Quantitative Genetics Analysis of Flowering Time and Leaf Development Traits with Candidate Genes in *Brassica rapa*

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List of abbreviations

AFLPs	Amplified fragment length polymorphism
BC	Backcross
CC	Chinese cabbage
cM	CentiMorgan
DH	Double Haploid
FT	Flowering Time
GA	Gibberellins
LD	Linkage Disequilibrium
LG	Linkage Group
LT	Leaf traits
PC	Pak Choi
PCR	Polymerase Chain Reaction
QTLs	Quantitative trait loci
RFLP	Random fragment length polymorphisms
RAPD	Random amplified polymorphic DNA
RILs	Recombinant inbred lines
SAM	Stem apical meristem
SSR	Simple sequence repeats

Abstract

The *Brassica rapa* species has been intensively studied due to the wide genomic diversity (various morphotypes), use as a model crop for studying polyploidy plants and their worldwide use as vegetables, oil source, animal feeds and condiments. Most agronomic traits including leaf traits and flowering time are polygenic and affected by prevailing environmental condition. SNP markers were used to assess the genetics of the leaf traits of *B. rapa*. 82 lines of double haploid population developed from cross between Chinese cabbage (CC048) and Pak choi (PC101) were established in the greenhouse. CC048 is a heading type while PC101 is non-heading. Twelve leaf traits were assessed at different times of development. Candidate genes for morphological leaf traits were obtained by exploiting their known functions in *Arabidopsis thaliana* and Chinese cabbage reference genome (Chiffu). On basis of SNP data obtained we mapped the QTLs associated with the traits. 211 out of 2793 market traits were associated with the traits evaluated at p-value < 0.05. Some of these markers were associated with functions underlying the trait while others showed false positives. The QTL analysis revealed the association of some genes under the study to the leaf trait at different development stages. 26 markers were identified to be associated with various leaf trait gene functions. These can be used in improvement of leaf traits through marker-assisted breeding.

Keywords: Candidate genes, leaf developmental stages, gene association, SNP, double haploid

1.0 INTRODUCTION

1.1.1 *Brassica* genome

Brassica and *Arabidopsis* belong to the *Brassicaceae* family. They diverged from a common ancestor about 17-18 million years ago (Hong et al., 2007). *Brassica* contains 338 genera and 3700 species (Zhao et al., 2007). Of these, the economically most important species are *B. rapa* (A genome n=10), *B. nigra* (B genome n=8) and *B. oleracea* (C genome n=9) which are diploid and *B. carinata* (BC genome, n=17), *B. juncea* (AB genome, n=18) and *B. napus* (AC genome, n=19) which are amphidiploid. Amphidiploid arose from interspecific hybridization of the diploid species (Li et al., 2009; Zhao et al., 2005). This relationship is described in figure 1, the "triangle of U" which has been widely used to understand the chromosomal evolution of *Brassica* genome from their common ancestor with *A. thaliana* since divergence (King 2005).

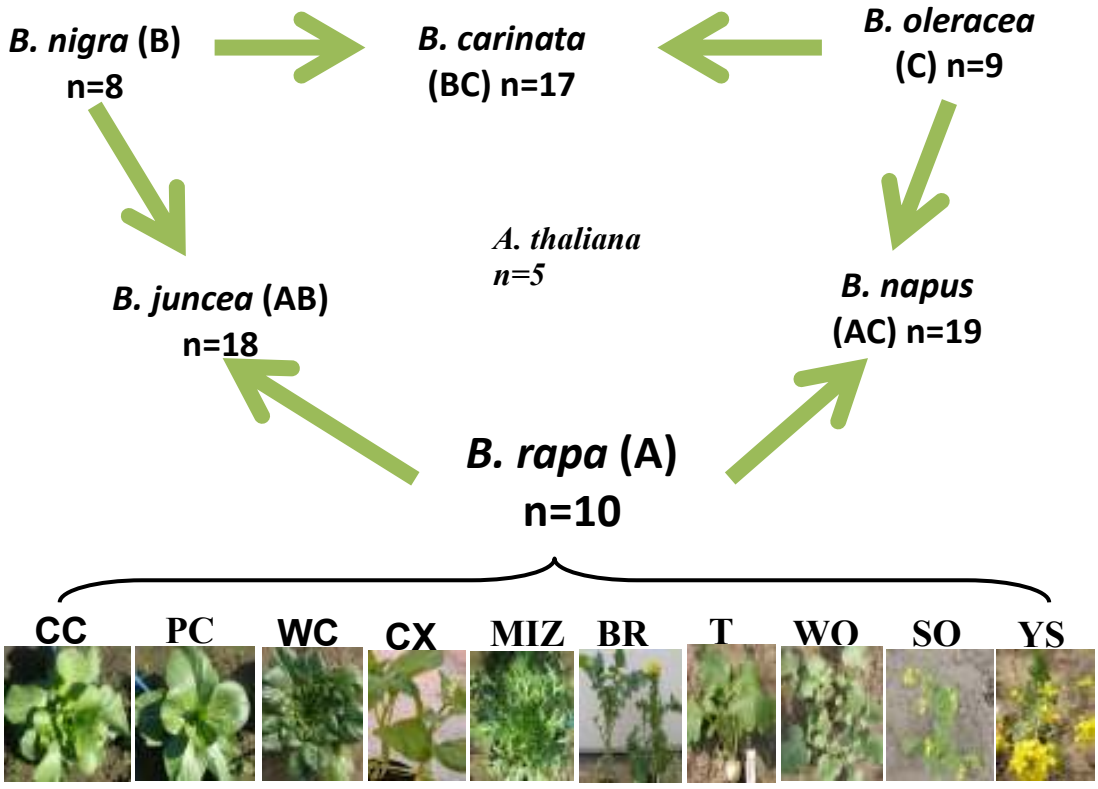


Figure 1. The "Triangle of U" diagram, showing the genetic relationships between the six species of the genus *Brassica. At the center is their close relative – A. thaliana. Source: Dong 2011 (Unpublished) with modifications*

Brassicas are cultivated for their great economic importance. They are good sources of vegetables, edible oil, condiments, biofuels and fodder. For instance, *Brassicas* account for 12% of total vegetable produced and consumed in the whole world. They are also a major source of edible oil and biofuel (Hong et al., 2007; Kim et al., 2006 and Mun et al., 2009). They are good source of protein, vitamin C, glucosinolates, dietary fibre, anti-cancer compounds and various minerals like magnesium, copper, phosphorous and calcium (Bell et al., 1999; Kim et al., 2006; Lou et al., 2009; Wang et al., 2011 and Zhao et al., 2007). With the sequencing of *Brassica* genome, it has been adopted as model crop for studying polyploid genome evolution (Wang et al., 2011). The triplication of *Brassica* genome due to polyploidization resulted in chromosomal rearrangements. The various studies done in the amphidiploid *Brassica's* will help in understanding the allotetra-polyploidy in various crops (Hong et al., 2007) and *B. rapa* genomic information is being used in studying the genome structure, function and evolution of other angiosperm (Toure et al., 2000).

1.1.2 Brassica rapa

Brassica rapa, one of the major economically important *Brassica* crops is believed to have different centers of diversity; Europe, South Asia (India etc), Africa and then far East Asia (China/Japan), but though the Mediterranean is suggested as the main origin (Toxopeus & Baas, 2004). The spread of *B.rapa* followed the trade routes from Mediterranean to Scandinavia to Germany then Central Europe and then to Asia. It reached China through Mongolia thousands of years ago (Mun et al., 2009; Toxopeus & Baas, 2004 and Zhao et al., 2005). Since then, very many subspecies and cultivar groups of *B. rapa* have risen exhibiting morphological diversity. They comprise the leafy, oil and turnip types (Chuang et al., 2004; Li, 1983; Opena et al., 1988). The leafy types include Chinese cabbage (*ssp. pekinensis*), pak choi (Chinensis), Mizuna, Japanese leafy types and Caixin. The oilseeds include, sarsons and toria's and winteroils, while the turnip are believed to be the oldest (Kim et al., 2006; Toxopeus & Baas, 2004 and Teutonico and Osborn 1994). The Chinese cabbage (CC) and Pak Choi (PC) are both leafy vegetable cultivated as annual vegetables though they are biennial. They differentiated from oilseed rape with distinctions between type related to the leaf width and cross-section of the leaf petiole (Dixon G. R., 2006; Toxopeus & Baas, 2004 and Zhao et al., 2005). In Chinese cabbages the leaves fold to make a head, while Pak choi forms no head. Almost all *B. rapa* accessions are heterozygous and heterogenous, caused by their self-incompatibility system. *B. rapa* has been adopted model crop to study polyploid genomes providing rich resource avenue for improving the crops quality and quantity.

1.2.1 Segregating populations for mapping purposes

The application of double haploid (DH) technique is used to develop homozygous lines quickly. The method to create DH populations developed from F1 plants supersedes other methods like F_2 and backcross (BC_1) (Zhang et al. 2011). F_2 and BC_1 though are easy to develop and quick, they possess a few disadvantages: 1. the resultant progenies are heterozygous and thus identical genotypes cannot be reproduced; 2. they are ephemeral, there is only one plant per genotype thus no replicates possible for accurate phenotyping. This mortal in nature makes them unusable for replication in multiple environments and years (Young N. 2000); 3. a large population is required and has quite poor resolution; 4. in both F_2 and BC_1, it is advantageous to have co-dominant markers instead of dominant markers to distinguish the heterozygous forms. Recombinant Inbred Lines (RIL) created by single seed descent from F_2 offspring results in lines which are almost homozygous. Each line differs from the other thus enabling the linkage analysis. RIL enables selection of desired characters on the population and thus the desired characters can be improved. RIL are faced by a disadvantage that they require many generations to develop and also some genome regions tend to remain heterozygous longer than expected in theory (Young N. 2000). The DH produced through a single meiosis is faster to develop resulting to fully homozygous lines which are genetically identical. Thus a large homozygous population is developed in much less time, promoting breeding programmes through efficient genetic analysis marker application. The lines are immortal thus they can be replicated in multiple locations. This allows for breeding trials over multiple years and environments. The DH population does not require co-dominant markers.

1.2.2 Genetic Maps

Genetic maps are valuable tools for studying genome organization, structure and evolution of species. They set the stepping stone for localizing genetic markers associated with agricultural important traits and breeding process like marker assisted selection (Semagn et al., 2006). Genetic maps have been applied in studying and understanding the evolution, phenotypic diversity and relation between various species including *Brassica* species and model plant - *Arabidopsis thaliana* (Hong et al., 2007 and Yu et al., 2009). Many agronomic important traits are quantitative and controlled by many genes each with a small effect making them to have complex genetic basis. Mapping of quantitative trait loci (QTLs) has enabled the investigation of the genetic variation within segregating populations in *Brassica* (Acquaah, 2010). The use of segregating population detects only the QTLs for which the parents differ

and due to low amount of recombination occurring, the QTL location extends over many centiMorgan (cM). Identifying the quantitative trait loci (QTLs) sets the starting point of studying and applying the molecular information of these complex traits. Various genetic maps have been developed in *B. rapa*. These maps have been used in quantitative trait loci (QTLs), mapping studies of agricultural important traits like flowering time, various diseases like downey mildew disease, morphological leaf traits, yield and leaf hairiness in *Brassicas* (Hong et al., 2007; Zhao et al., 2007; Li et al., 2009; Yan et al., 2009; Yu et al., 2011). Genetic maps are also valuable in identifying introgression between different genomes and genetic analysis of quantitative traits (Ge et al., 2011, Kim et al. 2009 and Yu et al., 2009). With the recent availability of the genome sequence of *B. rapa*, there are great possibilities to develop different types of markers. Based on various genetic markers, over 20 genetic maps of *Brassica rapa* have been developed (Hong et al., 2007). One possibility is to develop markers based on genes involved in the trait under study, also called candidate gene approach. Once QTLs are detected, plant breeders can use marker assisted selection for the traits under investigation (Toure et al., 2000).

1.3 Arabidopsis - Brassica synteny

Brassica and *Arabidopsis* are closely related species having extensive regions of gene collinearity (synteny). This phenomenon has enabled successful comparative studies between these two species (Hong et al., 2007). The syntenic relation and level between the *Arabidopsis thaliana* and *Brassicas* has enabled the studies of genetics and morphological variations in *Brassicas*. That is, the QTL study in *Brassica* is facilitated by its syntenic relationship with *Arabidopsis thaliana* genome (Pe´rez-Pe´rez et al, 2009). Their chromosomes are differentiated by the various chromosomal rearrangements (Hong et al, 2007). *Arabidopsis thaliana* has been well studied and fully sequenced. This has been made possible due to its small genome (125mbp) organized into 5 chromosomes containing 20,000 genes, many of which with known functions. The plant has short lifecycle (6 weeks from germination to production of mature seeds) with prolific seed production. It also harbors a large number of available mutant lines and genomic resources (http://www.arabidopsis.org/ and Meinke et al., 1998).

Brassica rapa has been preferred as model crop to study polyploidy crops prompting its intensive study. It is useful model crop for studying polyploidy having undergone genome triplication and has a relatively small genome (529mbp). The genome triplication led to

almost two-fold increase in the *B. rapa* gene number compared to *A. thaliana* depicting genome shrinkage with distinct organization of ancestral genome (Mun et at, 2009, Feng et al. 2012). Chromosomal rearrangements, including translocations, deletions, insertions, inversions, fusions and/or fissions and substitutions of *Arabidopsis*, resulted to the current "diploid" *Brassica* species with variations in chromosome number and phenotype (Hong et al., 2007 and Saito et al., 2006). *B. rapa* has compact genome with genes concentrated in euchromatic spaces (Wang et al. 2011, Mun et at, 2009 and Schranz et al., 2007). Thus, the genomic information of *Arabidopsis thaliana* can be applied in mapping of *Brassica* genomes. For instance, the syntenic relationship between *A. thaliana* and *Brassicas* enabled the mapping of clubroot resistance gene, Crr3, in *B. rapa* (Saito et al., 2006). This relationship enabled the cloning of gene responsible for hairiness and seed coat colour traits in *B. rapa* (Zhang et al., 2009).

The similarities and collinearity relationship between these two species has enabled the identification of candidate genes and successful studies in *Brassica* species through comparative genomics (Hong et al 2007).

1.4 Flowering time (FT) and leaf development (LT)

1.4.1 Flowering Time (FT)

Flowering time is a major component in the lifecycle of plant. It enables optimal utilization of resources available to the plant by ensuring that each developmental stage is at optimal in the environmental condition (Laurie D., 1997). Thus it contributes to the continuous existence of the plant through seed production, plant adaption and yield production (Laurie D., 1997 and Pe´rez-Pe´rez et al., 2009). The flowering time traits are sensitive to different environmental cues like the variations in temperature and day-length and respond according to these cues (Zhao et al,. 2010).

The accessions of model plant, *Arabidopsis thaliana* show natural variations in flowering time habits. Over 60 genes responsible for regulating flowering time has been identified in *Arabidopsis thaliana* and over 28 loci identified to affect natural variation in flowering time (Ehrenreich et al., 2009). These variations depict the different responses to vernalization which is often due to allelic variation at *FLOWERING LOCUS C (FLC)* and/or *FRIGIDA (FRI)* (Ehrenreich et al., 2009; Kim et al., 2005 and Salome et al., 2011). Many flowering time genes like *FLOWERING LOCUS C (FLC), CONSTANS (CO) and FLOWERING TIME (FT)* in *Arabidopsis* are located at Chromosome 5. In *Brassica rapa*, several QTLs have been identified in F_2 and recombinant inbred lines (RIL) populations (Lou et al, 2007). Of these

QTLs are *VFR1, VFR2, VFR3, FR1, FR2* and *FR3*. The QTL *VFR2* has large effect and its candidate gene is a homologue of FLC, which is a major flowering time gene in *Arabidospis* (Kim et al., 2005 and Lou et al., 2007). Lou et al (2007) noted that *VFR2* locates at the *BrFLC1* locus, *FRI* at *BrFLC2* and *FR2* at *BrFLC5*. These Br*FLC1,* Br*FLC2* and Br*FLC5* found in *B. rapa* are in the syntenic regions of *A. thaliana* and *FLC5* is in non-syntenic region. And thus, *BrFLC1* and *BrFLC2* are linked to the QTL for bolting time and flowering time. This shows that *FLC* contributes to the flowering time variation with similar role in Arabidopsis and Brassica (Li et al., 2009; Lou et al., 2007; Yang et al., 2006 and Yu et al., 2003). Different paralogues in *B. rapa* play a role different roles in modulating flowering time and the vernalization response. Also variation of flowering time within and between *B. rapa* morphotypes influences the breeding objectives (Zhao et al., 2010; Zhao et al., 2005; and Li et al., 2009).

1.4.2 Leaf development

1.4.2.1 Leaf Morphology

Leaves are determinate organs developing from the shoot apical meristem (SAM). They form through the speciation of leaf founder cells. The cells undergo proliferation, differentiation and expansion and these processes overlaps. Specification of adaxial- abaxial leaf cells leading to flat lamina is a main differentiation event (Barkoulas et al., 2007). Leaves are the main photosynthetic structures of plants. They determine the photosynthetic efficiency, respiration, evapotranspiration and form a source of various minerals and nutrients required by plant. They have three axes of asymmetry; proximal-distal, medial-lateral and adaxial-abaxial axes (Figure 2). The leaves of *Brassica* are divided into upper and lower leaf parts. The Upper part comprise of lamina and petiole while the lower part includes the leaf base and stipules (Piazza et al., 2005). Morphological leaf traits are of great importance in determining the quality and quantity of yield in *B. rapa*. (Li et al, 2010). Various morphological leaf traits like the leaf size, shape, leaf angle, leaf hairiness and leaf number influence various agronomic traits including the yield production, attractiveness of the crop as vegetables, resistance to diseases and pests and response to various plant stresses. Leaf hair can act as pest control as pest avoids plants with hairs. More leaves, larger lamina length and width can be interpreted as having more yields. Depending on preference smooth leaves can be preferred to hairy leaves. These quantitative traits are highly affected by environmental factors, including planting density and climatic conditions cues such as light, temperature, and moisture content (Yan et al 2009).

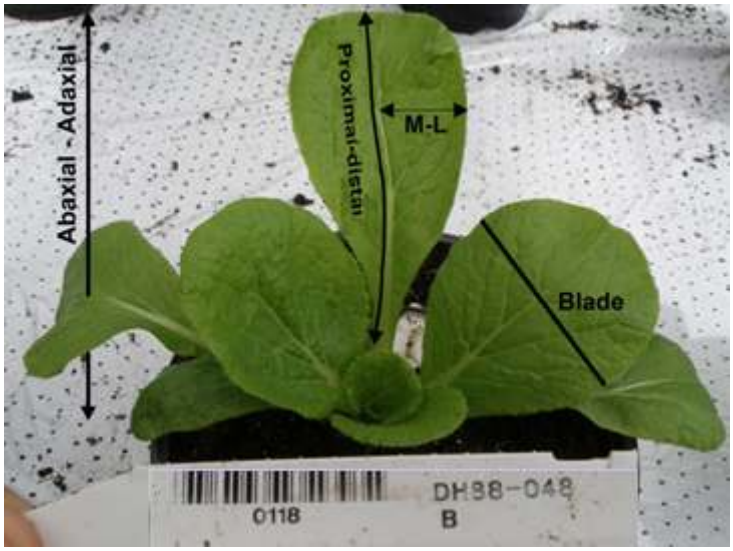


Figure 2. B. rapa picture showing the three axes of asymmetry within a leaf; the proximal–distal axis (from the leaf tip to point of attachment to the stem), Adaxial–abaxial axis (upper and lower sides respectively). The medial–lateral (M–L) axis spans the leaf, from the mid vein to the edge of the blade. Source (Piazza et al., 2005 with modifications)

1.4.2.2 Genes related to leaf development

The variation in leaf traits are due to the coordination between cell proliferation and cell expansion along the proximal–distal, medial–lateral and adaxial–abaxial axes (Pe´rez-Pe´rez et al, 2009). There are various genes involved in growth and development of leaves; KNOTTED-LIKE HOMEOBOX (KNOX) genes are confined to the SAM and are responsible for maintaining the meristem. KNOX genes include BREVIPEDICELLUS (BP), KNAT2 and KNAT6. They regulate the activities of cytokinin and gibberellin growth regulators. They elevate the cytokinin levels and depress the gibberellin (GA) levels. Thus, maintaining the cells in an indeterminate state. Early leaf initiation responds to down regulation of KNOX in the meristem. Auxins play a role in initiation of leaf and cell elongation. Auxins also delay leaf senescence. For instance, *Arabidopsis* plants treated with polar auxin transport (PAT) inhibitors led to a failure to initiate lateral organs while application of auxin (IAA) at SAM led to formation of new primordium. ASYMETRIC LEAVES1 (*AS1*) promote leaf development through down-regulation of the KNOX gene - *BP* (Barkoulas et al., 2007 and Piazza et al, 2005). Narrow Sheath (NS) genes are expressed at lateral edges of the leaf and are responsible for wide leaves. The lack of marginal domains results in development of narrow leaves. Homeodomain-leucine zipper containing proteins (HD-ZIPIII) genes promote the adaxial leaf identity. They include Phabulosa (PHB), Phavoluta (PHV) and Revoluta (REV) and they are expressed in the SAM (Piazza et al 2005). KANADI and YABBY (KAN and YAB) genes promote the abaxial identity. KAN activity is required for correct elaboration

of abaxial vascular identity. Adaxial-promoting HD-ZIPIIIs repress abaxial-promoting KAN and YAB genes and vice versa. YAB activity is required for leaf lamina expansion and it represses the KNOX1 at the leaf primordia (Ha et al., 2010). JAGGED (JAG) transcription factors regulate the growth of lateral organs. They slow down the arrest of cell division in the distal region of the lateral organs where they are expressed. Cincinnata (CIN) genes cause the excess growth of leaf margin leading to leaf curvature (Piazza, et al., 2005). Ha et al, 2010 in their study with *A. thaliana* found out that BLADE-ON-PETIOLE1 (BOP1), and BOP2 genes are required for correct leaf morphogenesis. They suppress both KNOX1 and YAB activity at the leaf base thereby upholding the suitable cellular condition for normal leaf differentiation (Figure 3).

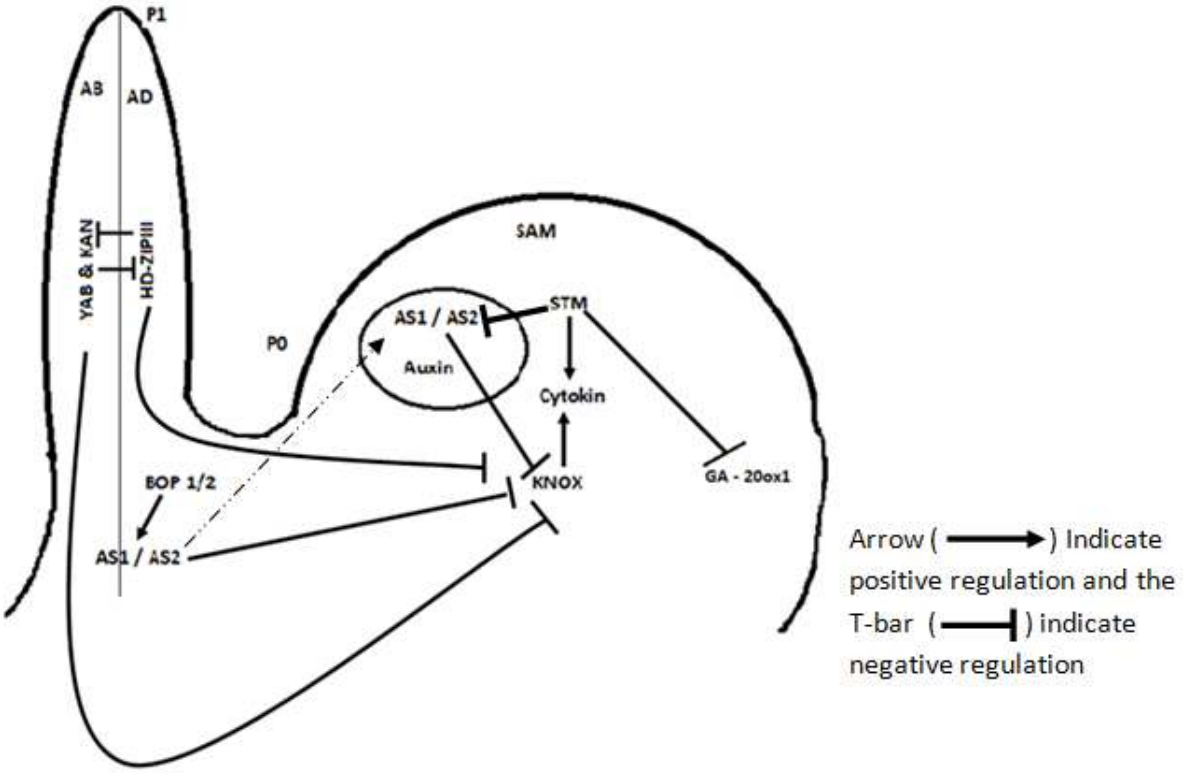


Figure 3. Model for *Arabidopsis* genes function during leaf development. The initiation of leaf is flagged at the flanks of the SAM. The *BOP1/2* induces AS1 which represses *KNOX* thus promoting leaf development in the primordium initiation (P0). *Auxins* aids in leaf development. *KNOX* is also down-regulated by *HD-ZIPIII* and *YAB* & *KAN*. *KNOX* promotes cytokinins. *YAB* & *KAN* are expressed in the abaxial (AB) domain of leaf primordial to promote abaxial fates and organ growth. *HD-ZIPIII* are expressed in the SAM, in the incipient leaf primordial and in the adaxial (AD) domain of old primordial (P1). *STM* down-regulates the *GA* biosynthesis gene *GA20ox1* (Adopted from Ha et al. 2010 and Piazza et al., 2005).

1.5 Leaf QTL studies

The quantitative trait loci (QTLs) associated with various morphological leaf traits have been mapped on linkage maps of *B. rapa*; ten QTLs and a syntenic region containing GIBBERELLIN 20 OXIDASE 3 has been detected to have influence in leaf morphological traits (Kubo et al, 2010; Pe´rez-Pe´rez et al, 2009 and Li et al., 2009). In an F2 population of *B. oleracea*, five QTLs explaining 45% of phenotypic variance in lamina length, and three which colocalized with QTLs affecting lamina width as well as 4 QTLs for petiole length explaining 49% of phenotypic variance have been identified (Pe´rez-Pe´rez et al, (2009).

Certain genes are expressed at certain growth stage of the plant. The development of leaf morphological traits is a result of action and interactions of multiple genes at different growth stages. The expression of these genes is affected by epistatis and environment (Yan et al 2009). These conditions necessitate the importance of understanding the dynamics of certain gene expression at different growth stage. In this study, the expression of various genes involved in growth of dynamic morphological leaf traits will be studied. These will be in line with the objective of this study which is to expound the genetics of length, width, and chlorophyll content of leaf in *B. rapa* by QTL mapping using a DH88 population from a cross between a Chinese cabbage and a Pak Choi.

1.6 Objectives of the study

Brassica rapa is closely related to model species *Arabidopsis*, and its triplicated genome structure with blocks with high synteny to *Arabidopsis* is well described based on comparative physical mapping and genome sequencing studies (Schranz et al. 2006, Wang et al. 2011). QTL mapping is an effective approach used to understand and locate the genetic loci governing complex traits in the genome. Although several QTL mapping and genetic studies have been carried out and documented in *B. rapa*, there is still less information available concerning the genetic analysis based SNP markers for identification of candidate genes controlling leaf and flowering time traits. (Yu et al., 2009)

The leaf trait and flowering time of *B. rapa* exhibits great variation, both depending on genotype and environment. Different *B. rapa* vegetables have different flowering times and thus different optimal growing regions; breeding for flowering time and leaf traits could result in varieties adapted to multiple environments (Zhao et al., 2010; Zhao et al., 2005; and Li et al., 2009). The genetics and inheritance of the morphological leaf traits and flowering time are directed by many genes and are highly influenced by environmental conditions (Lou et al.,

2007 and Yu et al., 2009). The studies directed in identifying the loci controlling morphological trait variations shows the complexity of genetics of these quantitative inherited traits in *B. rapa* (Lou et al., 2007). There is need to understand the basis of these variation for the application in breeding *B. rapa*. The genetic analysis of DH88 will assist in providing an understanding in genome structure and through the localization of QTLs provide basis for genetic studies concerning the agronomic traits of *B. rapa*. They will also help in breeding programs with the use of marker assisted selection (MAS). These will emphasis on leaf traits, which have great impact on the efficiency of photosynthesis which influence the yield quality and quantity of *Brassica*. In addition, leaf colour, size and shape define the marketability of leafy vegetables. The flowering time is very useful tools used by growers to know when and where to grow specific type *B. rapa* types. The crop is targeted to be used as model crop to study the angiosperm. This study was addressed by the following objectives:

- To develop genetic markers based on genes homologous to *Arabidopsis* leaf trait genes polymorphic between Chinese Cabbage and Pak Choi, parents of DH88
- To develop a genetic map of DH 88 using these genes complemented with AFLP markers
- To assess the quality of the DH88 genetic map by comparing genetic marker order of DH 88 based on SNPs in leaf and flowering time genes with physical order of these genes in the reference genome (Chiifu genome) and DH 68 map (YS-143×PC-175)
- Phenotyping of leaf traits – Leaf length, leaf width, lamina length, lamina width, petiole length, leaf index, plant diameter, plant height, leaf number, leaf color, heading and tillering. Also flowering time.
- To identify allelic variation in the leaf-gene-based markers correlating with the phenotypic variation

2.0 MATERIALS AND METHODS

2.1 Plant materials and growing condition

The plant material used in this study was a DH88 population. The Double haploid population derived from microspore culture of an F_1 cross between two DH lines derived from two accessions: Chinese cabbage – DH CC-048 (CGN06867) as the female parent originated from Soviet Union and Pak Choi – DH PC-101 (CGN13926) as the male parent originated from China and its cultivar name is Celery, Shantung or Peking. The two parents were obtained from Dutch Crop Genetic Resources Center (CGN).

The DH88 population was grown in the greenhouse in three blocks in a completely randomized design. 82 lines were established in block one, 80 lines in block two and 77 lines in block three. The DH lines and their parents were grown in Nergena greenhouse in Wageningen University from May to August 2012. The seeds were pre-germinated in normal wet filter paper at 25℃ in the dark for two days then transferred to nursery trays for culturing for two weeks. Seedlings with three to five true leaves were transplanted into the soil in pots of diameter 14 cm. And later on 45th day they were transferred to pots of Ø 21cm.

2.2 Phenotyping

A total of 12 morphological leaf traits, tillering, leaf color and flowering time trait were scored in this study. The scoring scheme is as described in table 1. The 3rd leaf was measured to evaluate LL, PL, LaL and LW, at 18, 22, 25, 30, 35 and 39 days after sowing using a ruler. The LI was calculated as a ratio (LaL/LW), to determine the leaf shape. These traits were measured as described in Figure 4. The plant diameter (PD) was determined by calculating the average distance through the centre of the two widest points. Plant height (PH) was determined by measuring the height of the plant from soil level to the highest point. PH was measured at 22, 25 and 39 days after sowing when the plant was expected to have vigorous growth. The density of leaf hairiness (H) on young leaves was qualitatively determined using a six-grade (0 - 5) scale as follows: 0 – hairless (entirely smooth), 1 - some hairs along the leaf edge, 2 - some hairs on the lower surface of leaf, 3 - dense hairs on the lower surface of leaf, 4 - dense hairs on lower surface and some sparsely hairs on the upper surface and 5 - dense hairs on both lower and upper leaf surfaces. The number of leaves (LN) was counted at 25 and 30 days after sowing.

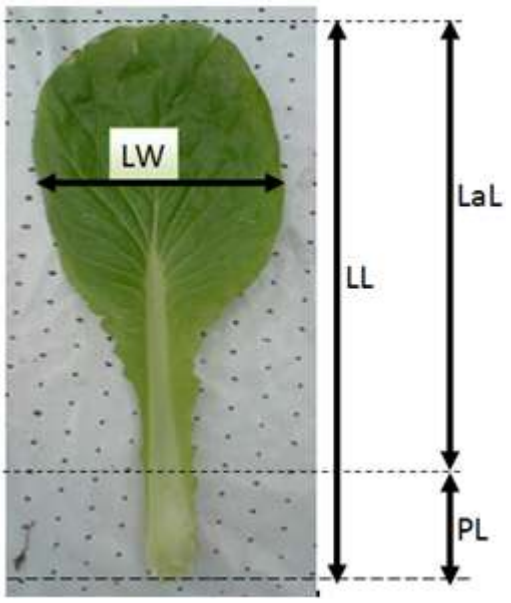


Figure 4. Diagram of *B. rapa* leaf illustrating different leaf parameters - leaf length (LL), lamina length (LaL), petiole length (PL) and lamina width (LW).

Table 1. Phenotypic traits scoring scheme: The table explains the scoring criteria for every trait of interest.

Trait name	**Code**	**Trait description**	**Scale**
Leaf length	LL	Length of entire 3rd leaf from the tip to the base of the leaf at 3 days interval consecutively from day 15 after sowing.	mm
Lamina length	LaL	Measured from the leaf tip to the start of leaf blade where first lobe appears. The 3rd leaf was measured at interval of 3 days consecutively from 15th day after sowing.	mm
Petiole length	PL	Length of leaf stalk from leaf base to start of leaf blade of 3rd leaf from 15th day after sowing at 3 days interval.	mm
Lamina width	LW	Widest part of the lamina of the 3rd leaf from 15th day after sowing at 3 days interval.	mm
Leaf index	LI	Calculated as ratio of Lamina length to Lamina width (LI=LaL/LW)	ratio
Plant diameter	PD	Cross-section average distance from 15th day after sowing for four times.	cm
Plant height	PH	Height of plant at 22, 25 and 30 days after sowing measured from the ground level to the highest point.	cm
Leaf number	LN	Number of leaves, at 25 and 30 days after sowing.	Number
Leaf Hairiness	LH	In a six-grade scale of 0-5 the density of hairiness on the young leaves.	0 - 5
Heading	HD	Progressive development of the head was evaluated at 56, 70, 84, 97, 108, 114 days after sowing using a four-grade scale	1 - 4
Leaf colour	LCh	Amount of Chlorophyll of the 4th leaf using portable Chlorophyll Meter (SPAD-502Plus).when measured?	Number
Tillers	T	The growth of tillers at the base of the plant. If present-1 and if absent-0	0 - 1
Flowering Time	FT	Days from transplanting to appearance of floral buds in the centre of the rosette	Days

The presence of tillers (T) was scored as 1 and absence of the tiller scored as 0 as shown in figure 5

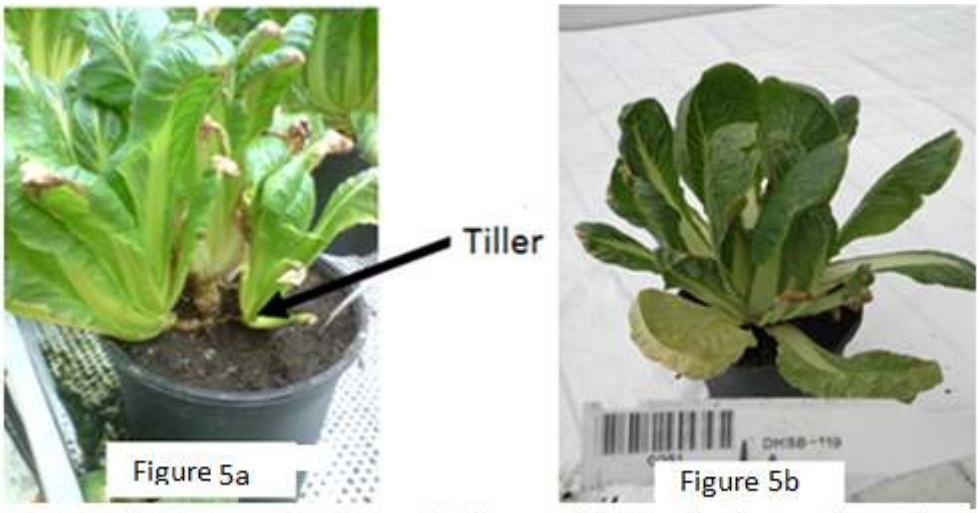


Figure 5a shows Brassica plant with tillers and figure 5b Plant without tillers

The heading (HD) was scored qualitatively using a four-grade (1-4) scale as follows: - 1 -No head at all, the leaves are wide open, 2 -The leaves curls inward as though forming a head though the head is not fully formed. 3- A head is formed but not completely closed at the top and 4- a well tight closed up head as shown in Figure 6.

Figure 6. A diagram showing the scoring scale of heading

The chlorophyll content of the 4th leaf was determined with a non-destructive method by use of Chlorophyll Meter (SPAD-502Plus). Three readings were taken at a time at different same leaf positions and their average determined and recorded.

Flowering time (FT) was scored in terms of days from sowing to appearance of floral buds in the centre of the rosette.

The average measurements were calculated as mean value among the three blocks for each trait at every scoring time.

2.3 DNA extraction

The DH88 population of 82 lines was studied. Young, not expanded leaves from every line were harvested for DNA extraction. The harvested plant materials were preserved in liquid nitrogen (LN_2) and later were ground still in LN_2 using mortar and pestle. The DNA was extracted using CTAB according to RETCH 1.3 Protocol (Gert van Arkel & Maarten Nijenhuis) with some modifications as described in appendix 1, according to Van der Beek et al (1992). The coster plates were incubated in water bath at 65^0C for 1 hour and then ice-cold for 15 min, then the chloroform: isoamyl alcohol (24:1) was added. The mixture was centrifuged and supernatant transferred into new tubes. One volume of isopropanol was added and centrifuged to precipitate the DNA. The product was allowed to dry. After drying, the DNA pellet was dissolved into 100 µl E-buffer (10.5mg RNAse) to get rid of RNA in the DNA samples (Park T. 2007). The genomic DNA quality was tested in 1% (w/v) agarose gel and DNA concentrations were determined on a Nanodrop photometer (Thermo Scientific, Waltham, MA). The genomic DNA was diluted to 5-10ng/µl for use in PCR reaction in single marker analysis using the LightScanner and 50 and 100ng/µl for AFLP analysis.

2.4 Molecular marker analysis

2.4.1 High Melting Resolution Analysis (LightScanner)

PCR-based markers for morphological leaf traits and flowering time developed from the known functions in *Arabidopsis thaliana*. The information obtained in TAIR (http://www.arabidopsis.org/) and then blasted in *Brassica* database (http://brassicadb.org/brad/searchAll.php). The primers were named based on the gene nomenclature system of *Brassica* defined by Østergaard and King (2008), ordering from left to right (genus–species–gene–name–genome–locus). Primers were developed with homology to *Brassica rapa* genes homologous to Arabidopsis genes involved in leaf development. The PCR products based on these primers were first screened for polymorphisms in DH68, a cross between yellow sarson YS-143 (accession number: FIL500) as the female parent and pakchoi PC-175 (cultivar: Nai Bai Cai; accession number: VO2B0226) as the male parent. In this study, the CC and PC parents, and six progeny lines were screened for polymorphism for each marker using the LightScanner. LightScanner is a program which enables analysis of DNA melting points. It is a high resolution melting analysis which performs high-throughput gene scanning and mutation detection. The system does not require post-PCR reagent addition or separation and thus becomes time effective method (Idaho Technology Inc. and Yuan et al.,

2008). The PCR reactions were performed in 96-well plates. The PCR mixture (table 2) was loaded and PCR program was set as 30sec at 98°C, then 40 cycles of 10sec at 98°C, 10sec at 60°C, 30sec at 72°C and a final elongation step of 30sec at 72°C with 10^0C for indefinite time (Figure 7). After the PCR, the samples were subjected to temperatures between 70^0C and 95^0C in the LightScanner system to determine the melting point. The samples were grouped according to the melting point in a 1 unit minimum temperature difference and also 1unit maximum temperature difference (Figure 11). A total of 166 primers were screened for polymorphism between the two parental lines. The primers which showed polymorphic band pattern were used to amplify the DH 88 population DNA. The parental alleles were labeled as "a" for female parent (CC048) and "b" for male parent (PC101).

Table 2. The composition of master mix used for PCR reactions for LightScanner

Component	Volume 1x (µl)
5X Phire enzyme	0.1
Reaction buffer for Phire (5x)	2
LC-green	1
dNTPs	0.4
Forward Primer (diluted)	0.25
Reverse Primer (diluted)	0.25
MQ	4.5
DNA (5 – 10ng)	1
Total Volume	**9.5**
Mineral Oil	20

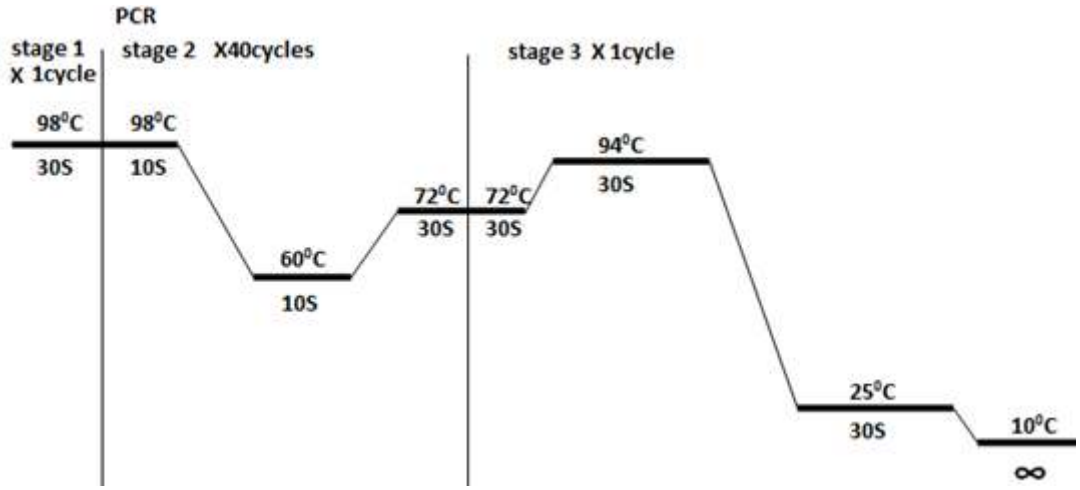


Figure 7. The diagram of PCR reaction flow for LightScanner

2.4.2 AFLP analysis

Amplified fragment length polymorphism (AFLP) analysis was performed based on Vos et al. (1995) and AFLP protocol released by The Laboratory of Plant Breeding, Wageningen University using *EcoRI* and *MseI* restriction enzymes. Digestion and ligation were performed simultaneously in a 50 µl volume containing 5 µl of DNA and other reagents as described in step 1 in table 3. Pre-amplification was performed in 20.08 µl reaction volume (step 2 in table 3). The program for pre-amplification consisted of an initial incubation period at 94^0C for 3 minutes followed by 24 cycles of 94^0C for 30 seconds, 56^0C for 30 seconds and 72^0C for 1 minute and final elongation at 72^0C for 5 minutes followed by 10^0C for indefinite time (figure 8a). Selective amplification was performed in 10.04 µl volume (step 3 in table3). The touchdown profile program was performed for initial incubation period at 94^0C for 3 minute, 12 cycles of 94^0C for 30 seconds, 65^0C for 30 seconds and 72^0C for 1 minute, then touchdown profile of 24cycles at 94^0C for 30 seconds, 56^0C for 30 seconds and 72^0C for 1 minute and final elongation at 72^0C for 5 minutes followed by 10^0C for indefinite time (figure 8b). Before selective amplification, the quality of pre-amplified product was checked in agarose gel to detect a smear of band at 500bp (figure 12). The selective *EcoRI* primers were labeled with fluorescent dye (blue buffer). Six primer combinations were used - E33M47, E31M60, E41M62, E33M51, E35M62 and E42M51 (table 6). The *Eco*RI primers were labeled with IRD-700 at their 5′ ends (Zhao et al., 2005). The selective amplification reaction product was mixed with equal volume of formamide-loading buffer. Then it was denatured for 5 min at 94°C before cooled on ice and run on a polyacrylamide gel using the *LI-COR* system 4200 DNA sequencer (*Li-Cor*, Lincoln, Neb.)

Table 3. The master mix for the 3 stages of AFLP PCR reactions

Step 1: Digestion and Ligation Reagent	Volume 1X (µl)	
5 Units EcoRI	0.5	
5 Units MseI	0.5	
EcoRI Adapter	1	
MseI Adapter	1	
Energy (ATP)	1	
Buffer	10	
Ligase Enzyme	1	
MQ	30	
250 - 500ng DNA	5	
STEP 2. Pre-amplification	DILUTION 20X Volume 1X (µl)	
30ng Unlabeled *Eco*-Primer	0.6	
30ng unlabeled *Mse* Primer	0.6	
5mM dNTPs	0.8	
10X Super Buffer	2	
5 Units/ul Taq-Polymerase	0.08	
MQ	11	
Diluted RL DNA	5	
TOTAL	20.08	
Step 3. Selective amplification	IRD 700 Volume 1X (µl)	IRD 800 Volume 1X (µl)
Unlabeled *Mse*-Primer	0.3	0.3
IRD-labeled *Eco*-Primer	0.5	0.6
5mM dNTPs	0.4	0.4
10X Super Buffer	1	1
5U/ul *Taq*-Polymerase	0.04	0.04
MQ	2.8	2.7
20X diluted Pre-amplified product	5	5

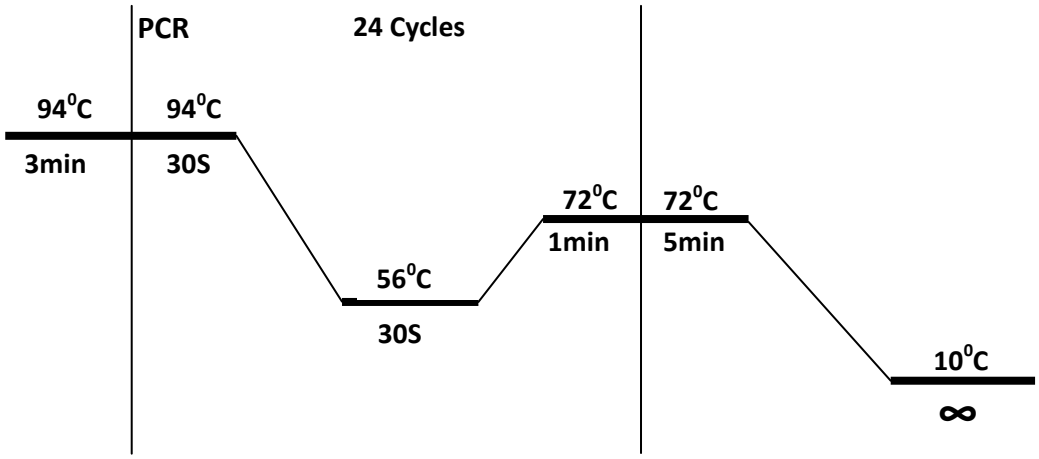


Figure 8a. Pre-Amplification PCR Reaction diagram

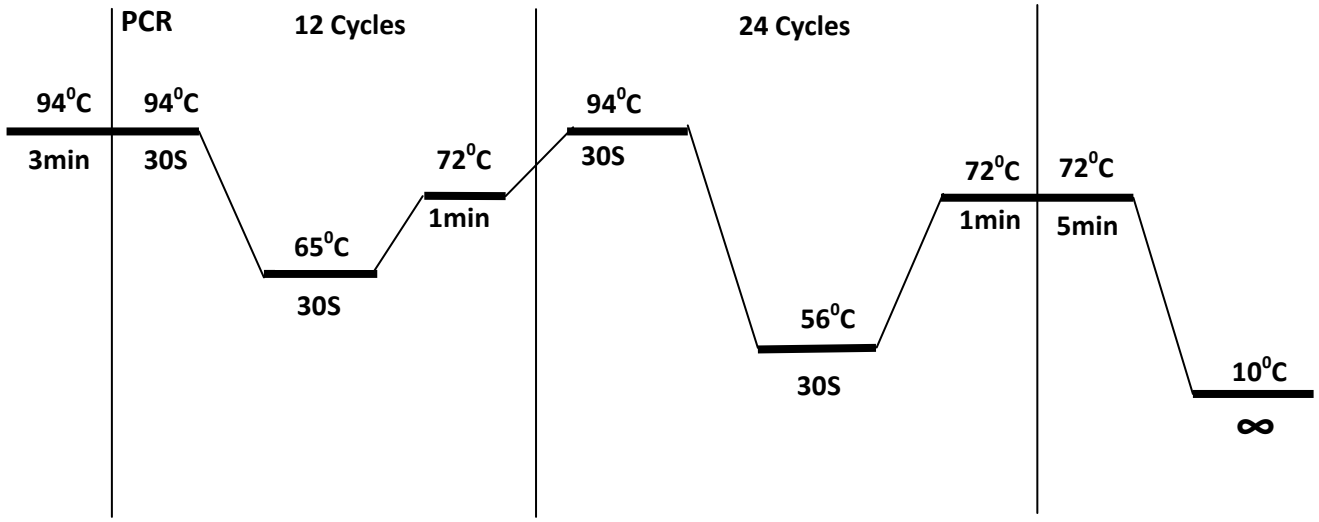


Figure 8b. Selective Amplification PCR protocol; the touchdown profile of the *Li-Cor* PCR

2.5 Data Analysis

Genetic map construction

The linkage map of DH88 was supposed to be constructed using JoinMap 4.0 based on single nucleotide polymorphism and amplified fragment length polymorphism (AFLP) (Pino del Carpio, 2010).

QTL Mapping Analysis

The population distribution was performed using The R Project for Statistical Computing (*R version 2.13.1)* and correlation analysis was performed using GenStat 14th edition statistical software. The *p-value* for each trait at specific growth time was determined. The p-*values* threshold was set at α=0.01 and α=0.05. This was done after the JoinMap failed due to too low numbers of the markers. The inadequacy of markers was caused by the failure of AFLP markers. Also Chinese cabbage and Pak choi are very close relatives both being leafy types and thus polymorphism rate was lower than for DH68.

3.0 RESULTS

3.1 Phenotypic Variation

In this study a Chinese cabbage DH line was used as female parent and a Pak choi DH line as male parent to develop the DH88 population. These plant materials differ in several traits; Chinese Cabbage is heading type with the following characteristics:- 1. Grows to a height of 200 – 500mm at vegetative stage and reaches 1.5m at reproductive stage, 2. It has a prominent taproot with dense fibrous lateral roots. 3 An enlarged rosette leaves arrangement forming short conical head. The leaf size varies between 200 -900mm by 150-350mm and their shape depend on the growth stage; the outer leaves are dark green, narrow ellipsoid with long laminae and winged petiole while inner heading leaves are whitish-green, broad and subcircular. 4. The flowering stem contains the lanceolate leaves which are smaller than heading leaves and they clasp to the stem. 5. The heading varies between wrapped-over and joined-up. The wrapped-over type, (Leaves overlap at the top of the conical head), are densely leaved, round headed and mature early. They are better adapted to warmer climates. The heading can be used to classify the Chinese cabbage depending on degree of heading; non-heading, half-heading to completely headed types. Pak Choi is non-heading type with the following characteristics: 1. They are dull green and smooth vegetative stage 2. It's height is 150 – 300mm tall, reaches 700mm at generative stage. 3. Its leaves are arranged in a spiral manner not forming a compact head but spreading in groups of 15-30 leaves. 4. Have enlarged, needlelike/flattened petioles of 15 – 40mm wide and 5 – 10mm thick. They grow upright forming subcylindrical bundle. 5. Their stems are tender, smooth, shiny green to dark green and articulate clasping. 6. The leaf blade greenish-white in colour is orbicular to obovate 70 – 200 by 70 – 200mm (Dixon G. R., 2006, Toxopeus & Baas, 2004 and Zhao et al., 2005).

Leaf characteristics including leaf hairiness, leaf number and dynamic leaf traits are important morphological traits distinguishing vegetable *B. rapa* morphotypes. The traits were scored as described in previous chapter. The normal distribution of these traits is shown in Appendix 2. The statistics for all observed traits are summarized in table 4. The DH88 population and their parents had slight differences in dynamic leaf traits. The LL, LaL, PL, LW, LI, PD, PH and LN traits which were scored quantitatively segregated continuously and the skewness values were below 1.0 in all stages except for plant height at stage 1. The data shows that the segregation of these quantitative dynamic leaf traits fit a normal distribution for all growth stages. The heading showed a progressive formation, with the number of plants that were

heading increasing with every scoring time. The DH88 population segregated for heading with 42% having no head, 47% had incomplete head formed and 11% had complete head formed. The PC101 did not form head as expected while CC048 exhibited a progressive heading and by the end of research time it had a complete head formed. Leaf color, leaf hairiness, heading and tillering traits were qualitatively scored thus had definite groups. The different grades of leaf hairiness frequency (0, 1, 3 and 4) had a normal segregation. The female parent, CC048 had a score value of 5 indicating high density of leaf hair on both adaxial and abaxial surfaces. The male parent, PC101 had a score of 0 meaning that it had no hair on either side neither on the leaf edge. The normal distribution graph shows two equal groups for leaf hairiness. The number of leaves increased rapidly making it impossible to count them without damaging them. This made it possible to have only consecutive two readings during plant development. This trait was also hampered by the interlocking of leaves and presence of short leaves at the apical point. The frequency graph skewed towards the right due to the tremendous increase in number of leaves for some DH lines showing distortive segregation. 19.5% of the DH88 population developed tillers while both parents did not have tillers. The intensity of chlorophyll was measured using SPAD to determine the leaf color. The range remained unchanged between the two readings and the amount increased in parents as well as in the DH88 population (Figure 9). The skewness was less than 1.0 and the data shows a segregation fitting a normal distribution. The minimum values were far below the parental values and maximum values were almost similar to that of parent PC101. The segregation of these traits was continuously distributed among all the DH lines, but showed significant transgressive segregation. The skewness for all the traits was less than 1, indicating their normal distribution. Additionally where transgressive segregation was observed, indicated that both parents transmitted favorable alleles for the trait. The first five stages of development of lamina width, and all stages of leaf length showed distortive segregation in favor of Pak choi genotype (figure 9 and appendix 2).

Table 4: Descriptive statistics of the DH88 population and the mean values of the parents. Std Dev. – Standard Deviation and CoV – Coefficient of Variance)

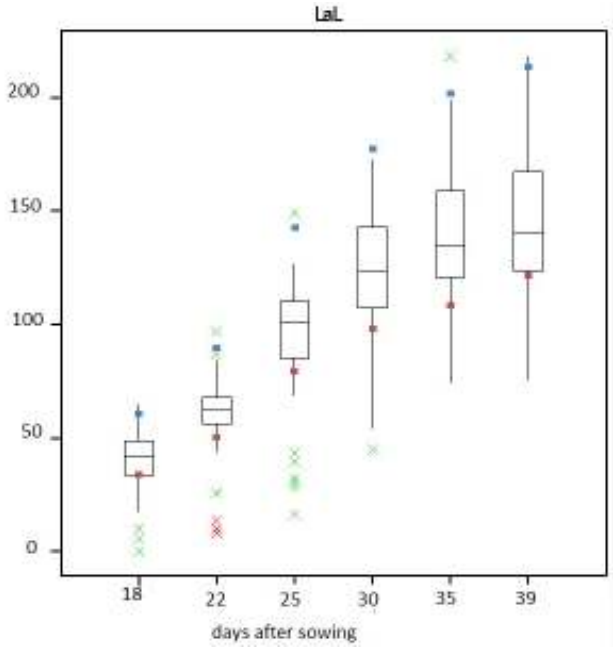
Table 4a – summary of leaf length (LL), lamina length (LaL), petiole length (PL), lamina width (LW), leaf index (LI), plant diameter (PD) and plant height (PH)

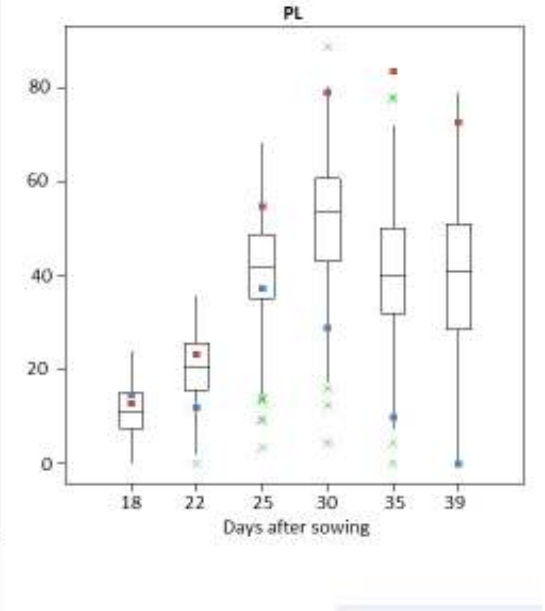
										PARENTS MEANS	
Trait	D.a.S*	Mean	Minimum	Maximum	Std Dev.	Range	CoV	Skewness	Kurtosis	CC-048	PC-101
	18	51.20	6.40	87.00	16.97	80.60	33.15	-0.91	1.29	78.50	50.00
	22	81.50	8.00	131.00	20.03	123.00	24.58	-1.53	4.25	103.33	77.67
	25	136.80	25.53	214.30	32.93	188.77	24.07	-1.54	3.37	179.33	137.00
LL	30	174.70	69.33	242.30	35.01	172.97	20.04	-1.12	1.71	206.00	178.00
	35	180.40	113.30	241.70	29.29	128.40	16.23	0.11	-0.75	213.33	198.33
	39	183.70	126.00	239.50	27.89	113.50	15.19	0.28	-0.86	214.00	198.33
	18	39.76	5.40	65.00	12.94	59.60	32.54	-0.95	1.50	63.00	36.50
	22	61.15	8.00	97.00	14.81	89.00	24.22	-1.45	3.89	90.67	49.67
LaL	25	95.80	16.47	149.00	23.57	132.53	24.60	-1.30	2.24	142.00	79.67
	30	122.00	44.77	173.00	26.96	128.23	22.11	-0.58	0.13	177.50	98.67
	35	138.50	74.00	218.00	27.25	144.00	19.67	0.31	-0.045	202.33	110.00
	39	144.00	76.00	218.50	28.88	142.50	20.05	0.07	-0.55	214.00	121.67
	18	11.20	1.00	24.00	5.66	23.00	50.52	0.13	-0.52	15.50	13.50
	22	19.86	0.00	35.67	8.33	35.67	41.92	-0.42	-0.14	12.67	28.00
PL	25	39.71	3.33	68.33	14.03	65.00	35.33	-0.64	0.41	37.33	57.33
	30	50.96	4.33	88.67	17.13	84.34	33.62	-0.47	0.089	28.50	79.33
	35	40.34	0.00	78.00	16.36	78.00	40.55	0.034	0.043	10.67	76.67
	39	39.43	0.00	79.00	17.47	79.00	44.30	-0.23	-0.30	0.00	88.33
	18	31.73	7.25	49.50	9.16	42.25	28.85	-1.30	2.64	48.00	32.00
	22	46.07	10.00	63.00	9.93	53.00	21.54	-1.72	3.98	58.67	41.00
LW	25	72.13	15.13	120.30	16.54	105.17	22.94	-123	2.95	94.67	66.33
	30	86.79	42.33	109.30	14.97	66.97	17.24	-0.99	0.55	109.33	83.33
	35	94.63	59.67	120.30	13.31	60.63	14.06	-0.36	-0.61	113.33	92.67
	39	96.96	68.50	135.50	15.09	67.00	15.56	0.049	-0.57	116.50	92.67
	18	1.21	0.74	1.31	0.27	0.57	22.04	-2.25	8.68	1.31	1.14
	22	1.32	0.80	1.54	0.17	0.74	12.69	0.064	1.82	1.55	1.21
LI	25	1.33	1.09	1.24	0.15	0.15	11.12	0.119	0.112	1.50	1.20
	30	1.40	1.06	1.58	0.17	0.53	11.96	0.098	-0.50	1.62	1.18
	35	1.47	1.24	1.81	0.21	0.57	14.22	0.16	-0.46	1.79	1.19
	39	1.49	1.11	1.61	0.22	0.50	14.92	-0.18	-0.64	1.84	1.31
	18	11.86	2.80	26.75	3.31	23.95	27.86	0.78	4.63	17.50	11.00
	22	13.84	5.25	21.67	2.57	16.42	18.54	-0.27	1.74	20.73	12.13
PD	25	19.18	6.23	30.20	3.95	23.97	20.57	-1.11	2.57	28.23	18.43
	30	25.71	11.03	43.00	5.45	31.97	21.18	0.006	1.37	36.70	24.57
	22	6.69	2.85	46.00	5.02	43.15	75.00	6.60	46.51	6.00	5.60
PH	25	9.59	2.50	14.37	2.20	11.87	22.90	-0.99	1.55	9.00	8.07
	30	13.76	4.00	19.90	3.12	15.90	22.68	-0.63	0.87	13.00	12.50

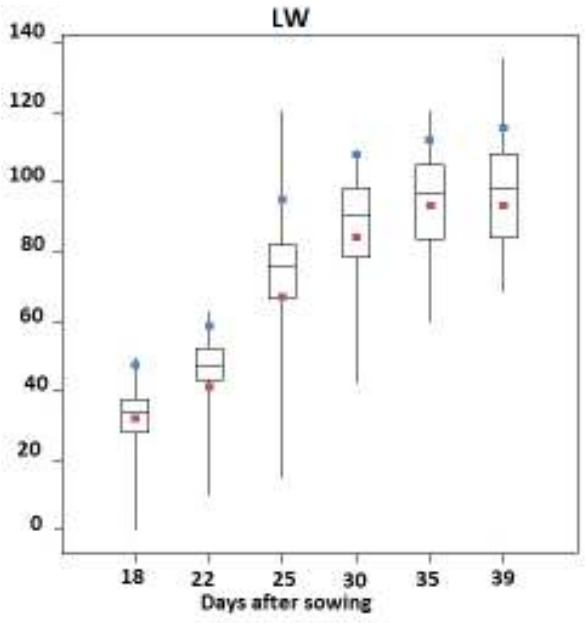
Table 4b: Statistical summary of leaf number (LN), leaf hairiness (LH), Tillers (T,) leaf chlorophyll (LCh), and Heading (HD)

										PARENTS MEANS	
Trait	D.a.S*	Mean	Minimum	Maximum	Std Dev.	Range	CoV	Skewness	Kurtosis	CC-048	PC-101
LN	25	3.60	1.00	5.00	1.86	4.00	17.16	-0.74	1.68	4.00	3.67
LN	30	10.85	5.00	14.00	0.68	9.00	18.85	-1.21	1.38	13.00	11.33
LH	25	2.26	0.00	4.67	1.67	4.67	73.69	0.02	-1.74	4.67	0.00
T	94	0.08	0.00	1.00	0.19	1.00	229.00	2.64	7.71	0.00	0.00
LCh	15	25.55	13.05	32.47	3.04	19.42	11.89	-0.53	2.31	24.97	28.40
	27	28.29	20.90	35.43	2.99	14.53	10.52	-0.07	-0.28	33.87	35.37
HD	56	1.09	1.00	2.00	0.22	1.00	19.78	2.79	7.52	1.67	1.00
	70	1.27	1.00	2.67	0.46	1.67	36.09	1.49	0.84	2.33	1.00
	84	1.48	1.00	3.33	0.68	2.33	45.78	1.19	0.056	3.00	1.00
	97	1.94	1.00	4.00	1.03	3.00	52.97	0.77	-0.78	3.67	1.00
	108	1.77	1.00	4.00	1.10	3.00	61.88	1.09	-0.43	3.67	1.00
	114	1.57	1.00	4.00	0.83	3.00	53.07	1.64	1.70	4.67	1.00

** D.a.S refers to Days after sowing*


LaL
200
150
100
50
0
18
22
25
30
35
39
days after sowing


PL
80
60
40
20
0
18
22
25
30
35
39
Days after sowing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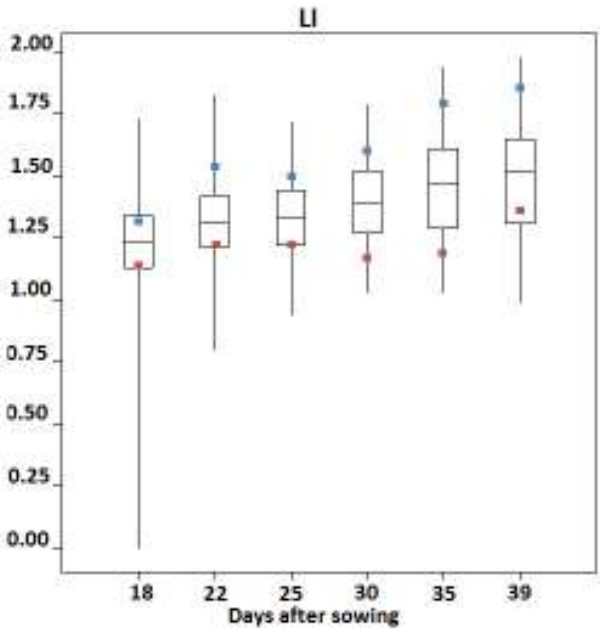
120
100
80
60
40
20
0
18
22
25
30
35
39
Days after sowing


LI
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
18
22
25
30
35
39
Days after sowing

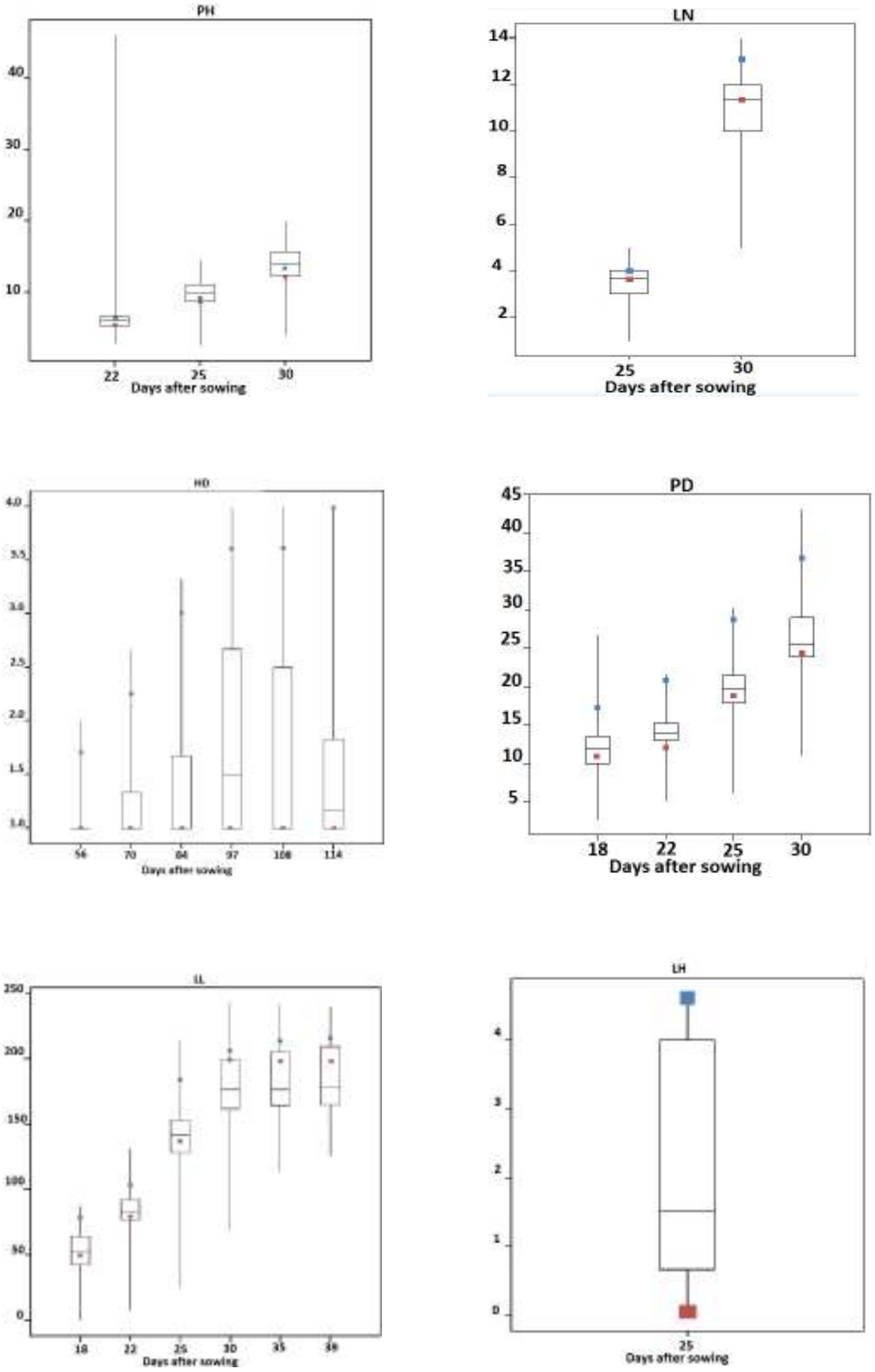
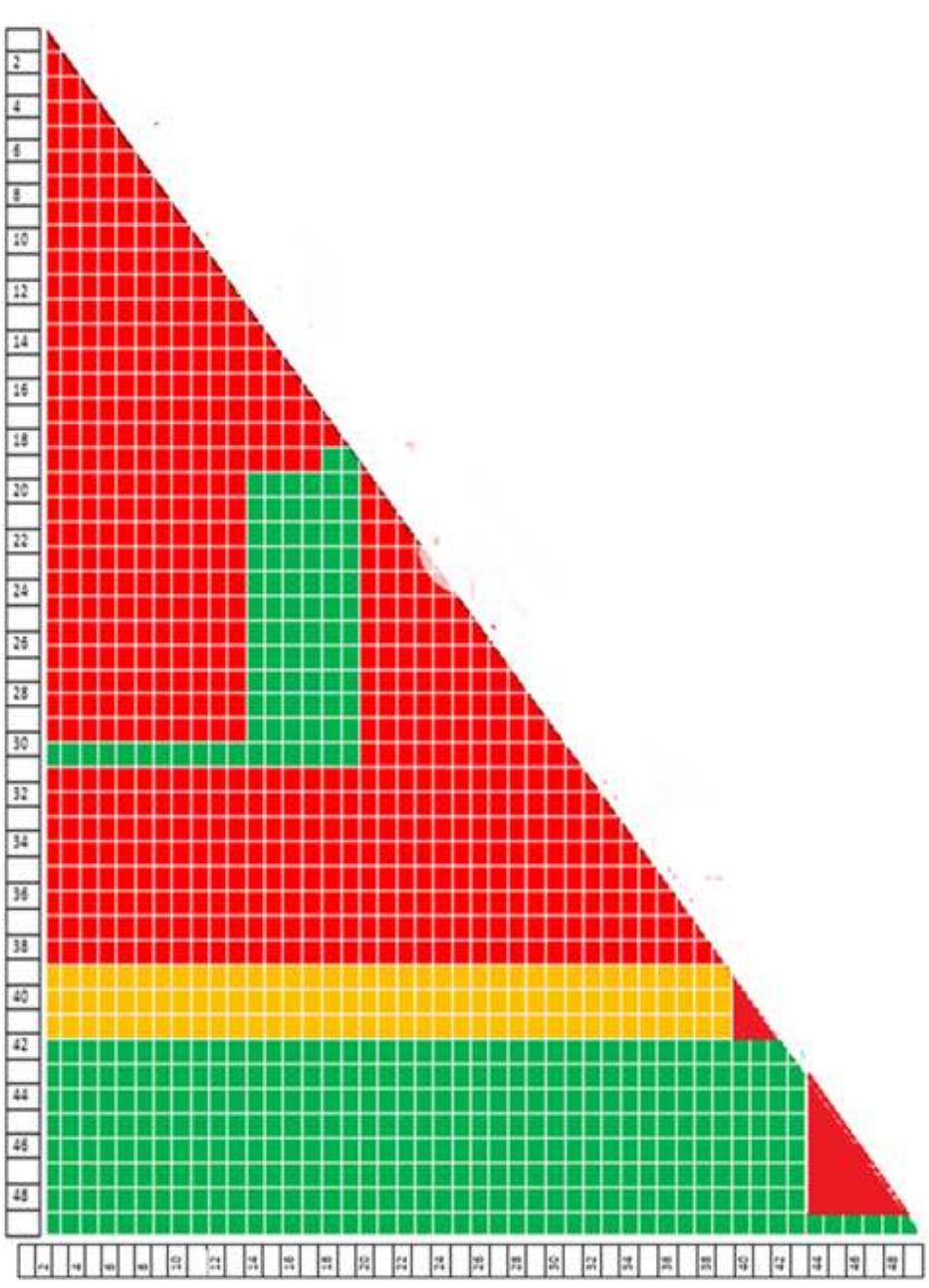


Figure 9: Diagram showing the box plots for the leaf traits of DH88 population and their parent lines indicated by the dots; ■ Pak Choi and ■ Chinese cabbage

The correlation analysis was done using GenStat 14th Edition, to determine the correlation strength among the traits under the study (Figure 10 and appendix 5). There is a strong positive correlation among leaf length, lamina length, petiole length, lamina width, leaf index, plant diameter and plant height. But the last two scoring stages of lamina width show a weak negative correlation with the lamina length, leaf index and petiole length. Leaf hairiness, heading and tillering had negative correlation with other traits.



Code	D.a.S.	Trait
1	18	LL
2	22	LL
3	25	LL
4	30	LL
5	35	LL
6	39	LL
7	18	LaL
8	22	LaL
9	25	LaL
10	30	LaL
11	35	LaL
12	39	LaL
13	18	LW
14	22	LW
15	25	LW
16	30	LW
17	35	LW
18	39	LW
19	18	LI
20	22	LI
21	25	LI
22	30	LI
23	35	LI
24	39	LI
25	18	PL
26	22	PL
27	25	PL
28	30	PL
29	35	PL
30	39	PL
31	18	PD
32	22	PD
33	25	PD
34	30	PD
35	22	PH
36	25	PH
37	30	PH
38	25	LN
39	30	LN
40	15	LCh
41	27	LCh
42	25	LH
43	56	HD
44	70	HD
45	84	HD
46	97	HD
47	108	HD
48	114	HD
49	94	T

Figure 10. A simplified correlation diagram among all traits. Red color indicate positive strong correlation, orange indicate positive weak correlation and green indicate neutral to weak negative correlation. (Simplified from appendix 5). D.a.S. – Days after sowing

Table 5. A table of markers genetically mapped in DH88, their corresponding genes in *B. rapa* and in *A. thaliana* as well as their functions – referenced from NCBI, and TAIR websites.

Corresponding AGI	**Gene Family**	***A.t* Chr**	***B.rapa* Gene**	***B.rapa* Chr.**	**Marker name**	**Gene Function**
AT3G05040	HST1	3	Bra040117	A01	BrHST1P1b	Specification of adaxial/abaxial axis, negative regulation of flower development, leaf morphogenesis
AT1G32240	KAN2	1	Bra021216	A01	BrKAN2P1b	Polarity specification of adaxial/abaxial axis, works together with KAN1 & KAN4 to regulate the proper localization of PIN1 in early embryogenesis
AT4G34160	CYCD3;1	4	Bra011501	A01	BrCycD3;1P1a	Involved in the switch from cell proliferation to the final stage of differentiation.
AT4G17695	KAN3	4	Bra040176	A01	BrKAN3P1b	Involved in abaxial-adaxial polarity, DNA binding transcription factor activity
AT3G13960	ATGRF5	3	Bra021521	A01	BrGRF5P1b	ATP binding in leaf morphogenesis and development
AT5G53950	CUC2	5	Bra022685	A02	BrCUC2P1b	Leaf development & morphogenesis. Mutants results in small leaves
AT1G68480	JAG	1	Bra033931	A02	BrJAGP1a	Proper lateral organ shape thus specify the abaxial cell fate and leaf morphogenesis
AT1G70510	KNAT2	1	Bra007920	A02	BrKNAT2P1a	KNAT2 acts synergistically with cytokinins and antagonistically with ethylene
AT5G07200	GA20OX3	5	Bra028706	A02	BrGA20OX3P1a	Gibberellins biosynthesis process
AT4G00220	NUB	1	Bra008514	A02	BrJAGP1c	Specification between lateral organs and meristem. When overexpressed it activates STM and KNAT1 but down-regulates PIN1
AT1G68480	JAG	1	Bra033931	A02	BrJAGP1b	Proper lateral organ shape thus specify the abaxial cell fate and leaf morphogenesis
AT1G70210	CYCD1;1	1	Bra007901	A02	BrCYCD1;1P1a	Encodes a D-type cyclin that physically interacts with CDC2A and its expression may contribute to spatial and temporal regulation of cell division in plants

AT1G73590	PIN1	1	Bra008105	A02	BrPIN1P1a	Involved in the determination of leaf shape by actively promoting development of leaf margin serrations.
AT4G37740	GRF2	4	Bra017851	A03	BrGRF2P2b	Responsible in leaf size development
AT4G08150	KNAT1	4	Bra000638	A03	BrKNAT1P1a	meristem initiation, Its repression by AS1 and Auxins results in the promotion of leaf fate
AT2G36985	ROT4	2	Bra023090	A03	BrROT4P1a	Involved in controlling polarity-dependent cell proliferation.
AT5G07200	GA20OX3	5	Bra005927	A03	BrGA20OX3P2a	Gibberellins biosynthesis process
AT2G44080	ARL	2	Bra000340	A03	BrARLP1a	Involved in cell expansion-dependent organ growth.
AT1G54390	ING2	1	Bra040776	A03	BrIng2P1a	
AT4G34580	COW1	4	Bra017663	A03	BrCow1P2a	Cell tip and development growth.
AT5G13910	LEP	5	Bra006222	A03	BrLEPP1a	Acts as a positive regulator of gibberellic acid-induced germination. Petiole differentiation and expansion. Leaf senescence
AT2G45190	YAB1	2	Bra000378	A03	BrYAB1P1a	Involved in abaxial cell type specification in leaves and fruits
AT2G27100	SE	4	Bra034322	A04	BrSEP1a	Regulation of adaxial/abaxial pattern formation, regulates meristem development
AT2G23380	CLF	2	Bra032169	A04	BrCLFP1b	Control of leaf morphogenesis - Mutants exhibit curled & involute leaves
AT1G48410	AGO1	1	Bra032254	A05	BrAGO1P1a	Auxin metabolic process & polar transport. Also leaf morphogenesis and lateral expansion
AT2G36985	ROT4	2	Bra005225	A05	BrROT4P2a	Involved in controlling polarity-dependent cell proliferation.
AT1G30490	PHV	1	Bra025036	A06	BrPHVP1a	Dominant PHV mutations cause transformation of abaxial leaf fates into adaxial leaf fates. Trichome morphogenesis

AT5G60690	REV	5	Bra038295	A06	BrREVP2b	Polarity specification of adaxial/abaxial axis, Trichome morphogenesis
AT2G27100	SE	4	Bra025684	A06	BrSEP2a	Regulation of adaxial/abaxial pattern formation, regulates meristem development
AT1G52150	CAN	1	Bra018948	A06	BrCNAP1a	A HD-ZIPIII member critical for vascular development and negatively regulates vascular cell differentiation
AT2G17950	WUS	2	Bra024485	A06	BrWUSP1b	Regulates WUS transcription. This indirectly activates CLAVATA3.
AT1G20610	CycB;3	1	Bra025831	A06	BrCycB2;3P1a	Cyclin-dependent protein kinase regulator activity and protein binding
AT1G69440	AGO7	1	Bra011904	A07	BrAGO7P1a	Involved in development timing and is localized in the mature rosette leaves and floral buds
AT1G69440	AGO7	1	Bra003999	A07	BrAGO7P2a	Involved in development timing and is localized in the mature rosette leaves and floral buds
AT5G16560	KAN1	5	Bra015130	A07	BrKAN1P2a	Adaxial/abaxial specification - i.e. it promotes the abaxial cells
AT2G45190	YAB1	2	Bra003309	A07	BrYAB1P3a	Involved in abaxial cell type specification in leaves and fruits
AT1G69690	TCP15	1	Bra004407	A07	BrTCP15P2a	Involved in the regulation of endoreduplication. Expressed at various leaf developmental stages
AT1G48410	AGO1	1	Bra014136	A08	BrAGO1P2a	Auxin metabolic process & polar transport. Also leaf morphogenesis and lateral expansion
AT1G48410	AGO1	1	Bra014136	A08	BrAGO1P2b	Auxin metabolic process & polar transport. Also leaf morphogenesis and lateral expansion
AT1G09700	HYL1	1	Bra030793	A08	BrHYL1P2a	Promote flower development and prevents leaf bending (hyponasty)
AT1G08465	YAB2	1	Bra030728	A08	BrYAB2P3a	Abaxial cell fate specification
AT4G34580	COW1	4	Bra034638	A08	BrCow1P3a	Cell tip and development growth.

AT1G16330	CYCB3;1	1	Bra016640	A08	BrCycB3;1P2a	Core cell cycle
AT4G14720	PPD2	4	Bra039720	A08	BrPPD2P2a	Deletion of the PPD locus increases leaf lamina size and results in dome-shaped rather than flat leaves.
AT1G20610	CycB;3	1	Bra016460	A08	BrCycB2;3P2a	Cyclin-dependent protein kinase regulator activity and protein binding
AT1G26770	AtEXP10	1	Bra024686	A09	BrEXP10P1b	For loosening cell wall and thus involved in multidimensional cell growth
AT2G26330	ER	2	Bra007759	A09	BrERP1a	Specification of organs originating from SAM. Polarity of adaxial/abaxial axis
AT2G26330	ER	2	Bra007759	A09	BrERP1b	Specification of organs originating from SAM. Polarity of adaxial/abaxial axis
AT1G32240	KAN2	1	Bra023254	A09	BrKAN2P3a	Polarity specification of adaxial/abaxial axis, works together with KAN1 & KAN4 to regulate the proper localization of PIN1 in early embryogenesis
AT1G32240	KAN2	1	Bra039528	A09	BrKAN2P4a	Polarity specification of adaxial/abaxial axis, works together with KAN1 & KAN4 to regulate the proper localization of PIN1 in early embryogenesis
AT1G23380	KNAT6	1	Bra024593	A09	BrKNAT6P2a	Expression is increased in AS and BOP1 leaf mutants.
AT5G66190	FNR1	5	Bra037182	A09	BrFNR1P2b	Encodes a leafy-type ferredoxin(NADPH) oxidaoreductase. Transfer of noncyclic electron transport pathway of photosynthesis activity.
AT3G50070	CYCD3;3	3	Bra036051	A09	BrCycD3;3P3a	Determines cell number in developing lateral organs. Mediate cytokinin effects in apical growth & development
AT3G50630	KRP2	3	Bra036095	A09	BrKRP2P2a	Encodes CDK inhibitor, negative regulator of cell division. And is expressed in leaf lamina base and leaf apex
AT1G01510	AN	1	Bra033258	A10	BrANP1b	Involved in polar cell expansion and leaf width direction
AT5G53950	CUC2	5	Bra003023	A10	BrCUC2P2a	Leaf development & morphogenesis. Mutants results in small leaves
AT5G07200	GA20OX3	5	Bra009285	A10	BrGA20OX3P4b	Gibberellins biosynthesis process

Flowering time (FT) was scored in terms of days from transplanting to the appearance of first open floral buds in the centre of the rosette. The flowering time was scored from the start of the experiment till 122 days from transplanting; then it will be continued by Xiao Dong to score the lines, which have not yet flowered. Those results are not included in this thesis. During this period 32 lines flowered and the rest did not flower. The earliest line to flower was DH88 241, which flowered after 33 days from transplanting in the 3 blocks. There was for certain genotypes variation in flowering time between blocks. For instance, in block 2 the male parent PC101 flowered after 69 days and 123 days after transplanting in block 1 but did not flower in block 3. The female parent CC048 flowered after 111 days in block 1, however in the other blocks did not flower. Both parental lines did not flower in block 3 (see table appendix 3).

3.2 Genetic Linkage map

One hundred and sixty six PCR-based single markers that could amplify genes related to morphological leaf characteristics were developed from *A. thaliana* information resource "TAIR" (http://www.arabidopsis.org/) utilizing the close relationship between *B. rapa* and *A. thaliana*. The functions of these genes to morphological leaf traits in *A. thaliana* are described in table 5. The primer design involved the following parameters: the target product size of 100 – 300bp, with primer size between 18 – 22bp in length. The melting temperature (Tm) was at range of 58 – 60oC and G-C % was maintained at 40% as the optimum level. The primers were named based on the gene nomenclature system of Brassica defined by Østergaard and King (2008), ordering from left to right (genus–species–gene–name–genome–locus). The *A. thaliana* genes have an annotation "AT" while *Brassica* genes have "Bra" (table 5)

One hundred and sixty six SNP markers were screened for polymorphism with the two parents. Only fifty seven markers were polymorphic between parents of DH88 and were used to profile the whole DH88 population under study using the LightScanner applications. Figure 11 shows the melting points and grouping of population according to these temperatures. The positions of these 57 *B. rapa* polymorphic markers were used to search in *Brassica* database (BRAD) http://brassicadb.org/brad/searchAll.php) to obtain their physical position on *B. rapa* scaffolds (in table 5 markers are arranged according to their map position).

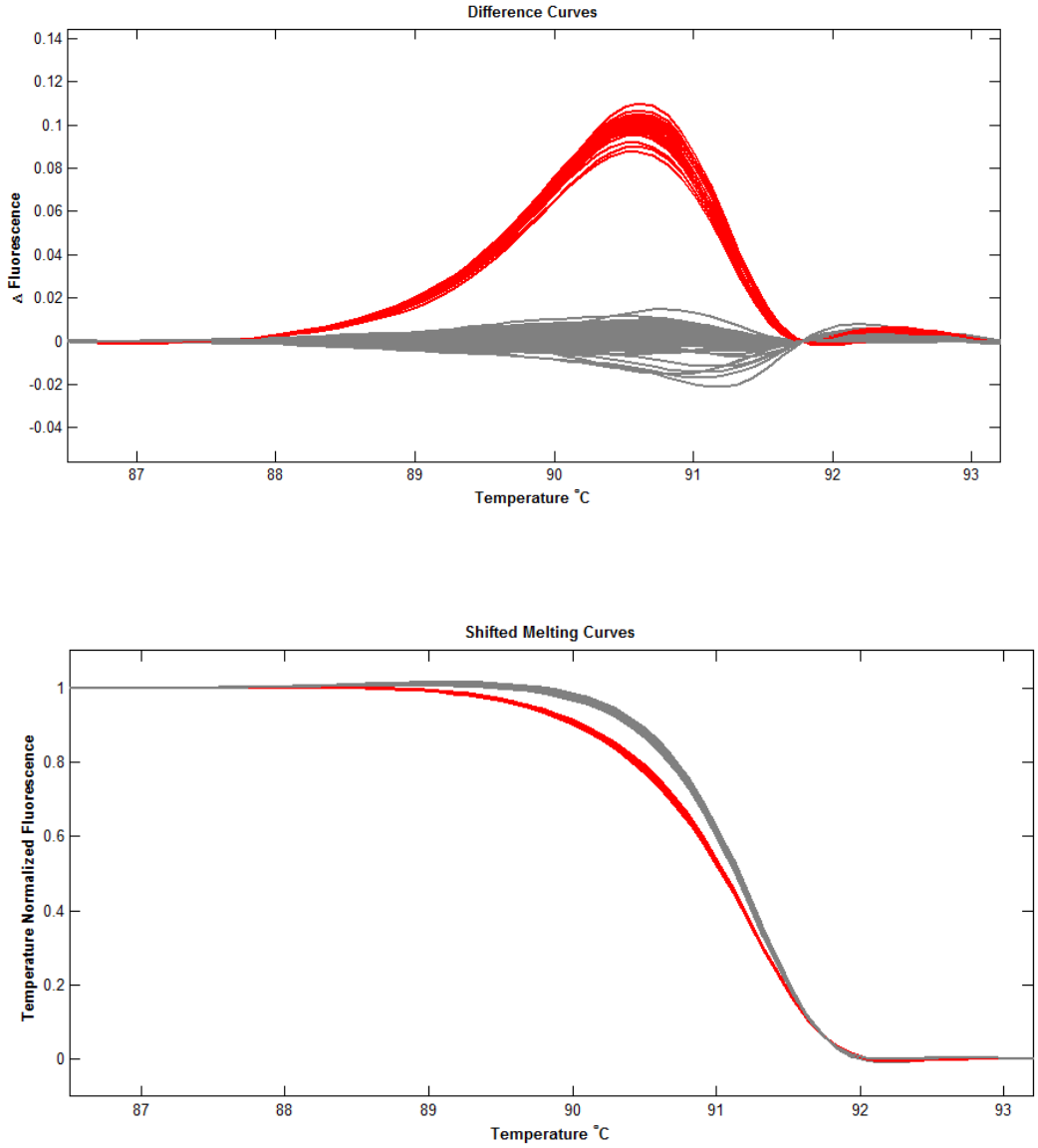


Figure 11. High resolution melting curves of allelic expression variants of a sample gene locus. The red curve represents one allele from one parent and grey curve represents the other allele from the other parent. The melting curve normalization was done with LightScanner software (Idaho Inc., UT, USA).

The lower graph shows the normalized curve at 1unit temperatue difference at minimum and maximum temperatures. Upper curve shows the groupings as per the temperature difference. Two clear distinct groups are observed.

Fifty seven SNP markers polymorphic over DH88 population were not enough to develop genetic map, more markers were required. The AFLP method was tested on 8 DH88 lines and the parental lines with six primer combinations (table 6). The AFLP - *Li-cor* protocol as described in Vos et al. (1995) and in The Protocols and Rules in Laboratory of Plant Breeding, Wageningen University (2008) was followed to the latter. Two DNA concentration levels of 50ng/µl and 100ng/µl were tested. Two dilution levels of 10X and 20X at digestion and ligation step as well as at pre-amplified stage were tested. This was done to try to diagnose why no marker products were produced. The

parental lines and 8 randomly selected DH88 lines were screened for polymorphism with 6 AFLP primer combinations (table 6).

Table 6. The primer combination for AFLP markers tested in this study

IRD	*EcoRI*	*MseI*
800	E33	M47
	E31	M60
	E41	M62
700	E33	M51
	E35	M62
	E42	M51

A combination of 100ng/µl and 20X dilution of pre-amplified without diluting the digestion and ligation product gave good results of a clear smear of bands around 500bp in agarose gel as shown in figure 12. This combination was used for selective amplification step, which unfortunately did not yield any marker results.

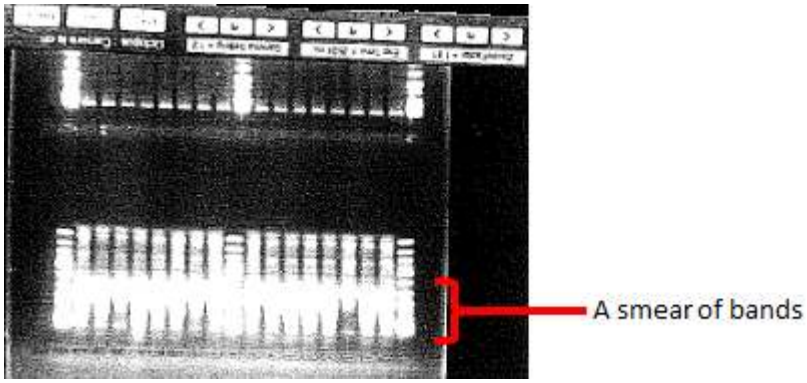


Figure 12. A diagram of agarose gel to check the quality of pre-amplified product for AFLP–*Li-Cor* gel showing a smear of band at 500bp.

3.3 Genetic Linkage Analysis:

The linkage association was determined by analyzing the phenotypic and genotypic data by one-way analysis of variance and the *p-value* calculated. The markers were considered as classes and the population lines while corresponding genotypes were considered as observations for the marker class. The distribution of the individual genetic marker types varied along the linkage groups. There were 10 linkage groups named orderly as LG01 to LG10. The variance for the phenotypic values was calculated and a p-value < 0.05 was set as being significant thus terming the marker to be linked to the trait. If the variance for the genotype class is significant, then the molecular marker used to define the genotype class is considered to be associated with the trait, and considered a QTL. In this study a total of forty-nine traits and 57 markers were evaluated enabling a combination of 2793 possible

markers trait association. Out of these, 211 marker trait associations were significant having a P-value < 0.05 across the all traits under study over all markers. A more strict p-value of <0.01 was used and significant values below this p-value were 52 marker trait associations while the remaining 159 were between 0.01> p <0.05. The analysis enabled the association of gene functions to their location on the chromosomes and the phenotype trait. The locations of the genes that affect the phenotype were indicated by the color segments and 57 SNP molecular markers were used in the linkage analysis for the DH88 population (Table (7). The vertical axis shows the marker name, with gene name, organized in order of chromosome (linkage group) and position on the physical map of the reference Chiifu genome (Wang et al, 2011). The horizontal axis shows the leaf traits in developmental order. The number for QTLs were 27, 21, 19, 13, 30, 19, 9, 12, 6, 13, 35, and 8 for the traits LL, LaL, PL, LW, LI, PD, PH, LCh, LN,H, HD and T respectively when summarized for all developmental stages. For the analysis the QTLs in the same linkage group were regarded as one QTL. The major QTLs for all traits were in LG01, LG02 and LG06 except for hairiness and tillering.

Table 7. Table of QTLs analysis; horizontal axis shows leaf trait at various development stages while vertical axis shows the markers (gene-name, Linkage group and Bra code) ranked according to their physical map position. Red indicates QTL at p-value<0.01 and green show QTL at p-value between 0.01 and 0.05

Table 8. Table of the functional linkage association of markers to leaf trait at various stages of leaf development. On a threshold of p-value<0.05, a total of 26 genes were associated with the functional trait gene.

Gene Family	*B.rapa* Gene	Linkage group	Marker name	Gene Function	Associated trait
HST1	Bra040117	LG01	BrHST1P1b	specification of adaxial-abaxial axis	LL, PL, LW, and LI
KAN2	Bra021216	LG01	BrKAN2P1b	polarity of adaxial-abaxial axia	LI and LCh
CYCD3;1	Bra011501	LG01	BrCycD3;1P1a	cell proliferation	LL, PL, PD, PH and LN
CUC2	Bra022685	LG02	BrCUC2P1b	leaf size	LaL
JAG	Bra033931	LG02	BrJAGP1a	lateral organ shape/abaxial-adaxial fate	HD
KNAT2	Bra007920	LG02	BrKNAT2P1a	cell division and antagonatic to ethylene	LL, LaL, LW, PD and HD
JAG	Bra033931	LG02	BrJAGP1b	proper lareal organs and temporal cell division	HD
CYCD1;1	Bra007901	LG02	BrCYCD1;1P1a	Cell division	T
GRF2	Bra017851	LG03	BrGRF2P2b	Responsible in leaf size development	LW
KNAT1	Bra000638	LG03	BrKNAT1P1a	repress AS1	LL, LI and PD
COW1	Bra017663	LG03	BrCow1P2a	Cell tip and development growth.	LaL and PD
SE	Bra034322	LG04	BrSEP1a	adaxial/abaxial pattern	LI
ROT4	Bra005225	LG05	BrROT4P2a	control cell porality	LL, LW, LI, PH and LN
SE	Bra025684	LG06	BrSEP2a	adaxial/abaxial pattern	LI
CAN	Bra018948	LG06	BrCNAP1a	HD-ZIPIII	PL and PD
WUS	Bra024485	LG06	BrWUSP1b	maintain meristem	LL, Lal, LW and LI
KAN1	Bra015130	LG07	BrKAN1P2a	abaxial cell	LL, LaL, LI and HD
AGO7	Bra011904	LG07	BrAGO7P1a	timing development	LI, LCh, HD and T
AGO7	Bra003999	LG07	BrAGO7P2a	timing development	LI, LCh, HD and T
AGO1	Bra014136	LG08	BrAGO1P2a	lateral expansion	HD
AGO1	Bra014136	LG08	BrAGO1P2b	lateral expansion	HD
PPD2	Bra039720	LG08	BrPPD2P2a	lamina size, leaf shape	LaL, LI and PD
AtEXP10	Bra024686	LG09	BrEXP10P1b	multidimensional cell growth	LaL and LW
CYCD3;3	Bra036051	LG09	BrCycD3;3P3a	lateral organ/cytokins	PH
AN	Bra033258	LG10	BrANP1b	leaf expansion and direction	PD
CUC2	Bra003023	LG10	BrCUC2P2a	Leaf size	LL, LaL and PD

4.0 DISCUSSION

Chinese cabbage (CC) and Pak choi (PC) are both leafy type vegetables of *B. rapa*. This makes them closely related phenotypically and genetically though they have few distinctive features like CC is heading, with hairy leaves while PC is non-heading with smooth leaves. Dynamic leaf traits and flowering time are important traits in the lifecycle of angiosperm crops. Leaf traits are the main trait for photosynthetic efficiency while flowering time defines the transition from vegetative to reproductive growth in plants. Chinese cabbage and Pak choi are cultivated for use as leafy vegetable making leaf traits important traits for study. Generally, the main objectives of breeding of *B. rapa* is to increase yield, improve agronomic traits as well as the quality (Zhao et al., 2007). The yield, agronomic traits and quality in *B. rapa* depends on the leaf traits. And thus among the traits under this study all were very important ; LL, LaL, PL, LW, PD, PH and LN have great influence on yield, hairiness (H) and heading influences the quality, while leaf colour and tillering influences the quality and yield. Table 5 shows the various markers, their corresponding genes in *B. rapa* and functions. The gene functions were studied by looking at their role in other species, to try to understand if they were the causative genes for the traits at different growth times.

The use of molecular markers offers infinite opportunities to explore, select and develop QTLs influencing the leaf traits in *B. rapa*. An important feature of molecular marker-facilitated analysis of QTL is the ability to dissect complex traits into individual components (Song et al 1995). In this study, the phenotypic and their genetic values of leaf traits were used for QTL analysis. There were 211 QTLs detected over all traits under the study with p-value below 0.05 (Table 7) though in this study fewer QTLs were expected based on the fact that we screened only a relative small DH population with a limited number of markers. These markers were mapped by use of LightScanner applications and are SNP markers, which offer important contributions to the growing number of gene-based markers. The small deletions and insertions results in different melting curves can bring confusion between the SNP and small indels. The results from this study demonstrate the usefulness of SNP markers in dissecting genetic components of quantitative traits in *B. rapa* (Song et al 1995). Various markers were associated to certain traits at different developmental stage linking the functions of the corresponding genes. From 166 SNP leaf trait gene markers developed, 57 were polymorphic in DH88 population. These markers were inadequate to construct the genetic map using the program Joinmap 4.0 (Kyazma, Wageningen, The Netherlands) (http://www.kyazma.nl). As no AFLP data were obtained, total marker numbers were too low to construct a genetic map. Instead, a semi physical map was constructed by placing the genetic markers, candidate leaf trait genes, on the physical map using annotated gene position number by blast the Brassica databases

(BRAD) (http://brassicadb.org/brad/search All.php). The 10 linkage groups of *B. rapa* were named LG01 to LG10 corresponding to the reference maps (http://www.brassica-rapa.org/BRGP/chromosomeSequence.jsp) of Chiffu genome (Appendix 4). Out of these 57 SNP markers, 26 were associated with the various traits using the known functional gene trait (Table 8).

The improvement of agronomic traits like leaf traits have been a major breeding objective in *Brassica species*. This is demonstrated by the enormous morphotypes of *B. rapa* developed since domestication. The analysis of QTLs controlling morphological leaf traits and heading has been intensively investigated. There are reports concerning the relationship between different morphotypes in terms of the leaf morphology in *B. rapa*. Different genes are expressed at different growth stages. The use of single development stage can make genetic information be undetected (Yan et al 2009). In this study multiple development stages were evaluated to help in making detailed genetic information. For instance the gene Bra011501 involved in cell proliferation and Bra040117 involved in leaf morphogenesis were both in LG01 and could be associated with leaf length from 2nd and 3rd stages till 6th stage respectively. The gene Bra014136 in LG08 involved in auxin process and lateral expansion can be associated with leaf index in the first four stages of development. Also leaf traits are quantitative traits and respond to both environmental and hormonal cues. The several QTLs found illustrate that the leaf traits under this study were quantitatively inherited and could be controlled by multiple genes. These QTLs together with fact that the mean values of the DH88 population were quite close to the parental values for all traits or at the middle of parental values (Table 4). Additionally the significant variation and normal distribution of all traits measured in this study suggest the suitability of the population for QTL analysis. That is, it explains a polygenic inheritance in DH88 population.

In order to evaluate the marker trait association, we checked whether the markers were detected in different leaf developmental stages. We looked at the *p-value* and tried to link the function with the gene trait. With this, various markers seemed to be associated with the traits. For instance CycD3;1 gene (Bra011501) in LG01 was significantly associated with LL from 2nd measurement till the 6th measurements, while HST1 (Bra040117) was significantly associated with LL from 3rd stage till 6th stage. These two genes are responsible for adaxial/abaxial axis and leaf morphogenesis (TAIR-http://www.arabidopsis.org). The CUC2 gene (Bra022685) in LG02 with the function of leaf development & morphogenesis was associated with lamina length, significant at stage 5 and 6. Mutants in CUC2 result in small leaves. This marker was also associated with plant height and is significant at stage 1 only. The QTLs having only one time-point per trait being significant and the one which cannot be associated with the gene functions should be treated with caution; we cannot

exclude that these are false positive. The PPD2 gene (Bra039720) in LG08 involved in lamina size in *A. thaliana*, can be associated with final stage of lamina length had a QTL for leaf hairiness and thus the gene very unlikely explains leaf hair trait.

The leaf chlorophyll content was positively correlated to leaf index, leaf length and lamina length. This can be concluded as being due to enhancement of photosynthesis efficiency. In a similar study Dong et al 2007 found out that high chlorophyll content was positively correlated with photosynthetic capacity. A QTL for the gene KAN2 (Bra021216) in LG01 was associated with leaf index from 2nd stage to 4th stage and with leaf chlorophyll at 3rd stage of leaf development.

Positive additive effects means that the Chinese cabbage provides alleles for head formation and Pak choi provide for non-heading alleles, it is satisfying finding offspring with various head "types" as per the scale of heading 0-4. Chinese cabbage had bigger LL, LaL and LW and the diameter as expected thus presumed it provided alleles to increase the sizes of these traits. Some QTLs for LL, LaL and LW were found on the same linkage group. These traits have strong correlation (figure 10). Though still it is possible that parent 2 (Pak choi) with smaller sizes of these traits also provided alleles for size increment. This shows that the alleles increasing the LL, LaL and LW come from the two parents collectively (Yan et al, 2009). Three QTLs for plant height were mapped in F2 population of turnip rape and yellow sarson and six QTLs in RIL of Chinese cabbage (Song et al 1995 and Yu et al, 2003). In this study, we identified five QTLs for PH with a major QTL at LG07. The genetic effects of these QTLs varied at different measuring stages. This could be to the effect of gene action being discrete at distinct growth stage. This gene action divulges the genetic basis for the creation of the transgressive segregation for these traits. In this study, the marker for gene YAB1 in LG07 (BraYAB1P3a) paralogue was associated with plant height throughout development. This marker was associated also with petiole length, plant diameter, leaf number and leaf chlorophyll. This could be due to its influence and effect in meristem structural organization and vegetative growth (Tair- http://www.arabidopsis.org). Leaf index determined as a ratio between LaL and LW explains the leaf shape, which has an influence in photosynthesis efficiency influencing the chlorophyll intensity. The gene KAN2 (Bra021216) in LG01 colocates for the leaf index and leaf chlorophyll (leaf colour). Gene CNA (Bra018948) in LG06 is associated with both petiole length and plant diameter.

There were no AFLP bands observed from *Li-cor* gel despite a clear smear of band in agarose gel of the primary amplification, which indicated that the quality of pre-amplification product was good. This could have been due to various defect in the process, which include use of probably expired

primers/reagents, the primers could probably not amplify in the selective amplification. Also could be due to a technicality failure.

The flowering time showed variation within some DH lines in the three blocks (Appendix 3). These observations are hard to explain, as all blocks were in the same greenhouse compartment, only plants in block 1 were more spaced compared to those in block 2 and 3. This valuation can be partly be explained by this inhomogeneity of the environment among the blocks and probably the selfing during pollination was not always 100%.

5.0 CONCLUSION AND RECOMMENDATION

The aim of this study was to combine the phenotypic leaf scores and marker data in order to identify the genomic regions that effect the leaf traits in the *B. rapa*. In order to evaluate the marker trait association, we checked whether they were detected in different developmental stages, we looked at p value and tried to link function with gene traits. Twenty-six SNP markers associated with the leaf traits were identified in this study. These markers can be used useful for genetic mapping and QTL analysis of these important leaf traits which have great impact on quantity and quality of *B. rapa*. *Brassica rapa* contributes greatly to survival of man all over the world due to their great use. The characterization of the gene functions can be helpful in marker assisted breeding and introgression. This will speed up the breeding programs of *Brassica* species. Leaf traits are main economic part of these leafy vegetable and thus the markers found to be associated with these morphological traits, and development of tillers, further study is recommended to improve the genetic map with SNP markers in multiple seasons.

Flowering time is a major component in the lifecycle of plants and is very useful tools to growers. The growers used it as a guide to know where and when to grow which morphotype of *B. rapa*. Thus study for flowering time should be continued to ascertain the genes involved in this important trait.

The DH88 is an interesting population, as many QTLs were identified and trait segregation should be subjected for further studies to develop a genetic map. AFLP though it yields enormous marker, it is very expensive and time consuming. Application of SNP markers is more preferred as they are relatively less expensive and requires less time.

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APPENDICES

Appendix 1.

DNA extraction method using CTAB according to RETCH 1.3 protocol (Gert van Arkel & Maarten Nijenhuis)

For better results use this protocol for young/ immature non frozen plant material

Day before harvesting the plant material:

Put the 96 micronic tubes in a blue holder and mark them well

Add 2 steel balls to every tube and Check if there is enough Isolation buffer and other solutions *(volumes)*

DNA isolation day:

Put the water bath on and set it to 65°C

Put the 70% ethanol to the -20°C

Put the isolation buffer at 65°C (pipetting becomes easier)

Put two leaf disc in a tube by closing the tube

Place the already done tubes onto ice leave 2 tubes empty for calibration.

Put the entire blue holder with the tubes into liquid nitrogen

Place the blue holder without the transparent lid into a black and white adapter. The 2 round extensions should be facing one way and fixate in the RETCH machine.

Check the boxes with samples if they are really fixed.

Mill the samples for 30 seconds at 30 Hz for three times and changing the direction of the holders

Put the entire blue holder with the tubes back into liquid nitrogen

Centrifuge the boxes briefly in order to get the powder in the bottom of the tube

Add 400 µl of isolation buffer to the tubes once finished.

Place the blue holder without the transparent lid in the special designed clamp and tight the 4 nuts *(This will prevent that the lids will pop off after incubation at 65°C)*

Incubate at 65°C in a water bath for 60 minutes

Cool the clamp containing the tubes for 15 minutes on ice-water

Work in the fumehood for these steps.

Add 400 µl of chloroform: isoamyalcohol (24 : 1) to the tubes and mix by inversion for 5 minutes

Separate the phases by centrifuging at 4600 rpm for 20 minutes

During the centrifuging fill new tubes with 300 µl isopropanol

Pipette 2x 175 µl of the water phase in tube that contains 300 µl isopropanol

Mix the content carefully by inversion *(Look if you can see the something moving inside the solution)*

Pellet the DNA by centrifuging for 15 minutes at 4600 rpm

Discard the supernatant

Add 300 µl of ice cold 70% ethanol

Centrifuge for 5 minutes at 4600 rpm

Discard the supernatant and let the pellet dry overnight in the fumehood

Next day dissolve the pellet in 100 µl of MQ containing 4 µg/ml RNase

Let the tubes containing DNA stand on the lab bench for 3 hours

(The RNase will get rid off the RNA in your DNA sample)

Check the DNA quality of a 1% agarose gel by putting:

2 µl of isolated DNA

8 µl of MilliQ

2 µl of loading buffer

Load 12 µl on the gel

Check the DNA concentration on the NANOdrop; Concentration: is in ng/µl i.e

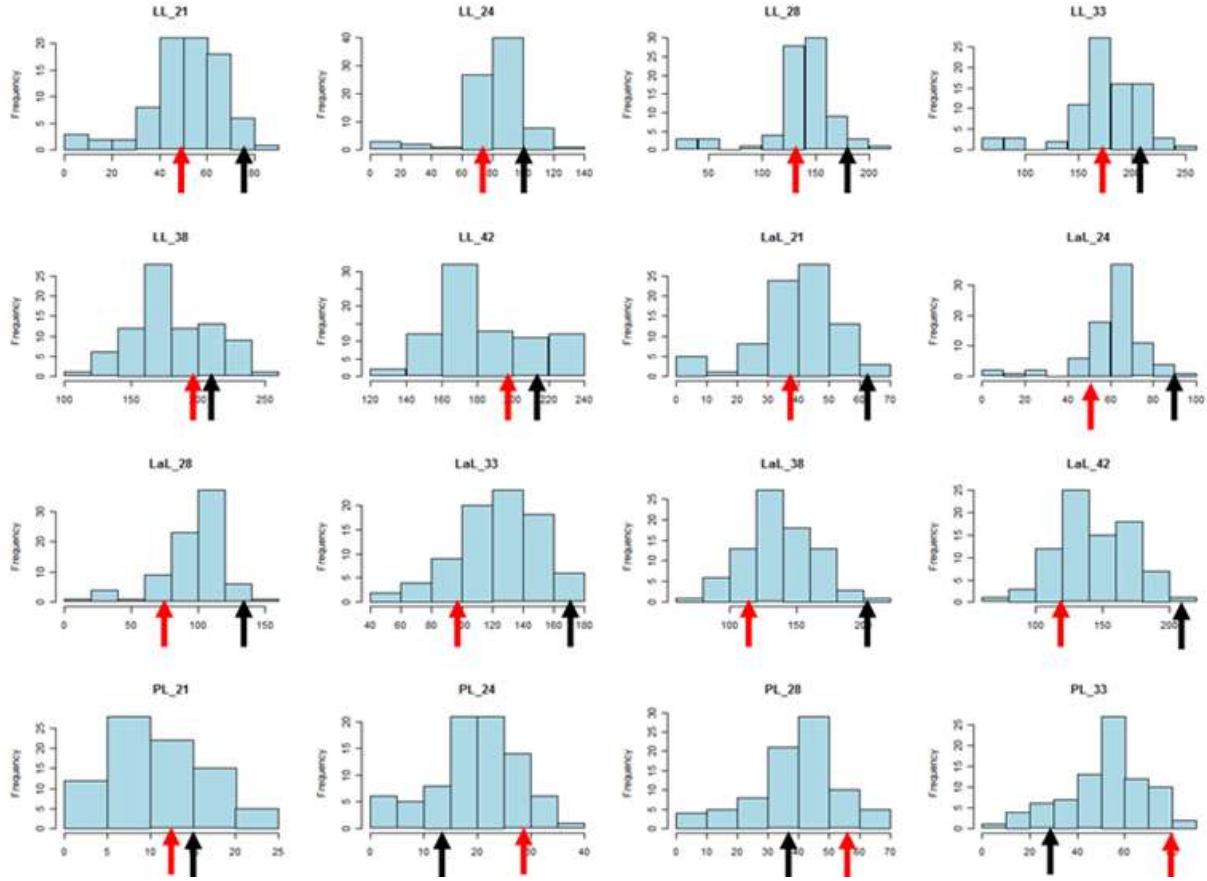
260/280: a value of 1.8 is considered as pure DNA values of 2.0 or higher are considered as pure RNA

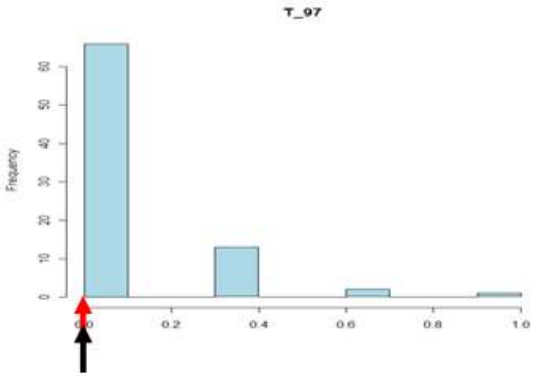
Lyses buffer	**500 ml**
Tris-HCl 1M (pH 7.5)	100 ml
EDTA 0.5 M (pH 8.0)	5 ml
NaCl 5 M	200 ml
MQ	195 ml
CTAB	10 g

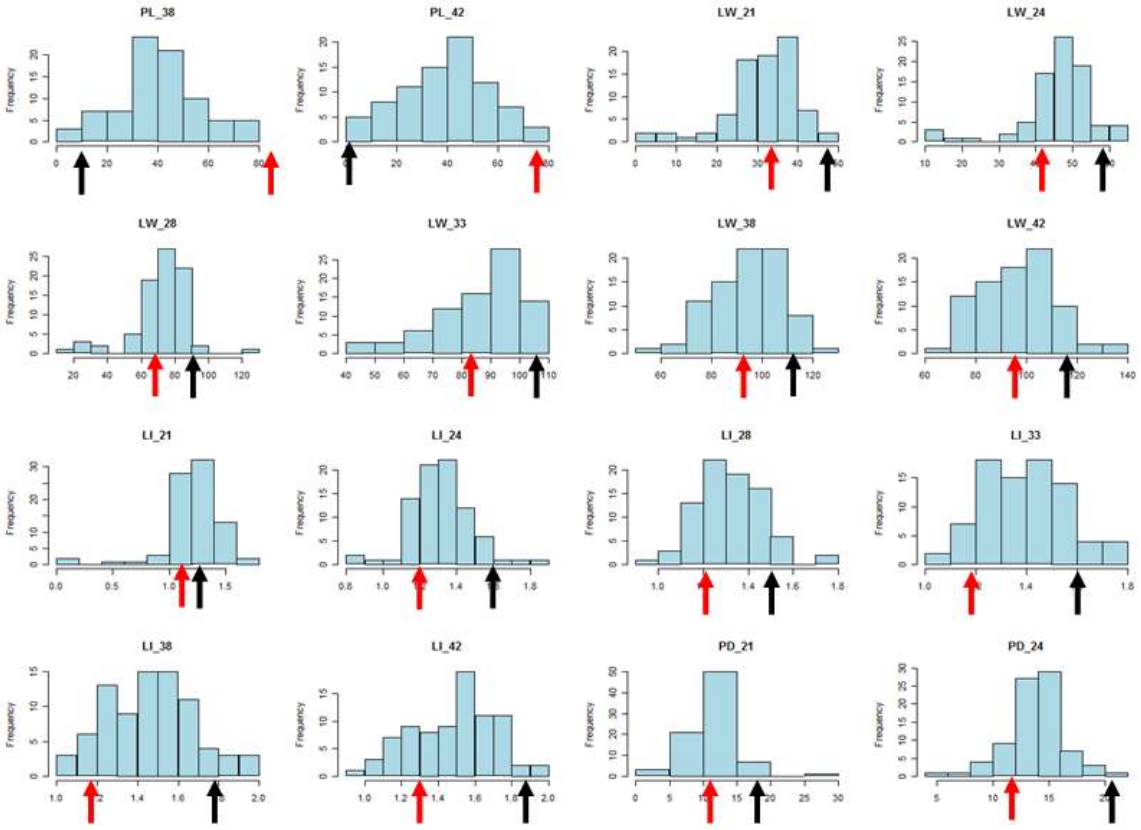
Extraction buffer	**500 ml**
Tris-HCl 1M (pH 7.5)	50 ml
EDTA 0.5 M (pH 8.0)	5 ml
MQ	445 ml
Sorbitol	31.9 g
Sarkosyl 5%	

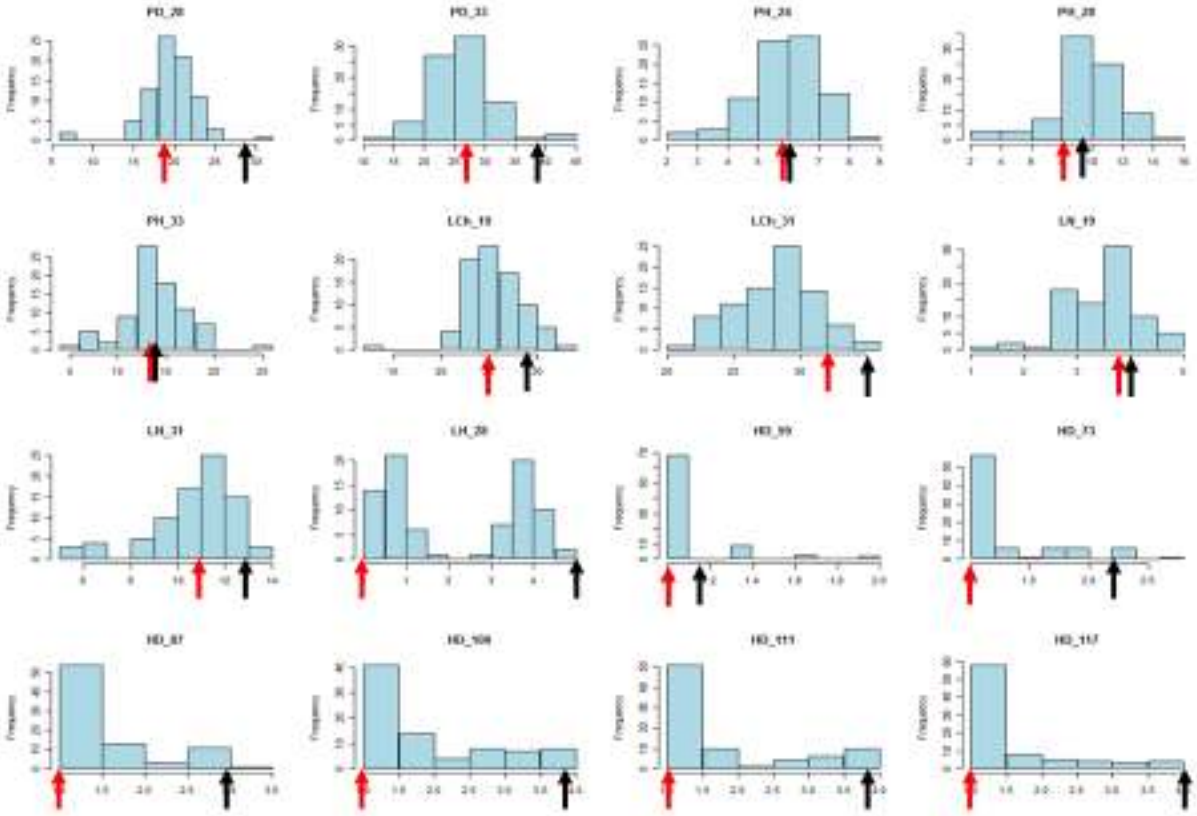
Isolation buffer	**200 ml**
Lyses buffer	84.0 ml
Extraction buffer	84.0 ml
Sarkosyl 5%	33.5 ml
Sodium bisulfide	500 mg

Appendix 2. Diagram showing the Segregation of morphological leaf traits of DH88 populationand their parent line indicated by arrows; ↑ Pak Choi and ↑ Chinese cabbage






PL_38
PL_42
LW_21
LW_24
LW_28
LW_33
LW_38
LW_42
LI_21
LI_24
LI_28
LI_33
LI_38
LI_42
PD_21
PD_24
Frequency

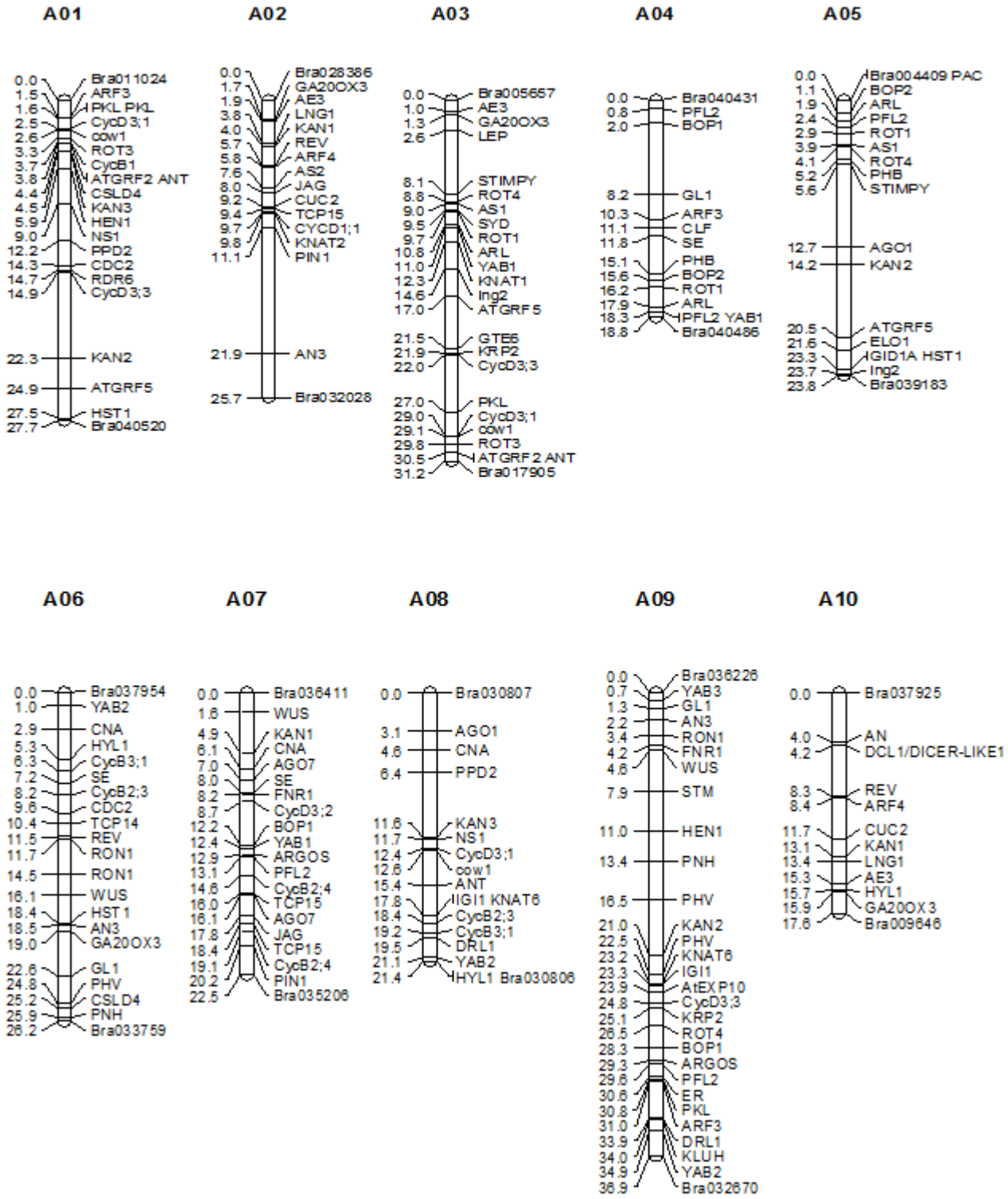

PH_33
LCh_19
LCh_31
LN_19
LH_31
LH_39
HD_59
HD_73
HD_87
HD_108
HD_111
HD_117
Frequency

Appendix 3. Table showing the flowering time for the lines which flowered and their respective days after transplanting (D.a.T* - Days after Transplanting)

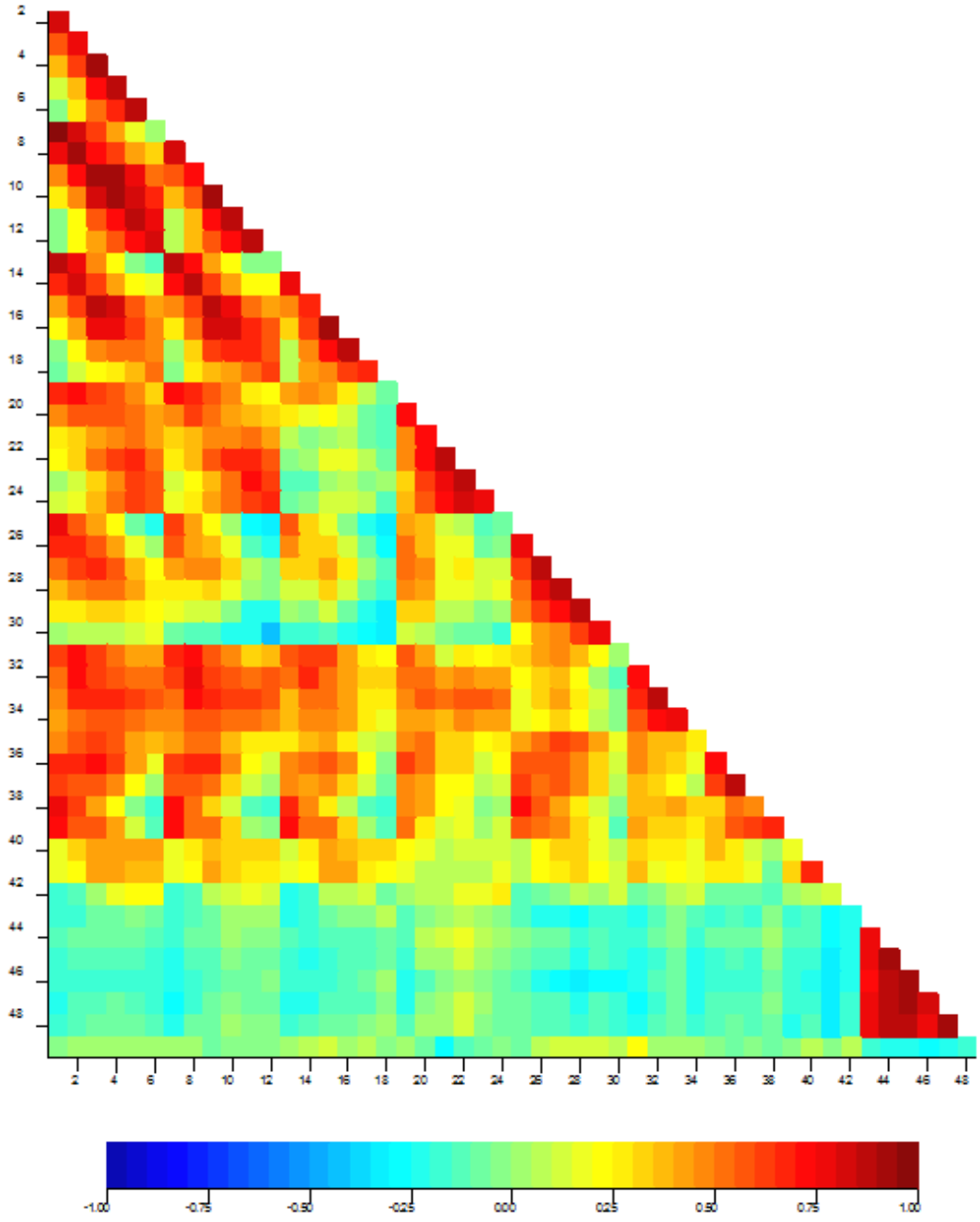
Block 1		Block 2		Block 3	
Pedigree	D.a.T*	Pedigree	D.a.T*	Pedigree	D.a.T*
DH88 241	33	DH88 241	33	DH88 241	33
DH88 134	49	DH88 134	49	DH88 134	49
DH88 242	50	DH88 242	62	DH88 242	56
DH88 041	54	DH88 041	50	DH88 041	58
DH88 025	52	DH88 025	52	DH88 025	54
DH88 157	56	DH88 157	52	DH88 157	58
DH88 079	54	DH88 079	54	DH88 079	54
DH88 245	54	DH88 245	54	DH88 245	54
DH88 129	58	DH88 129	62	DH88 129	58
DH88 176	69	DH88 176	58	DH88 176	69
DH88 118	66	DH88 118	66	DH88 118	60
DH88 232	66	DH88 232	73	DH88 232	61
DH88 237	69	DH88 237	61	DH88 237	62
DH88 019	62	DH88 019	61	DH88 019	69
DH88 164	66	DH88 164	66	DH88 164	90
DH88 183	66	DH88 183	86	DH88 183	88
DH88 170	66	DH88 170	88	DH88 170	76
DH88 209	76	DH88 209	87	DH88 209	69
PC101	**123**	**PC 101**	**69**		
DH88 128	78	DH88 128	72	DH88 128	108
DH88 114	72	DH88 114	89	DH88 114	76
DH88 095	89	DH88 095	87	DH88 095	72
DH88 132	76	DH88 132	75	DH88 132	75
DH88 224	75	DH88 224	88	DH88 224	76
		DH88 119	76	DH88 119	76
DH88 147	76	DH88 147	121	DH88 147	102
		DH88 135	88	DH88 135	79
DH88 188	87	DH88 188	103	DH88 188	82
		DH88 021	82	DH88 021	104
DH88 044	92				
DH88 229	121	DH88 229	92	DH88 229	92
DH88 160	99			DH88 160	111
DH88 115	103				
DH88 216	108				
DH88 021	108				
DH88 048	111				
CC048	**111**				
		DH88 086	121		
		DH88105	133	DH88 105	111

D.a.T* – days after transplanting

Appendix 4. The physical map of Chiifu Genome (Wang et al, 2005)



Appendix 5. The correlation diagram showing the correlation of all traits to each other. Red color shows positive correlation, and light blue indicate weak negative to neutral correlation.



Code	D.a.S.	Trait
1	18	LL
2	22	LL
3	25	LL
4	30	LL
5	35	LL
6	39	LL
7	18	LaL
8	22	LaL
9	25	LaL
10	30	LaL
11	35	LaL
12	39	LaL
13	18	LW
14	22	LW
15	25	LW
16	30	LW
17	35	LW
18	39	LW
19	18	LI
20	22	LI
21	25	LI
22	30	LI
23	35	LI
24	39	LI
25	18	PL
26	22	PL
27	25	PL
28	30	PL
29	35	PL
30	39	PL
31	18	PD
32	22	PD
33	25	PD
34	30	PD
35	22	PH
36	25	PH
37	30	PH
38	25	LN
39	30	LN
40	15	LCh
41	27	LCh
42	25	LH
43	56	HD
44	70	HD
45	84	HD
46	97	HD
47	108	HD
48	114	HD
49	94	T

D.a.S. – Days after Sowing