001). During this process changes can be seen in the chloroplast, which includes the swelling of thylakoids, the appearance of lipid droplets and plastoglobuli, a decline in the size and number of starch granules and the unstacking of the thylakoid grana (Ghosh *et al.*, 2001). During the disintegration of chloroplasts remobilisation of amino acids from proteins also occurs, this includes the Chl *a/b* binding proteins and the release of phototoxic Chl, therefore the mechanism of chlorophyll degradation exists to avoid photooxidative damage from Chl in its free form with Hortenstiener (2006) describing the breakdown of Chl as a vital detoxification mechanism for plant development and survival.

1.4.2 Chlorophyll degradation during senescence.

Chlorophyll degradation in higher plants is the main visual cue for senescence due to the loss of green colour. The elucidation of the chlorophyll (Chl) degradation pathway has only been possible in the last decade with the identification of stay-green mutants and the discovery of the structure of the final Chl breakdown product a nonfluorescent Chl catabolite (NCC), in barley (Hortensteiner, 2006). Current knowledge has identified seven steps that are involved in the breakdown of Chl to NCCs that are subsequently transported to the vacuole (Figure1.6).

1.4.3 The chlorophyll degradation pathway.

1. *The conversion of chlorophyll b to chlorophyll a*

Evidence from early studies by Folly and Engel (1999) and Ito *et al,* (1994) in etioplasts from barley and cucumber proposed that Chl *b* is converted to Chl *a* before degradation by the reduction of the formyl group at C7 of Chl *b* to the methyl group of Chl *a* (Scheumann *et al.*, 1999). This hypothesis is further supported by the isolation of Chl catabolites that are all, apart from one, derived from Chl *a* (Curty and Engel, 1996; Scheumann *et al.*, 1998; Folly and Engel, 1999) and the specificity of pheophorbide *a* oxygenase (PAO) for the *a*-form of Chl (Scheumann *et al.*, 1998). Therefore, for Chl *b* to be degraded it must be converted to Chl *a* by Chl *b* reductase inside the thylakoid membrane which can occur as part of the Chl cycle (Scheumann *et al.*, 1999; Hortensteiner, 2006). The Chl cycle consists of a series of reactions that enables the interconversion of Chl *a* and Chl *b* catalysed by NADPH-dependent Chl *b* reductase and ferredoxin-dependent 7^1-OH-Chl *a* reductase (Rudigher, 2002; Hortensteiner, 2006). Chl *b* reductase has been identified from the rice stay green mutant, *non-yellow coloring1* (*nyc1*), which exhibits impaired chlorophyll degradation and breakdown of light-harvesting complex II (Kusaba *et al*., 2007; Sata *et al.,* 2009). Two genes, *NYC1* and *NOL* (NYC2-like), have been molecularly characterised, and encode a membrane-localised short chain dehydrogenase/reductase (SDR) that is believed to represent Chl *b* reductase in rice (Kusaba *et al*., 2007; Sata *et al.,* 2009).

2. *Dephytylation of chlorophyll (Chl) to chlorophyllide (Chlide).*

The conversion of Chl to chlorophyllide (Chlide) and phytol is catalysed by the hydrolysis of an ester bond by the enzyme chlorophyllase (CHL). Two research

groups have successfully cloned the gene encoding CHL in citrus fruit (Jakob-Wilk *et al.,* 1999), *A. thaliana* and *Chenopodium album* (Tsuchiya *et al.,* 1997, 1999) respectively. Matile *et al*. (1997) and Jakob-Wilk *et al.* (1999) propose that CHL is located in the chloroplast inner envelope membrane as isolation of envelope membranes from chloroplasts and gerontoplasts have been shown to contain CHL activity. Therefore, for Chl to be degraded by CHL transportation of Chl pigments from the thylakoid membrane to the envelope is required; this is hypothesized to occur via plastoglobules (Guiamet *et al*., 1999; Hortensteiner, 2006). Guiamet *et al*. (1999) examined the chloroplast of senescing leave from soybean and reported that many plastoglobuli were found to contain Chl or Chl derivative, the structures were also found to protrude through the chloroplast envelope into the cytoplasm. However, more recently studies in *Arabidopsis* by Schenk *et al.* (2007) found that the two CHLs present in the model plant were not essential for chlorophyll breakdown, although Tsuchiya *et al.* (1999) demonstrated that the two CHLs identified in *Arabidopsis* did exhibit CHL activity. Therefore, to date, molecular cloning of the genuine CHL from *Arabidopsis* has not been carried out yet (Hortensteiner, 2009).

3. The removal of magnesium by a metal chelating substance (MCS).

Original proposals suggested that Mg-dechelation, the third step in the Chl pathway, was catalysed by an Mg-dechelatase enzyme associated with the chloroplast membrane (Costa *et al*., 2002; Hortenstiener, 2006). However, conflicting evidence suggests that the removal of Mg^{2+} from Chlide occurs via a low-molecular weight metal chelating substance (MCS), although to date the structure and molecular nature of MCS has not been characterised. Findings from Kunieda *et al.* (2005) show that

only MCS displays Mg-dechelation activity for Chlide when testing the Mg-dechelation activity of MCS and Mg-releasing proteins (MGRs) in extracts from mature leaves of *C. album* in the presence of the native substance, Chlide, and an artificial substrate, Mg-chlorophyllin *a* (Chlin). Similar findings have been reported by Suzuki *et al*. (2005) in radish cotyledons.

4. *The conversion of pheophorbide a* (*pheide a*) *to red chlorophyll catabolite (RCC).*

The visible loss of green colour associated with chlorophyll occurs with the opening of the tetrapyrrole ring of pheophorbide *a* (pheide *a*) at the α-mesoposition between C4 and C5, this reaction is a two step process, the first of which involves the conversion of pheide *a* into a red chlorophyll catabolite (RCC) intermediate catalysed by pheophorbide *a* oxygenase (PAO) (Barry, 2009; Hortensteiner, 2006; Pruzinska *et al.*, 2003). The gene encoding the enzyme PAO from *Arabidopsis thaliana (AtPaO)* has been characterised by Pruzinska *et al.* (2003) to be a Rieske-type iron sulphur protein, identical to the *accelerated cell death 1* (*ACD1*) gene in *Arabidopsis*, which is expressed during senescence and is localised at the envelope membrane of gerontoplasts (Oberhuber *et al.*, 2003; Hortenstiener, 2006). PAO shows specificity for pheide *a* as pheide *b* is shown to be a competitive inhibitor. Reactions catalysed by PAO require reduced ferredoxin (Fd) as a source of electrons, with generation of Fd occurring either by NADPH through the pentose phosphate cycle or from photosystem I.

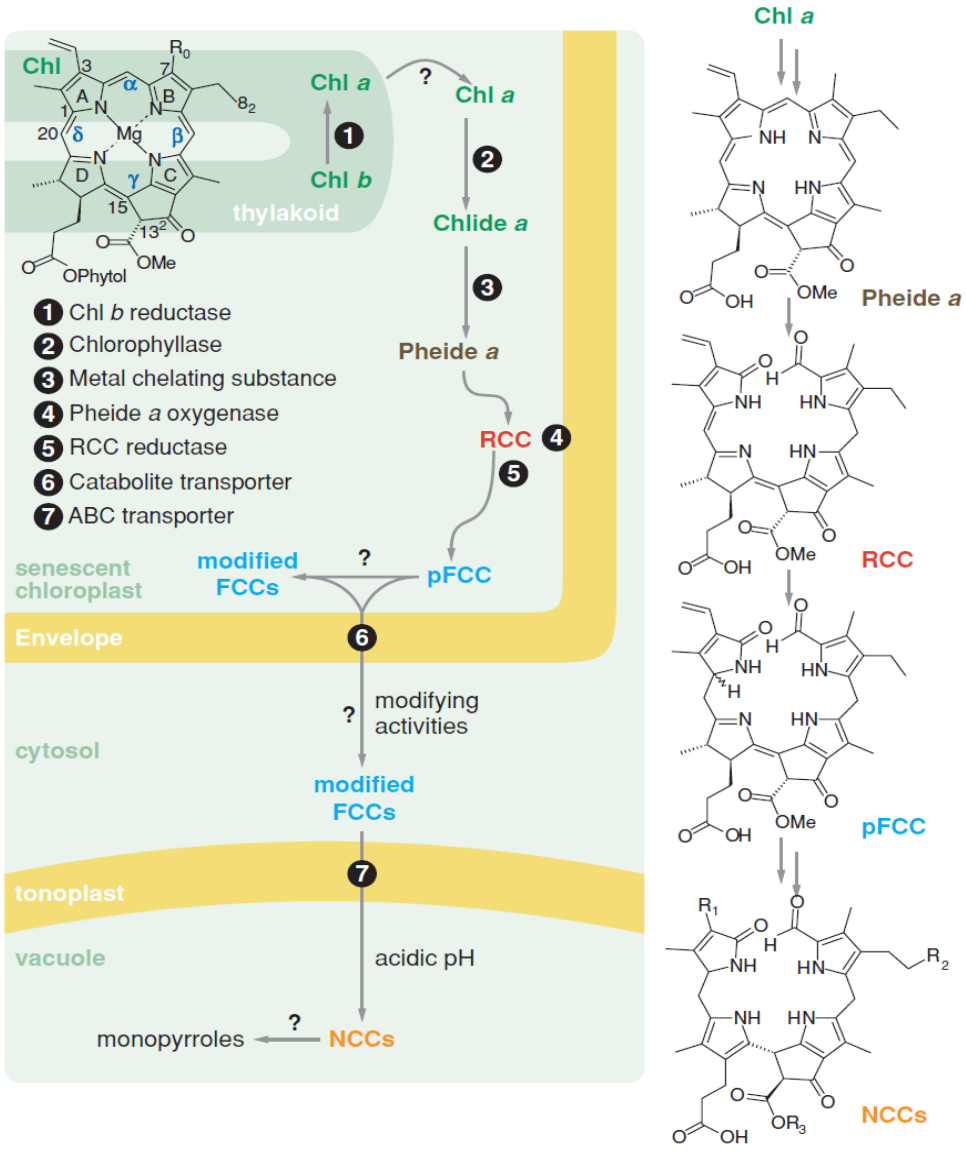


Figure 1.6: Taken from Hortensteiner (2006): Topographical model of the chlorophyll breakdown pathway of higher plants and the chemical structures of chlorophyll and chlorophyll catabolites. Question marks indicated putative (enzymatic) reactions. Pyrrole rings (*A-D*), methane bridges ($\alpha - \delta$) and carbon atom are labelled in Chlorophyll structure (*top left*). $R_0 = CH_3$, chlorophyll *a*; R_0 = CHO, chlorophyll *b*. R_1-R_3 in NCCs indicate the sites of species-specific modifications.

5. *The conversion of red chlorophyll catabolite (RCC) to primary fluorescent chlorophyll catabolite (pFCC).*

The second enzymatic reaction causing the loss of visible green colour in the pathway involves the reduction of the C20/C1 double bond of RCC to primary fluorescent catabolite (pFCC) by red chlorophyll catabolite reductase (RCCR), which

is located in the stroma (Wuthrich *et al*., 2000). As with PAO, the reaction is ferredoxin dependent but in contrast with PAO the enzyme is constitutively expressed at all stages in leaf development. Reactions catalysed by RCCR produce two forms of pFCC (pFCC-1 and pFCC-2), which are stereoisomeric at C1 and define the structures of NCCs, the end product of chlorophyll degradation (Wuthrich *et al*., 2000).

6. *Modifications of primary fluorescent chlorophyll catabolite (pFCC).*

Modification of pFCC occurs before the exportation and further modification of FCCs in the cytosol (Wuthrich *et al.,* 2000). Modifications summarised by Hortenstiener (2006) include: Dihydroxylation of the vinyl group of pyrrole A (R_1), hydroxylation at C8 accompanied by glycosylation and/or malonyltion (R_2) and C13 demethylation (R_3).

7. *The conversion of modified fluorescent chlorophyll catabolite (FCC) to nonfluorescent chlorophyll catabolite (NCC).*

The final step in the chlorophyll degradation pathway is the conversion of modified fluorescent chlorophyll catabolite (FCC) to nonfluorescent chlorophyll catabolite (NCC), which occurs in the vacuole. It is proposed that FCCs are transported across the tonoplast via ATP-dependent transport systems. Once inside the vacuole, FCCs are non-enzymatically converted to NCCs due to the acidic vacuolar pH.

1.4.4 Chlorophyll degradation and stay green mutants

Stay-green mutants and the identification of the stay-green (*SGR/SID*) gene have been particularly useful in identifying the genes encoding enzymes that catalyse

chlorophyll breakdown (Barry, 2009; Hortensteiner, 2009). Five types of stay green phenotypes (Type A-E) have been described by Thomas and Howarth (2000). Type A and type B are classed as functional stay greens: type A stay-greens show a delay in the onset of senescence, but after initiation senescence proceeds at a normal rate, whereas in type B stay-greens senescence occurs at a slower rate, but is initiated on schedule. In, contrast, type C-E are classed as cosmetic stay-greens and retain green colour. Type C stay-greens undergo normal senescence but chlorophyll degradation is impaired. Type D stay-greens retain green colour because of tissue death and type E stay-greens may contain more chlorophyll and have an increased photosynthetic capacity and therefore stay green due to enhanced pigments levels. The study of type C stay-green mutants in different plant species, has been helpful in elucidation of the chlorophyll breakdown pathway and the cloning of the stay green gene (Hortensteiner 2009; Barry, 2009). The stay green gene has been identified in mutants of *Festuca pratensis* (Bf993), *Festuca/Lolium* introgressions (*y*), rice (*sgr*), tomato (*gf*), bell pepper (*cl*), pea (JI2775) and Arabidopsis (*nye1-1*) (Vicentini *et al*., 1995; Ren *et al*., 2007; Jiang *et al*., 2007); Park *et al*., 2007; Armstead *et al*., 2006; Sata *et al*., 2007; Armstead *et al*., 2007; Barry *et al*., 2008; Aubry *et al.*, 2008). Stay green proteins (SGR) have been shown to be highly conserved, with defects in SGR originally proposed to be associated with reduced PAO activity and/or expression (Hortensteiner, 2009). However, an inhibitor study in the pea mutant (JI2775) by Aubry *et al.* (2008) demonstrated that SGR acts independently of PAO. Therefore, Hortensteiner (2009) proposes that the function of SGR proteins could be involved in the disassembly of the light-harvesting II complex, and that the absence of SGR during senescence leads to retention of chlorophyll.

1.4.5. Chlorophyll degradation and plant growth hormones

Ethylene and cytokinin are two plant growth hormones that influence the regulation of chlorophyll degradation during senescence. The biosynthesis of ethylene requires 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase enzymes, which catalyse the two step conversion of *S*-adenosylmethionine to ACC and ACC to ethylene respectively (Higgins *et al*., 2006). Endogenous ethylene has been shown to be regulator of chlorophyll loss as the application or presence of ethylene stimulates chlorophyllase (CHL) activity, an enzyme involved in the first step of Chl breakdown (Kasai *et al*., 1996; Gong and Mattheis, 2002; Suzuki *et al.*, 2005; Jacob-Wilk *et al.,* 1999). A study by Jacob-Wilk *et al.* (1999) using ethylene treatments in citrus report on an increase in the transcript level of Chlase which had the effect of accelerating Chl breakdown. Ethylene inhibitors such as 1-Methylcyclopropene (1-MCP) have also been used to investigate ethylene-stimulated chlorophyll catabolism in broccoli. Findings by Gong and Mattheis (2003) show that Chl degradation is accelerated by the exposure to ethylene, which stimulated Chlase production. However, treatments with 1-MCP followed by exposure to ethylene lead to the reduction of Chlase activity in broccoli. A more recent study by Buchert *et al*. (2010) examining the expression of broccoli Chlase genes *BoCLH1* and *BoCLH2* in response to ethylene treatment found that the expression patterns of the genes differed from what was expected. The expression of *BoCLH1* was found to decrease by ~80% after the initial day of the experiment regardless of treatment with ethylene, whereas the expression patterns of *BoCHL2* were irregular. When compared to the control, similar expression levels were observed on day three of induced senescence but lower expression levels were found on day four and day five of the experiment (Buchert *et al*., 2010). In contrast to ethylene, the plant hormone cytokinin delays

senescence by preventing chlorophyll loss (Gapper *et al*., 2005). Studies using 6-benzylaminopurine (6-BAP) cytokinin treatments in broccoli have been shown to both reduce chlorophyllase, Mg-dechelatase, and peroxidase-linked chlorophyll degradation (Costa *et al.,* 2005).

1.5 Methods to delay post harvest senescence

Retention of green colour in harvested vegetables is often an indicator of quality for consumers (Jacobsson *et al*., 2003). Post harvest factors that affect the quality of fresh produce include respiration rate, the presence of ethylene and storage conditions; temperature, relative humidity (RH), light and the composition of the surrounding atmosphere (Jacobsson *et al*., 2003). These factors promote degradation, such as the loss of colour and texture and are limiting factors of shelf life. To preserve market quality of fresh produce and delay senescence, storage conditions are often manipulated to find the optimum conditions for the product being stored. Other approaches to delay senescence include packaging and treatments.

1.5.1 Storage conditions

In order to sustain quality in fruits and vegetables the respiration rate must be controlled. The quality of actively respiring produce is often compromised due to internal sugar and fatty acid reserves within plant cells being consumed by oxidative metabolism during transport, retailing and storage (Uchino *et al*., 2004; Fonseca *et al*., 2002). To control the respiration rate of fresh produce after harvest, refrigeration, controlled atmosphere (CA) and modified atmosphere packaging (MAP) can be used. Schouten *et al*. (2008) states that to maintain the post harvest quality of

harvested broccoli, storage conditions with low temperatures (0-4 ^{o}C) and high relative humidity (98-100 %) are required. High temperatures during storage are very detrimental to fresh produce as metabolic processes increase two to three-fold for every 10^{o}C rise in temperature. Therefore, storage at low temperatures retards metabolic processes, such as respiration, and increases shelf life (Sandhya, 2010). Low RH also has an unfavourable effect on fresh produce and can cause transpiration damage, increased respiration and desiccation leading to an unmarketable product (Sandhya, 2010). Controlled atmosphere (CA) storage endeavours to reduce respiration, oxidative tissue damage, discolouration and ethylene sensitivity so that shelf life can be extended. Schouten *et al.* (2008) reports that by combining oxygen ($O_{2)}$ levels of 1-2 % with carbon dioxide (CO_2) levels of 5-10 % at low temperatures (0-5 ^{o}C) the appearance of broccoli can be maintained during storage.

1.5.2 Packaging

The use of packaging aims to prolong shelf life by reducing water loss and creating an ideal atmosphere to maintain colour, texture and the appearance of fresh produce (Jacobsson *et al*., 2004). Types of packaging commonly used include modified atmosphere packaging (MAP), which are often polymeric films that vary in their permeability to O_2, CO_2, ethylene, water vapour and other gases such as nitrogen (N_2) (Jacobsson *et al*., 2004). If optimum atmospheric conditions inside are reached using MAP, respiration rates and the production and sensitivity to ethylene are reduced (Fonseca *et al*., 2002). Many different types of polymeric films are commercially available and all vary in their effectiveness to delay senescence. A study by Jacobsson *et al.* (2004) to determine the effect of three different types of

MAP on weight, texture and colour in fresh broccoli, found that low density polyethylene (LDPE) packaging was more effective at prolonging the shelf life of broccoli compared to polypropylene (OPP) and polyvinyl chloride (PVC) packaging, with the deterioration occurring fastest in PVC packaging.

1.5.3 Treatments

Another approach to delay post harvest yellowing in broccoli is to apply physical or chemical treatments. Physical treatments are non-damaging methods that use heat shock or cold shock for a short duration (Zhang *et al*., 2009). Heat shock treatment (HST) involves exposure to hot air or hot water for a short period of time, wereas cold shock treatment (CST) involves brief immersion in ice water (Zhang *et al*., 2009). It has been reported in the literature that HST can delay yellowing in post harvest broccoli florets (Tian *et al*., 1996; Zhang *et al*., 2009).

Other physical treatments to delay senescence include the use of UV-B and UV-C irradiation. Exposure of broccoli to UV light has been shown to suppress chlorophyll degradation, which delays the onset of yellowing. Aimla-or *et al*. (2010) investigated the effects of UV-B irradiation doses of 4.4-13.1 KJ m^{-2} on chlorophyll degradation and chlorophyll degrading enzymes in broccoli. They found that a dose of 8.8 KJ m^{-2} was sufficient in delaying yellowing and the decrease of chlorophyll *a* and *b* as well as suppressing chlorophyll-degrading peroxidises (Aimla-or *et al*., 2010). Similar findings were reported when using UV-C irradiation in broccoli, Costa *et al.* (2006), report that an irradiation dose of 10 KJ m^{-2} preserving the green colour of broccoli heads, retained chlorophyll content and reduced the activity of chlorophyll peroxidise and chlorophyllase.

Chemical treatments are used to prevent undesirable colour changes and to maintain texture in harvested fruits and vegetables. Common chemical treatments include edible coatings and natural plant antimicrobials and antioxidants (Oms-Oliu, 2010). In broccoli chemical treatments such as ethanol vapour, 1-methylcyclopropene (1-MCP) and 6-benzylaminopurine (BAP) have been used to improve the shelf life of broccoli (Able *et al*., 2002; Costa *et al*., 2005; Asoda *et al*., 2009). Both ethanol vapour and 1-MCP aim to inhibit ethylene production, which promotes the loss of green colour in broccoli. A study by Asoda *et al*. (2009) found that the use of ethanol vapour pads was effective in suppressing ethylene responsiveness and ethylene biosynthesis in broccoli as senescence was delayed and the expression of broccoli 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase genes was suppressed. Similar findings are reported in studies using 1-MCP, which acts as an ethylene inhibitor. The use of 6-BAP as a chemical cytokinin treatment has been shown in a study by Costa *et al*. (2005) to be effective in prolonging the shelf life of broccoli. Plants treated with 6-BAP were found to have reduced rates of chlorophyll degradation due to diminished activities of enzymes involved in chlorophyll catabolism such as chlorophyllase, Mg-dechelatase and peroxidises associated with chlorophyll bleaching (Costa *et al*., 2005).

1.5.4 Preharvest factors

Pre-harvest factors that aid the extension of shelf life are often over looked with attention focusing on methods that attempt to delay senescence post harvest, however, studies considering pre-harvest factors, such as water stress in broccoli, have also shown to have an effect on prolonging shelf life (Zaicovski *et al*., 2008). Wurr *et al*. (2002) demonstrated that exposing broccoli plants to water stress during

floret maturation resulted in greener, more turgid heads after harvest compared to broccoli plants that were irrigated daily. Water stress has also been associated with increased cytokinin content, the effects of which, Szekacs *et al*. (2000) and Zhu *et al*. (2004) observed to cause better retention in green colour during storage.

The methods described here all attempt to delay senescence and prolong the shelf life of fruit and vegetables but the cost, safety and effectiveness of each method is variable. An alternative approach to improving the shelf life of vegetable is to use a genetic approach, which aims to utilize naturally occurring variation in shelf life for cultivar development and improvement.

1.6 A genetic approach to delay postharvest senescence and improve quality in crops.

Post harvest senescence, quality loss, and reduced nutritional status of crops, are of high concern for growers, retailers and consumers. Kader (2002) states that a high percentage of harvested fruit and vegetables are never consumed, estimating post harvest losses of between 5% and 25% for fruit and vegetables. To reduce post harvest losses in broccoli, shelf life needs to be extended, and a logical approach to do this is to utilise the natural variation displayed for quantitative traits such as shelf life, within broccoli cultivars.

1.6.1 Quantitative traits

Naturally occurring phenotypic variation observed in crops and other species is often due to genetic differences causing minor variations in several genes (Kearsey, 1998; Maloof, 2003). Traits that display this variation are often referred to as quantitative traits. Kearsey (1998) describes such traits as displaying continuous variation in a

population, which is usually normally distributed. In crop species, quantitative variation is present for many agronomically important traits such as yield, quality and disease resistance (Asins, 2002). Quantitative variation arises from both the combined effect of many segregating genes, termed polygenes, and interaction with environmental factors (Asins, 2002; Kearsey and Farquhar, 1998). Therefore, by understanding the genetic basis of quantitative variation and selecting genotypes with desirable characteristics, improvements in crops for traits of interest can be made through conventional plant breeding.

Evidence for genetic based variation in quantitative traits

Post harvest senescence, shelf life and other quality traits affecting the marketability of fresh produce show quantitative characteristics and are therefore expected to be influenced by genotype. A study reported in a paper by Buchanan-Wollaston *et al.* (2003) investigating the shelf life phenotypes of doubled haploid (DH) lines derived from a commercial broccoli variety Marathon, found that although the DH lines used in the study were derived from same genetic background, the shelf life performance of genotypes varied from 59 - 101 hours in storage. This suggests that genotype influences shelf life due to recombination during microspore culture resulting in different combinations of genes in the DH lines created.

1.6.2 Mapping populations

Mapping population are often created experimentally by selecting and crossing contrasting parental lines for a trait of interest. By utilising natural phenotypic and allelic variation in traits of interest within mapping populations, improved shelf life and metabolite content can be selected for and bred into existing commercial

cultivars. Mapping populations can be utilized in backcrossing programmes for cultivar development, the construction of genetic linkage maps, QTL analysis and plant breeding using marker-assisted selection. Types of mapping populations commonly used include doubled haploids (DH), recombinant inbred lines (RILs), F_2 populations and backcross populations.

Doubled Haploids

Doubled haploid (DH) plants are homozygous diploids produced by microspore culture, in which spontaneous chromosome doubling occurs during the culturing process. The use of DH lines to study agronomic traits has many advantages; populations of DH lines are homozygous and 'genetically fixed' therefore, the performance of DH lines can be evaluated in replicated field trials indefinitely (Pink *et al.*, 2008). This allows the collection of vast amounts of phenotypic and quantitative data over many growing seasons. Doubled haploid lines are also useful in the construction of genetic linkage maps, as they can easily be scored, as only one parental genotype will segregate at each locus (Snape and Simpson, 2004). Many published *B.oleracea* maps such as the integrated *B.oleracea* map produced by Sebastian *et al.* (2000) use DH lines. The integrated map consists of marker information from the AxG population and the NxG population. The AxG population was produced from a cross between a DH line A12DH of *B.alboglabra* and a DH line GDDH33, derived from the F1 broccoli cv. Green Duke. The NxG population was produced from a cross between DH lines derived from the cauliflower cv. Nedcha (CA25) and a DH line derived from the Brussels sprout cv, Grower (AC498). As the AxG population and the MGDH population in this study both share the parental genotype GDDH33 the vast marker information produced from the

integrated map can be utilised. Another advantage of DH lines is the identification of QTLs associated with quantitative traits. In this study, this has the potential to identify senescence associated genes (SAGs) and vitamin C biosynthesis genes (VCT) found within mapped QTL regions (Buchanan-Wollaston *et al.*, 2003). Chen *et al.* (2008) reports on the identification of 42 SAGs that are associated with the senescence of broccoli florets, these include ethylene related genes, metabolic related genes and genes with other functions, but the location of these genes in the genome is unknown.

1.6.3 Genetic Markers

Genetic Markers are defined by Kumar (1999) as being specific locations on chromosomes which act as landmarks for genome analysis. The locations that the markers occupy within the genome are termed loci (Collard *et al.*, 2005). Genetic markers represent genetic differences between individual species or organisms and are often near or linked to genes controlling traits of interest. However, they do not affect the phenotype of the trait (Collard *et al.*, 2005). Three types of genetic markers exist: morphological (visible), biochemical and molecular (DNA) markers (Collard *et al.*, 2005), although only molecular markers will be reviewed here.

Molecular (DNA) Markers

Molecular markers or DNA markers exist due to the occurrence of variation at the DNA sequence level, which can be caused by mutations in the form of point mutations, rearrangements (insertions and deletions) and errors in DNA replication (Jones *et al*., 2009). Molecular markers are highly abundant and are widely used in genetic analysis as they have the advantage of being neutral; they do not affect the

phenotype and are often located in the non-coding regions of DNA (Jones *et al*., 2009). Molecular markers can be classified into six distinct types:

(1) **Hybridisation based markers**: restriction fragment length polymorphism (RFLP) and oligonucleotide fingerprinting (Gupta *et al.,* 1999).

(2) **PCR based markers**: microsatellite or simple sequence repeat (SSR), random amplified polymorphic DNA (RAPD) which can be converted into sequence characterised amplified regions (SCAR), amplified fragment length polymorphism (AFLP), inter-simple sequence repeat amplification (ISA), cleaved amplified polymorphic sequence (CAP), amplicon length polymorphism (ALP), sequence- specific amplification polymorphism (S-SAP), inter-simple sequence repeat (ISSR), variable number tandem repeat (minisatellite) (VNTR), sequence tagged sites (STS) and sequence amplification of microsatellite polymorphic loci (SAMPL).

(3) **DNA sequencing based markers**: single nucleotide polymorphism (SNP)

(4) **Expressed gene markers within the genome**: expressed sequence tags (ESTs), sequence-related amplification polymorphism (SRAP) and target recognition amplification protocol (TRAP).

(5) **DNA chip markers**: Microarrays and diversity array technology (DArT).

(6) **Other marker systems**: single – strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), temperature gradient electrophoresis (TGGE) and methylation – sensitive PCR.

In this study, two PCR based molecular markers; SSRs and AFLPs were chosen to screen the parental genotypes (Mar34 and GD33) of the MGDH mapping population for the presence of polymorphic loci. Therefore, these types of markers will be

reviewed here. The advantages and disadvantages of other types of commonly used molecular markers are summarised in table 1.2.

Microsatellites (SSRs)

Microsatellites or simple sequence repeats (SSRs) are polymorphic loci present in nuclear DNA comprising of repeating units of 1-6 base pairs that can be repeated up to 100 times, this number varies in different lines therefore size can be used as a marker with flanking sequence primers (Tonguc and Griffithis, 2004). The most abundant types of SSR in plants is the (AT)n dinucleotide repeat (Jones *et al*., 2009). SSR markers are co-dominant and molecular polymorphisms occur as a result of differences in the number of repeats between plant varieties, which, can be detected using polymerase chain reaction (PCR). Using SSRs as molecular markers has many advantages, as they are easily detectable by PCR, they require low molecular weight DNA; they are highly abundant and are located throughout eukaryote genomes. Microsatellites were chosen to genotype the MGDH population in this study due to the abundance of publically available SSRs designed specifically for the *Brassica* species (Lowe *et al.*, 2004; Szewe-McFadden *et al.*, 1996; Lagercrantz *et al.*, 1993).

Amplified Fragment Length Polymorphisms (AFLPs)

The AFLP technique developed by Vos *et al*. (1995) produces a unique, reproducible DNA fingerprint (or profile) for each individual, by generating dominant markers from the digestion of total genomic DNA with restriction enzymes, followed by selective amplification of DNA fragments by PCR (Meudt and Clarke, 2007). The advantages of this technique are: (1) the production of multiple polymorphic markers (30-50) from a single-primer combination (2) the markers generated are often found

to be widely distributed throughout out the genome, permitting an evaluation of genome-wide variation (3) no prior sequence information is required (4) they are effective when genetic variability is low and (5) they allow the rapid generation of data (Meudt and Clarke, 2007). Disadvantages of the technique include high set up costs and moderate time and labour investment due to the technical difficulty of the system (Jones *et al*., 2009). The main applications of AFLPs are in high-resolution mapping and marker assisted selection (MAS). Due to the narrow genetic background of a broccoli x broccoli interspecies cross, AFLPs were chosen as an appropriate marker type for use this study.

Table 1.2: List of the different molecular marker types: hybridisation, PCR, DNA sequencing, expressed genes, DNA chip and other marker systems and their advantages and disadvantages summarised in Jones *et al*. (2009).

Marker	Advantages	Disadvantages
RFLP	Co-dominant marker, Highly reproducible	Require microgram amounts of DNA Low multiplex ratio Time and labour extensive.
CAP	Insensitive to DNA methylation No requirement for radiolabelling Co-dominant marker	Informative PCR products
RAPD	Time and labour effective No prior knowledge of sequences required No requirement for radiolabelling	Dominant marker Not easily transferred between sp Poor reliability and reproducibility
S-SAP	Targets DNA sequence of genes, retrotransposons or sequences of interest	Dominant marker, Sequence must be known for primer design
ISSR	Technically simple No prior genomic information required	Dominant marker
VNTR	Multi-allelic forms of single loci	Low resolution fingerprints in plants
STS	Useful in mapping (map alignment)	Requires sequence knowledge
	Co-dominant marker	
SCAR	May be dominant or co-dominant, better reproducibility that RAPDs	
SAMPL	Co-dominant marker, high multiplex ratio extensive levels of polymorphism	Stutter bands/peaks Blurred banding/traces
SNP	Highly abundant, high throughput, easily automated, time, cost & labour effective	Biallelic
EST	Useful for gene discovery, reveals novel transcripts, easy to detect and sequence	Error-prone Depends on isolation of mRNA
SRAP	Reveals genetic diversity among related sp Co-dominant marker, highly reproducible Targets coding sequence, simple to use High throughput No prior knowledge required	Complex to analyse Detects null alleles
TRAP	Exploits EST sequence from public databases Highly informative, simple to use Uses markers targets to a specific gene	Requires cDNA/EST sequence information for primer design
Microarrays	Scans whole-genome, High throughput Genotype-phenotype relationship can be established Expression analysis of many genes	Expensive, Technically difficult Requires gene sequence data
DArT	High throughput Detects single base changes and INDELs No prior sequence knowledge required	Dominant markers Technically difficult
SSCP	Detects DNA polymorphism/mutations in DNA fragments at multiple sites	Temperature dependent Sensitive to pH
DGGE	Uses sequence differences to separate individual sequences for a mixture of microbes	500 bp size limitation Difficult to separate fragments that differ by 1 or 2 bp
TGGE	Very similar to DGGE but more reliable Uses a temperature gradient	Limited use in plants Technically difficult
Methylation Sensitive PCR	Detects DNA methylation sites	

1.6.4 Genetic linkage maps

Genetic linkage maps constructed using molecular markers exist for many species including humans, animals and crops and provide invaluable tools to study the organisation of genomes (Schmidt *et al*., 2001). The construction of genetic linkage maps involves the ordering of molecular markers, the assignment of markers to linkage groups and the estimation of relative genetic distances between markers, based on the calculation of recombination frequencies from all pairwise combinations (Jones *et al*., 1999). Referring to basic concepts of segregation and recombination, where a recombination frequency of 1% is equivalent to one arbitrary map unit (Centimorgan, cM), markers that are located in close proximity will have smaller recombination frequencies and therefore shorter map distances (cM) compared to markers that are located further apart (Jones *et al*., 1999). Markers that map together to create one linkage group are assumed to be located in a single chromosome; hence the number of different linkage groups resulting from the mapping process should reflect the basic chromosome number of the species (Jones *et al*., 1999).

1.6.5 Quantitative trait loci (QTL) and QTL analysis.

Quantitative trait loci (QTL) refer to genomic regions that are associated with the variation in complex, quantitative traits (Borevitz and Chory, 2004). Detecting and locating loci affecting quantitative traits relies on the combined analysis of phenotypic data and genotype information on the segregation of markers from individuals or lines (Asins, 2002). QTL analysis makes use of mapping populations in which a cross has been made between parental lines that differ for one or more quantitative traits (Asins, 2002). By analysing the segregation of the progeny, QTL

can be linked to DNA markers (Asins, 2002). Other factors to consider for QTL analysis are the size of a mapping population and the number of markers used in the genotyping process. Mapping population size should be large enough to capture the variation of the trait of interest so that the phenotype - genotype relationship can be determined. To create a genetic linkage map with sufficient marker saturation and even coverage, an appropriate number of markers need to be selected; ideally the number and type chosen should span as much of the genome as possible.

The identification of QTL can serve many purposes. In plant breeding, the goal is often to identify loci that improve crop quality or yield, for the introduction of the beneficial alleles into existing commercial cultivars, for the production of elite breeding lines (Borevitz and Chory, 2004). Alternative uses of QTL are to determine the number, location and interaction of QTL or to identify candidate genes underlying the QTL. To locate and determine the number QTL that explain the variation for a trait, computer programs such as MapQTL$^{(R)}$ and QTL cartographer are used. These software programs calculate the positions of QTL in a genetic linkage map in mapping populations of diploid species (Van Ooijen *et al*., 2002). Common QTL mapping approaches include: interval mapping, MQM mapping and marker regression.

Interval mapping (single QTL model) overview.

Detecting QTL by using the interval mapping method was first developed by Lander and Botstein (1989). This method determines the likelihood that a single QTL is segregating (H1) for each position in the genome (i.e. every centimorgan) against the likelihood that no QTL are segregating (H0), by calculating a likelihood ratio

statistic called the LOD score (logarithm of the odds). If the LOD score exceeds a predefined significance threshold (*P>0.05) in a linkage group a segregating QTL is detected. However, determining the LOD threshold in a QTL analysis is difficult due to linkage, as closely linked markers have equivalent test statistics as a result of neighbouring positions in the genome not being independent (Van Ooijen, 1999). Therefore, appropriate LOD threshold often will depend on the genome size and linkage group lengths (cM) of the species in which the QTL analysis is being carried out.

Multiple QTL model (MQM)

The MQM mapping technique developed by Jansen (1993, 1994) and Jansen and Sam (1994) is an extension of interval mapping, based on multiple QTL models. The MQM approach first identifies putative QTLs by using interval mapping; markers that are in close proximity to the detected QTLs are then selected as cofactors for use in MQM mapping. The cofactors act to reduce the residual variance as they are simultaneously fitted whilst the method performs a one dimensional search over the genome. Therefore, if a QTL detected in interval mapping explains a large proportion of the total variance, selecting cofactors linked to the QTL in MQM mapping will allow the detection of other segregating QTL masked by the original QTL.

1.7 Transgenics as an alternative genetic approach to improve shelf life in broccoli.

The production of transgenics through genetic transformation provides an alternative genetic approach to delaying senescence in broccoli by *Agrobacterium*-mediated

transformation of genes (Chen *et al*., 2008). Targets for transformation in broccoli include: ethylene biosynthesis genes (ACC synthase and ACC oxidase), an ethylene receptor gene (*boers*), a gene encoding chlorphyllase (*BoCHL1*) and a cytokinin biosynthesis gene (*ipt*).

1.7.1 Transformation of ethylene related genes.

The use of transgenics as a strategy to block ethylene production or confer ethylene insensitivity has been reported in tomato, *Arabidopsis* and broccoli (Wilkinson *et al*., 1997; Chen *et al*., 2008; Gapper *et al*., 2005; Higgins *et al*., 2006). Studies that attempt to block ethylene production in broccoli aim to disrupt the expression of ethylene biosynthesis genes (ACC synthase and ACC oxidase). A study by Higgins *et al*, (2006) reports on producing transgenic broccoli with improved shelf life of up to 2 days, by co-transforming *Brassica oleracea ACC synthase 1* and *ACC oxidase 1* and *2* in sense, and antisense orientations in GDDH33, using a *A. rhizogens* Ri vector. Broccoli transformed with all constructs were shown to produce significantly less postharvest ethylene and also showed a reduction in chlorophyll loss when compared to untransformed plants.

Ethylene insensitivity in *Arabidopsis* is conferred by the *etr1-1* gene that encodes a mutated ethylene receptor, Wilkinson *et al*. (1997) was able to introduce the mutated receptor from *Arabidopsis* by transformation into petunia and tomato. The expression of the gene lead to an impaired response to ethylene, which resulted in delays in fruit ripening, flower senescence and flower abscission. Ethylene insensitivity has also been seen in transgenic broccoli created by Chen *et al*. (2003) through site directed mutagenesis of the broccoli ethylene-sensor-response (*boers*)

gene, which was found to delay the senescence in broccoli by 1-2 days in detached leaves and harvested broccoli florets.

1.7.2 Other examples of broccoli transgenics.

Targeting genes for transformation that control chlorophyll catabolism and cytokinin production have also been successful in extending the shelf life of broccoli. Transgenic broccoli with antisense chlorophyllase (*BoCHL1*) described in a study by Chen *et al*. (2008) was observed to improve shelf life by 1-2 days with transgenics exhibiting slower yellowing, based on chlorophyll retention, when stored at 20^{o}C in the dark. Chen *et al*. (2001) also report on retarding postharvest yellowing in broccoli via *Agrobacterium tumefaciens*-mediated transformation of the cytokinin synthesising *IPT* (isopentenyltransferease) gene, as transformed plants where shown to retain chlorophyll in detached leaves and florets after 4 days of storage at 25^{o}C.

1.8 Nutritional Value of broccoli.

Consumers include broccoli in their diets as it is a vegetable with many health promoting properties (Leja *et al.,* 2001). Broccoli is known as a 'functional food' as it contains high levels of vitamins, minerals, glucosinolates and flavonoids (Vallejo *et al.,* 2003; Heimler *et al.,* 2006). These phytochemicals are known to have a protective role against cancer and cardiovascular disease (Bengtsson *et al.,* 2005; Vallejo *et al.,* 2004). However, during post-harvest senescence, the remobilisation of nutrients occurs which causes a decline in the nutritional status of the crop.

1.8.1 Nutrient remobilisation.

The remobilisation of nutrients during developmental and induced leaf senescence is a highly regulated active process that is controlled by many genes, termed senescence associated genes (SAGs), which are up regulated during senescence (Bernhard and Matile, 1994; Buchanan-Wollaston, 1997; Thompson *et al*., 1998; Quirno *et al*., 2000; Yoshida, 2003; Guiboileau *et al*., 2010). Molecular approaches using the model system *Arabidopsis thaliana* have characterised SAGs to be genes encoding protease, lipase and nuclease enzymes involved in the degradation of macromolecules such as proteins, lipids and nucleic acids respectively (Bernhard and Matile, 1994; Buchanan-Wollaston, 1997; Thompson *et al*., 1998; Quirno *et al*., 2000; Yoshida, 2003; Guiboileau *et al*., 2010). The breakdown of macromolecules provides resources for the survival of the plant by recycling nutrients from mature senescing leaves to younger parts of the plant, developing organs and growing seeds, or alternatively, remobilised nutrients are stored for the next growing season (Bernhard and Matile, 1994; Buchanan-Wollaston, 1997; Thompson *et al*., 1998)

Protein degradation and nitrogen remobilisation.

The chloroplasts of a leaf cell are the first organelle targeted for breakdown during senescence as they are a very rich source of protein. Protein degradation occurs via the action of protease enzymes or the ubiquitin pathway which degrades proteins into amino acids such as glutamine and asparagines (Buchanan-Wollaston, 1997). The amino acids are then further catabolised for nitrogen remobilisation and transported via the phloem to developing organs in the plant during senescence (Quirno *et al*., 2000). Nitrogen assimilation is catalysed by the enzyme glutamine synthase (GS) which detoxifies ammonia produced by the deamination of amino acids and

catabolism of nucleic acids by converting it to exportable glutamine (Bernhard and Matile, 1994).

Carbohydrate metabolism.

Carbohydrates such as sucrose and glucose are metabolised during senescence to generate ATP for cellular respiration through the TCA cycle.

Nucleic acid breakdown.

Nucleic acids are broken down by nucleases, RNases and phosphatases in senescing leaves to provide a source of transportable carbon, nitrogen and inorganic phosphorus (Buchanan-Wollaston 1997;). Initially, nucleic acids are degraded into sugars, purines and pyrimidines which are further modified by uricase and xanthine oxidases and directed to developing parts of the plant.

Lipid degradation.

Lipids are highly abundant in plant membranes and are degraded to provide an energy source for respiration and to drive nutrient degradation and remobilisation during the senescence process (Thompson *et al*., 1998; Buchanan-Wollaston, 1997). Lipids are converted to sugars via gluconeogenesis and the glyoxylate cycle with excess sugar that is not required for respiration exported to actively growing parts of the plant.

1.8.2 Glucosinolates

Glucosinolates are sulphur containing plant secondary metabolites that are hydrolysed by myrosinase upon tissue damage such as cutting, chewing or digestion

(Mithen, 2001). The hydrolysis products of glucosinolates include 3 main classes described by Wallsgrove and Bennett (1995) and Jones *et al.* (2006): aromatic (derived from phenylalanine), aliphatic and alkenyl (derived from methionine) and indole (derived from tryptophan). Table 1.3 summarises glucosinolates found in broccoli and their relative abundance (Jones *et al.*, 2006). The hydrolysis products of glucosinolates are often toxic to insects and animals; their presence within plants is believed to act as a form of chemical plant defence to deter damage by herbivores (Shroff *et al.*, 2008). The chemical structure of glucosinolates and their different side chain modifications are also responsible for the array of characteristic flavour compounds found in edible crops (Mithen, 2001). Bitterness in Brussels sprouts is due to 2-hydroxy-3-butenyl and 3-butenyl glucosinolates, where as in Salad rocket

Table 1.3: Dietary glucosinolates found in broccoli (*Brassica oleracea* var. *italica*) and their percentage relative abundance (RA).

Glucosinolate	Systematic Name	Class	RA (%)
Glucoraphanin	4-Methylsulphinylbutyl	Aliphatic	55.5
Glucobrassicin	3-Indolylmethyl	Indolyl	8.6
Gluconapin	3-Butenyl	Alkenyl	7.8
Progoitrin	(2R) 2-Hydroxy-3-butenyl	Alkenyl	7.8
Napoleiferin	2-Hydroxy-4-pentenyl	Indolyl	5.5
4-Methoxyglucobrassicin	4-Methoxy-3-indolylmethyl	Indolyl	3.1
Gluconasturtiin	2-Phenylethyl	Aromatic	3.1
Glucobrassicanapin	4-Pentenyl	Indolyl	2.3
Glucoalyssin	5-Methylsulphinylpentyl	Aliphatic	1.6
4-Hydroxyglucobrassicin	4-Hydroxy-3-indolylmethyl	Indolyl	1.6
Neoglucobrassicin	N-Methoxy-3-indolylmethyl	Indolyl	1.6
Sinigrin	2-Propenyl	Alkenyl	0.8
Glucoiberin	3-Methylsulphinylpropyl	Aliphatic	0.7

the strong pungent flavour is caused by a volatile methylthioalkyl glucosinolate (4-methylthiobutyl isothiocyante) (Mithen, 2001). In humans however, glucosinolates and their breakdown products have been shown to have chemoprotective properties and play a role in cancer prevention (Mithen, 2001). Isothiocyanates are a group of

glucosinolate hydrolysis products known to have many chemoprotective effects including: induction of phase 2 enzymes, which are involved in the detoxification of xenobiotic compounds, antioxidant properties, a capacity to induce apoptosis in cancer cells, the inhibition of *Helicobacter pylori* and the ability to ease hypertension (Krul *et al.,* 2002). Sulforaphane, the breakdown product of glucoraphanin, is the most potent inducer of phase two enzymes and is found in high abundance in broccoli (Johnson, 2002). Findings by Charron *et al.* (2005) also suggest that the content of metabolites, in particular glucosinolates, varies with genotype. Levels of total glucosinolates and indole glucosinolates varied between different *B.oleracea* cultivars. Similar findings were also reported by Jeffery *et al.* (2003) who showed that the synthesis of aliphatic glucosinolates from ten broccoli varieties is primarily regulated by genotype (60%), compared to the environment (5%).

1.8.3 Flavonoids

Flavonoids are naturally occurring phenolic compounds found in fruit and vegetables and can be categorised into 5 classes: flavonols, anthocyanins, hydroxycinnamates, flavanones, flavan-3-ols and the related oligomeric procyanidins (Proteggente *et al.,* 2002). The flavonoid class anthoycyanins are responsible for the orange, red and blue colour seen in vegetables, fruits, flowers and plant storage tissues (Merken and Breecher, 2000). These different pigments attract insects and animals resulting in pollination and seed dispersal (Merken and Breecher, 2000). Dietary flavonoids have many health benefits; they are hydrogen donating radical scavengers, they have activity against allergies, inflammation, viruses, carcinogens, cancer and AIDS (Merken and Breecher, 2000). Broccoli contains large amounts of the flavonoids kaempferol and quercetin (Neilson *et al.*, 1997). Kaempferol is considered to have

anti cancer properties by inducing apoptosis (Yoshida *et al.*, 2008). Quercetin and Kaempferol are also known to be potent free radical scavengers, as antioxidants they may protect against cardiovascular disease (Winkler *et al.*, 2007).

1.8.4 Vitamins and Minerals

Cruciferous vegetables, including broccoli, are a good source of dietary essential vitamins and minerals such as (Table 1.4): vitamins A, C, E, folate, calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), iron (Fe) and zinc (Zn).

1.8.5 Vitamin C.

Unlike humans, plants have the ability to synthesize L-ascorbic acid (vitamin C), which is crucial for the modulation of plant development (Valpuesta and Botella, 2004). L-ascorbic acid in plants is also crucial for photosynthesis, membrane electron transport, cell expansion and the scavenging of reactive oxygen species (ROS) under normal and stress conditions (Wheeler *et al*., 1998; Nishikawa *et al*., 2003). Humans however, have to acquire vitamin C through consumption of fruits and vegetables (Leskova *et al.*, 2006; Kurilich *et al.*, 1999). Vitamin C in plants refers to both the reduced and oxidised forms of ascorbate and is highly abundant in plant tissues, with concentrations of 1-5 mM found in leaves and 25 mM found in chloroplasts (Wheeler *et al*., 1998). Vitamin C is an essential part of the human diet and has many physiological roles within the body; it acts as a co-factor to enzymes involved in the synthesis of collagen, carnitine and neurotransmitters (Naidu, 2003). Vitamin C is also associated with general health and well being, it has been shown to have a role in the prevention and relief of common colds and is essential for wound healing and repair (Naidu, 2003). The benefits of vitamin C also prevent diseases such as cancer and atherosclerosis (Nadiu, 2003). The recommended daily allowance

for vitamin C is 100-120 mg per day for adults (Naidu, 2003). A 100g serving of raw broccoli provides 89.2 mg of Vitamin C with 100g of cooked broccoli (boiled) providing 64.9 mg of vitamin C (Table 1.4). However, it is unclear how stable vitamin C content is once broccoli has been harvested and when is the optimal time (days) after harvest to consume broccoli to benefit most from the active phytochemicals it contains.

Table 1.4 The nutritional value of raw and cooked broccoli (*Brassica oleracea* var. *italica*) in 100g edible portions according to the USDA nutritional database.

		100g edible portion	
Nutrient	**Units**	**Raw**	**Cooked**
Minerals			
Calcium, Ca	mg	47	40
Iron, Fe	mg	0.73	0.67
Magnesium, Mg	mg	21	21
Phosphorus, P	mg	66	67
Potassium, K	mg	316	293
Sodium, Na	mg	33	41
Zinc, Zn	mg	0.41	0.45
Copper, Cu	mg	0.049	0.061
Manganese, Mn	mg	0.21	0.194
Selenium, Se	mcg	2.5	4
Vitamins			1.6
Vitamin C, total ascorbic acid	mg	89.2	64.9
Thiamin	mg	0.071	0.063
Riboflavin	mg	0.117	0.123
Niacin	mg	0.639	0.553
Pantothenic acid	mg	0.573	0.616
Vitamin B-6	mg	0.175	0.2
Folate, total	mcg	63	108
Folate, food	mcg	63	108
Folate, DFE	mcg_DFE	63	108
Choline, total	mg	18.7	40.1
Betaine	mg	0.1	0.1
Vitamin A, RAE	mcg_RAE	31	77
Carotene, beta	mcg	361	929
Carotene, alpha	mcg	25	-
Cryptoxanthin, beta	mcg	1	-
Vitamin A, IU	IU	623	1548
Lutein + zeaxanthin	mcg	1403	1080
Vitamin E (alpha-tocopherol)	mg	0.78	1.45
Tocopherol, beta	mg	0.01	0.01
Tocopherol, gamma	mg	0.17	0.25
Vitamin K (phylloquinone)	mcg	101.6	141.1

1.9 Ascorbate (vitamin C) metabolism

Ascorbate metabolism in higher plants involves the synthesis, recycling and the degradation of ascorbate (Figure 1.7).

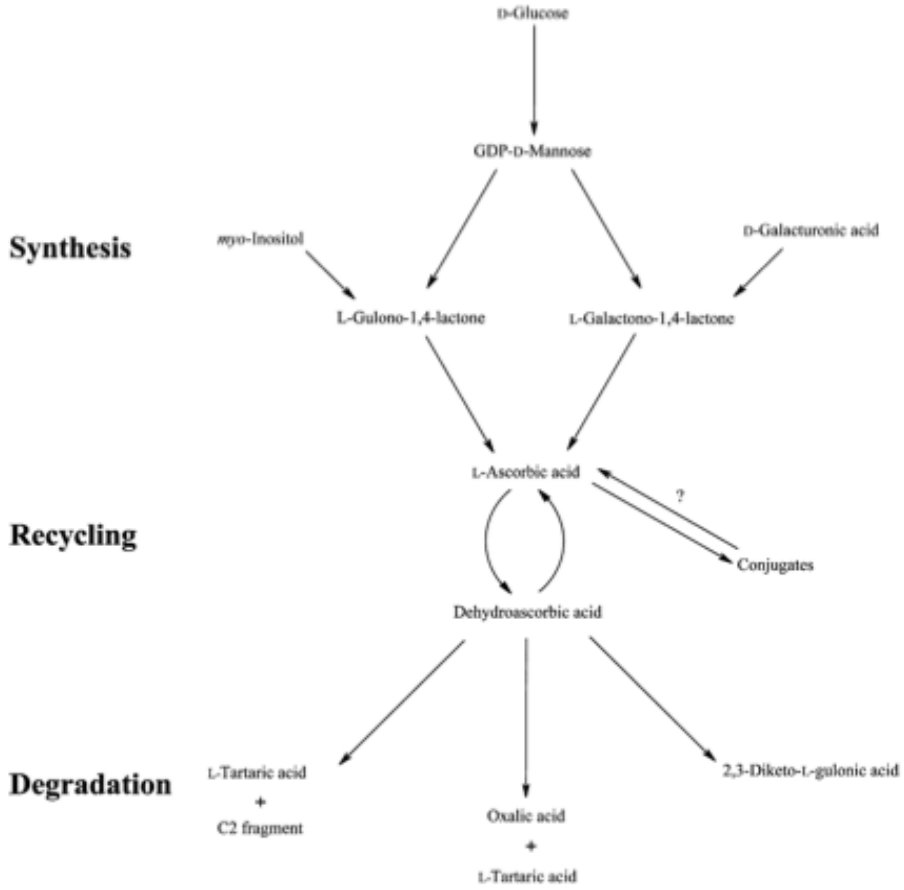


Figure 1.7: An overview of ascorbate metabolism including biosynthsis, recycling and degradation pathways taken from Hancock and Viola (2005).

1.9.1 Ascorbate biosynthesis; the Smirnoff-Wheeler pathway.

The elucidation of the biosynthesis pathway of L-ascorbic acid (AsA) in higher plants has been facilitated by the identification of AsA-deficient mutants in *Arabidopsis.* At present, the most widely accepted pathway for ascorbate metabolism in the scientific community is the Smirnoff-Wheeler pathway, although alternative pathways have been described in the literature.

In vitro studies by Wheeler *et al*. (1998) using the model system *Arabidopsis thaliana* and pea seedlings led to the proposal of the Smirnoff-Wheeler pathway for ascorbate biosynthesis in higher plants. Wheeler *et al*. (1998) provided evidence for a pathway involving GDP-D-mannose, GDP-L-galactose, L-galactose and galactono-1, 4-lactone (Figure 1.8) by the *in vitro* synthesis of ascorbate from GDP-D-mannose via the intermediates outlined. Precursors for ascorbate biosynthesis were identified as D-mannose and L-galactose through the use of feeding and radiolabelling experiments. The application of universally labelled GPD-D-mannose as a substrate in extracts of pea embryonic axes and in ammonium-sulphate precipitates in *A. thaliana* allowed the detection of GDP-mannose-3, 5-epimerase activity that can interconvert D-mannose and L-galactose. L-galactose was confirmed as a precursor of ascorbate biosynthesis by applying L-galactose to leaves of *A. thaliana* and the embryonic axes of germinating pea seedlings, which had the effect of increasing the ascorbate concentration in these tissues. An enzyme detected in cell-free extracts of *A. thaliana* leaves and pea seedlings, L-galactose dehydrogenase, that catalyses the oxidation of L-galactose to L-galactono-1, 4-lactone at the C1 position, without carbon skeleton inversion was also identified. The reaction was shown to be driven

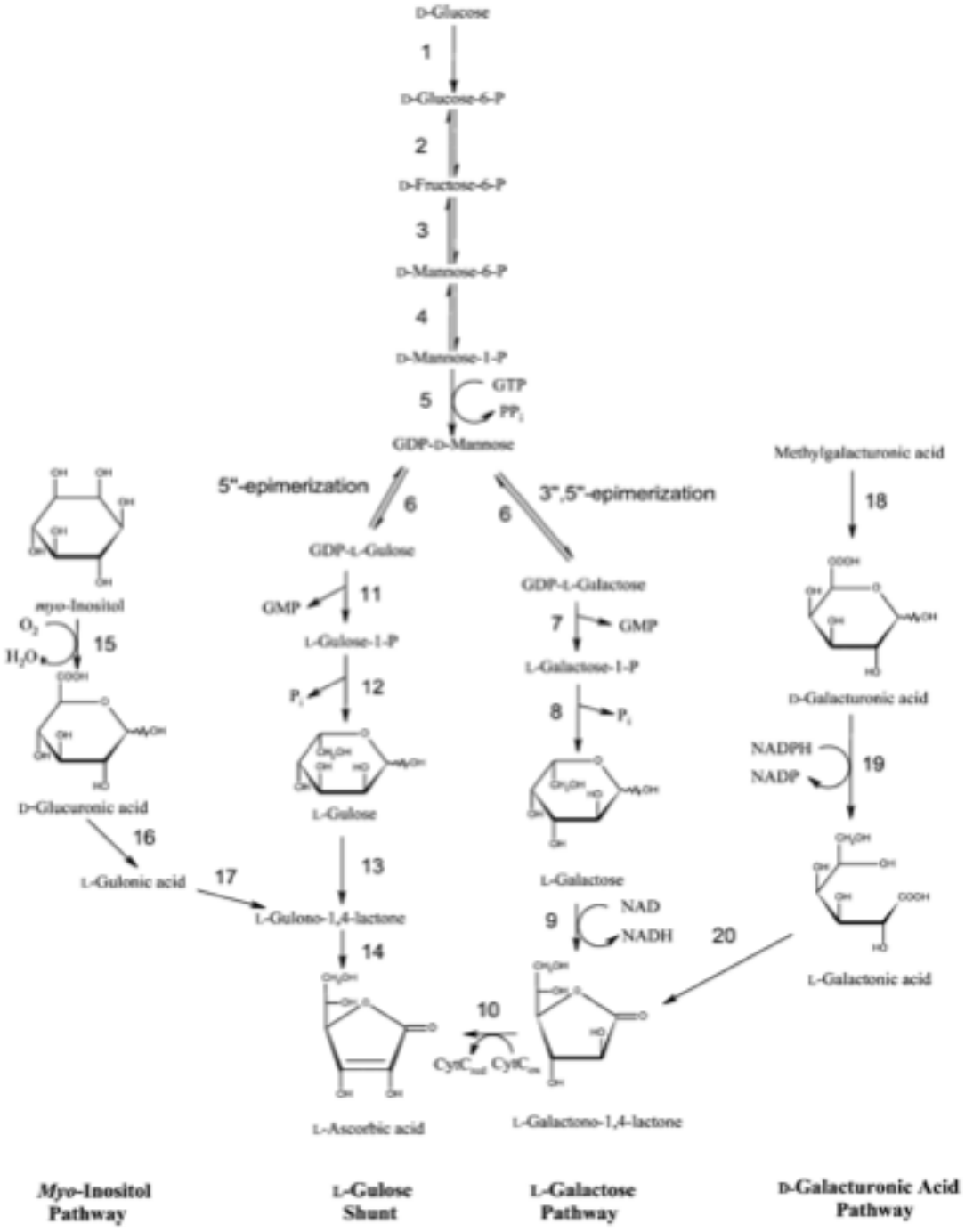


Figure 1.8: Taken from Hancock and Viola (2005). AsA biosynthetic pathways in plants: the L-galactose AsA biosynthetic pathway, the L-Gul shunt, the galacturonic acid pathway and the myo-inositol pathway. Reactions are catalyzed by 1. hexokinase 2. phosphoglucose isomerase, 3. phosphomannose isomerase, 4. phosphomannose mutase, 5. GDP-D-mannose pyrophosphorylase, 6. GDP-D-mannose-3,5-epimerase, 7. GDP-L-galactose pyrophosphatase, 8. L-galactose-1-phosphate phosphatase, 9. L-galactose dehydrogenase, 10. L-galactono-1,4-lactone dehydrogenase, 11. GDP-L-gulose pyrophosphatase, 12. L-gulose-1-phosphate phosphatase, 13. L-gulose dehydrogenase, 14. L-gulono-1,4-lactone dehydrogenase, 15. myo-inositol oxidase, 16. glucuronic acid reductase, 17. aldonolactonase, 18. (pectin) methylesterase, 19. galacturonic acid reducatase, 20. aldonolactonase.

by the reducing power of NAD+. Purification of the reaction product and GC-mass spectrometry (MS) was carried out to verify L-galactono-1, 4-lactone as the reaction product, GC-MS detected one peak that identical to authentic L-galactono-1, 4-lactone (Wheeler *et al*., 1998).

1.9.2 Identification of VTC genes controlling ascorbate biosynthesis from mutant studies in Arabidopsis thaliana.

Four *VTC* genes have been identified in *Arabidopsis,* from mutant studies that encode enzymes in the ascorbate biosynthesis pathway. The first ascorbate deficient mutant in *Arabidopsis* (*vtc 1-1*) was characterised by Conklin *et al*. (1999) to identify the *VTC1* gene. The *Arabidopsis vtc 1-1* mutant is sensitive to ozone and is suggested to be defective in ascorbate biosynthesis as it contains ~25 % of wild type ascorbate concentrations. Conklin *et al*. (1999) demonstrated that the *VTC1* locus in *Arabidopsis* encodes the enzyme GDP-mannose pyrophosphorylase by feeding experiments, mapping of the *VTC1* gene and complementary studies. *Arabidopsis* paralogous genes *VTC2* and *VTC5* have also been identified by Linster *et al.* (2007) to encode an enzyme in the Smirnoff-Wheeler ascorbate biosynthesis pathway. Linster *et al*. (2007) characterised the genes as a histidine triad (HIT) enzyme of the GalT/Apa1 Branch possessing GDP-L-Gal-1 phosphorylase activity for the conversion of GDP-L-galactose phophorylase to L-galactose-1-phosphatae.

Additional studies by Conklin *et al.* (2006) also involved the characterisation of another low ascorbate mutant from *Arabidopsis (vtc4-1)* which exhibits reduced ascorbate biosynthesis to identify the *VTC4* gene, which encodes a L-Gal-1-P phosphatase. This enzyme catalyses the conversion of L-Galactose-1-P to L-Galctose. More recently, the Conklin group have identified the *VTC3* gene from

Arabidopsis using map-based cloning of the *vtc3-1* and *vtc3-2* mutations. The gene is annotated as a novel dual function peptide with a protein kinase and protein phophatase domain. Therefore, Conklin proposed that the *VTC3* gene is a regulatory protein involved in a signal transduction pathway for ascorbate biosynthesis (Abstract, from the plant biology 2010 conference).

1.9.3 Alternative pathways

Three alternative pathways for ascorbate metabolism have been reported in the literature that provide an explanation for observations that are not accounted for by the Smirnoff-Wheeler pathway. These include the D-galacturonic acid pathway, the L-gulose shut and the myo-inositol pathway (figure 1.8).

The D-galacturonic acid pathway.

Evidence for an alternative L-ascorbic acid biosynthesis pathway involving D-galacturonic acid has been reported from studies in strawberry fruit (Figure 1.8). Agius *et al.* (2003), demonstrated that the biosynthesis of L-ascorbic acid in strawberry fruit could occur through D-galacturonic acid by isolating a strawberry gene encoding a NADPH-dependent enzyme D-galacturonate reductase (*GalUR*). To further confirm the involvement of *GalUR* in ascorbate metabolism, Agius *et al*. (2003) over expressed the enzyme in transgenic lines of *A. thaliana* that were engineered to ectopically express *GalUR,* this caused an increase in the levels of ascorbic acid compared to control plants. Furthermore, Agius *et al*. (2003) found that feeding *A. thaliana* plants with D-galacturonic acid also led to elevated levels of ascorbate in transgenic lines, with levels of ascorbate in control plants observed to be unaffected.

The L-gulose shunt

Studies in *Arabidopsis* by Woluka and Van Montagu (2003) led to the proposal of an alternative L-gulose (L-Gul) pathway for ascorbate biosynthesis in plants through the characterisation of *Arabidopsis* GDP-mannose 3', 5'-epimerase (Figure 1.8). Woluka and Van Montagu (2003) found that the epimerase catalysed the formation of an intermediate GDP-L-Gulose through HPLC analysis of the reaction products and *Arabidopsis* cell suspensions. To further support the role of L-gulose in ascorbate biosynthesis *Arabidopsis* plant cells were supplied with cold L-Gul or L-gulono-1, 4-lactone, both were found to increase levels of ascorbate, confirming that L-Gul and L-gulono-1, 4-lactone are converted to L-ascorbic acid by *Arabidopsis* cell suspensions. Woluka and Van Montagu (2003) also propose L-Gulono-1, 4-lactone dehydrogenase as the enzyme that catalyses the final conversion of L-gulono-1, 4-lactone to L-ascorbic acid, with high concentrations of the enzyme found in the cytosolic fraction from potato tubers, inferring the existence of differently localised isozymes.

The myo-inositol pathway

Myo-inositol (MI) has been proposed by Lorence *et al*. (2004) to be a precursor for ascorbate biosynthesis in *Arabidopsis* (Figure 1.8). Molecular evidence supporting an alternative pathway involving MI was achieved from the cloning of an ORF encoding a MI oxygenase (MIOX) indentified in chromosome four (*miox4*) of *Arabidopsis* which Lorence *et al*. (2004) used to constitutively express the *miox4* ORF in *Arabidopsis* plants. Plant over expressing *miox4* were found to have a 2- to 3-fold increase in ascorbate content in leaves compared with wild type plants.

1.9. 4 Ascorbate recycling: ascorbate-glutathione cycle.

The recycling of L- ascorbic acid (AsA) occurs through the ascorbate-glutathione cycle (AsA-GSH), which acts to detoxify reactive oxygen species (ROS) that can cause oxidative damage in plant cells (Figure 1.9). Isoforms of the enzymes involved in the pathway have been found in various organelles that produce ROS such as mitochondria, chloroplasts, persoxisomes and the lumen of the phloem sieve tubes (Hancock and Viola, 2005). The cycle removes ROS through the simultaneous partial oxidation of AsA to monodehydroascorbate (MDHA) and the reduction of H_2O_2 to water and dioxygen by ascorbate peroxidise (Hancock and Viola, 2005). The partially oxidised radical MDHA is then either reduced back to AsA by monodehydroascorbate reductase (MDHAR) which uses NAD(P)H generated from photosynthesis or disproportionates non-enzymatically into the fully oxidised radical dehydroascorbate (DHA) (Hancock and Viola, 2005). To complete the cycle DHA is reduced to AsA by dehydroascorbate reductase (DHAR) which uses reduced GSH as an electron donor (Hancock and Viola, 2005). The reduction of the oxidised form of GSH (GSSG) is catalysed by glutathione reductase (GR) using NADPH as a cofactor (Hancock and Viola, 2005).

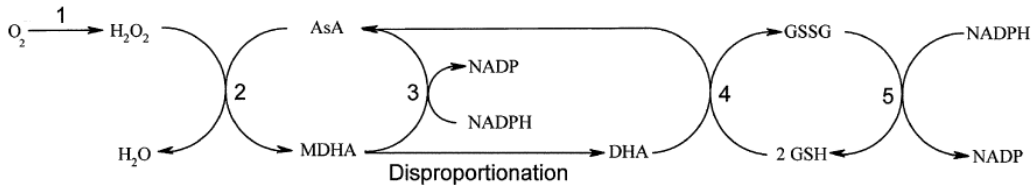


Figure 1.9: The ascorbate-glutathione cycle taken from Hancock and Viola (2005). Reactions are catalyzed by 1. superoxide dismutase, 2. ascorbate peroxidase, 3. monodehydroascorbate reductase 4. dehydroascorbate reductase , 5. glutathione reductase.

1.9.5 Ascorbate Degradation

The degradation of ascorbate occurs through the cleavage of the L-ascorbic acid carbon skeleton to produce oxalate, L-threonate and tartrate (Figure 1.10) (Davey *et*

al., 2000; Hancock and Viola, 2005). Radiolabelling studies have shown that the formation of tartrate occurs through the cleavage of the C4/C5 bond of L-ascorbic acid (or DHA) via 5-keto-L-idonic acid whereas the formation of oxalate and L-threonate is derived from the cleavage of L-ascorbic acid at C2/C3 position (Davey *et al*., 2000, Handcock and Viola, 2005). Oxalate is derived from the C1-2 fragment with L-theronate deriving from the C3-6 can be further oxidised to produce L-tartrate (Davey *et al*., 2000; Handcock and Viola, 2005).

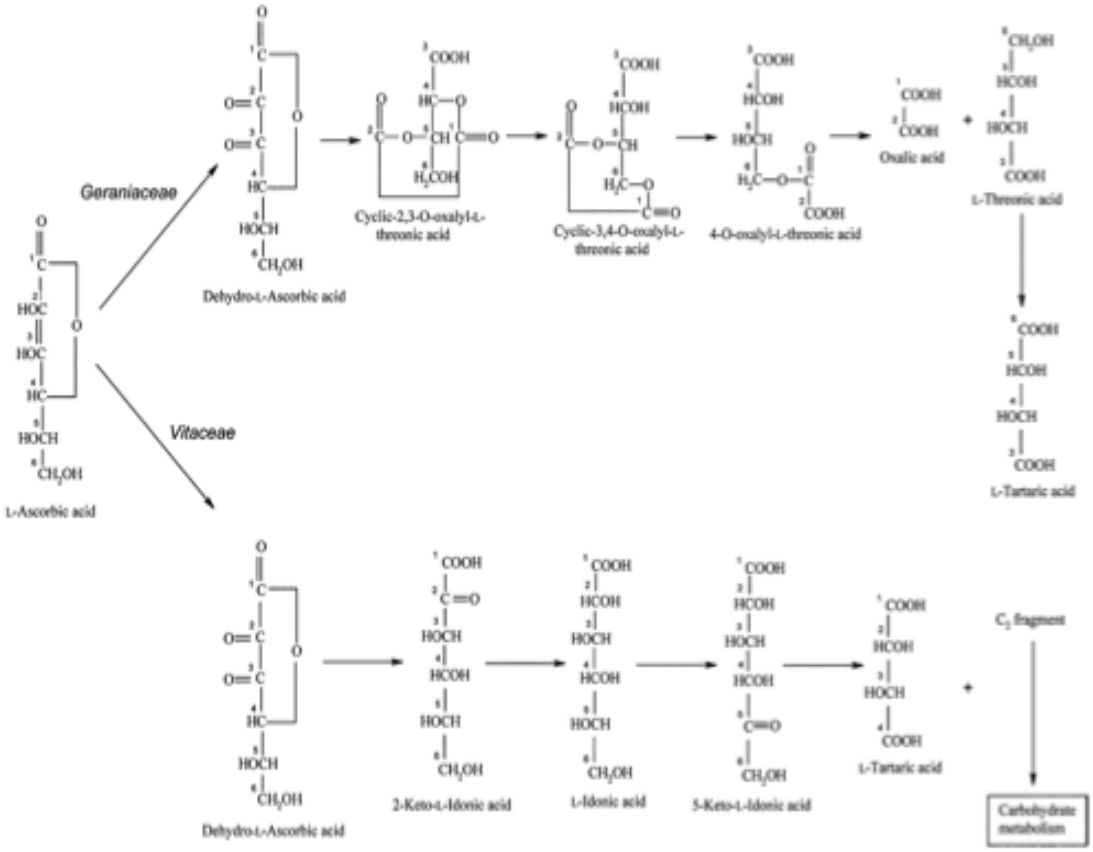


Figure 1.10: The ascorbate catabolism pathways taken from Hancock and Viola (2005). The pathway for vitaceous plants (grape), catabolism proceeds via derivative of L-idonate resulting in the synthesis of L-tartrate and a C2 fragment. The pathway for geraniaceous plants (lemon geranium), DHA undergoes internal bond rearrangements prior to hydrolysis to form oxalate and L-threonate which is subsequently oxidized to tartrate.

1.11 Project Aims

- To develop a new broccoli x broccoli framework linkage map (Chapter 3, Part I)
- To confirm that significant genetic variation exists within the MGDH population for shelf life and morphological traits (Chapter 3, Part II).
- To identify a suitable quantitative method for assessing head yellowing and colour change in broccoli (Chapter 4).
- To confirm that significant genetic variation exists within the MGDH population for vitamin C content at harvest and to assess the stability of vitamin C levels during storage in a sub-set of genotypes (Chapter 5, Part II).
- To carry out a QTL analysis for the identification of QTLs relating to shelf life, morphological traits and vitamin C content and stability (Chapter 3, Part III and Chapter 5, Part II).

CHAPTER 2:
MATERIALS & METHODS

2.1 Plant Material

2.1.1 Commercial Lines: Marathon & Green Duke.

Marathon and Green Duke (Figure 2.1) broccoli are F_1 hybrid commercial cultivars bred by Sakata Seed Corporation (Uchaud, France). Marathon is a widely grown Calabrese variety; it produces a medium uniform high dense dome head of fine blue-green buds. The crop has a high standard of performance, yield and quality and will grow in most soil types and environmental conditions. Marathon has a growing season of May-July in the UK, with harvesting occurring in the months of July-October. Green Duke is also a Calabrese variety, described as an early hybrid that produces a small-medium, compact semi domed head. The buds are medium sized, and green in colour. When grown, plants are compact and highly uniform in habit with slender leaves and therefore are suitable for close planting.

A). Marathon **B). Green Duke**

Figure 2.1: A). The broccoli cultivar Marathon. B). The broccoli cultivar Green Duke.

2.1.2 Production of Mar34 & GD33 doubled haploids (DH).

Doubled haploid (DH) genotypes Mar34 and GD33, were derived from the commercial lines Marathon and Green Duke, respectively, by isolating and culturing microspores from the F_1 hydrid plants for the production of DH plants outlined in figure 2.2

2.1.3 Mar34 x GD33 Doubled Haploid (DH) mapping population.

The doubled haploid lines used throughout the project were developed by Mathas (2004) from an F_1 created by crossing the parental lines GD33 and Mar34 by hand pollination following emasculation of the female. The lines were selected to produce DH lines due to their different shelf life performances. Bud donor F_1 (Mar34 x GD33) plants were used in microspore culture (Figure 2.2) to develop doubled haploid plantlets which were grown to maturity and allowed to self-pollinate to produce seed for subsequent field trials.

2.2 Field Trial Experiments

All replicated field trials were carried out on site at Warwick HRI, UK (latitude: 52.183. Longitude 1.583) during the summers of 2007 and 2008 in the experimental field sites Sheep Pens (West) and Big Cherry respectively. Planting consisted of two guarded replicate field blocks arrange in plots. Each plot contained 24 DH genotypes or 24 F_1 hybrid (Marathon) with 50 cm^2 plant spacing (Appendix A).

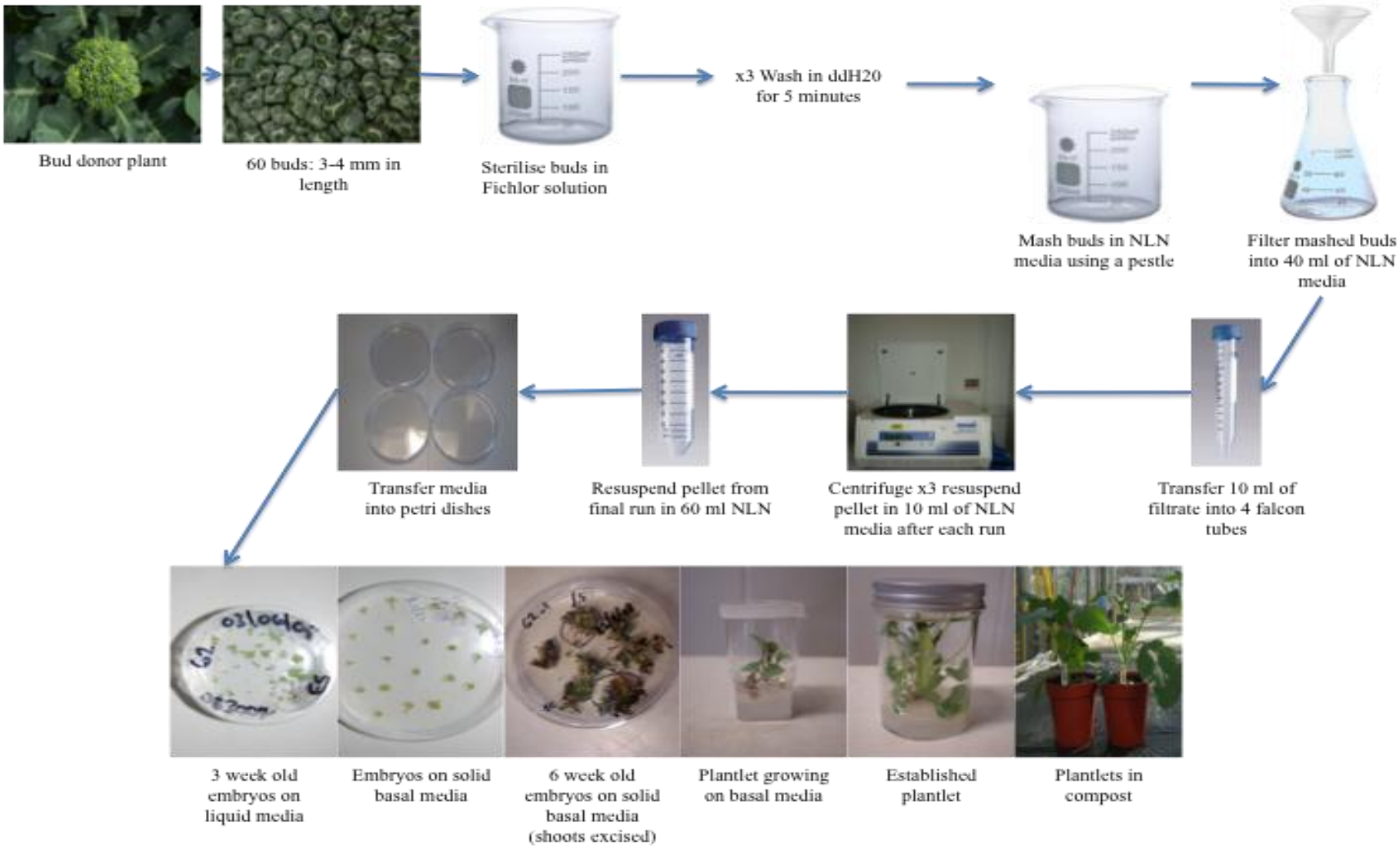


Figure 2.2: A diagramtic representation of the microspore culture process to produce DH lines from broccoli buds. Microspore were isolated from 3-4 mm buds in NLN media, when embryos had formed they were transferred onto basal media for plantlet establishment.

2.2.1 Trial design

The randomisation of planting in the field was carried out by using an alpha design (Patterson and Williams, 1976) created by Dr. James Lynn and Dr. Peter Walley (Appendix A) using the computer programme ALPHA^{+} (Williams and Tablot, 1993). The use of an alpha design in field experiments aims to allow sufficient randomisation of genotypes across the field environment and aims to control plot variation. This allows for valid estimation of error variation and unbiased estimates of means and variances (Masood *et al*., 2008).

2.3 Growing conditions

2.3.1 Sowing

Seed were sown into 'Hassy 308' trays loose filled with Levington F_1 compost (Levington Horticulture, UK). One seed was sown per cell and four lines were sown per tray. A total of 75 seed per genotype were sown into 20 trays in 2007, with 50 seed per genotype sown into ten trays in 2008. Seeds were handled with forceps and pushed down ~5 mm into the soil and covered before being watered.

2.3.2 Glasshouse conditions

Trays containing seed were placed under natural light conditions in a glasshouse for germination, with a day temperature of 16 ^{o}C and a night temperature of 14 ^{o}C. The compartment was vented if temperatures exceeded 18 ^{o}C in the day and 16 ^{o}C in the night. Shade and thermals screens were used to shade the plants if outside light levels were above 300 w/m^2. Once in the glasshouse, trays were given a Basilex drench, to protect against fungal infection. When most plants were at the first true leaf stage, a nutrient solution of 50 ppm KNO_3 was applied to aid and maintain growth. The seedlings were transferred to the cold frame one week prior

to transplanting and covered with plastic netting (Figure 2.3). A boost feed of 200ppm KNO_3 and NH_4NO_3 was applied two days before planting.

A). **B).**

Figure 2.3: Photographs of broccoli plantlets used in the 2007 field trial. A). Plantlets growing in 308 hassy trays in the glasshouse. B). Plantlets in a cold frame for hardening off before transplanting into the field.

2.3.3 Field Preparation

The land was prepared by marking out rows and applying a nitrogen dressing of Equity (chlorpyrifos, 10 ml/litre) onto the ground. Two field replicates were marked out (Figure 2.4). Netting was erected to protect the trial and oscillating irrigation was placed down the outer side of each trial replicate after planting. Dovetail, Plenum WG, Alphox and Hallmark pesticides were applied to control aphids and whitefly. Herbicides Alpha Propachlor and Dacthal were also used. Slug pellets (Lynx) were put down after evidence of leaf damage. Four weeks after planting a 100kg/ha nitrogen topdressing was applied.

2.3.4 Harvesting & storage procedure

Once broccoli heads had formed, field replicates were visually inspected each day to determine if the heads had reached harvest maturity. Broccoli heads were deemed to be mature based when the buds were fully developed. All heads were picked before the buds began to open. Broccoli heads picked for harvesting were of similar size, representative of the plot and taken from the central 'guarded' core within each plot. When harvesting stalks were cut, using secateurs, just above soil level and any outer leaves removed. The stalks were measured and re-cut to 15 cm in length. Trimmed heads were placed into plastic bags labelled with genotype; plot number and a unique consecutive plant number. The bags containing heads were placed into crates for transportation to the field lab for shelf life evaluation and trait assessment. After assessment, crates were stored overnight in a 4^{o}C cold room.

Once the heads for shelf life assessment had been cooled overnight they were transferred to a shelf life room (Figure 2.4) set at 14 ^{o}C with a 16-hour light period to mimic supermarket storage conditions. The shelf life room was illuminated by six (four side lights and two ceiling lights) 1.5 m Orsam L 58W/640, cool white tube lights (Osram, Germany). Cut heads were removed from the plastic bags and placed in seed trays (approximately ten heads per tray) and arranged randomly on metal shelves. Each shelf was illuminated by two parallel 1 m Osram L 36w/835 Lumilux white tube lights (Osram, Germany).

A). **B).**

Figure 2.4: Photograps of broccoli plants grown in the 2007 field trial A). Broccoli plantlets transplanted in the field and B). Harvested broccoli heads in storage in the shelf life facility.

2.4 Shelf life evaluation and trait assessment

2.4.1 Spectrophotometer Readings

Change in colour during storage was measured using a hand held Konica Minolta spectrophotometer device (Model: CM5031) (Figure 2.5). The spectrophotometer measurements enable the quantification of head yellowing. The measuring instrument of the Minolta has a 3 mm aperture and measures colour based on reflectance from the surface of the broccoli head. The Minolta was set to measure colour in co-ordinates using the Hunter Lab colour scale. Before the colour measurements, the spectrophotometer was calibrated using the white surface inside the aperture cap. Head discolouration (yellowing) was measured by performing a total of five colour readings from each broccoli head per day until at least day six or until the heads failed (visually perceived as yellow).

Figure. 2.5: The Konica Minolta hand held spectrophotometer CM5031 used to quantitatively measure the colour of harvested broccoli genotypes and colour change during storage.

The L, a, b readings from each day were then used to calculate chroma and hue angle for each broccoli head using the following equations:

$H = \arctan(b/a)$

$C = \sqrt{(a^2+b^2)}$

where 'a' is the red-green colour co-ordinate and 'b' is the yellow-blue colour co-ordinate. Chroma is a measurement of the intensity of colour, whereas hue angle measures colour in a similar way to human visual perception of colour.

2.4.2 Visual assessment: Wurr quality scoring system.

Each head was visually assessed and given phenotype scores for turgor, head colour, bud break, bud elongation and floret looseness using a scoring system devised by Wurr *et al.* (2001), Table 2.1. Phenotypic scores were given at harvest (day 0) until day 6 or the day of colour failure.

Table 2.1: The scoring system developed by Wurr *et al.* (2001) to assess broccoli quality for the traits stem turgor, head colour, bud compactness, bud elongation and floret looseness.

	Score			
Character	**0**	**1**	**2**	**3**
Stem Turgor	Firm	Slightly Flaccid	Very Flaccid	
Head Colour	Green	Yellow	Bronze	
Bud Compactness	Closed	Opening & yellow petals	Opening & green/ white sepals	Fully open
Bud Elongation	Uniform	Slightly uneven	Individual buds extending	
Floret Looseness	Firm	Florets beginning to separate	Florets wide apart	
	ACCEPTABLE		**UNACCEPTABLE**	

2.4.3 Weight & Weight Loss

Each head was weighed using digital scales at harvest (day 0) and daily, until day six in storage or the day of colour failure. Using the measures of weight during storage, two measures of weight loss could be calculated. Relative weight loss was expressed as the percentage of weight lost between harvest and colour failure whereas absolute weight loss was expressed as the overall amount of weight (g) that was lost between harvest and colour failure.

2.4.4 Head & Peduncle diameter.

Head and peduncle diameter (mm) was recorded by taking two measurements at right angles each broccoli head and peduncle at harvest (day 0) (Figure 2.6).

A). **B).**

Figure 2.6: Diagram of the diameter measurements for A) head diameter. B) peduncle diameter.

2.5 Statistical Analysis

All statistical analyses were performed using GenStat 12th edition (Payne *et al.*, 2007; VSN International, Oxford, UK). Initially, the data was plotted in GenStat to determine if the data had a normal distribution and evenly distributed variances. Data that did not display a normal distribution or uneven variances were $\log_{10}$ transformed to normalise and equalise the variance of the data.

2.5.1 Residual Maximum Likelihood (REML) analysis.

All experimental data, unless stated, was subject to Residual Maximum Likelihood (REML) analysis (Patterson and Thompson, 1971: Thompson and Welham, 2000). REML analysis is a generalised ANOVA suitable for unbalanced designs. Due to uneven sample sizes as a result of some plantlets failing to establish in the field, balanced designs were not possible. Therefore REML analysis was deemed most suitable to analyse the data. To test for significance and assess the contributions of individual terms in the fixed model, a Wald statistic is generated. As the variance

parameters in REML are estimated, the Wald statistic tests the true value of the parameter based on the sample estimate by producing an asymptotically distributed chi-square value.

2.5.2 Correlation analysis

Phenotypic data collected in both growing seasons (2007 and 2008) was subject to correlation analysis. A correlation coefficient (*r*) was generated to measure the linear relationship between traits in a correlation matrix. The correlation coefficient (*r*) ranges from -1 to +1 ($r = 0$, no correlation, $r = -1$, negative correlation and $r = +1$, positive correlation). The *r*-value generated is used to test the null hypothesis, which states that traits are not correlated. The null hypothesis was rejected if the *r*-value exceeded the tabulated *r*-value at the 5 % significance level.

2.6 QTL analysis

Quantitative trait loci (QTL) analysis was carried out on predicted trait means generated from REML analysis in conjunction with the software package MapQTL V4.0 (Van Ooijen *et al.,* 2002). Positions of putative QTLs on the linkage map were detected by using interval mapping (IM) and multiple QTL model (MQM) mapping. The significance thresholds for QTLs were determined by performing a permutation test, which permutes the trait data over individual genotypes whilst keeping the marker data fixed. The permutation test consisted of 1000 iterations to provide a null distribution from which 95% significance LOD threshold was obtained at both linkage group and genome wide levels. A QTL was declared significant if it

exceeded the LOD threshold for genome wide significance and declared suggestive if it exceeded the LOD threshold at linkage group level.

Initially, significant QTLs were detected via a single QTL model using interval mapping. This identified markers that were most closely linked to QTL for selection as cofactors for MQM mapping. Multiple QTL model (MQM) mapping performs in a similar manner to IM but takes into account the presence of other QTLs (cofactors) by statistically controlling the effect(s). After MQM mapping if new QTL(s) were identified the marker(s) associated with the QTL(s) would be incorporated into the next round of MQM mapping until no new QTL were detected. The output of MQM mapping was used to map the positions of genome wide significant QTLs on the linkage map. One and two LOD support intervals around each significant QTL were estimated by taking positions left and right of the peaks that had LOD values one and two less that the maximum LOD at the peak.

CHAPTER 3:

CHARACTERISATION OF GENETIC LOCI CONTROLLING SHELF LIFE IN BROCCOLI

Part I:

Linkage map construction.

3.1. Introduction

The construction of genetic linkage maps through the mapping of molecular markers is only possible if genetic variation is present. Genetic variation arises from differences in DNA sequence caused by mutations and repetitive nucleotide repeats. To construct a new broccoli x broccoli linkage map, genotype information has been compiled from a doubled haploid mapping population derived from a F_1 interspecific cross between two doubled haploid broccoli genotypes (DHGD33 and DHMar34). The parental genotypes of the mapping population have been confirmed by Mathas (2004) to contain sufficient genetic variation from genotyping the individuals using publicly available microsatellites (SSRs) and amplified fragment length polymorphisms (AFLP). This study aims to screen the mapping population with additional SSRs designed for the *Brassica* species and AFLP combinations to improve upon the skeleton linkage map produced by Mathas (2004). The linkage map will be used as a tool, in combination with trait data to identify QTL related to quality traits in broccoli.

Aims

- To confirm polymorphisms are present, at the DNA level, between the parental genotypes DHMar34 and DHGD33 for chosen publicly available SSRs and AFLP molecular markers.
- To genotype the population with markers confirmed to be polymorphic from the parental screen.
- To use the genotype information to create a new broccoli x broccoli framework linkage map.

3.1.2 Materials & Methods.

3.1.2.1 Plant material

The MGDH mapping population consists of a total of 154 DH genotypes developed by Mathas (2004) using microspore culture (see section 2.1.2). Screening for DNA polymorphisms was performed using microsatellite and AFLP markers on the parents of the population initially to identify polymorphic markers, followed by the screening of 94 genotypes from the MGDH mapping population. Segregation data was scored with Genemarker V.1.5 (Softgenetics) to provide a genotype matrix for all genotypes and all markers for the construction of a genetic linkage map.

3.1.2.2 DNA extraction

DNA extraction was performed on single harvested leaves from DH genotypes growing in a glasshouse which had been frozen in liquid nitrogen and stored at -80 ^{o}C. A DNeasy 96 plant mini kit (Qiagen, UK) was used, following the manufacturers guidelines, to isolate genomic DNA from frozen leaf material. Purified DNA was rehydrated in TE buffer (pH 8.0) and stored at -20 ^{o}C.

3.1.2.3 DNA quantification

After extraction, DNA products were visualised on a 1.2 % agarose gel, containing 0.5 µg/ml ethidium bromide (Figure 3.1.1). Three microlitre aliquots of DNA mixed with 2 µl of loading dye were run on the gel for 30 minutes at 80 V in 0.5 x TAE buffer. To quantify DNA levels, bands with varying intensities: low, medium and high from the gel (Figure 3.3.1) were chosen and the extracted DNA related to these was used to quantify the concentration of DNA. The DNA concentration from the

chosen samples was quantified using a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, USA). Using the information for the selected bands, all DNA samples were diluted to concentration of 10 ng/µl and stored at -20 ^{o}C. This DNA dilution plate was used for genotyping with microsatellite (SSR) markers.

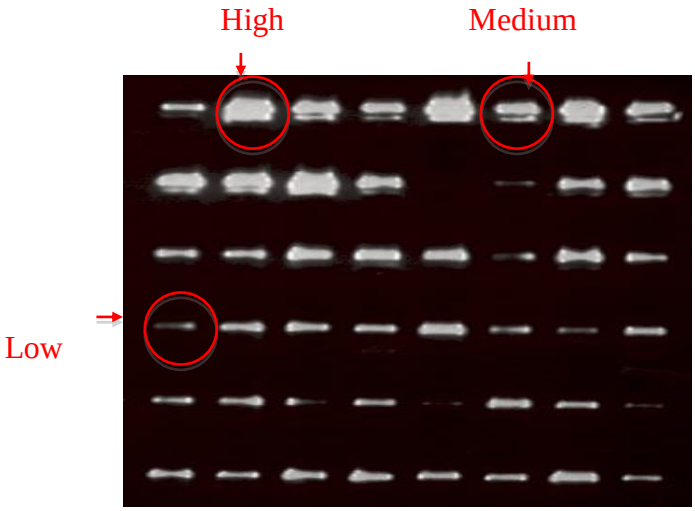


Figure 3.1.1: A gel picture of extracted DNA; high, medium and low intensity bands (circled in red) were selected to determine the DNA concentration of the extracted DNA, so that DNA could be diluted to 10 ng/ml.

3.1.1.4 GenomiPhication

Aliquots from the 10ng/µl DNA dilution plate were amplified to increase the DNA concentration using a GenomiPhi V2 DNA amplification kit (GE Health Care, UK) following the manufacturers guidelines. The GenomiPhi kit allows microgram quantities of DNA to be generated from nanogram starting amounts. Whole genome amplification is achieved by utilising bacteriophage Phi29 DNA polymerase to exponentially amplify single- or double-stranded DNA templates during isothermal strand displacement. After genomiPhication DNA samples were quantified as described above and diluted to a concentration of 100 ng/µl and stored at -20 ^{o}C. This DNA was used for AFLP analysis.

3.1.2.5 Genotyping of the MGDH mapping population.

Genotyping using microsatellite (SSR) markers:

A 10 µl PCR reaction was used to test microsatellite markers for polymorphisms between parental genotypes Mar34 and GD33 with the following reactants and concentrations shown in Table 3.1.1. The reaction mixture was denatured at 95 ^{0}C for 15 minutes, followed by 35 cycles of amplification at 94 ^{0}C for 30 seconds, 56 ^{0}C for 30 seconds and 72 ^{0}C for 30 seconds in a GeneAmp PCR system 9700 (Applied Biosystems, USA). The final cycle was at 72 ^{0}C for 7 min followed by a holding temperature of 20 ^{0}C for 30 minutes. The PCR products were run on 1.5 % agarose gel, containing 0.5 µg/ml ethidium bromide to stain, with 0.5 x TBE gel buffer for approximately 30 minutes at 80 V. The gel was viewed and photographed with a UV illuminator (Mini Darkroom UV Transilluminator, UVP).

Genotyping using fluorescently labelled microsatellite (SSR) markers.

Once polymorphisms had been confirmed using agarose gels, the forward primers of the microsatellite markers were fluorescently labelled with one of five different flurochrome moieties HEX, FAM, VIC, NED or PET (Applied Biosystems, USA). The PCR reactions were repeated, to include the parental genotypes Mar34 and GD33 and 94 genotypes from the MGDH population, using the same parameters as described above but including a fluorescently labelled forward primer. The PCR products with different fluorescent labels were combined for multiplexing, so that the PCR products from up to five reactions could be analysed simultaneously using an ABI 3130xl capillary sequencer (Applied Biosystems, USA), with LIZ-500 as the size standard and the fragment range set at 50 to 500 base pairs. The parental peak

sizes for each microsatellite marker were determined by viewing the labelled PCR products in Genemarker V1.5. To genotype the MGDH population, scoring of the PCR products for all markers was conducted using the peak information from the parental genotypes.

Genotyping using Amplified Fragment Length Polymorphism (AFLP) markers:

An AFLP core reagent kit (Invitrogen, UK) was used following the manufacturers guidelines in conjunction with the protocol developed by Vos *et al.* (1995) to generate AFLP fragments from parental DNA using nine fluorescently labelled E*coR*I/M*se*I

Table 3.1.1: Reactants used in the PCR master mix for the genotyping of the parental genotypes (DHMar34 and DHGD33) and the MGDH population.

Reactant	Supplier	Initial Conc.	Volume (µl)	Final Conc.
PCR Buffer	Qiagen	10 X*	1	1 X**
dNTPs	Invitrogen	2 mM	1	200 µM
HotStarTaq Polymerase	Qiagen	5 Units/µl	0.08	0.04 Units/µl
Forward Primer	Invitrogen	5 µM	0.6	300 nM
Reverse Primer	Invitrogen	5 µM	0.6	300 nM
ddH_20	-	-	5.72	-
DNA (10 ng/µl)	-	-	1	-
			Total: 10	

* 15 mM $MgC1_2$

** 1.5 mM $MgCl_2$

primer combinations (Table 3.1.2.). DNA (100ng/µl) was digested using E*coR*I and M*se*I restriction enzymes and adapter sequences were added using adapter ligation solution and T4 DNA ligase provided in the kit to create primer binding sites for preamplification. DNA fragments were preamplified using E*coR*I and M*se*I primers with one selective nucleotide at the 3' end (E+A and M+C). The preamplified

products were diluted 1:20 in TE buffer. Selective amplification was then carried out using touchdown PCR (Table 3.1.3) with nine different AFLP combinations.

Table 3.1.2: Nine E*coR*I/M*se*I primer combinations used for selective amplification of DNA fragments in the AFLP protocol.

Primer Combinations	
E11M50	E+AA/M+CAT
E11M54	E+AA/M+CCT
E11M61	E+AA/M+CTG
E11M48	E+AA/M+CAC
E11M59	E+AA/M+CTA
E11M47	E+AA/M+CAA
E11M62	E+AA/M+CTT
E11M49	E+AA/M+CAG

Table 3.1.3: Touch-down PCR temperatures and durations used for the genotyping of parental genotypes DHMar34, DHGD33 and the MGDH population. The starting annealing temperature (*) was 65oC; this was lowered by 1^{o}C per cycle during touch-down PCR. The letters m = minutes and s = seconds.

	Touch-down PCR				PCR					
Temperature (^{o}C)	94	94	65*	72	94	56	72	72	4	15
Duration	5 m	30 s	30 s	30 s	30 s	30 s	30 s	20 m	2 m	∞
Cycle	-		x 12			x 25		-	-	-

The M*se*I primers were 5'end labelled with the fluorescent dye FAM (Applied Biosystems, USA). Fragments were analysed using an ABI 3100 capillary sequencer with the size standard LIZ 500 (Applied Biosystems, USA). Polymorphic products and peak sizes were viewed and scored using Genemarker V1.5. The AFLP primer combinations were repeated with each individual in the mapping population and each individual was genotyped based on the peak information from the parental traces.

3.1.2.6 Linkage map construction using JoinMap V 4.0

Linkage map construction was carried in JoinMap V4.0 (Van Ooijen and Vorrips, 2006) using parameters for a DH population. A test for independence within JoinMap was used to group linked loci into linkage groups, the test produces a LOD score as a test statistic and was carried out at several significance thresholds of increasing stringency. Increasing the stringency tests the extent to which loci remained linked at higher LOD scores, as branching of groups can occur at higher LOD scores. In this experiment, linkage groups were selected by choosing the highest LOD score before any branching had occurred within the linkage, therefore LOD scores ranged from 3.5-8.5. To calculate the map distances in centimorgans (cM) between markers in each linkage group the Haldane mapping function (Haldane, 1919) was used.

3.1.3 Results

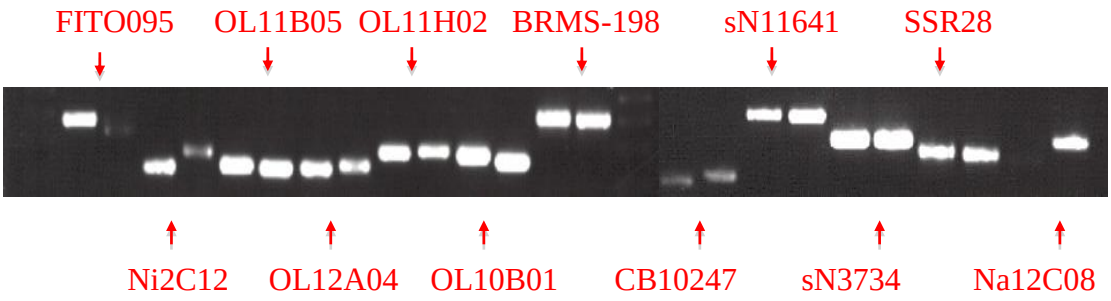
3.1.3.1 Genotyping with fluorescently labelled Microsatellite (SSR) and AFLP markers.

From the initial parental screen of 64 microsatellites markers (Appendix B) developed for the *Brassica* species, 35 were identified as being polymorphic between the parental genotypes DHGD33 and DHMar34 (Table 3.1.4). Microsatellite markers that were confirmed to be polymorphic, produced peaks or bands with distinct size differences (bp) as shown in examples of gel images and chromotograms from the genotyping process (Figure 3.1.2).

Table 3.1.4: List of SSRs confirmed to show polymorphism between the parental genotypes DHMar34 and DHGD33. Parental allele peaks positions for each marker, fluorescent label and multiplex group is also listed.

SRR Marker	Peak Positions		Multiplex Group	Fluorescent Label
	Mar-34	GD33		
Na10D09	156	147	1	FAM
Na12C08	315	271	1	FAM
MB4	0	66	1	HEX
0L10D08	172	186	1	HEX
BN83B1	0	185	2	FAM
BRAS-021	0	208	2	PET
Na12G12	156	131	2	NED
FITO284	304	359	2	PET
BRAS069	196	199	3	FAM
BRMS-071	289	239	3	FAM
0L12G04	124	100	3	HEX
FITO203	276	313	4	FAM
Ra2E12	143	148	4	HEX
0L10B01	181	165	4	FAM
BRMS-042	0	106	4	HEX
OL11B05	139	135	5	FAM
FITO285	185	192	5	VIC
Ni2C12	127	170	5	NED
FITO222	385	414	5	NED
OL11H02	190	196	6	FAM
OL11H06	192	206	6	VIC
OL12A04	132	142	6	NED
Na12C03	208	272	6	NED
A77096	204	0	6	PET
BRAS-026	0	122	7	NED
sN3734	288	286	7	FAM
Ra2G09	254	0	7	NED
BRMS-098	151	171	8	VIC
BRMS-296	146	141	8	NED
BRMS-027	236	0	8	FAM
BRMS-063	238	237	8	NED
sN11641	414	406	9	FAM
SSR28	239	226	9	VIC
Na12F03	325	312	9	NED
FITO095	278	239	10	FAM

A).



B).

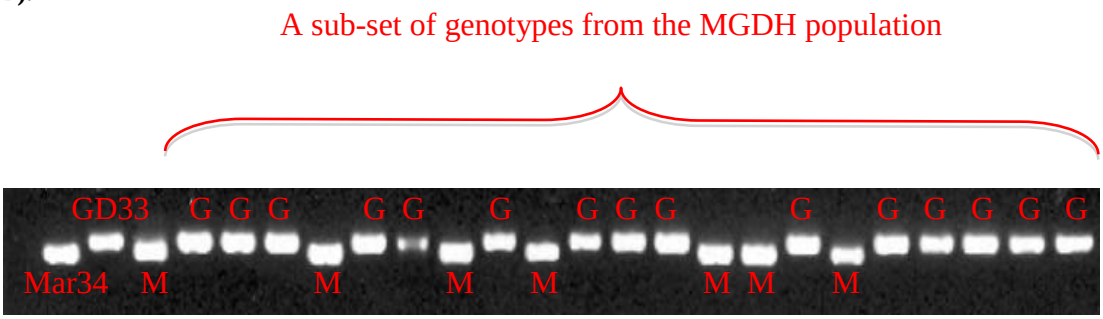


Figure 3.1.2: Gel images of (A) polymorphic bands produced from the parental screen with 12 SSR loci and (B) polymorphic bands produced with SSR loci Na12C08 from parental genotypes Mar34 (M) and GD33 (G) and the segregation of parental alleles in a subset of the MGDH population and (C) A chromatogram from Genemarker V1.5 displaying the polymorphic peaks produced from SSR OL10B01 for the parental genotypes Mar34 and GD33 and the segregation of parental alleles in genotypes MG111 and MG116(27/9) from the MGDH population.

C).

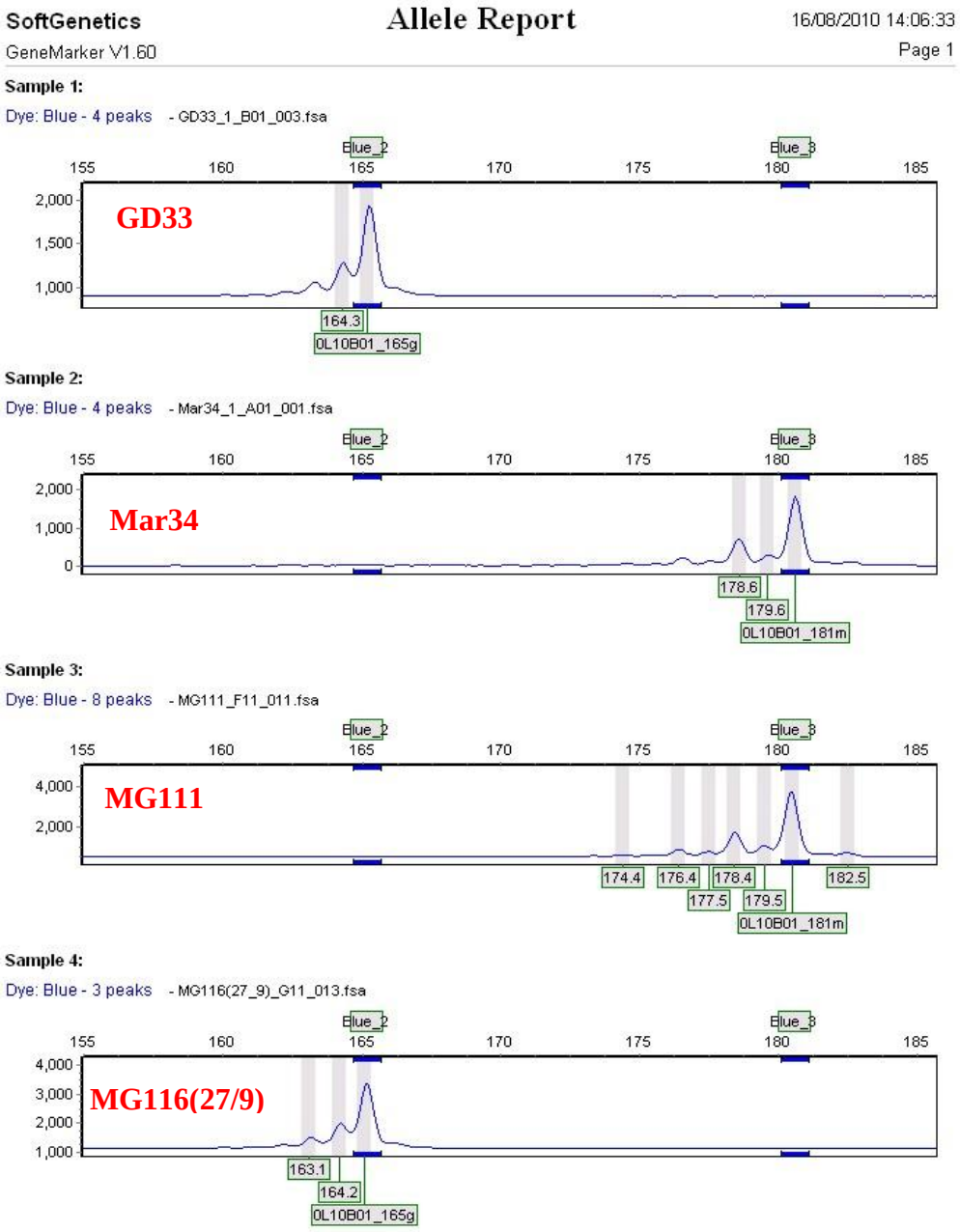


Figure 3.1.2: *Continued.*

A total of seven AFLP combinations were also found to show some polymorphism between the parental genotypes (Figure 3.1.3), however the different combinations varied in the number of polymorphic loci identified. The primer combination E11M48: AA/CAC generated the most polymorphic fragments with primer combination E11M54: AA/CCT producing the least (Table 3.1.5).

Table 3.1.5: Summary of the number of polymorphic loci identified between the parental genotypes DHMar34 and DHGD33 using seven selective AFLP primer combinations.

AFLP Combination	No of polymorphic loci
E11M48: AA/CAC	26
E11M49: AA/CAG	11
E11M50: AA/CAT	15
E11M59: AA/CTA	23
E11M61: AA/CTG	15
E11M54: AA/CCT	10
E11M62: AA/CTT	11
TOTAL	**111**

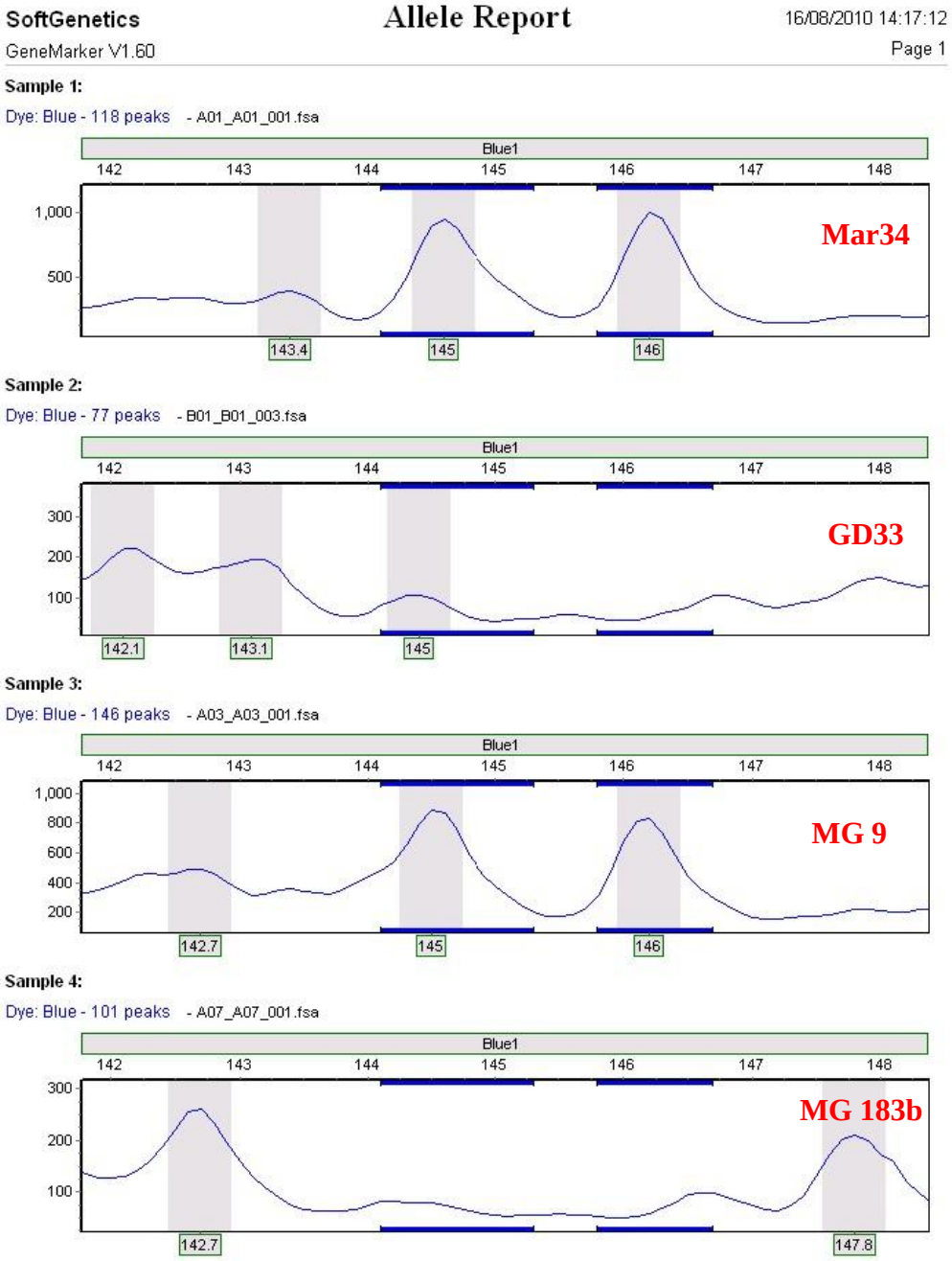


Figure 3.1.3: A chromatogram from Genemarker V1.5 displaying the polymorphic peaks produced from AFLP combination E11M59 (AA+CTA) for the parental genotypes Mar34 and GD33 and the segregation of parental alleles in genotypes MG 9 and MG 183b from the MGDH population.

3.1.3.2 Genotype code

Genotype data was obtained using 35 fluorescently labelled SSRs and seven fluorescently labelled AFLP combinations to produce a colour coded genotype matrix containing 146 polymorphic loci (Figure 3.1.4). The letters A, B (denoting parental genotypes A = Mar34, B = GD33) or U (unscored) are colour coded green, yellow or white respectively indicating the parental allele segregating for each individual genotype from all markers screened with members of the MGDH mapping population (Figure 3.1.4).

A).

		Genotypes							
		MG111	MG116(27/9)	MG16	MG1826	MG1831	MG185B	MG188B	MG284
		1	2	3	4	5	6	7	8
Markers	Na10D09	A	B	B	B	A	A	B	B
	Na12C08	A	B	A	B	A	A	A	B
	MB4	B	B	B	B	A	B	B	A
	0L10D08	B	B	B	A	B	B	B	A
	BN83B1	B	A	A	A	U	B	A	A
	BRAS-021	A	A	A	B	B	B	B	B
	Na12G12	A	B	A	B	A	B	B	A
	FITO284	B	A	A	A	B	B	A	A

Key

A = Mar34 allele

B = GD33 allele

U = Unscored

Figure 3.1.4: (A) An example of a sub section of the colour coded genotype matrix for all markers (SSRs and AFLPs) and genotypes in the MGDH population. The letters A (green), B (yellow) and U (white) denote Mar34 alleles, GD33 alleles and unscorable segregation data respectively.

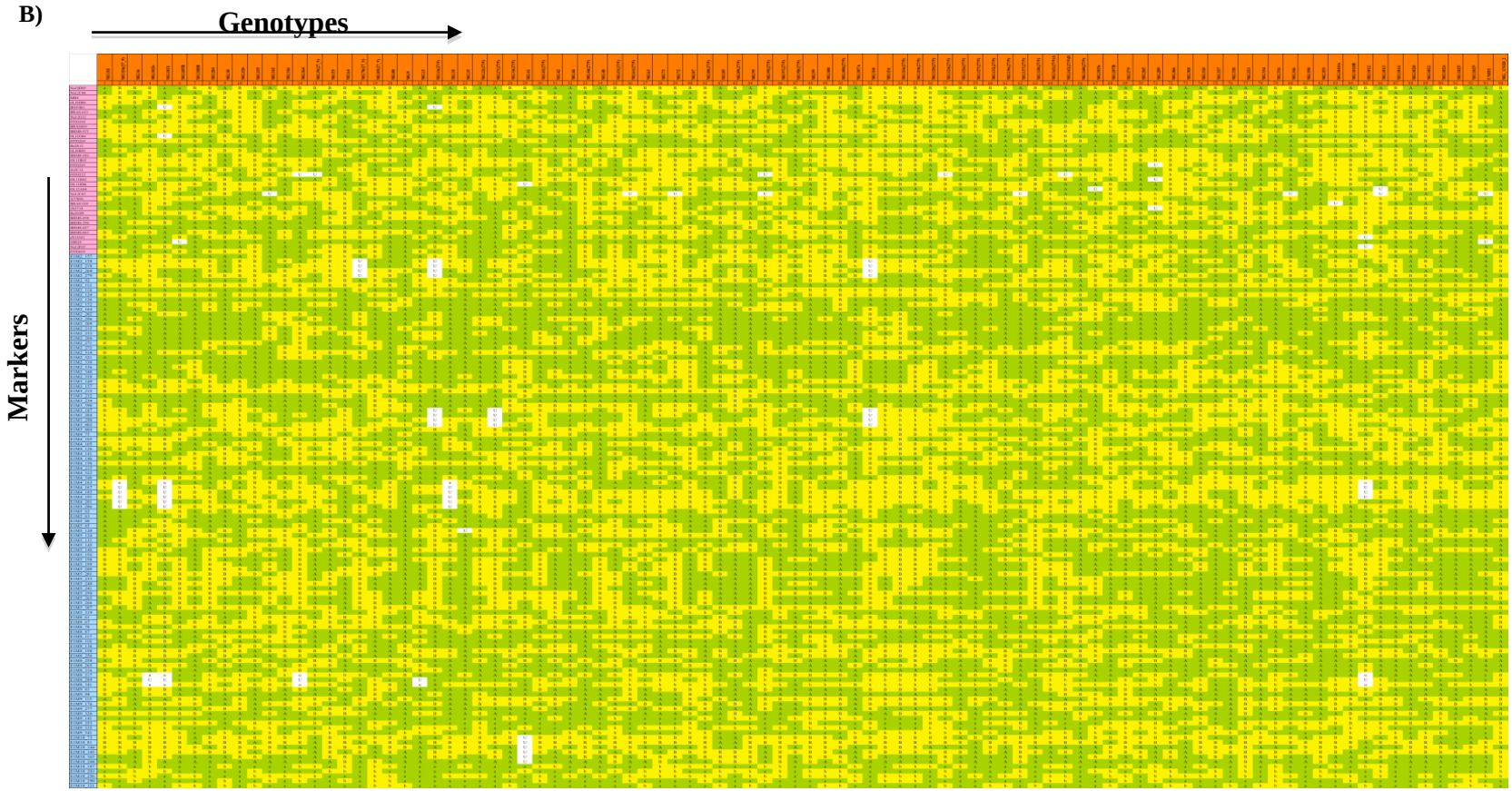


Figure 3.1.4: (B) The colour coded genotype matrix for all markers (SSRs and AFLPs) and all genotypes in the MGDH population. The colours yellow and green represent the segregation of GD33 alleles and Mar34 alleles for the population for each marker respectively. Unscorable segregation data was coloured white.

3.1.3.3 Linkage map construction.

A total of 94 genotypes from the MGDH mapping population were screened with SSR and AFLP markers to generate a broccoli x broccoli framework linkage map (Figure 3.1.5). The framework linkage map produced consists of a total of 80 markers (25 SSR and 55 AFLP loci) mapping to nine linkage groups. The total map length is 712.9 cM which equates to ~79.2 % coverage of the *B. oleracea* genome. Clustering of all loci did not occur and a total of 66 loci remain unmapped (10 SSR and 56 AFLP loci).

3.1.3.4 Linkage group ordering and assignment.

Linkage groups were ordered and assigned according to the universal reference nomenclature convention (www.brassica.info) in which a consensus was agreed to assign consistent linkage group/chromosome nomenclature to diploid *Brassica* genomes in the 'triangle of U' known as A, B and C (Table 3.1.6). By using informative *Brassica* SSRs that have been mapped onto published *Brassica* linkage maps, seven linkage groups have been identified and assigned with the correct nomenclature (Table 3.1.7). The two unassigned linkage groups are LG 4 and LG 8, which are the equivalent to *B. oleracea* linkage groups O4 and O8, but cannot be distinguished in this map using published information. However, using a personal communication from Dr. Peter Walley (unpublished data) the unassigned linkage groups were resolved.

The smallest linkage group is LG 8 (O8) which is 30.2 cM and only contains four loci, whereas the largest linkage group, LG 1 (O1) spans 132.0 cM and contains 15 loci. However, marker saturation of linkage groups does not necessarily coincide with linkage

Table 3.1.6: Correct nomenclature for assigned linkage groups/chromosomes of the *Brassica* species in the 'triangle of U'.

B. *nigra*	**B. *juncea***	**B. *oleracea***	**B. *napus***	**B. *rapa***
	A1 (J1)		A1 (N1)	A1 (R1)
	A2 (J2)		A2 (N2)	A2 (R2)
	A3 (J3)		A3 (N3)	A3 (R3)
	A4 (J4)		A4 (N4)	A4 (R4)
	A5 (J5)		A5 (N5)	A5 (R5)
	A6 (J6)		A6 (N6)	A6 (R6)
	A7 (J7)		A7 (N7)	A7 (R7)
	A8 (J8)		A8 (N8)	A8 (R8)
	A9 (J9)		A9 (N9)	A9 (R9)
	A10 (J10)		A10 (N10)	A10 (R10)
		C1 (O1)	C1 (N11)	
		C2 (O2)	C2 (N12)	
		C3 (O3)	C3 (N13)	
		C4 (O4)	C4 (N14)	
		C5 (O5)	C5 (N15)	
		C6 (O6)	C6 (N16)	
		C7 (O7)	C7 (N17)	
		C8 (O8)	C8 (N18)	
		C9 (O9)	C9 (N19)	
B1	B1 (J11)			
B2	B2 (J12)			
B3	B3 (J13)			
B4	B4 (J14)			
B5	B5 (J15)			
B6	B6 (J16)			
B7	B7 (J17)			
B8	B8 (J18)			

group length, as when comparing the largest linkage group, LG1 (O1), with LG 7 (O7), which has similar numbers of loci (14) loci there is a size difference of 70.9 cM. Overall the linkage map has an average marker interval of 9.5 cM, although the average marker intervals for individual linkage groups vary due to linkage group length and marker saturation (Table 3.1.8).

Table 3.1.7: List of informative SSR markers from published *Brassica* linkage maps that enabled the assignment and ordering of linkage groups in the broccoli x broccoli framework linkage map.

Linkage Group	Marker	Species	Reference Map	Reference LG	Mapping Population
1 (O1)	sN11641	B. *napus*	BnaN-fo-61-9	N11	fo-61-9
1 (O1)	FITO095	B. *oleracea*	BolAG	O1	A12DHd x GDDH33
1 (O1)	Na12C08	B. *napus*	BnaN-fo-61-9	N11	fo-61-9
1 (O1)	sN3734	B. *napus*	BnaN-fo-61-9	N11	fo-61-9
2 (O2)	Ra2G09	B. rapa	BnaN-fo-61-9	N12	fo-61-9
2 (O2)	Ni2C12	B. *napus*	BnaN-fo-61-9	N13	fo-61-9
3 (O3)	OL11B05	B. *napus*	BnaN-fo-61-9	N13	fo-61-9
3 (O3)	BRAS-069	B. *napus*	BnaN-fo-61-9	N13	fo-61-9
5 (O5)	Na12G12	B. *napus*	BnaN-fo-61-9	N15	fo-61-9
6 (O6)	MB4	B. *oleracea*	BolAG	O6	A12DHd x GDDH33
6 (O6)	FITO203	B. *oleracea*	BolAG	O6	A12DHd x GDDH33
7 (O7)	Na12F03	B. *napus*	BnaN-fo-61-9	N17	fo-61-9
9 (O9)	OL11H06	B. *napus*	BnaN-fo-61-9	N19	fo-61-9
9 (O9)	OL10D08	B. *oleracea*	BolAG	O9	A12DHd x GDDH33
9 (O9)	OL12A04	B. *napus*	BnaN-fo-61-9	N19	fo-61-9
9 (O9)	BN83B1	B. *oleracea*	BolAG	O9	A12DHd x GDDH33

Table 3.1.8: Summary of the LOD score, linkage group length, number of loci, loci type and average marker interval of each linkage group in the broccoli x broccoli linkage map.

Linkage Group	LOD	Length (cM)	Number of loci	Number of SSRs	Number of AFLPs	Av. Marker Interval (cM)
1 (O1)	5.0	132.0	15	4	11	8.8
2 (O2)	6.0	60.3	6	2	4	10.1
3 (O3)	4.0	70.0	12	5	7	5.8
4 (O4)	5.5	96.7	7	2	5	13.8
5 (O5)	5.5	126.7	8	1	7	15.8
6 (O6)	8.5	39.9	4	2	2	10.0
7 (O7)	6.5	61.1	14	3	11	4.4
8 (O8)	3.5	30.2	4	1	3	7.6
9 (O9)	3.5	96.0	10	5	5	9.6
TOTAL	**-**	**712.9**	**80**	**25**	**55**	**9.5**

3.1.3.5 Linkage map quality

Segregation distortion.

To determine the degree of segregation distortion, chi-squared tests were used in JoinMap® to determine the genotype frequencies of each locus mapped within the nine linkage groups (Table 3.1.9). Overall, 65 % of mapped loci exhibit significant segregation distortion (**P>0.05*-****P>0.001*) from the expected 1:1 Mendelian ration for a doubled haploid population**.** The loci displaying segregation distortion favoured GD33 alleles (56 %) over Mar34 alleles (44 %), with a higher proportion of segregation distortion being observed in the mapped AFLP loci (71 %) compared with the mapped SSR loci (29 %) (Table 3.1.10). Segregation distortion was observed within all linkage groups which all contain distorted loci biased towards one parent. Loci in linkage groups LG2 (O2), LG3 (O3), LG4 (O4), LG 5 (O5) and LG6 (O6) are biased towards GD33 alleles where as linkage groups LG7 (O7) and LG8 (O8) are biased towards Mar34 alleles (Figure 3.1.6). The remaining linkage group (LG 1 (O1), has distorted loci segregating for both parents, however there is more of a bias towards GD33 alleles. Loci showing segregation distortion appear to be clustered (figure 3.1.6), this implies some selection during the production of the MGDH population during microspore culture for regeneration and seeding.

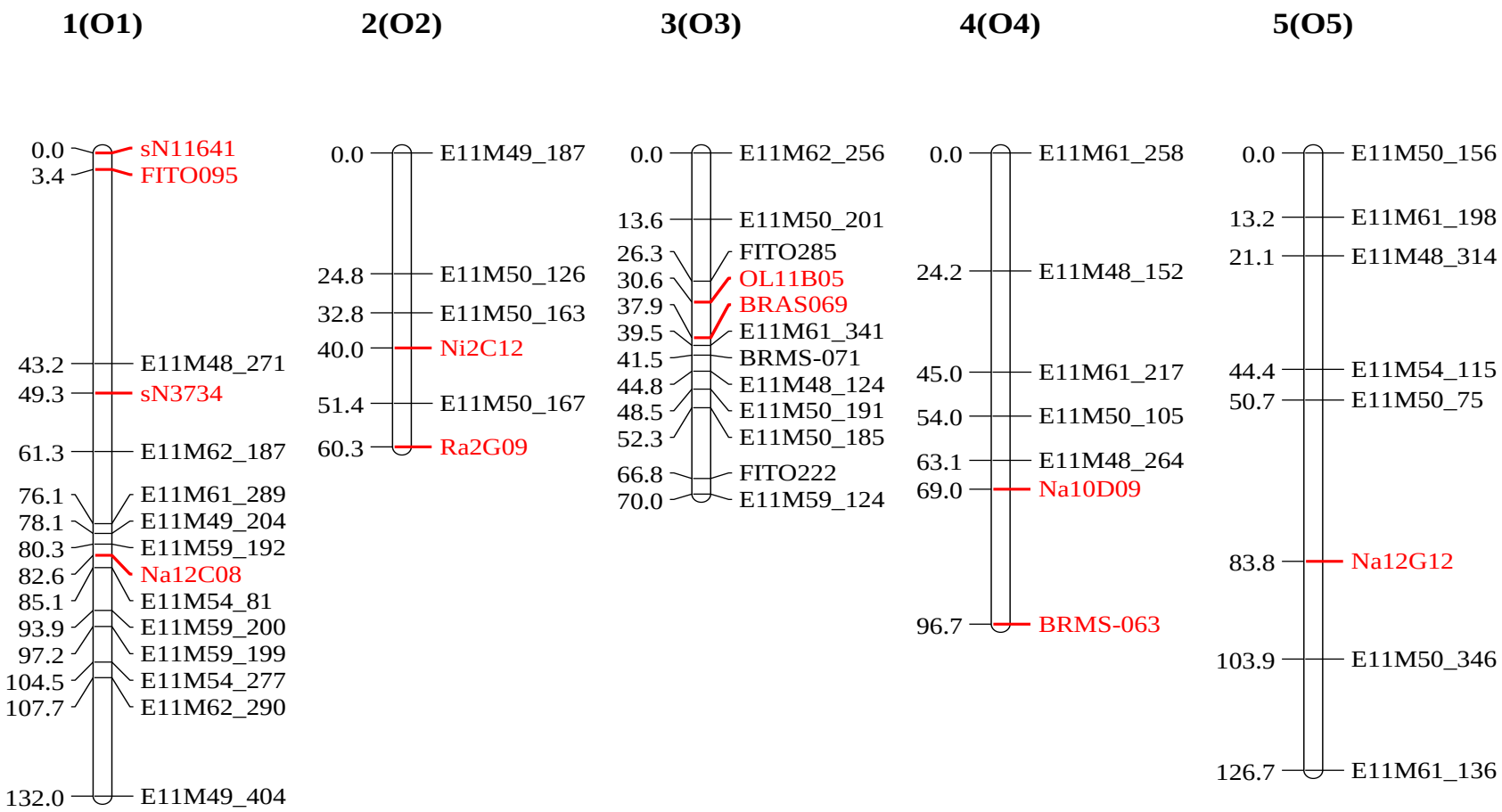


Figure 3.1.5: The broccoli x broccoli framework linkage map, linkage groups O1-O5. Informative SSR loci that were used to identify and order linkage groups according to correct nomenclature for the *Brassica* species are highlighted in red.

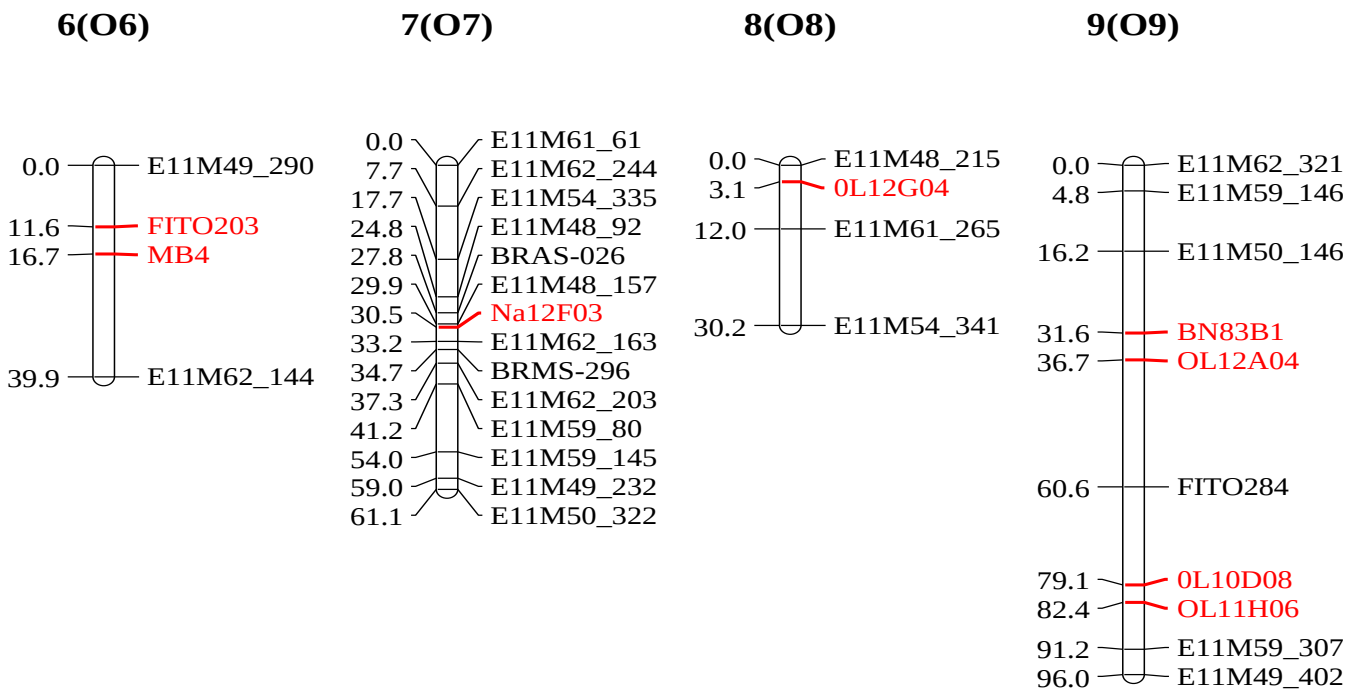


Figure 3.1.5: The broccoli x broccoli framework linkage map, linakge groups O6-O9. Informative SSR loci that were used to identify and order linkage groups according to correct nomenclature for the *Brassica* species are highlighted in red.

Table 3.1.9: Summary of the chi-square test for loci displaying segregation distortion within each linkage group. The proportion of alleles segregating are shown as a, b and u this corresponds to Mar34 alleles, GD33 alleles and unscorable alleles**.** Significance levels are represented as * (*P>0.05*), ** (*P>0.01*) and *** (*P>0.001*).

Linkage Group	Locus	a	b	u	X^2	df	Signif.	Direction
LG1(O1)	E11M59_199	59	35	0	6.13	1	*	Mar34
	E11M49_404	34	60	0	7.19	1	**	GD33
	E11M54_81	60	34	0	7.19	1	**	Mar34
	E11M61_289	57	32	5	7.02	1	**	Mar34
	E11M62_187	60	34	0	7.19	1	**	Mar34
	E11M49_204	59	32	3	8.01	1	**	Mar34
	Na12C08	63	31	0	10.89	1	***	Mar34
	E11M59_192	64	30	0	12.3	1	***	Mar34
	E11M62_290	72	22	0	26.6	1	***	Mar34
	E11M54_277	73	21	0	28.77	1	***	Mar34
LG2(O2)	E11M49_187	31	60	3	9.24	1	**	GD33
	Ra2G09	31	63	0	10.89	1	***	GD33
LG3(O3)	E11M62_256	32	62	0	9.57	1	**	GD33
	E11M50_201	31	60	3	9.24	1	**	GD33
	FITO285	26	67	1	18.08	1	***	GD33
	OL11B05	15	79	0	43.57	1	***	GD33
	BRAS069	25	69	0	20.6	1	***	GD33
	E11M61_341	15	74	5	39.11	1	***	GD33
	BRMS-071	12	82	0	52.13	1	***	GD33
	E11M48_124	16	78	0	40.89	1	***	GD33
	E11M50_191	18	72	4	32.4	1	***	GD33
	E11M50_185	16	74	4	37.38	1	***	GD33
	E11M48_124	30	64	0	12.3	1	***	GD33
LG4(O4)	E11M61_258	57	37	0	4.26	1	*	Mar34
	E11M50_105	36	58	0	5.15	1	*	GD33
	E11M48_264	33	58	3	6.87	1	**	GD33
	Na10D09	34	60	0	7.19	1	**	GD33
	BRMS-063	32	62	0	9.57	1	**	GD33
LG5(O5)	E11M50_346	36	58	0	5.15	1	*	GD33
	E11M50_156	21	73	0	28.77	1	***	GD33
	E11M61_198	27	67	0	17.02	1	***	GD33
	E11M48_314	21	73	0	28.77	1	***	GD33

Table 3.1.9 *continued*.

Linkage Group	Locus	a	b	u	X^2	Df	Signif.	Direction
LG6(O6)	FITO203	35	59	0	6.13	1	*	GD33
	MB4	34	60	0	7.19	1	**	GD33
LG7(O7)	E11M62_163	57	36	1	4.74	1	*	Mar34
	BRMS-296	60	34	0	7.19	1	**	Mar34
	E11M62_203	61	33	0	8.34	1	**	Mar34
	BRAS-026	59	34	1	6.72	1	**	Mar34
	E11M48_157	60	34	0	7.19	1	**	Mar34
	Na12F03	59	34	1	6.72	1	**	Mar34
	E11M62_244	77	16	1	40.01	1	***	Mar34
	E11M59_80	65	29	0	13.79	1	***	Mar34
	E11M49_232	78	16	0	40.89	1	***	Mar34
	E11M50_322	73	21	0	28.77	1	***	Mar34
LG8(O8)	E11M61_265	60	34	0	7.19	1	**	Mar34
	0L12G04	68	25	1	19.88	1	***	Mar34
	E11M48_215	70	24	0	22.51	1	***	Mar34
LG9(O9)	E11M62_321	64	30	0	12.3	1	***	Mar34
	0L10D08	30	64	0	12.3	1	***	GD33
	OL11H06	26	67	1	18.08	1	***	GD33
	E11M59_307	19	75	0	33.36	1	***	GD33
	E11M49_402	30	61	3	10.56	1	**	GD33

Table 3.1.10: Summary of the number of distorted loci, allele preference and distorted marker type within each linkage group in the broccoli x broccoli linkage map.

Linkage Group	No loci	Distorted AFLPs	Distorted SSRs	% Distortion GD33 alleles	% Distortion Mar34 alleles	% of non-distorted Loci
1 (O1)	15	9	1	6.7	60.0	33.3
2 (O2)	6	1	1	33.3	0.0	66.7
3 (O3)	12	8	3	91.7	0.0	8.3
4 (O4)	7	3	2	71.4	0.0	28.6
5 (O5)	8	4	0	50.0	0.0	50.0
6 (O6)	4	0	2	50.0	0.0	50.0
7 (O7)	14	7	3	0.0	71.4	28.6
8 (O8)	4	2	1	0.0	75.0	25.0
9 (O9)	10	3	2	50.0	0.0	50.0

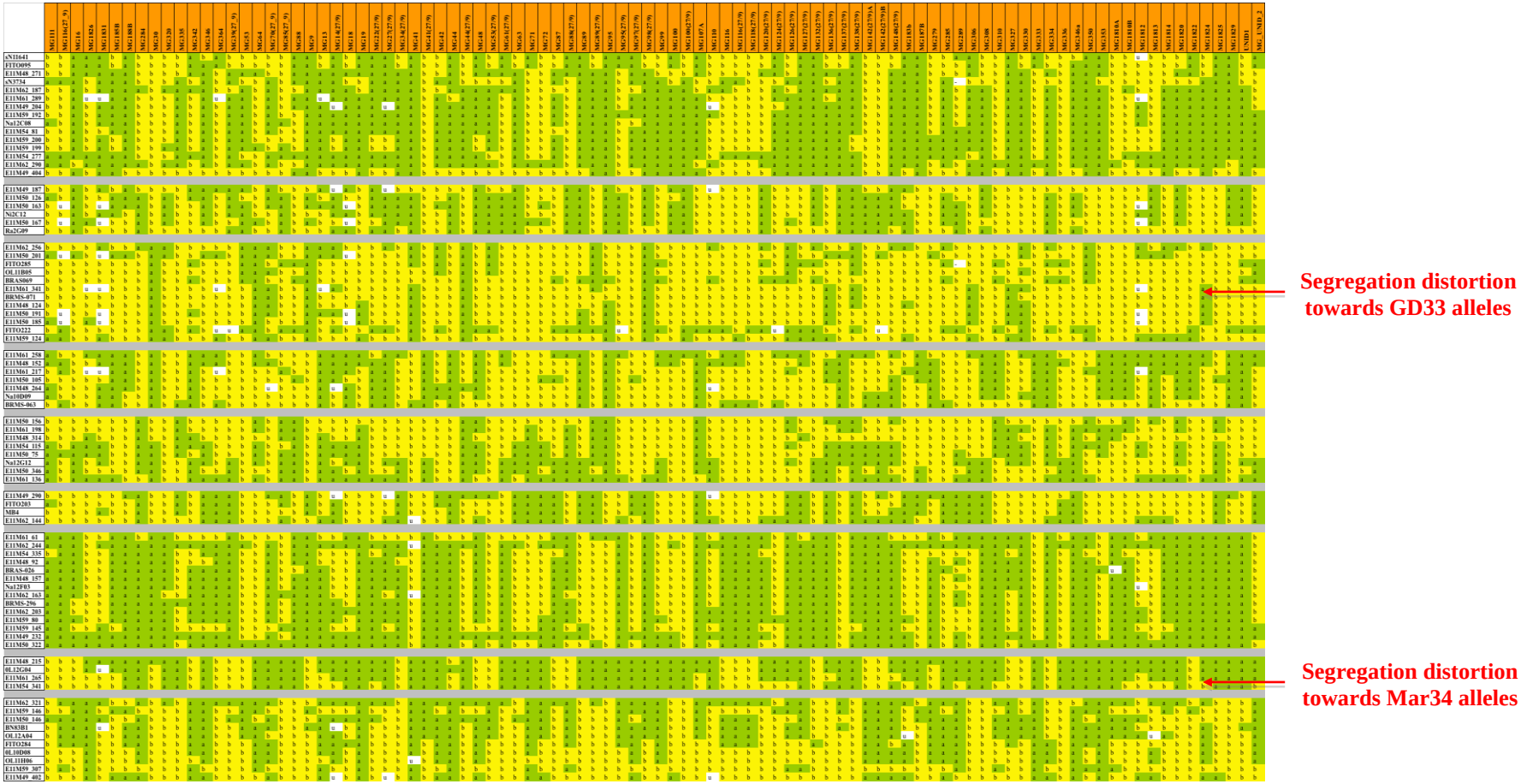


Figure 3.1.6: Colour coded genotype matrix representing the segregation of alleles for each marker in the nine linkage groups of the broccoli x broccoli linkage map. Green areas for markers in each linkage group represents segregation distortion for Mar34 alleles where as yellow regions represents segregation distortion for GD33 alleles.

Strong, Weak and Suspect linkages

To determine the quality of the data and the accuracy of scoring, thresholds for recombination frequencies can be set in Joinmap V4.0 so that strong, weak and suspect linkages between loci can be identified. The linkage map created in this study has strong linkages (LOD >10.00) associated with loci in all linkage groups with none of the nine linkage groups having suspect linkages. Four linkage groups (LG 1 (O1), LG 3 (O3), LG 5 (O5) and LG 9 (O9) do contain loci that have weak linkages however, weak linkages in these linkage groups tend to be associated with loci that are separated by a unsaturated region in the map.

3.1.4 Discussion

The linkage map produced in this study consists of nine linkage groups, contains 80 loci (25 SSRs and 55 AFLPs) and has a map distance of 712.9 cM, which covers a high proportion (79.2%) of the *B.oleracea* genome. The estimated map length of the *B.oleracea* genome is 900 cM in accordance with a personal communication by G.H. Jones (unpublished data), which estimates map length based on a chiasma frequency of 18 chiamata per meiosis (Sebastian *et al*., 2000). Mathas (2004) constructed a skeleton broccoli x broccoli linkage map based on 94 individuals from the same MGDH population. This map contained fewer markers overall (65) but also had nine linkage groups. The total map length was 346 cM equating to coverage of a smaller percentage (38.3 %) of the *B.oleracea* genome compared to the new map. The linkage map in this study increases the genome coverage by 366.9 cM, extends the lengths and improves the marker saturation of most of the linkage groups. Common SSR markers are present between both linkage maps, although the number of SSR

markers mapped (7) is low in the original skeleton map compared to 25 in the new map

3.1.4.1 Comparison with published B.oleracea linkage maps

Many genetic linkage maps have been published for the *Brassica* species, with 12 reference linkage maps being available for *B. oleracea* (Slocum *et al.,* 1990; Landry *et al.,* 1992; Kianian and Quiros 1992; Ramsay *et al.,* 1996; Bohuon *et al.,* 1996; Camargo *et al.,* 1997; Hu *et al.,* 1998; Sebastian *et al.,* 2000; Lan and Peterson, 2000; Li *et al.,* 2003; Babula *et al.,* 2003; Goa *et al.,* 2007). Linkage maps published pre 2000 are all based on RFLP or RAPD markers therefore, comparisons can be only drawn on linkage map length, linkage group number and loci number. All papers report that the linkage maps constructed contain nine linkage groups, which represent the nine haploid chromosomes of *B.oleracea*, with the exception of the linkage map produced by Kianian and Quiros (1992), which has 11 linkage groups. Over all, map distances (cM) vary between 747 cM- 1738 cM in the published linkage maps: 747 cM (Kianian and Quiros, 1992), 820 cM (Slocum *et al.,* 1990), 875 cM (Bohuon *et al.,* 1996), 921 cM (Camargo *et al.,* 1997), 1112 cM (Landry *et al.,* 1992) and 1738 cM (Hu *et al.,* 1998). The number of markers incorporated in each linkage map also varies between 108 – 303 markers. The linkage map in this study has a similar map distance (712.9 cM) to those constructed pre 2000 however, it contains less markers (80) overall.

Brassica oleracea maps published post 2000 consist of a combination of marker types, including RFLPs, AFLPs, SSRs, ESTs, SRAPs and BAC ends. The most notable linkage map produced is the integrated *B. oleracea* linkage map constructed

by Sebastian *et al*. (2000). The integrated map combines two independent linkage maps through common loci (RFLPs, AFLPs and SSRs) from two different F_1-derived doubled haploid populations of *B. oleracea*. The populations used for map integration were the AxG, A12 x GD33 population (Chinese Kale (*var. alboglabra)* x Broccoli (*var. italica)* cross) and the NxG population (Cauliflower (*var. botrytis*) x Brussels Sprout (*var. gemmifera*) cross). The high-density consensus map produced contains 547 markers mapping to nine linkage groups. The total map length is 893 cM (99% genome coverage) with an average locus interval of 2.6 cM. The genome coverage is greater in the integrated map compared to the linkage map produced in this study, and the marker density is much higher, which is reflected in the small average locus interval of 2.6 cM compared to 9.5 cM. The two maps share one common SSR locus, BN83B1 found on linkage group nine. The integrated map also has the advantage of having its nine linkage groups physically assigned to the respective *B.oleracea* chromosome by Howell *et al*. (2002) using fluorescent *in-situ* hybridisation (FISH). Another published linkage map that shares common loci with the linkage map from this study is the map constructed by Gao *et al*. (2007). The map is highly saturated with 1257 markers mapping to nine linkage groups with a map length of 703 cM. The common SSR loci found between the maps are: MB4, OL11B05, OL12A04 and OL11H06. The presence of common loci within published maps allows the anchorage to consensus maps. This can help to establish correct marker order and linkage group orientation, rectify variation between common markers on different maps and enable comparisons between maps to be made more easily (Sebastian *et al*., 2000).

The linkage map produced in this study has sufficient genome coverage but does not have as high levels of marker saturation compared with some of the published maps. To improve the marker saturation of the map more markers need to be incorporated; this could be achieved by integrating the map with another *B. oleracea* map that share common loci or by selecting publicly available markers (SSRs, AFLPs, ESTs, SCRAPs) used in published maps that are known to map in specific linkage groups for the targeted improvement of poorly saturated linkage groups.

3.1.4.2 Informative markers

The ability to produce informative marker loci is restricted in this study due to the relatively narrow genetic background of a broccoli x broccoli cross. As a result of a cross of this type polymorphisms are more likely to be present in low abundance compared to mapping populations derived from wider crosses. The two types of markers (SSRs & AFLPs) used in the map construction differ in their ability to produce polymorphic loci, AFLP markers in general generated more polymorphic loci than the SSRs. All of the AFLP primer combinations produced polymorphic loci whereas many of the SSRs failed to do so, with only 35 out of the 64 SSRs screened producing informative loci. In general, polymorphisms were harder to detect reliably in the AFLP traces compared with the SSR traces due to a low peaks heights. In total only 55% of the markers were mapped to genomic loci. The number of loci unable to be positioned on the map included those detected by 10 SSRs and 56 AFLPs. Comparing the type of loci that were mapped, 31% (25) are SSRs and 69% (55) are AFLPs. One reason for why loci are not incorporated into linkage maps is that the unmapped loci do not reach the linkage LOD threshold at the lowest

stringency and therefore are not significantly associated with any of the mapped markers within the linkage groups (Van Oojen, 2006).

3.1.4.3 Possible causes of linkage map anomalies

Published *B.oleracea* linkage maps and the linkage map produced in this study contain linkage groups with regions that are unsaturated with markers, inflated linkage group lengths i.e. groups that are much larger than 100 cM and linkage groups with short map distances. The causes of some of these anomalies can be explained by genotyping errors, missing values and recombination hotspots.

Genotyping errors

Errors in genotyping occur when the genotype determined by molecular analysis does not correspond with the real genotype of the individual of interest (Bonin *et al*., 2004; Pompanon *et al*., 2005). This can be due to human error, chance or technical difficulties and can occur at any stage in the genotyping process (sampling, DNA extraction, molecular analysis, scoring, data analysis). The main type of genotyping errors associated with microsatellite (SSRs) and AFLP molecular markers are allelic dropouts or false allele amplifications (SSRs) and differences in peak intensities or peak shifts (AFLPs). This can cause bias, especially in mapping studies where undetected genotyping errors can result in incorrect map orders and inflation of map lengths (Bonin *et al*., 2004; Hackett and Broadfoot, 2002). Other genotyping errors include missing genotype values, when these are present in marker data they have the effect of disguising the number of true recombination events along the chromosome, which can disrupt map order and map length (Hackett and Broadfoot, 2002).

Missing values can cause shorter map lengths when markers are more widely separated, especially if segregation distortion is present.

Recombination hotspots

Large intervals that exist in linkage maps that cause gaps between markers could indicate regions of high recombination, which may prove difficult to saturate regardless of how many new markers are added to the map (Sebastian *et al*., 2000). The integrated RFLP and AFLP *B. oleracea* map produced by Sebastian *et al*. (2000) contains gaps, a large interval of 31.2 cM is present at the top of LG2 and another gap occurs between 43.5 cM and 64.4 cM in LG6. Gaps are also present in the linkage map produced in this study.

3.1.4.4 Segregation distortion.

Segregation distortion occurs when segregation ratios deviate from the expected 1:1 Mendelian ratio and is a common occurrence in DH mapping populations. In this study 55% of the mapped loci are distorted, with all linkage groups exhibiting segregation distortion. Distorted loci favour GD33 alleles in six linkage groups with distorted loci in the remaining three linkage groups favouring Mar34 alleles. In comparison to the skeleton linkage map produced by Mathas (2004), where 64% of the loci mapped were distorted, the overall level of segregation distortion is lower. Other *B. oleracea* linkage maps that use DH mapping populations also report finding allelic imbalances in mapped loci, for example Iniguez-Luy *et al*. (2009) found allelic imbalances in 49% (139/279) of the loci mapped. Segregation distortion is often associated with selection during tissue culture, responsiveness and survival during microspore culture and the ability to produce seed (Hackett and

Broadfoot, 2003; Pink *et al*., 2008). When creating DH genotypes using microspore culture, GD33 is the responsive parent, therefore this may account for the levels of segregation distortion found within linkage groups favouring GD33 alleles. Where as linkage groups that favour Mar34 alleles could be due to the better seeding ability of this genotype. Compared to an F_2 population, higher levels of segregation distortion in DH populations are often observed. G. Teakle (unpublished) and Voorrips *et al*. (1997) report on finding significant segregation distortion at 65% of the loci in the AG DH mapping population and a cabbage x broccoli DH population respectively (Pink *et al*., 2008). Whereas, segregation distortion is only reported in 5-12% of loci in F2 mapping populations (Pink *et al*., 2008; Slocum *et al*., 1990; Kianon and Quiros, 1992; Landry *et al*., 1992). In *B. oleracea* two putative QTL for microspore culture responsiveness that correlate to two areas of segregation distortion in the AG DH population have been identified (Pink *et al*., 2008). Pink *et al*. (2008) propose that linkage drag of the loci linked to the QTL for responsiveness is one cause of segregation distortion in DH *Brassica* populations.

In summary, the construction of genetic linkage maps is an essential tool for QTL analysis, map-based cloning of genes and marker-assisted breeding (Jansen *et al.*, 2001). These approaches allow for the location of QTL or genes controlling traits of interest for the improvement of agronomically important traits (Sebastian *et al.*, 2000), as well as providing a resource to investigate *Brassica* evolution, taxonomy and synteny with related species and *Arabidopsis thaliana* (Sebastian *et al*., 2000).

Conclusions:

- DNA polymorphisms exist between the parental genotypes, DHMar34 and DHGD33, of the MGDH population. Therefore the MGDH population is suitable for genetic (QTL) analysis and linkage map construction.
- The linkage map constructed has the advantage of being based on a cross from within a crop; therefore markers associated with traits of interest will have a direct effect on crop improvement in broccoli.
- The broccoli x broccoli linkage map provides high genome coverage of the *B. oleracea* genome. However, due to the narrowness of the cross, the marker saturation is low and requires improvement.

CHAPTER 3:

CHARACTERISATION OF GENETIC LOCI CONTROLLING SHELF LIFE IN BROCCOLI

Part II:

Shelf life evaluation and morphological trait assessment of the MGDH population.

3.2.1 Introduction

The appearance and texture of fresh produce is a major contributing factor to determine whether consumers will buy a product. The marketability of broccoli is mainly affected by yellowing, as a result of chlorophyll degradation, and a loss of firmness caused by compromised stem turgor and dehydration. The parents of the MGDH mapping population differ in their post harvest shelf life performances. GD33 exhibits rapid post harvest senescence with the onset of yellowing occurring within three days in storage at 14^{o}C. In contrast, Mar34 retains its green colour and has a shelf life of up to nine days in storage at 14^{o}C. The parental genotypes also have distinct morphological differences; the good shelf life performer Mar34 produces a medium-large uniform compact head, which is green-blue in colour, where as GD33 produces a medium sized, branched head that is pale green in colour. GD33 is also more susceptible to loss of turgor and the loosening of florets, which causes the product to be unacceptable for retail. To determine the amount of natural variation present for quality traits in the MGDH population, shelf life and morphological traits were assessed. This will enable the identification of genotypes that have desirable quality characteristics for breeding and in conjunction with the map produced in the previous section (Chapter 3, Part I) will allow for the identification of QTL controlling quality traits in broccoli.

Aims

- To confirm that natural variation is present for the trait shelf life within the parental genotypes Mar34 and GD33.
- To determine the shelf life phenotypes of the MGDH population.

- To quantify colour and colour change in the MGDH population using a spectrophotometer.
- To assess the natural variation in the MGDH population for morphological and quality traits.
- To determine whether morphological traits influence shelf life.

3.2.2 Materials & Methods

3.2.2.1 Plant material

The plant material used in the trait assessment field trial conducted in 2007 is described in section 2.1.

3.2.2.2 Shelf life evaluation and morphological trait assessment of the MGDH mapping population.

Due to seed availability only 73 genotypes from the MGDH population grown in the field experiment in 2007, were assessed for their shelf life phenotypes and morphological traits, as described in section 2.4. Mature broccoli heads were harvested between 01/08/07 and 17/08/07 from two field trial replicates (76 plots per replicate). Each plot contained 24 broccoli plants (4 rows x 6 plant arrangement) and a total of eight broccoli heads were harvested per plot. The central six 'guarded' heads were harvested for shelf life evaluation and morphological trait assessment, and two of the outer guard heads were harvested as extra samples for metabolite analysis. All assessments were carried out at harvest (day 0) and daily, until at least day six, or the day that heads failed due to yellowing. All of the 12 heads harvested for each genotype were visually inspected and judged individually for head yellowing, heads failed if any yellowing present.

Bud material was cut on day 0 (harvest), using a scalpel, from the two guard heads harvested as samples for metabolite analysis. Bud material was placed into a 50 ml Falcon tube and frozen in liquid nitrogen before being stored at -80^{o}C, this material was used in Chapter 5.

3.2.2.3 Statistical analysis

All phenotypic data was analysed using REML, as described in section 2.5.1, with genotype as the fixed effect and rep/row/col as the random effects. Correlations between traits were established using the correlation analysis function in GenStat as described in section 2.5.2.

3.2.3 Results

3.2.3.1 REML analysis

The REML analyses for all phenotypic traits took into account the position of genotypes within the field. However, for all traits, the position of the genotypes in each field replicate had little or no effect on the variation present, since the estimated variance components for the trial design (rep/row/col) are much lower than the residual variance model for each traits.

3.2.3.2 Shelf life evaluation of the MGDH population.

Consumers use visual cues when purchasing fresh produce and associate the green colour in broccoli with good quality. The presence of yellowing in broccoli heads can incite an unfavourable consumer response resulting in the consumer opting not to purchase the product due to compromised quality. To replicate the consumer

response, the MGDH population were visually assessed for post harvest yellowing each day during storage, to the time it took for individual plants in the MGDH population to be classed as unacceptable for retail due to the presence of yellowing.

3.2.3.3 Visual assessment of head yellowing.

Genotypes in the MGDH population were scored daily for visual signs of head yellowing using the Wurr scoring system (section 2.4.2). Using the results for head colour an average could be calculated for the day in storage when the onset of post harvest yellowing was observed for each genotype. The population as a whole exhibits significant differences (****P<0.001*) in their post harvest shelf life phenotypes, in terms of the time taken (days) for genotypes to become yellow after harvest (Wald $_{[73]}$ = 713.25). The MGDH population displays natural variation in shelf life length with a 3-fold variation (2.73-9.79 days) in the time taken (days) for heads to become yellow after harvest (figure 3.2.1). The poorest performer is the parental genotype GD33, becoming yellow 2.73 ± 0.82 days after harvest. The parental genotype Mar34 is one of the best shelf life performers only becoming yellow 9.09 ± 0.55 days after harvest, however three genotypes show similar shelf life performance to Mar34 (MG107A = 9.26 ± 0.92, MG1831 = 9.43 ± 0.47 and MG532 = 9.79 ± 0.69) and stayed green for longer after harvest. During the visual assessment for head yellowing a total of 27 genotypes in the population were observed to have purple heads, due to the presence of anthocyanins. However, the presence of purple pigments in broccoli heads did not seem to affect the shelf life of genotypes during storage, as genotypes with purple heads fall within the whole range of the distribution for the trait for days to yellowing (Figure 3.2.1).

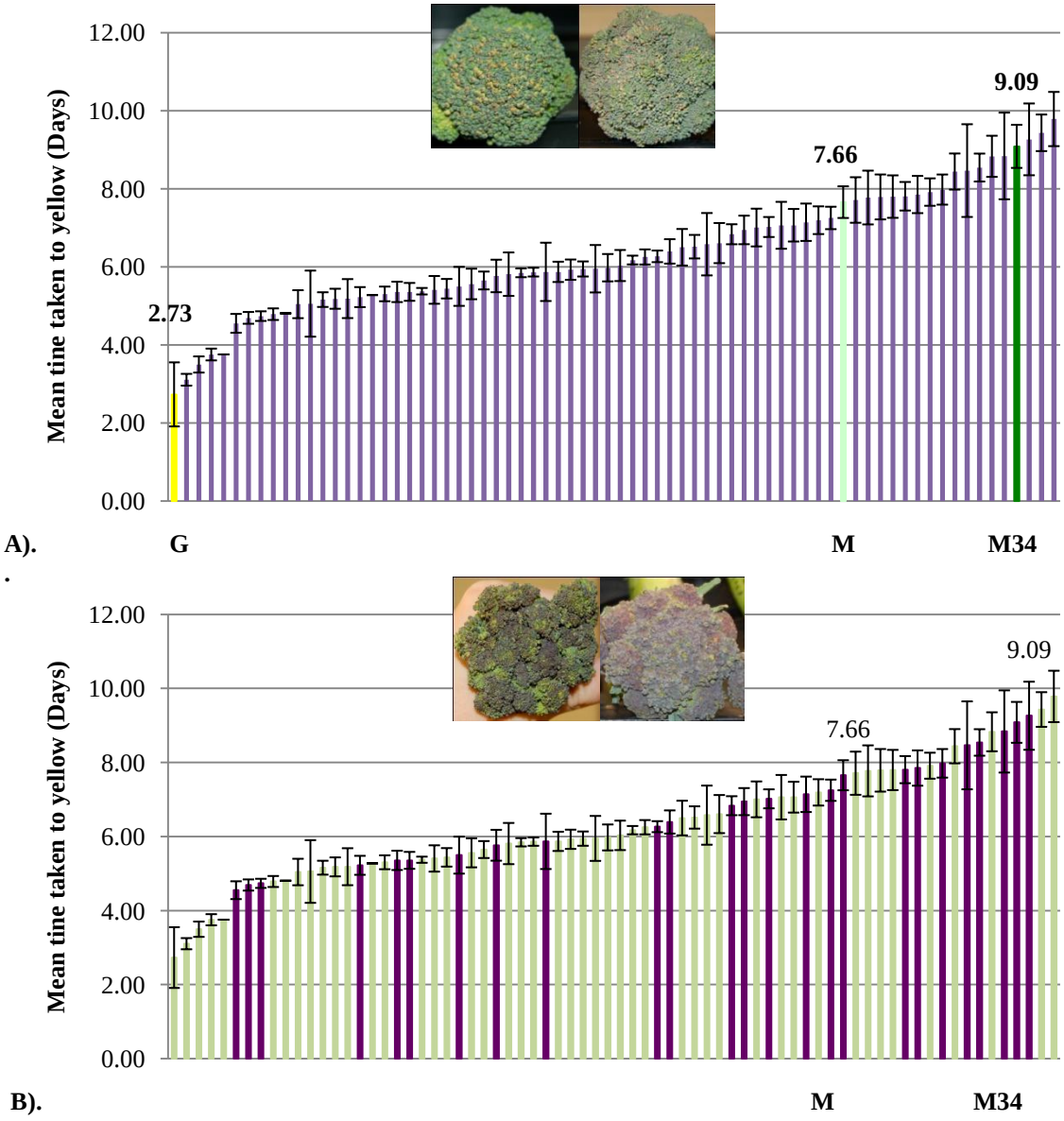


Figure: 3.2.1: Predicted means (from REML) of A.) the time taken (Days) for genotypes in the MGDH population to become yellow and B.) the spread of genotypes with purple heads within the distribution for days to yellowing (n = 72). Error bars represent the s.e.m (standard error of the mean). Genotypes that were observed to have purple heads are highlighted in purple. M = Marathon, M34 = Mar34 and G = GD33.

3.2.3.4 Quantitative assessment of head yellowing.

Consumers associate head yellowing in broccoli with a loss in quality and are reluctant to make a purchase if visible yellowing is present. In this study, visual assessments have been used to replicate the consumer response and to establish the day in storage that genotypes in the MGDH population are classed as unacceptable

for retail due to yellowing. However, the perception of yellowing is subjective, with consumers varying in their response to when they judge produce as unacceptable. Therefore, as an alternative approach, quantitative measurements of head colour and colour change could provide a more accurate and robust method to measure the presence and extent of yellowing in broccoli.

A hand held spectrophotometer (make/model) was used to quantitatively measure the colour and colour change of genotypes in the MGDH population as described in section 2.4.1. A REML analyses was carried out on all of the Hunter *Lab* colour co-ordinates (L, a, b, hue angle and chroma), as described in section 2.5.1, on all days during storage. The MGDH population exhibit significant differences (****P<0.001*) for all colour co-ordinates on day 1 after harvest and on all days during storage. To establish the days where most variation in the colour co-ordinates is occurring, the day when 50 % of the population had failed for yellowing was selected. In this study, 50 % of the genotypes were classed as visibly yellow between day five and day six in storage; therefore colour change must be occurring on these days and also the preceding day (day 4). The work presented here will focus on the change in the colour co-ordinates on day one after harvest and on day four, day five and day six in storage.

Yellow-Blue colour scale (b)

To deduce the extent of post harvest yellowing in broccoli, changes in the yellow-blue colour scale (+b = yellow, -b = blue) should be the most informative colour co-ordinate and allow for a quantitative measurement of yellowing in broccoli. In general, the b-value does not seem to be affected by the presence of anthocyanins, as genotypes with purple heads fell within the whole range of the distribution on the yellow-blue scale.

At all time points (day 1, 4, 5 and 6), the poor performing parental genotype GD33 has the highest b-value (Figure 3.2.2); whereas the b-value for the good performer Mar34 is towards the mid-lower end of the distribution for the MGDH population. The commercial genotype Marathon (from which Mar34 is derived) also has a high b-value, falling within the higher extreme of the distribution at each time point, even though the onset of yellowing in this genotype does not occur until day eight in storage. During storage, the position of GD33 within the distribution remains the same, where as the positions of Mar34 and Marathon change due to an increase in their b-values of 0.77 and 1.53 respectively, between day one and day six in storage. Initially, the b-value for Mar34 decreases between day one and day four before increasing to 5.96 on day six.

The b-value increases, between day one and day six in storage, for the majority (79%) of the genotypes in the MGDH population, however in 15 genotypes the b-value decreases. On day one after harvest, when broccoli heads are all still visually green, the range for the b-value in the MGDH population is 1.14-11.05.This range is larger than expected at this stage in storage, as colour differences are not observed (all look green). By day six in storage, when ~50% of the MGDH population have visually failed due to yellowing the range for the b-value has only marginally increased to 1.5-11.74, although much more variation in colour is being observed visually. This suggests that the spectrophotometer is detecting yellowing and colour change in broccoli heads during storage to some extent, but it would be expected that at failure, when yellowing is present, genotypes would have higher b-values than

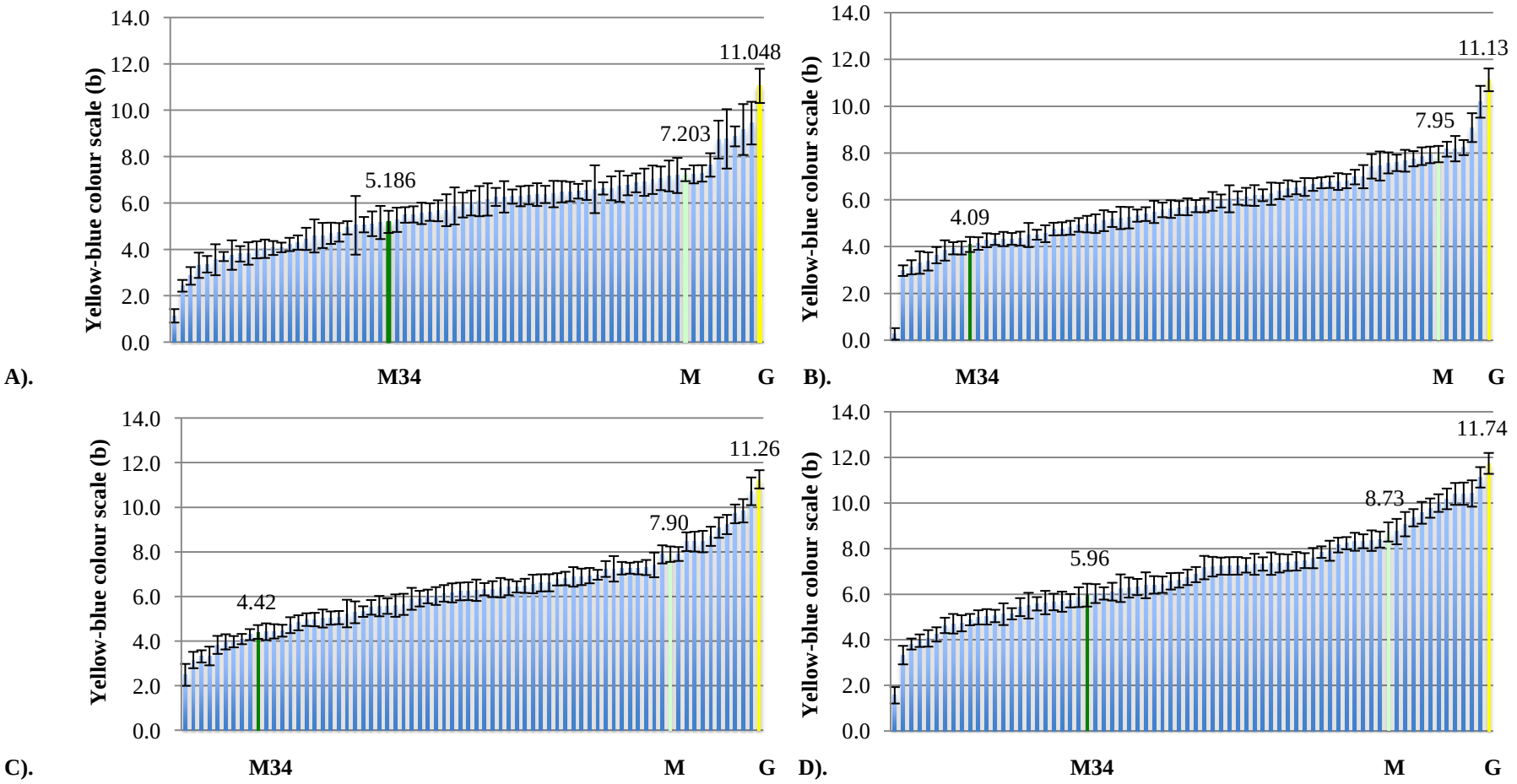


Figure 3.2.2: Predicted means (from REML) for the yellow-blue colour scale (b) of genotypes in the MGDH population A.) On day one in storage B). On day four in storage, C.) On day five in storage and D.) On day six in storage (n = 72). Error bars represent the s.e.m (standard error of the mean). The parental lines (G = GD33 & M34 = Mar34) and the commercial line (M = Marathon) are highlighted.

those that remain green. This was not always the case. For example, on day six in storage genotype MG41(27/9) had a relatively low b-value of 4.06 even though it had failed for visible yellowing on day five, where as genotype MG453 had a high b-value of 8.75 but was still visually green on day six. Hence, the b-value does not accurately discriminate between broccoli heads that are visually green and broccoli heads that are visually yellow.

The five genotypes that displayed the most change in the yellow-blue colour scale between day one and day six are: MG419 (+6.53), MG539 (+5.09), MG136 (27/9) (+4.29), MG120(27/9) (+3.52) and MG526 (+3.34). These genotypes were distributed evenly within the yellow-blue colour scale for the MGDH population on day one after harvest, suggesting rapid chlorophyll degradation may be occurring in these genotypes. The trait 'days to yellowing' and the b-value show an inverse relationship as they are negatively correlated (Table 3.2.1), with the highest correlation between the two traits occurring on day six (***$P<0.001$, $r_{[60]} = -0.66$). This suggests there is a weak association between high b-values and short shelf life or longer shelf life and low b-values. However, this relationship is not truly reflected in the data (Table 3.2.2).

Table 3.2.1: Correlation matrix for the colour co-ordinates L, a and b, on day four, day five and day six in storage. Significance levels are represented as: ***P<0.001 (pink), **P<0.01 (orange) and *P<0.05 (yellow). L = Lightness, H = Hue angle, C = Chroma, DY = Days to Yellowing, D4 = Day four, D5 = Day five and D6 = Day six.

	L_D4	a_D4	b_D4	H_D4	C_D4	L_D5	a_D5	b_D5	H_D5	C_D5	L_D6	a_D6	b_D6	H_D6	C_D6	DY
L_D4	-															
a_D4	***-0.65***	-														
b_D4	***0.58***	***-0.75***	-													
H_D4		*-0.34*		-												
C_D4	***0.63***	***-0.87***	***0.97***		-											
L_D5	***0.86***	***-0.53***	***0.48***		***0.53***	-										
a_D5	***-0.53***	***0.88***	***-0.60***	*-0.37*	***-0.72***	***-0.56***	-									
b_D5	***0.48***	***-0.63***	***0.88***	-0.24	***0.85***	***0.60***	***-0.61***	-								
H_D5		-0.26	-0.25	***0.72***			*-0.34*	***-0.41***	-							
C_D5	***0.53***	***-0.75***	***0.87***		***0.89***	***0.64***	***-0.77***	***0.97***	-0.24	-						
L_D6	***0.80***	***-0.51***	***0.48***		***0.51***	***0.88***	***-0.42***	***0.56***		***0.56***	-					
a_D6	***-0.49***	***0.83***	***-0.53***	***-0.41***	***-0.66***	***-0.45***	***0.91***	***-0.46***	***-0.44***	***-0.62***	***-0.41***	-				
b_D6	***0.42***	***-0.57***	***0.78***		***0.76***	***0.54***	***-0.49***	***0.90***	***-0.39***	***0.85***	***0.68***	***-0.44***	-			
H_D6			-0.27	***0.70***			*-0.31*	***-0.44***	***0.85***	-0.27		***-0.44***	***-0.51***	-		
C_D6	***0.49***	***-0.71***	***0.81***		***0.82***	***0.57***	***-0.64***	***0.90***	-0.23	***0.90***	***0.69***	***-0.62***	***0.97***	*-0.34*	-	
DY	-0.29	0.25	***-0.48***		***-0.43***	***-0.40***		***-0.60***	***0.40***	***-0.51***	***-0.51***		***-0.66***	***0.56***	***-0.58***	-

Table 3.2.2: The b-values of the MGDH population on day six in storage, when ~50% of the population have failed due to head yellowing, and the day on which individual genotypes in the population failed (DOF). Red text indicates genotypes that are still green in storage. An asterix (*) represents genotypes with the presence of purple pigments.

Genotype	DOF	b-value	Genotype	DOF	b-value
GD33	3	11.74	MG10027/9	6	6.74
MG419	5	11.13	MG308	6	6.61
MG183B	5	10.42	MG435	5	6.57
MG12027/9	3	10.41	MG18	6	6.42
MG526*	5	10.40	MG407*	6	6.41
MG13627/9	5	10.18	MG406*	7	6.39
MG12427/9	4	10.00	MG438	6	6.31
MG8727/9	5	9.78	MG1824	7	6.29
MG12627/9	5	9.57	MG543*	7	6.26
MG1814	6	9.35	MG107A*	9	6.11
MG539*	5	9.07	MG1810A	6	6.03
MG453*	8	8.75	MG415*	6	6.03
Marathon*	8	8.73	M34*	9	5.96
MG462	5	8.40	MG562*	6	5.87
MG422*	5	8.35	MG10	7	5.71
MGUNID2*	6	8.32	MG450*	7	5.67
MG440*	5	8.30	MG1831	9	5.66
MG301	5	8.24	MG433	9	5.63
MG72	5	8.16	MG1427/9	8	5.58
MG1812	5	7.91	MG44*	8	5.50
MG99	6	7.85	MG560*	7	5.43
MG469	6	7.58	MG100	8	5.14
MG13727/9	7	7.47	MG334	7	5.12
MG95	6	7.45	MG1829	8	5.04
MG1825	6	7.38	MG89	7	5.01
MG87	5	7.36	MG532	10	4.98
MG116*	8	7.34	MG1826	6	4.88
MG9527/9	7	7.33	MG528*	7	4.72
MG418*	5	7.31	MG454	7	4.70
MG1813	3	7.27	MG1822	7	4.63
MG71	5	7.24	MG521*	8	4.24
MG927/9	6	7.24	MG4127/9*	5	4.06
MG63	6	7.23	MG416*	9	3.96
MG430*	5	7.21	MG353	8	3.81
MG187B	8	7.17	MG410*	8	3.33
MG1810B	4	6.86	MG475*	9	1.56

Lightness (L)

The L-value of genotypes in the MGDH population determines how dark or light broccoli heads are at harvest and during storage on a 0-100 scale (0 = black, 100 = white). It is assumed that, as broccoli starts to senesce postharvest, the L-values will increase to indicate the increase in yellowing.

The range for lightness (L) in the MGDH population shows an increase from harvest (28.45 - 38.73) and between the time points day four (30.03 - 39.30), day five (31.08 - 39.51) and day six (32.13 - 40.08) in storage, suggesting that the population is becoming lighter (Figure. 3.2.3). The parental genotypes, Mar34 (good shelf life performer), GD33 (poor shelf life performer) and the commercial genotype Marathon all become lighter during storage with an increase in lightness of 2.94, 2.30 and 1.35 respectively, between day one and day six. The poor shelf life performer GD33 and the commercial genotype Marathon both fall within the higher extreme of the distribution for lightness. Interestingly, Marathon is the lightest genotype throughout storage although visually it appears to be darker than GD33 and does not fail in storage until day eight. The parental genotype GD33 fails earliest for yellowing, by day three in storage, it has the 4th highest L-value on day one and on day 4 in storage increasing to the 2nd highest L-value on day 5 and day 6. The L-value for Mar34 falls within the middle of the distribution for lightness for all time points except day six, with the L-value shifting towards the mid-high end of the distribution.

Overall, 95% of the population exhibit an increase in lightness between day one and day six, with three genotypes showing a decrease in lightness. The five genotypes that increase the most for lightness are: MG419 (+7.8), MG120(27/9) (+6.9), MG539 (+5.7), MG136(27/9) (+5.4) and MG422 (+5.0) with the exception of MG422, these

genotypes also show the most increase on the yellow-blue scale (b) between day one and day six.

The colour co-ordinates L and b are positively correlated, with the highest correlation seen on day six in storage (Table 3.2.1) (***$P<0.001$, $r_{[60]} = 0.68$). In general, genotypes that have high b-values are also found in the higher extreme of the distribution for lightness. However, the trait 'days to yellowing' does not seemed to be influenced by lightness with only a weak correlation being found between these traits (***$P<0.001$, $r_{[60]} = -0.51$) (Table 3.2.1).

Red-Green colour scale (a)

The 'a' co-ordinate measures colour on a red-green colour scale (+ a = red, -a = green). The range for the a-value in the MGDH population becomes more negative at all time points during storage (Figure 3.2.4). Negative values on this colour scale indicate that genotypes in the MGDH population are becoming less red and therefore greener after harvest and during storage, contradicting what is visually seen as heads senesce. However, the Hunter *Lab* colour co-ordinates work on the principle of an opponent colour scale, with an increase or decrease in the yellow-blue colour scale (b) causing an opposite effect in the red-green colour scale (a). This is supported by negative correlation (***$P<0.001$ $r_{[60]} = -0.75$, day four) between the two colour co-ordinates (Table 3.2.1). The a co-ordinate shows no correlation with the trait 'days to yellowing. The a-value of genotypes ranges from -0.43 to -5.97 on day one, after harvest, and from -1.91 to -7.24 by day six in storage (Figure 3.2.4). The positions of the commercial and parental genotypes, Marathon, Mar34 and GD33, all fall at the more negative extreme of the distribution at all time points on the red-green colour scale (a). Comparing the three genotypes, GD33 has the most negative a-value compared to Mar34, which has the least negative a-value. Overall, the a-value

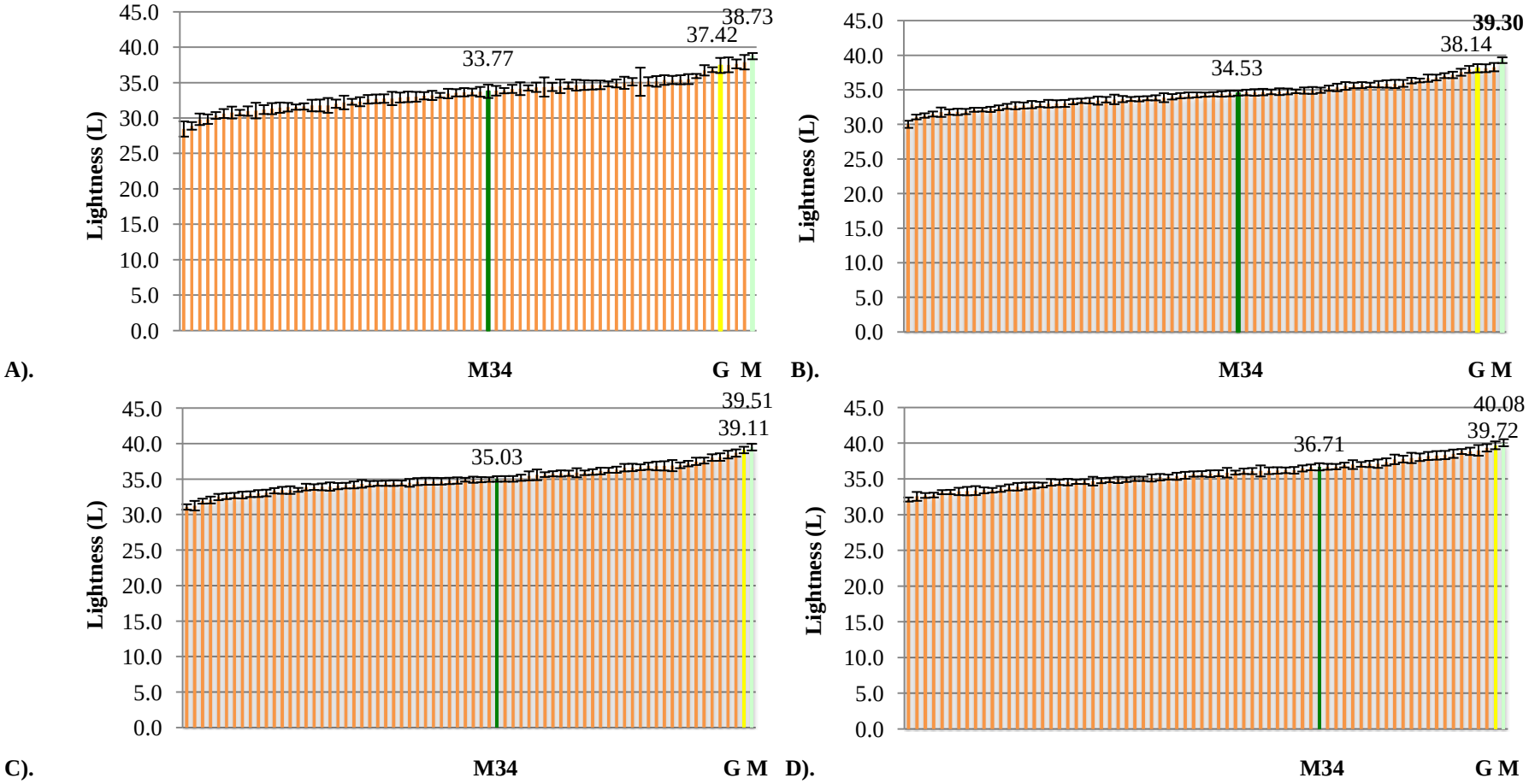


Figure 3.2.3: Predicted means (from REML) for Lightness (L) of genotypes in the MGDH population A.) On day one B). On day four. C). On day five and D). On day six in storage (n =72). Error bars represent the s.e.m (standard error of the mean). The parental lines (G = GD33 & M34 = Mar34) and the commercial line (M = Marathon) are highlighted.

decreases for 88% of genotypes in the population, although, the a-value for nine genotypes increases. Most genotypes that display an increase on the red-green colour scale (a) also show a decrease on the yellow-blue scale (b). Therefore, genotypes display an inverse relationship when comparing a-values with b-values. In general, genotypes that have high b-values have more negative a-values. For example, the parental genotype GD33 has one of the most negative a-values on day one (-5.97) and at day six (-6.38) but, also has the highest b-value (11.05, day one and 11.74, day six) and is one of the lightest (37.42, day one and 39.72, day 6) genotypes at these time points. Some genotypes that have purple heads appear redder on the red-green colour scale (a) having small negative a-values, although in general genotypes with purple heads are evenly distributed throughout the red-green (a) colour scale.

Hue angle (h^o)

Hue angle is the colour co-ordinate that is most similar to how humans visually perceive colour. Different primary colours are represented by a colour wheel: 0^o = red, 90^o = yellow, 180^o = green and 270^o = blue. On day one after harvest the hue angle for the MGDH population ranges from 91.5 – 134.8 with the range increasing slightly to 102.40 – 139.90 on day six in storage (Figure 3.2.5). An increase in the range for hue angle contradicts what is expected as it infers that genotypes have become greener when visually yellowing is present in ~50 % of the population by day 6 in storage. The poor performing parental genotype GD33 falls within the mid-lower extreme of the distribution for hue angle, whereas Mar34, the good performing

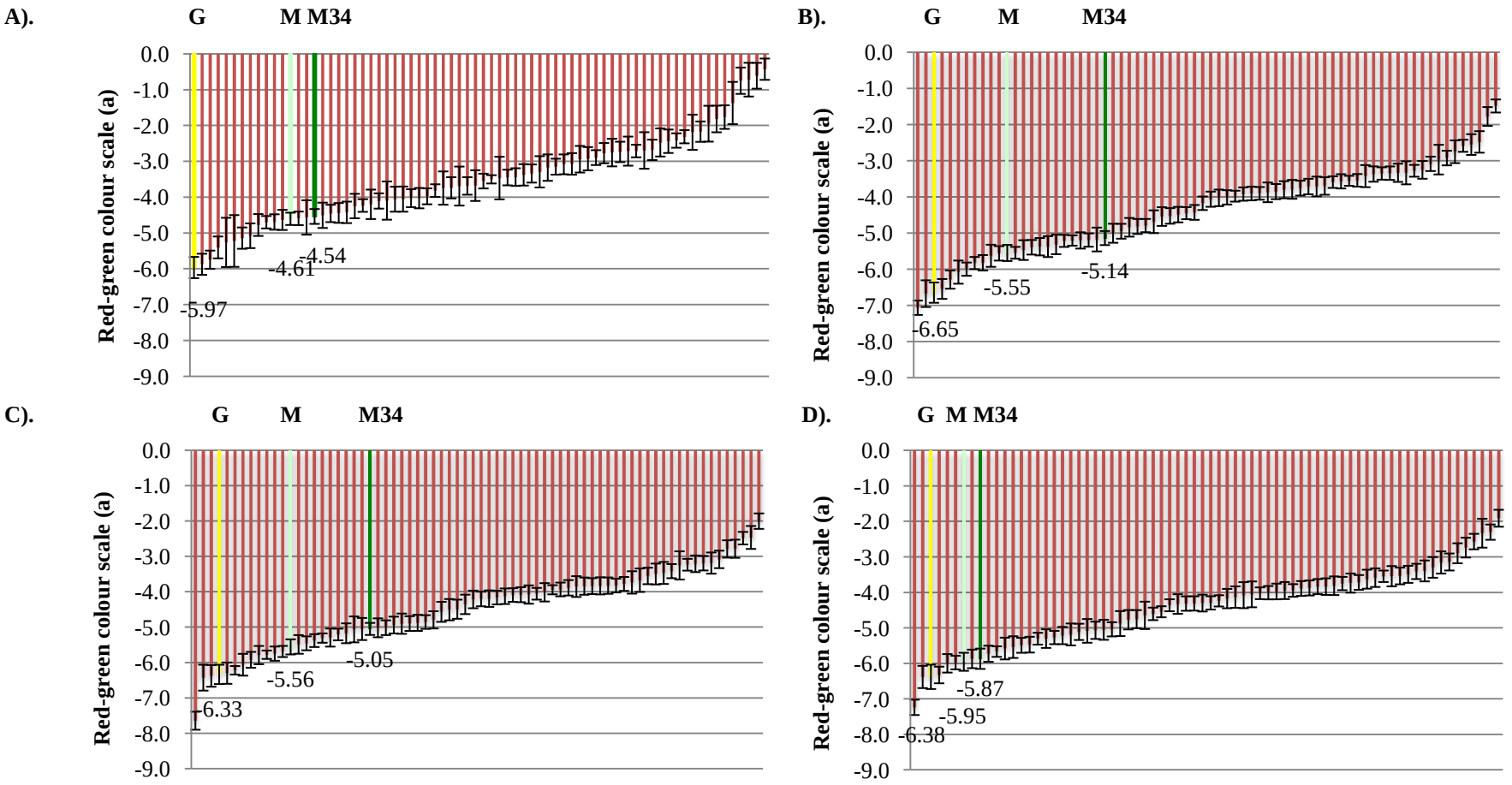


Figure 3.2.4: Predicted means (from REML) for the red-green colour scale (a) of genotypes in the MGDH population A.) On day one. B). On day four. C). On day five and D). On day six in storage (n= 72). Error bars represent the s.e.m (standard error of the mean). The parental lines (G = GD33 & M34 = Mar34) and the commercial line (M = Marathon) are highlighted.

parental genotype, fall at the high extreme of the distribution. Initially, the hue angle for GD33 and Mar34 increases between day one and day four in storage before decreasing to 119.90 and 137.40 respectively by day six. Visually, GD33 fails for head yellowing by day three in storage and has one of the highest L- and b-values on the lightness (L) and yellow-blue (b) colour scales, suggesting it is lighter and more yellow than other genotypes in the population. However, GD33 does not have one of the lowest hue angles. In fact out of the five genotypes that appear most yellow on the yellow-blue (b) colour scale only two genotypes (MG120(27/9) and MG419) also have the lowest hue angles. Hue angle shows no correlation with lightness, but shows a weak correlation with the 'b' co-ordinate (***$P<0.001$ $r_{[60]}$ = -0.51) and the trait 'days to yellowing' (***$P<0.001$ $r_{[60]}$ = 0.56) on day six in storage (Table 3.2.1)

Chroma (C)

Chroma (C) measures the intensity of colour in terms of how vivid or dull a colour appears, on a 0-60 scale (0 = dull, 60 = vivid). The range for chroma (C) in the MGDH population increases during storage from 2.12-12.59 on day one in storage to 3.06-13.55 on day six in storage, suggesting that the colour of broccoli heads is becoming more vivid over time (Figure 3.2.6). The pattern for chroma values closely resembles the values from the yellow-blue (b) colour scale and at all time points chroma is strongly positively correlated with the 'b' co-ordinate (***$P<0.001$ $r_{[60]}$ = 0.97) (Table 3.2.1). Chroma is also negatively correlated with the 'a' co-ordinate from the red-green colour scale, with the highest correlation occurring on day five in storage (***$P<0.001$ $r_{[60]}$ = -0.77) (Table 3.2.1). This suggests that an inverse relationship exists between chroma values and a-values. The poor shelf life performer, GD33, has the highest chroma value at all time points throughout storage

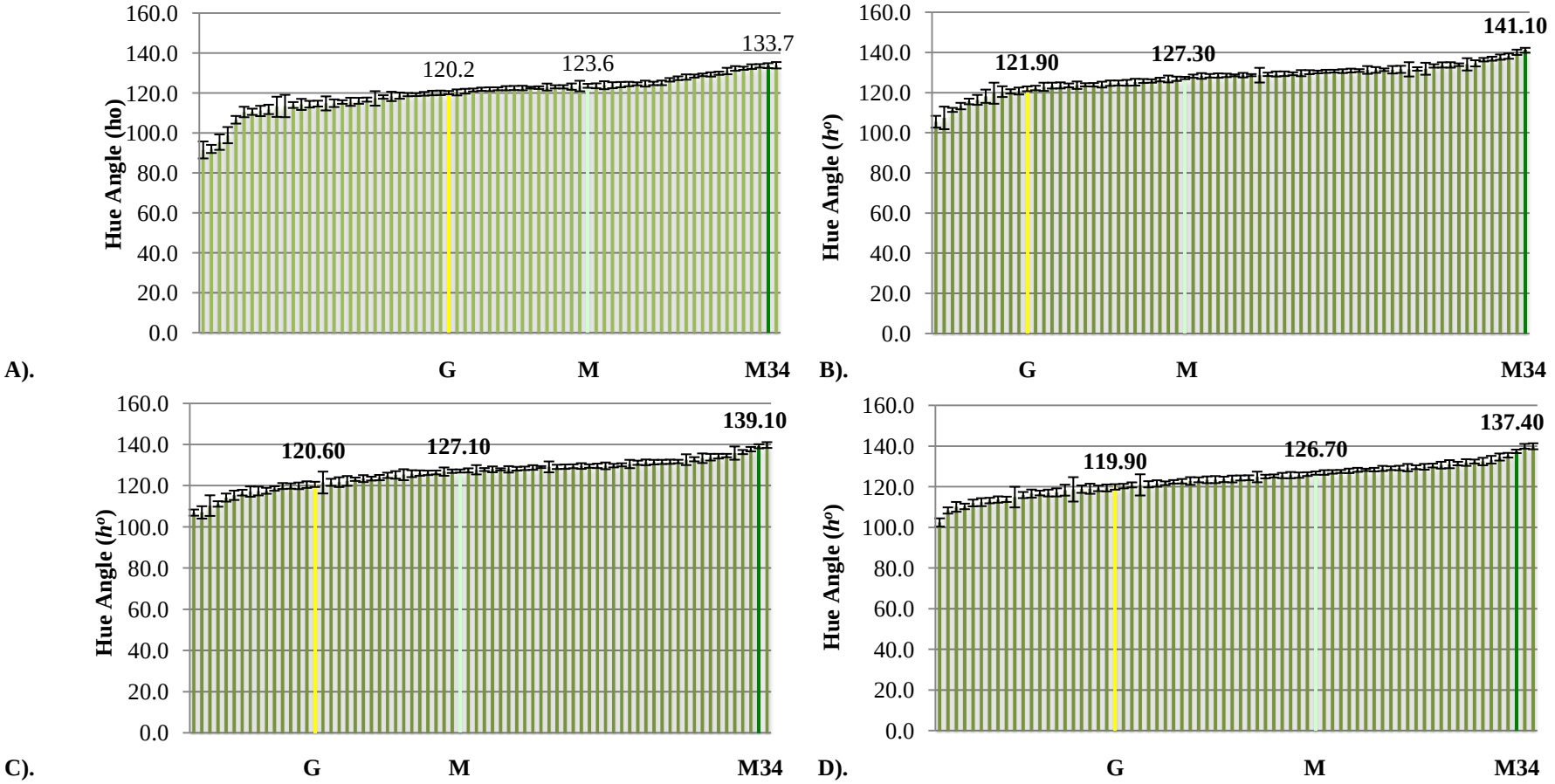


Figure 3.2.5: Predicted means (from REML) for the hue angle (h^o) of genotypes in the MGDH population A.) On day one. B). On day four. C). On day five and D). On day six in storage (n= 72). Error bars represent the s.e.m (standard error of the mean). The parental lines (G = GD33 & M34 = Mar34) and the commercial line (M = Marathon) are highlighted.

and displays an increase in chroma of 0.96 between day one and day six in storage. The commercial genotype Marathon also falls within the higher extreme of the distribution for chroma and shows an increase of 2.12 in chroma between day one and day six in storage. The good shelf life performer Mar34 falls within the mid-low range of the distribution for chroma and initially exhibits a decrease in chroma between day one and day four and between day four and day five before showing an increase on day six in storage. Overall, the chroma values for Mar34 increase by 1.32 to 8.20 on day six in storage.

In summary, visual assessments of colour and colour change in broccoli appear to be more reliable than quantitative measurements using a spectrophotometer, as the numerical values for the colour co-ordinates fail to distinguish between broccoli heads that are green and broccoli heads that have begun to turn yellow. Therefore, an alternative quantitative method and the optimisation of the spectrophotometer as a tool to quantify yellowing in broccoli will be explored in the next chapter (Chapter 4).

3.2.3.5 Morphological trait assessment of the MGDH population.

Consumers expect value for money when they purchase fresh produce and are influenced by factors such as size, weight and the appearance of a product. In broccoli, consumers expect a green, medium-large compact uniform head, with closed buds and a firm texture. To evaluate the morphological features of individuals in the MGDH population, traits such as head diameter, peduncle diameter, weight, weight loss, stem turgor, bud compactness, bud elongation, floret looseness and head colour were assessed. This will identify genotypes that have

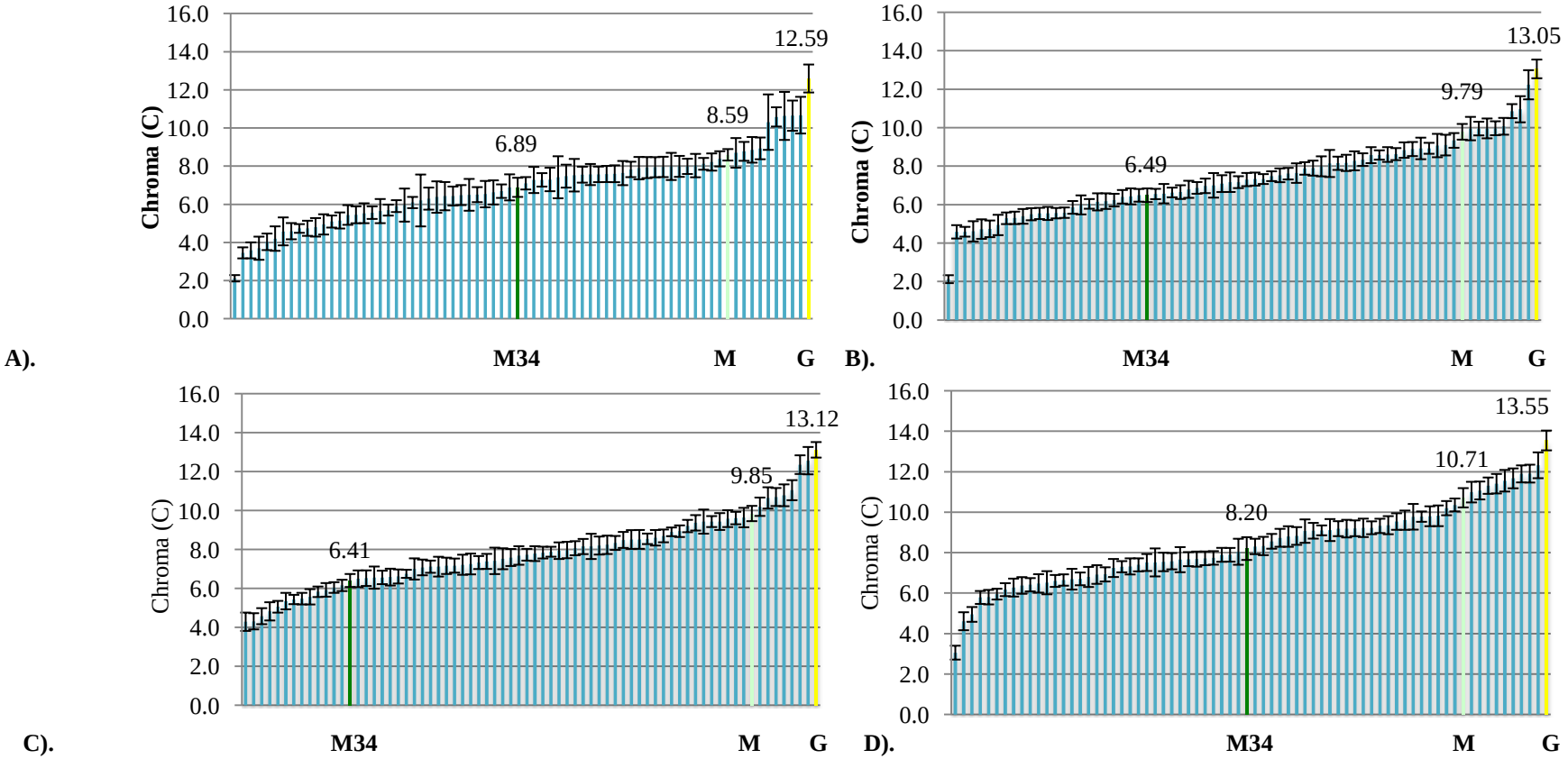


Figure 3.2.6: Predicted means (from REML) for the chroma (C) of genotypes in the MGDH population A.) On day one. B). On day four. C). On day five and D). On day six in storage (n =72). Error bars represent the s.e.m (standard error of the mean). The parental lines (G = GD33 & M34 = Mar34) and the commercial line (M = Marathon) are highlighted.

multiple desirable characteristics such as extended shelf life, retained firmness and acceptable head size and quality for the use in breeding for the improvement of quality traits in broccoli and also identify whether there are any undesirable associations, which is important to know for breeding.

1. *Head diameter (mm) & peduncle diameter (mm)*

The MGDH population display significant differences (****P<0.001*) for the traits head diameter (Wald $_{[72]}$ = 463.46) and peduncle diameter (Wald $_{[72]}$ = 465.20 at harvest (day 0). For both traits, the commercial genotype Marathon has the largest head and peduncle diameter (Figure 3.2.7), with the parental genotype GD33 having one of the smallest head and peduncle diameters (mm). In general, head diameter and peduncle diameter (mm) seem to be proportionate as the traits are positively correlated (Table 3.2.5) (****P<0.001*, $r_{[60]}$ = 0.68).

2. *Weight (g) and weight loss (g^{-1})*

Genotypes in the MGDH population show significant differences (****P<0.001*) in weight, at harvest, (Wald $_{[72]}$ = 640.75) and in absolute weight loss (Wald [72] = 569.27) and relative weight loss (Wald [72] = 172.25) during storage (Figure 3.2.8). Weight has a strong positive correlation (Table 3.2.5) with both head diameter and peduncle diameter (****P<0.001*, $r_{[60]}$ = 0.81 and 0.86 respectively) whereas absolute weight loss displays a strong negative correlation with weight and peduncle diameter and a weak negative correlation with head diameter (****P<0.001* $r_{[60]}$ = -0.82, -0.71 and -0.60 respectively). The commercial genotype Marathon had the heaviest head (160.48g) and the largest head and peduncle diameters, where as the parental genotype GD33 falls within the lower extremes of the distribution for weight, head diameter and peduncle diameter. Genotypes that are larger and

therefore heavy tend to lose more absolute weight during storage than smaller, lighter genotypes. Marathon and Mar34 lost 67.08 g and 39.65 g during storage compared to a loss of 12.07 g for GD33. To determine the amount of weight lost without the influence of size as a factor, relative weight loss was calculated (%). The parental genotypes GD33, Mar34 and the commercial genotype Marathon lose 42%, 44% and 48% of their starting weight (g) at harvest respectively by the time they reach failure due to head yellowing. Although correlations are present in the MGDH population between various morphological traits, only relative weight loss shows a positive correlation with the trait 'days to yellowing' (***$P<0.001$ $r_{[60]} = 0.76$) (Table 3.2.3).

Table 3.2.3: **Correlation matrix for morphological traits**. Significance levels are displayed as: ***P>0.001 (pink), **P>0.01 (orange) and *P>0.05 (yellow). HD = Head diameter (mm), PD = Peduncle diameter (mm), Wt = Weight (g), A_WtL = Absolute weight Loss (g^{-1}), R_WtL = Relative weight loss (%) and DY = Days to yellowing.

	HD	PD	Wt	A_WtL	R_WtL	DY
HD	-					
PD	***0.68***	-				
Wt	***0.81***	***0.86***	-			
A_WtL	***-0.60***	***-0.71***	***-0.82***	-		
R_WtL	*-0.37*	*-0.32*	*-0.31*		-	
DY				***-0.48***	***0.76***	-

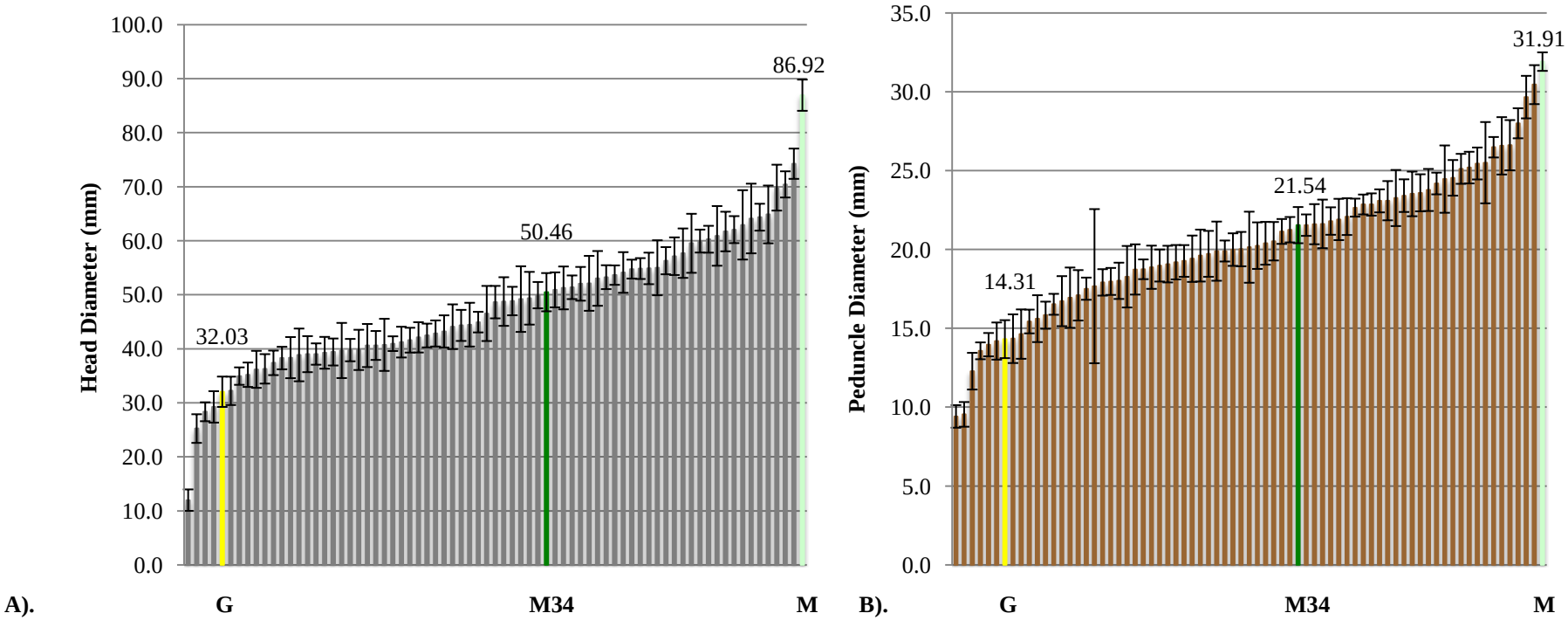


Figure 3.2.7: Predicted means (from REML) for the MGDH population for the traits A.) Head diameter (mm) B.) Peduncle Diameter (n = 72). Error bars represent the s.e.m (standard error of the mean). The parental lines (G = GD33 and M34 = Mar34) and the commercial line (M = Marathon) are highlighted.

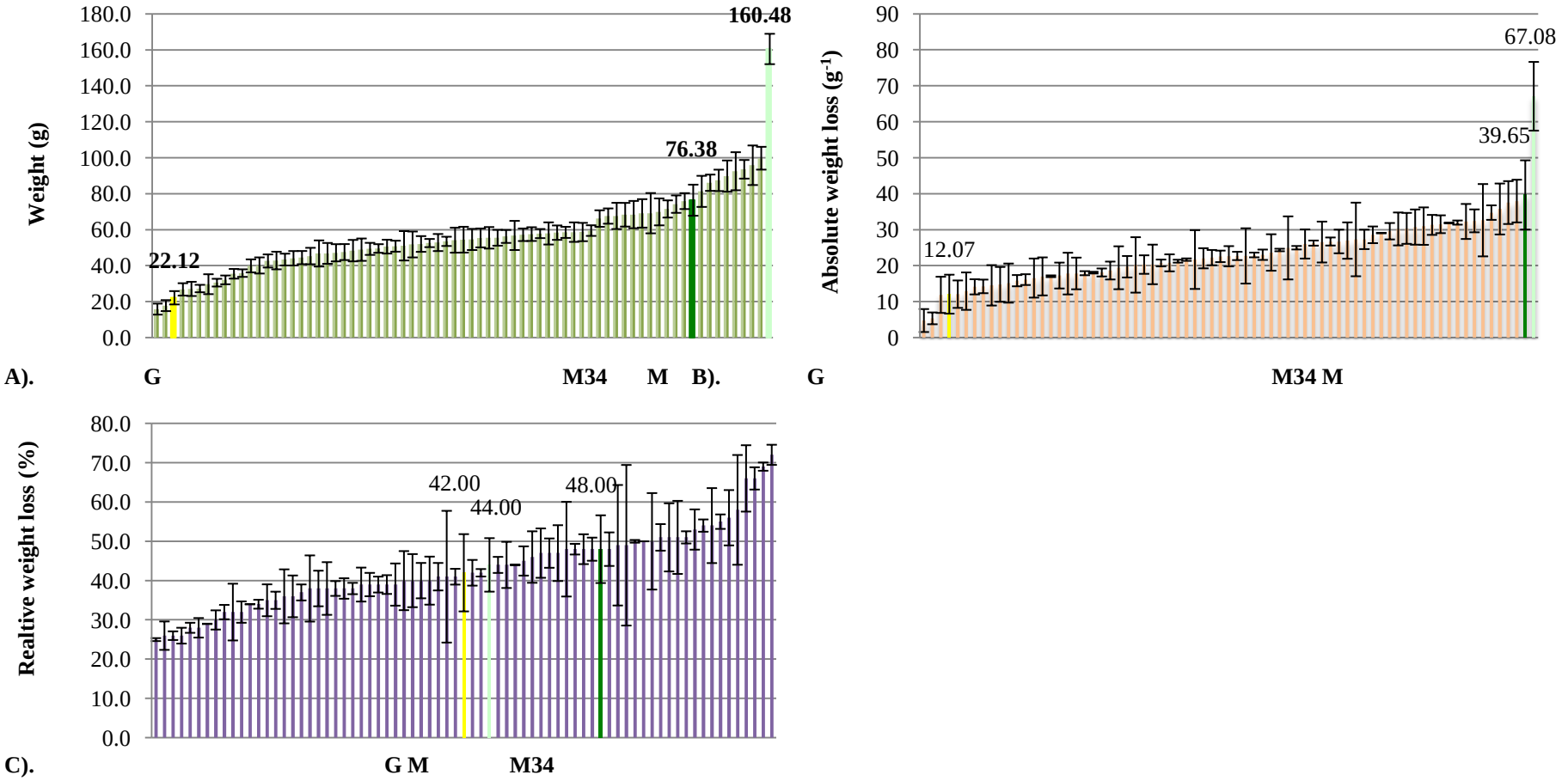


Figure 3.2.8: Predicted means (from REML) for the MGDH population for the traits A.) Weight (g), B.) Absolute weight Loss (g^{-1}) and C.) Relative weight loss (%) of original weight (n =72). Error bars represent the s.e.m (standard error of the mean). The parental lines (G = GD33 and M = Mar34) and the commercial line (M = Marathon) are highlighted.

3.2.3.6 Wurr broccoli quality scoring system.

The scoring system developed by Wurr *et al.* (2001) was used to assess the quality of genotypes in the MGDH population, for head colour, stem turgor, bud compactness, bud elongation and floret looseness. Each genotype was given a numerical score of 0, 1, 2 or 3 according to the guidelines for each trait (Table 3.2.4). On day one after harvest, most genotypes are acceptable for retail for all traits, with the exception of the trait bud compactness where 28 genotypes fail due to the opening of the flower buds (Figure 3.2.9). Changes in the acceptability was observed between day four and day six in storage, with genotypes in the population becoming unacceptable for retail for the traits stem turgor, bud elongation and floret looseness. The biggest change in scores can be seen for the traits stem turgor and floret looseness, with 86 % and 15 % of genotypes being classed as unacceptable for retail for these traits by day six in storage. A small proportion of genotypes (4 %) are also classed as unacceptable for retail due to bud elongation (3) by day six in storage.

Table 3.2.4: Photographic examples of genotypes assessed using the Wurr quality scoring system for head colour, bud compactness, bud elongation and floret looseness. Descriptions highlighted in red indicate when broccoli heads would be classed as unacceptable for retail for each quality trait.

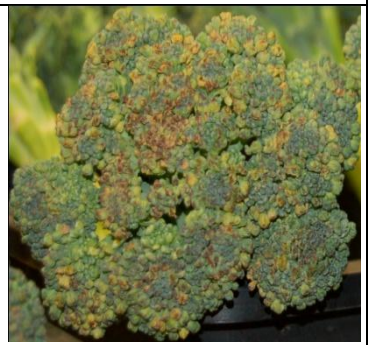
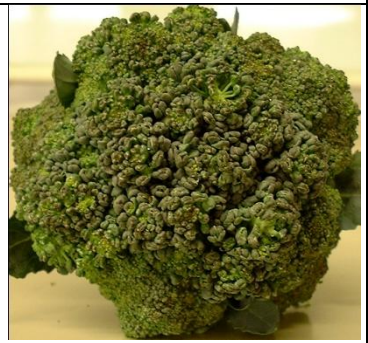
	0	1	2	3
Head Colour	 **Green**	 **Yellow**	 **Bronze**	
Bud Compactness	 **Closed**	 **Opening + yellow petals**	**This trait was not observed therefore a picture in unavailable** **Opening + green/white sepals**	**This trait was not observed therefore a picture in unavailable** **Fully open**

Table 3.2.4: *Continued*

	0	1	2	3
Bud Elongation	 **Uniform**	 **Slightly uneven**	 **Individual buds extending**	
Floret Looseness	 **Firm**	 **Florets beginning to separate**	 **Florets wide apart**	

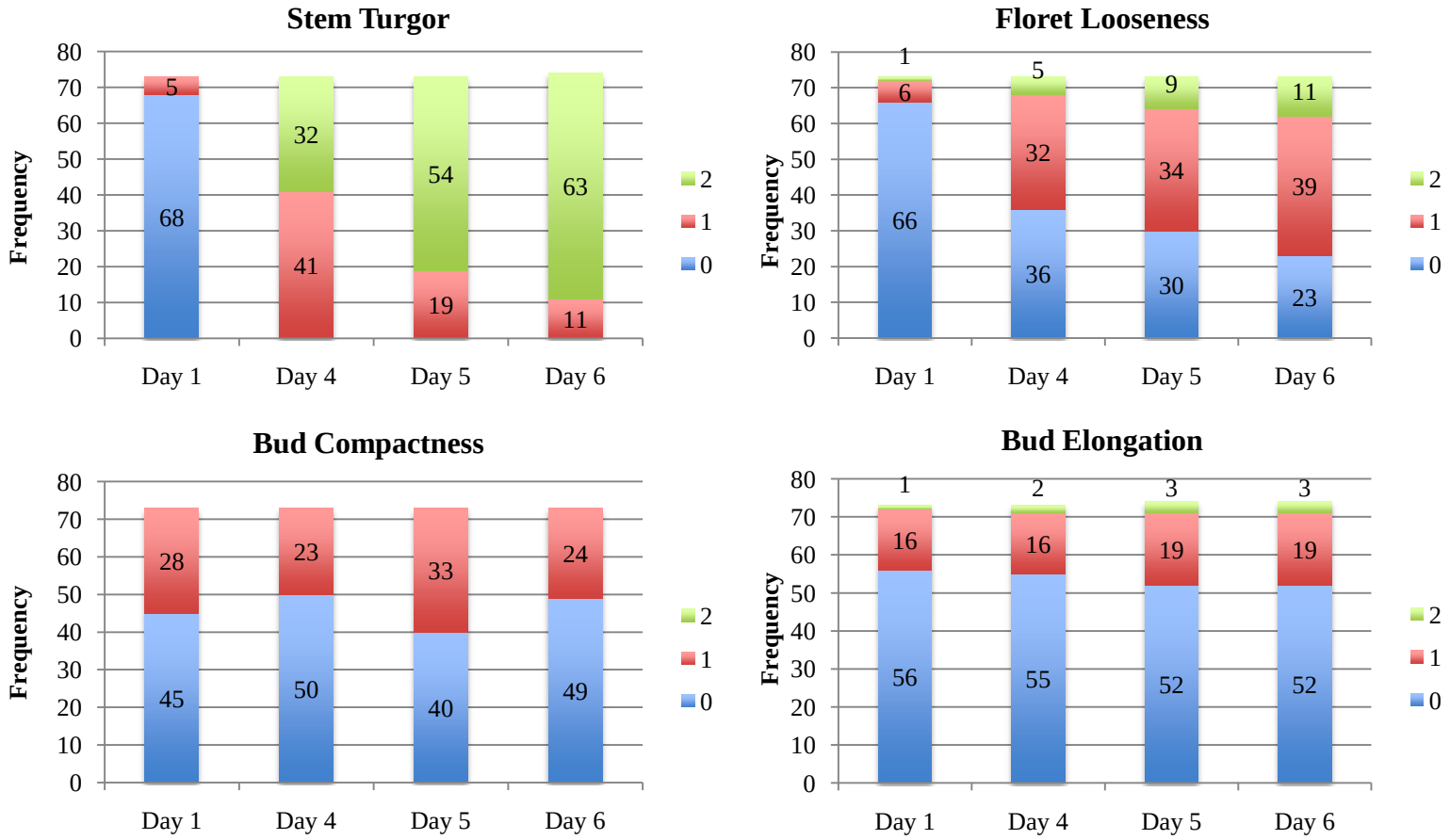


Figure 3.2.9: Frequency bar charts showing the quality scores of genotypes in the MGDH population based on the Wurr scoring system for the traits stem turgor, floret looseness, bud compactness and bud elongation on day one, day four, day five and day six in storage (n =72). For all traits, with the exception of bud compactness, a score of zero and one indicated that genotype were still acceptable for retail where as a score of two indicated that genotypes were classed as unacceptable for retail. For the trait bud compactness a score of one or above indicated that genotypes were unacceptable for retail.

Stem Turgor

Stem turgor exhibits the most change between day one after harvest and day six in storage for genotypes in the MGDH population (Figure 3.2.9). Out of the 63 genotypes that are classed as unacceptable for retail, 49 fail for stem turgor before becoming yellow. Out of the remaining genotypes, ten fail for both stem turgor and head yellowing simultaneously and three genotypes become yellow before the loss of stem turgor. Out of the 49 genotypes that are unacceptable for retail due to stem turgor, 67 % (Figure 3.2.10) fail for the trait one or two days before visible signs of head yellowing. However, two genotypes (MG532 and MG433) are classed as unacceptable for retail due to stem turgor five days before becoming yellow in storage. This indicates that stem turgor is a major contributing factor to the marketability of broccoli.

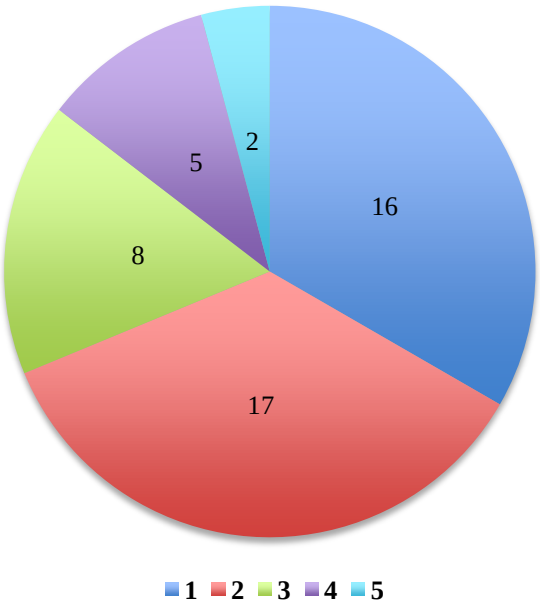


Figure 3.2.10. A pie chart showing the proportion of genotypes that are classed as unacceptable for retail due to stem turgor 1 (blue), 2 (red), 3 (green), 4 (purple) and 5 (cyan) days before visible head yellowing.

However, the approach used to measure stem turgor in the MGDH population is very simplistic and crude. A more sophisticated measure of stem turgor would be required to investigate this trait further.

Bud compactness

Opening of the flower buds to expose yellow petals was observed in 28 genotypes on day one after harvest (Figure 3.2.9) causing these genotypes to be classed as unacceptable for retail before failing for colour. In general, the number of genotypes with open flower buds remains constant during storage.

Floret looseness

Another trait that causes genotypes to become unacceptable for retail before head yellowing is floret looseness. In total, 11 genotypes fail due to floret looseness by day six in storage (Figure 3.2.9). However, only one genotype (MG124(27/9) was classed as unacceptable for retail due to floret looseness alone. For the remaining ten genotypes, three failed for both stem turgor and floret looseness simultaneously, five genotypes failed for stem turgor before the florets become loose and two genotypes become yellow before failing for floret looseness. Therefore, this trait tends to be influenced by changes in stem turgor rather than being a contributing factor to the marketability of broccoli alone.

Correlation between traits.

Traits that affect the marketability of broccoli do show some weak correlation with other morphological traits (Table 3.2.5), for example stem turgor displays a weak positive correlation with floret looseness on day four (***$P<0.001$ $r_{[60]} = 0.42$) and

with absolute weight loss on day 5 in storage (***$P<0.001$ $r_{[60]} = 0.38$), where as the trait floret looseness exhibits a weak positive correlation with both head diameter (***$P<0.001$ $r_{[60]} = 0.43$) and bud elongation (***$P<0.001$ $r_{[60]} = 0.59$). Bud compactness did not correlate with any of the morphological or quality traits assessed and none of the traits from the Wurr quality scoring system are correlated with the 'trait days to yellowing'.

Table 3.2.5: Correlation matrix for morphological traits and Wurr quality traits at harvest and on day four , day five and day six in storage. Significance levels are displayed as: ***P<0.001 (pink), **P<0.01 (orange) and *P<0.05 (yellow). HD = Head diameter, PD = Peduncle diameter, Wt = Weight, A_WtL = Absolute weight loss, R_WtL = Relative weight loss, ST = Stem Turgor, BC = Bud Compactness, BE = Bud Elongation, FL = Floret Looseness, DY = Day to Yellowing, D4 = day four, D5 = day five, D6 = day six

	HD	PD	Wt	A_WtL	R_WtL	ST_4	BC_D4	BE_D4	FL_D4
HD	-								
PD	***0.68***	-							
Wt	***0.81***	***0.86***	-						
A_WtL	***-0.60***	***-0.71***	***-0.82***	-					
R_WtL	*-0.37*	*-0.32*	*-0.31*		-				
ST_D4	0.24					-			
BC_D4							-		
BE_D4	0.26				-0.28			-	
FL_D4	***0.42***				*-0.32*	***0.42***		***0.59***	-
ST_D5		-0.27	*-0.30*	***0.38***		***0.52***			
BC_D5							***0.62***		-0.21
BE_D5					-0.26		-0.25	***0.84***	***0.62***
FL_D5	***0.42***				*-0.30*	***0.41***		***0.51***	***0.83***
ST_D6		*-0.37*	*-0.36*	*0.36*		*0.36*			
BC_D6	-0.23						***0.58***	*-0.32*	-0.26
BE_D6	0.23				***-0.38***			***0.79***	***0.50***
FL_D6	***0.43***				*-0.32*	*0.33*		***0.50***	***0.70***
DY				***-0.48***	***0.76***	*-0.31*		*-0.30*	*-0.30*

	ST_D5	BC_D5	BE_D5	FL_D5	ST_D6	BC_D6	BE_D6	FL_D6	DY
ST_D5	-								
BC_D5		-							
BE_D5			-						
FL_D5		-0.25	***0.59***	-					
ST_D6	***0.70***				-				
BC_D6		***0.65***	*-0.37*	-0.25		-			
BE_D6			***0.91***	***0.48***		*-0.32*	-		
FL_D6			***0.56***	***0.85***		-0.29	***0.48***	-	
DY	*-0.30*		*-0.30*	-0.27			***-0.42***	-0.26	-

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3.2.4 Discussion.

To evaluate the natural variation present in the MGDH population for shelf life, morphological and quality traits, a combination of visual and quantitative assessments were used.

3.2.4.1 Visual assessments of colour and colour change in the MGDH population.

Shelf life evaluation was carried out using visual assessments to replicate the consumer response and determine the day in storage when the onset of postharvest yellowing occurred. In this study, the MGDH population exhibited significant differences in natural variation for shelf life, with the onset of postharvest yellowing being observed in genotypes as early as day three and as late as day ten after harvest when stored at 14^{o}C. Postharvest yellowing is one of the main limiting factors for the marketability of broccoli and occurs due to chlorophyll degradation, which can be promoted by factors including storage conditions, respiration rate, the production of plant hormones and other gaseous compounds such as ethylene. Chlorophyll degradation is mediated by enzymatic reactions and loss of green colour is associated with the opening of the chlorophyll backbone by the conversion of pheophorbide *a* (breakdown product of chlorophyll *a*) to red chlorophyll catabolite (RCC) by pheophorbide oxygenase *a* (PAO) (Barry, 2009). Yamauchi *et al.* (1997) investigated the *in vitro* chlorophyll degradation in stored broccoli florets to elucidate the pathway of chlorophyll degradation by HPLC analysis of the degradative products. The findings indicate that in broccoli florets chlorophyllase is present to degrade chlorophyll to chlorophyllide *a* which is subsequently degraded

by phaeophorbide to pyrophaephorbide and converted into low molecular weight colourless compounds (Yamauchi *et al*., 1997).

The gaseous compound ethylene is also reported to have an important role in chlorophyll degradation and the regulation of induced senescence (Nishikawa *et al*., 2005), with the presence of exogenous ethylene being reported to accelerate yellowing in many *Brassica* species (Able *et al*., 2002; King and Morris, 1994). The use of ethylene inhibitors such as 1-Methylcyclopropene (1-MCP), has demonstrated the detrimental effect that ethylene has on broccoli shelf life. Application of 1-MCP in broccoli has been shown to delay yellowing and decrease respiration to prolong shelf life (Able *et al*., 2002; Blankenship and Dole, 2003) Ethylene production can be induced by mechanical wounding when vegetables are cut during harvesting. In this study, up to 500 harvested broccoli plants were stored in an enclosed shelf life facility allowing for the accumulation of endogenous ethylene. As ethylene acts to promote yellowing and increase respiration leading to deterioration in broccoli, genotypes in the MGDH population that display rapid postharvest yellowing may be more susceptible to the presence of endogenous ethylene.

The trait 'days to yellowing' is positively correlated with relative weight loss ($r = 0.76$). This suggests that genotypes that lose higher proportions of weight have a shorter shelf life. Rapid weight loss mainly occurs through water loss from evaporation, with the stress of water deficiency in harvested plants being linked with induced senescence (King and O'Donoghue, 1995). Ethylene production can also be triggered as a stress response (Yu and Yang, 1980); therefore senescence in genotypes that lose a high proportion of weight during storage may be accelerated.

Delayed senescence was observed in ~50% of the MGDH population with genotypes remaining green for up to ten days after harvest when stored at 14^{o}C. Differences in

the rate of senescence indicate that genotype is a large contributing factor to shelf life and suggests that genotypes with delayed senescence may show evidence of the stay green phenomenon reported in the literature. Five types of stay green phenotypes exist as described in Thomas and Smart (1993) and Thomas and Howarth (2000): 1. The initiation of senescence is delayed but then proceeds at the normal rate (Type A); 2. Senescence is initiated on time but proceeds at a slower rate (Type B); 3. Senescence is initiated and proceeds normally but green pigmentation from chlorophyll is retained (Type C); 4. Green colour is retained by processing methods such as boiling, freezing or drying (Type D) and 5. Intensely green genotypes which have increased photosynthetic capacity undergo normal senescence but appear to stay green due to elevated chlorophyll levels (Type E). Genotypes in the MGDH population that stayed green for a longer period during storage could exhibit any of the stay green phenotypes described with the exception of type D. Further experiments would be required to classify the stay green phenotypes in the MGDH population. Individuals in the population were observed to vary in green colour at maturity (type E) inferring that they differ in chlorophyll content, therefore genotypes that appear greener at harvest probably contain more chlorophyll than paler genotypes and require more chlorophyll degradation before a yellowing phenotype is observed (Thomas and Howarth, 2000). Currently, supermarkets issue a two-day retail period for broccoli, with a requirement for an improvement in shelf life by a minimum of one day to fit in with supermarket logistics for other green produce. The identification of genotypes with delayed senescence could provide a resource to improve shelf life in broccoli by conventional breeding.

3.2.4.2 Quantitative measurements of colour and colour change in the MGDH population.

As an alternative approach to visual assessments, of colour and colour change in broccoli, which can be subjective, a spectrophotometer was used to attempt to quantify yellowing. The Hunter *Lab* colour co-ordinates measure the lightness (L), and the colour of an object on a red-green (a) colour scale and a yellow-blue (b) colour scale. The L, a, b values can then be used to calculate hue angle, which is similar to how humans perceive colour, and chroma which shows the vividness/dullness of an object. However, the results obtained from genotypes in the MGDH population were ambiguous since the numerical values failed to effectively distinguish between broccoli heads that were visually green and broccoli heads that were visually yellow. Possible reasons for anomalies in the data include: the variation in the green colour of genotypes at maturity, the uneven surface of a broccoli head, floret looseness, exposed flower buds, bronzing of buds and the small number of readings taken per head (5) due to restraints on time. This has been addressed in more detail in Chapter 4.

3.2.4.4 Morphology.

Consumers stipulate produce to be of a reasonable size and weight when they make a purchase and expect produce to have good storing ability with minimal quality and weight loss before consumption. The MGDH population exhibits significant natural variation in head size, peduncle size and weight at harvest, and weight loss (absolute and relative) during storage, with correlations being observed among these traits. As expected, weight is positively correlated with both head ($r = 0.81$) and peduncle ($r = 0.86$) diameter, showing that both the size of the head and the peduncle influence

weight, with larger diameters being associated with heavier weight. Head diameter and peduncle diameter also show a positive correlation (r = 0.68) although the relationship is weaker compared to the correlations with weight. In general, for the majority of the population head size and peduncle size were proportionate. However, some genotypes were observed to have large peduncles but small head sizes. Head width has been reported by Lan and Paterson (2000) to be associated with the branching pattern, in particular the length of the apical shoot and first branches and the acute angle of the curd since QTL for curd related traits were found to map in the same chromosomal regions as head width. This study only measured head diameter, however to understand the genetic control of head morphology in more detail, measurements used by Lan and Paterson (2000) such as first rank branching, the number of side branches, apical shoot length and first branch length should be considered. Water stress has also been shown to influence head diameter and weight in broccoli with Wurr *et al*, (2002) reporting a reduction in head size and weight when water stress was applied for 2-3 weeks after head initiation. Absolute weight loss is also influenced by weight, head and peduncle size as these traits are all negatively correlated, suggesting that larger broccoli heads may lose more weight through water loss due to evaporation and increased surface area compared to smaller broccoli heads. In contrast, it is speculated that larger peduncle sizes may help to reduce weight loss due to the retention of water in the stem but this would need to be investigated further.

3.2.4.5 Bud morphology

Bud morphology in the MGDH population was also assessed by using the Wurr scoring system for traits such as bud elongation and bud compactness. Bud

elongation was not a limiting factor for the marketability of genotypes in the population as only three genotypes were observed to have elongated buds. Some genotypes were observed to have 'pheasant eye' bud arrangement; however these were classed as slightly uneven and therefore still acceptable to consumers. In contrast, bud compactness did cause 38 % of the population to be classed as unacceptable for retail due to opening of the flower buds to expose yellow petals, before failing for colour. Bud opening compromises the quality of broccoli by causing heads to have a stale appearance (Wurr *et al*., 2002). Changes in bud morphology have been shown to be influenced by preharvest factors. A study by Wurr *et al*. (2002) investigating the effect of water stress on bud morphology found that changes in bud elongation and bud compactness were observed when applying water stress for different durations and at different times after head initiation. Water stress applied for 14 days and 21 days after head initiation was shown to have a deleterious effect and promote bud elongation compared to the control, whereas bud compactness was not affected. Bud elongation and bud compactness were also found to be affected when water stress was applied at three different stages after head initiation. When water stress was applied two weeks after head initiation bud elongation and bud compactness was improved as broccoli heads lasted longer in storage before failing for these traits compared to the control and early (at head initiation) and late (4 weeks after head initiation) water stress treatments.

3.4.4.6 Changes in texture and quality.

Texture is another important attribute of vegetables, as consumers associate firmness with freshness and quality, and reject vegetables that appear limp or soft (Rico *et al*., 2007). Textural quality in fresh produce is determined by a combination of factors

including genotype, growing conditions, postharvest handling and storage (Toivonen and Brummell, 2008). Water loss promotes loss of turgor, which influences the rigidness, or elasticity of the plant cell wall (Toivonen and Brummell, 2008). Vegetables have firm tissue as they consist of cells with thickened secondary walls, when tissue failure occurs it is usually as a result of the primary cell wall rupturing (Toivonen and Brummell, 2008). In vegetables such as broccoli, loss of textural quality is associated postharvest senescence or natural ageing, water loss, wounding and a reduction in turgor (Toivonen and Brummell, 2008). Changes in texture leading to loss of firmness in the MGDH population were associated with water loss and compromised stem turgor, which resulted in genotypes with rubbery stems and flaccid heads with the florets separating. A high proportion (86%) of genotypes in the MGDH population were classed as unacceptable for retail, due to loss of stem turgor, by day six after harvest when stored at 14^{o}C, indicating that stem turgor is a limiting factor for the marketability of broccoli. Changes in texture resulting in the loss of firmness can also be caused by cell wall modifications induced by postharvest senescence and wounding. Softening is caused by enzymatic degradation of cell wall pectin to polygalacturonic acid by pectin methylesterase (PME) and polygalacturonase (PG) with the genes encoding these enzymes shown to be upregulated during leaf senescence.

Conclusions:

- The MGDH population displays significant and measurable natural variation for morphological traits, colour co-ordinates and the trait ‘days to yellowing’. Therefore these traits are suitable for genetic analysis.
- The trait stem turgor is a limiting factor for the marketability of broccoli and many genotypes failed for this before yellowing. Therefore the genetic control of this trait will be important when breeding for improved quality in broccoli.
- The shelf life of genotypes in the MGDH population are not influenced by morphological traits, therefore breeders can make improvements to shelf life without compromising on size and weight.
- Colour co-ordinates from the MGDH population do not produce reliable quantitative measurements for head yellowing. Alternative methods will be investigated and the use of the spectrophotometer optimised in Chapter 4.

CHAPTER 3:

CHARACTERISATION OF GENETIC LOCI CONTROLLING SHELF LIFE IN BROCCOLI

Part III:

QTL analysis of shelf life and morphological traits.

3.3.1 Introduction

The mapping of quantitative trait loci (QTL) relies on variation to be present both at the genotype and phenotype level. The previous sections demonstrated that variation is present in the MGDH population at both the DNA level, by the use of molecular markers to construct a genetic linkage map, and at the phenotype level as genotypes showed variation for colour, morphological and quality traits. By locating QTL within the genetic linkage map for traits of interest, identification of the regions in the genome that contain genes controlling the trait concerned can be achieved. Furthermore, the QTL regions provide targets for fine scale mapping to delimit the confidence interval of the QTL with the eventual aim of indentifying candidate gene(s) determining the trait. QTL mapping also enables the selection of markers linked to QTL for use in marker-assisted selection for the improvement of quality traits in broccoli through conventional breeding. To date many QTL studies in *Brassica* haved focused on agronmonic traits such as seeding vigour (Finch-Savage *et al*, 2005 and 2010; Bettey *et al*, 2000;), flowering time (Axelsson *et al*, 2010; Camargo *et al*, 1996; Bohuon *et al*, 1998; Uptmoor *et al*, 2008), disease/insect resistance (Nagaoka et al, 2010; Camargo *et al*, 1995; Voorrips *et al*, 2003; Sarfraz *et al*, 2006), plant morphology (Maloof *et al*, 2003; Lan and Paterson, 2000 and 2001; Sebastian *et al*, 2002). However, there are no comprehensive studies in broccoli that have located QTL assicated with quality traits and shelf life. Therefore the aim of this chapter was to identify QTL linked to improved shelf life and quality traits in broccoli.

Aims

- To map QTL(s) relating to shelf life, head yellowing and morphological traits.

- To determine the beneficial allele for traits of commercial importance.
- To identify markers for use in marker assisted selection to breed for improved quality in broccoli.

3.3.2 Materials and Methods

QTL analysis was carried out as described in section 2.6 on all traits described in the previous chapter (Chapter 3, Part II).

3.3.3 Results

The REML analyses carried out in Chapter 3, Part II, confirmed that genetic variation was present for all traits assessed in the 2007 field trail. By using the adjusted means from REML, QTL analysis can be carried out to determine the location of putative QTL in the *B.oleracea* genome controlling quality traits in broccoli.

3.3.3.1 Variation for traits between parents of the MGDH population.

Using the predicted means for all traits assessed in the 2007 field trial, variation among the parental genotypes can be determined by comparing the position of the parents in each trait distribution (Figure 3.3.1). The parental genotype Mar34 falls within the higher tail of the distribution for all traits when compared with GD33, confirming that Mar34 is the better performing genotype for most quality traits in broccoli. This is also true for the quality traits assessed using the Wurr quality scoring system, as Mar34 does not fail for stem turgor, bud compactness, bud elongation or floret looseness during storage, whereas GD33 fails for both stem turgor and floret looseness during storage.

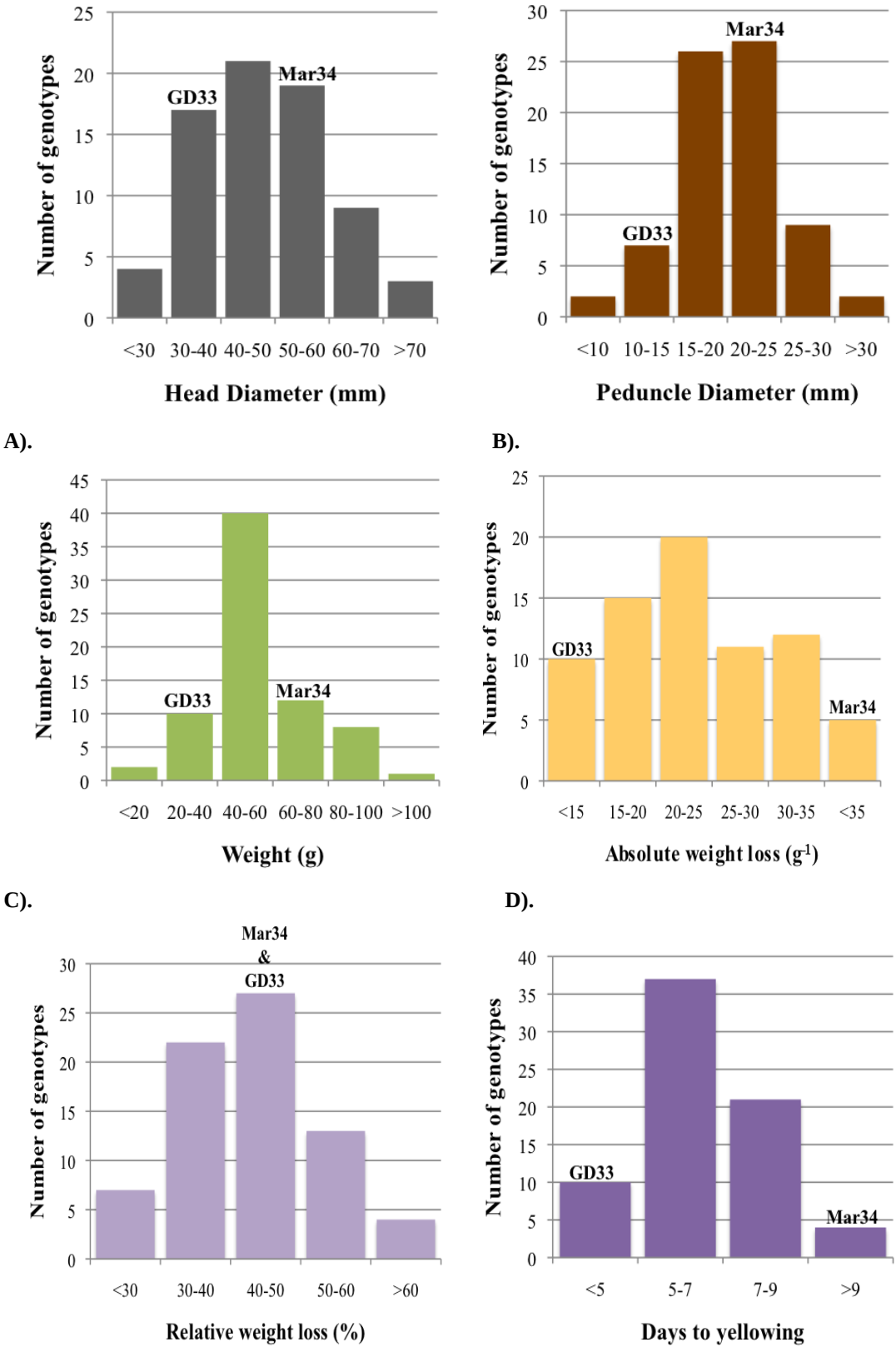
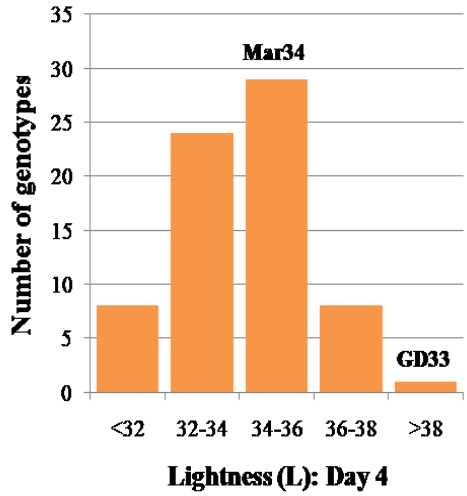
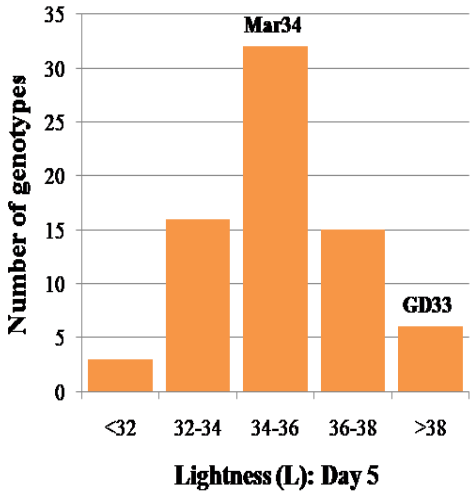


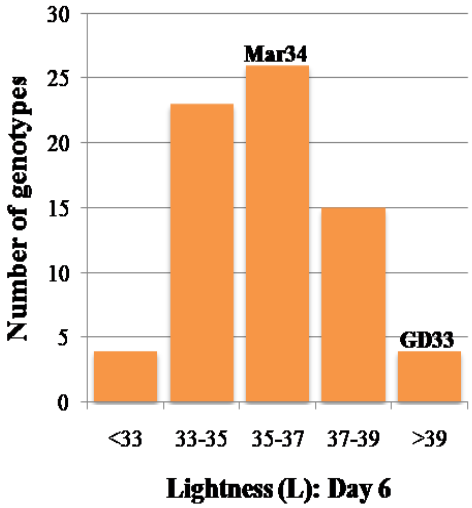
Figure 3.3.1: Histograms of traits assessed in the 2007 field trial (= 72): A). Head diameter. B). Peduncle diameter. C). Weight. D). Absolute weight loss. E). Realtive weight loss and F). Days to yellowing.



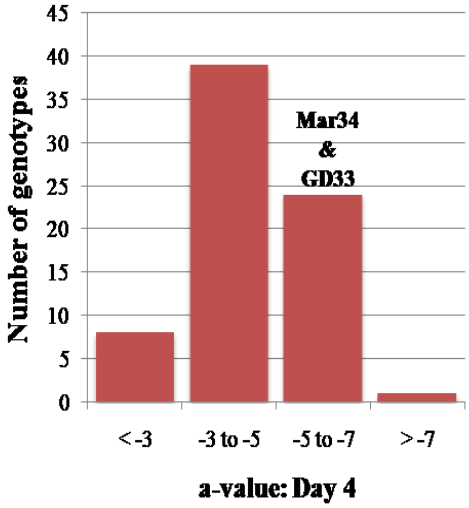
G).



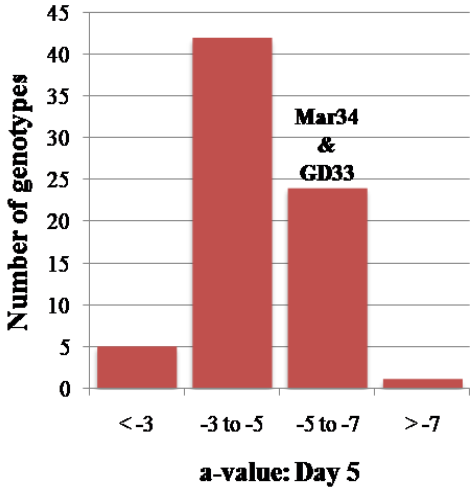
H).



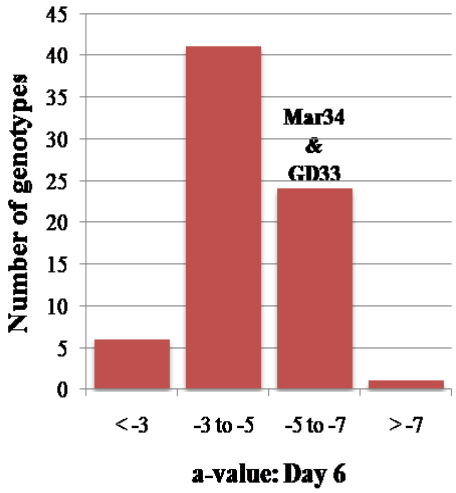
I).



J).

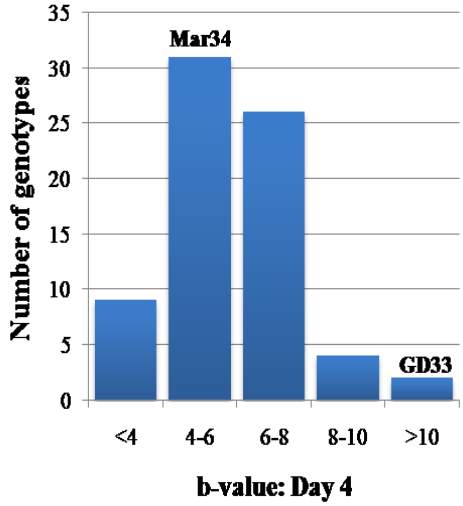


K).

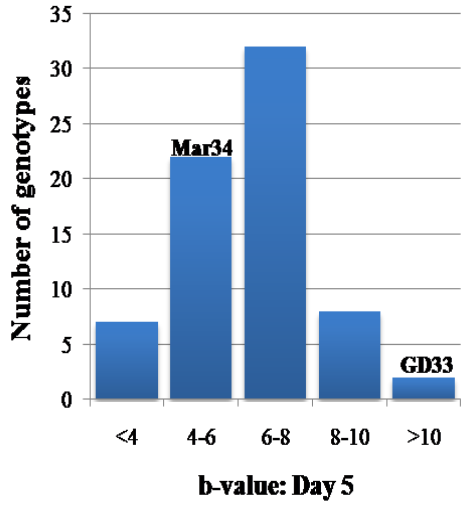


L).

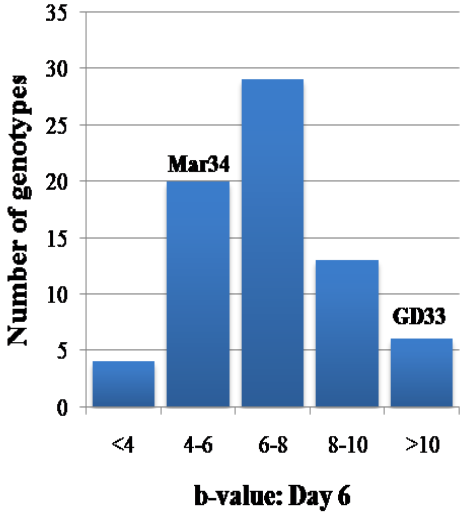
Figure 3.3.1 Histograms of traits assessed in the 2007 field trial (n = 72): G). Lightness on Day 4. H). Lightness on day 5. I). Lightness on day 6. J). a-value on day 4. K). a-value on day 5 and L). a-value on day 6



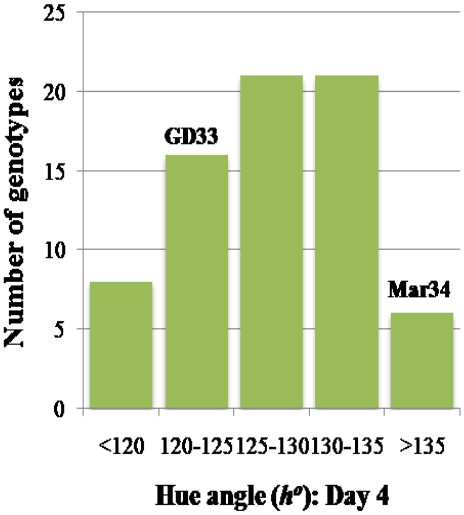
M).



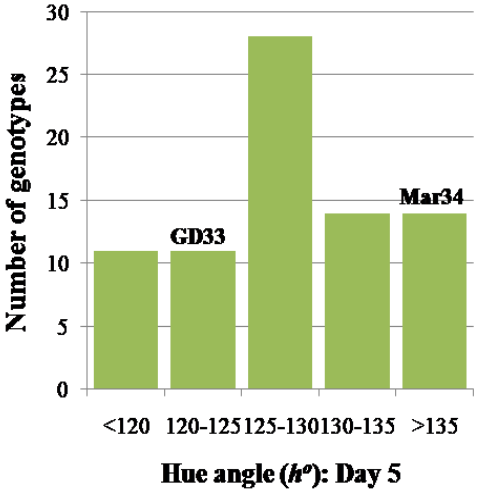
N).



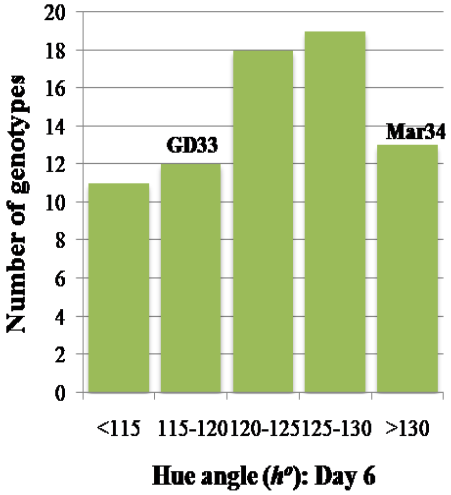
O).



P).

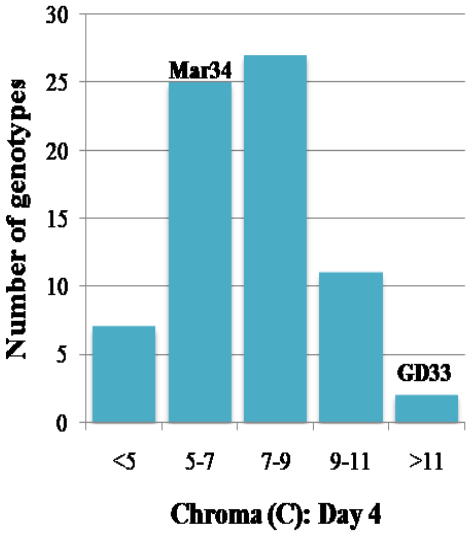


Q).

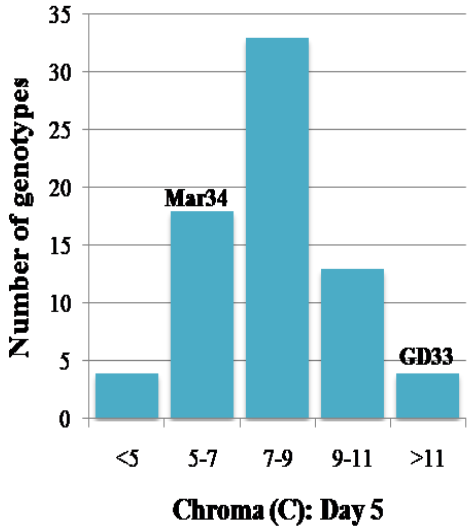


R).

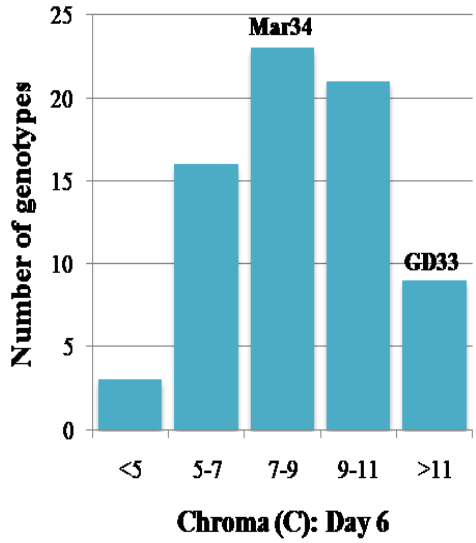
Figure 3.3.1 Histograms of traits assessed in the 2007 field trial (n = 72): M). B-value on Day 4. N). B-vaule on day 5. O). B-value on day 6. P). Hue angle on day 4. Q). Hue angle on day 5 and R). Hue angle on day 6



S).



T).



U).

Figure 3.3.1 Histograms of traits assessed in the 2007 field trial (n = 72): S). Chroma on Day 4. T). Chroma on day 5. U). Chroma on day 6.

3.3.3.2 QTL detection and location

Quantitative genetic analysis was carried in MapQTL 4.0 (Van Oojen *et al.*, 2002) using predicted means for all traits generated from REML analyses. Both a single QTL (interval mapping) and a multiple QTL model (MQM mapping) were used to

detect significant putative QTL in the broccoli x broccoli linkage map for quality traits in broccoli.

3.3.3.3 LOD significance thresholds

Genome wide LOD significant thresholds at **P>0.05, **P>0.01* and ****P>0.001* were calculated using a permutation test in MapQTL V4.0 (Van Ooijen *et al.,* 2002) for all traits, with the exception of the Wurr quality scores, where LOD significant threshold were calculated from a paper published by Van Ooijen (1999).

3.3.3.4 Single QTL model (Interval mapping)

Interval mapping (IM) was carried out using MapQTL V4 (Van Ooijen *et al.,* 2002) on all traits assessed in the 2007 field trial: head diameter (*HD*), peduncle diameter (*PD*), weight (*Wt*), absolute weight Loss (*AWtL*), relative weight loss (*RWtL*), days to yellowing (*DY*), stem turgor (*ST*), bud compactness (*BC*), bud elongation (*BE*), floret looseness (*FL*), presence of purple pigments (*P*), Lightness (*L*), colour on a red-green colour scale (*a*), colour on a yellow-blue colour scale (*b*), hue angle (*HA*) and chroma (*C*). IM detected a total of 27 genome wide significant (LOD ≥ 2.8) QTLs (Appendix C) QTL for 13 of the 15 traits; *HD* and *PD* were the only traits that did not produce significant QTL at the genome wide level. To improve the robustness of the QTL detected with a single QTL model and to minimise the chance of type I (false positives i.e. no QTLs are present) and type II errors (QTLs that are missed/masked by the presence of other QTLs) that occur in QTL mapping (Jansen, 1994), MQM mapping was carried out using the closest markers from IM linked to QTL as cofactors.

3.3.3.5 Multiple QTL model (MQM mapping)

MQM mapping increased the number of genome wide significant QTL detected from 27 to 40 for traits assessed in the 2007 field trial (Table 3.3.1). QTL were detected in all linkage groups, with the exception of LG O8, for the following traits (Figure 3.3.2): *Wt* (4 QTL), *AWtL* (1 QTL), *RWtL* (3 QTL), *DY* (4 QTL), *ST* (4 QTL), *BC* (1 QTL), *BE* (2 QTL), *FL* (4 QTL), *P* (1 QTL), *a* (4 QTL), *b* (6 QTL), *HA* (3 QTL) and *C* (3 QTL).

Table 3.3.1: QTLs detected by MQM mapping for colour, morphology and quality traits in the MGDH mapping population from the 2007 field trial.

Trait[a]	LG[b]	Position[c] (cM)	Marker[d]	LOD[e]	Beneficial Allele[f]	Variance[g] (%)	QTL Label[h]
Wt	O1	104.5	E11M54_277	3.6**	Mar-34	12.1	*Wt_1*
	O2	40	Ni2C12	5.1***	GD33	18.8	*Wt_2*
	O3	37.9	BRAS_069	4.1**	Mar34	14.5	*Wt_3*
	O9	36.7	OL12A04	5.0***	Mar34	18.7	*Wt_4*
AWtL	O9	36.7	OL12A04	4.1**	Mar34	20.7	*AWtL_1*
RWtL	O1	104.5	E11M54_277	3.7**	Mar34	13.9	*RWtL_1*
	O3	66.8	FTIO222	4.7***	Mar34	19	*RWtL_2*
	O9	36.7	OL12A04	8.5***	Mar34	44	*RWtL_3*
DY	O1	3.4	FITO095	4.1**	Mar34	13.9	*DY_1*
	O2	40	Ni2C12	5.3***	Mar34	19.3	*DY_2*
	O7	41.2	E11M59_80	3.9**	Mar34	13.1	*DY_3*
	O7	61.1	E11M50_322	3.8**	Mar34	12.8	*DY_4*
ST(d4)	O6	16.7	MB4	3.0*	Mar34	20.3	*ST(d4)_1*
	O9	31.6	BN83B1	3.7**	Mar34	26.3	*ST(d4)_2*
ST(d5)	O6	11.6	FITO203	2.9*	Mar34	19	*ST(d5)_1*
ST(d6)	O3	18.6	E11M50_201	PF***	Mar34	95	*ST(d6)_1*
BC(d5)	O3	37.9	BRAS069	3.4*	GD33	32.3	*BC(d5)_1*
BE(d5)	O7	61.1	E11M50_322	3.9**	GD33	33.7	*BE(d5)_1*
BE(d6)	O1	112.7	E11M62_290	3.1*	GD33	12.8	*BE(d6)_1*
FL(d5)	O2	50	E11M50_167	4.7***	Mar34	28.4	*FL(d5)_1*
	O6	16.7	MB4	4.1**	Mar34	23.3	*FL(d5)_2*
	O7	61.1	E11M50_322	3.6**	Mar34	21.5	*FL(d5)_3*
	O6	0	E11M49_290	2.9*	Mar34	22.2	*FL(d6)_1*
P	O1	112.7	E11M62_290	PF***	GD33	100	*P_1*
	O1	117.7	E11M62_290	PF***	GD33	100	*P_1*
a(d4)	O4	68.1	Na10D09	5.7***	GD33	37.3	*a(d4)_1*
a(d5)	O1	80.3	E11M59_192	2.8*	Mar34	17.5	*a(d5)_1*
a(d6)	O1	80.3	E11M59_192	5.4***	Mar34	30.4	*a(d6)_1*
	O4	68.1	Na10D09	3.0*	GD33	14.5	*a(d6)_2*
b(d4)	O3	52.3	E11M50_185	3.0*	Mar34	9.1	*b(d4)_1*
	O4	63.1	E11M48_264	7.3***	Mar34	29.5	*b(d4)_2*

Table 3.3.1: *Continued.*

Trait[a]	LG[b]	Position[c] (cM)	Marker[d]	LOD[e]	Beneficial Allele[f]	Variance[g] (%)	QTL Label[h]
b(d6)	O4	63.1	E11M48_264	7.7***	Mar34	15	*b(d6)_1*
	O5	0	E11M50_156	4.0**	Mar34	5.8	*b(d6)_2*
	O5	103.9	E11M50_346	3.0*	Mar34	4.5	*b(d6)_3*
	O6	39.9	E11M62_144	9.2***	Mar34	19.6	*b(d6)_4*
	O9	96	E11M49_402	9.4***	Mar34	20.5	*b(d6)_5*
HA(d4)	O1	80.3	E11M59_192	3.7**	Mar34	33.7	*HA(d4)_1*
HA(d5)	O1	78.1	E11M49_204	5.9***	Mar34	46.3	*HA(d5)_1*
HA(d6)	O1	78.1	E11M49_204	5.4***	Mar34	34.9	*HA(d6)_1*
C(d4)	O3	44.8	E11M48_124	3.1**	Mar34	14.1	*C(d4)_1*
	O4	63.1	E11M48_264	4.7***	Mar34	27	*C(d4)_2*
C(d6)	O4	63.1	E11M48_264	5.3***	Mar34	29.3	*C(d6)_1*

[a] Trait abbreviations: *Wt* (g); *AWtL*, absolute weight loss (g^{-1}); *RWtL*, relative weight loss (%); *DY*, days to yellowing; *ST*, stem turgor; *BC*, bud compactness; *BE*, bud elongation; *FL*, floret looseness; *P*, Purple; *a*, red-green colour scale; *b*, yellow-blue colour scale; *HA*, hue angle; *C*, chroma [b] LG: Linkage group containing QTL.[c] Position (cM) of the QTL peak in the linkage group [d] Marker: Closest marker to the QTL peak .[e] LOD: log of the odds score for genome wide significant QTL.[f] The parental allele that causes an increase in the trait value. [g] The percentage variation explain by the QTL. [h] The QTL label given to QTL mapped in the broccoli x broccoli linkage map.

3.3.3.6 Morphological traits.

Weight and weight loss

A total of eight putative QTL located in six linkage groups were mapped for morphological traits weight, relative weight loss, and absolute weight loss (Figure 3.3.2). For the trait weight four genome wide significant QTL were detected in LG O1 (*Wt_1*), LG O2 (*Wt_2*), LG O3 (*Wt_3*) and LG O9 (*Wt_4*). Each QTL for the trait weight explains 12.1-18.8 % of the genetic variation for the trait with the beneficial alleles coming from the parental genotype Mar34. Three QTL that were detected for weight using MQM mapping were also present in IM, with a new QTL

in LG O3 being detected after MQM mapping. Four genome wide significant QTLs were also detected for the traits relative weight loss and absolute weight loss in LG O1 (*RWtL_1*), LG O3 (*RWtL_2*) and LG O9 (*RWtL_3, AWtL_1*). Each QTL for the trait relative weight loss explains between 13.9-44.0 % of the genetic variation present, with the QTL for the trait absolute weight loss explaining 36.7 % of the genetic variation present. For all QTL mapped, the beneficial allele associated with these traits comes from the good performer Mar34. Out of the three QTLs mapped for relative weight loss, two were also present using IM, with QTL *RWtL_1* being detected after MQM mapping. For the trait absolute weight loss two QTL were detected using IM, QTL *AtWL_1* was retained after MQM mapping but the other QTL was lost. QTL for measures of weight loss co-localise with two of the QTL mapped for weight on LG O1 (*Wt_1* and *RWtL_1*) and LG O9 (*Wt_4*, *RWtL_3* and *AWtL_1*). QTL for the trait *Wt* also co-localise with QTL *DY_2* in LG O2 and *BC(d5)_1* in LG O3.

3.3.3.7 Wurr broccoli quality scoring system.

In total, eleven putative QTL were detected in five linkage groups for quality traits assessed on day four, day five and day six in storage using the Wurr scoring system (Figure 3.3.2).

Stem Turgor

MQM mapping retained all QTL for the trait stem turgor that were present in IM. In total, four QTL were mapped in LG O3 (*ST(d6)_1*), LG O6 (*ST(d4)_1* and *ST(d5_1*) and LG O9 (*ST(d4)_2*) (Figure 3.3.2). The beneficial allele for all QTL detected for the trait stem turgor comes for the good performer Mar34. Two QTL for the trait *ST* (*ST(d4)_1* and *ST(d5)_1*) overlap with two QTL for the trait floret looseness

(*FL(d5)_2* and *FL(d6)_2*) in LG O6. QTL *ST(d4)_2* is also found to overlap with QTL mapped for weight, relative weight loss and absolute weight loss in LG O9. QTL detected for stem turgor on day four in storage explains 20.3-26.3% of the genetic variation for the trait, whereas QTL detected for day five in storage and day six in storage explain 19.0% and 95% of the genetic variation respectively.

Bud Compactness

MQM mapping confirmed the single QTL detected for the trait *BC* on day five in storage that was also present using IM (Figure 3.3.2). QTL *BC(d5)_1* maps in LG O3 with the GD33 providing the beneficial allele for the trait. The QTL accounts for 32.3% of the genetic variation present and co-localises with QTL *Wt_3* at map position 39.5 cM in the linkage group.

Bud Elongation

Two QTL detected in LG O1 and LG O7 for the trait *BE* on day five and day six in storage were confirmed by MQM mapping (Figure 3.3.2). QTL *BE(d5)_1* and QTL *BE(d6)_1* account for 33.7% and 12.8% of the genetic variation associated with the trait on day five and day six in storage, with the beneficial alleles coming from GD33. Both QTL co-localise with QTL for other traits with QTL *BE(d5)_1* co-localising with QTL *DY_4* and QTL *FL(d5)_3* in LG O7 and QTL *BE(d6)_1* co-localising with QTL *P_1* in LG O1.

Floret Looseness

IM mapping detected a total of five QTL for the trait FL during storage, after MQM mapping two QTL from IM mapping were retained and two new QTL were detected.

In total, four QTL were mapped for the trait *FL* in three linkage groups LG O2 (*FL(d5)_1*), LG O6 (*FL(d5)_1* and *FL(d6)_1*) and LG O7 (*FL(d5)_2*) with three QTL detected on day five in storage and one QTL detected on day six in storage (Figure 3.3.2). For all QTL, the Mar34 provides the beneficial allele for the trait. All QTL for the trait *FL* overlap or co-localise with QTL for other quality traits. QTL *FL(d5)_1* that maps in LG O2 explains 28.4 % of the genetic variation present for the trait and overlaps with QTL *DY_2* and *Wt_2* that also map in LG O2. Both QTL for *FL* that map in LG O6 explain 22.2 and 23.2 % of the genetic variation associated with the trait and co-localise with QTL *ST(d4)_1* and *ST(d5)_1*. Similarly QTL FL(d5)_2 that maps in LG O7 explains 21.5 % of the genetic variation present and co-localises with QTL for the traits *DY* and *BE*.

3.3.3.8 Colour and colour change.

Days to yellowing

MQM mapping allowed the detection of three new QTL (*DY_1*, *DY_3* and *DY_4* for the trait *DY* and retained QTL *DY_2* identified in IM. In total, four putative QTL were mapped in three linkage groups (Figure 3.3.2): LG O1 (*DY_1*), LG O2 (*DY_2*) and LG O7 (*DY_3* and *DY_4*), with each QTL explaining between 12.8 – 19.3 % of the genetic variation associated with the trait. For all QTL, beneficial allele for the trait *DY* comes from the good shelf life performer Mar34. Two QTL, *DY_2* and *DY_4* co-localise with QTL for other quality traits. QTL *DY_2* co-localises with QTL *Wt_2*, *FL(d5)_1* and *FL(d6)_1* between map positions 40.0 cM in LG O2. Similarly, QTL *DY_4* co-localises with QTL *BE(d5)_1* and QTL *FL(d5)_3* at map position 61.1 cM.

Presence of purple pigments.

Genotypes in the MGDH population were observed to have purple heads due to the presence of anthocyanins. Both IM and MQM mapping detected QTL for the trait *P* in LG O1 (Figure 3.3.2). A single QTL was mapped that explains 100% of the genetic variation, however this may be due to to numerical scores used. Mar34 appears purple in storage and therefore provide the deleterious alleles for this trait.

Hunter Lab colour co-ordinates.

A total of 17 putative QTL were detected for the colour co-ordinates (*L*, *a*, *b*, *HA* and *C*) on day four, day five and day six in storage, with the exception of Lightness (L) that failed to produce significant QTL after MQM mapping (Figure 3.3.2).

The 'a' co-ordinate

The 'a' co-ordinate produced four significant QTL (*a(d5)_1*, *a(d6)_1*, *a(d4)_1* and *a(d6)_2*) mapping in LG O1 and LG O4, with a single QTL being detected on day four and day five in storage and two QTL being detected on day six in storage. QTL *a(d5)_1* and *a(d6)_1* co-localise at map position 80.3 cM in LG O1 and explain 17.5 % and 30.4 % of the genetic variation associated with the trait. For both QTL the beneficial allele comes from the good performer Mar34. Linkage group O4 contains QTL *a(d4)_1* and *a(d6)_2*, which also co-localise at map position 68.1 cM. Each QTL explains 37.3 % and 14.5 % of the genetic variation present with the beneficial alleles associated with the trait coming from the poor performer GD33.

The 'b' co-ordinate

MQM mapping retained three out of five QTL that were identified using IM and detected three new QTL for the 'b' co-ordinate. In total, six significant QTL were mapped in five linkage groups for the 'b' co-ordinate on day four and day six in storage (Figure 3.3.2). QTL *b(d4)_1* and *b(d4)_2* map in LG O3 and LG O4 respectively with each QTL accounting for 9.1% and 29.5% of the genetic variation associated with the trait. The beneficial allele for the trait *'b'* comes from the good performing genotype Mar34.

Hue Angle

For the trait *HA* a single QTL was detected for each day in storage in LG O1 (Figure 3.3.3). The three QTL mapped co-localise between map positions 78.1 and 80.3 cM. In all cases the beneficial allele for the trait is associated with the good performer Mar34, with each QTL explaining 33.7 %, 46.3 % and 34.9 % of the genetic variation for hue angle on day four, day five and day six respectively. QTL for hue angle also co-localise with QTL for the 'a' co-ordinate that map in LG O1.

Chroma

Three QTL were detected for the trait *C* on day four and day six in storage (Figure 3.3.2), with one QTL mapping in LG O3 (*C(d4)_1*) and two QTL mapping in LG O4 (*C(d4)_2* and *C(d6)_1*). All QTL detected for the trait *C* co-localise with QTL for the 'b' co-ordinate. For all QTL mapping in LG O4, the allele associated with QTL is from the poor performer GD33, whereas the QTL mapped in LG O3 is associated with the good performer Mar34. Each QTL accounts for 14.1 % (*C(d4)_1*), 27.0 % (*C(d4)_2*) and 29.3 % (*C(d6)_1*) of the variation associated with the trait.

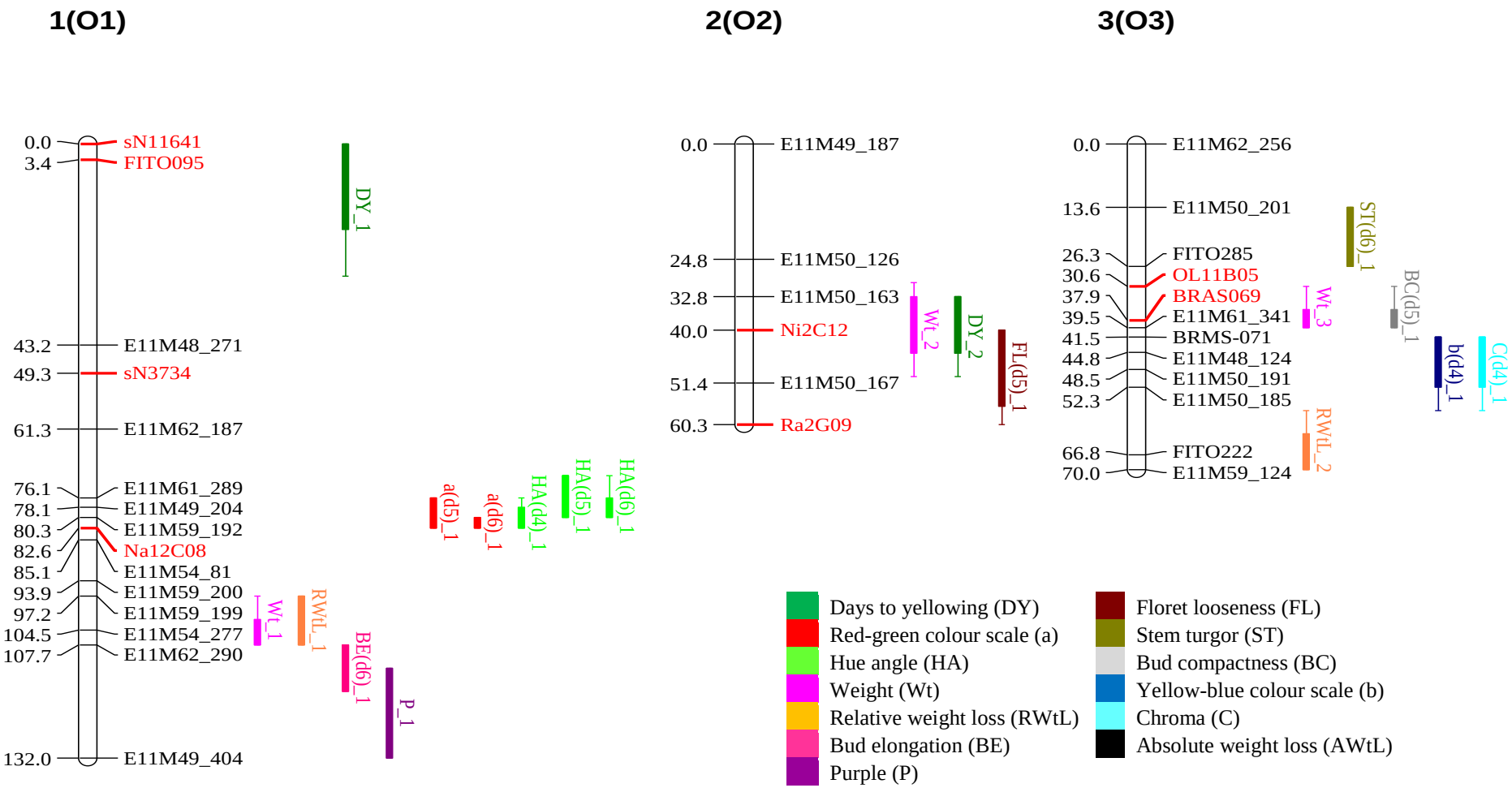


Figure 3.3.2: The broccoli x broccoli linkage map, linkage groups O1-O3, showing the positions of QTL for shelf life, colour co-ordinates and morphological traits.

Informative SSR markers that enabled the ordering and assignment of linkage groups are highlighted in red.

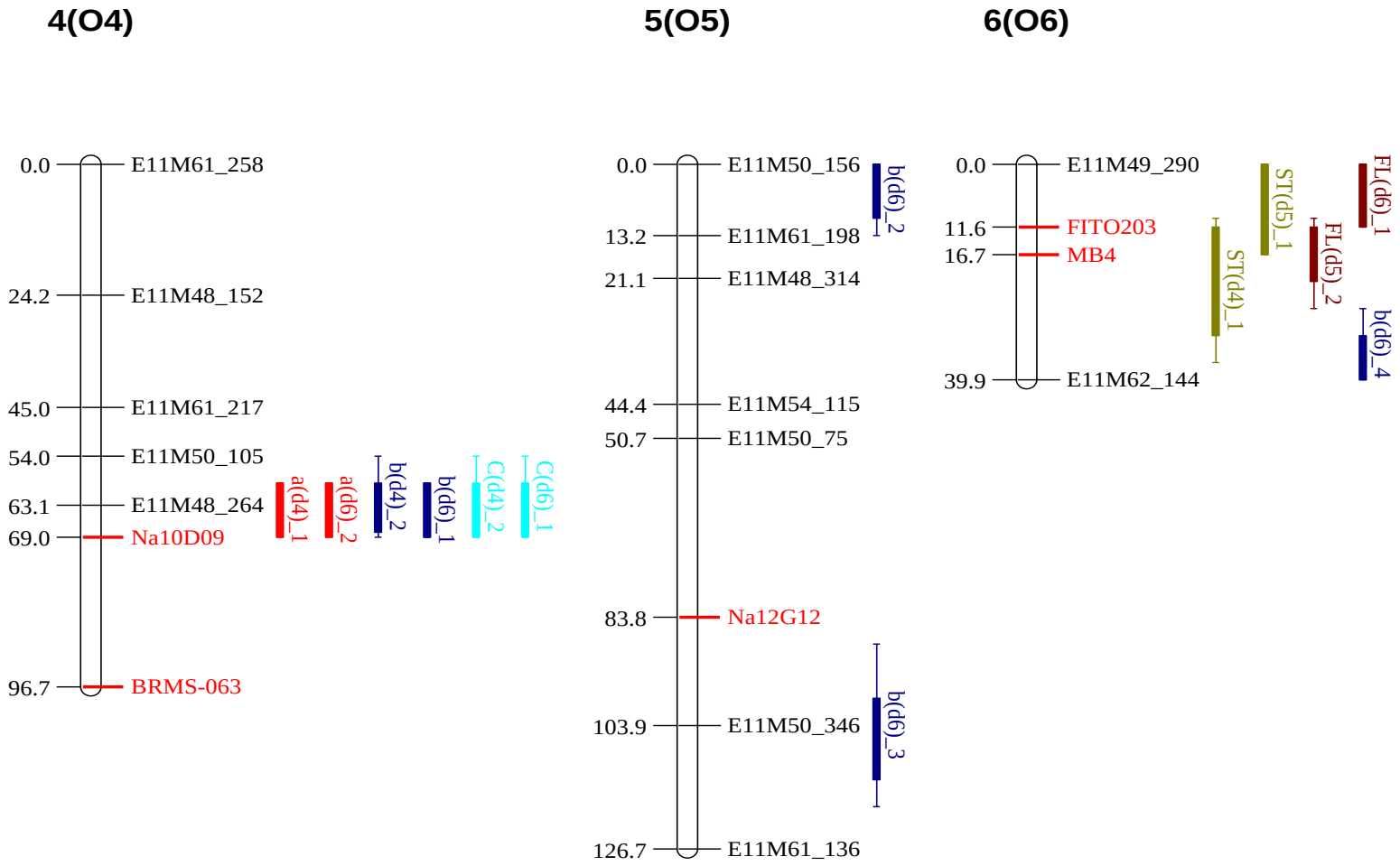


Figure 3.3.2: *Continued*. Linkage groups O4-O6.

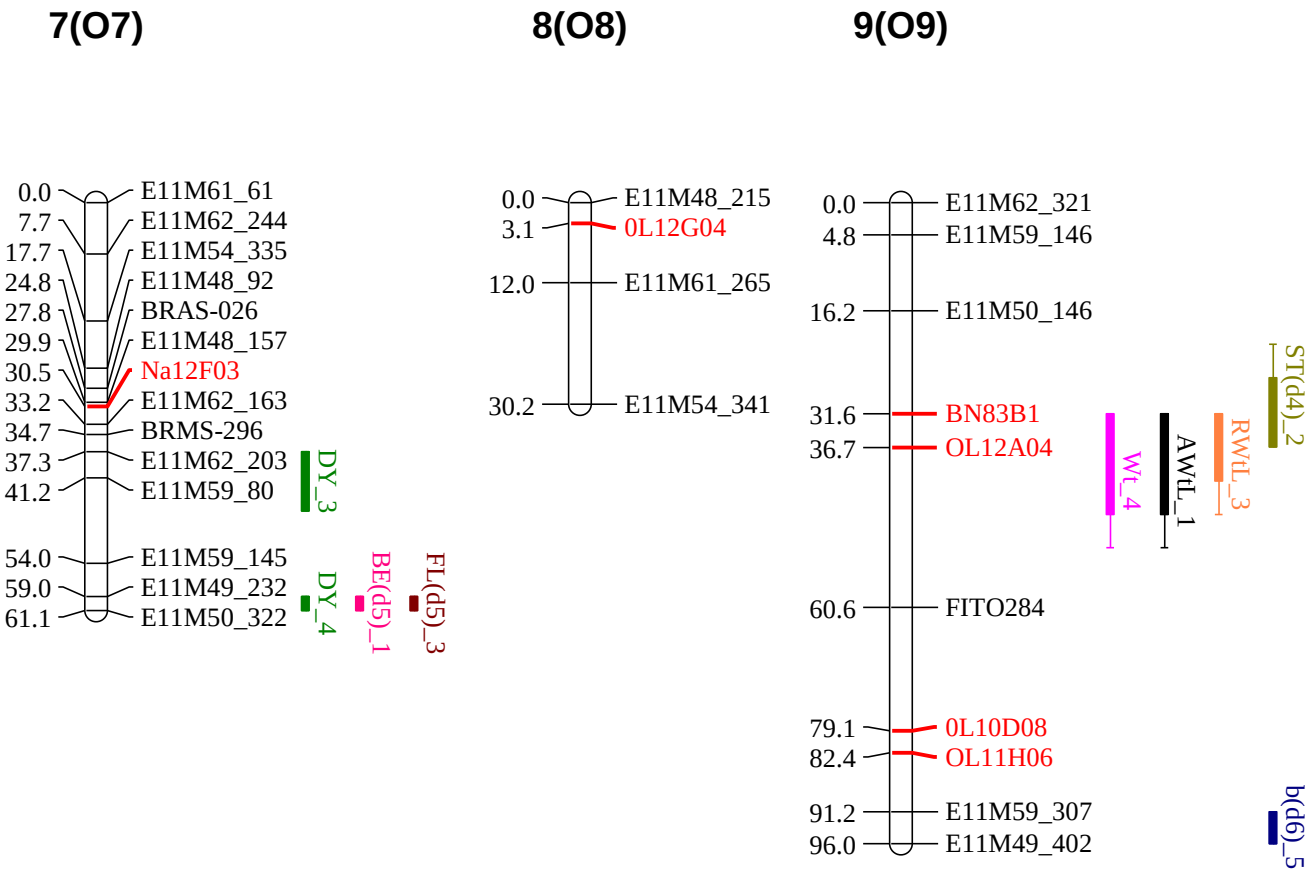


Figure 3.3.2: *Continued.* Linkage groups O7-O9.

3.3.4 Discussion

Using shelf life, colour and morphological trait data generated for the MGDH population, a total of 40 putative QTL were detected using a multiple QTL model (MQM mapping). In total, 25 new QTL were detected, 15 QTL were retained and 14 QTL were lost after MQM mapping, indicating the occurrence of both type I and type II errors. These errors are known to be common in QTL analysis when using a single QTL model, such as interval mapping (Jansen, 1994). Type I error refers to when a presence of a QTL is indicated at a location where no QTL is actually present, whereas type II error refers to QTL not being detected or missed by interval mapping (Jansen, 1994). Simulation studies have proven that a reduction in the occurrence of errors can be achieved by using MQM mapping, as the selection of marker co-factors diminishes the unexplained variance associated with nearby QTL, therefore minimising the chance of errors and maximising the power for detection of QTL (Jansen, 1994).

3.3.4.1 Shelf life and colour QTL

Shelf life QTL representing the trait 'days to yellowing' were detected in three linkage groups and accounted for a total of 59.1% of the variation for the trait. In most cases, with the exception of QTL *DY_3*, the Mar34 allele was associated with increased shelf life. QTL for shelf life did not co-localise with any of the QTL detected for colour, which could be explained by the low correlation coefficients between these traits and the observation in Chapter 3, Part II. The spectrophotometer readings did not seem to produce reliable results and did not distinguish between broccoli heads that were observed to be green and those that had become yellow.

QTL for colour co-ordinates map into six linkage groups, with clustering occurring in LG O1 (71.3-82.6 cM), LG O3 (41.5-57.3 cM) and LG O4 (54.0-69.0 cM). QTL for colour co-ordinates that co-localise also have high correlation coefficients. To determine colour and colour change in broccoli the most informative colour co-ordinates should be 'b', which measures colour on a yellow-blue scale, and hue angle, which is similar to how humans perceive colour. QTL for hue angle all co-localise in LG O1 and explain up to 46.3 % of the variation for the trait with the alleles from the good shelf life performer Mar34 being linked to retention of green colour throughout storage. QTL for the b co-ordinate are more dispersed throughout the genome and map into five linkage groups, in total 38.6 % and 65.4% of the variation is explained for trait on day four and day six in storage with beneficial alleles for the trait associated with both parents. In this study, the onset of visible postharvest yellowing occurred on day nine in storage for the parental genotype Mar34, with the b-values for this genotype being low compared to genotypes that had turned yellow. Therefore, Mar34 provides the ideal genotype associated with the b co-ordinate with two QTL mapped in LG O5 and LG O6 containing beneficial alleles from Mar34.

3.3.4.2 QTL for morphological traits.

Morphological QTLs for *Wt*, *RWtL* and *AWtL* map into four linkage groups and account for a total of 64.1%, 76.9% and 20.7% of the variation associated with each trait. Clustering of morphological QTL was found in LG O1 and LG O9 with Mar34 alleles being responsible for improved weight. The beneficial allele associated with QTL mapped for *RWtL* and *AWtL* also come from Mar34, however Mar34 was observed to lose more weight during storage compared to GD33, but when the factor

of size and weight was removed both parental genotypes are observed to lose a similar percentage of weight during storage. The trait weight also co-localises with other QTL mapped for quality traits, therefore selecting for improved weight may have undesirable effects due to the presence of linked QTL associated with undesirable traits.

Although MQM mapping improves the power for detecting QTL, for all traits, with the exception of QTL detected for *P* and *ST* on day six, much of the variation still remains unexplained, indicating that QTL with smaller effects have not been detected or that the variance unaccounted for may be due to the environment. Agronomic and quality traits in crops are often complex and are known to be controlled by several genes.

3.3.4.3 Clustering of QTL.

Out of the 40 QTL detected by MQM mapping in this study many co-localise, with clustering occurring in seven linkage groups for morphological traits: *Wt*, *AWtL* and *RWtL*, colour co-ordinates: *L*, *a*, *b*, *HA* and *C* and quality traits: *ST*, *BE* and *FL*. In most studies, QTL that overlap are considered separately but potentially QTL hotspots could indicate the presence of a single QTL that has pleiotrophic effects (Zhang *et al*., 2007). QTL hotspots that contain beneficial alleles from one parent that are indicated to improve desirable traits could be utilised by breeders, as the selection of one QTL with the ideal genotype could lead to the improvement of other traits of interest (Zhang *et al*., 2007). However, selection for desirable traits and an improved genotype may be difficult if QTL that co-localise have alleles for both desirable and undesirable traits. In this situation fine mapping would be required to

determine the nature of QTL hotspots to confirm whether there are multiple QTLs or a single QTL with pleiotrophic effects (Zhang *et al*., 2007). In this study, two QTL hotspots for morphological traits (*Wt* and *RWtL*) and colour co-ordinates (*a* and *HA*) in LG O1 contain beneficial alleles from the parent associated with the improvement of these traits, therefore selection of the ideal genotype in each hotspot could improve other desirable traits that have co-localised in the same region.

3.3.4.4 Segregation distortion at QTL intervals.

Segregation distortion was observed in most linkage groups when constructing the broccoli x broccoli linkage map for the MGDH population, this could have implications when identifying markers linked to QTL for the improvement of quality traits in broccoli via marker assisted selection. For all traits, except absolute weight loss, the Mar34 allele causes an increase in the trait values and therefore is the ideal genotype to select for to improve quality traits in broccoli. For traits such as *ST*, *BE* and *FL* the nearest marker linked to the QTL shows segregation distortion for the GD33 allele. Therefore, the use of MAS selection for the improvement of traits is important if segregation distortion is present as breeders can positively select for the beneficial alleles.

3.3.4.5 Confidence intervals of QTL.

The confidence intervals of QTL mapped in this study vary from 2.1 cM (smallest) – 28.4 cM (largest). To delimit the confidence intervals further marker saturation in some QTL regions needs to be improved. Where there is sufficient marker saturation fine-mapping of QTL is required to confirm the position of the QTL before employing marker-assisted selection.

3.3.4.6 Robustness of QTL.

Detection and mapping of QTL in this study is based on trait data generated from one growing season, however QTL effects can be influenced by environment. Plant species show phenotypic plasticity (gene expression changes depending on the environment) as they have the ability to adapt to local environments (Asins, 2002). Therefore to test the robustness of the QTL mapped for morphology, quality and shelf life, evaluation of the MGDH population needs to be carried out in another environment or repeated over many growing seasons to determine genetic x environmental (GxE) interactions.

In summary, the QTL mapped for quality traits in this study provide an indication of the regions in the *B.oleracea* genome that may contain genes controlling traits of interest. However, the confidence intervals of QTLs and the marker saturation in QTL regions require improvement to increase the potential of identifying candidate genes underlying the QTL for quality traits in broccoli.

Conclusions:

- QTL controlling quality traits assessed in the 2007 field trial have been successfully positioned in the broccoli x broccoli linkage map.
- QTL identified in this study provide targets for fine-scale mapping, marker assisted selection, and regions for the identification of candidate genes underlying the trait.

CHAPTER 4:
EVALUATION OF QUANTITATIVE METHODS TO ASSESS COLOUR AND COLOUR CHANGE IN BROCCOLI.

4.1 Introduction

Visible head yellowing affects the marketability of broccoli leading to rejection along the retail chain and subsequently high levels of waste for growers, retailers and consumers. To improve shelf life and delay yellowing in broccoli, natural variation within the MGDH population, combined with QTL analysis is being used to identify regions in the genome associated with delayed yellowing. In this study, visual assessments of head yellowing have been used for QTL analysis, however visual assessments are subjective and the perception of head yellowing could vary from person to person. A spectrophotometer device was also used to quantify colour and colour change during the 2007 field trial (Chapter 3, Part II), however the data generated was inconsistent visual observations of head yellowing. Therefore, the use of a spectrophotometer to quantitatively measure head yellowing in broccoli needs further optimisation. This chapter aims to identify a suitable method to reliably quantify colour change in broccoli for use in a QTL analysis. To deduce if quantitative measures of head yellowing in broccoli are more informative than visual assessment, three approaches for colour measurement were tested and compared: 1) Image J analysis using the RGB colour model. 2) Spectrophotometer readings using Hunter *Lab* colour space and 3) visual assessment.

Aims

- To compare visual assessment of head colour (yellowing) with quantitative measures of head colour to deduce which method(s) are most informative to assess time of colour change.

- To compare Image J analysis of photographs with spectrophotometric Minolta readings to determine the most reproducible method to quantitatively measure head yellowing and colour change.
- To determine the optimum number of readings to take (5, 10, 15) when using a Minolta device for an accurate measurement of head yellowing and colour change.

4.2 Materials & Methods

4.2.1 Broccoli samples

Ten shop bought broccoli heads were used to evaluate three different methods of measuring head discoloration (yellowing) and colour change over time.

4.2.2 Experimental Conditions

Broccoli heads were labelled 1-10 using merit strung tickets and placed in an open cardboard box. The box containing the broccoli heads was left on a lab bench at room temperature to allow the heads to naturally senesce. Heads were assessed for discoloration (yellowing) and colour change from purchase (Day 0) until Day 7 using three different methods.

4.2.3 Quantitative assessment of head yellowing: Image J analysis

Lighting

Broccoli heads were illuminated using two 56 cm parallel lamps (each containing 2 Bell 240v/150w GLS clear triple life tungsten bulbs, British Electric Lamps UK). Both lamps were situated 30 cm above the broccoli head and at an angle of 45^{O} to the sample.

Digital camera and image acquisition

Images of individual broccoli heads were taken each day using a colour digital camera, model D50 (Nikon, Japan). Broccoli samples were placed upright in a beaker on a white background with the camera located vertically over the broccoli head at a distance of 75 cm. When capturing images the following camera settings were used: Programmed auto (P) mode, centred dynamic focus area, no zoom, no flash, with images stored in JPEG format. The white balance was set for incandescent light.

Image processing

Images for each broccoli head from day 0 –day 7 were ordered into folders by head number. To view and process the RGB values for images the software programme Image J was used. The elliptical tool was used to select an area of white background in each image so that the RGB values for the background could be used to normalise each image. To process the colour of the images, the polygon tool was used to draw around the circumference of the broccoli head in each image. The RGB values for the broccoli heads were then determined using the colour histogram function.

Scripts developed in R by Mr. Stuart McHattie (personal communication) were used to generate normalised red, green and blue values from the pixels in each image for each head over the time course. To determine the secondary colours of magenta, yellow and cyan, scripts developed in R were used to calculate the ratio of the primary colours from each image each day.

4.2.4 Quantitative assessment of head yellowing: Spectrophotometer Readings.

A hand held Konica Minolta spectrophotometer as described in section 2.4.1 was used to measure head discolouration (yellowing) and colour change by performing a total of 15 colour readings from each broccoli head per day.

Visual Assessment.

Each broccoli head was visually assessed using a scoring system developed by Wurr *et al.* (2001), as described in section 2.4.2. As part of the visual assessment the weight (g) of all broccoli heads was also recorded each day.

4.2.6 Statistical Analysis

All statistical analysis was carried out in GeneStat 12th edition (Payne *et al.,* 2007)

Fitting of logistic curves.

Logistic curves ($Y = a + c /(1 + e^{-b(X-m)})$) were fitted to each broccoli head in the two different sets of colour data using the non-linear regression model in GenStat. The model used each colour variable from the two quantitative methods (L, a, b, C, HA and red, green, blue, yellow, magenta and cyan) as the response variate (Y) and day as the explanatory variate (X).

T-tests

Two sided paired T-tests compared the means of each colour measurement between days. If the *P-value* exceeded the 95% confidence interval the null hypothesis was rejected. The null hypothesis assumes that the differences of the means of the two samples being compared will equal zero.

Discriminant analysis

Discriminant analysis was carried out on spectrophotometer readings (*L, a, b*) taken daily from purchase (Day 0) until day 7 in storage. Discriminant analysis obtains scores for group means using a cononical variates analysis (CVA). The purpose of discriminant analysis is to assign objects or events to one or several groups based on a set of measurements obtained from each observation (Friedman, 1989).

4.3 Results

4.3.1 Quantitative measurement of head yellowing: Image J analysis

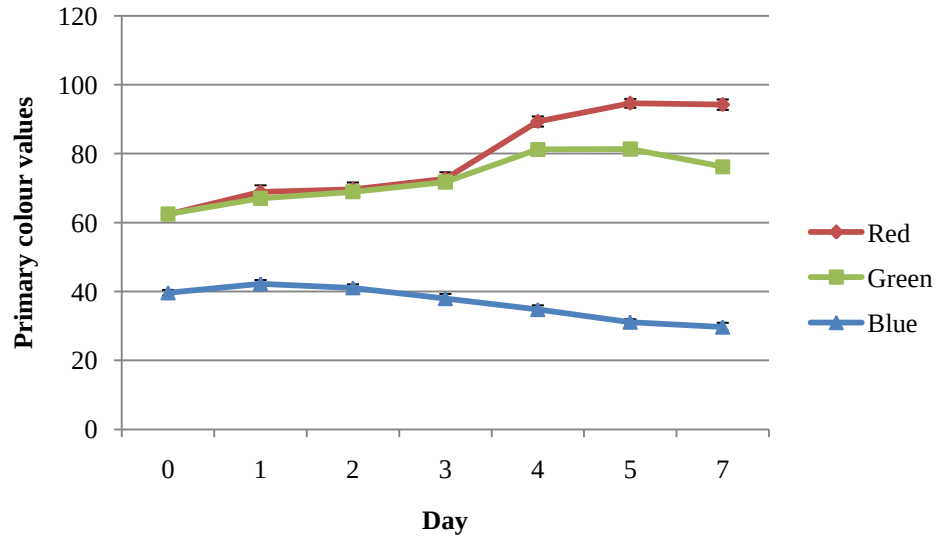
Red, Green and blue (RGB) values.

During storage the mean for blue colour in the broccoli heads decreased, where as the means for green and red colour increased (Figure 4.1). Blue colour started to decrease in broccoli heads from day two and continued to decrease until day seven, most change occurred between day 4 and day 5. Initially, the red and green colour in broccoli heads increased at a similar rate from purchase (day 0) until day three. The largest increase in red and green colour occurred between day three and day four. After day four the mean green value plateaued and then decreased, where as the mean red value continued to increase then plateaued between day five and day seven.

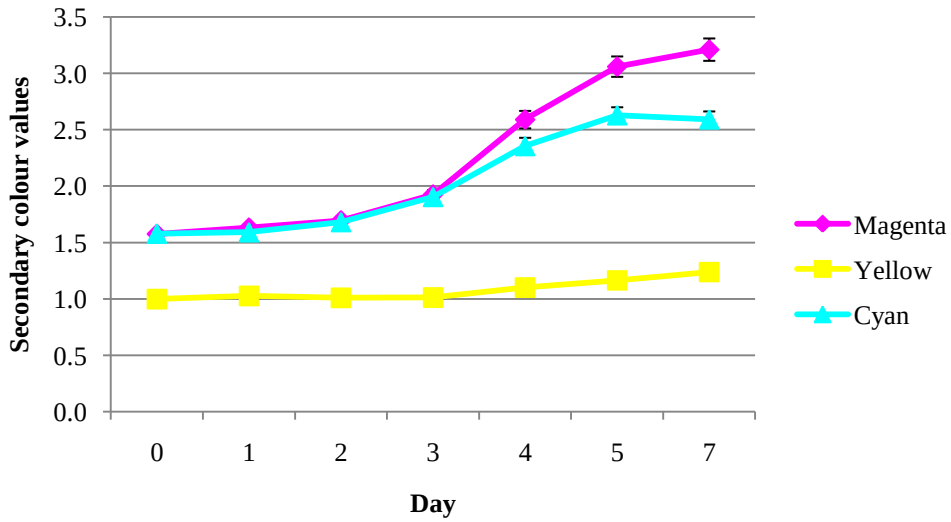
Magenta, yellow and cyan (MYC) values.

The mean for magenta, yellow and cyan in the broccoli heads increased after purchase (Figure 4.1). Yellow colour in broccoli heads only began to increase three days after purchase (day 0) and continued to increase until day seven. Magenta and cyan colour in broccoli heads followed a similar pattern, and increased gradually

between day one and day three. After day three the mean value for magenta colour increased rapidly until day seven, the biggest increase was observed to occurr between day three and day four. The mean value for cyan colour also rapidly increased after day three, and continued to increase until day five, where it started to decrease. The largest increase in cyan values also occurred between day three and day four after purchase.



A).



B).

Figure 4.1: Means for A). primary (RGB) colours and B). secondary colours (MYC) in 10 broccoli heads over 7 days after purchase (n = 10). Error bars represent the s.e.m (standard error of the mean)

4.3.2 Quantitative measurement of head yellowing: Spectrophotometer readings

Lightness (L)

The mean for lightness (L) increased from day one until day five after purchase, the largest increase in lightness (L) was observed to occur between day three and day four. (Figure 4.2) Over time the L-value became more positive indicating that broccoli heads were becoming lighter when left to senesce at room temperature.

Red-green colour scale (a)

The a-value expresses colour change in broccoli heads on a red-green scale (+a = red, -a = green). Overall there is little change in the a-value after purchase (Figure 4.2). Initially the mean of the a-value decreased during storage (day 0-3), before increasing between day three and day seven. From day three the a-value is became more positive suggesting that the broccoli heads had become less green.

Blue-yellow colour scale (b)

The b-value expresses colour change in broccoli heads on a blue-yellow scale (+b = yellow, -b = blue). In general, the broccoli heads are become more yellow after purchase as the b-value was more positive (Figure 4.2). The mean of the b-value began to increase on day one after purchase and continued to increase until day five, where it peaks. Most change in the b-value occured between day two and day five after purchase, with the largest increase seen to occurr between day two and day three.

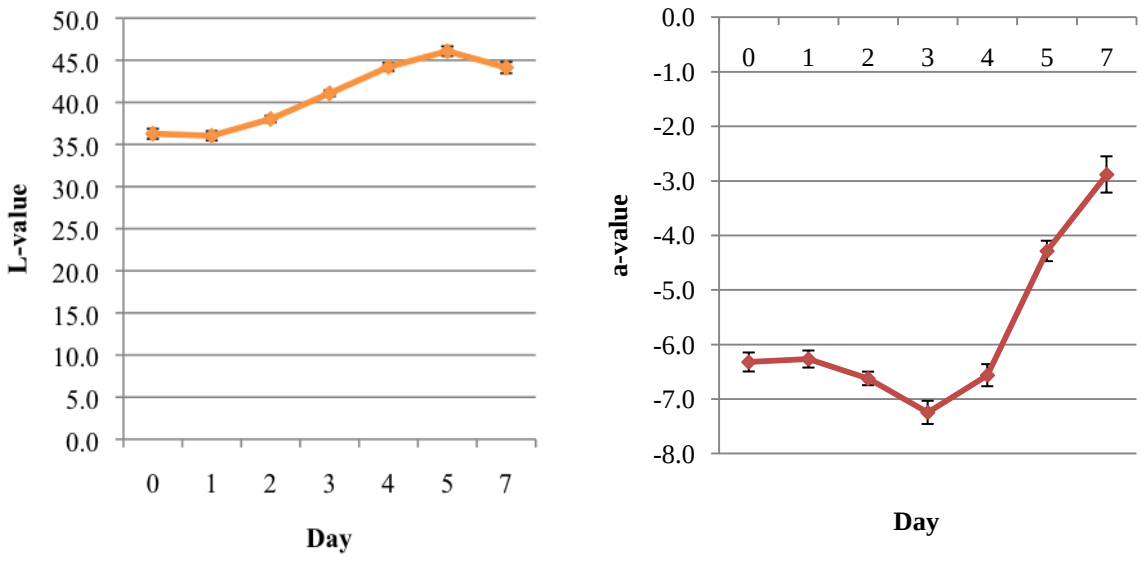
Hue angle (HA)

Hue angle establishes where an object lies in the opponent colour spectrum (Red = 0^o, Yellow = 90^o, Green = 180^o, Blue = 270^o). The mean for hue angle in all broccoli

heads decreased over time (Figure 4.2). Heads had a green-yellow hue at purchase (Day 0) with a mean hue angle of 131^{o}. As heads senesced the hue angle decreased, on day seven the mean hue angle was 100^{o} suggesting that the broccoli heads had a yellow hue. Most change is hue angle occurred between day two and day five after purchase, with the largest decrease in hue angle observed between day four and day five.

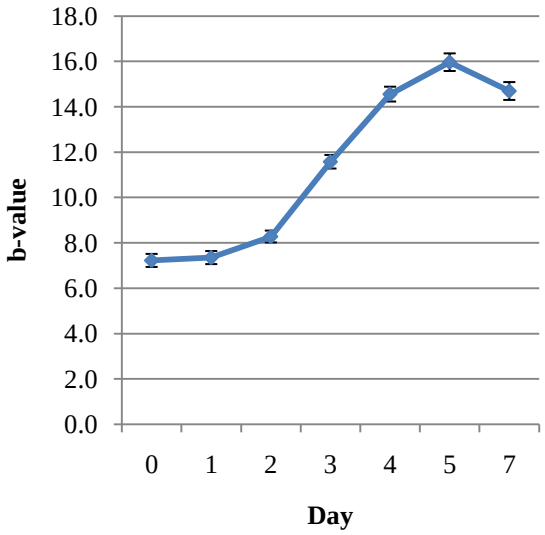
Chroma (C)

Chroma represents how dull or vivid the colour of an object is. The mean for chroma over time followed a similar trend to the b-values. Chroma started to increase on day one after purchase and continued to increase until day five (Figure 4.2). Most change in chroma occurred between day two and day five, with the biggest increase in chroma observed between day two and day three. This suggests the chromacity of colour being measured from the broccoli heads had become more vivid during senescence.

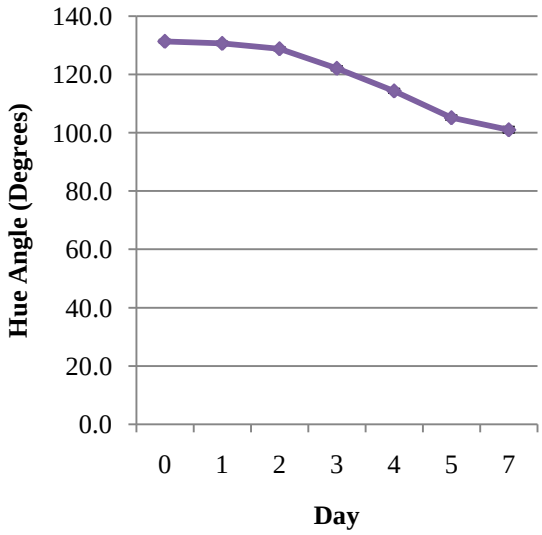


A). **B).**

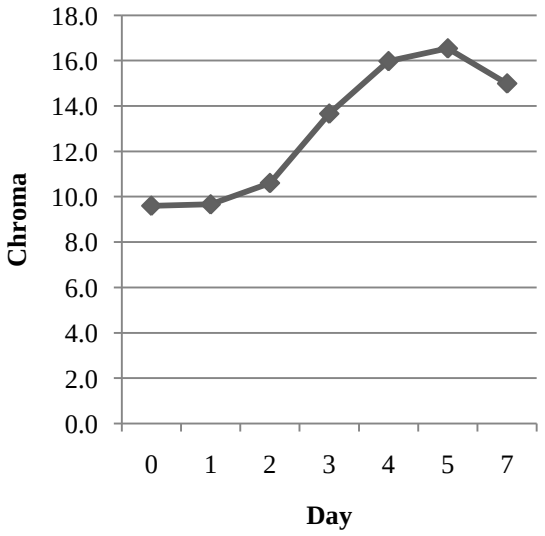
Figure 4.2: Mean values for colour co-ordinates for ten broccoli heads during storage (n = 10) A). L-value B). a-value. C). b-value D). hue angle and E). chroma. Error bars represent the s.e.m (standard error of the mean)



C).



D).



E).

Figure 4.2: *Continued.*

4.3.3 Fitting of logistic curves

Logistic curves were fitted to the both sets of colour data to provide an estimate for which day the most change in each colour variable was occurring (Figure 4.3) This was estimated by identifying the time (day) of the maximum gradient (M) from the slope of the graph using the logistic regression equation ($Y = a + c\ (1\text{-}e^{-b(X+m)}$). As this chapter is aimed at quantifying yellowing in broccoli, the data presented here

will focus on the ‘b’ co-ordinate (yellow-blue colour scale) and hue angle (equivalent to human perception of colour) from the spectrophotometer readings and measurements of yellow colour from Image J analysis.

Table 4.1: Time of the maximum gradient of the slope (M) calculated from the logistic curve equation for ten broccoli heads during storage to determine the day in storage when most change in yellow colour had occured using Image J analysis (yellow) and spectrophotometer readings (hue angle and the ‘b’ co-ordinate)

	Time (day) of the maximum gradient of the slope (M)		
Head	**Yellow**	**b**	**Hue Angle**
1	4.24 ± 0.16	2.50 ± 0.29	3.34 ± 0.07
2	4.49 ± 0.31	3.00 ± 0.23	3.72 ± 0.23
3	4.85 ± 0.93	2.90 ± 0.13	3.93 ± 0.22
4	4.71 ± 1.03	2.85 ± 0.13	4.21 ± 0.20
5	4.07 ± 0.13	3.21 ± 0.43	3.59 ± 0.35
6	4.90 ± 0.16	3.07 ± 0.25	3.87 ± 0.18
7	4.26 ± 0.28	2.65 ± 0.25	3.79 ± 0.18
8	4.52 ± 0.18	3.09 ± 0.16	3.52 ± 0.15
9	4.36 ± 0.21	3.48 ± 0.25	4.13 ± 0.12
10	4.43 ± 0.43	2.39 ± 0.43	3.72 ± 0.24

Quantifying head yellowing using the Image J method indicated that the steepest point of the slope (M) on all graphs for yellow colour was on day four (Table 4.1), this infers the change in yellow colour had occurred on this day after purchase. The maximum gradient (M) identified from the graphs using the spectrophotometer data for all broccoli heads estimates that the most change had occurred between day two and day three for the b-coordinate (yellow-blue colour scale) and day three and day four for hue angle (equivalent to human colour perception). This suggests that the change in yellowing was detected earlier with this method compared to Image J analysis.

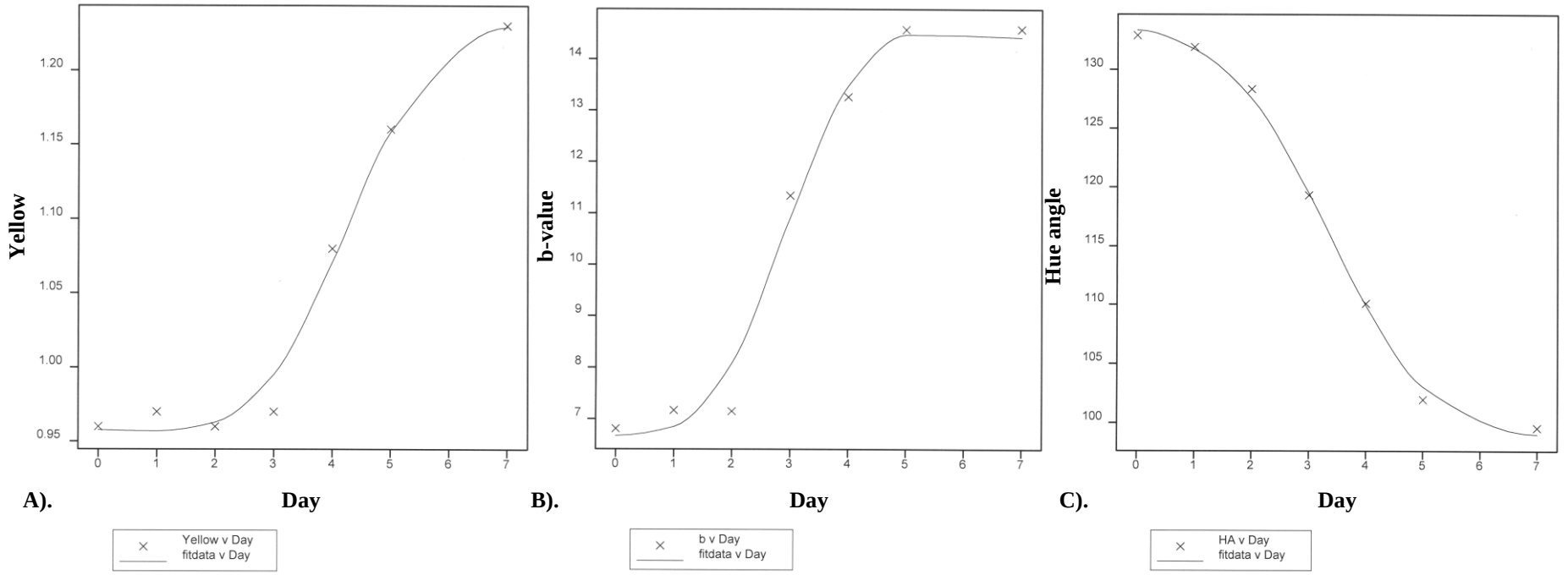


Figure 4.3: Examples of the logistic curves fitted to the colour data from ten broccoli heads during storage for the calculation of the maximum gradient of the slope which indicates the day in storage when most colour change is occurring. A). Yellow values from the RGB colour model calculated in Image J. B). B-values from spectrophotometer readings and C). Hue angle calculated using spectrophotometer readings.

4.3.4 Paired T-tests

Paired t-tests were used to determine the day in storage after purchase at which the most significant colour change was first occurring for both quantitative methods of measuring head colour in broccoli heads.

Image J analysis: RGB colour model

Paired t-tests compared the red, green and blue colour of broccoli heads between days (Table 4.2) showed that the most significant difference (****P<0.001*) in mean values was found between day three and day four after purchase. The most significant difference (****P<0.001*) in the mean values for yellow colour in broccoli heads was also found between day three and day four, whereas the most significant difference (****P<0.001*) in mean values for magenta and cyan were found earlier, between day two and day three and between day one and day two, respectively.

Spectrophotometer readings: Hunter Lab colour scale

Comparing the means for L, a, b, hue angle and chroma between days (Table 4.2) showed that the means for the b-value, hue angle chroma were most significantly different (****P<0.001*) between day two and day three after purchase, where as the most significance difference (****P<0.001*) in the means for lightness (L) was seen earlier, between day one and day two after purchase. The mean for the a-value was most significantly different (****P<0.001*) between day four and day five.

Weight

The mean value for weight significantly changed between all days (****P<0.001*) (Table 4.2).

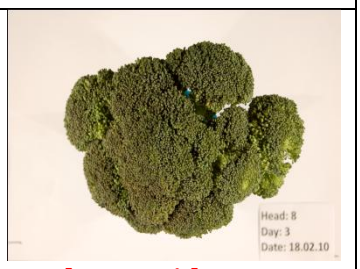
Table 4.2: Probability values from paired t-test comparing means for different colour measurements (Image J and spectrophotometer readings) from ten shop bought broccoli heads between different days in postharvest storage (df =9). Significance effects shown as *P<0.05, **P>0.01 and ***P>0.001.

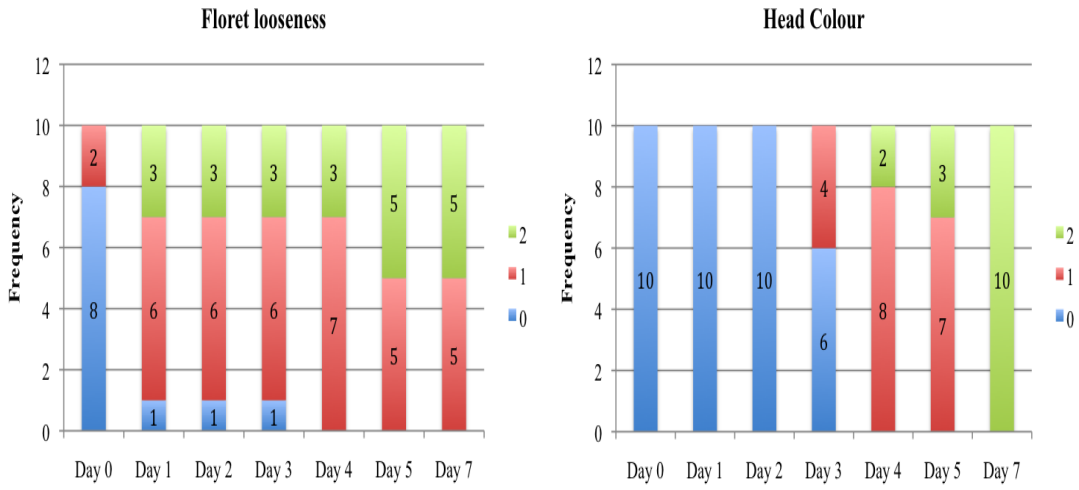
	Day					
	0 and 1	**1 and 2**	**2 and 3**	**3 and 4**	**4 and 5**	**5 and 7**
Red	**0.002	0.581	0.08	***<0.001	**0.003	0.803
Green	**0.006	0.181	0.068	***<0.001	0.899	**0.007
Blue	-	0.169	*0.015	***<0.001	**0.005	0.069
Magenta	**0.004	**0.002	***<0.001	***<0.001	***<0.001	*0.014
Yellow	**0.004	0.964	0.311	***<0.001	***<0.001	***<0.001
Cyan	0.168	***<0.001	***<0.001	***<0.001	***<0.001	0.844
L	0.549	***<0.001	***<0.001	**0.001	*0.013	*0.023
a	0.748	**0.002	*0.017	*0.028	***<0.001	**0.007
b	0.712	**0.003	***<0.001	***<0.001	*0.013	*0.010
Hue angle	0.342	*0.014	***<0.001	***<0.001	***<0.001	**0.009
Chroma	0.866	**0.002	***<0.001	***<0.001	0.256	**0.007
Weight	***<0.001	***<0.001	***<0.001	***<0.001	***<0.001	***<0.001

4.3.5 Wurr quality scoring system.

The ten shop bought broccoli heads were assessed daily for quality traits such as stem turgor, head colour, bud compactness, bud elongation and floret looseness using the Wurr quality scoring system (section 2.4.1). On all days, from purchase and during storage broccoli heads remained acceptable for retail for the traits stem turgor, bud compactness and bud elongation, scoring zero at all time points. In contrast the traits floret looseness and head colour caused broccoli heads to become unacceptable for retail from day one and day three in storage respectively (Table 4.3). All broccoli heads fail for head colour by day four in storage, where as half of the broccoli heads fail for floret looseness by day five in storage (Figure 4.4).

Table 4.3 Photographic images of the Wurr quality scoring system used to assess the ten shop brought broccoli head for head colour and floret looseness. Text highlighted in red indicates when heads were classed as unacceptable for retail.

	0	1	2
Head Colour	 **Green**	 **Yellow**	 **Bronze**
Floret Looseness	 **Firm**	 **Florets beginning to separate**	 **Florets wide apart**



A). **B).**

Figure 4.4: Frequency bar charts showing the quality scores for ten shop bought broccoli heads based on the Wurr scoring system for A). stem turgor and B). floret looseness (n = 10). For the trait head colour a score of one or more indicated that genotype were classed as unacceptable for retail whereas for the trait floret looseness a score of two or more indicated that genotypes had failed.

4.3.6 Discriminant analysis

The output from the discriminant analysis assigned spectrophotometer readings (*L*, *a*, *b*) into groups by day (Figure 4.5). Groups formed with readings on day 0 (purchase), day 1 and day 2 overlap, this suggests that the *L*, *a*, *b* values on these days and therefore the colour of broccoli heads are similar, as visually broccoli head are all green on these days. Groups formed with *L*, *a*, *b* readings on day 5 and day 7 also overlap again suggesting that the colour of broccoli heads on these days are comparable, as all broccoli heads are visually yellow on these days. In contrast, *L*, *a*, *b* readings from broccoli heads on day 3 and day 4 do not overlap with any other day and form two distinct groups. This infers that the readings and the colour of broccoli heads on these days differ from the readings on any other day, which could suggest that change in colour may be occurring most on these days.

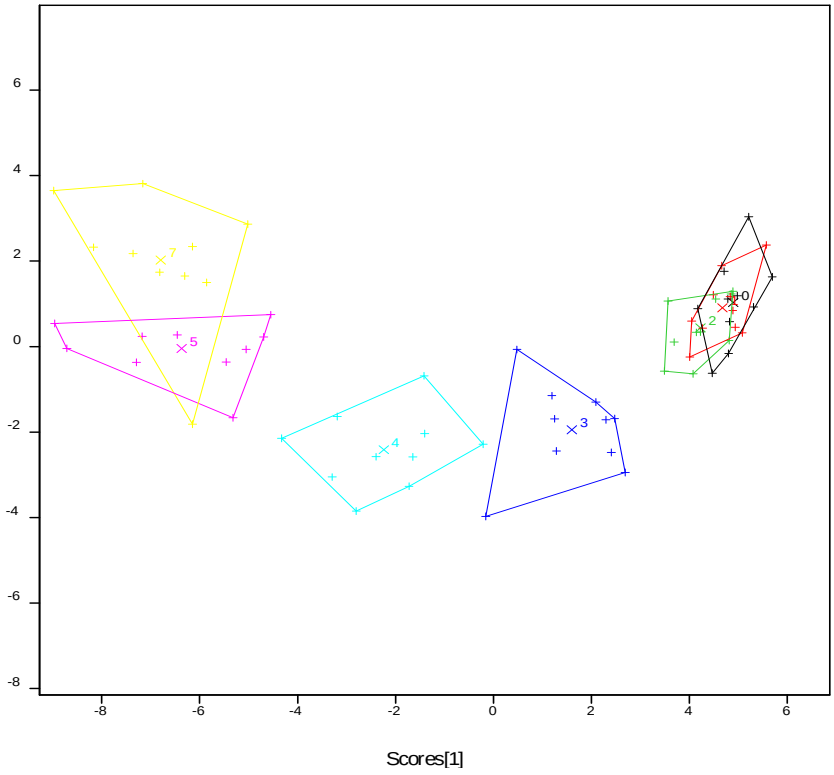


Figure 4.5: Groupings for 15 spectrophotometer readings (*L, a, b*) from discriminant analysis for ten broccoli heads during storage. The different colours represent the day the readings were taken: Purchase (day 0) = black, day 1 = red, day 2 = green, day 3 = blue, day 4 = cyan, day 5 = magenta and day 7 = yellow

4.4.7 Visual assessment of head yellowing.

Four individual broccoli heads (head 1, 5, 7 & 9) failed, on day 3 after purchase due to head yellowing (Figure 4.6). All broccoli heads had failed visually for head yellowing four days after they were purchased.

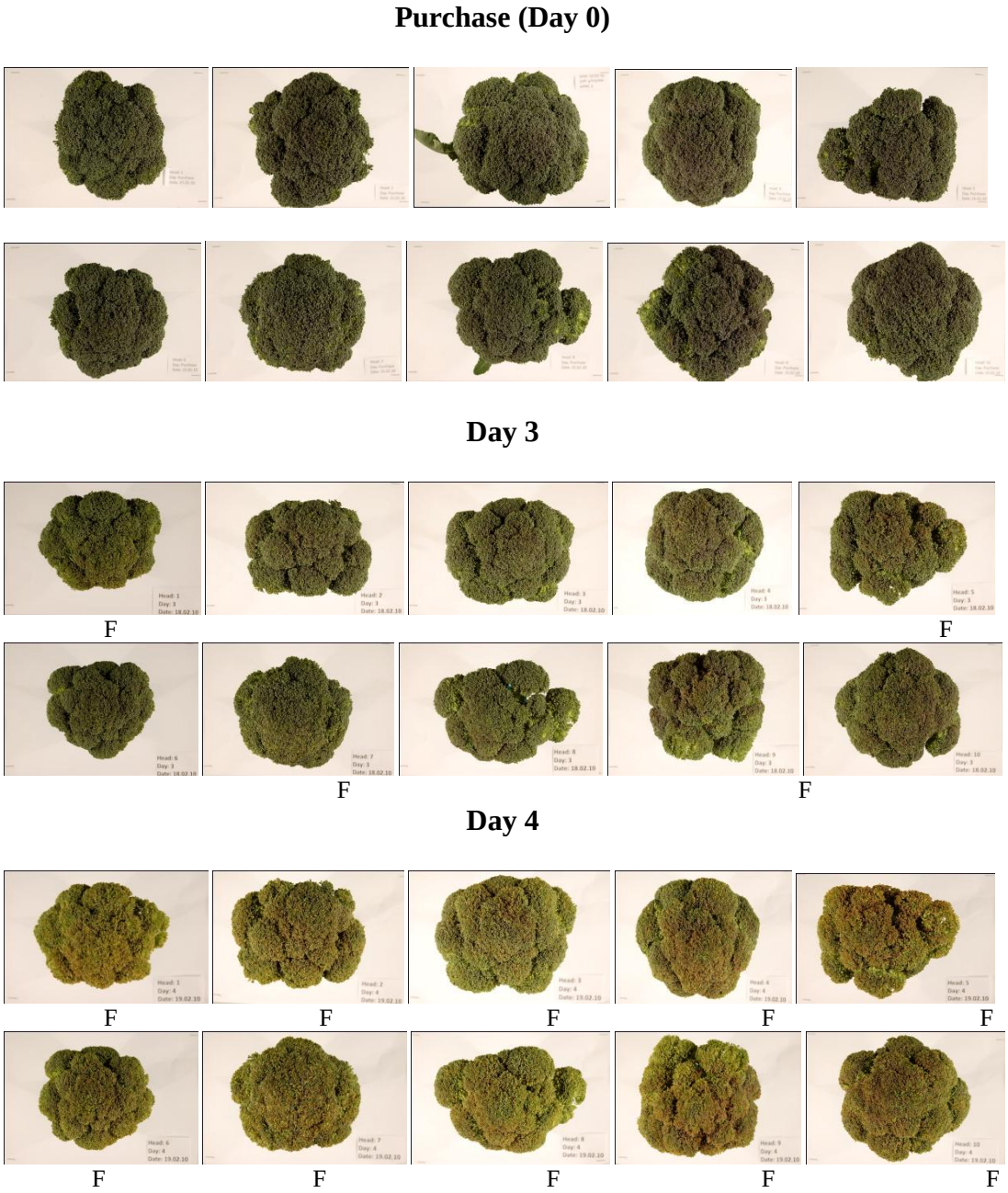


Figure 4.6: Photographs of displaying the colour change of ten individual broccoli heads at purchase (day 0),and during day 3 and day 4 in storage. F indicates failure due to head discoloration.

Weight

After purchase the mean weight of all broccoli heads decreases each day in a linear manner (Figure 4.7).

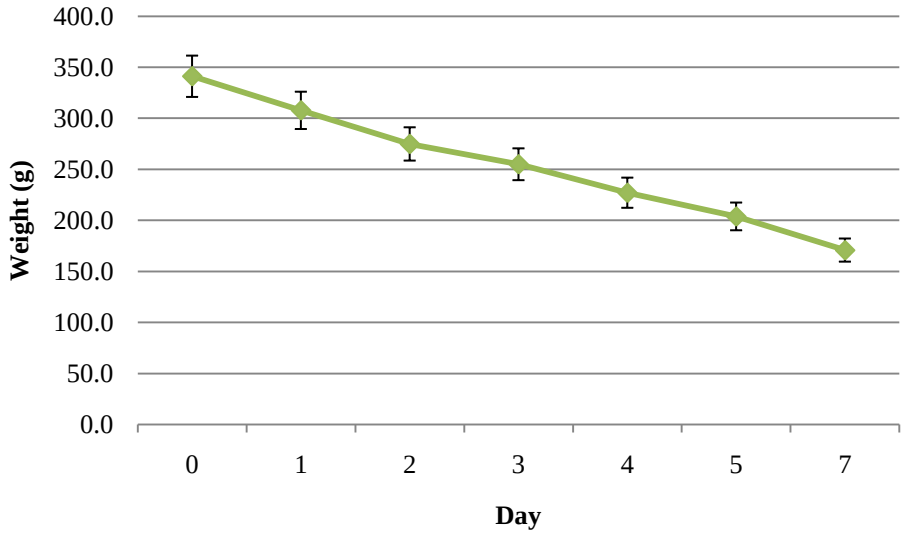


Figure 4.7: Mean for weight (g) for ten broccoli heads at purchase and over seven days in storage (n = 10). Error bars represent the s.e.m (standard error of the mean)

4.4.8 Spectrophotometer reading optimisation.

A comparative study was carried out to determine the optimum number of spectrophotometer readings, 5, 10 or 15, required to accurately reflect the colour change in broccoli heads over time. By comparing the variances for lightness (L), red-green colour scale (a), yellow-blue colour scale (b), (Table 4.4) from 10 heads used in the study the consistency of the data for 5, 10 and 15 readings can be observed (Figure 4.8). In general, for all colour parameters 15 readings improved the variance of the data, as the variance of the data was reduced in most cases when 15 spectrophotometer readings were taken compared to five or ten (Table 4.4).

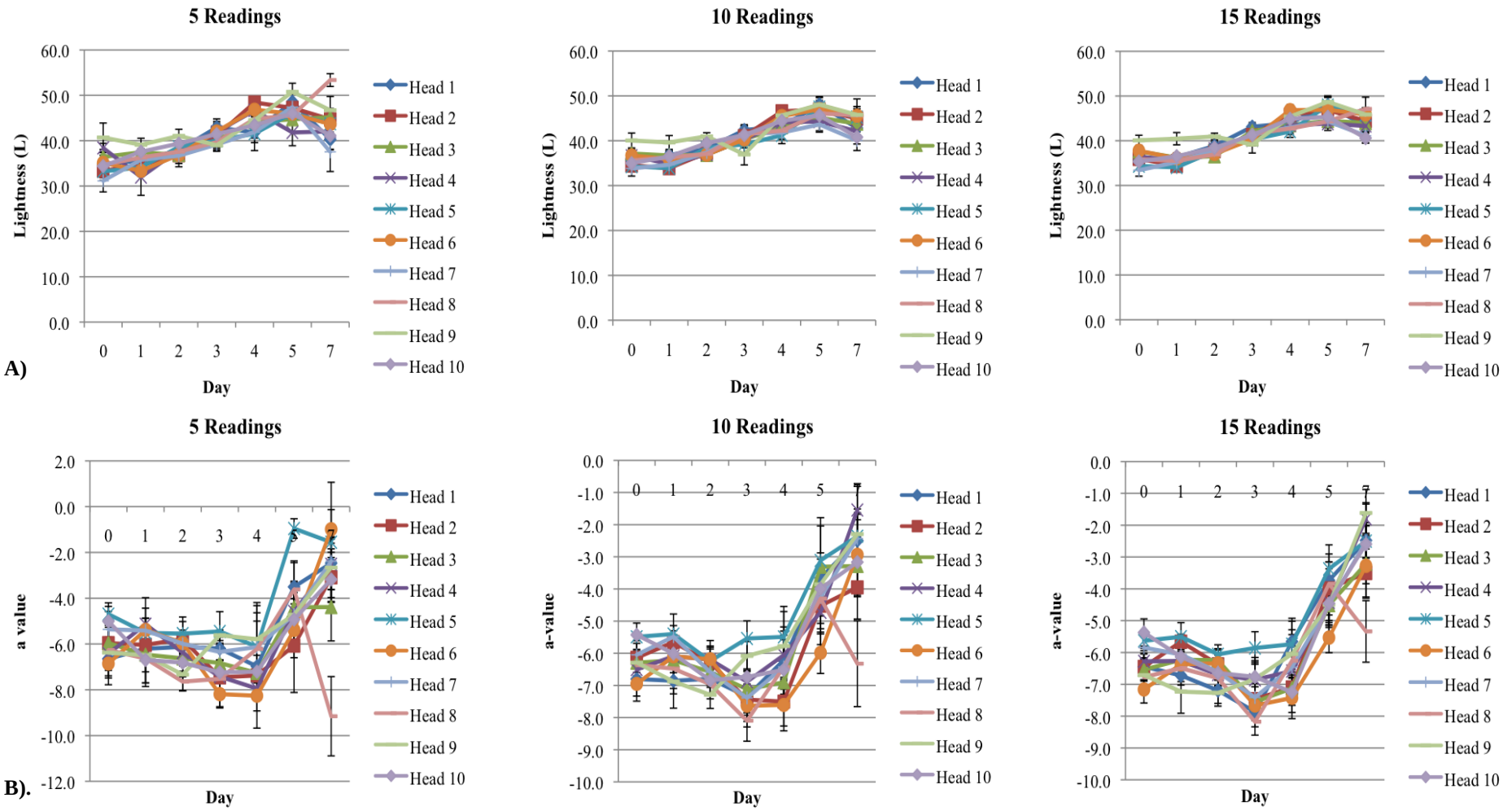


Figure 4.8. Individual head means from ten broccoli heads based on an increasing number of spectrophotometer readings (5, 10 and 15) for A). Lightness, B). a-value and C). b-value. Error bars represent the s.e.m (standard error of the mean)

C).

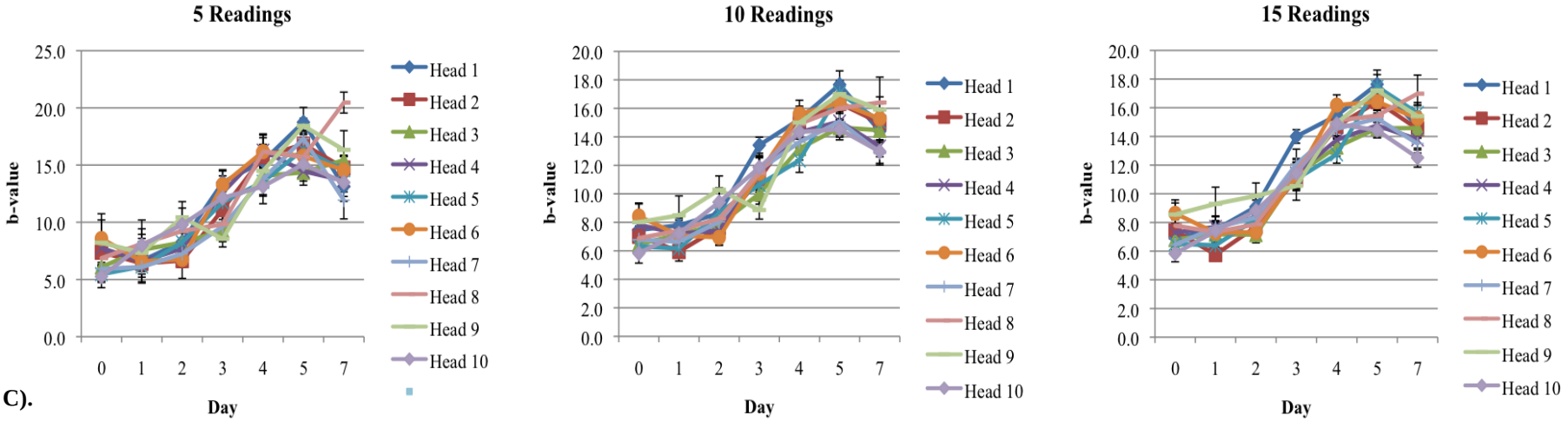


Figure 4.8: *Continued.*

Table 4.4: The range of the variance for 5, 10 and 15 spectrophotometer readings (L, a and b) taken from ten shop brought broccoli heads during storage.

	5			10			15		
Day	**L**	**a**	**b**	**L**	**a**	**b**	**L**	**a**	**b**
0	5.19-50.80	0.56-6.13	2.58-32.30	8.76-28.80	1.83-4.70	2.71-18.05	7.75-20.64	1.40-4.38	2.63-15.79
1	0.44-80.79	0.01-7.66	0.01-21.64	5.33-45.27	1.21-6.84	1.31-18.65	5.68-33.90	0.84-6.91	1.09-20.32
2	0.13-16.52	0.71-4.81	1.57-12.13	6.30-17.70	1.01-4.66	2.62-14.48	5.43-19.49	1.01-3.66	3.59-12.19
3	1.48-41.18	0.27-9.40	1.67-19.96	4.84-54.74	2.01-8.14	3.16-24.12	12.81-53.81	2.20-6.87	3.33-18.77
4	1.50-69.22	0.96-17.94	2.11-12.09	11.86-39.80	3.49-11.56	4.96-13.38	10.86-49.85	2.61-10.07	4.27-11.86
5	3.05-42.49	0.91-20.92	2.09-8.42	5.63-43.39	3.48-23.34	2.98-11.82	6.91-50.00	3.39-19.53	2.91-14.74
7	4.19-94.17	3.10-34.31	0.45-14.18	16.49-131.17	4.21-26.68	5.11-31.80	16.85-104.04	3.89-20.36	5.89-25.35

4.4 Discussion

Broccoli heads failed due to head yellowing on day three and day four after purchase using visual assessment. This supports the output from discriminant analysis of the spectrophotometer readings, as *L, a, b* readings taken on day three and day four form two distinct groups suggesting that colour change in broccoli heads is occurring between these days. Groups for *L, a, b* readings on earlier (day 0, 1 and 2), when all heads are visually green, and later (day 5 and 7) days after purchase, when all heads are visually yellow, overlap inferring that the colour of broccoli on these days is similar.

Spectrophotometer readings detect storage related colour change in broccoli heads earlier than image J analysis and visual assessment of head discoloration (yellowing). Significant changes in lightness (L) were detected between day 1 and day 2, and significant changes in hue angle, chroma and yellow-blue colour (b) were detected a day later, between day 2 and day 3. The alternative quantitative method using image J analysis first detected significant changes in red, green, blue and yellow colour between day 3 and day 4. This suggests that spectrophotometer readings are more sensitive to colour change, whereas image J analysis is more similar to visual assessment. Differences in sensitivity could be due to the output from image J analysis providing an average of colour from an image rather than individual readings from broccoli heads. However, as some broccoli head were susceptible to floret looseness, the spectrophotometer aperture there is a chance that readings would have been taken from the gaps between florets, at the base of the bud material, which is lighter in colour.

Although spectrophotometer readings have the advantage of being more sensitive, the method is less reproducible than image J analysis. Stringent set up and camera settings produce more consistent colour values where as spectrophotometer readings are more variable. The use of photographic images also removes any bias, as the colour of the whole head is being analysed, whereas a readings taken with a spectrophotometer represent a random sample of the head. Variation in spectrophotometer readings could be due to differences in handling and selection of the surface area where readings are to be taken. To minimise the variation in *L, a, b* readings a minimum of ten readings is required. Depending on the number of samples being processed, spectrophotometer readings can also be time consuming.

Conclusions

- Spectrophotometer readings are more sensitive than image J analysis and visual assessment for detecting the first colour change in broccoli.
- A minimum of ten spectrophotometer readings is necessary to obtain sufficient consistency and accuracy of the *L, a, b* data.
- Image J analysis provides a more reproducible method for measuring head discoloration (yellowing) in broccoli.

CHAPTER 5:

QUANTIFYING POSTHARVEST VITAMIN C LEVELS AND STABILITY IN BROCCOLI.

Part I:

Shelf life evaluation and morphological trait assessments of a sub-set of genotypes in the MGDH population.

5.1.1 Introduction

Shelf life evaluation and morphological traits assessment was carried out on broccoli genotypes that had not been assessed in the previous growing season and on a sub-set of genotypes that were evaluated in 2007. The assessment of new lines has enabled the identification of additional genotypes with desirable characteristics, such as size, weight, bud quality and colour retention, for the potential use in a breeding or backcross programme. Assessing genotypes over two growing seasons has also tested the robustness of traits and has indicated which traits were more susceptible to environmental conditions.

Aims

- To evaluate the shelf life phenotypes, morphological and quality traits of additional genotypes from the MGDH population.
- To determine the robustness of shelf life and morphological traits in genotypes grown in two different seasons.

5.1.2 Materials & Methods

5.1.2.1 Collection of metabolite samples from a sub-set of the MGDH mapping population (2008).

In total, 40 genotypes were grown in replicated field trials in 2008. Genotypes selected consisted of 20 new genotypes for shelf life evaluation and morphological trait assessment and 20 genotypes, previously grown in 2007, which were collected as for metabolite analysis. The sub-set of genotypes selected for metabolite analysis represented the distribution for the trait 'days to yellowing' in the DH population.

Mature broccoli heads were harvested between 28/07/08 and 11/08/08 from two field replicates (80 plots per replicate) in the experimental field site Big Cherry. Each plot contained 24 broccoli plants from two different genotypes (6 row x 4 plant arrangement). A total of 15 heads were harvested per plot. From the eight central 'guarded' heads a total of seven heads were harvested for metabolite analysis. To assess the shelf life and morphological traits of the new genotypes eight heads were harvested from the outer guard plants. Seven heads were used for shelf life evaluation and morphological trait assessment and one head was collected for metabolite analysis.

All 40 genotypes were subject to shelf life evaluation and morphological trait assessment before the processing of heads collected for metabolite analysis. Bud material from the 20 genotypes collected for metabolite analysis was harvested from one head per day from day 0 (harvest) until day 6. Buds were removed using a scalpel and the harvested material was placed into a 50 ml Falcon tube and immersed in liquid nitrogen before being stored at -80^{o}C.

5.1.2.2 Statistical analysis.

The trait days to yellowing was analysed using REML analysis as described in section 2.5.1. A Wilcoxon matched pairs test was also carried out to compare the ranks of genotypes assessed in both growing seasons for the traits head diameter, peduncle diameter and weight (at harvest).

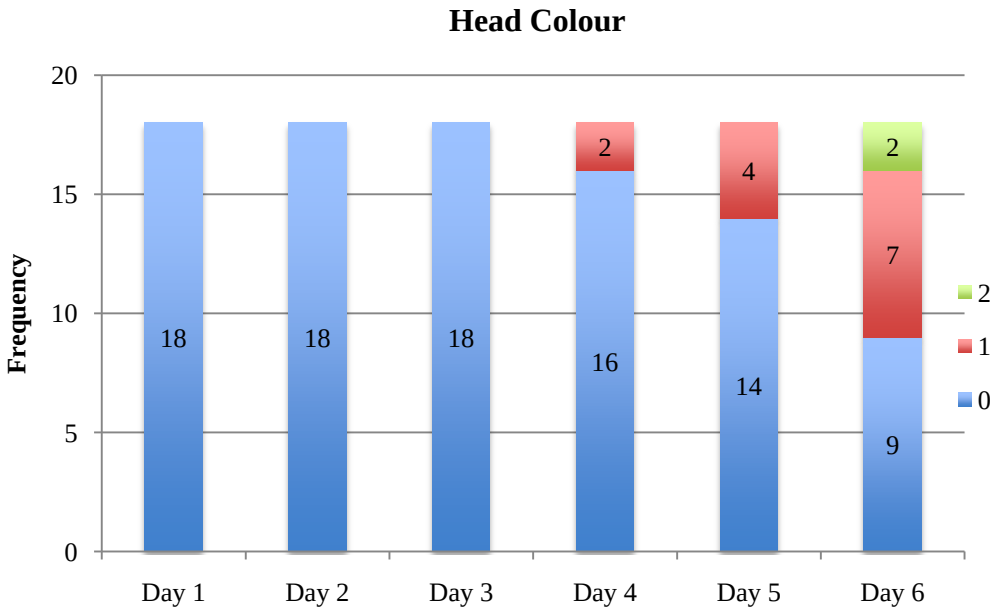
5.1.3 Results.

As a detailed trait analysis was carried out in Chapter 3, Part II, the data presented here will focus on the shelf life performances of the additional genotypes, as this is the main trait of interest, and the comparison of lines grown over two different seasons (2007 and 2008). Additional analysis can be seen in Appendix D.

5.1.3.1 Visual assessments of head yellowing for additional genotypes.

Visual assessments of head yellowing were carried out using the Wurr system as described in section 2.4.2, to determine the day in storage when the onset of postharvest yellowing occurs for the additional genotypes. In the first six days in storage after harvest yellowing first becomes present on day four in storage for two genotypes (MG279 and MG333), by day six in storage 50 % of the genotypes have become yellow, with two genotypes observed to have turned bronze (Figure 5.1.1). The new genotypes display significant (****P<0.001*) natural variation for the trait 'days to yellowing' with genotypes ranging from 4.86 - 13.61 days before the onset of postharvest yellowing occurs, with genotype MG19 remaining green for the longest during storage and MG333 becoming yellow the quickest during storage (Figure 5.1.1).

A).



B).

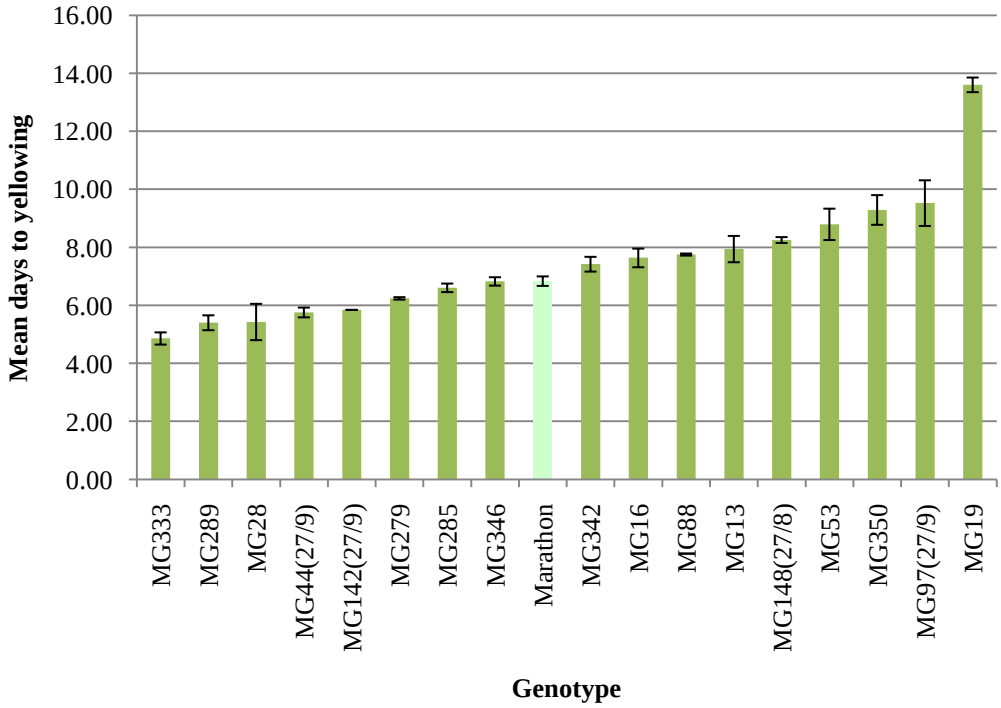


Figure 5.1.1 A). Frequency bar chart showing the quality scores for head colour based on the Wurr scoring system for a sub-set of the MGDH population. Scores of 0 = green, 1 = yellow and 2 = bronze. **B). Predicted means (from REML) of the time taken (Days) for a sub-set of genotypes from the MGDH population to become yellow**. Error bars represent the s.e.m (standard error of the mean).

Estimation of 'days to yellowing' for genotypes collected as metabolite sample.

Due to destructive sampling of genotypes harvested for metabolite analysis 'days to yellowing' was estimated. To estimate the trait 'days to yellowing' was the mean score for head colour for each genotype was calculated over the first six days in storage (Table 5.1.1). The estimated shelf life for genotypes collected as metabolite samples ranges from 4.25 to +6.00 days in storage.

Table 5.1.1: Predicted means (from REML) and estimated means for the traits 'days to yellowing' for genotypes grown in both seasons (2007 and 2008).

	Days to Yellowing	
Genotype	**2007**	**2008**
GD33	2.73	+5.00
MG120(27/9)	3.11	5.00
MG526	4.74	+6.00
MG87(27/9)	4.81	5.00
MG1812	5.16	5.00
MG87	5.44	5.25
MG99	5.65	5.25
MGUNID2	5.77	5.38
MG100(27/9)	5.85	5.00
MG95	5.86	5.25
MG308	5.87	5.75
MG10	7.01	4.25
MG406	7.25	+6.00
Marathon	7.66	+6.00
MG1829	7.77	5.75
MG353	7.91	+6.00
MG433	8.54	+6.00
MG475	8.83	+5.00
Mar34	9.09	+6.00
MG532	9.79	+6.00

Comparing estimates for the trait 'days to yellowing' to the predicted means for shelf life in 2007 most genotypes display similar performances in storage across seasons (2007/2008). For example, genotypes that last in storage for six days or more in

2007 are also estimated to have a shelf life greater than six days in 2008, with the exception of MG1829 and MG10. The poor shelf life performer GD33 has a shelf life of 2.73 days in 2007, however in 2008 GD33 maintains green colour and has an estimated shelf life of +5.00 days in storage.

5.1.3.2 Comparison of lines grown in both growing seasons (2007/2008).

In general, genotypes were observed to be larger and heavier in 2008 when comparing the traits head diameter, peduncle diameter and weight over both growing seasons (Figure 5.1.2). To determine if the changes in ranks were significant different between the growing seasons, Wilcoxon matched-pairs test were carried out in GeneStat. The data from both seasons was ordered by sorting genotypes by largest values to smallest values for each trait (head diameter, peduncle diameter and weight) and each genotype was given a rank of 1-20 in both years, with 1 representing the highest rank (largest value) and 20 representing the lowest rank (smallest value). For all traits the rank order of genotypes for weight, head diameter and peduncle diameter changes between the two growing seasons (Table 5.1.2). The rank for the commercial genotype Marathon remains the same for all traits in both seasons, whereas the parental genotypes GD33 and Mar34 both increase in rank for all traits between 2007 and 2008. GD33 exhibited the biggest increase in rank for head diameter and weight between 2007 and 2008, whereas MG1812 and MG526 showed the largest decrease in rank for head diameter and weight respectively. For the trait peduncle diameter Mar34 showed the largest increase in rank, with MG475 and MG87 decreasing the most in rank between growing seasons.

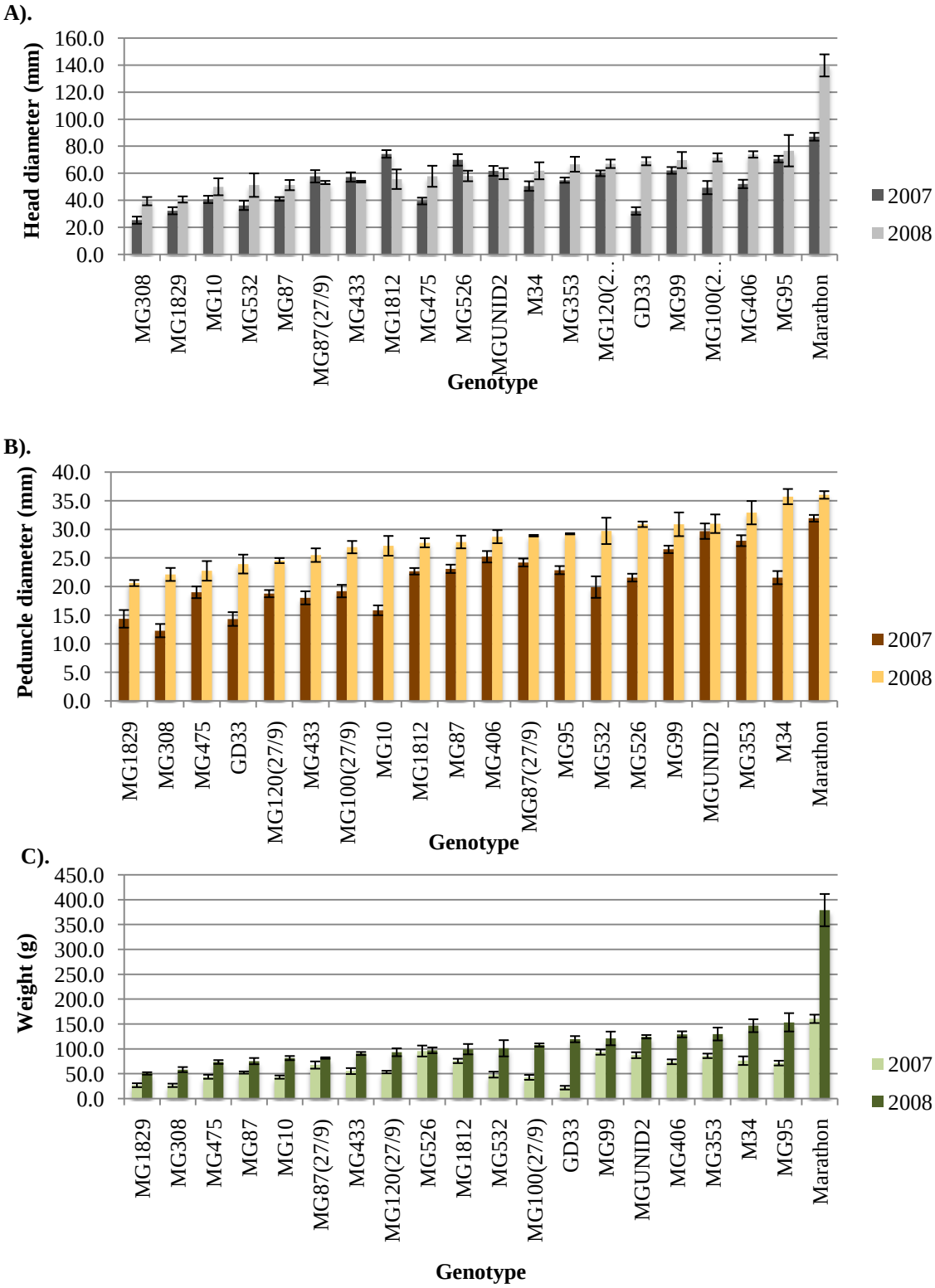


Figure 5.1.2 A comparison of the predicted means (from REML) for genotypes in the MGDH population grown in 2007 and 2008 for the traits A). Head diameter (mm), B). Peduncle diameter (mm) and C). weight (g). Error bars represent the s.e.m (standard error of the mean).

Table 5.1.2: Wilcoxon signed rank test to determine the change in rank order for genotypes assessed in both growing seasons for the traits head diameter (HD), peduncle diameter (PD) and weight (Wt) at harvest. A positive rank indicated a increase in rank, a negative rank indicated a decrease in rank and a where ranks remained the same a score of zero was given.

	Wilcoxon Signed Ranks		
Genotype	**HD**	**PD**	**Wt**
Marathon	0.0	0.0	0.0
MG1812	-14.0	-9.5	-10.5
MG95	1.5	0.0	14.0
MG526	-10.5	15.0	-16.0
MG99	0.0	-6.5	-10.5
MGUNID2	-7.5	-6.5	-3.5
MG120(27/9)	0.0	-2.5	-1.5
MG87(27/9)	-10.5	-9.5	-13.0
MG433	-9.0	2.5	-6.5
MG353	3.5	0.0	1.5
MG406	12.0	15.0	6.5
M34	5.5	17.0	6.5
MG100(27/9)	13.0	-2.5	15.0
MG87	-3.5	-12.0	-10.5
MG10	-5.5	12.0	0.0
MG475	7.5	-12.0	-6.5
MG532	0.0	15.0	10.5
MG1829	-1.5	-6.5	-3.5
GD33	15.0	6.5	17.0
MG308	0.0	2.5	0.0

5.1.4 Discussion

The 2008 field trial assessed the shelf life and morphological traits of 20 new genotypes and 20 genotypes that had been assessed in the previous growing season. As with the shelf life evaluation and morphological trait analysis carried out in 2007, similar findings were found when analysing the 2008 trait data, since significant differences were found among genotypes for weight, head diameter, peduncle diameter, weight loss (relative and absolute) and the trait 'days to yellowing'. As a detailed discussion of the relationship between traits can be seen in Chapter 3 Part II, this discussion will focus on the quality phenotypes of the new genotypes assessed and the robustness of traits over two growing seasons.

5.1.4.1 Head colour.

Shelf life evaluation of the new genotypes identified three genotypes (MG350, MG97(27/9) and MG19) that retained green colour for nine days or more during storage, with MG19 out performing all genotypes assessed in both 2007 and 2008 by remaining green for 13.61 days in storage. This indicates that MG19, MG350 and MG97(27/9) are desirable genotypes to use for the introduction of alleles conferring extended shelf life by conventional breeding. Compared to genotypes grown in 2007 the onset of postharvest yellowing was first observed a day later, on day four in storage for genotypes grown in 2008, although as in 2007, 50% were observed to have failed for yellowing by day six. Most genotypes grown in both seasons show similar postharvest shelf life performances, indicating that the trait is robust and not largely affected by different environmental condition over growing seasons. However, the trait 'days to yellowing' could only be estimated due to destructive

metabolite sampling, therefore to truly test the robustness of the trait additional experiments are required.

5.1.4.2 The influence of environment.

A study by Wurr *et al*. (2002) demonstrated that preharvest factors such as, water stress can have a detrimental affect on the postharvest quality of broccoli. However, when comparing the weather data for both growing seasons (Table 5.1.3) only average rainfall and the amount of sunshine were different between growing seasons. In general, genotypes grown in both seasons perform slightly better for shelf life and were heavier and larger in 2008 compared to 2007, with genotype showing consistency for morphological traits over growing seasons. The difference in shelf life performance may be explained by environmental differences however a comparison of daily weather data, instead of an average would be required to determine if temperature, amount of sunshine and rainfall did have a significant influence on morphological traits.

Table 5.1.3: Average rainfall, sunshine and temperature for the 2007 and 2008 growing seasons

	Rainfall	**Sunshine**	**Temperature (^{o}C)**		
	(mm)	**(Hrs)**	**Min**	**Average**	**Max**
2007	2.25	6.11	15.51	20.29	10.72
2008	3.38	4.58	15.93	20.38	11.48

Conclusions:

- The shelf life performances and morphological traits of genotypes grown in both seasons are robust as the phenotypes are consistent in both years.
- From the new genotypes evaluated for shelf life and morphological traits, six additional genotypes have been identified that have desirable characteristics for the use in the breeding of improved quality traits in broccoli.

CHAPTER 5:

QUANTIFYING POSTHARVEST VITAMIN C LEVELS AND STABILITY IN BROCCOLI.

Part II:

Vitamin C quantification of the MGDH population.

5.2.1 Introduction

Vitamin C is used as a generic term to describe biologically active forms of L-ascorbic acid (AO) and L-dehydroascorbic acid (DHA). In humans, Vitamin C is an important dietary nutrient that must be acquired through the consumption of fruits and vegetables. The recommended daily allowance (RDA) of vitamin C for adults in the UK is 60 mg/ml with commercial broccoli demonstrated to contain high levels of vitamin C, as different cultivars ranged from 22.61-119.80 mg in 100g edible portions (Kulrich *et al*, 1999). This suggests that vitamin C content is dependent on genotype. Therefore, to determine if vitamin C content was a suitable trait for genetic analysis, total vitamin C (TA) levels of the MGDH population were quantified at harvest. The postharvest stability of vitamin C was also evaluated by quantifying TA, AO and DHA in sub-set of the MGDH population at four time points (day 0, 2, 4 and 6) during storage. This will enable the identification of QTLs associated with vitamin C content at harvest and vitamin C stability during storage.

Aims

- To demonstrate that the MGDH population exhibits natural variation for the trait vitamin C content (at harvest) and to confirm the trait is suitable for genetic analysis.
- To establish if vitamin C content is associated with morphological traits and shelf life.
- To determine the stability of vitamin C during storage in a sub-set of genotypes.
- To map QTL(s) relating to vitamin C levels at harvest and vitamin C stability during storage.

5.2.2 Materials and Methods

5.2.2.1 Vitamin C Quantification at harvest: High Performance Liquid Chromatography (HPLC).

All chemicals used, except where stated were purchased from Sigma-Aldrich Ltd. UK.

Sample Material

Broccoli bud material was collected from one head per plot during the 2007 shelf life field trial (see section 3.2.3.1). This provided tissue samples, from the edible portion of broccoli, for total ascorbate quantification at harvest (day 0) from four biological replicates per genotype.

Tissue Extraction

A 0.5 g sample of frozen broccoli bud material was homogenised in 10 mls of 2% meta-phosphoric acid using a pestle and mortar. The homogenate was filtered into a 15 ml Falcon tube using Munktell filter paper (125 mm) placed inside a funnel. Two mls of the filtrate was transferred to a new 15 ml Falcon tube and 1 ml of L-cysteine (40g/l) was added. The pH was adjusted to 7 using trisodium phosphate solution (200g/l) on an Ultrabasic pH meter (Denver Instruments, UK) and left at room temperature for 5 minutes. The pH was then lowered to 2.8 using 2 % meta-phosphoric acid. The final volume was adjusted to 10 ml using ddH_20. The tissue extraction was repeated to provide a total of 2 technical replicates per biological sample. Extracts were stored at 4^oC before HPLC analysis.

Preparation of standards

Ascorbate standards of 2.5, 5, 10, 50 and 100 µg/ml were made by diluting an ascorbate stock solution (1mg/ml) All standards were made to a final volume of 10 ml. Standards were stored at 4^{o}C before HPLC analysis. Fresh batches of standards were made before each run on the HPLC.

Ascorbate quantification using High Liquid performance Chromatography (HPLC)

One millilitre of the extracted ascorbate solution was transferred into 1.5 ml screw cap amber vials (Agilent Technologies, Germany) and loaded onto a Hewlett-Packard HPLC 1100 series system (Agilent Technologies, Germany) fitted with a UV-visible detector. A 30µl volume of ascorbate solution was injected into the system. Separation of peaks was achieved by isocratic elution on a RP-C18 (250mm x 4mm; 5µm) column (HiChrom Ltd. UK) with a mobile phase of 0.1 M KH_2PO_4 made up with 10 % HPLC grade methanol containing N-cetyl-N, N, N-trimethylammonium bromide (CTAB) and a flow rate of 0.7 ml/min. Ascorbate was detected at 265 nm and quantified against a standard curve of ascorbate.

Calculation of total ascorbate in 100g edible portions.

To calculate the total ascorbate in 100g edible broccoli portions samples were multiplied by 5 to give the concentration in the original extract (µg/ml) and then multiplied by 10 to give the concentration in 0.5 g. This was then multiplied by 200 to give the concentration in a 100 g portion (µg/ml) and divided by 1000 to give the total ascorbate in 100 g edible portion in mg/ml.

5.*2.2.2 Ascorbate Quantification of a subset of the MGDH population during storage: Plate assays.*

Sample Material

Broccoli bud material was harvested from 20 genotypes grown in the 2008 metabolite field trial (see section 5.1.2.1). This provided tissue samples from the edible portion of broccoli to quantify ascorbate, dehydroascorbate and total ascorbate during storage. Assays were performed on 4 biological replicates per genotype, sampled at day 0, 2, 4 & 6.

Tissue extraction.

A 0.2 g sample of frozen broccoli bud material was ground to a fine powder using an electronic grinder (Dremel, UK) in a 2 ml Eppendorf tube (Eppendorf, Germany). Ground samples were homogenised in 1 ml of 0.1 M HCL/1 mM EDTA using a whirlimixer (Fisherbrand, UK). The homogenate was centrifuged at 13,000 rpm for 2 minutes; the supernatant was retained for each assay. The tissue extraction was repeated using the same biological material to provide 2 technical replicates per head. The extract was kept on ice until required.

Preparation of sodium ascorbate standards.

A 5 mM (5mg/5ml) stock solution of sodium ascorbate was made using 0.1 M HCL/1 mM EDTA, standards were prepared as a dilution series from the stock solution, Table 5.2.1. The standards were stored at 4^{o}C and discarded at the end of each day.

Table 5.2.1: The dilution series for sodium ascorbate standards used for ascorabate quantification using the plate assay method.

nmol ascorbate per assay	0	10	20	30	40	50
Ascorbate (ml)	0	0.05	0.1	0.15	0.2	0.25
HCL/EDTA (ml)	0.5	0.45	0.4	0.35	0.3	0.25

5.2.2.3 Assay for total ascorbate (Ascorbate & dehydrascorbate).

Twenty micro-litre aliquots of 5 mM dithiothreitol (DTT) were transferred into Immuno 96 microwell™ plate, with maxiSorp F96 surface (nunc™, UK), to this 20 µl of either standard or extract were added. The plate was covered with a gas permeable adhesive seal (Thermo Scientific, UK) and placed in a binder incubator for 15 minutes at 42°C. After incubation 10 µl of 5% N-ethylmaleimide (NEM) was added to the wells containing standard or sample and incubated for 1 minute at room temperature to inactivate any excess DTT. To each well, 80 µl of a colour reagent solution (2.2 ml colour reagent A: 4.6% TCA, 15.3% H_3PO_4 and 0.6% $FeCl_3$; 0.8 ml colour reagent B: 4% 2,2 bipyridyl in 70% ethanol) was added. Plates were resealed and incubated at 42°C for 45 minutes. For each sample a blank assay was included by omitting bipyridyl (2.2 ml colour reagent A and including 0.8 ml blank colour reagent B: 70% ethanol). This was used to counteract the effect of red pigment produced by the presence of anthocyanins. After incubation the absorbance at 550 nm was recorded using a GENios fluorometer (Genesis workstation 150, Tecan UK)

5.2.2.4 Assay for Ascorbate only.

A 20 µl aliquot of 0.4 M phosphate buffer (pH?) was transferred into a Immuno 96 microwell™ plate, with maxiSorp F96 surface (nunc™, UK), to this 20 µl of either standard or extract and 80 µl of colour reagent (2.2 ml colour reagent A: 4.6% TCA, 15.3% H_3PO_4 and 0.6% $FeCl_3$; 0.8 ml colour reagent B: 4% 2,2 bipyridyl in 70%

ethanol) was added. A blank for each sample was included on the plate by omitting bipyridyl (2.2 ml colour reagent A and 0.8 ml blank colour reagent B: 70% ethanol). The microtitre plate was covered with an adhesive gas permeable seal (Thermo Scientific) and incubated for 45 minutes in a binder incubator set at 42^{o}C. After incubation the absorbance at 550 nm was recorded using a GENios fluorometer (Genesis workstation 150, Tecan UK)

Sample correction.

To counteract the effect of red pigments (anthocyanins) being present in the extract the blank samples from each assay were used to correct the corresponding colour sample by subtracting the blank.

Calculating the concentration of ascorbate and total ascorbate from corrected samples.

The standards for each assay were plotted on a line graph (x-axis = absorbance (OD, 550 nm), y-axis = concentration (nmol) and a polynomial trend line fitted to each graph. To determine the concentration of ascorbate and total ascorbate in samples assayed the absorbance (OD, 550 nm) values were substituted into the equation of the line ($y = m(x^2) + mx + c$).

Estimation of dehydroascorbate (DHA).

Dehydroascorbate (DHA) was estimated by subtracting the ascorbate only concentration from the total ascorbate concentration for each sample.

Calculation of ascorbate and total ascorbate in 100g edible portions.

To calculate the ascorbate and total ascorbate concentration in a 100 g edible broccoli portion the corrected absorbance (OD, 550 nm) readings for each sample was multiplied by 5, to give the ascorbate and/or total ascorbate concentration (mg/ml) per gram of tissue. This was then multiplied by 100 to give the concentration (mg/ml) in a 100 g edible portion.

5.2.2.5 Statistical analysis.

Vitamin C data was analysed using REML analysis as described in section 2.5.1.

5.2.3 Results

5.2.3.1 Vitamin C quantification (at harvest) of genotypes in the MGDH population using HPLC.

To determine if vitamin C is a suitable trait for genetic analysis, levels of total vitamin C at harvest, in the MGDH population were quantified using a HPLC approach (Figure 5.2.1). Genotypes in the MGDH population exhibit significant differences (****P>0.001*) in total vitamin C levels (Wald $_{[70]}$ = 131.80), at harvest, in 100g edible portions. Overall, the MGDH population displays 3-fold natural variation for the trait (Figure 5.2.1), with total vitamin C levels for genotypes ranging from 66.90-170.00 mg/ml in 100g edible portions. The commercial genotype Marathon falls within the lower range of the distribution for total vitamin C at harvest, where as the parental genotype Mar34 falls within the mid range of the distribution. Due to lack of tissue sample for the parental genotype GD33 in the field, total vitamin C levels for this genotype at harvest could not be quantified.

5.2.3.2 Correlation among traits.

A correlation analysis was carried out to determine if total vitamin C levels (at harvest) were correlated with any of the traits assessed in the 2007 field trial. No correlation was found between total vitamin C levels (at harvest) and the Wurr quality traits or the colour co-ordinates, although a weak negative correlation (****P<0.001*, $r_{[70]}$ = -0.38 (PD), ***P<0.01*, $r_{[70]}$ = -0.36 (Wt)) was found between vitamin C levels (at harvest) and the traits peduncle diameter and weight (Table 5.2.2). Similarly, a weak positive correlation (***P<0.01*, $r_{[70]}$ = 0.36) was found between total vitamin C levels (at harvest) and absolute weight loss (Table 5.2.2).

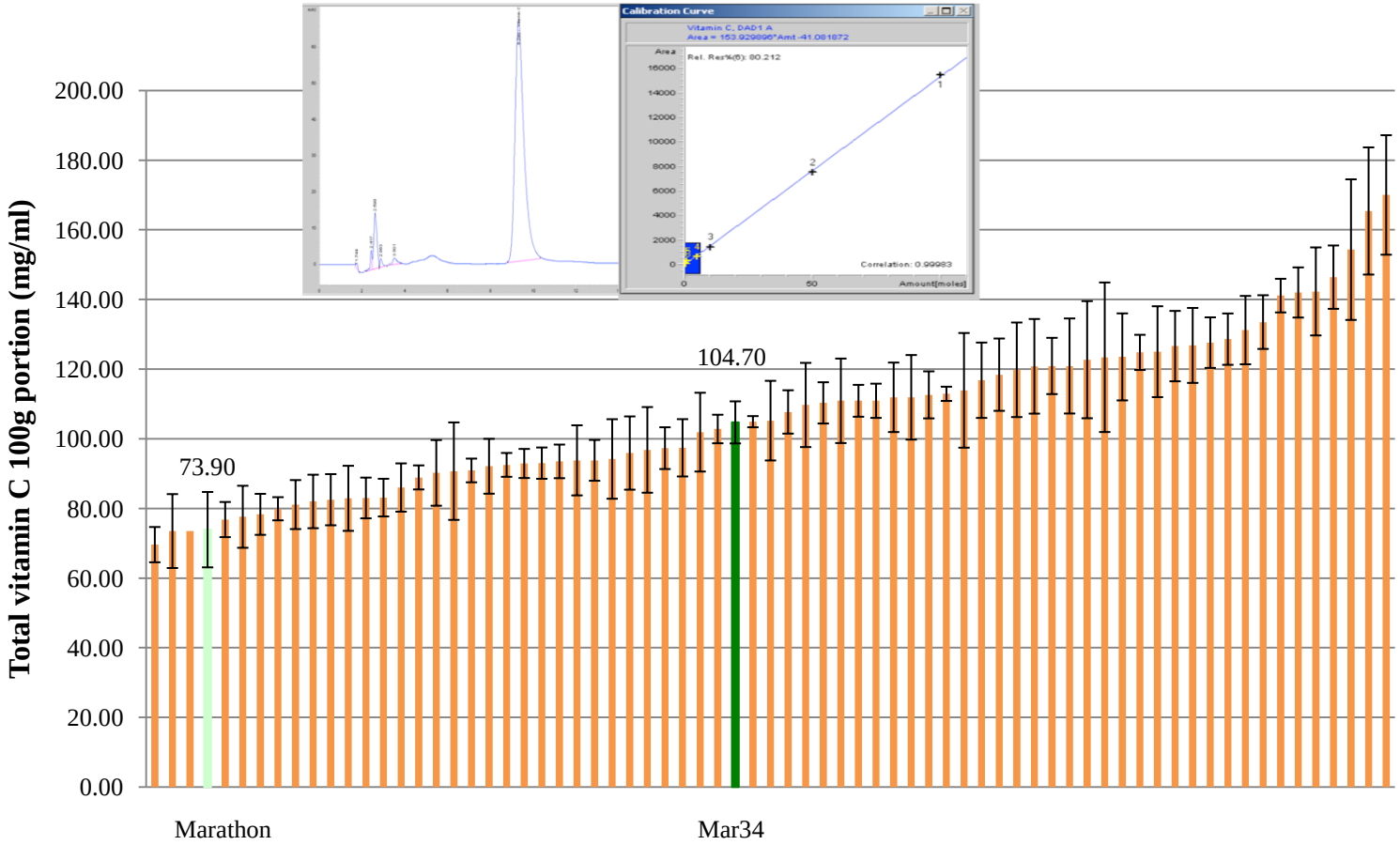


Figure 5.2.1: Predicted means (from REML) for total vitamin C levels in 100g edible portions from genotypes in the MGDH population with an example of the HPLC trace and standard curve used to quantify total vitamin C levels (n = 70). Error bars represent the standard errors of the means (s.e.ms). The commercial genotype Marathon and the parental genotype Mar34 are highlighted.

Table 5.2.2: Correlation matrix for morphological traits and total vitamin C content, at harvest. Significance levels are displayed as: ***P<0.001 (pink), **P<0.01 (orange) and *P<0.05 (yellow). HD = head diameter (mm), PD = peduncle diameter (mm), Wt = weight (g), RWtL = relative weight loss (%), AWtL = absolute weight loss (g-1), DY = days to yellowing and VitC_H = total vitamin C levels at harvest.

	HD	PD	Wt	RWtL	AWtL	DY	VitC_H
HD	-						
PD	***0.65***	-					
Wt	***0.79***	***0.86***	-				
RWtL	*-0.37*	*-0.32*	*-0.31*	-			
AWtL	***-0.59***	***-0.7***	***-0.82***		-		
DY	-0.23			***0.79***	***-0.46***	-	
VitC_H		***-0.38***	*-0.36*		*0.36*		-

*5.2.3.2 Vitamin C quantification during storage in a subset of genotypes from the MGDH population using plate assays***.**

To quantify ascorbate levels throughout storage two plate assays were used (figure 5.2.2); the ascorbate only assay measures the amount of reduced ascorbate in a sample whereas the total ascorbate assay measures the amount of ascorbate and dehydroascorbate (DHA, the oxidised form of ascorbate) by using dithiothreitol (DTT) to reduce DHA to ascorbate. DHA can then be estimated from the difference between the two assays. Levels of all forms of ascorbate where compared in a sub-set of genotypes from the MGDH population to determine the stability of ascorbate during storage.

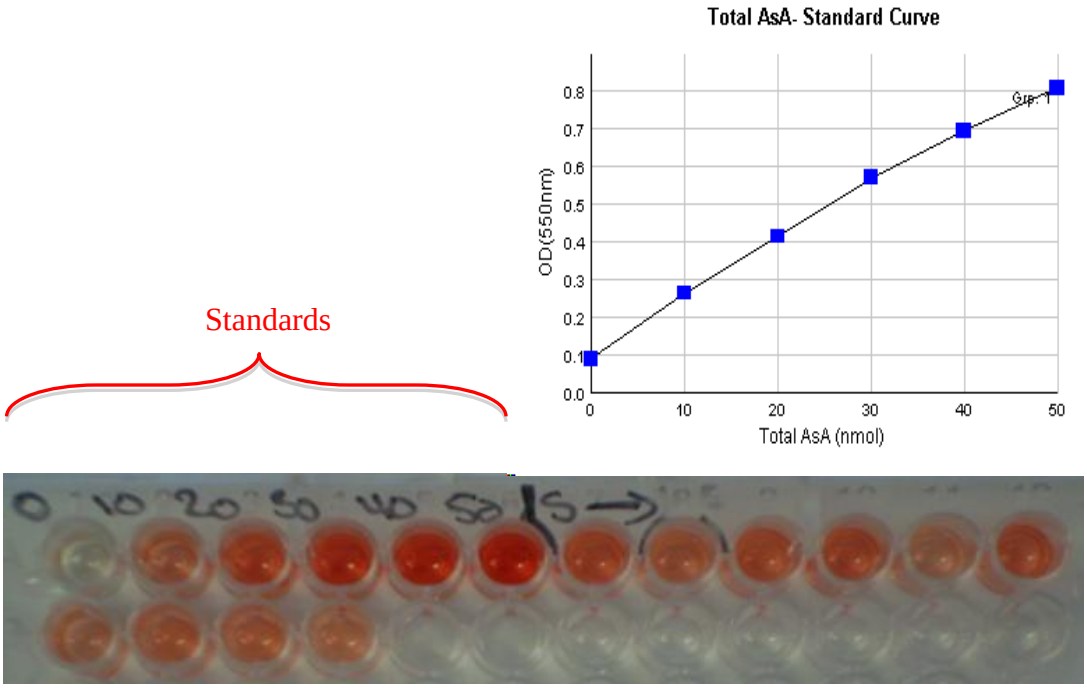


Figure 5.2.2: A photographic representation of the colour change reaction to quantify ascorbate in a sub-set of genotypes in the MGDH population and an example of an ascorbate standard curve used to quantify ascorbate from individuals in the population. The standard curve is produced by plotting the optical density (OD) of samples measured at 550 nm against the concentration of ascorbate from the standards.

5.2.3.3 Statistical analysis.

REML analysis was carried out on all measures of ascorbate (TA, AO and DHA) on each day of storage (0, 2, 4 and 6). An amount of total ascorbate (TA) in 100g edible portions was found to be significantly different within genotypes from a subset of the MGDH population on all days in storage. However, amounts of ascorbate (AO) and dehydroascorbate (DHA) in 100g edible portions was only found to be significantly different between genotypes on day two and day four for AO and at harvest (day 0) , day four and day six for DHA.

5.2.3.4 Total Ascorbate.

At harvest (Day 0), levels of total ascorbate in the sub-set of genotypes ranges from 43.49 - 172.99 mg/ml in a 100g edible portion (Figure 5.2.4), with the parental genotypes, Mar34 and GD33 and the commercial genotype Marathon containing the least total ascorbate compared to other genotypes assayed. On day two in storage, eight genotypes exhibit an increase in total ascorbate levels of 104.8 – 306.2 %, whereas the remaining genotypes assayed show a decrease in total ascorbate of 0.89 – 55.47 % between harvest (day 0) and day two in storage. Between theses two time points, the positions of the parental and commercial genotypes within the distribution change, GD33 remains at the lower extreme of the distribution whereas Marathon and the good shelf life performer Mar34 are located in the middle and the higher extreme of the distribution respectively. Both Mar34 and Marathon show an increase in total ascorbate content of 89.68 and 36.26 mg/ml. The range for total ascorbate on day four in storage is 29.82 – 90.68 mg/ml in a 100g edible portion. All genotypes, with the exception of MG10 and MG99 show a decrease in total ascorbate levels between day two and day four in storage. On this day the parental genotype GD33 remains at the lower end of the distribution, whereas Mar34 falls within the mid-lower and of the distribution and Marathon falls within the mid-high end of the distribution. By day six in storage, total ascorbate levels for most genotype have decreased by ~50% compared to harvest levels of total ascorbate. The range for total ascorbate on day six is 18.13 - 65.22 mg/ml in a 100g edible portion, with Marathon and GD33 falling at the lower end of the distribution and Mar34 falling at the mid-higher end of the distribtion for total ascorbate content. Genotypes that exhibited a decrease in total ascorbate levels at all time points during storage show an overall decrease in total ascorbate of 10.05-80.45 % between harvest and day six in storage,

with MG475 losing the most total ascorbate during storge, however this genotype also had the highest total ascorbate level at harvest. In general, total ascorbate levels start to decrease as soon as broccoli is harvested, with the exception of those genotypes that show an increase between harvest (day 0) and day two. This show levels of total ascorbate are higher between harvest (day 0) and day two in storage.

Comparison of genotypes grown in both seasons for total ascorbate.

In general, the rank order of genotypes grown in two different growing seasons changes with genotypes differing in realtive levels of total ascorbate at harvest between 2007 and 2008. Two genotypes, MG10 and Marathon, had the same rank in 2007 and 2008, whereas MG95 exhibited the largest decrease in rank and MG406 exhibited the largest decrease in rank between the growing seasons (Table 5.2.3). Out of the remaining genotypes 5 increase in rank and six decrease in rank between 2007 and 2008.

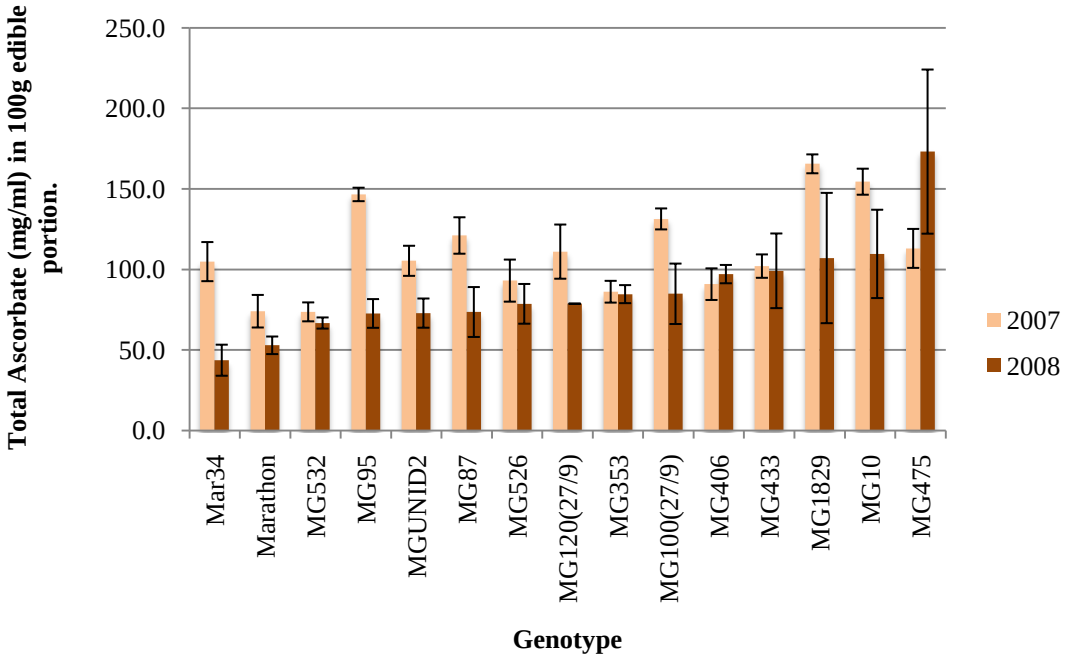


Figure 5.2.3: A comparison of predicted means (from REML) for genotypes in the MGDH population grown in 2007 and 2008 for total vitamin C content at harvest (n = 15). Error bars represent the s.e.m (standard error of the mean).

Table 5.2.3: Wilcoxon signed rank test to determine if the rank order changes for total vitamin C content at harvest between two different growing seasons. Negative scores indicated a decrease in rank, positive scores indicated a increase in rank and a score of zero represent no change in rank.

Genotype	Rank	Genotype	Rank
MG1829	-3.5	Mar34	-10.0
MG10	0.0	MG433	10.0
MG95	-13.0	MG526	3.5
MG100(27/9)	-3.5	MG406	12.0
MG87	-7.5	MG353	10.0
MG475	7.5	Marathon	0.0
MG120(27/9)	-1.0	MG532	3.5
MGUNID2	-6.0		

5.2.3.5 Ascorbate (AO) and dehydroascorbate (DHA).

Relative levels of ascorbate (AO) and dehydroascorbate fluctuate in individual genotypes during storage (Figure 5.2.5). In general, genotypes that exhibited an increase in total ascorbate between harvest and day two in storage, as seen in the parental genotype Mar34 and the commercial genotype Marathon, also exhibited an increase in dehydroascorbate (DHA) and ascorbate (AO), however the increase in AO is smaller in most genotypes compared to DHA. Three genotypes do not fit in with this trend are MG433, MG353 and Marathon. MG433 and MG353 exhibit a decrease in DHA and AO respectively, whereas AO levels in Marathon only show a marginal increase between these timpoints during storage. Between day two and day four in postharvest storage, levels of DHA and AO decrease in most of the eight genotypes that display a peaking pattern for total ascorbate during storage. This is pattern is also seen for AO between day four and day six in storage, whereas levels of DHA between these time points show a varied response betrween different genotypes: DHA levels in two genotypes (Mar34 and MG353) reamin constant, six genotyes exhibit a marginal decrease in DHA (MGUNID2, MG87, Marathon, MG526, MG120(27/9) and MG308) and the remaining three genotypes (MG1829, MG532 and MG433) display a marginal increase in DHA level between these

timepoints. Genotypes that display a decrease in total ascorbate over all time points during storage, as seen in the parental genotype GD33, show a larger overall decrease in DHA compared to AO between harvest and day two in storage, with the exception of MG353 and GD33. DHA levels bewteen these timepoints in GD33 remains constant whereas DHA in MG353 increases. The general trend for AO levels in genotypes between harvest (day 0) and day two is to remain stable or to exhibit a slight decrease, although two genotypes MG1829 and MG10 display an increase in AO between these timepoints. DHA levels in genotypes that show the same trend during storage as GD33, show a variable response between day two and day four in storage with DHA levels in 50% of genotypes increasing with the remaining 50% showing a decrease in DHA, whereas AO levels in all genotypes decrease between these timepoints. Between day four and day six of postharvest storage levels of AO decrease again for all genotypes with the exception of MG120(27/9). Levels of DHA between these timepoints display an erratic response with five genotypes exhibiting an increase in DHA levels and three genotypes exhibiting a decrease in DHA levels.

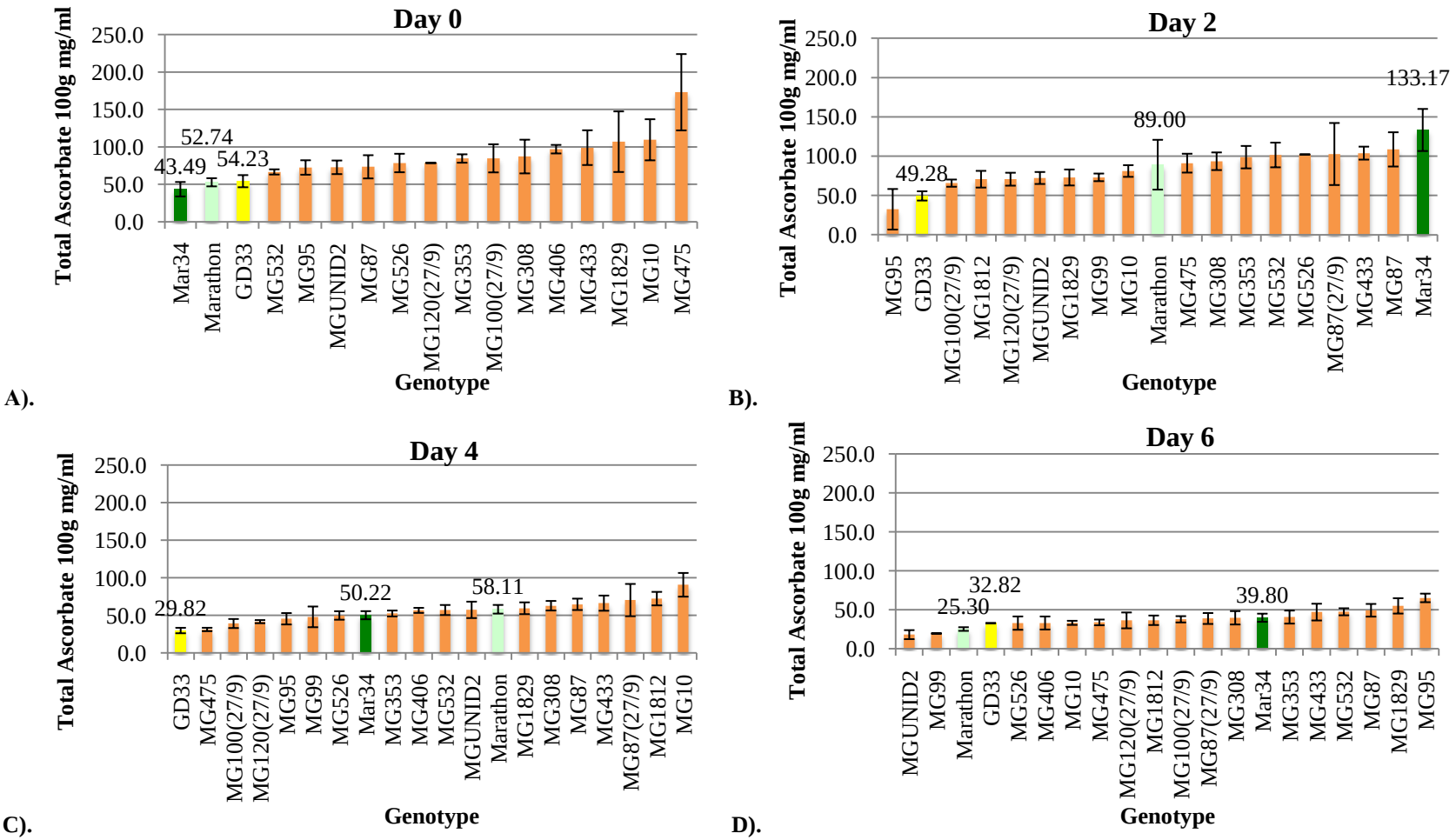


Figure 5.2.4: Predicted means (from REML) for total ascorbate levels, using the plate method, of a sub-set of genotypes in the MGDH population during storage A). day 0 (harvest), B). day 2, C). day 4 and D). day 6 in storage (n= 20). Error bars represent the s.e.m (standard error of the mean).

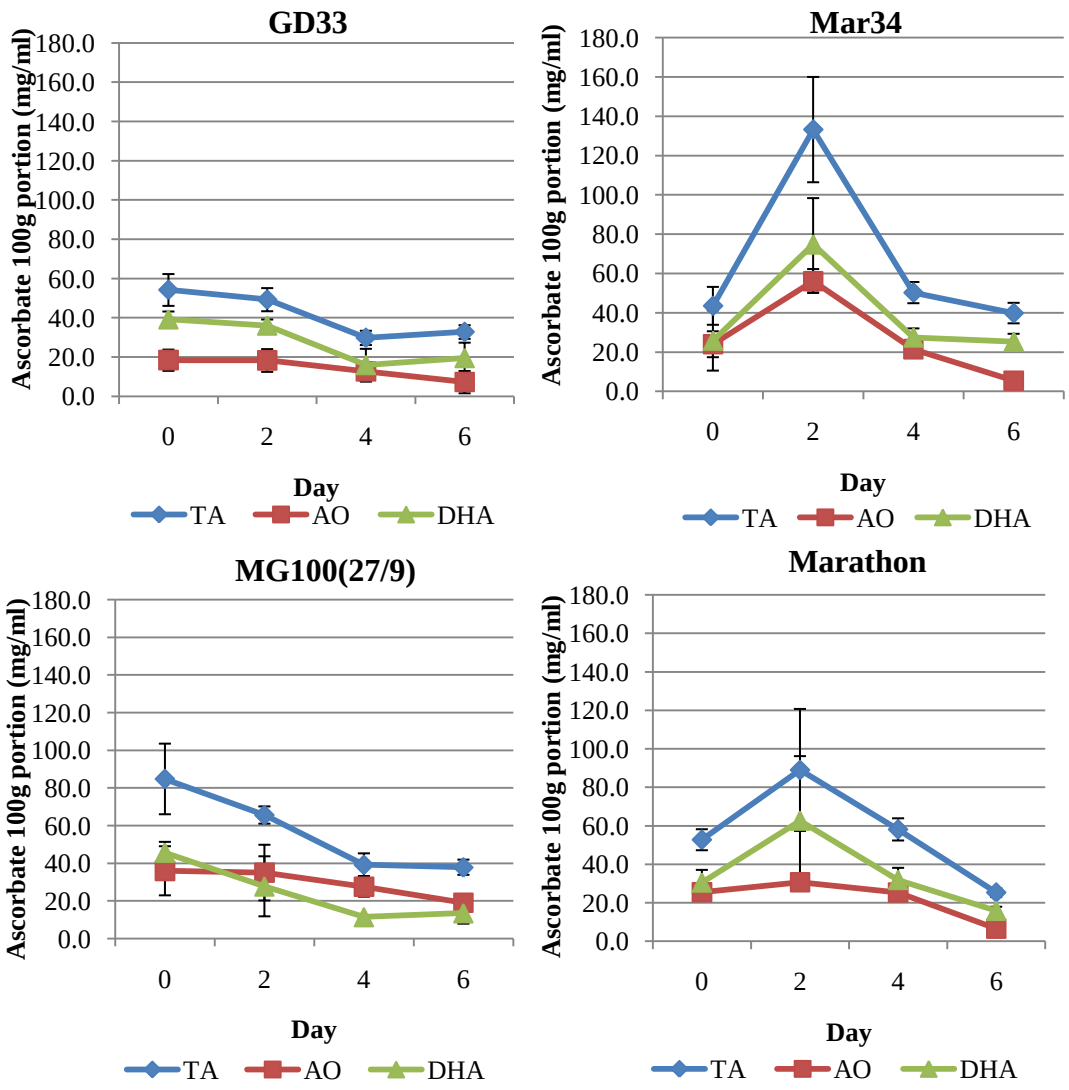


Figure 5.2.5: Predicted means (from REML) for measures of ascorbate (TA, AO and DHA) during storage in a sub-set of genotpes in the MGDH population (n = 8). Error bars represent the s.e.m (standard error of the mean). TA = total ascorbate (AO + DHA), AO = ascorbate only and DHA = dehydroascorbate)

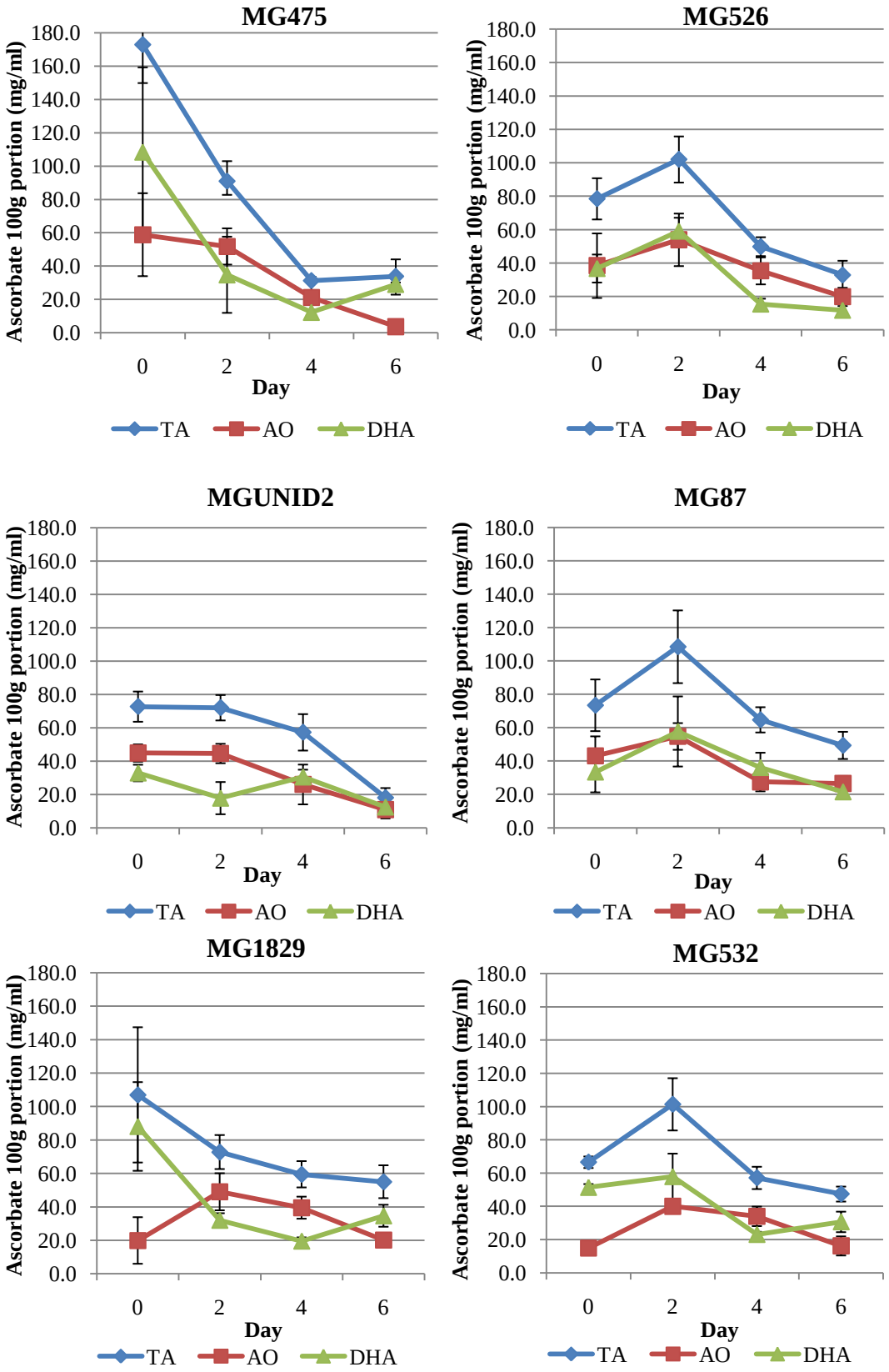


Figure 5.2.5: *Continued*

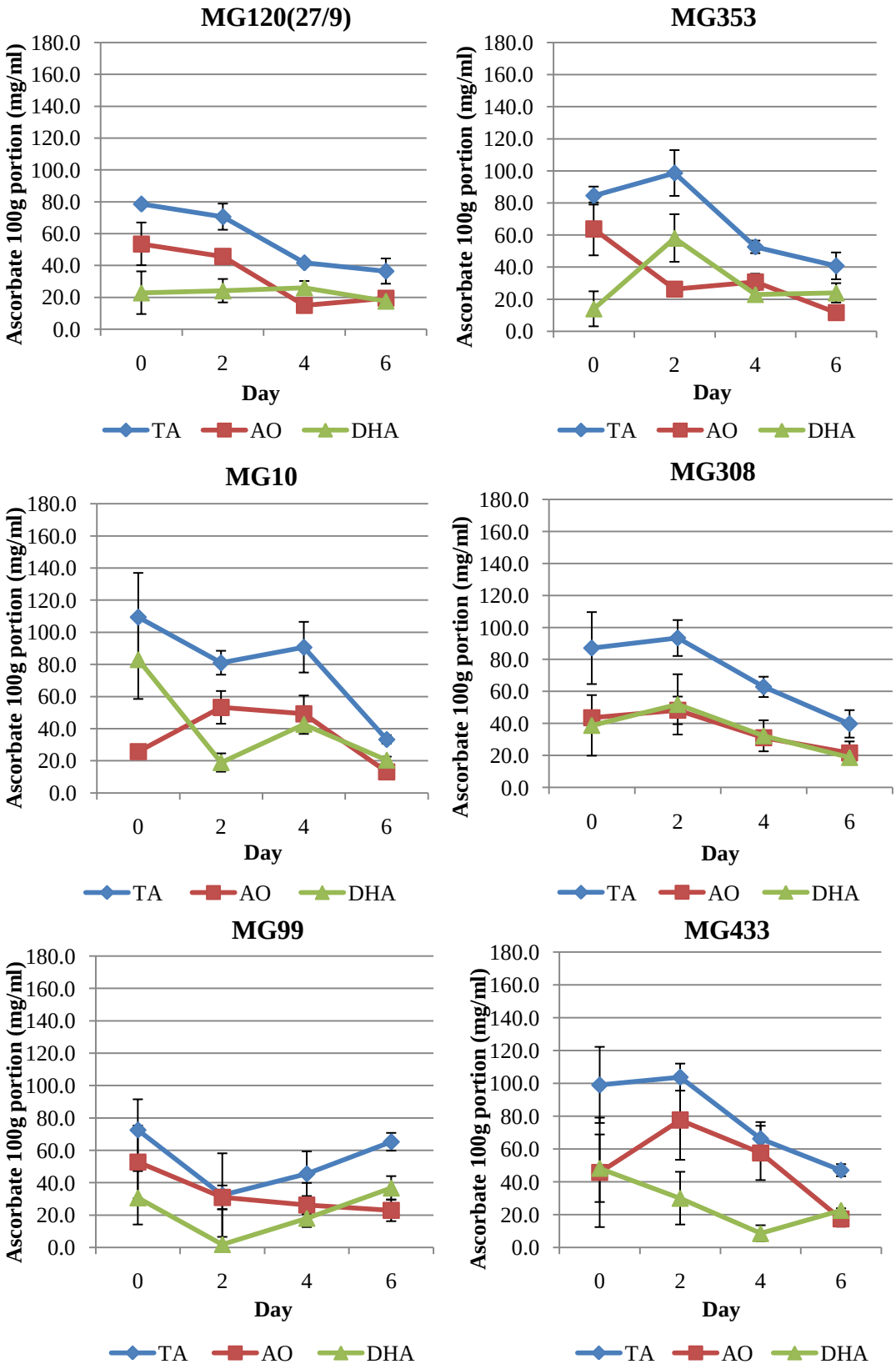


Figure 5.2.5: *Continued*

5.2.3.6 QTL analysis of total vitamin C content, at harvest, in the MGDH population.

The distribution for Vitamin C at harvest show a normal distribution (Figure 5.2.6), therefore QTL analysis was conducted on the predicted means generated from REML analysis in MapQTL V.4 (Van Ooijen *et al*., 2002) using both the interval mapping (IM) and MQM mapping functions as described in section 2.6. MQM mapping confirmed three QTL detected for the trait total vitamin C levels in LG O1 and LG O9 (Figure 5.2.7). Two QTL detected in LG O1 account for 17.1 % and 19.4 % of the variation associated with the trait where as the QTL detected in LG O9 is accountable for 15% of the variation associated with the trait (Table 5.2.4). For all QTL mapped the parental genotype Mar34 provides the beneficial allele for the trait. Each vitamin C QTL co-localises with other QTL mapped for quality traits in broccoli. QTL *VitC_h_1* and *VitC_h_2* co-localise with QTL *DY_1* and QTL for *HA* and *'a'* in LG O1 and QTL *VitC_h_3* co-localises with QTL *ST(d4)_2* and QTL for *Wt* and weight loss in LG O9.

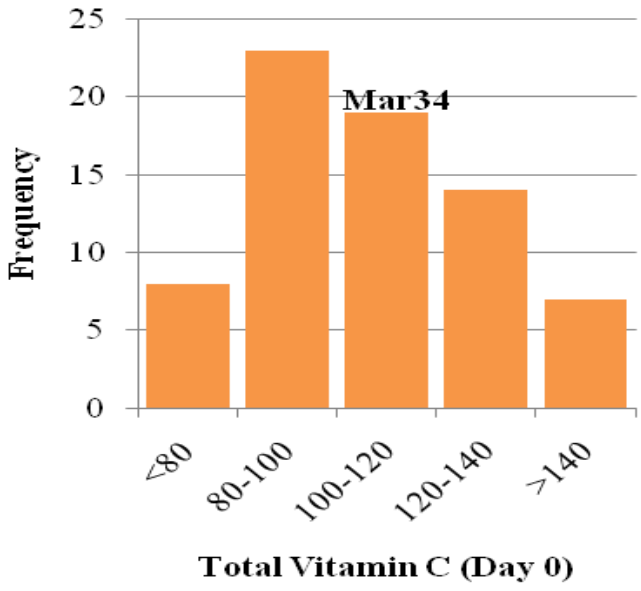


Figure 5.2.6: A histogram showing the distribution of genotypes for total vitamin C content at harvest (n = 20).

Table 5.2.4 QTLs detected by MQM mapping for vitamin C content at harvest in the MGDH population from the 2007 field trial.

Year	Trait[a]	LG[b]	Position[c] (cM)	Marker[d]	LOD[e]	Beneficial Allele[f]	Variance[g] (%)	QTL Label[h]
2007	*VitC_h*	O1	3.4	FITO095	4.2***	Mar34	17.1	*VitC_h_1*
		O1	78.1	E11M49_204	4.6***	Mar34	19.4	*VitC_h_2*
		O9	14.8	E11M50_146	3.9	Mar34	15	*VitC_h_3*

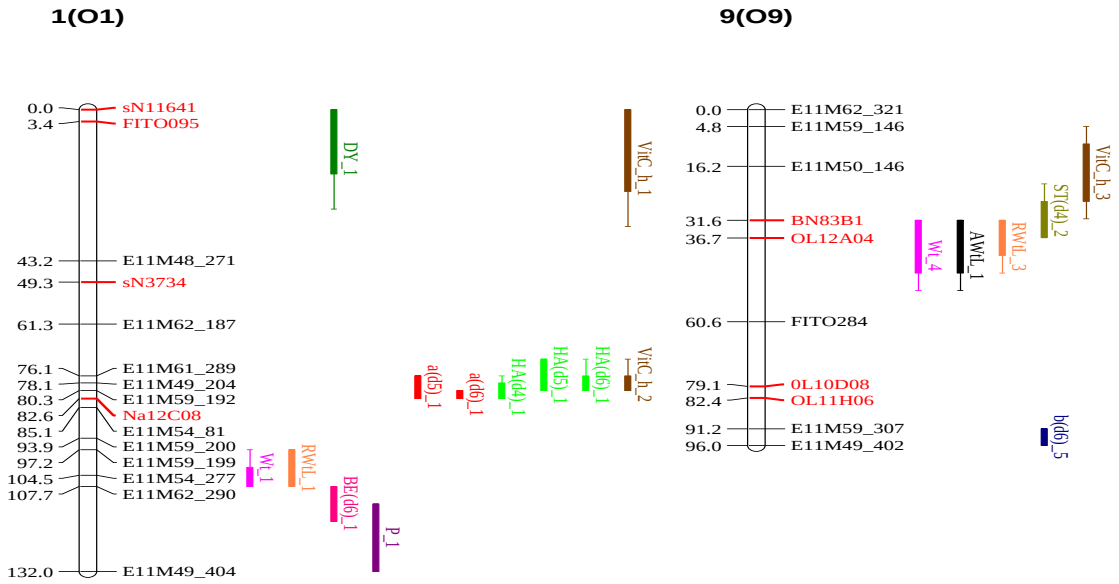


Figure 5.2.7: Linkage group O1 and O9 from the broccoli x broccoli linkage map showing the location of QTLs detected for morphological and shelf life traits evaluated in Chapter 3, Part III and new QTLs detected for vitamin C content at harvest in the MGDH population using MQM mapping. DY = Days to yellowing, Wt = Weight, RWtL = Relative weight loss, BE = Bud elongation, a = red-green colour scale, HA = hue angle, P = purple, VitC_h = vitamin C at harvest, AWtL = absolute weight loss, ST = stem turgor and b = yellow-blue colour scale.

5.2.3.7 QTL analysis of ascorbate levels during storage in a subset of genotypes from the MGDH population.

REML analysis was carried out on measures of ascorbate (TA, AO and DHA) during storage confirmed that genetic variation was present within genotypes from a sub-set of the MGDH population for all measure of ascorbate on all days in storage for TA, on day two and day four in storage for AO and on day zero (harvest), day four and day six in storage for DHA. Therefore, QTL analysis will be carried out on these time points for each measure of ascorbate.

Variation for measures of ascorbate during storage between the parental genotypes of the MGDH population.

Using the predicted means (from REML) for measures of ascorbate during storage, variation among the parental genotypes can be determined by comparing the position of the parental genotypes in each distribution for all measures of ascorbate (TA, AO and DHA) (Figure 5.2.8). The parental genotype Mar34 falls with the higher tail of the distribution for most measures of ascorbate on most days in storage. The only exception to this is for total ascorbate (TA) at harvest (day 0), as GD33 has a higher total ascorbate content at harvest than Mar34, and measures of total ascorbate and dehydroascorbate on day six and harvest respectively, as both parents have similar values for TA and DHA and fall in the same bin on these days.

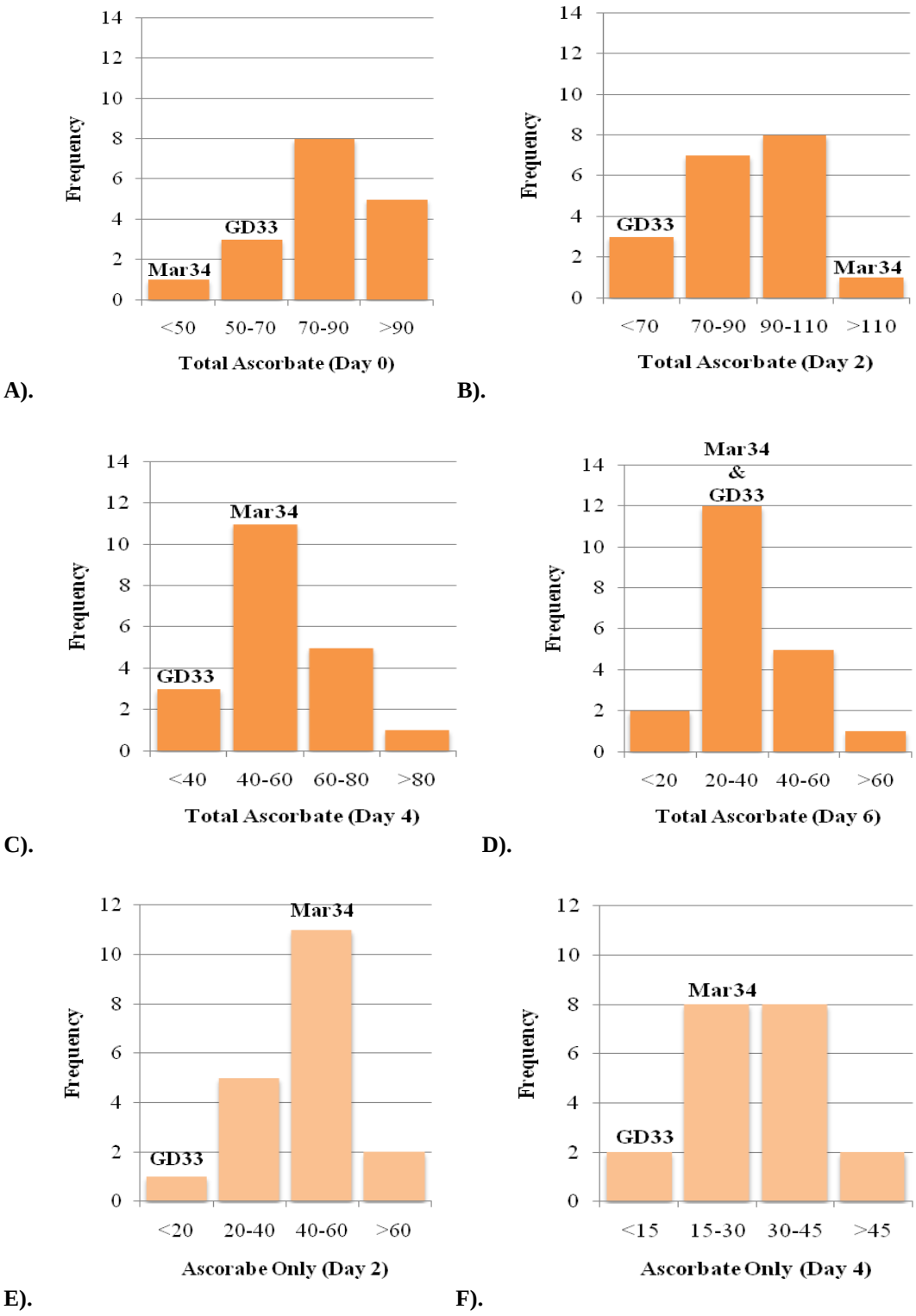
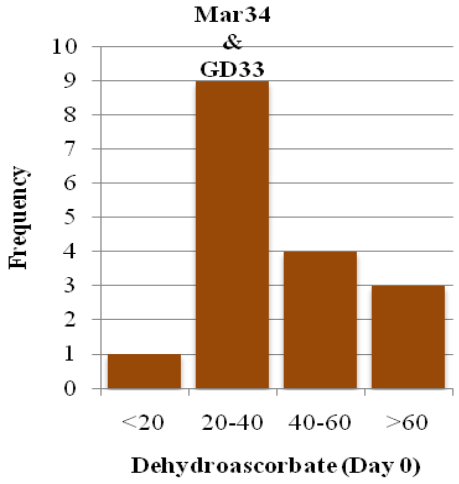
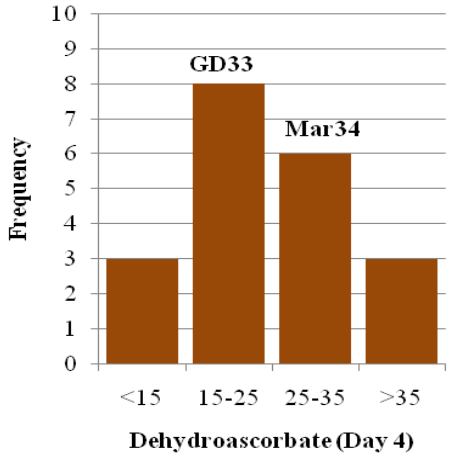


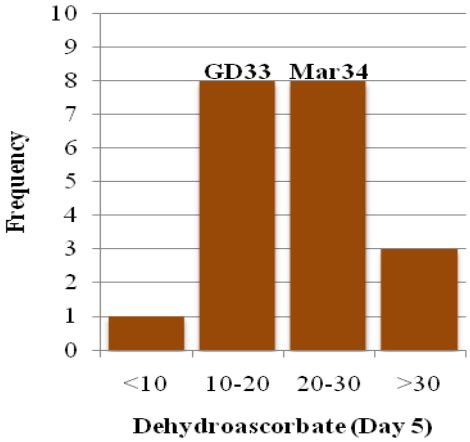
Figure 5.2.8: Histograms showing the distribution of the parental genotypes for measure of ascorbate (n =20). A-D: total ascorbate on day 0, 2, 4 and 6. E-F: ascorbate only on day 2 and day 4. G-I: dehydroascorbate on day 0, 4 and 6.



G).



H).



I).

Figure 5.2.8 *Continued*

QTL analysis: Interval mapping.

Interval mapping was carried out on significant (from REML analysis) measurements of ascorbate (TA, AO and DHA) during storage. Interval mapping identified a total of five genome wide significant QTL and eight QTL significant at the linkage group level for measurements of ascorbate levels during storage (Table 5.2.5). The QTL detected provide an indication of possible QTL locations for different measurements of ascorbate during storage, as MQM mapping could not be carried out to confirm QTL detected using IM, or to identify new QTL due to the presence of singularity errors. Singularity errors occur when the expected trait

values for the QTL genotypes can't be estimated causing the calculated LOD probability for one or more QTL genotypes to equal zero. Errors of this type can be exacerbated by missing trait values, in this study ascorbate measurements were only carried out on a small number (20) of genotypes with 74 genotypes having missing trait values. Therefore, the location of only the genome wide significant QTL for measures of ascorbate will be included on the broccoli x broccoli linkage map, to indicate possible QTL positions for measures of ascorbate during storage.

Out of the five genome wide significant (*$P<0.05$*) QTLs that were detected using interval mapping, two QTL were detected for total ascorbate (TA) on day four in storage, a further two QTL were detected for dehydroascorbate (DHA) at harvest and on day four in storage, and one QTL was detected for ascorbate (AO) only on day two in storage (Figure 5.2.9). QTL *AO(D2)_1* is located in LG O7 and co-localises with the QTL detected for TA on day four and QTL for floret looseness, bud elongation and days to yellowing. The second QTL for TA on day four is located in LG O6 and co-localises with QTLs for DHA on day four, floret looseness, stem turgor and the colour co-ordinate 'b'. Linkage group O1 contains the other QTL detected for DHA ion day four in storage, which is found to overlap with QTL for purple colour and bud elongation.

Table 5.2.5 QTLs detected by interval mapping (IM) for measure of ascorbate (TA, AO and DHA) during storage.

Year	Trait[a]	LG[b]	Position[c] (cM)	Marker[d]	LOD[e]	Beneficial Allele[f]	QTL Label[g]
2008	*TA(D0)*	O4	96.7	BRMS-063	2.6	Mar34	
	TA(D2)	O3	48.5	E11M50_191	1.6	GD33	
	TA(D4)	O2	56.4	Ra2G09	1.6	GD33	
		O6	0.0	E11M49_290	3.1*	Mar34	*TA(D4)_1*
		O7	51.2	E11M59_145	2.8*	Mar34	*TA(D4)_2*
	TA(D6)	O3	26.3	FITO285	1.7	GD33	
	AO(D2)	O5	49.4	E11M50_75	2.1	GD33	
		O6	0.0	E11M49_290	1.6	Mar34	
		O7	59.0	E11M49_232	3.1*	Mar34	*AO(D2)_1*
	AO(D4)	O3	0.0	E11M62_256	1.8	GD33	
	DHA(D0)	O1	107.7	E11M62_290	2.9*	GD33	*DHA(D0)_1*
	DHA(D4)	O6	39.9	E11M62_144	2.8*	Mar34	*DHA(D4)_1*
	DHA(D6)	O4	84.0	BRMS-063	2.1	Mar34	

[a] Trait abbreviations: *TA*, total ascorbate (AO +DHA); *AO*, ascorbate only; *DHA*, dehydroascorbate; *D0*, day one; *D2*, day two; *D4*, day four and *D6*, day six. [b] LG: Linkage group containing QTL. [c] Position (cM) of the QTL peak in the linkage group [d] Marker: Closest marker to the QTL peak .[e] LOD: log of the odds score for genome wide significant QTL.[f] The parental allele that causes an increase in the trait value. [g] The QTL label given to QTL mapped in the broccoli x broccoli linkage map.

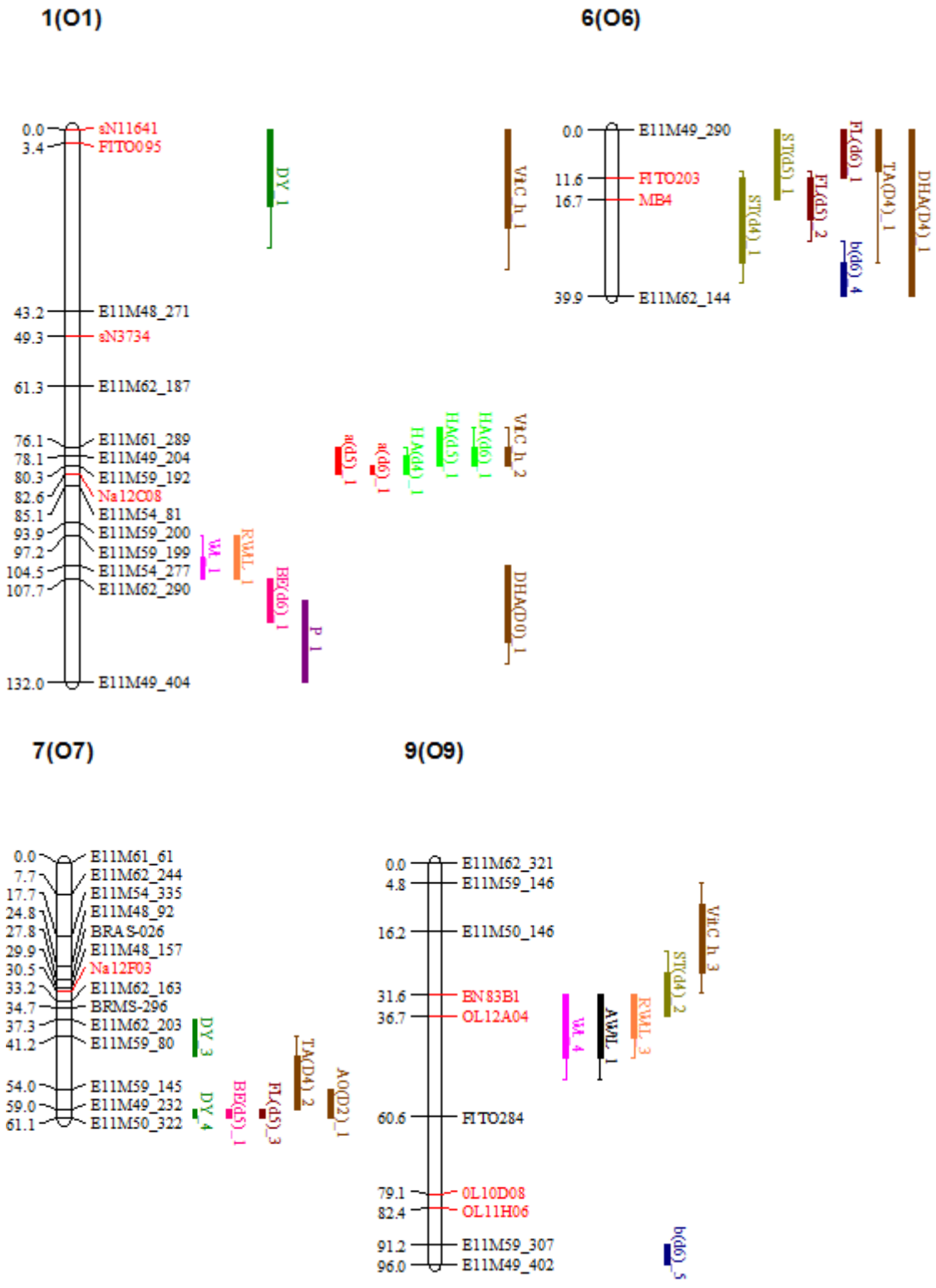


Figure 5.2.9: Location of ascorbate (TA, AO and DHA) QTLs detected using interval mapping in linkage groups O1, O6, O7 and O9 from the broccoli x broccoli linkage map. TA = total ascorbate, AO = ascorbate only, DHA = dehydroascorbate, D0 = day 0, D2 = day 2 and D4 = day 4

5.2.3.8 Identification of the Arabidopsis VTC1 gene ortholog in broccoli

Five genes (*VTC 1-5*) encoding enzymes in the ascorbate biosynthesis pathway have been identified through mutant studies in *Arabidopsis*. The *VTC1* gene (At2g39770) encodes the enzyme GDP-mannose pyrophophorylase (Table 5.2.6) which catalyses the conversion of D-Mannose-1-P to GDP-D-Mannose in the Smirnoff-Wheeler ascorbate biosynthesis pathway.

Table 5.2.6: Gene features and the position in base pairs (bp) of the *Arabidopsis VTC1* vitamin C biosynthesis gene (At2g39770) encoding the enzyme GDP-mannose pyrophophorylase.

VTC1: At2g39770	
Gene Feature	**Co-ordinates (bp)**
ORF	700-2040
5' utr	1-136
5' utr	675-699
Coding region	700-810
Coding region	892-954
Coding region	1043-1282
Coding region	1369-2040
Exon	1-136
Intron	137-674
Exon	675-810
Intron	811-891
Exon	892-954
Intron	955-1042
Exon	1043-1282
Intron	1283-1368
Exon	1369-2284
3' utr	2041-2284

To identify the *VTC1* gene ortholog in broccoli the *VTC1* cDNA sequence from *Arabidopsis* was used to BLAST search within *Brassica* EST, GSS and BAC collections. In total, 48 contigs were identified that showed homology to the *VTC1* gene. All contigs were aligned in SeqMan (DNASTAR) and grouped according to common polymorphims, this identified polymorphisms specific to the *Brassica* A (*B. rapa*), B (*B. nigra*) and C (*B. oleracea*) genomes. Due to the triplicated nature of *Brassica* primers were designed to target three groups identified due to common

polymorphisms, therefore three sets of primers (Table 5.2.7) were designed to target the three copies of the *VTC1* ortholog in *B.oleracea.* The primers were amplified with parental DNA and products were visualised on an agarose gel. All of the three primer combinations produced visible products however, there were no size differences found between the products.

The PCR products targeting the *VTC1* ortholog were used for sequencing using an automated ABI capillary sequencer to determine if polymorphisms existed between the parental genotypes at the sequence level. The sequencing reactions were viewed in 4Peaks (Mekentosj.com) and aligned in MegAlgin V 8.0.2 (DNASTAR, Inc.) (Figure 5.2.10) to look for polymorphisms between parental DNA (Figure 5.2.11). From the three primer combinations used, only group one was found to be polymorphic and contained 24 SNPs (Figure 5.2.12). To map the *VTC1* gene ortholog in the broccoli x broccoli linkage map the MGDH population would have genotyped by amplifying the primers with DNA from the population, and visualising the products on an SSCP gel. However, due to time constraints this couldn't be carried out.

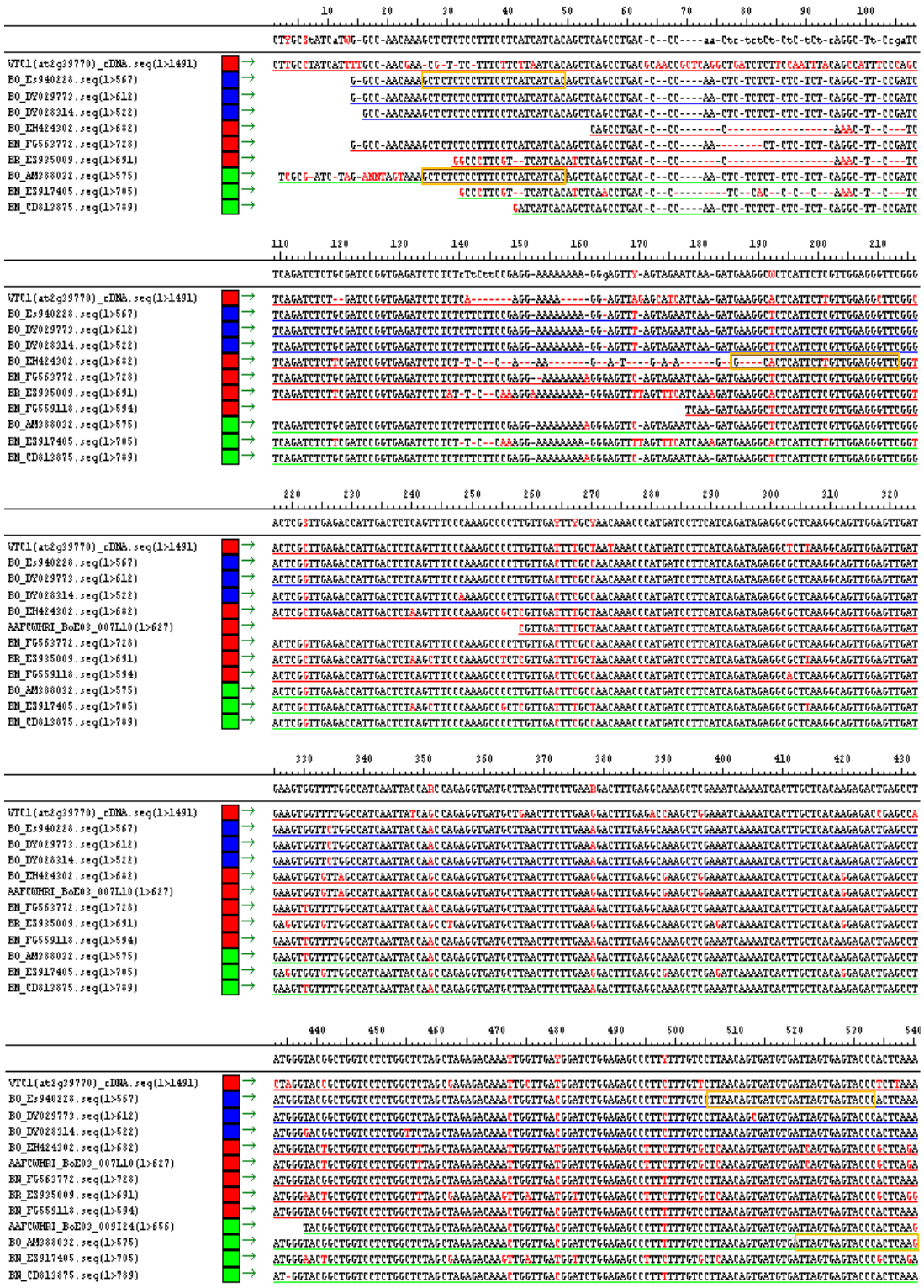


Figure 5.2.10: Contig alignment in SeqMan of orthologous *Brassica oleracea* ESTs from a BLAST search using *Arabidopsis* cDNA for the *VTC1* gene. Sequences were grouped into three groups (1 = red, 2 = green and 3 = blue) based on common polymorphisms. Primers were designed (boxed in orange on the diagram) for each group for amplification with parental DNA.

Figure 5.2.10: *continued.*

Table 5.2.7: Three sets of primers designed to target the *VTC1* gene ortholog in broccoli. The forward and reverse sequence, predicted product size and if the primers produced a polymorphic product is shown for each group.

Grp	EST	Forward	Reverse	Size (bp)	Product	Polymorphic
1	EH424302	GCACTCATTCTTGTTGGAGGGTTC	TCTTTCTCTATTGAGGTTGGTCTTAGC	~1200	Y	Y
2	AM388032	GCTCTCTCCTTTCCTCATCATCAC	TGAGCTTTGTGAAACTCAATCATT	~1200	Y	N
3	ES940228	GCTCTCTCCTTTCCTCATCATCAC	GTGAGCTTTGTGAAATTCAATCATCTCT	~1200	Y	N

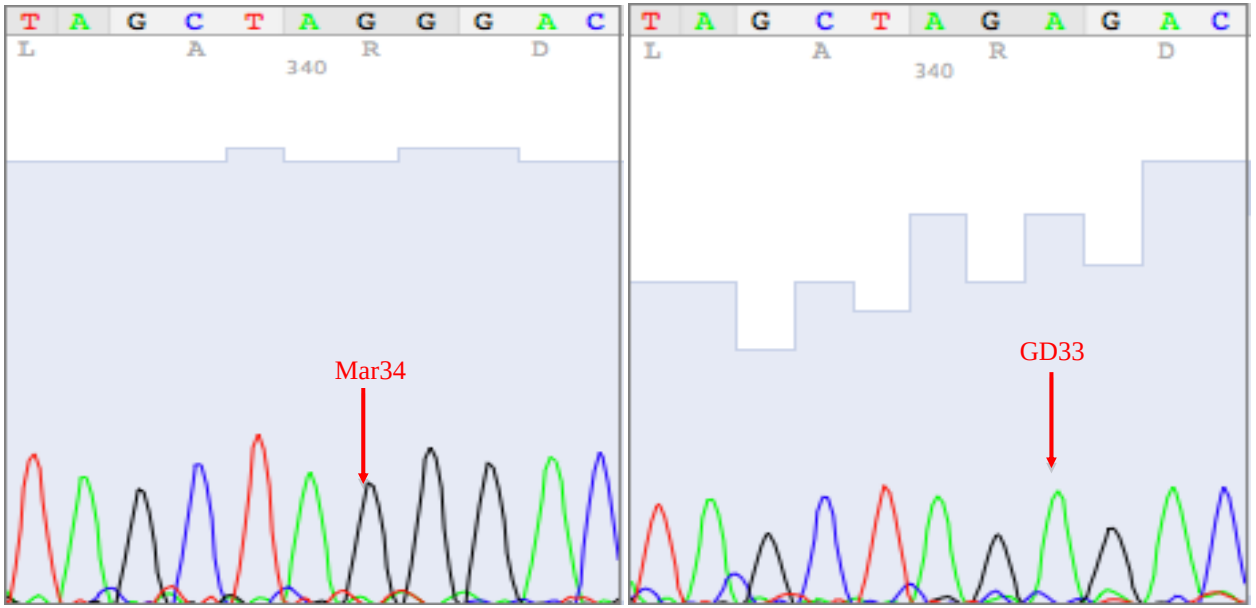


Figure 5.2.11: DNA sequence traces of the parental genotypes viewed in 4Peaks showing a SNP at position 342 bp in the PCR product. Mar34 has the nuclotide base G whereas GD33 has the nuclotide base A**.**

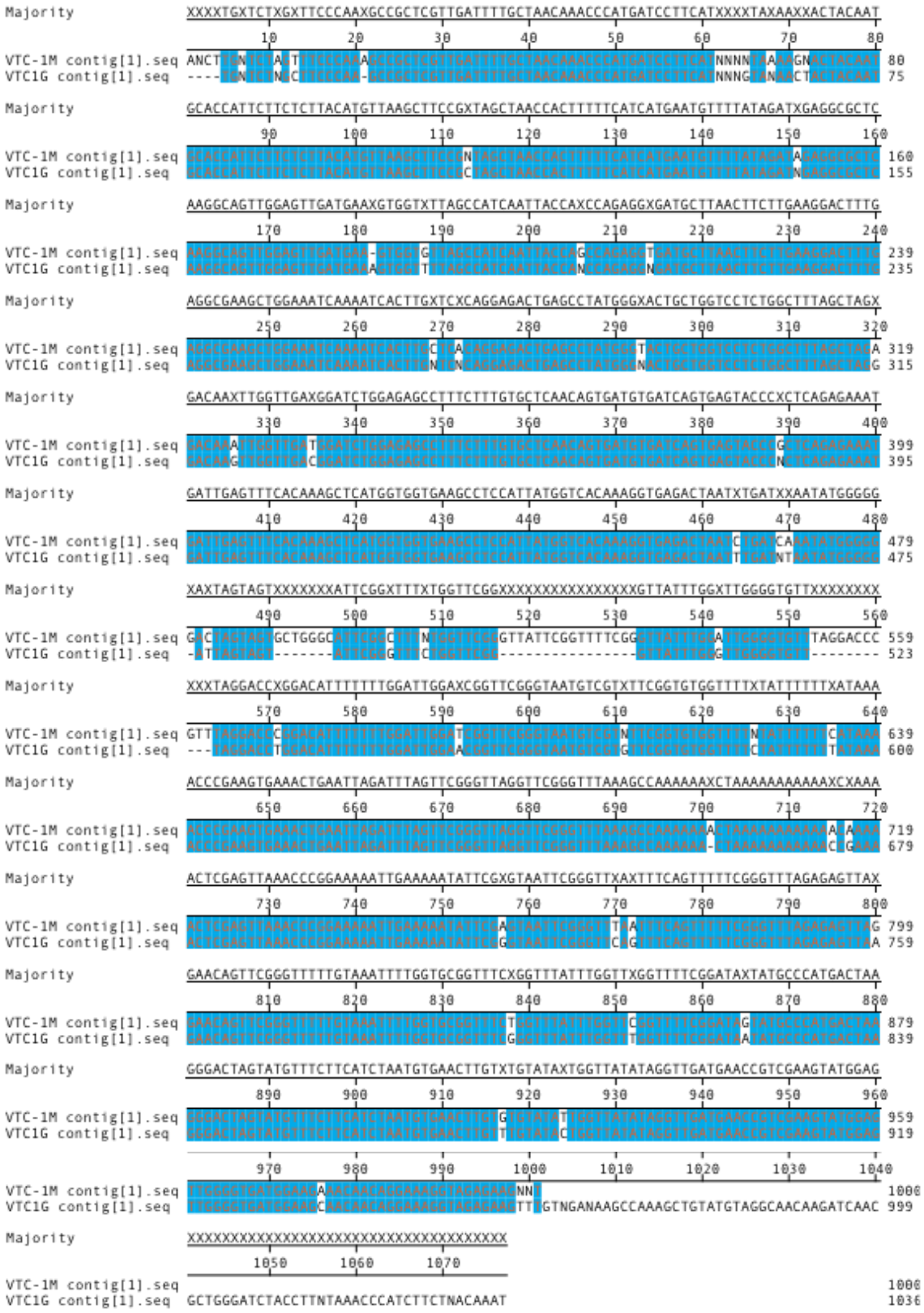


Figure 5.2.12: The sequence alignment of parental DNA sequencse amplified with primers (Group 1) designed to target the *VTC1* gene ortholog in broccoli.

5.2.4 Discussion

The MGDH population displays natural variation for total vitamin C content at harvest, indicating that Vitamin C content is influenced by genotype. This is supported by similar findings from a study by Kulrich *et al.,* (1999), in which the edible portions of 50 broccoli accessions were analysed to determine the variation in ascorbate content at harvest. Kulrich *et al.* (1999) found that the different accessions varied from 22.61 – 119.80 mg for ascorbate content at harvest in 100g edible portions. Genotypes in this study were found to have a higher range for ascorbate of 66.90-170 mg in a 100g edible portion. Kulrich *et al.* (1999) also determined how much of the variability between accessions was accounted for by genetic differences by analysis of the variance, and concluded that 55% of the total variation was associated with genetic differences among broccoli accessions, with the remainder of the variation being accounted for by variation among replicates.

5.2.4.1 Preharvest and post harvest factors affecting total vitamin C content in crops.

Vitamin C content in horticultural crops has been shown to be influenced by both preharvest and postharvest factors. Preharvest factors that influence vitamin C content described by Lee and Kader (2000) include cultural practices and climatic conditions. The main climatic conditions that could have affected the vitamin C content in this study are light intensity and average temperature. As the precursors for ascorbate metabolism are sugar products derived from photosynthesis, intensity of light during the growing season can influence total vitamin C content (Lee and Kader, 2000). In general, higher light intensity is associated with increased vitamin C levels in horticultural crops (Lee and Kader, 2000). Temperature is another influencing factor for vitamin C content in crops with the optimal temperatures for

vitamin C composition being crop dependent (Lee and Kader, 2000). Preharvest cultural practices that affect vitamin C content include irrigation, with moderate water deficit increasing vitamin C content in broccoli, this may simply be as a result of reduced water availability in the crop leading to an increase in the concentration of ascorbate. However studies investigating the effects of water deficit on fruit concluded that increased ascorabe concentration can not solely be explained by a concentration effect, suggesting that there is an underlying physiological mechanism resulting in higher ascorbate levels in reponse to moderate water deficit (Cui *et al*, 2008)).

Harvesting methods and the maturity of the crop at harvest can also affect vitamin C content in crops (Lee and Kader, 2000). As broccoli is harvested when it is immature, it is still undergoing rapid preharvest growth. Vitamin C has been indicated to have a role in cell wall biosynthesis and growth (Davey *et al*., 2000) therefore; vitamin C levels may be the highest in broccoli at harvest. All harvesting methods involve wounding, which promotes the synthesis of jasmonates (JA) and ethylene. These plant hormones have been demonstrated to regulate ascorbate metabolism. In general, harvesting causes vitamin C levels in vegetables to decrease in response to physical injury.

The vitamin C content of horticultural crops is also influenced by postharvest factors such as storage conditions (temperature and relative humidity) and processing methods (cutting and trimming). High storage temperature has been shown by to have a detrimental effect on vitamin C content in crops (Lee and Kader, 2000). Similarly, the trimming of vegetables, to remove the inner and outer leaves, has also been observed to reduce vitamin C content in crops (Lee and Kader, 2000). Leaves are highly abundant in vitamin C as they contain many chloroplasts, which provide

the sugar precursors for ascorbate metabolism through photosynthesis, therefore the removal of leaves from crops may promote vitamin C degradation (Nishikawa *et al*., 2004).

5.2.4.2 The stability of vitamin C during storage.

Levels of total vitamin C in this study were observed to degrade quickly after harvest with relative levels of ascorbate and DHA fluctuating throughout storage. Vanderslice *et al.* (1990) found that DHA levels increased in broccoli during storage, however, in this study, levels of DHA were found to be variable throughout storage. The ascorbate pool is balanced by the rate of biosynthesis and breakdown of ascorbate, with the ascorbate pool in broccoli genotypes in this study diminishing during storage. Enzymes involved in the breakdown of ascorbate include ascorbate peroxidase (APX) and ascorbate oxidase (AO). Ascorbate can be readily oxidised by both AO and APX into an intermediate form, monodehyroascorbate (MDHA) or a fully oxidised form dehydroascorbate (DHA) which is either recycled back to ascorbate through the ascorbate-glutathione (AsA-GSH) cycle, irreversibly hydrolysed to 2, 3-diketo-L-gulonic acid (2, 3-DKG) or degraded. If DHA is not recycled back to ascorbate it can be further degraded to oxalate, L-threonate, L-tartrate and other products.

The recycling of ascorbate via the AsA-GSH cycle defends against oxidative damage from reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), produced by photosynthesis and photorespiration (Smirnoff, 2000). Harvesting can induce stress conditions in the cell, which causes an increase in the production of ROS. Other stress conditions that accelerate the production of ROS include dehydration, chilling, high temperature, intensive light and wounding (Nishikawa *et al*., (2003b). APX acts

to detoxify ROS through the simultaneous reduction H_2O_2 to H_2O coupled with the oxidation of ascorbate.

Jasmonates (JA) are a family of plant hormones that regulate plant stress responses and have been indicated to influence AsA metabolism under oxidative stress (Suza *et al*., (2010). Suza *et al*. (2010) investigated the effects of wounding and methyl jasmonate (MeJA) treatment on AsA metabolism in *Arabiodpsis*. Exogenous MeJA treatment was found to enhance AsA in *Arabidopsis* leaves in wild type (WT) plants compared to the Arabidopsis *acx1/5* mutant, in which JA synthesis is impaired. Mutant plants were observed to have decreased levels of AsA, suggesting that JAs are regulators of AsA metabolism (Suza *et al*., 2010). Furthermore, transcript data showed that AsA biosynthetic genes were induced by exogenous MeJA (Suza *et al*., 2010). Wounding, which activates JA signalling, was also found to upregulate genes involved in AsA biosynthesis in WT leaf tissue but failed to induce the transcript of AsA biosynthesis genes in the *acx1/5* mutant (Suza *et al*., 2010). However, Suza *et al*, (2010) concluded that induction of AsA genes in response to wounding could be due to changes in cell wall formation, as AsA has been inferred to have a role in cell wall biosynthesis, rather than as a response to JA signalling.

5.2.4.3 Ascorbate metabolism studies in broccoli.

Ascorbate metabolism studies by Nishikawa *et al*. (2003a, 2003b) have investigated the expression of enzymes involved in ascorbate synthesis and breakdown in broccoli florets and stem tissue. Two cytosolic ascorbate peroxidise genes (*BO-APX1* and *BO-APX2*) have been identified in broccoli by Nishikawa *et al*. (2003a) which differed in expression in different tissue samples from broccoli (stem tissue and floret tissue). Transcript levels of *BO-APX2* were found to increase in florets

after harvest whereas transcript levels of *BO-APX1* decreased (Nishikawa *et al*., 2003a). In stem tissue, transcript levels of both enzymes increased after harvest in which Nishikawa *et al*. (2003a) proposed that induction of the transcript was caused by oxidative stress at the cut portion from wounding at harvest. Nishikawa *et al*. (2003a) also demonstrated that both enzymes showed high affinity for ascorbate and H_2O_2 and concluded that the loss of ascorbate postharvest was related to high, continuous APX activity and gene expression.

Nishikawa *et al*. (2003b), also studied the gene expression of other ascorbate metabolism genes including ascorbate oxidase (AO), L-galactono-1, 4-loctone dehydrogenase (GLDH), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) in stem tissue and floret tissue in broccoli. Gene expression of *BO-APX1*, *BO-APX2*, *BO-AO*, *BO-MDHAR2* and *BO-GR* in the cytosol exhibited an increase in broccoli florets (Nishikawa *et al*., 2003b). In contrast, mRNA levels of chloroplastic *APX*, *BO-sAPX* (stromal) and *BO-tbAPX* (thylakoid bound) were observed to decrease 12 hours after harvest with *BO-MDHAR1* and *BO-DHAR* decreasing 24 hours after harvest (Nishikawa *et al*., 2003b). From these findings, Nishikawa *et al*. (2003b) postulated that differences in chloroplastic and cytosolic gene expression may be due to the breakdown of the ROS-scavenging system in the chloroplast, as a result of chloroplast degradation, leading to the inactivation of enzymes (*APX*, *MDHAR*, *DHAR*) caused by excess H_2O_2 and the depletion of ascorbate under stress conditions, as well as the redistribution of ascorbate into the cytosol to protect cells against oxidative damage under stress conditions induced by harvesting and postharvest senescence.

Nishikawa *et al*. (2003b) also studied to ascorbate levels in the stem tissue, which remained unchanged after harvest, even though similar gene expression patterns were observed in both stem and floret tissue. Nishikawa *et al*. (2003b) assumed that loss of ascorbate after harvest is influenced by the number of chloroplasts and the regulation of genes involved in the ROS-scavenging system therefore, the retention of ascorbate may be due to the reduced number of chloroplasts found in the stem. Other factors contributing to the reduction in AsA during storage is proposed by Nishikawa *et al*. (2003b) to be associated with a reduction in ascorbate biosynthesis due to sugar deficiency. The biosynthesis of ascorbate requires sugar precursors generated from photosynthesis. However, the photosynthetic capacity of broccoli becomes reduced after harvest resulting in a loss of sugar. This was supported by a rapid decrease in the expression of *BO-GLDH*, which catalyses the synthesis of ascorbate from L-galactono-1, 4-lactone, in broccoli postharvest (Nishikawa *et al*., 2003b). Furthermore, Nishikawa *et al.* (2004) investigated the relationship between sucrose and AsA metabolism and found that both sucrose and AsA content decreased in harvested broccoli florets with the leaves removed after 48 hours. Sucrose feeding experiments were also found to upregulate the biosynthesis and expression of genes associated with AsA metabolism in chloroplasts, therefore Nishikawa *et al*. (2004) concluded that sugar deficiency may disrupt AsA biosynthesis and the ROS-scavenging system in chloroplasts leading to AsA degradation.

5.2.4.4 QTL analysis of vitamin C content and stability at harvest and throughout storage.

Three QTLs were detected that account for 51.5% of the variation associated with the trait vitamin C content at harvest; for each QTL the beneficial allele associated

with the trait belongs to the good shelf life performer Mar34. QTLs detected for vitamin C content in harvest all co-localise with other QTL for the trait 'days to yellowing' and head colour (Hue angle and the colour co-ordinate 'a'). However, no correlations were found between these traits. The co-localisation of these QTL may be due to the association of photosynthesis in the chloroplast providing sugar precursors for ascorbate metabolism, as a reduction in ascorbate pool size of crops postharvest is found to coincide with chlorophyll degradation, as a result of the dismantling of chloroplasts. The confidence intervals for two of the vitamin C QTLs at harvest (*VitC_h_1* and *VitC_h_3*) are large and require refining. As both of the QTL are located in linkage groups that are poorly saturated with markers, targeted saturation of these linkage groups with additional markers should delimit the confidence interval of the QTLs.

Five QTLs for levels of ascorbate during storage were detected in three linkage groups, using interval mapping (IM). However these QTL could not be confirmed using MQM mapping due to a high percentage of missing values causing singularity errors. Therefore, the QTL detected only provide possible locations for QTLs relating to vitamin C stability during storage. To determine if the QTL detected are true QTLs the measurement of ascorbate levels during storage would need to be carried out on the whole MGDH population. The identification of QTL relating to vitamin C in broccoli content allows the potential to identify candidate genes underlying the QTL, such as the *VTC* genes identified in *Arabidopsis* that are involved in ascorbate metabolism. Work was undertaken to identify and map the *VTC1* gene ortholog from *Arabidopsis* in broccoli. Primers were designed that would have been used to genotype the MGDH population using SSCP gels. This would have provided genotype data so that a marker for the *VTC1* gene ortholog could have

been positioned in the broccoli x broccoli linkage map. Ideally, the *VTC1* marker would have fell within a QTL for vitamin C content as this would have indicated it as a candidate gene underlying the QTL. However this work could not be completed due to time constraints.

Conclusions:

- The MGDH population was confirmed to exhibit significant natural genetic variation for vitamin C content at harvest.
- Vitamin C is unstable after harvest and is quickly degraded during storage, with the parental genotypes displaying distinct degradation patterns, which can be observed in the sub-set of genotypes from the MGDH population.
- Significant QTLs were detected for vitamin C levels at harvest but QTLs for vitamin C content and stability during storage could only be detected with a single QTL model due to the small sample size. Therefore, vitamin C quantification during storage would need to be repeated using the whole population.

CHAPTER 6:
GENERAL DISCUSSION

6.1 General Discussion

Demand for fresh broccoli in the UK has increased in the last decade due to increased public awareness of the nutritional benefits associated with the crop. However, the quality of broccoli is compromised by rapid postharvest senescence at ambient temperatures causing undesirable head yellowing and a loss of texture due to dehydration. Loss of nutrients during storage also results in a lower quality product for the consumer. Approaches to prolong the shelf life of broccoli include controlled atmosphere storage, packaging and treatments. However, an alternative approach is to use genetics to utilise the naturally occurring allelic variation to produce broccoli genotypes that have an extended shelf life and retain firmness and nutrient levels postharvest. The main aim of this study was to investigate the genetic control of shelf life and vitamin C content in broccoli, so that these quality traits can be improved in broccoli through conventional breeding.

The MGDH population created by Mathas (2004) from a F_1 cross between a good shelf life performer Mar34, and a poor shelf life performer GD33, was confirmed in this study to exhibit significant genetic variation for head yellowing, vitamin C content and morphological traits and can therefore be used as a tool to investigate the genetics of shelf life and quality in broccoli. The MGDH population was also confirmed to show DNA polymorphism by genotyping with SSR and AFLP markers and a new improved broccoli x broccoli linkage map was created provided good coverage of the *Brassica oleracea* genome. However, to further improve the linkage map the marker saturation of some linkage groups requires improvement. Recently, Syngenta have screened the MGDH population with their SSR platform to produce a broccoli linkage map that incorporates more markers (310) and covers ~100% of the *B. oleracea* genome (Dr. Walley, personal communication). As the two maps

contain common markers in all linkage groups, they could be anchored together, which could aid the saturation of markers in gaps in individual linkage groups. The integrated *Brassica* map produced by Sebastian *et al*. (2000) is an example of an anchored map in which two linkage maps, from the AxG and NxG populations, have been combined. The anchoring of maps can also clarify marker order and ensure linkage groups are in the right orientation. Alternatively, the linkage map produced in this study could be anchored to other published *Brassica* maps that have common markers, such as the integrated map by Sebastian *et al*. (2000) or a more recent high density linkage map produced by Gao *et al*. (2007). Another approach to improve the saturation of linkage groups would be to select markers that map into specific linkage groups from published maps, for the targeted improvement of poorly saturated linkage groups. This may also help to incorporate unmapped markers if there is sufficient linkage. Furthermore, an improved map would be of more use in identifying syntenous regions with *Arabidopsis* by comparative mapping, which would enhance the potential of identifying candidate genes controlling senescence (head yellowing), vitamin C metabolism and morphological traits in broccoli.

The postharvest yellowing phenotypes of the MGDH population, vitamin C content and stability and other quality characteristics such as weight, weight loss, turgor and bud quality exhibited significant natural variation for all traits. This has allowed the identification of desirable genotypes that had good storing ability, were of a commercial size and weight and retained quality postharvest. In total, 50% of the population were observed to stay green for longer than six days in storage. However, further characterisation is required to establish if these individuals exhibited cosmetic or functional stay green phenotypes. Cosmetic stay greens retain chlorophyll and therefore keep a green appearance during storage even though photosynthetic

capacity is lost, whereas the photosynthetic capacity of functional stay greens is retained but the initiation of senescence is delayed or progresses at a slower rate (Hortensteiner, 2009). Experimental approaches to determine the stay green phenotypes of individuals include determining the protein content of genotypes and chlorophyll assays.

In this study, the shelf life of the MGDH population was evaluated by visual assessment of head yellowing. However, as visual assessment can be subjective, spectrophotometer readings were also taken, to provide a quantitative measurement of head colour and colour change during storage. However, the colour co-ordinate data failed to distinguish clearly between genotypes that had remained green and those that had become yellow. Therefore, in a separate experiment, the number of spectrophotometer readings was increased to 15, to determine if inconsistencies in the data were due to the restricted number of readings (5) taken in 2007. Increasing the number of readings was seen to improve the variance of the data in the mini experiment. This suggests that accuracy of taking the readings may be adversely affected by time constraints. Spectrophotometer readings were also compared to an alternative approach that quantifies colour by calculating RGB values from photographic images of broccoli heads in Image J. The spectrophotometer was found to be more sensitive than image J in detecting colour change, with differences detectable a day earlier. However, image J data are more reproducible and are less liable to bias since the whole of the broccoli head is used to calculate colour. Colour is a complex trait, and quantifying colour change using co-ordinates (L, a, b, Chroma and Hue angle) or RGB values may be too subtle when compared to the processing power of the human eye. The eye is exceptionally good at processing a number of colour cues to determine what is acceptable (green) and what is unacceptable

(yellow) with the variation in acceptability being explained by the variation between people's vision.

Stem turgor was also identified as an important phenotypic characteristic that influenced the marketability of broccoli, with several genotypes being classed as unacceptable for retail due to compromised stem turgor before becoming yellowing in storage. However, the method to assess stem turgor in this study was very crude, with the loss of stem turgor being judged by the amount of flexibility and movement of the stem when shaken. Therefore, to accurately measure stem turgor a more sophisticated approach should be employed such as the measurement of tensile strength and compression

The MGDH population also exhibited natural variation for vitamin C levels at harvest, with vitamin C levels in a sub-set of the population degrading quickly during postharvest storage. Vitamin C is an antioxidant that detoxifies ROS, with ROS production increasing under stress conditions, such as postharvest senescence, which is induced rapidly once broccoli is harvested. Therefore, a decline in vitamin C levels may be due to recycling through the ascorbate-glutathione cycle. A decline in photosynthetic capacity has also been indicated to reduce the pool size of ascorbate, as biosynthesis of ascorbate will decrease due to the reduction of sugar precursors generated from photosynthesis. QTL for vitamin C content (at harvest) have been mapped in this study. However, problems were encountered when carrying out QTL analysis on vitamin C stability during storage due to the small sample size. Therefore, to improve the detection of QTL associated with vitamin C content during storage, vitamin C assays would need to be repeated on the whole of the MGDH population.

In total, 48 significant QTL were detected for head yellowing (4), colour co-ordinates (17), morphological traits (17), bud quality (2) and vitamin C content (3) and stability (5) postharvest. However, there are unaccounted sources of variation associated with all QTL. The remaining variation may be explained by the environment and G x E interactions or by undetected QTL. To determine if the QTLs are environmentally robust, additional field trials would need to be carried out in a different environment. The MGDH population is currently being grown in Enkhuizen in the Netherlands, this will provide additional phenotype data to test the robustness of the original QTLs detected for head yellowing and morphological traits and may identify new QTLs that are dependent on the environment.

The confidence intervals of many QTLs detected in this study are also large, however these may be improved through the anchoring of linkage maps and the remapping of QTL or by fine-scale mapping of QTL by backcrossing. A backcrossing programme exists at HRI that has involved the selection of genotypes containing beneficial alleles associated with QTL mapped for the trait 'days to yellowing'. Ten genotypes have been selected and crossed to the microspore receptive parent GD33, to produce new DH backcross lines. Three generations of backcrossing have been carried out to date, which should allow for fine mapping of QTL by introducing recombination into the region of interest. Delimiting the confidence intervals of QTLs will also provide smaller target regions to search for candidate genes underlying the trait. Many senescence associated genes involved in processes such as ethylene biosynthesis, chlorophyll degradation and other metabolic processes have been identified in broccoli (Chen *et al.*, 2008) however the location of these in the genome is unknown. However, with the current advances in the multinational *Brassica* genome sequencing projects that use high throughput

illumina, 454, BAC ends and shotgun sequencing techniques, the identification of candidate genes will become easier, as the detection of QTLs will lead directly to sequence data. Currently, the following *B. oleracea* morphotypes are being resequenced in the UK, Canada, USA, Australia and China: cauliflower, Chinese kale, kohl rabi, cabbage, broccoli and brussel sprout. It is proposed that by the end of 2010 two *B.oleracea* genomes will be fully sequenced with the alignment data available in the public domain. It is estimated that the sequencing of these genomes will cover the whole genome (~600 Mb), which are expected to contain ~44,000 genes. As part of the UK effort, the parents of the mapping population used in this study are being resequenced to provide x20 fold coverage of the genome. (Dr. G. Barker, update as of 'sequencing and resequencing' discussion at the *Brassica* 2010: 17th Crucifer Genetics Workshop).

QTL analysis has identified beneficial and deleterious alleles associated with all traits assessed in this study. For most of the QTL identified for shelf life and quality traits in broccoli, the parental genotype Mar34 provides the beneficial allele. This information can be utilised by breeders to improve shelf life and quality traits in broccoli through marker-assisted selection (MAS), by selecting for beneficial alleles and selecting against deleterious alleles linked to QTL for these traits. In some cases, QTL for desirable traits in this study co-localise, and contain beneficial alleles linked to QTL from one parent, this may suggest the presence of a single QTL that has pleiotrophic effects (Zhang *et al.*, 2007), which could have the advantage of allowing the selection of multiple traits by breeders. However, if both undesirable and desirable traits co-localise fine mapping may be required to determine the nature of the QTL clusters. A consideration before employing MAS to improve shelf life in broccoli is to determine if selecting for this trait will have deleterious effects on other

agronomically important traits, such as weight and size. However, in the case of QTL for the trait ‘days to yellowing’ correlations between phenotype data for other morphological traits is low, which would suggest that breeders could improve shelf life without compromising on other traits.

REFERENCES.

4Peaks by A. Griekspoor and Tom Groothuis, mekentosj.com.

Able, A.J., Wong, L.S., Prasad, A., O'Hare, T.J. (2002). 1-MCP is more effective on floral *brassica* (*Brassica oleracea* var. *Italica L.*) than a leafy *brassica* (*Brassica rapa* var. *Chinensis*). *Postharvest Biology and Technology* 26: 147-155

Agius, F., Gonzalez-Lamothe, R., Caballero, J. L., Munoz-Bianco, J., Botella, M. A. and Valpuesta, V. (2003). Engineering increased vitamin C levels in plants by overexpression of a D-galaxturonic acid reductase. *Nature Biotechnology* 21: 177-181

Aiamla-or, S., Kaewuksaeng, S., Shigyo, M., Yamauchi, N. (2010). Impact of UV-B irradiation on chlorophyll degradation and chlorophyll-degrading enzyme activities in stored broccoli (*Brassica oleracea* L. *Italica* Group) florets. *Food Chemistry* 120: 645-651

Albanese, D., Russo, L., Cinquanta, L., Brasiello, A. and Di Matteo, M. (2007). Physical and chemical changes in minimally processed green asparagus during cold-storage. *Food Chemistry* 101: 274-280.

Axelsson, T., Shavorskaya, O. and Langercrantz, U. (2001). Multiple flowering time QTLs within several *Brassica* species could be the result of duplicated copies of one ancesteral gene. *Genome* 44: 856-864.

Armstead, I., Donnison, I., Aubry, S., Harper, J., Hortensteiner, S., James, C., Mani, J., Moffet, M., Ougham, H., Roberts, L., Thomas, A., Weeden, N., Thomas, H., and King, I. (2007). Cross-species identification of Mendel's *I* locus. *Science* 315: 73

Armstead, I., Donnison, I., Aubry, S., Harper, J., Hörtensteiner, S., James, C., Mani, J., Moffet, M., Ougham, H., Roberts, L.,Thomas, A., Weeden, N., Thomas, H., King, I. (2006). From crop to model crop: identifying the genetic basis of the satygreen mutation in the Lolium/Festuca forage and amenity grasses. *New Phytologist* 172: 592-597.

Asins, M.J. (2002). Present and future of quantitative trait locus analysis in plant breeding. *Plant Breeding* 121: 281-291

Asoda, T., Terai, H., Kato, M., and Suzuki, Y. (2009). Effects of postharvest ethanol vapour treatments on ethylene responsivness in broccoli. *Postharvest Biology and Technology* 52: 216-220

Aubry, S., Mani J., and Hortensteiner, S. (2008). Stay-green protein, defective in Mendel's green cotyledon mutant, acts independent and upstream of pheophorbide *a* oxygenase in the chlorophyll catabolic pathway. *Plant Molecular Biology* 67: 243-256

Babula. D., Kaczmarck. M., Barakat. A., Delseny. M., Quiros, C. F. and Sadowski. J. (2003). Chromosomal mapping of *Brassica oleracea* based on ESTs from *Arabidopsis thaliana*: complexity of the comparative map. *Molecular Genetics and Genomics* 268: 656-665.

Barry, C.S. (2009). The stay-green revolution: Recent progress in deciphering the mechanisms of chlorophyll degradation in higher plants. *Plant Science* 176: 325-333.

Barry, C.S., McQuinn, R.P., Chung, M., Besuden, A., and Giovannoni, J. (2008). Amino acid subsitutions in homologs of the STAY-GREEN protein are responsible for the green-flesh and chlorophyll retainer mutants of tomato and pepper. *Plant Physiology* 147: 179-187.

Basic Horticulutral Statistics 2010. DEFRA

Bengtsson, G.B., Schoner, R., Lombardo, E., Schoner, J., Borge, G.I.A. and Bilger, W. (2006). Chlorophyll fluorescence fro non-destructive measurement of flavonoids in broccoli. *Postharvest Biology and Technology* 39: 291-298.

Bernard, W. R. And Matile, P. (1994). Differential expression of glutamine synthetase genes during the senescence of *Arabidopsis thaliana* rosette leaves. *Plant Science* 98: 7-14.

Bettey, M *et al* (2000). Quantitative genetic analysis of seed vigour and pre-emergence seedling growth traits in *Brassica oleracea*. *New Phytologist* 148: 277-286.

Blankenship, S.M., and Dole, J.M. (2003). 1-Methylcyclopropene: a review. *Postharvest Biology and Technology* 28: 1-25.

Bohuon. E. J. R., Keith. D. J., Parkin. L. A. P., Sharpe. A. G. and Lydiate. D. J. (1996). Alignment of the conserved C genomes of *Brassica oleracea* and *Brassica napus. Theoretical Applied Genetics* 93: 833-839.

Bohuon, E. J .R. *et al* (1998). The association of flowering time quantitative trait loci with duplicated regions and candidate loci in *Brassica oleracea*. *Genetics* 189: 393-401.

Bonin. A., Bellemain. E., Eidesen. B., Pompanon. F., Brochmann. C. and Taberlet. P. (2004). How to track and assess genotyping errors in population genetic studies- review. *Molecular Ecology* 13: 3261-3273.

Borevitz, J.O., and Chory, J. (2004). Genomic tools for QTL analysis and gene discovery. *Current Opinion in Plant Biology* 7: 132-136

Brash, D.W., Charles, C.M., Wright, S. and Bycroft, B.L. (1995). Shelf-life of stored asparagus is strongly related to postharvest respiratory activity. *Post Harvest Biology and Technology* 5: 77-81.

Brosnan, T. and Sun, D. (2001). Precooling techniques and applications for horticultural products. *Journal of Refrigeration* 24: 154-170.

Buchanan-Wollaston, V. (1997). The molecular biology of leaf senescence. *Journal of Experimental Botany* 48: 181-199.

Buchanan-Wollaston, V. and Ainsworth, C. (1997). Leaf senescence in *Brassica napus*: cloning of senescence realted genes by subtractive hydridisation. *Plant Molecular Biology* 33: 821-834.

Buchanan-Wollaston, V., Earl, S., Harrison, E., Mathas, E., Navabpour, S., Page, T. and Pink, D. (2003). The molecular analysis of leaf senescence- a genomics approach. *Plant Biotechnology Journal* 1: 3-22.

Buchert, A.M., Civello, P.M., and Martinez, G.A. (2010). Chlrophyllase versus pheophytinase as candidates for chlorophyll dephytilation during senescence of broccoli. *Journal of Plant Physiology*

Camargo. L. E. A., Savides. L., Jung. G., Nienhuis. J. and Osborn. T. C. (1997). Location of the Self-incompatability locus in an RFLP and RAPD map of *Brassica oleracea. Heredity 88 (1)*

Camargo, L. E. A., Williams, P. H. and Osborn, T. C. (1995). Mapping of quantitative trait loci controlling resistance of Brassica oleracea to *Xanthomonas campestris* pv. *campestris* in the field and glasshouse. *Phytopathology* 85: 1296-1300.

Camargo, L. E. A. And Osborn, T. C. (1996). Mapping loci controlling flowering time in *Brassica oleracea*. *Theoretical Applied Genetics* 92: 610-616.

Charron, C.S., Saxton, A.M. and Sams, C.E. (2005). Relationship of climate and genotype to seasonal variation in the glucosinolate-myrosinase system. I. Glucosinolate content in ten cultivars of Brassica oleracea grown in fall and spring seasons. *Journal of the Science of Food and Agriculture* 85: 671-681.

Chen, L.O., Hwang, J., Charng, Y., Sun, C., and Yang, S. (2001). Transformation of broccoli (*Brassica oleracea* var. *Italica*) with isopentenyltransferase gene via Agrobacteriumn tumefaciens for post-harvest yellowing retardation. *Molecular Breeding* 7: 243-257.

Chen, L.O., Lin, C., Kelkar, A.M., Chang, Y., and Shaw, J. (2008). Transgenic broccoli (*Brassica oleracea* var. *Italica)* with antisense chlorophyllase (*BoCLH1*) delays postharvest yellowing. *Plant Science* 174: 25-31

Chen, Y., Chen L.O., Saw, J. (2008). Senescence-associated genes in harvested broccoli florets. *Plant Science* 175:137-144

Collard, B.C.Y., Jahufer, M.Z.Z., Brouwer, J.B., and Pang, E.C.K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica* 142: 169-196

Conklin, P. L., Pallanca, J. E., Last, R. L., and Smirnoff, N. (1997). L-Ascorbic acid metabolism in the ascorbate-deficient Arabidopsis mutant vtc1. *Plant Physiology* 155: 1277-1285

Conklin, P.L., Gatzek, S., Wheeler, G.L., Dowdle, J., Raymond, M.J., Rolinki, S., Isupov, M, Littlechild, J.A., and Smirnoff, N. (2006). Arabidopsis thaliana VTC4 encodes L-Galactose-1-P phosphatase, a plant ascorbic acid plant biosynthetic enzyme. *The Journal of Biological Chemistry* 281: 15662-15670.

Conklin, P.L., Norris, S.R., Wheeler, G.L., Williams, E.H., Smirnoff, N., and Last, R.L. (1999). Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. *Proceeding for the National Academy of Science of America* 96: 4198-4203

Conklin, P.L., Saracco, S.A., Norris, S.R., and Last, R.L. (1999). Identification of ascorbic acid-deficient Arabidopsis thaliana mutants. *Genetics*: 154: 847-856

Costa, L., Vicente, A.R., Civello, P.M., Chaves, A.R., and Martinez, G.A. (2006). UV-C treatment delays postharvest senescence in broccoli florts. *Postharvest Biology and Technology* 39: 204-210

Costa, M. L., Civello, P. M., Chaves, A. R. amd Martinez, G. A. (2002). Characterization of Mg-dechelatase activity obtsined from Fragaria x ananassa fruit. *Plant Physiological and Biochemistry* 40: 111-118

Costa, M.L., Civello, P.M., Chaves, A.R., Martinez, G.A. (2005). Effect of ethephon and 6-benzylaminopurine on chlorophyll degrading enzymes and a peroxidase0linked chlorophyll bleaching during post-harvest senescence of broccoli (Brassica oleracea L.) at 20oC. *Postharvest Biology and Technology 35*: 191-199

Coupe, S.A., Sinclair, B.K., Watson, L.M., Heyes, J.A. and Easton, J.R. (2002). Analysis of acid invertase gene expression during the senescence of broccoli florets. *Journal of Experimental Botany* 28: 1045-1056

Cui, N., Du, T., Kang, S., Li, F., Zhang, J., Wang, M. and Li, Z. (2008). Regulated deficit irrigation improved fruit quality and water use effiency of pear-jujube trees. *Agricultural Water Management* 95: 489-497.

Curty, C. and Engel, N. (1996). Detection, isolation and structure elucidation of a chlorophyll a catabolite from autumnal senescent leaves of Cercidiphyllum Japonicum. *Phytochemistry* 42: 1531-1536

Davey, M. W., Van Montagu, M., Inze, D., Sanmartin, M., Kanellis, A., Smirnoff, N., Benzie, I. J. J., Strain, J. J., Favell, D., and Fletcher, J. (2000). Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. *Journal of the Science of Food and Agriculture* 80: 825-860.

Downs, C.G., Somerfield, S.D. and Davey, M.C. (1997). Cytokinin treatment delays senescence but not sucrose loss in harvested broccoli. *Postharvest Biology and Technology* 11: 93-100.

Drake, R., John, I., Farrel, A., Cooper, W., Schuch, W. and Grierson, D. (1996). Isolation and analysis of cDNAs encoding tomato cysteine proteases expressed during leaf senescence. Plant Molecular Biology. *Plant Molecular Biology* 30: 755-767.

Finch-Savage et al. (2005). Sensitivity of *Brassica oleracea* seed germination to hypoxia: A QTL analysis. *Plant Science* 164: 753-759.

Finch-Savage, W.E. et al (2010). Towards a genetic understanding of seed vigour in small- seeded crops using natural variation in *Brassica oleracea*. *Plant Science* 179: 582-589.

Folly, P. and Engel, N. (1999). Chlorophyll b to chlorophyll a conversion precedes chlorophyll degradation in Hordeum vulgare L. *The Journal of Biological Chemistry* 31: 21811-21816.

Fonseca, S., Oliveira, F.A.R., Brecht, J.K. (2002). Modelling respiration rate of fresh fruits and vegetables for modified atmosphere packages: a review. *Journal of Food Engineering* 52: 99-119

Friedman, J.H. (1989). Regularized discriminant analysis. *Journal of the American Statistical Association* 84 (405), 165-175

Gao. M., Li. G., Yang. B., Qiu. D., Farnham. M. and Quiros. C. (2007). High-density Brassica oleracea linkage map: identification of useful new linkages. *Theoretical Applied Genetics* 115: 277-287.

Gapper, N.E., Coupe, S.A., McKenzie, M.J., Scott, R.W., Christey, M.C., Lill, R.E., McManus, M.T. and Jameson, P.E. (2005). Senescence-associated down-regualtion of 1-aminocyclopropane-1-carboxylate (ACC) oxidase delays harvest-induced senescence in broccoli. *Functional Plant Biology*. 891-901.

Genemarker V5.0 Softgenetics

Ghosh, S., Mahoney, S.R., Penterman, J.N., Peirson, D., and Dumbroff, E.B. (2001). Ultrastructural and biochemical changes in chloroplasts during Brassica napus senescence. *Plant Physiology and Biochemistry* 39: 777-784

Gong, Y. and Mattheis, P. (2003). Effect of ethylene and 1-methylcyclopropene on chlorophyll catabolism of broccoli florets. Plant Growth Regulation 40: 33-38
Guiamet, J. J., Pichersky, E. and Nooden, L. D. (1999). Mass exodus from senescing soybean chloroplasts. *Plant Cell Physiology* 40: 986-992.

Guiboileau, A., Sormani, R., Meyer, C. and Masclaux-Daubresse, C. (2010). Senescence and death of plant organs: Nutrient recycling and developmental regulation. *Plant Biology and Pathology* 333: 382-391.

Gupta, P.K., Varshney, R.K., Sharma, P.C., and Ramesh, B. (1999). Molecular markers and their applications in wheat breeding. *Plant Breeding* 118: 369-390

Hackett, C.A., and Broadfoot, L.B. (2003). Effects of genotyping errors, missing values and segregation distortion in molecular marker data on the construction of linkage maps. *Heredity* 90: 33-38

Haldane, J.B.S. (1919). The combination of linkage values, and the calculation of distances between the loci of linked factors. *Journal of Genetics* 8: 299 – 309.

Hancock, R.D. and Viola, R. (2005). Biosynthesis and catabolism of L-ascorbic acid in plants. *Critical Reviews in Plant Sciences* 24: 167-188.

He, Y. and Gang, S. (2002). A gene encoding an Acyl hydrolayse is involved in leaf senescence in Arabidopsis. *Plant Cell* 14: 805-815.

He, Y., Fukushige, H., Hildebrand, D.F., and Gan, S. (2002). Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. *Plant Physiology* 128: 876-884.

Heimler, D., Vignolini, P., Dini, P.G., Vincieri, F.F. and Romani, A. (2006). Antiradical activity and polyphenol composition of local Brassicaceae edible varieties. *Food Chemistry* 99: 464-469.

Higgins, J.D., Newbury, H.J., Barbara, D.J., Muthumeenakshi, S., and Puddephat, I.J. (2006). The production of marker-free genetically engineered broccoli with sense and antisense SCC synthase 1 and ACC oxidases 1 and 2 to extend shelf-life. *Molecular Breeding* 17: 7-20

Hodeges, D.M. and Foreny, C.F. (2000). The effects of ethylene, depressed oxygen and elevated carbon dioxide on antioxidant profiles of senescing spinach leaves. *Journal of Experimental Botany* 51: 645-655.

Hong, Y., Wang, T.W., Hudak, K.A., Schade, F., Frose, C.D. and Thompson, J.E. (2000). An ethylene-induced cDNA encoding a lipase expressed at the onset of senescence. *Proceedings of the National Acadamey of Science, USA* 97: 8717-8722.

Hortensteiner, S. (2006). Chlorophyll degradation during senescence. *The Annual Review of Plant Biology* 57: 55-77.

Hortensteiner, S. (2009). Stay-green regulates chlorophyll and chlorophyll binding protein degradation during senescence. *TRENDS in Plant Science* 14: 155-162

Hu. J., Sadowski. J., Osborn. T. C., Landry. B. S. and Quiros. C. F. (1998). Linkage group alignment from four independent Brassica oleracea RFLP maps. *Genome* 41, 226-235.

Iniguez-Lay. F. L., Lukens. L., Farnham. M. W., Amasino, R. M. and Osborn. T. C. (2009). Development of public immortal mapping populations, molecular

markers and linkage maps for the rapid cycling Brassica rapa and B. oleracea. *Theoretical Applied Genetics* 123: 31-43.

Ito, H., Takaichi, S., Tsuji, H. and Tanaka, A. (1994). Properties of synthesis of chlorophyll a from chlorophyll b in cucumber etioplasts. *The Journal of Biological Chemistry* 35: 22034-22038.

Jacob-Wilik, D., Holland, D., Goldschmidt, E.E., Riov, J., and Eyal Y. (1999). Chlorophyll breakdown by chlorophyllase: isolation and functional expression of the Chlase 1 gene from ethylene-treated Citrus fruit and its regulation during development. *The Plant Journal* 26: 653-661.

Jacobsson, A., Nielsen, N., and Sjoholm., I. (2003). Effects of type of packaging material on shelf-life of fresh broccoli by means of changes in weight, colour and texture. *European Food Research and Technology* 218: 157-163

Jacobsson, A., Nielsen, T., and Sjoholm, I. (2004). Effects of type of packaging material on shelf-life of fresh broccoli by means of changes in weight, colour and texture. *European Food Research and Technology* 218: 157-163

Jansen, R.C. (1993). Interval mapping of mulitple quantitative trait loci. *Genetics* 135: 205-211

Jansen, R.C. (1994). Controlling the type I and type II errors in mapping quantitative trait loci. *Genetics* 138: 871-881

Jansen, R.C., and Stam, P. (1994). High resolution of quantitative traits into multiple loci via interval mapping. *Genetics* 136: 1447-1455

Jansen. J., de Jong. A. G. and van Ooijen. J. W. (2001). Constructing dense genetic linkage maps. *Theoretical Applied Genetics* 102: 1113-1122.

Jeffery, E.H., Brown, A.F., Kurilich, A.C., Keck, A.S., Matusheski, B.P., Klein, B.P. and Juvik, J.A. (2003). Variation in content of bioactive components in broccoli. *Journal of Food Consumption and Analysis* 16: 323-330.

Jiang, H., Li, M., Liang, N., Yan, H., Wei, Y., Xu, X., Liu, J., Xu, Z., Chen, F., and Wu, G. (2007). Molecular cloning and function analysis of the stay green gene in rice. *The Plant Journal* 52: 197-209

Johnson, I.T. (2002). Glucosinolates: Bioavailability and importance to health. *International Journal for Vitamin and Nutrition Research* 72: 26-31.

Jones, N., Ougham, H., and Thomas, H. (1997). Markers and mapping: we are all geneticists now. *New Phytologist* 137: 165-177

Jones, N., Ougham, H., Thomas, H., and Pasakinskiene, I. (2009). Markers and mapping revisited: finding your gene. *New Phytologist* 183: 935-966.

Jones, R.B., Faragher, J.D. and Winkler, S. (2006). A review of the influence of postharvest treatments on quality and glucosinolate content in broccoli (Brassica oleracea var. italica) heads. *Postharvest Biology and Technology* 41: 1-8.

Kader, A.A. (1992) Postharvest Biology and Technology: An Overview". In: Kader, A.A (ed.), Postharvest Technology of Horticultural Crops. University of California. Division of Agriculture and Natural Resources. Publication No.3311. pp 15 – 20.

Kasai, Y., Kato, M., and Hyodo, H. (1996). Ethylene biosynthesis and its involvement in senescence of broccoli florets. *Journal of Japananese Society of Horticultural Science* 65: 185-191

Kearsey, M.J. (1998). The principles of QTL analysis (a minimal mathematics approach). *Journal of Experimental Botany* 49: 1619-1623.

Kianian. S. F. and Quiros. C. F. (1992). Generation of a Brassica oleracea composite RFLP map: linkage arrangements among various populations and evolutionary implications. *Theoretical Applied Genetics* 84: 544-554.

King, G.A. and O'Donoghue, E.M. (1995). Unravelling senescence: New opportunities for delaying the inevitable in harvested fruit and vegetables. *Trends in Food Science and Technology* 6: 385-389.

Krul, C., Humblot, C., Philippe, C., Vermeulen, M., Nuenen, M., Havenaar, R. and Rabot, S. (2002). Metabolism of sinigrin (2-propenyl glucosinolate) by the human colonic microflora in a dynamic in vitro large-intestinal model. *Carcinogenesis* 23: 1009–1016.

Kumar, L.S. (1999). DNA markers in plant improvements: An overview. *Biotechnology Advances* 17: 143-182.

Kunieda, T., Amano, T. and Shioi, Y. (2005). Search for chlorophyll enzyme, Mg-dechelatase, from extracts of Chenopodium album with native and artificial substrates. *Plant Science* 169: 177-183.

Kurilich, A.C., Tsau, G.J., Brown, A., Howard, L., Klein, B.P., Jeffery, E.H., Kushad, M., Wallig, M.A. and Juvik, J.A. (1999). Carotene, tocopherol and ascorbate contents in subspecies of Brassica oleracea. *Journal of Agricultural Food and Chemistry* 47: 1576-1581.

Kusaba, M., Ito, H., Morita, R., Iida, S., Sato, Y., Fujimoto, M., Kawasaki, S., Tanaka, R., Hirochika, H., Nishimura, M., and Tanaka, A. (2007). Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. *The Plant Cell* 19: 1362-1375.

Lagercrantz U., Ellegren H., Andersson L., (1993). The abundance of various polymorphic microsatellite motifs diffres between plants and vertebrates. *Nucleic Acids Research* 21: 1111-1115

Lan. T. and Paterson, A.H. (2000). Comparative mapping of quantitative trait loci sculpting the curd of Brassica oleracea. *Genetics* 155, 1927-1954.

Lan, T. H. and Paterson, A. H. (2001). Comparative mapping of QTLs determining the plant size of *Brassica oleracea*. *Theoretical Applied Genetics* 103: 383-397.

Lander, E.S., and Botstein, D. (1989). Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* 121: 185-199

Lee, S. K. and Kader, A. A. (2000). Preharvest and postharvest factors influencing vitamin C content of horticutural crops. *Postharvest Biology and Technology* 20: 207-220.

Leja, M., Mareczek, A., Starzynska, A. and Rozek, S. (2001). Antioxidant ability of broccoli flower buds during short-term storage. *Food Chemistry* 72: 219-222.

Leskova, E., Kubikova, J., Kovacikova, E., Kosicka, M., Porubska, J. and Holcikova, K. (2006). Vitamin losses: Retention during heat treatment and continual chnages expressed by mathetical models. *Journal of Food Consumption and Analysis* 19: 252-276.

Li, G., Gao, M., Yang, B. and Quiros, C.F. (2003). Gene for gene alignment between the Brassica and Arabidopsis genomes by direct transcriptome mapping. *Theoretical Applied Genetics* 107: 168-180.

Lim, P.O., Woo, H.R. and Nam, H.G. (2003). Molecular genetics of leaf senescence in Arabidopsis. *TRENDS in Plant Science* 8: 272-278.

Linster, C. L., and Clarker, S.G. (2008). L-Ascorbate biosynthesis in higher plants: the role of VTC2. *TRENDS in Plant Science* 13: 567-573.

Linster, C.L., Gomez, T.A., Christensen, K.C., Adler, L.N., Young, B.D., Brenner, C., and Clarke, S.G. (2007). Arabidopsis VTC2 encodes a GDP-L-Galsctose phosphorylase, the last unkown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. *Journal of Biological Chemistry* 26: 18879-18885

Lorence, A., Chevone, B. I., Mendes, P. and Nessler, C. L. (2004). Myo-Inositol oxygenase offer a possible entry point into plant ascorbate biosynthesis. *Plant Physiology* 134: 1200-1205.

Lowe, A.J., Moule, C., Trick, M. and Edwards, K.J. (2004). Efficient large-scale development of microsatellites for marker and mapping application in Brassica crop species. *Theoretical and Applied Genetics* 108: 1103-1112.

Lowe. A. J., Jones. A. E., Raybould. A. F., Trick. M., Moule. C. L. and Edwards. K. J. (2002). Transferability abd genome specificity of a new set of microsatellire primers among Brassica species of the U triangle.

Lukens, L., Zou, F., Lydiate, D., Parkin, I. and Osborn, T. (2003). Comparison of Brassica oleracea genetic map with the genome of Arabidopsis thaliana. *Genetics* 164: 359-372.

Maloof, J.N. (2003). QTL for plant growth and morphology. *Current Opinion in Plant Biology* 6: 85-90

Masood, M. A., Farqooq, K., Mujahid, Y. and Anwar, Z. A. (2008). Improvement in precison of agricultural filed experiments through design and analysis.

Mathas, E. (2004). The identification and mapping of post harvest traits in broccoli (Brassica oleracea var. italica). PhD Thesis University of Birmingham.

Matile, P., Schellenberg, M. and Vicentini, F. (1997). Localization of chlorophyllase in the chloroplast envelope. *Planta* 201: 96-99

Merken, H.M. and Beecher, G.R. (2000). Measurement of food flavonoids by high-performance liquid chromotography: A review. *Journal of Agricultural Food and Chemistry.* 577-599.

Mithen, R. (2001). Glucosinolates-biochemistry, genetics and biological activity. *Plant Growth Regulation* 34: 91-103.

Mureika, J.R. (2005). Fractal dimension in perceptual colour space: A comparison study using Jackson Pollock's art. *Chaos* 15

Naidu, K.A. (2003). Vitamin C in human health and disease is still a mystery? *Nutrition Journal* 2: 1-10.

Nagaoka, M.A. et al (2010). Identification of QTLs that control clubroot resistance in *Brassica oleracea* and comparative analysis of clubroot resistance genes between *B. rapa* and *B. oleracea. Theoretical Applied Genetics* 120: 1335-1346.

Nakabayashi, K., Ito, M., Kiyosue, T., Shinozaki, K. and Watanabe, A. (1999). Identification of clp Genes Expressed in Senescing Arabidopsis Leaves. *Plant Cell Physiology* 40: 504-514.

Nishikawa, F., Iwama, T., Kato, M., Hyodo, H., Ikoma, Y., and Yano, M. (2005). Effect of sugars on ethylene synthesis and responsiveness in harvested broccoli florets. *Postharvest Biology and Technology* 36: 157-165

Nishikawa, F., Kato, M., Hydo, H., Ikoma, Y., Sugiura, M. and Yano, M. (2003b). Ascorbate metabolism in harvested broccoli. *Journal of Experimental Botany* 54: 2439-2448.

Nishikawa, F., Kato, M., Hydo, H., Ikoma, Y., Sugiura, M. and Yano, M. (2004). Effect of sucrose on ascorbate level and expression of genes involved in the ascorbate biosyntheis and recycling pathway in harvested broccoli florets. *Journal of Experimental Botany* 56: 65-72.

Nishikawa, F., Kato, M., Wang, R., Hydo, H., Ikoma, Y., Sugiura, M. and Yano, M. (2003a). Two ascorbate peroxidases from broccoli: identification, expression and characterization of their recombinant proteins. *Postharvest Biology and Technology* 27: 147-156.

Nixon, J. (2006). Testing for segregation distortion in genetic scoring data from backcross or doubled haploid populations. *Heredity* 96: 290-297.

Oberhuber, M., Berghold, J., Breuker, K., Hortensteiner, S., and Krautler, B. (2003). PNAS 100: 6910-6915

Ostergaard, L. and King, G.J. (2008). Standardzed gene nomenclature for the Brassica genus. *Plant Methods* 4: 10.

Palma, J.M., Sandalio, L.M., Corpas, F.J., Romero-Puertas, M.C., McCarthy, I. and del Rio, L.A. (2002). Plant proteases, protein degradation, and oxidative stress: role of peroxisomes. *Plant Physiology and Biochemistry* 40: 521-530.

Park, J.H., Oh, S.A., Kim, Y.H., Woo, H.R. and Nam, H.G. (1998). Differential expression of senescence-associated mRNAs during leaf snescence induced by different senescence-inducing factors in Arabidopsis. Plant Molecular Biology 37: 445-454.

Park, S., Yu, J., Park, J., Li, J., Yoo, S., Lee, N., Lee, S., Jeong, S., Seo, H.S., Koh, H., Jeon, J., Park, Y., and Paek, N. (2007). The senescence-induced staygreen protein regulates chlorophyll degradation. *The Plant Cell* 19: 1649-1664

Patterson, H.D. and Williams, E.R. (1976). The efficiecy of incomplete block designs. *Biometrika* 63: 83-92.

Pink, D., Bailey, L., McClement, S., Hand, P., Mathas, E., Buchanan-Wollaston, V., Astley, D., King, G., and Teakle, G. (2006). Doubled haploids, markers and QTL analysis in vegetable brassicas. *Euphytica*

Pompanon, F., Bonin. A., Bellemain. E. and Taberlet. P. (2005). Genotyping errors: causes, consequences and solutions. *Nature Reviews*

Proteggente, A.R., Pannala, A.S., Paganga, G., Buren, L.V., Wagner, E., Wiseman, S., De Put, F.V., Dacombe, C. and Rice-Evans, C.A. (2002). The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C compositon. *Free Radicals Research*. 217-233.

P**ruzinska, A., Tanner, G., Anders, I., Roca, M., and Hortensteiner, S. (2003).** Chlorophyll breakdown: Pheophorbide a oxygenase is a Rieske-type iron-sulfur protein, encoded by the accelerated cell death 1 gene. *PNAS* 100: 15259-15264

Qui, G., Morris, J. and Fan, X. (2007). Visual guided navigation for image retrieval. *Pattern Recognition* 40 (6), 1711-1721.

Quirino, B. F., Noh, Y., Himelblau, E. and Amasino, R. (2000). Molecular aspects of leaf senescence. *Trends in Plant Science* 5: 278-282.

Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009.

Ren, G., Liao, Y., Zhou, X., Cao, Y., Zhao, H., Ge, X., and Kuai, B. (2007). Identification of a novel chloroplast protein AtNYE1 regulating chlorophyll during leaf senescence in Arabidopsis. *Plant Physiology* 144: 1429-1441.

Rudiger, W. (2002). Biosynthesis of chlorophyll b and the chlorophyll cycle. *Photosynthesis Research* 74: 187-193.

Ryu, S.B. and Wang, X. (1995). Expression of Pholpholipase D during castor bean leaf senescence. *Plant Physiology* 108: 713-719.

Sandhya. (2010). Modified atmosphere packaging of fresh produce: Current status and future needs. LWT- *Food Science and Technology* 43: 381-392

Sarfraz, M., Dosdall, L. M. and Keddie, B. A. (2006). Diamondback moth-host plant interactions: Implications for pest management. *Crop Protection* 25: 625-639.

Sato, Y., Morita, R., Katsuma, S., Nishimura, M., Tanaka, A., and Kusaba, M. (2009). Two short-chain dehydrogenase/reductases, NON-YELLOWING COLOURING 1 abd NYC1-LIKE, are required for cholorophyll b and light-harvesting complex II degradation during senescence in rice. *The Plant Journal* 57: 120-131

Sato, Y., Morita, R., Nishimura, M., Yamaguchi, H., and Kusaba, M. (2007). Mendel's green cotyledon gene encodes a positive regulater of the chlorophyll-degrading pathway. *Proceedings of the National Academy of Sciences of the United States of America* 104: 14169-14174.

Schenk, N., Schelbert, S., Kanwischer, M., Goldschmidt, E.E., Dormann, P., and Hortensteiner, S. (2007). The chlorophyllase AtCHL1 and ATCHL2 are not essential for senescence-related chlorophyll breakdown in Arabidopsis thaliana. *FEBS Letters* 581: 5517-5525.

Scheumann, V., Schoch, S. and Rudiger, W. (1999). Chlorophyll b reduction during senescence of barley seedlings. *Planta* 209: 364-370.

Scheumann, V., Schocu, S. and Rudiger, W. (1998). Chlorophyll a formation in the chlorophyll b reductase reaction requires reduced ferredoxin. *The Journal of Biological Chemistry* 52: 35102-35108.

Schmidt, R., Acarkan, A., and Boivin, K. (2001). Comparative structural genomics in the Brassucacea family. *Plant Physiology and Biochemistry* 39: 253-262

Schouten, R.E., Zhang, X., Verschoor, J.A., Otma, E.C., Tijskens, L.M.M., and Kooten, O. (2009). Development of colour of broccoli heads as affected by controlled atmosphere storage and temperature. *Postharvest Biology and Technology* 51: 27-35.

Sebastian, R.L., Kearsey, M.J and King, G.J. (2002). Identification of quantitative trait loci controlling developmental characteristics of Brassica oleracea L. *Theoretical and Applied Genetics* 104: 601-609.

Sebastian. R. L., Howell. E. C., King. G. J., Marshall. D. F. and Kearsey. M. J. (2000). An integrated AFLP and RFLP Brassica oleracea linkage map from two morphologically distinct doubled-haploid mapping populations. *Theoretical Applied Genetics* 100, 75-81.

Sebastian, R. L., Kearsey, M. J. and King, G. J. (2002). Identification of quantitative trait loci controlling developmental characteristics of *Brassica oleracea* L. *Theoretical Applied Genetics* 104: 601-609.

Serrano, M., Martinez-Romero, D., Guillen, F., Castillo, S. and Valero, D. (2006). Maintenance of broccoli quality and functional properties during cold storage as affected by modified atmosphere packaging. *Post Harvest Biology and Technology* 39: 61-68.

Shaaban, S.A. and Deeb, S. S. (1998). Functional Analysis of the Promoters of the Human Red and Green Visual Pigment Genes. *Investigative Ophthalmology & Visual Science* 39 (6).

Shroff, R., Vergara, F., Muck, A., Svatos, A. and Gershenzon, J. (2008). Non-uniform distribution of glucosinolates in Arabidopsis thaliana leaves has important consequences for plant defense. *Proceedings of the National Academy of Sciences, USA 105*: 6196-6201.

Slocum. M. K., Figdore. S. S., Kennard. W. C., Suzuki. J. Y. and Osborn. T. C. (1990). Linkage arrangements of restriction fragment length polymorphism loci in Brassica oleracea. *Theoretical Applied Genetics* 80, 57-64.

Smart, C.M., Hosken, S.E., Thomas, H., Greaves, J.A., Blair, B.G. and Schuch, W. (1995). The timing of maize leaf senescence and characterisation of senescence-related cDNAs. *Physiologica Plantarum* 93: 673-682.

Smirnoff, N. (2000). Ascorbate biposynthesis and function in photoprotection. *Philosophical Transactions of the Royal Society B: Biological Sciences* 335;1455-1464

Soloman, S.G. and Lennie, P. (2007). The machinery of colour vision. *Nature Reviews Neuroscience* 8, 276-286.

Suwabe, K., Iketani, H., Nunome, T. and Kage, T. (2002). Isolation and characterisation of microsatellites in Brassica rapa L. *Theoretical Applied Genetics* 104: 1092-1098

Suza, W. P., Avila, C. A., Carruthers, K., Kulkarni, S., Goggin, F. L., and Lorence, A. (2010). Exploring the impact of wounding and jasmonates on ascorbate metabolism. *Plant Physiology and Biochemistry* 48: 337-350.

Suzuki, T., Kunieda, T., Murai, F., Morioka, S. and Shioi, Y. (2005). Mg-dechelation activity in radish cotyledons with artifcal and native substrates, Mg-chlorophyllin *a* and chlorophyllide *a*. *Plant Physiological and Biochemistry* 43: 459-464

Suzuki, Y., Asoda, T., Matsumoto, Y., Terai, H. and Kato, M. (2005). Suppression of the expression of genes encoding ethylene biosynthetic enzymes in harvested broccoli with high temperature treatment. *Postharvest Biology and Technology* 36: 265-271.

Szekacs, A., Hegedus, G., Tobias, I., Pogany, M., Barna, B., 2000. Immunoassays for plant cytokinins as tools for the assessment of environmental stress and for plant cytokinins as tools for the assessment of environmental stress and disease resistance. *Analytica Chimica Acta* 421: 135–146

Szewe-McFadden. A. K., Kresovich. S., Bliek. S. M., Mitchell. S. E. and McFerson. J. R. (1996). Identification of polymorphic, conserved simple sequence repeats (SSRs) in cultivated Brassica species. *Theoretical Applied Genetics* 93, 534-538.

Thomas, H., and Howarth, C.J. (2000). Five ways to stay green. *Journal of Experimental Botany* 51: 329-337.

Thomas, N., and Smart, C.M. (1993). Crops that stay green. *Annual of Applied Biology* 123: 193-219

Thompson, J. E., Froese, C. D., Madey, E., Smith, M. D. And Hong, Y. (1998). Lipid metabolism during plant senescence. *Progress in Lipid Research* 37: 119-141.

Tian, M.S., Davies, L., Downs, C.G., Liu, X.F., and Lill., R.E. (1995). Effects of floret maturity, cytokinin and ethylene on broccoli yellowing after harvest. *Postharvest Biology and Technology* 6: 29-40

Toivonen, P.M.A., and Brummell, D.A. (2008). Biochemical bases of appearance and texture changes in fresh-cut fruit and vegetables. *Postharvest Biology and Technology* 48: 1-14.

Tsuchiya, T., Ohta, H., Masuda, T., Mikami, B., Kita, N., Shioli, Y., and Takamiya, K. (1997). Purification and chatacterisation of two isozymes of chlorophyllase from mature leaves of Chenopodium album. *Plant Cell Physiology* 38: 1026-1031.

Tsuchiya, T., Ohta, H., Okawa, K., Iwamatsu, A., Shimade, H. and Msauda, T. (1999). Cloning of chlorophyllase, the key enzyme in chlorophyll degradation: finding of a lipase motif and the induction by methyl jasmonate. *Proceedings of the National Academy of Sciences* 96: 15362-15367

U N: Genome analysis in Brassica with special reference to the experimental formation of B. napus and peculiar mode of fertilization. *Japanese Journal of Botany* 1935, 7:389-452.

Uchino, T., Nei, D., Hu. W., and Sorour, H. (2004). Development of a mathematical model for dependence of respiration rate of fresh produce on temperature and time. *Postharvest Biology and Technology 34*: 285-293

Uptmoor, R. et al. (****). Crop model based QTL environments and QTL based estimation of time to floral induction and flowering in *Brassica oleracea*. *Molecular Breeding* 21: 205-216.

Vallejo, F., Tomas-Barberan, F. and Garcia-Viguera, C. (2003). Characterisation of flavonols in broccoli (Brassica oleracea L. Var. Italica) by liquid chromatography-UV diode array detection-electrospray ionisation mass spectrometry. *Journal of Agricultural and Food Chemistry*. 3029-3034.

Valpuestra, V. and Botella, M.A. (2004). Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. *TRENDS in Plant Science*. 573-577.

Van Ooijen, J. W., Boer, M. P, Jansen, R. C. and Maliepaard, C. (2002). MapQTL® 4.0, Software for the calculation of QTL positions on genetic maps. Plant Research International, Wageningen, the Netherlands.

Van Ooijen, J.W., 2006. JoinMap ® 4, Software for the calculation of genetic linkage maps in experimental populations. Kazyma B.V., Wageningen, Netherlands.

Van Ooijen, V.W., Boer, M.P., Jansen, R.C. and Maliepaard, C., 2002. MapQTL ® 4.0, Software for the calculation of QTL positions on genetic maps. Plant Research International, Wageningen, Netherlands.

Van Ooijen. J. W. (1999). LOD significance thresholds for QTL analysis in experimental populations of diploid species. *Heredity* 83, 613-624.

Vanderslice, J.T., Higgs, D.J., Hayes, J.M., and Block, G. (1990). Ascorbic acid and dehydroascorbic acid content of foods-as-eaten. *Journal of Food Composition and Analysis* 3: 105-118.

Vicentini, F., Hortensteiner, S., Schellenberg, M., Thomas, H., and Matile, P. Chlorophyll breakdown in senescent leaves: identification of the biochemical lesion in a stay-green genotypes of Fesuca pratensis Huds. *New Phytologist* 129: 247-252.

Voorrips, R.E., Jongerius, M. C. and Kanne, H. J. (2003). Quantitative trait loci for Clubroot resistance in *Brassica oleracea.*

Vos, P., Hogers, R., Bleeker, M., Reijens, M., Van de lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kulper, M., and Zabeau. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4467-4414

Wallsgrove, R.M., Bennett, R.N., 1995. The biosynthesis of glucosinolates in Brassicas. In: Wallsgrove, R.M. (Ed.), Amino Acids and their Derivatives in Higher Plants. Cambridge University Press.

Wheeler, G.L., Jones, M.A., and Smirnoff, N. (2008). The biosynthetic pathway of vitamin C in higher plants. *Nature* 393: 365-369

Wilkinson, J.Q., Lanahan, M.B., Clark, D.G., Bleecker, A.B., Chang, C., Meyerowitz, E.M., and Klee, H.J. (1997). A dominant mutant receptor from Arabidopsis confers ethylene insensitivity in heterologous plants. *Nature Biotechnology* 15: 444-448

Williams, E.R. and Talbot, M. (1993) ALPHA+. Experimental designs for variety trials. Design user manual. CSIRO, Australia, and SASS, Edinburgh.

Winkler, S., Faragher, J., Franz, P., Imsic, M. and Jones, R. (2007). Glucoraphanin and flavonoid levels remain stable during simulated transport and marketing of broccoli (Brassica oleracea var. italica) heads. *Postharvest Biology and Technology* 43: 369-375.

Wolucka, B. A. and Van Montagu, M. (2003). GDP-mannose 3', 5'-epimerase fores GDP-L-gulose, a putative intermediate for the de Novo biosynthesis of vitamin C in plants. *The Journal of Biological Chemistry* 48: 47483-47490.

Wolucka, B. A. and Van Montagu, M. (2007). The VTC2 cycle and the de novo biosyntheisi pathway for vitamin C in plants: An opinion. *Phytochemistry* 68: 2602-2613.

Woo, H.R., Chung, K.M., Park, J.H., Oh, S.A., Ahn, T., Hong, S.H., Jan, S.K. and Nam, H.G. (2001). ORE9, an F-Box Protein That Regulates Leaf Senescence in Arabidopsis. *Plant Cell* 13: 1779-1790.

Wurr, D.C.E., Hambidge, A.J., Fellows, J.R., Lynn, J.R. and Pink, D.A.C. (2002). The influence of water sress during crop growth on the postharvest quality of broccoli. *Postharvest Biology and Technology*. 193-198.

Wuthrich, K.L., Bovet, L., Hunziker, P.E., Donnison, I.S., and Hortensteiner, S. (2000). Molecular cloning, functional expression and characterisation of RCC reductase involved in cholorphyll catabolism. *The Plant Journal* 21: 189-198.

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Yamauchi, N., Harada, H. and Watada, A.E. (1997). In vitro chlorophyll degradation in stored broccoli (*Brassica oleracea* L. Var. *Italica Plen*.) florets. *Postharvest Biology and Technology* 12. 239-245.

Yonguc, M., and Griffiths, P.D. Genetic relationships of Brassica vegetables determined using database derived simple sequence repeats. *Euphytica* 137: 193-201

Yoshida, S. (2003). Molecular regulation of leaf senescence. *Current Opinion in Plant Biology* 6: 79-84.

Yoshida, S., Ito, M., Nishida, I. and Watanbe, A. (2001). Isolation and RNA Gel Blot Analysis of Genes that Could Serve as Potential Molecular Markers for Leaf Senescence in Arabidopsis thaliana. *Plant Cell Physiology* 42: 170-178.

Yoshida, T., Konishi, M., Horinaka, M., Yasuda, T., Goda, A.E., Taniguci, H., Yano, K., Wakada, M and Sakai, T. (2008). Kaempferol sensitizes colon cancer cells to TRAIL-induced apoptosis . *Biochemical and Biophysical Research Commuinications* 375: 129-133.

Yu, Y., and Yang, S.F. (1980). Biosynthesis of wound ethylene. *Plant Physiology* 66:281-285

Zaicovski, C.B., Zimmerman, T., Nora, L., Nora, F.R., Silva, J.A., Rombaldi, C.V. (2008). Water stress increases cytokini biosynthesis and delays postharvest yellowing of broccoli florets. *Postharvest Biology and Technology* 49: 436-439

Zhang, F.Z., Wagstaff, C., Rae, A., Sihota, A.K., Keevil, W., Rothwell, S.D., Clarkson, G.J.J., Michelmore, R.W., Truc, M.J., Dixon, M.S., and Taylor, G. (2007). QTLs for shelf life in lettuce co-locate with those for leaf biophysical properties but not with those for leaf developmental traits. *Journal of Experimental Botany* 58: 1433-1449

Zhang, Z., Nakano, K., Maezawa, S. (2009). Comparison of the antioxidant enzymes of broccoli after cold or heat shock treatment at different storage temperatures. *Postharvest Biology and Technology* 54; 101-105

Zhu, L.H., van de Peppel, A., Li, X.Y., Welander, M., (2004). Changes of leaf water potential and endogenous cytokinins in young apple trees treated with water potential and endogenous cytokinins in young apple trees treated with or without paclobutrazol under drought conditions. *Scientia Horticulturae* 99: 133–141.

Zhuang, H., Hildebrand, D.F. and Barth, M.M. (1997). Temperature influenced lipid peroxidation and deterioration in broccoli buds during postharvest storage. *Postharvest Biology and Technology* 10: 49-58.

APPENDIX A: Field trial designs and genotypes assessed (2007 and 2008).

	Rep 1					Rep 2				
	A	B	C	D		E	F	G	H	
1	57	44	61	45	1	61	22	68	71	
2	53	54	48	68	2	16	3	58	30	
3	25	36	15	24	3	64	38	53	56	
4	17	8	40	26	4	29	73	34	8	
5	74	19	28	41	5	70	62	49	25	
6	10	34	11	51	6	11	7	33	23	
7	30	29	32	16	7	43	4	20	74	
8	46	72	50	12	8	65	48	5	21	
9	33	6	37	18	9	57	24	54	45	
10	60	21	73	38	10	9	10	12	2	
11	13	52	71	66	11	31	59	52	13	
12	2	7	31	67	12	19	47	46	36	
13	70	43	62	65	13	41	39	66	63	
14	55	58	74	69	14	1	51	40	37	
15	47	27	5	8	15	32	17	74	6	
16	1	9	23	74	16	35	74	26	27	
17	42	56	49	64	17	44	72	55	18	
18	22	59	35	63	18	67	69	50	42	
19	20	14	39	4	19	60	15	14	28	2m

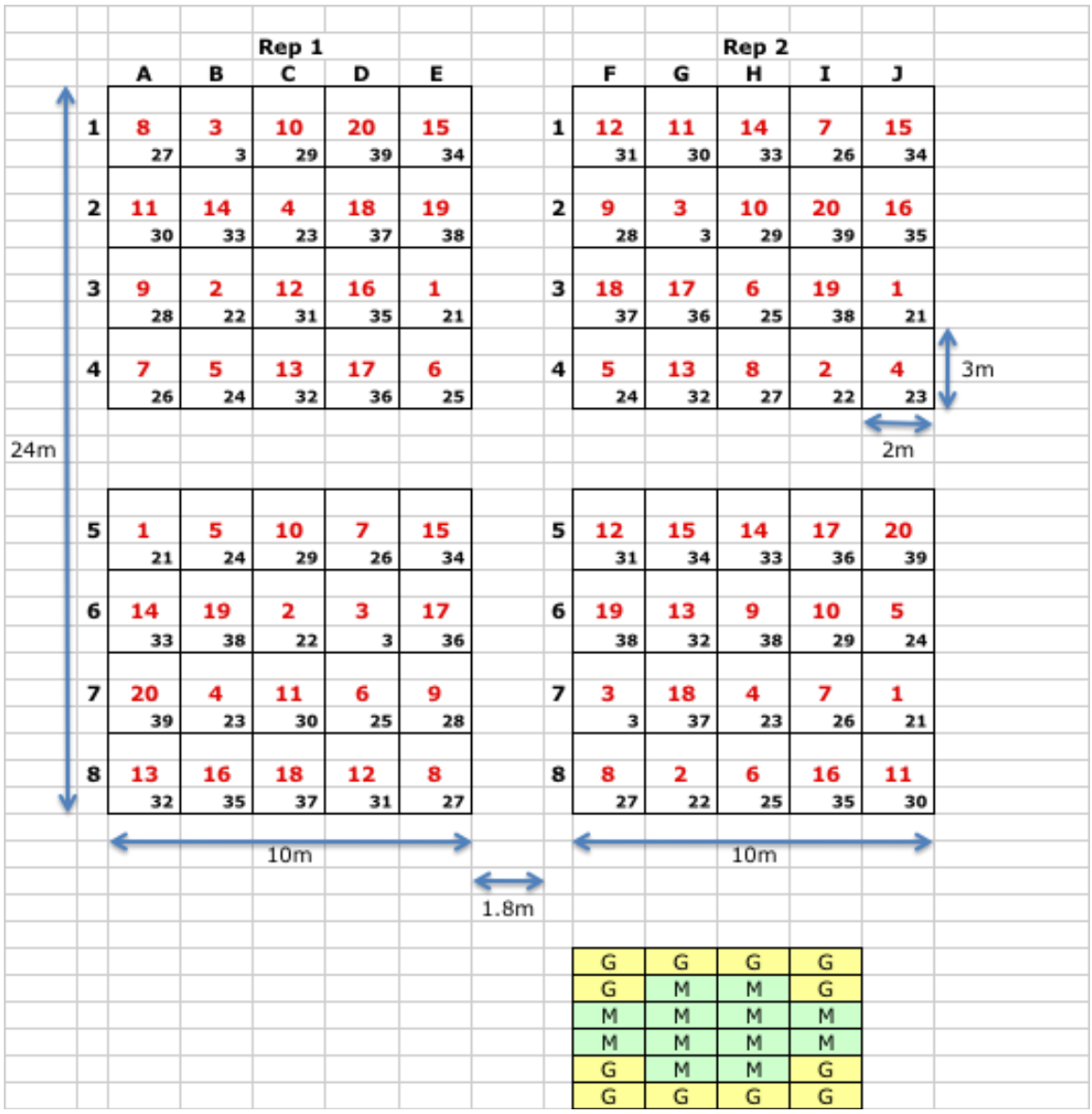
3m

G	G	G	G	G	G
G	S	S	S	S	G
G	S	S	S	S	G
G	G	G	G	G	G

The 2007 field trial layout consisting of 76 plots in two replicate blocks. Each plot was 3m x 2m in size and contained 24 plant in a 4 row x 6 plant column arrangement. The central six heads (T) were harvested for shelf life evaluation and morphological trait assessment and the remaining broccoli plants served as guard plants (G).

List of genotypes assessed for shelf life and morphological traits in the 2007 field trial.

Treatment No	Genotype	Treatment No	Genotype
1	MG 9 27/9	38	MG 1824
2	MG 10	39	MG 1825
3	MG 14 27/9	40	MG 1826
4	MG 18	41	MG 1829
5	MG 41 27/9	42	MG 1831
6	MG 44	43	MG UNID 2
7	MG 63	44	MG 406
8	MG 71	45	MG 407
9	MG 72	46	MG 410
10	MG 87	47	MG 415
11	MG 89	48	MG 416
12	MG 87 27/9	49	MG 418
13	MG 95	50	MG 419
14	MG 95 27/9	51	MG 422
15	MG 98 27/9	52	MG 430
16	MG 99	53	MG 433
17	MG 100	54	MG 435
18	MG 100 27/9	55	MG 438
19	MG 107A	56	MG 440
20	MG 116	57	MG 450
21	MG 120 27/9	58	MG 453
22	MG 124 27/9	59	MG 454
23	MG 126 27/9	60	MG 462
24	MG 136 27/9	61	MG 475
25	MG 137 27/9	62	MG 521
26	MG 183B	63	MG 532
27	MG 187B	64	MG 539
28	MG 301	65	MG 543
29	MG 308	66	MG 562
30	MG 334	67	MG 469
31	MG 353	68	MG 526
32	MG 1810A	69	MG 528
33	MG 1810B	70	MG 560
34	MG 1812	71	MG 564
35	MG 1813	72	GD 33
36	MG 1814	73	Mar34
37	MG 1822	74	Marathon



The 2008 field trial layout consisting of 80 plots in two replicate blocks with treatment numbers for metabolite samples highlighted in red and guard plants highlighted in black. Each plot was 3m x 2m in size and contained 24 plant in a 6 row x 4 column plant arrangement. The central seven heads (M) were harvested as metabolite samples and eight of the outer guard plants (G) were harvested to assess the shelf life and morphological traits of the new genotypes.

List of genotypes collected as metabolite samples (green) and new genotypes assessed for shelf life and morphological traits (yellow) in the 2008 field trial.

Treatment No	Genotype	Treatment No	Genotype
1	Mar-34	21	MG13
2	GD33	22	MG16
3	Marathon (M)	23	MG19
4	MG10	24	MG44 27/9
5	MG87	25	MG53
6	MG8727/9	26	MG88
7	MG10027/9	27	MG97 27/9
8	MG308	28	MG142 27/9
9	MG353	29	MG148 27/9
10	MG406	30	MG279
11	MG475	31	MG285
12	MG532	32	MG289
13	MG95	33	MG333
14	MG99	34	MG342
15	MG120_27/9	35	MG346
16	MG1812	36	MG350
17	MG1829	37	MG441
18	MG_UNID_2	38	MG447
19	MG_433	39	MGB23
20	MG_526	40	MG417

Metabolite samples

Guards

APPENDIX B: List of SSRs used to genotype the parents (Mar34 and GD33) of the MGDH mapping population to determine if polymorphism was present.

SSR	Species	Forward Primer	Reverse Primer	LG	Ref
A77096	*Bn*	TAGGACACGTGACAAAACTTCAT	TATCGATGGTATCAAAGAATGGA	N08	1
BRAS069	*Bn*	CTCGATCTTCCCCTGCTTTC	GTTGAGCCAATCTACGGTTCC	O3	1
BRAS038	*Bn*	TGAGCTGTTACCACTTTCT	AAAACACTTATTTCTTTCCTG	O3	1
BRAS019	*Bn*	CTCAAGACAAACGACCAGTAA	GAGAAGAAATCGCCAAGA	O7	1
BRAS026	*Bn*	ATTACAAAAATGCCCTGAC	TAAGTGATCTTCTCTCCAACA	N01	1
BRAS021	*Bn*	ACCGTTGAGATCAATCCCTAT	CATCTTCCTTAATCGAAACCC	N04	1
BRAS063	*Bn*	-	-	N05	1
BN83B1*	*Bn*	GCCTTTCTTCACACCTGATAGCTA	TCAGGTGCCTCGTTGAGTTC	O9	2
BRMS-027	*Br*	GCAGGCGTTGCCTTTATGTA	TCGTTGGTCGGTCACTCCTT	N06	3
BRMS-042	*Br*	GGATCAGTTATCTGCACCACAA	TCGGAATTGGATAAGAATTCAA	R03	3
BRMS-244	*Br*	GTAGAGTACTTTGCGAGGCAAGGAT	AGGATTCTTTACTCTCTGCAGCTTT	UN	4
BRMS-201	*Br*	GTAAATAACAGTTCTGCCTCTGCTC	CTGCTGAATTAATTGCTGCTTCT	UN	4
BRMS-252	*Br*	ACTGGACTTATGTCTGAACAAGGAC	CTGGCCAACATCAACATATAAACTA	UN	4
BRMS-309	*Br*	CAAGAGCAAGTTTGAAACAAACGAT	CATCAGTTCTTGATATGCTAGGTGA	R06	4
BRMS-198	*Br*	CGAGAGCAGTTAGGAAGCTTATAGA	AGAGATACTCTGTCCTCCACCTCTT	UN	4
BRMS-071	*Br*	CAAAGCGAGAAAGTGCAGTTGAGAG	TCCACGAAACTACTGCAGATTGAAA	R03	4
BRMS-098	*Br*	TGCTTGAGACGCTGCCACTTTGTTC	CATTCCTCCCCACCACCTTCACATC	UN	4
BRMS-296		CATCCTAATGTTGCTGAGAAAGAGG	TATATGAAACCGATGAAGCTCCTTT	R07	4
CB10003	*Bn*	ACGGTGCCGAATCTCAACG	AAATGGGTCACAGCCGAGAA	N13	1
CB10416	*Bn*	GCTGTTGCTGTAGGTTTGA	GAGCCAGCGTTGATAAGA	N02	1
CB10247	*Bn*	TCCCAACAAAAGAGTCCA	CAGCGAACCGAGTCTAAA	UN	1

1: Piquemal *et al.* (2005). 2:Szewec-McFadden *et al*. (1996). 3: Suwabe *et al.* (2002). 4: Matsumoto *et al*. 5: Osborn and Inguiez. 6: Langercrantz *et al.* (1993). 7: Lowe *et al.* (2003). 8: Lim *et al.*

SSR	Species	Forward Primer	Reverse Primer	LG	Ref
FIT0300	*Bn*	GCTTTCCTACATCCTCAAGTC	CTTCTTCTTCCTCTCATTCGT	UN	5
FIT0203	*Bn*	AAGTCGTTAGGCGAATCTG	ATTGAAGAGGAAGAAGGAGAA	O6	5
FIT0043	*Bo and Br*	AAAGTCGTGGGAAGTATCGT	AGGTGTAAGGATGGTGGTAGT	UN	5
FIT0282	*Bo and Br*	CAGAGGTGGAGTGAGAAAGA	GATGAAGAATGGAACCCTAAA	O2/O5	5
FIT0100	*Bo and Br*	GATGAGAGAAGGAAACCCTAA	ACAGCAGGAGAAGAGAGAGAA	UN	5
FIT0222	*Bo and Br*	GCACACTCCACTACACGAA	ACCATTCAACCACTCAAATC	UN	5
FIT0314	*Bo and Br*	-	-	UN	5
FIT0345	*Bo and Br*	-	-	UN	5
FIT0285	*Bo and Br*	TGAAGATGGGACTCAAACA	GATGAAGCAGAGAATGACAAG	UN	5
FIT0095	*Bo*	AGATTTCATCCACAGCCTC	TTTGATTCTTGCGTTCTCTC	UN	5
FIT0284	*Bo and Br*	AGCAATAAGCCAGAAACTTG	GTTCATCATCACAACTCTAACCT	UN	5
MB4*	UN	TGTTTTGATGTTTCCTACTG	GAACCTGTGGCTTTTATTAC	O6	6
Na12H09*	*Bn*	AGGCGTCTATCTCGAAATGC	CGTTTTTCAGAATCTCGTTGC	O2/O3	7
Na10D09	*Bn*	AAGAACGTCAAGATCCTCTGC	ACCACCACGGTAGTAGAGCG	N09	7
Na12C08*	*Bn*	GCAAACGATTTGTTTACCCG	CGTGTAGGGTGATCTAGATGGG	O1	7
Na12F03	*Bn*	GGCGACATAGATTTGAACCG	TCCACTTTCTCTCTCTTCCCC	O7	7
Na12C03	*Bn*	ATCGTTGCCATTAGGAGTGG	ACCAAATTAACCCTCTTTGC	N12	7
Na12G12*	*Bn*	GAGTGACATCGAAAATCAGATAGC	CCTAAATGGAAAGGCTTGGC	N15	7
Ni2C12	*Bni*	ACATTCTTGGATCTTGATTCG	AAAGGTCAAGTCCTTCCTTCG	N12	7
Ni4F09	*Bni*	CTGTTATGCAAGGTCATCGC	TGTTCCAGGTGAAGAAACCG	N18	7
Ni2F02	*Bni*	TGCAACGAAAAAGGATCAGC	TGCTAATTGAGCAATAGTGATTCC	N15	7

1: Piquemal *et al.* (2005). 2:Szewec-McFadden *et al*. (1996). 3: Suwabe *et al.* (2002). 4: Matsumoto *et al*. 5: Osborn and Inguiez. 6: Langercrantz *et al.* (1993). 7: Lowe *et al.* (2003). 8: Lim *et al.*

SSR	Species	Forward Primer	Reverse Primer	LG	Ref
O|12_G04*	*Bo*	CGAACATCTTAGGCCGAATC	GGTTAACCTGCGGGATATTG	N18	7
OL10H04	*Bo*	TCACCCCTCTATATCCACCC	CAGAATCTGCCTGAACATCG	N17	7
OL10D08*	*Bo*	TCCGAACACTCTAAGTTAGCTCC	GAGCTGTATGTCTCCCGTGC	O9	7
OL|1B05	*Bo*	TCGCGACGTTGTTTTGTTC	ACCATCTTCCTCGACCCTG	N13	7
O|12A04	*Bo*	TGGGTAAGTAACTGTGGTGGC	AGAGTTCGCATACTCTGGAGC	N19	7
OL|1H02	*Bo*	TCTTCAGGGTTTCCAACGAC	AGGCTCCTTCATTTGATCCC	N14	7
O|10B01	*Bo*	CCTCTTCAGTCGAGGTCTGG	AATTTGGAAACAGAGTCGCC	N17	7
OL|1H06	*Bo*	TCCGAACACTCTAAGTTAGCTCC	TTCTTCACTTCACAGGCACG	N19	7
Ra2E12	*Br*	TGTCAGTGTGTCCACTTCGC	AAGAGAAACCCAATAAAGTAGAACC	O3	7
RA2G09	*Br*	ACAGCAAGGATGTGTTGACG	GATGAGCCTCTGGTTCAAGC	N01	7
sN12352_b	*Bn*	TCATTGAATTGATTTCCTCACG	CAACTGTCAATGGCGAGAGA	N18	-
sN12264	*Bn*	ATCTTTTAACCGCCTCCACC	GATCACGATGTGTCGATTGC	UN	-
sNRD71	*Bn*	AAGACATGCACAGCAACAGC	CCTCATCCACAACGTCATCTT	N04	-
sN0464_b	*Bn*	CCAAAGCAGGACAATCTCATC	CCGGCTCTTGTTTTATGGTT	N14	-
sN3603	UN	AACCAGTCATCACCGAAAGG	AGAAGTCTGGAGCAATTTCCA	N6	-
SSR27	UN	GCATGCAAGCTTGGAACTGAT	CAGTCACGCTTTCTGACGAAAA	UN	8
SN4513	UN	-	-	UN	
SSR03	UN	TTTGACATCGTGCAATGCTA	TTGGGCTGGTCCTGAAGATA	O3	8
SN2189	UN	-	-	N18	-
SN11641	UN	-	-	N11	-
SN3734	UN	-	-	O1	-
SSR28	UN	-	-	UN	8

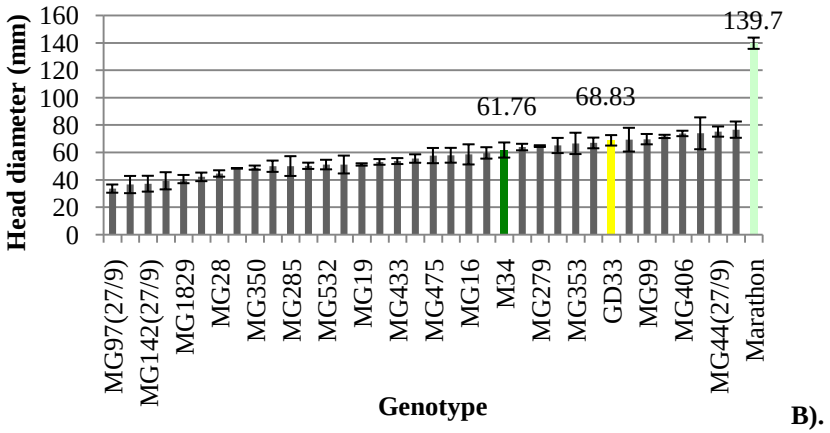
1: Piquemal *et al.* (2005). 2:Szewec-McFadden *et al*. (1996). 3: Suwabe *et al.* (2002). 4: Matsumoto *et al*. 5: Osborn and Inguiez. 6: Langercrantz *et al.* (1993). 7: Lowe *et al.* (2003). 8: Lim *et al.*

Appendix C: **Genome wide significant QTLs detected using interval mapping for traits assessed in the 2007 field trial.**

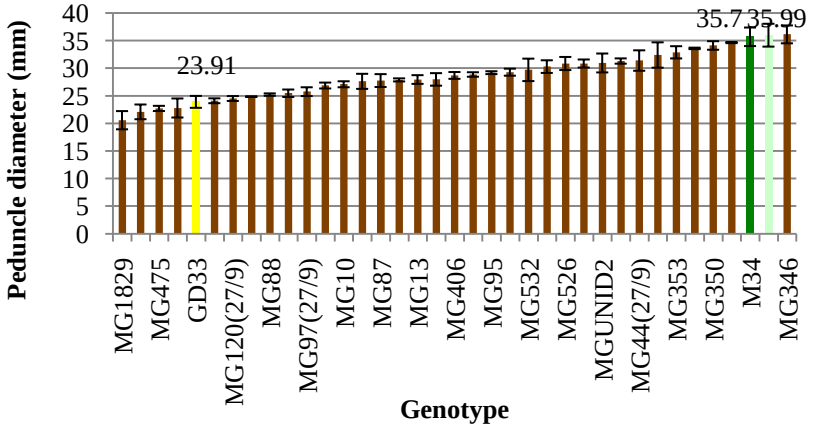
Trait[a]	LG[b]	Position[c] (cM)	Marker[d]	LOD[e]	Beneficial Allele[f]	Variance[g] (%)
Wt	O2	40	Ni2C12	3.3*	GD33	31.8
AWtL	O2	37.8	Ni2C12	3.2*	GD33	34.2
	O9	36.7	OL12A04	3.3*	Mar-34	31.8
RWtL	O9	41.7	OL12A04	2.8*	Mar-34	26
ST(d4)	O9	31.6	BN83B1	3.1*	GD33	30.2
ST(d5)	O3	18.6	E11M50_201	***	GD33	100
	O6	11.6	FITO203	3.2*	Mar-34	30.8
ST(d6)	O3	18.6	E11M50_201	***	GD33	100
	O3	23.6	E11M50_201	***	GD33	100
	O3	57.3	E11M50_185	***	GD33	100
BC(d5)	O3	37.9	BRAS069	3.4*	Mar-34	32.3
BE(d6)	O1	112.7	E11M62_290	3.5**	GD33	87.2
P	O1	112.7	E11M62_290	***	GD33	100
	O1	117.7	E11M62_290	***	GD33	100
L(d5)	O6	39.9	E11M62_144	3.3*	Mar-34	32.2
L(d6)	O4	69	Na10D09	2.8*	GD33	28.5
a(d4)	O4	69	Na10D09	3.7**	GD33	35.2
a(d5)	O4	69	Na10D09	2.8*	GD33	28.3
a(d6)	O4	69	Na10D09	2.8*	GD33	28.4
b(d4)	O4	63.1	E11M48_264	3.0*	GD33	31
b(d5)	O4	63.1	E11M48_264	2.9*	GD33	27
b(d6)	O4	10	E11M61_258	2.8*	GD33	36.5
	O4	63.1	E11M48_264	3.6**	GD33	35.6

[a] Trait abbreviations: *Wt* (g); *AWtL*, absolute weight loss (g^{-1}); *RWtL*, relative weight loss (%); *DY*, days to yellowing; *ST*, stem turgor; *BC*, bud compactness; *BE*, bud elongation; *FL*, floret looseness; *P*, Purple; *a*, red-green colour scale; *b*, yellow-blue colour scale; *HA*, hue angle; *C*, chroma [b] LG: Linkage group containing QTL.[c] Position (cM) of the QTL peak in the linkage group [d] Marker: Closest marker to the QTL peak .[e] LOD: log of the odds score for genome wide significant QTL.[f] The parental allele that causes an increase in the trait value. [g] The percentage variation explain by the QTL. [h] The QTL label given to QTL mapped in the broccoli x broccoli linkage map.

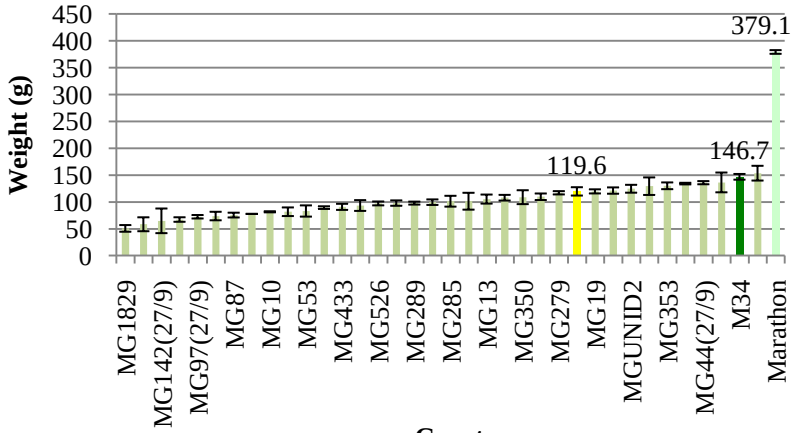
APPENDIX D: Additional data for Chapter 5, Part II.



A).



B).



C).

Figure A1: Predicted means (from REML) for a sub-set of the MGDH population for the traits A). head diameter (mm), B). peduncle diameter and C). Weight (g)

Error bars represent the s.e.m (standard error of the means).

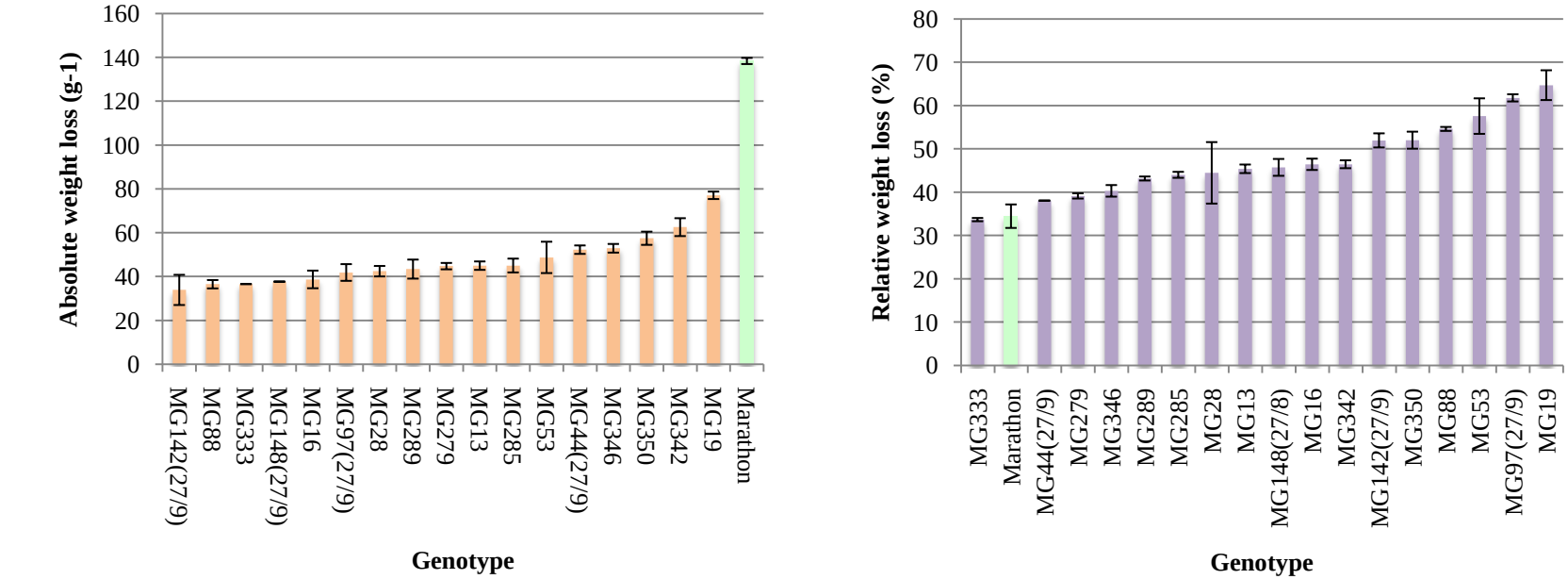


Figure A2: Predicted means (from REML) for a sub-set of the MGDH population for the traits A). Absolute weight loss and B). relative weight loss. Error bars represent the s.e.m (standard error of the means).

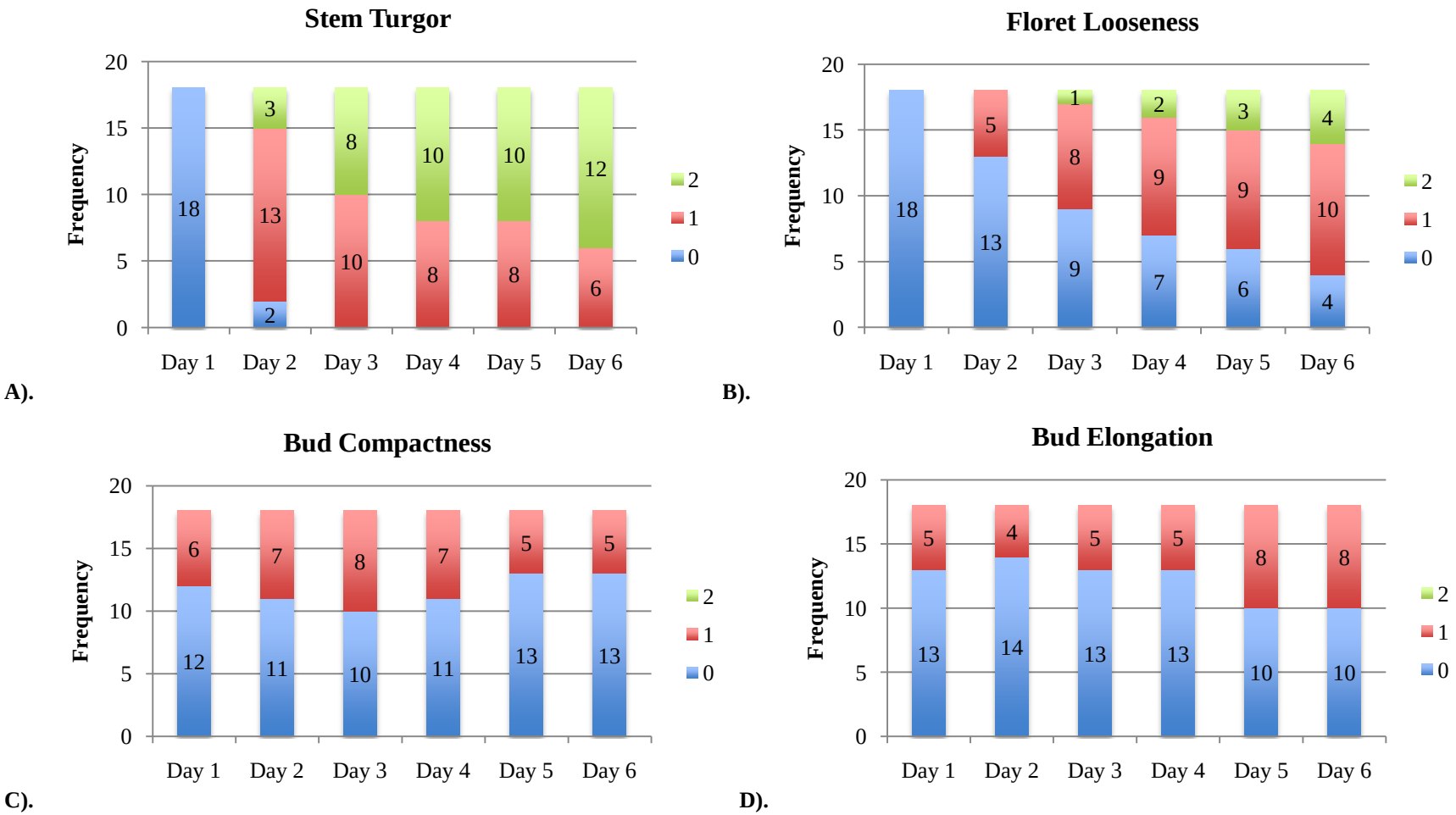


Figure A3: Frequency bar charts of quality scores for a sub-set of genotypes from the MGDH population assessed for the traits A). Stem turgor, B). Floret looseness, C). Bud compactness and D). Bud elongation using the Wurr scoring system.