Morphological and Genetic Characterization of The Leafy Head of

Chinese Cabbage

(*Brassica rapa*)

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Chapter 1

General Introduction

Brassicaceae

The Brassicaceae family is one of the largest families in the angiosperm plant kingdom, which comprises 370 genera and over 4000 species in some 10 poorly defined tribes (Bonnema et al., 2011). Several species of great scientific, economic and agronomic importance are included, such as the model species *Arabidopsis thaliana*, as well as many widely cultivated species, for example *Brassica oleracea* (cabbage, cauliflower, turnips), *B. rapa* (Chinese cabbage, turnips, pak chois) and *B. napus* (oilseed rape, rutabaga, canola).

A. thaliana is a model plant species for its small genome size ($2n=10$), short generation cycle and ease of transformation (Koornneef & Scheres, 2001). The work on *A. thaliana* has revolutionized our knowledge in almost every field of modern plant biology. Many public resources are available and the molecular regulation of many biological processes is well studied with the function of many genes known. The genus *Brassica* is a monophyletic group, which is evolutionarily closely related to this model crucifer plant *A. thaliana*, and is reported to have diverged almost 14.5-20.4 million years ago (Hong et al., 2008; Cheng et al., 2013). Therefore, comparative experiments between most *Brassica* plants and *A. thaliana* are generally very informative to understand the molecular regulation of phenotypic variation and developmental processes. However, several cultivated *Brassica* species have special phenotypes, like tubers (turnips), leafy heads (cabbage or Chinese cabbage) and enlarged inflorescences (curds of cauliflower), which are absent in *A. thaliana*.

Triangle of U species

Brassica species (broccoli, cabbage, canola) and *A. thaliana* share a common ancestor ~43 million years ago (MYA) (Mark et al., 2010). After divergence, a whole genome triplication (WGT) event occurred between 13-17 million years ago (MYA) from which the different *Brassica* species evolved (Town et al. 2006). The genomes of some *Brassica* species (eg. *B. oleracea*, *B. rapa* and *B. napus*) are sequenced and supports the theory for the comparative genomic study for *Brassica* species (Wang et al., 2011; Chalhoub et al., 2014; Liu et al., 2014; Parkin et al., 2014). The three different sub-genomes have evolved at different rates, with the Least Fractionated (LF) retaining most genes and, Medium Fractionated 1 (MF1) and Most Fractionated 2 genomes (MF2) having lost more genes (Wang et al., 2011; Cheng et al., 2012). The rates of genome fractionation, block reshuffling and chromosome reduction in these three sub-genomes are different (Cheng et al., 2012; Tang et al., 2012).

In addition to WGT and hybridization, domestication and breeding also contributed to the extreme morphotypes in *Brassica* (Bonnema et al., 2011; Cheng et al., 2016). The *Brassica* genus includes six major economically important interrelated species, each with considerable morphological variation as the result of local selection and breeding. The three diploid species are *B. rapa* ($2n=20$, genome AA), *B. nigra* ($2n=16$, genome BB) and *B. oleracea* ($2n=18$, genome CC). From the cytogenetic relationships of the *Brassica* species it is evident that there are three amphidiploids derivatives, *B. juncea* ($2n=36$, genome AABB, brown mustard), *B. carinata* ($2n=34$, genome BBCC), *B. napus* ($2n=38$, genome AACC, oilseed rape), evolved after hybridization and polyploidization of two of the diploid species (Ashraf & McNeilly, 2004). The genetic relationship between species in the *Brassica* family is described by the confirmed evolutionary theory called the ‘Triangle of U’ (U, 1935) (Figure 1).

Figure 1. ‘Triangle of U’ described the genetic relationship between six *Brassica* species. According to the theory, three diploid ancestral species *B. rapa* (AA), *B. nigra* (BB) and *B. oleracea* (CC) are represented by different colors. Three amphidiploid species *B. juncea* (AABB), *B. napus* (AACC), *B. carinata* (BBCC) derived from each pair of the three diploid species (U, 1935).

Recently, it was proposed to expand the triangle of U into a multi-vertex model by speciation and hybridization. Several new species or crop types were suggested to be added, such as *Raphanus*, *Rapistrum* and *Sinapis* (Cheng et al. 2017). Most *Brassica* species display an enormous variation, ranging from leafy heads, large storage tubers, enlarged arrested inflorescences to abundant seed yields. The model species *Arabidopsis* lacks this huge variation (Koch et al., 2008).

Brassica rapa

Brassica rapa originated in the Middle east/crescent belt area and is cultivated worldwide in most continents (Li, 1981; Gomez-Campo & Prakash, 1999; Bonnema et al., 2011). The genome of *B. rapa* ($n=10$) is diploid and is the first *Brassica* genome that was sequenced. In total, the estimated genome size of *B. rapa* is 485 Mb and the genome contains 41020 genes (Wang et al., 2011; Cai et

al., 2017). *Brassica* vegetable crops are characteristic for their lower levels of saturated fat and high values of fiber, secondary metabolites like glucosinolates and vitamins, which contribute to a healthy diet.

Sub-species in *Brassica rapa*

B. rapa consists of various subspecies that are often referred to as morphotypes: ssp. *chinensis* (pak choi), ssp. *pekinensis* (Chinese cabbage), ssp. *parachinensis* (caixin), ssp. *rapa* (turnip), ssp. *trilocularis* (yellow sarson), ssp. *oleifera* (oil seeds), ssp. *narinosa* (wutacai) and ssp. *nipposinica* (mizuna) (Gómez-Campo, 1999) (**Figure 2**). These subspecies display enormous variation, such as leafy heads, enlarged hypocotyls/roots, stems, inflorescences, axillary shoots and oilseeds.

Figure 2. Morphotypes of sub-species in *B. rapa*

Five important *B. rapa* crops are the leafy vegetable crops ssp. *pekinensis*, ssp. *chinensis*, ssp. *parachinensis*, the tuber crop ssp. *rapa* and the oilcrop ssp. *oleifera* (Chi-meng, 1979; Van Wyk, 2005). Pak choi (ssp. *chinensis*), which originated in Southeast Asia has long been cultivated in China and Japan. The edible part of pak choi is the young plant with green small leaves and white petioles. The closely related Chinese flowering cabbages (ssp. *parachinensis*) have elongated flowering shoots and fleshy stems with pak choi like leaves which form the edible parts. Turnips (ssp. *rapa*), as one of the oldest cultivated tuber crops, have a fleshy and globe shaped hypocotyl as a storage organ. Their tubers and young leaves are cooked as dishes in Europe. Tubers are also used as fodder crop (Reiner et al., 1995; Vogl-Lukasser et al., 2007). There are in fact two different groups of turnips, suggesting that they were domesticated twice: the Japanese turnips are annual, while European turnips are biannual (Gómez-Campo, 1999; Zhang et al., 2014). Chinese cabbages (ssp. *pekinensis*) represent an important leafy vegetable crop with a long history of cultivation in East Asia. The flat leaves fold together to form a leafy head. The general theory is that heading Chinese cabbages have been domesticated from non-heading leafy pak choi in China (Tan, 1979; Li, 1981). Oil

seed (*ssp. oleifera*) types are annual (sarsons) or biannual (winter oils). The seeds are used for edible oil extraction (Kuvshinov et al., 1999).

The different *B. rapa* accessions were clustered into three groups based on genetic diversity studies (Zhao et al., 2005 & 2010). Broccoletto accessions and most European origin turnips and biannual oils were assigned to one group. Annual oil type accessions, mainly from India and Bangladesh were assigned to the second group and group three is a group of Asian crop types. However, like in the study of Pino Del Carpio et al. (2011), group three (Asian crop types) is represented by two sub groups, one comprising of Chinese cabbage, and the other of Asian turnips and pak choi.

Heading and non-heading leafy vegetables in *B. rapa*

Two important leafy vegetables are the non-heading pak choi (*B. rapa* ssp. *chinensis*) and the heading Chinese cabbage (*B. rapa* ssp. *pekinensis*) (**Figure 3A**). When we compare the vegetative developmental stages of heading Chinese cabbage and the non-heading pak choi, the Chinese cabbage develops first a rosette, followed by a leaf folding stage and finally forms a leafy head (Yu et al., 2000). In contrast, the pak choi does not form a leafy head after rosette stage and forms clusters of loose green leaves with long fleshy petioles (Hanson et al., 2009). Compared to the flat non-heading pak choi leaves, Chinese cabbage heading leaves grow upward with white and broad mid vein and display inward curvature (**Figure 3B**). Chinese cabbages have been domesticated for some clear advantages for consumers and traders, such as their high yield and good cold tolerance, ease in transport and long-time storage (Daly & Tomkins, 1997). As I am interested in leafy head development in Chinese cabbage, I will first review what is known about leaf development in the model plant *A. thaliana*.

Figure 3. *Developmental stages and leaf phenotypes for heading Chinese cabbage and non-heading pak choi. (A) Developmental stages from seedling stage to mature plants. (B) Leaf phenotypes for mature Chinese cabbage and pak choi. Upper panel is Chinese cabbage, lower panel is pak choi.*

Leaf development

Leaves are important plant organs and provide the basis for plant growth. Through photosynthesis, leaves absorb sunlight and convert it into energy (Gonzalez et al., 2012). For food crops, improvement of the capacity and efficiency of this process plays a major role in increasing in yield production (Zhu et al., 2010; Harbinson et al., 2012; Van Rooijen et al., 2017). The photosynthesis capacity is strongly influenced by the leaf size and leaf shape and also leaf orientation (Demmig-Adams et al., 2017). In addition, leaves sense the external environment, like light intensity, temperature and day length (Parkhurst & Loucks, 1972; Haworth & McElwain, 2008; Song et al., 2013; Sun et al. 2018).

Leaf cellular structure

The cells of a leaf are arranged as a sandwich, with two outer epidermal layers surrounding the mesophyll zone in the middle (**Figure 4**). The epidermis provides the leaf with a waxy layer and protects against water loss. The only way for gas exchange is through stomata, with the majority positioned on the lower epidermis of the leaf. The tissue between the epidermal cells (mesophyll layers), contains large numbers of chloroplasts that turn sunshine into carbohydrates. The mesophyll layer is composed of the palisade layer with palisade cells located on the adaxial side (upper side) of the leaf and the spongy parenchyma cells located at the leaf abaxial (lower) side. The palisade cells are tightly packed, oblong, with one small end facing the upper epidermis and the long ends squeezed in side-by-side. They have been arranged to maximally capture the sunlight and contain many chloroplasts. The spongy cells are irregularly shaped, less ordered, loosely arranged and leaves large intracellular spaces for carbon dioxide (CO₂) and oxygen (O₂) exchange. Among the spongy tissue, the vascular bundles are formed which are composed of two different types of “transport ” tissue, xylem and phloem vessels. These two tissues consist of typically long and slender cells. The xylem cells transport water and dissolved minerals to the leaves, while the phloem transports the photosynthetic products to the other parts of the plant.

Figure 4. Cellular structure of *C3* plant leaf, showing the cuticle, epidermis cells, palisade parenchyma, spongy parenchyma, vascular bundles, stomata and guard cells. Left part is a cartoon diagram (leaf figure is modified from <http://redslime.typepad.com/i/adaptations-of-plant-leaves.html>), right part is a *B. rapa* (Chinese cabbage) leaf cross section.

Leaf developmental processes and genetic control in *Arabidopsis*

The development of a leaf is a complex process that is regulated by several pathways. In *A. thaliana*, the leaves are initiated from the shoot apical meristem (SAM) and growth involves six overlapping and interconnected phases: leaf primordium initiation, leaf polarity formation (adaxial/abaxialization), cell division, transition phase, cell expansion and cell differentiation into stomata, vascular tissue and trichomes (Kalve et al., 2014). During these processes, several hormonal pathways, like auxin, cytokinin (CK) and gibberellic acid (GA) pathways regulate the development of the leaf. Auxin maxima define leaf initiation and auxin gradients play a role in ad/abaxial polarity and leaf growth (Qi et al. 2014).

The SAM is organized in three layers: central zone (CZ), peripheral zone (PZ) and rib zone (RZ). The cells in the peripheral zone can differentiate into leaf primordia. This cell differentiation decision involves dynamic gradients of the signaling molecule auxin (Benková et al., 2003) and is governed by the plant hormones auxin, cytokinin and gibberellin. The genes *AUXIN RESISTANT (AUX1)* and *PIN-FORMED1 (PIN1)*, Class I *KNOTTED1-LIKE HOMEBOX (KNOX1)* and *GA20-oxidases* play important roles in leaf initiation. KNOX proteins initiate and maintain the SAM by promoting cytokinin biosynthesis and repressing GAs by down-regulating the *GA20-oxidase* gene (Sakamoto et al., 2001; Jasinski et al., 2005; Byrne, 2012). The influx carrier *AUX1* and efflux carrier *PIN1* orient the

auxin transport from cell to cell to create auxin maxima (Bayer et al., 2009; Guenot et al., 2012) thereby promoting leaf primordia formation.

After leaf initiation, the developing leaf primordium establishes upper (adaxial), middle and lower (abaxial) domains. The adaxial-abaxiality reflects positional differences in the leaf blade, which are established by the leaf polarity pathway (**Figure 5A**). *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*) and *PHABULOSA* (*PGD*), which encode class III *HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZIPIII*) transcription proteins, determine the adaxial fate and have an antagonistic relationship with the abaxial determinants *KANADI* (*KAN1*, *KAN2* and *KAN4*) (Byrne, 2006; Ariel et al., 2007). Together with *KAN*, members of the *YABBY* gene family (*FIL*, *YAB2*, *YAB3* and *YAB5*) and *ARF3*, *ARF4* are involved in leaf abaxial fate specification (Bowman et al., 2002; Eshed et al., 2004). *KAN* and *ARF3-ARF4* proteins have overlapping roles in the formation of partially adaxialized leaves (Husbands et al., 2009). The expression of *FIL* is up-regulated by both *KAN* and *ARF3-ARF4* and inversely *KAN*, *ARF3* and *ARF4* are positively regulated by the activity of *FIL* (Eshed et al., 2004; Bonaccorso et al., 2012). Both the adaxial and abaxial determinants are regulated by small regulatory *RNAs-microRNAs* (*miRNAs*) or trans-acting short interfering *RNAs* (*ta-siRNAs*). *miR165* and *miR166* target the *HD-ZIPIII* genes on the adaxial side (Kidner & Martienssen, 2004). *ta-siRNA* (*TAS3*) accumulates on the adaxial side and forms a gradient that determines leaf polarity by targeting auxin-response transcription factors, *ARF3* and *ARF4* on the abaxial side (Hunter et al., 2006). This *TAS3-ta-siRNA* pathway is activated by RNA-dependent RNA polymerase 6 (*RDR6*), *SUPPRESSOR OF GENE SILENCING3* (*SGS3*) and *DICER-LIKE4* (*DCL4*).

The final leaf size and shape is determined by leaf cell division and cell expansion (Meyerowitz, 1997; Tsukaya, 2003) (**Figure 5B**). Cytoplasmic growth plays a crucial role in controlling a range of cell division and expansion events that are regulated by the Target of Rapamycin (TOR) pathway (Sablowski & Dornelas, 2014). On the basis of increasing the cell volume by cytoplasmic growth, the cell division cycle is operated by the activity of the CDKs-CYC (cyclin dependent kinases-cyclin) complex (Francis & Sorrell, 2001; Dissmeyer et al., 2009; Kalve et al., 2014). The *AINTEGUMENTA* (*ANT*) family proteins, GRF-INTERACTING FACTOR (*GIF*), GROWTH-REGULATING FACTOR (*GRF*) and *TEOSINTE BRANCHED1/CYCLOIDEA/PCF* (*TCP*) transcription factors are essential to promote cell division (Lee et al., 2009; Kim et al., 2003; Palatnik et al., 2003; Rodriguez et al., 2010). In the cell expansion phase, roles for the cell wall associated proteins *EXPANSINS* (*EXPs*), which regulate cell elongation, the TOR

pathway and ARGOS LIKE (ARL) have been described (Sampedro & Cosgrove, 2005). The cell division, which increases the number of cells, is accompanied by continued cell expansion. Some factors and genes control the transition between cell division and expansion. For example, ORGAN SIZE RELATED1 (ORS1) positively regulates cell division as well as expansion (Feng et al., 2011). ErbB-3 EPIDERMAL GROWTH FACTOR RECEPTOR BINDING PROTEIN (EBP1) positively regulates leaf growth and promotes cell division and expansion (Horvath et al., 2006). Conversely, AUXIN RESPONSE FACTOR 2 (ARF2) represses leaf growth by affecting cell division and expansion (Schruff et al., 2006). Whereas, the phytohormones like auxin, gibberellins (GA), cytokinin (CK) and abscisic acid (ABA) also impact cell division and expansion (Perrot-Rechenmann, 2010; Hu et al., 2000).

A.

B.

Figure 5. Molecular interactions for leaf development in Arabidopsis. (A) Leaf adaxial-abaxial polarity pathways: HD-ZIPIII–KANADI, miR166/165–HD-ZIPIII and TAS3–ta-siRNA–ARF3/ARF4 pathways are involved. All these three pathways converge on the YABBY gene family. (B) Leaf cell division and expansion pathways. (Modified from Iwasaki et al., 2013 and Kalve et al., 2014.)

Leafy head formation in Chinese cabbage

Chinese cabbages represent an important leafy vegetable with high yield and great storability (Liu et al. 2014). The heading trait is a major determinant of Chinese cabbage quality. The Chinese cabbage leafy heads have different shapes and sizes, which were found to be associated with morphological features of rosette leaves. Many environmental factors affect the formation of the leafy head, such as temperature, daylight, light intensity, auxin concentration and nutrition (Ito & Kato, 1957; Wang et al., 2012). In addition, the genetic background affects leafy head formation and head shape and color (Mao et al., 2014). Understanding the regulation of leafy head development is therefore of vital agronomic importance.

Leafy head structure

The Chinese cabbage leafy head consists of inner yellow-green crinkly leaves that are folded around each other. The plant goes through four stages of vegetative growth: seedling, rosette, folding and heading stage (Chen, 1984). At seedling stage, primary and juvenile leaves are round and have long petioles. At rosette stage, the plant forms a rosette by extending large and round rosette leaves, with increasing leaf base angle. At the end of the rosette stage, folding leaves with short leaf petioles are differentiate to form the leafy head. Compared to non-heading leafy types, the folding leaves are more inwards curving standing up and remain metabolically active during the entire head development in order to supply the head with photosynthetates (Wang et al., 2012). Following the rosette and folding stages, the heading leaves, which are folded around the shoot apexes, orientate extremely upward and curve inwards giving rise to the leafy head. Different head shapes can be distinguished, depending on the cultivar. The Chinese cabbage leafy head shape is divided into four groups: round, oblong, cylindrical and cone-like shape (**Figure 6**). Recent studies showed that the shape and size of rosette and folding leaves influence the shape of the Chinese cabbage leafy head (Mao et al., 2014).

Figure 6. Chinese cabbage different leafy head shapes. (A) Round head shape. (B) Oblong head shape. (C) Cylindrical head shape. (D) Cone-like head shape. (Pictures from Mao et al., 2014.)

Genetic studies of leafy head formation

As a quantitative trait, multiple genes control the head formation in Chinese cabbage. However, the understanding of genetics and molecular mechanisms for this trait is still limited. The complete genome of *B. rapa* has been sequenced, which provides opportunities to genetically study the head formation in Chinese cabbage (Wang et al., 2011). Several quantitative genetic studies identified quantitative trait loci (QTLs) for heading related traits, including head top leaf overlap shape, head weight, head diameter and head height. In Yu et al.'s (2013) paper, 18 QTLs for six head traits were detected and three candidate genes were selected; *BrGL1* for trichome formation, *BrESR1* and *BrSAW1* for the leaf wing trait. In the study of Inoue et al., (2015), four QTLs for head leaf overlap shape, head height, head diameter and head height to head diameter ratio were detected under different growth conditions, but no candidate genes were proposed. Several classes of small RNAs (*microRNAs* and *siRNAs*) serve as guides for post-transcriptional and epigenetic regulation of target genes with functions in plant developmental processes (Kidner and Martienssen, 2004; Laufs et al., 2004). In leafy heads of China cabbage, accumulation of *microRNA319* reduces the expression level of the *BrTCP4* gene, which controls cell proliferation in developing leaves and associates with a cylindrical head shape (Mao et al., 2014; Schommer et al., 2014). Another microRNA, *microRNA156*, targets *BrSPL9-2*, controlling the heading time of Chinese cabbage. Over-expression of *microRNA156* prolongates the juvenile and early adult phases, thus delaying the folding stage (Wang et al., 2013).

Furthermore, in a large re-sequencing study of both *B. rapa*, and *B. oleracea*, bioinformatic analyses revealed genomic regions or selective sweeps for the domestication trait leafy head formation (Cheng et al., 2016). In these regions, several candidate genes were identified in the ab/adaxial leaf formation pathway, such as the Auxin Response Factors (*ARF*) *BrARF3.1* and *BrARF4.1*, the *KANADI* transcription factors *BrKAN2.1* and *BrKAN2.2* and *BrRDR6*.

BrARF3 in *B. rapa*

As a single non-synonymous SNP in the C-terminal region of *BrARF3.1* was highly associated with heading Chinese cabbages (**Figure 7**) in a large association

mapping panel, we decided to investigate how allelic variation of this gene relates to both leaf size, shape, and folding and leafy head formation in Chinese cabbages. ARFs are well known auxin-mediated response factors that bind to the promoters of auxin response genes (Guilfoyle & Hagen, 2001). Typical ARFs consist an N-terminal B3 DNA binding domain (DBD), middle region-Auxin response super family domain (MR) and C-terminal protein-protein interaction domain (AUX/IAA domain) (Guilfoyle & Hagen, 2007; Boer et al., 2014). ARF-DBD binds to specific DNA sequences to regulate transcription of different target genes. ARF-MR targets the reporter genes and acts as an activation or repression domain. These two domains alone are not influenced by auxin. The ARF-AUX/IAA domain interacts with Aux/IAA by amino acid sequence motifs III and IV and is required for an auxin response (Tiwari et al., 2003).

Figure 7. Auxin response factor (ARF) family information. (A) Phylogenetic tree of *B. rapa* and *A. thaliana* ARFs. (B) Conserved protein domains of ARFs. (C) BrARF3.1 C-terminal SNP ratio in 806 *B. rapa* heading and non-heading accessions.

Arabidopsis has 23 *ARF* genes and these genes are expressed in a wide variety of tissues and organs. Based on the *A.thaliana ARF* gene family, 33 *BrARF* genes were identified in the *B. rapa* genome and divided into four subgroups (Mun et al., 2012; Huang et al., 2015) (**Figure 7A**). All *BrARFs* consist of an N-terminal DBD and an auxin response super family domain at the middle region. However, not all *ARF* have the C-terminal AUX/IAA domain. Like *AtARF3* and *AtARF17*, *BrARF3.1*, *BrARF3.2* and *BrARF17.1*, *BrARF17.2* also lack the C-terminal AUX/IAA domain (**Figure 7B**). In presence of auxin, *ARF3* and *ARF17* cannot be repressed or activated by the Aux/IAA repressor (Tiwari et al., 2003). Interestingly, *BrARF3.1* (Bra005465) and *BrARF4.1* (Bra002479) cluster together in group II based on their sequence similarity and were both identified as candidate genes in selective sweeps for leaf heading traits (Cheng et al., 2016). A C-terminal amino acid difference between heading (Chinese cabbage) and non-heading *B. rapa* accessions was identified and used to design a SNP marker assay to genotype the *BrARF3.1* alleles in 806 *B. rapa* accessions. 90% of the heading accessions (Chinese cabbage) carry a “G” allele (heading allele), and 73% non-heading accessions carry a “C” allele (non-heading allele) which leads to a significant association between heading and allelic variation in *BrARF3.1* (**Figure 7C**). The G/C allele difference changes the amino acid from Glutamine (Gln) to Histidine (His) in the *ARF3.1* protein.

Objectives and outline of the thesis

The aim of this thesis is to elucidate molecular pathways underlying the domestication trait leafy head formation in *B. rapa* and to investigate the role of *BrARF3* in this. As a quantitative trait, the heading trait is still not fully understood. In this thesis I followed several approaches to study leafy head formation. First, I described the morphology of Chinese cabbage during development at both whole plant and the leaf cellular level. To study the genetic regulation of head formation, Quantitative Trait Loci (QTLs) were detected for both rosette leaf and leafy head traits in biparental populations. To study the molecular regulation of leafy head formation, gene expression was profiled during Chinese cabbage development and compared to gene expression during development of non-heading pak choi. To study the role of the *BrARF3* gene in leafy head formation, functional studies of *BrARF3* were conducted. The functional studies included over-expression studies in *A. thaliana*, and protein interaction studies with different *B. rapa* *ARF3* protein alleles. Below both the used plant materials and all these aspects are shortly described within the context of the different chapters.

Plant materials used in this thesis: The population genetic experiments were conducted in China. The Chinese cabbage collection consisted of breeding materials from Hebei Agricultural University (HAU) and commercial varieties from different provinces in China. The Doubled Haploid (DH) population was generated in Wageningen using available DH parental lines: CC-48 (*B.rapa* ssp. *pekinensis*: CGN06867) as father line and non-heading pak choi PC-101 (*B.rapa* ssp. *chinensis*: CGN13926) as mother line. These genotypes were selected for their high response in microspore culture. In chapters 3 where we phenotyped whole plant and leaf development and in chapter 4 where we analysed the transcriptome in Chinese cabbage and pak choi during their development, we used homozygous genotypes, either DH lines generated in Wageningen or inbred lines obtained from others. In chapter 3 and 4 the used pak choi PC-024 was a DH line developed in our group, while the early heading Chinese cabbage CC-Z16 with smooth leaves and a round head was provided by the Institute of Vegetables and Flowers (IVF-CAAS) (Prof. Wang Xiaowu). This Chinese cabbage genotype was chosen for its smooth leaf phenotype and similar developmental speed compared to the PC-024 DH line. In addition we used the homozygous inbred Chinese cabbage line Chiifu in chapter 4, which was sequenced as reference genome (Wang et al. 2011) and in chapter 3 we used the DH line CC-A03, provided by HAU (Prof. Zhao Jianjun). CC-A03 is a late heading line with a cylindrical head and rugose leaves. From this line, an EMS mutant population is available at HAU. In chapter 5, we cloned *BrARF3.1*, *BrARF4.1*, *BrKAN2.1*, *BrKAN2.2*, *YAB1.1* and *YAB1.2* from PC-024 and CC-Z16. In addition, *Arabidopsis thaliana* Col-0 was used for functional studies.

Chapter 2: The correlation between rosette leaf traits and head formation was determined by using 152 Chinese cabbage accessions. Hereafter, QTL analysis was performed to link genotypic data (molecular markers) and phenotypic data (head formation related trait measurements) in two parental populations from a cross between non-heading pak choi and Chinese cabbage (Doubled Haploid population and F2 population). QTL explaining heading and heading related traits were identified.

Chapter 3: Compared to non-heading pak choi, which goes through seedling and rosette stages only, Chinese cabbage has two extra stages: folding and heading. The difference in morphological development at the full plant scale was described during the vegetative growth period for heading Chinese cabbages and non-heading pak choi. Specific developmental stages were defined and RNA was isolated from central emerging leaves for gene expression profiling using a *B. rapa*

60-mer custom made microarray. Differences in transcript abundance among genes between developmental stages and morphotypes are identified and annotated. Furthermore, the co-expression of genes related to heading formation is shown by comparing differences between the pak choi on the one hand and the two very different Chinese cabbage genotypes on the other hand. Mapman enrichment categories that define the different co-expressed genes are being discussed, with special focus on 'cell', 'cell-wall' and 'photosynthesis' related genes.

Chapter 4: The leafy head of Chinese cabbage is composed of a number of heading leaves. Certain seedling and rosette leaf traits, like leaf size, shape, degree of blistering, leaf curvature and leaf waviness, are considered to be closely related to the formation of the leafy head. To understand these different leaf morphologies, the shapes, sizes, density and patterns and arrangements of cells that make up the palisade and spongy tissues were investigated using optical microscopy from different leaf segments. We then used quantitative real time PCR to profile gene expression of genes associated with leaf ad/abaxial formation and leaf cell division/expansion. The combined data are discussed and related to the differences in rosette leaves between Chinese cabbages and pak choi.

Chapter 5: In this chapter we investigated the role of *BrARF3.1* in leaf and leafy head development. Over-expression of both the heading and non-heading alleles of *BrARF3.1* in *A. thaliana* resulted in a clear phenotype for the non-heading alleles only. The alleles differ in only four amino acids (aa), with one located between the ARF3 auxin response factor domain and B3 DNA binding domain, two located in the ARF3 B3 DNA binding domain and one located at the protein's C-terminus. We over-expressed two different chimeric constructs of *BrARF3.1* in *A. thaliana* to understand which mutation caused the strong phenotype. These same constructs were used in yeast two hybrid (Y2H) protein-protein interaction studies to test interaction with selected transcription factors. Interestingly, the aa change in *BrARF3.1* that affects its interaction with other proteins, corresponds to the aa change that causes phenotypes in *Arabidopsis* over-expression plants.

Chapter 2

Genetic analysis of Chinese cabbage reveals correlation between rosette leaves and leafy heads

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Abstract

To understand the genetic regulation of the domestication trait leafy-head formation of Chinese cabbages, we exploit the diversity within *Brassica rapa*. To improve our understanding of the relationship between variation in rosette-leaves and leafy heads, we phenotyped a diversity set of 152 Chinese cabbages. This showed correlation between rosette-leaf traits and both head traits and heading capacity. Interestingly, the leaf number of the mature head is not correlated to heading degree nor head shape. We then chose a non-heading pak choi genotype to cross to a Chinese cabbage to generate populations segregating for the leafy head traits. Both a large F2 (485 plants) and a smaller Doubled Haploid (88 lines) mapping population were generated. A high density DH-88 genetic map using the *Brassica* SNP array and an F2 map with a subset of these SNPs and InDel markers was used for quantitative trait locus (QTL) analysis. Thirty-one quantitative trait loci (QTLs) were identified for phenotypes of rosette-leaves in time and both heading degree and several heading traits. On chromosome A06 in both DH-88 and F2-485 QTLs for rosette leaf length and petiole length at different developmental days and an F2 QTL for head height co-located. Variation in head height, width and weight all correlate with variation in heading degree with co-locating QTLs respectively on chromosome A03, A05 and A08 in F2-485. The correlation between rosette-leaf and heading traits provides not only insight in the leaf requirements to form a head, but also can be used for selection by Chinese cabbage breeders.

Introduction

B. rapa is an economical important species that includes many morphotypes that are cultivated as vegetable, oil and fodder crops (Prakash & Hinata, 1980; Gomez-Campo, 1999; Hanelt, 2001). It contributes many important leafy vegetable crops grown worldwide, with main production and consumption area's in Asia and Europe (Dixon, 2007; Ge et al., 2011). The two most important *B. rapa* leafy morphotypes are Chinese cabbage with leafy heads and pak choi's with flat smooth leaves and fleshy petioles that do not form a head. The general hypothesis is that Chinese cabbages have been domesticated from non-heading leafy pak choi's in China (Li, 1984). Compared to the non-heading accessions, the heading types can be transported easily, stored long time and have better cold tolerance and higher yield (Daly & Tomkins, 1997).

Chinese cabbages go through four stages of vegetative growth: seedling, rosette, folding and heading stage (Ge et al., 2011). During seedling stage the leaves are round and have long petioles. Rosette leaves (RL) differentiate at the rosette stage and become large and round, with short petioles. At the end of the rosette stage, the folding leaves (FL) are also large and round with short petioles, but curve more inwards and thereby form a mould for further head development. Unlike species that do not form a leafy head, the rosette and folding leaves in heading plants remain metabolically active during the entire head development in order to supply the head with the products by photosynthesis (Yu et al., 2000; Wang et al., 2012). Leafy heads of Chinese cabbage are formed following the rosette and folding stages, when heading leaves develop an extreme upward and inward curvature giving rise to the leafy head that in effect becomes a nutrient storage organ. However, details of leaf development are rarely described and the correlation between variation of leaves in rosette/folding stage with timing of head formation and head shape is still unclear. As far as we know, only few genetic studies have been conducted on variation in rosette leaf traits and the relationship with head formation. Mao et al. (2014) phenotyped the rosette leaves and head shape at the heading stage and found a correlation between their morphology. Generally round heads had flat rosette leaves, cylindrical heads had rosette leaves with wavy margins and cone-like heads had rosette leaves that curved inwards. The same team also recently published about the effects of microRNAs on leaf curvature, which is also highly relevant to understand leafy head formation in Chinese cabbage (Wang et al., 2014). In addition, several genomic studies are conducted that describe gene expression profiles during Chinese cabbage development (Wang et al., 2012; Wang et al., 2013, Gau et al.,

2017). When these are combined with phenotyping of both the complete plant and the anatomy of leaves and meristems, our understanding of leafy head formation will increase.

Head formation is a quantitative trait, controlled by multiple genes, however genetic mechanism of head formation is still unclear. Currently, few studies have identified quantitative trait loci (QTLs) for heading traits in Chinese cabbage, such as head weight, head diameter, head height and number of leaves. Kubo et al. (2010) constructed a map based on a mapping population consisting of 188 F₂ plants derived from a cross between a Chinese cabbage and a vegetable turnip. For head formation, two QTLs were detected in both 2005 and 2007, with no common head formation QTLs detected. Ge et al. (2011) phenotyped 154 F₃ families and identified twenty-seven QTL distributed over all nine linkage groups for gross weight, head weight, head length, head width, numbers of wrapper leaves and head forming leaves. Inoue et al. (2015) evaluated eight traits over 2 years (2010 and 2011) for a F₂ population of 188 plants from a cross between two Chinese cabbage cultivars. They reported QTL for four heading traits (degree of head-top-leaf overlap, head height, head weight and the ratio of head height to head diameter) on linkage groups A1, A5, A7 and A8 in both years. Xiao et al. (2013) used a genetical genomics approach to study variation in leaf shape and size in *B. rapa*. For this purpose they used a DH population derived from a cross between non-heading pak choi and an oil type, yellow sarson. They identified several candidate genes for leaf variation and also revealed correlations between leaf traits and both plant architecture and flowering time.

In this paper we aimed to understand further the complexity of head formation during Chinese cabbage development. To achieve this goal, we performed the first global analysis of the correlation between leaf traits during development and head formation at heading stage using 152 Chinese cabbage cultivars. The data will substantially increase knowledge of Chinese cabbage heading related traits during leafy head development and provide research directions for QTL studies of heading related traits. We also generated an F₁ population derived from a cross between heading Chinese cabbage (CC-48) and non-heading pak choi (PC-101). A doubled haploid population (DH-88) and an F₂ population (F2-485) were developed from F₁ by microspore culture and self-pollination respectively. DH-88 and F2-485 linkage maps were constructed using 66 DH-88 and 485 F2-485 individuals for QTL analysis to detect loci related to rosette leaves related traits at rosette stage and leafy head formation at heading stage.

Materials and Methods

Plant materials

Chinese cabbage genotypes for morphological traits analysis

A collection of 152 *B. rapa* L. ssp. *pekinensis* (Chinese cabbage) accessions from different locations of China was used in this study (**Supplemental Table S1**). Among them, 69 accessions were inbred lines and 83 accessions were hybrid varieties (F1). Plants were grown in a random block design in the field in Hebei province in China, September 2013. Three blocks with fifteen plants of each accession were used for collection of morphological trait data.

DH-88 and F2-485 mapping populations

The mapping populations used in this study consisted of a doubled haploid population (DH-88) and an F2 population (F2-485) (**Figure 1A**). The DH-88 and F2-485 have the same father line, heading Chinese cabbage CC-48 (*B. rapa* ssp. *pekinensis*: CGN06867) and mother line, non-heading pak choi PC-101 (*B. rapa* ssp. *chinensis*: CGN13926). The F1 was obtained from a cross between CC-48 and PC-101. The 66 lines of DH-88 were obtained by F1 plant microspore culture. From this F1, 485 F2 plants were produced after selfing.

Figure 1. The strategy for constructing the Chinese cabbage (DH-88 and F2-485) populations (A) and description of the measurements of leaf and heading traits (B). Head degree (HDe), head diameter (HD), head height (HH), core length (CL) and core width (CW) were recorded for the leafy heads. Leaf length (LL), leaf width (LW), leaf mid vein length (LVL), leaf mid vein width (LVW) and leaf mid vein thickness (LVT) were recorded for leaves.

For QTL analyses, the experiments were conducted in Hebei Agriculture University, Hebei, China. Sixty-six DH-88 lines, plus their parental lines and their F1's were sown in seeding soil in the greenhouse, transplanted September 2014 to an open field at the Hebei Agriculture University, and grown under short day conditions (11 h photoperiod) until December 2014. The 485 F2 seeds were sown on the same experimental field September 2015, and grown under natural conditions until November 2015.

Trait evaluations and phenotypic data analyses

The collection of 152 Chinese cabbage accessions were evaluated for 17 agronomic traits during heading stage and the traits scored in each individual plant are listed in **Table 1**. Five plants as biological replicates for each accession were evaluated for their agronomic traits.

For QTL analysis, 4 traits of DH-88 and eight traits of the F2-485 population were evaluated at rosette and heading stages. In rosette stage, the 6th rosette leaf was phenotyped for leaf width (LW), leaf length (LL), leaf petiole length (PL) and leaf trichomes (LT) at 36, 41, 44, 47, 50 and 53 days after sowing for the DH-88 and 30, 34, 37, 41, 44 and 48 days after sowing for the F2-485 population. At the heading stage, the degree of heading (HDe) was classified on a 1-4 scale (1.non-heading, 2.leaf up curling, 3.loose heading, 4.tight heading) by visual observation before maturity of the heads for both the DH-88 and F2-485 population (Figure. 1B). At maturity, head diameter (HD), head height (HH) and head fresh weight (HW) were measured after discarding the loose outer leaves of all F2-485 plants at the same time. In DH-88 population, five biological replicates per DH line were used for traits observation.

The mean, standard deviation and variance were calculated for all traits by SPSS (Statistical Package for the Social Sciences, IBM) software. R package ("corrplot" package: <http://www.R-project.org>) with Pearson correlation coefficient was used to calculate the correlation coefficients between traits and between populations.

Genetic map construction and QTL mapping

DNA extraction

Genomic DNA was extracted from young leaves of DH-88 and F2-485 plants by the CTAB method (Murray & Thompson, 1980; Rogers & Bendich, 1985). Subsequently, the DNA was checked on gel, diluted to the same concentration with water and used to screen for SNP markers.

DH-88 and F2-485 population genetic maps

TraitGenetics (Germany) developed a 60K *B. napus* array. The parental genotypes CC-48 and PC-101 were screened for this array, which resulted in 5795 polymorphic SNPs, which were used to construct the genetic map. The DH-88 individuals were genotyped for a set of 5795 SNPs covering the 10 linkage groups. A subset of 899 specific SNPs was used for constructing the DH88 genetic linkage map (**Supplemental Table S2**) in this study. The F2-485 genetic map was based on 99 InDel markers and 36 SNP markers (**Supplemental Table S3**). The InDel markers were designed using the *B. rapa* data base (<http://brassicadb.org/brad/>) and SNP markers were selected from the TraitGenetics set. SNP markers were used to anchor DH-88 and F2-485 populations. InDel markers were used for F2-485 genotyping by Native-PAGE method. SNP genotyping was carried out by high resolution melting analysis of small amplicons and HRMA was performed on 96-Well LightScanner System. Genetic maps for both populations were constructed by using JoinMap4.1 (Van Ooijen and Voorrips, 2006). After creating population nodes, LOD scores 8.0 to 10.0 were used to assign the markers into linkage groups (LGs) and Kosambi's mapping function (Kosambi, 2016) was used to construct genetic maps. Consensus linkage groups of the two populations were constructed with common markers in both populations and drawn using Mapchart 2.3 (Voorrips, 2002).

DH-88 and F2-485 population QTL mapping of heading related traits

Based on the genetic map, QTL mapping analysis was performed using MapQTL6 (van Ooijen, 2009). QTL regions for testing traits were identified by interval mapping (IM) analysis. Based on the results of IM, multiple- QTL model mapping (MQM) was performed using the closest markers as cofactors. A QTL was considered significant when LOD thresholds were estimated with a genome-wide confidence level $p < 0.005$ (Doerge & Churchill, 1996).

Results

Diversity and correlation analyses of 17 heading related traits scored over the 152 Chinese cabbage accessions

Phenotypic data for seventeen traits scored for the diversity set of 152 Chinese cabbages are shown in **Table 1**. All the quantitative traits showed normal distribution (**Supplemental Figure S1**). The coefficient of variation for “head-top-leaf overlap level” (HtO, 0.65) was higher than that of other traits, while

coefficient of variation for “heading degree” (HDe, 0.13) was the lowest among the seventeen investigated traits.

Table 1. List of 17 evaluated phenotypic traits of 152 Chinese cabbage accessions, their ranges and descriptive statistics

Traits	Description	Range	Mean	SD ¹	CV ²
HDe	Head Degree: <i>Degree of heading leaf curvature at the time of harvest</i>	1-3	2.88	0.37	0.13
HtO	Head top Overlap: <i>Overlap degree of the top of heading leaf</i>	1-4	2.01	1.30	0.65
PL (cm)	Plant Length: <i>Length of the plant at the time of harvest</i>	18.5-75.0	39.84	9.37	0.24
PD (cm)	Plant Diameter: <i>Width of the plant at the time of harvest</i>	29.0-92.0	58.44	11.67	0.20
PW (kg)	Plant Weight: <i>Weight of the plant at the time of harvest</i>	0.37-7.69	2.96	1.25	0.42
LL (cm)	Leaf Length: <i>Length of the rosette leaf</i>	24.0-81.0	42.80	8.21	0.19
LW (cm)	Leaf Width: <i>Width of the rosette leaf</i>	16.0-42.0	27.51	5.23	0.19
LVW (cm)	Leaf mid Vein Width: <i>Width of the rosette leaf mid vein at the widest point</i>	3.5-28.0	6.94	2.40	0.35
LVL (cm)	Leaf mid Vein Length: <i>Length of the rosette leaf mid vein at the longest point</i>	10.3-60.0	23.81	6.59	0.28
LVT (cm)	Leaf mid Vein Thickness: <i>Width of the rosette leaf mid vein at the thickest point</i>	0.57-2.4	1.14	0.32	0.28
HD (cm)	Head Diameter: <i>Width of the leafy head without external leaves</i>	8.0-23.5	14.26	3.02	0.21
HH (cm)	Head Height: <i>Length of the leafy head without external leaves</i>	12.0-65.0	30.69	9.56	0.32
HW (kg)	Head Weight: <i>Weight of the leafy head without external leaves</i>	0.22-4.52	1.88	0.76	0.40
CW (cm)	Core Width: <i>Width of the core of leafy head</i>	1.6-6.0	3.75	0.85	0.23
CL (cm)	Core Length: <i>Length of the core of leafy head</i>	1.65-11.0	5.02	2.11	0.42
HI (ratio)	Head Index: <i>The ratio of leafy head length and leafy head width</i>	0.85-5.7	2.27	0.95	0.41
HLN	Head Leaf Number <i>Extant number of leaves of leafy head without external</i>	11-69	33.16	9.98	0.30

¹Std. Deviation; ² Coefficient of Variation (CV) = (Std. Deviation / Mean) * 100%

The 17 traits were divided into three clusters based on the outcome of principal component analysis (**Figure 2A**). HDe, CL, HD were classified as “heading capacity traits” with regard to the Chinese cabbage head formation. Thirteen traits (LVL, LL, PL, HtO, HI, HH, CW, PW, HW, LVW, LVT, PD and LW) were classified as “head type traits”. Based on the Eigen Value, CW, PW, HW, LVW, LVT, PD, LW traits mainly belong to the Head Trait component, but are also partly related to “Head

capacity trait” component. This is also supported by Pearson correlation analysis (**Figure 2B**). Significant phenotypic correlations were found within the head type trait and within the heading capacity groups. CL and HDe clustered into one group and LVL, LL, PL, HtO, HI, HH formed another group, with significant correlation among these traits. The traits shared between Head Trait components and Heading capacity component grouped together based on their correlation rate. Only head leaf number (HLN) was negatively correlated with other traits in both component and correlation analysis.

Figure 2. Principal component (A) and Pearson correlation (B) Pearson correlation analysis for 17 traits in 152 Chinese cabbage accessions.

QTL analysis in DH-88 and F2-485

Linkage map construction

Construction of DH-88 linkage maps

The 5795 SNPs were profiled over the DH-88 population, which resulted in 899 bins distributed over ten linkage groups spanning a total genetic distance of 1469.5cM and average distance between markers is 0.92cM. The linkage groups correspond to the Chinese cabbage reference map. We only found one inversion of adjacent SNP bins located at the bottom of A10 and few inconsistent bins located in chromosomes A02 and A07, respectively, compared to the Chiifu Chinese cabbage reference genome (**Supplemental Figure S2**).

Construction of F2-485 linkage map

The DH-88 population consists of only 66 DH lines, limiting mapping precision and detection of significant QTLs. In order to identify significant quantitative trait loci, a large F2-485 population with 485 F2 plants was generated and a linkage map was constructed. A variety of different types of molecular markers were used for the construction of this linkage map. These included 36 SNPs and 99 InDel markers distributed over the ten linkage groups spanning a genetic distance of 1145.7cM with an average distance of 8.93cM between markers (**Supplemental Figure S3**). In general, marker order was in agreement with the Chinese cabbage genome sequence v1.5 (<http://brassicadb.org/brad/>) with few exceptions. There were 36 common SNPs markers between DH-88 and F2-485 linkage maps (**Supplemental Table S4**). Common markers assist in comparison of the QTL positions on the two different linkage maps.

Correlation analysis of rosette leaf and heading traits in both DH-88 and F2-485

Development of rosette leaves in DH-88 and F2-485

Rosette leaves were phenotyped six times during their development; rosette leaf length and width continuously increase throughout the development (**Figure 3**). In DH-88, LL increased significantly between 36DAS (days after sowing) to 47DAS, but growth ceased 50DAS as there was no significantly increase between 50DAS and 53DAS ($P=0.146$). For LW, growth ceased after 47DAS ($P=0.167$). In F2-485, LL increased significantly during all time points measured, while for LW no significant difference was seen between 44DAS and 48DAS ($P=0.763$), and we can conclude that leaf growth ceased at 44DAS. Correlations between LL, LW and PL at different DAS with the trait “Final heading degree” are shown in Figure 3B. In both DH-88 and the F2-485 population, LL and PL are not significantly correlated with heading degree. LW however is positively correlated with “Final heading degree” in both DH-88 and F2-485. In DH-88, the correlation between LW and heading degree was clear from 36DAS to 53DAS, while this correlation was only significant at 37DAS in F2-485.

Correlation coefficients among DH-88 population rosette leaf traits with Heading Degree

Day	36	41	44	47	50	53
LL	0.180	0.168	0.197*	0.183	0.174	0.157
LW	0.277**	0.358**	0.290**	0.283**	0.337**	0.314**
PL	-0.201	-0.192	-0.246*	-0.216	-0.155	-0.179

**Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

Correlation coefficients among F2-485 population rosette leaf traits with Heading Degree

Day	30	34	37	41	44	48
LL	0.156	0.100	0.022*	0.156	0.111	0.32
LW	0.114	0.055	0.016*	0.006**	0.002**	0.067*
PL	0.829	0.169	0.244	0.653	0.330	0.084

**Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

Figure 3. Sixth rosette leaf traits analysis in DH-88 and F2-485 populations throughout development. Box plots of the rosette leaf length (LL), leaf width (LW), petiole length (PL), grouped by growth days (the days after sowing: DAS). Correlation analysis between rosette leaf traits and “Final heading degree”.

Head trait analysis in F2 population

At the heading stage, the heading degree (HDe) was evaluated for 66 DH-88 lines and 485 F2 plants, while HH, HW and HWe were only evaluated in the F2-485 population (**Supplemental Table S5**). In the F2 population, the variance was higher for HH than for HW and HWe. The correlation coefficient analyses showed that HH, HW, HWe and HD were significantly correlated with each other. Correlation between HH and HD was lower, and not significant at the 0.01 level.

Detection of QTLs

QTL analyses for both leaf traits at the rosette stage and heading traits were performed for the DH-88 and F2-485 populations (**Table 2**).

In the rosette stage, QTLs for rosette leaf area length (LL), leaf width (LW) and petiole length (PL) were detected for both the DH-88 and F2-485 population. For LL one QTL was detected in DH-88 (R_DHLL1), while four QTLs (R_F2LL2, 3, 4, 5) were detected in the F2-485. R_F2LL4 and R_F2LL5 had high LOD scores (9.93 and 9.65), and explained around 18% of the phenotypic variation. There was one LW QTL detected on A03 in DH-88 that explained 33% of the phenotypic variation and one on A04 in the F2 that explained 6.8%. For PL, 6 QTLs were detected in DH-88 on A06. Four QTLs on A06 for PL in F2-485 were identified for different developmental days. The QTL region of A06 showed multiple QTL for LL and PL.

In the heading stage, two QTLs for head height (H_F2HH1 and H_F2HH2), one for head width (H_F2HW1) and two for head weight (H_F2HWe1 and H_F2HWe2) were detected in the F2-485. For the heading degree trait, a total of five QTL in four LGs in F2-485 and two on two LGs in DH-88 were detected. Of the QTL detected for the HDe trait, H_F2De7 showed a higher LOD (8.54) value on A08 in F2-485. We found that the HDe QTL region of A03 and A08 were detected in both DH-88 and F2-485, while the HDe QTL on A04 and A05 were only detected in the F2-485 population.

In both DH-88 and F2-485, a major QTL for leaf trichomes (LT) was detected on A06, explaining 64% and 57.8% of the variation.

Table 2. Quantitative trait loci found in DH-88 and F2-485 population

QTL	StaAe	LG	Position (cM)	Nearest markers	LOD	R ² (%)
R_DHLL1A6	41D	A06	46.907-49.574	Bn-A06-p3934890(46.907)-Bn-A06-p4623401(49.574)	3.14	28.0
R_F2LL2A6	37D	A06	56.150	Bn-A06-p4452778(46.310)-A06-22-2(57.157)	4.48	8.9
R_F2LL3A6	41D	A06	55.150	Bn-A06-p4452778(46.310)-A06-22-2(57.157)	7.08	13.7
R_F2LL4A6	44D	A06	49.310	Bn-A06-p4452778(46.310)-A06-4(52.150)	9.93	18.7
R_F2LL5A6	48D	A06	55.150	A06-4(52.150)-A06-22-2(57.157)	9.65	18.3
R_DHLW1A3	47D	A03	84.414-89.414	Bn-A03-p14684924(84.414)-Bn-A03-p15627698(88.414)	3.87	33.3
R_F2LW2A4	44D	A04	11.211	A04-17-2(8.211)-A04-6(12.356)	3.38	6.8
R_DHPL1A6	36D	A06	53.349	Bn-A06-pp4056446(44.241)-Bn-A06-p5845542(53.682)	3.35	29.6
R_DHPL2A6	41D	A06	43.537	Bn-A06-p3496759(41.537)-Bn-A06-p3986320(45.574)	3.75	32.4
R_DHPL4A6	44D	A06	44.241	Bn-A06-p4056446	4.42	37.0
R_DHPL6A6	47D	A06	43.537	Bn-A06-p3496759(41.537)-Bn-A06-p3986320(45.574)	3.72	32.2
R_DHPL7A6	50D	A06	46.574	Bn-A06-p4056446(44.241)-Bn-A06-p4623401(49.574)	3.35	29.5
R_DHPL8A6	53D	A06	48.241	Bn-A06-p4021930	3.04	27.3
R_F2PL9A6	37D	A06	49.310	Bn-A06-p4452778(46.310)-A06-4(52.150)	8.14	15.5
R_F2PL10A6	41D	A06	49.310	Bn-A06-p4452778(46.310)-A06-4(52.150)	10.06	19
R_F2PL11A6	44D	A06	44.394	Bn-A06-p4452778(46.310)	5.97	11.6
R_F2PL12A6	48D	A06	49.310	Bn-A06-p4452778(46.310)-A06-4(52.150)	5.82	11.5
R_DHLT1A6	--	A06	147.610	Bn-A06-p21896359(147.610)	9.75	64
R_F2LT2A6	--	A06	127.7-132.9	Bn-A06-p22910339(106.266)	9.1	57.8
H_F2HH1A6	Head	A06	32.085	A06-43-1(31.085)-zyj6-6-2(59.563)	9.74	9.1
H_F2HH2A3	Head	A03	16.071	B03-11-1(10.071)-B03-12-2(19.627)	6.41	6.1
H_F2HW1A5	Head	A05	60.708	A05-7(60.708)	5.76	5.6
H_F2HWe1A8	Head	A08	10.000	A08-1-1(0.000)-Zyj8-2-2(10.978)	6.49	6.0
H_F2HWe2A6	Head	A06	62.563	Zyj6-6-2(59.563)-Zyj6-10-2(75.643)	5.89	5.5
H_DHHD1A3	Head	A03	73.394	Bn-A03-p12863642(72.394)-Bn-A03-p12903591(73.728)	4.09	34.8
H_DHHD2A8	Head	A08	8.140	Bn-A08-p1847328	3.39	29.9
H_F2HD3A3	Head	A03	8.000	zyj3-3-2(0.000)-Bn-A03-p5439400(10.071)	5.90	5.5
H_F2HD4A4	Head	A04	25.311	Zjy4-7(17.311)-zyj4-9-2(28.172)	4.46	4.2
H_F2HD5A5	Head	A05	57.664	B05-2-1(54.664)-zyj5-18(58.964)	4.28	4.0
H_F2HD6A5	Head	A05	77.975	zyj5-20-2(71.975)-A05-16-2(78.111)	4.91	4.6
H_F2De7A8	Head	A08	7.000	A08-1-1(0.000)-zyj8-2-2(10.978)	8.54	7.8

Co-localization of QTLs between rosette leaf and head traits

For the F2-485 population both rosette leaf and head traits were recorded, while for DH-88 population we measured rosette leaf traits and heading degree only. A total of 9 QTLs in rosette stage and 11 QTLs in heading stage were detected in the F2-485. Several QTLs were found to co-localize (**Figure 4A**). The QTL region of A03, A04, A05, A06 and A08 showed multiple QTLs for two till five traits. Head trait QTL H_F2HH2 and H_F2HDe3 were located at the top position of A03 (0.000-16.071cM). Heading degree and rosette leaf width H_F2HDe4 and R_F2LW2 were mapped closely linked on the top of LG A04. The bottom part of A05 (60.708-77.975cM) showed overlapping QTL for head weight and heading degree traits: H_F2HW1 and H_F2HDe5, H_F2HDe6. QTL for both rosette leaf and head traits R_F2LL (2, 3, 4, 5), R_F2PL (9, 10, 11, 12), R_F2LH2, H_F2HH1 and H_F2HWe2 were clustered at the interval of 32.085-62.563cM region on F2-485 LG A06. QTL for head traits H_F2HDe7 and H_F2We1 were detected at the top of A08 around 10.978cM.

The positions of the rosette leaf RLL, RPL, leaf trichomes LT and heading degree HDe QTLs in this study were compared between DH-88 and F2-485 maps (**Figure 4B**). A QTL for rosette leaf length (RLL: 46.8-49.5cM) was mapped on DH88 LG A06. QTLs for RLL in F2 (46.3-57.2cM) were detected on LG A06 below the common marker Bn-A06-p4452778. The SNP marker Bn-A06-p4452778 was linked with the QTL for RPL in both DH-88 (41.5-53.5cM) and F2-485 (33.4-52.2cM) in this study. The percentage of phenotypic variation explained by RPL QTLs ranged from 64% in DH-88 and 57.8% in F2-485. QTLs for leaf trichomes (LT) were mapped on LG A06 and a comparison of map positions could be made using the common Bn-A06-p22910339 marker in the maps. QTL for heading degree (HDe) in DH-88 (5.4-9.5cM) and F2-485 (0.0-11.0cM) on the top of LG A08 overlap, as they were linked to the common marker Bn-A08-p4528943.

A

RLL: Rosette Leaf Length
 RLW: Rosette Leaf Width
 RPL: Rosette Petiole Length
 HDL: Head Degree
 HH: Head Height
 HW: Head Width
 HWE: Head Weight
 LT: Leaf Trichomes

B

Figure 4. Quantitative trait loci hotspots for the DH-88 and F2-485 population. (A) QTLs are represented by colored vertical bars positioned at the right of each linkage group. Common markers between DH-88 and F2-485 are indicated by “Sxx” and marker information, which is also shown in **Supplemental Table S4**. (B) Zoom in of QTL hotspots of the rosette traits RLL (green), RPL (yellow), leaf trichomes LT (red) and heading degree HDe (black) on DH-88 and F2-485 on A06 and A08.

Discussion and Conclusion

Heading is an important breeding trait, as size and shape of the Chinese cabbage heads determine their market share. In addition, the heading trait is an important domestication trait that was domesticated around 1600 years ago (Tan, 1979; Li, 1981). Identification of QTL and the underlying causal genes can both shed light on the mutations and pathways that were selected for during domestication, but will also generate tools for breeders to select for optimal size and shape of Chinese cabbages.

Head formation related traits

So far, only few studies are published that study the genetics of leafy head formation (He et al., 2000; Wang et al., 2012; Yu et al., 2013), but only very few

combined morphological variation at the rosette stage with variation in heading degree and shape. Mao et al. (2014) demonstrated that variation in expression of *TCP4* miRNA (*miRNA319*) explained head shape and the size, shape and curvature of rosette leaves. They showed clear correlation between morphology of leaves in rosette stage and final leafy head shape. In this study, we also extensively phenotyped leaf development during rosette stage and phenotyped the leafy head in a diverse collection of Chinese cabbage hybrids and inbred lines. This allowed us to evaluate variation and correlation between rosette and heading traits. We found that rosette leaf petiole- and leaf blade length were not correlated with final heading degree (HDe). However, rosette leaf width measured at several developmental stages was correlated with HDe during plant development in F2-485 and DH88 populations. This correlation was weaker at early rosette stage and increased following the head formation process. The observation that rosette leaf width, which largely determines size and heading degree, are correlated fits with the physiology of Chinese cabbage. The special feature of heading Chinese cabbages is that younger leaves that initiate and grow inside the head are not photosynthetically active. As a consequence the outer leaves should remain active until harvest to generate photosynthetates and stimulate N uptake (Wang et al. 2012). Also, the redistribution from older outer leaves to the young inner leaves forming the head is of importance (Lammerts van Bueren & Struik, 2017). At later rosette stages when the head growth increases, the rosette leaves are thus very important and their width may affect the growth of the head.

A number of leafy head related traits were studied in the heading stage of the collection of Chinese cabbage accessions. By phenotype variation analysis, we found that in Chinese cabbage the variation in the extent to which the outer heading leaves overlap each other (HtO) was the largest, while the trait Heading Degree (HDe) showed little variation in the collection. We showed clear correlations among traits, with a cluster of traits related to heading capacity and traits related to the shape of the head. The core of a Chinese cabbage head is the compact stem from which the leaves branch off. The shorter the core is, the denser the head. We detected strong correlation between core length (CL) and heading degree (HDe). Surprisingly, head leaf number (HLN) was not significantly correlated to the core length or to other head related traits. In this study the head weight was measured, however head density (weight divided by volume) was not determined, a trait that is likely related to both leaf number and core length. Currently we developed tools to analyze 3D images that make it possible to calculate head shape, volume and density.

Co-localized QTLs

By phenotyping a large F₂ population derived from a cross between a PC and a CC parent for both rosette and heading traits and in addition phenotyping rosette traits in a DH population generated from the same cross, we found that QTLs for leaf size traits LL, LW, PL, the leaf trichome trait (LT) and leafy head traits HH, HW, HWe, HDe collocated in many cases. This was expected based on the correlations found between these traits.

For rosette leaves traits, common QTLs for leaf length (RLL), petiole length (RPL) and leaf trichome (LT) traits were identified on LG A06 in both the DH-88 and the F₂-485 populations. However several QTLs are found in a single population only. The two populations have very different sizes (485 F₂ plants and 65 DH lines), which affects statistical power to detect QTL. In addition, F₂ phenotypic data are based on single plants while DH data are based on several biological repeats of the same DH line. This, and the fact that all loci are homozygous in the DH, while in F₂ plants homo- and heterozygotes both occur, and dominance relationships of the traits are generally unknown, can all affect QTL detection. QTLs for rosette leaf width (RLW) were identified on chromosome A03 in the DH-88 and on chromosome A04 in the F₂-485. Very interestingly, the RLW QTLs all co-located with heading degree (HDe) QTL. This as discussed based on their correlation, matches the physiological explanation that rosette leaves are the source of nutrients for the growing leafy head that serves as a sink. Colocation of QTL for RLL and RPL on LG A06 in both F₂-485 and DH-88 population illustrates that total leaf length is correlated to petiole length.

For head related traits in the F₂-485, we detected three QTLs for head length (HH) on LG A03, A06, A08, one QTL for head width (HW) on LG A05 and two QTLs for head weight (HWe) on LG A06, A08. Another QTL study in F₃ families from a cross between small-and large heading Chinese cabbage lines identified QTLs for head related traits on the same linkage groups: HH on LG A03 and for HWe on LG A06 and A08 (Ge et al., 2011). In yet another study a head width QTL on LG A05 was detected (Yu et al., 2013). In the study of Yu et al. (2013) HW was phenotyped using 150 recombinant inbred lines from a cross between heading and non-heading Chinese cabbage in both 2011 and 2012 and one HW QTL on LG A05 was identified. In our study, we found that the head traits height, width and weight (HH, HW and HWe) were highly correlated with heading degree (HDe) and their QTLs co-located on LG A03, LG A05 and LG A08. Very interestingly, colocation of QTL for rosette leaves traits (RLL, RPL) and head related traits (HH, HWe) were identified on LG A06 in F₂-485. This again shows the relation

between rosette leaves and leafy heads, where rosette leaves provide the nutrients for the growing leafy head.

In conclusion, we provide information on the relationship between leaf characteristics at rosette stage and leafy head formation. Colocation of QTL for heading related traits and for rosette leaf and heading traits suggests pleiotropic gene regulation of these complex traits. Knowledge about the relationship between the size and shape of rosette leaves that affect heading-degree, -size and -shape, is relevant for breeders that select for optimal quality of Chinese cabbage leafy heads.

Supplementary Tables and Figures

Table S1. 152 Chinese cabbage hybrids and inbred lines used for morphological trait analysis

Table S2. Genes of corresponding to 899 SNP makers in DH-88 map

Table S3. Details of the 135 InDel/SNP markers on the map of F2-485

Table S4. The common SNP marker (S1-S10) between DH-88 and F2-485

Common marker	Linkage group	Primer number in F ₂ / DH-88	Common marker	Linkage group	Primer number in F ₂ / DH-88
S1a	A01	B01-15-2/ Bn-A01-p994722	S7a	A07	B07-12-2/ Bn-A07-p7027400
S1b	A01	B01-10-1/ Bn-A01-p6376105	S7b	A07	B07-3-1/ Bn-A07-p10099786
S1c	A01	B01-5-2/ Bn-A01-p24655110	S7c	A07	B07-8-2/ Bn-A07-p14958606
S2a	A02	B02-2-1/ Bn-A02-p3422005	S7d	A07	B07-6-1/ Bn-A07-p13639374
S2b	A02	B02-5-1/ Bn-A02-p19683093	S7e	A07	B07-9-2/ Bn-A07-p19977445
S2c	A02	B02-7-1/ Bn-A02-p25181474	S8a	A08	B08-3-2/ Bn-A08-p4528943
S3a	A03	B03-11-1/ Bn-A03-p5439400	S8b	A08	B08-11-2/ Bn-A08-p12761022
S3b	A03	B03-12-2/ Bn-A03-p6919500	S8c	A08	B08-7-1/ Bn-A08-p14447288
S3c	A03	B03-3-2/ Bn-A03-p8629992	S8d	A08	B08-1-2/ Bn-A08-p17586676
S3d	A03	B03-9-2/ Bn-A03-p16812953	S8e	A08	B08-2-1/ Bn-A08-p20085504
S3e	A03	B03-15-2/ Bn-A03-p20560569	S9a	A09	B09-17-2/ Bn-A09-p1243032
S5a	A05	B05-6-1/ Bn-A05-p1671823	S9b	A09	B09-7-1/ Bn-A09-p2044817
S5b	A05	B05-7-1/ Bn-A05-p2898061	S9c	A09	B09-15-2/ Bn-A09-p6632506
S5c	A05	B05-15-2/ Bn-A05-p6337412	S9d	A09	B09-13-1/ Bn-A09-p30002747
S5d	A05	B05-2-1/ Bn-A05-p18453140	S9e	A09	B09-8-1/ Bn-A09-p29348596
S6a	A06	B06-2-2 /Bn-A06-p4452778	S10a	A10	B10-4-2/ Bn-A10-p9186877
S6b	A06	B06-4-1/ Bn-A06-p22910339	S10b	A10	B10-6-1/ Bn-A10-p15031810

Table S5A. Summary statistics for the head traits in F2-485

Trait	PC-101	CC-48	Mean	S.D	Variance
HH	-	52.4	34.57	5.081	25.81
HW	-	15.2	16.42	3.124	9.759
HWe	-	2.5	1.688	0.674	0.454

Table S5B. Correlation coefficients of head traits in F2-485

Trait	HW	HWe	HDe
HH	0.214**	0.434**	0.118*
HW	1	0.638**	0.273**
HWe		1	0.348**

Figure S1. Distribution analysis for PL, PD, PW, LL, LW, LVW, LVL, LVT, HD, HH, HW, CW, CL, HI and HLN traits.

Figure S2. Genetic linkage map (orange) and Physical map (Chiifu reference genome: blue) of DH-88 consisting of 10 linkage groups with 1603 SNP markers.

Figure S3. Genetic map of the F2-485 consisting of 10 linkage groups with 36 SNP and 99 InDel markers. Common markers between DH-88 and F2-485 are pink.

Tables S1, S2, S3 and Figure S1 could be found on:

https://www.frontiersin.org/articles/10.3389/fpls.2018.01455/full?utm_source=Email_to_authors&utm_medium=Email&utm_content=T1_11.Se1_author&utm_campaign=Email_publication&field=&journalName=Frontiers_in_Plant_Science&id=411309

Chapter 3

Genome wide transcriptome analysis reveals molecular pathways involved in leafy head formation of Chinese cabbage (*Brassica rapa*)

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Abstract

Chinese cabbage plants go through seedling and rosette stage before formation of their leafy head. Pak choi plants resemble Chinese cabbage at their seedling stage, but in their rosette stage the leaves are different from Chinese cabbage leaves. The latter increase in size and have shorter petioles. In order to understand the molecular pathways that play a role in leafy head formation, transcript abundance of young emerging leaves was profiled during development of two Chinese cabbage genotypes and a single pak choi genotype. The two Chinese cabbages differed in many aspects, among others earliness, leaf size and -shape, leaf numbers and leafy head shape. Genome wide transcriptome analysis clearly separated the seedling stages of all three genotypes and all pak choi rosette time points from the later developmental stages (rosette, early heading and heading) of both Chinese cabbages. Weighted correlation network analysis (WGCNA) and hierarchical clustering using Euclidean distance allowed identification of gene clusters with transcript abundance patterns distinguishing the two Chinese cabbages from pak choi. Three clusters included genes with transcript abundance affected by both genotype and developmental stage, while two clusters showed only genotype effects. This included a genotype by developmental stage cluster highly enriched with the MapMan category photosynthesis, with high expression during rosette and early heading in Chinese cabbages and low expression in the heading inner leaves that are not exposed to light. The other clusters contained many genes in the MapMan categories Cell and Cell wall, showing again differences between pak choi and both Chinese cabbages. We discuss how this relates to the differences in leaf blade growth between Chinese cabbage and pak choi, especially at the rosette stage. Overall, comparison of the transcriptome between leaves of two very different Chinese cabbages with pak choi during plant development allowed the identification of specific gene categories associated with leafy head formation.

Introduction

Chinese cabbage (*Brassica rapa* ssp. *pekinensis*), is a widely cultivated and economically important vegetable in Asia, composed of a large number tightly wrapped heading leaves surrounding the shoot apexes (He et al., 2000). The leafy head is the storage organ of Chinese cabbage and is formed after the rosette stage (Wang et al., 2012). Head shapes of Chinese cabbages vary depending on the cultivars. Developmental growth stages for Chinese cabbage have been described at the morphological level, including germination, seedling, rosette and heading stages (Ke, 2010). At seedling stage, primary and juvenile leaves are round and have long petioles. At rosette stage, rosette leaves (RL) become large and round with short petioles, which begin to fold upward. After rosette stage, incurving process of the leaves continues until heading leaves (HL) are arranged tightly around each other to form leafy heads as storage organs for nutrients. The heading leaves are wrinkled with an upward curvature with broad midveins. Both the size, shape and degree of incurvature heading leaves define the final head size and shape.

We also found that size and shape of heading leaves correlates to that of the rosette leaves (chapter 2 in this thesis). However, the exact division between each developmental stage is not clear and the molecular mechanism during leafy head formation is still not well studied. In the study by Wang et al., (2012), a wide range of transcriptional events and interesting gene expression patterns were described analyzing RNA-sequencing data from rosette leaves and folding leaves of a typical heading Chinese cabbage (inbred line Fushanbaotou). Their results showed that stimuli like carbohydrate levels, light and hormones play important roles in leafy head development and that regulation of transcription factors, protein kinases and calcium play important roles in this developmental process. In yet another study a global analysis of microRNAs was performed in rosette and heading stage of Chinese cabbage (Wang et al, 2013). Besides identification of many conserved and novel miRNA's, they also annotated the target genes of miRNA with differential expression patterns between rosette and heading stages, which included transcription factors, F-box proteins, protein kinases, auxin and Ca signalling proteins, illustrating their roles in leafy head formation.

The external leaves of Chinese cabbage leafy head are green (Li et al., 2018). The inner head leaves are not exposed to the light and can be white, orange or yellow (Li et al., 2012). These yellow inner leaves accumulate higher lutein and carotene content and involve not only lutein and carotenoid synthesis but also other major metabolic processes, such as chlorophyll synthesis and degradation (Wang et al.,

2012; Li et al., 2018). These processes may also be important for leafy head formation during heading stage. By composite interval mapping analysis, three QLTs have been identified controlling the yellow color of the inner leaves in *B. rapa* (Li et al., 2012). However, little is known about the genetic function and metabolic contribution to the development of yellow leaves.

In our study, we profiled transcript abundance in more developmental stages, including late seedling, rosette, early heading and heading stage. This was done for two different Chinese cabbage genotypes, an early and a late heading type with diverse head shapes and a non-heading pak choi. The non-heading pak choi (*Brassica rapa* ssp. *Chinensis*) morphotype is closely related to heading Chinese cabbage as concluded from genetic diversity studies (Zhao et al., 2005; Wang et al., 2011; Cheng et al., 2016). Pak choi does not form leafy heads, and the flat green leaves with fleshy petioles that grow as a rosette represent the consumed part of this leafy vegetable. In addition, the inner leaves remain green during pak choi development. By comparing heading Chinese cabbage with non-heading pak choi, numerous genes that may specifically relate to head formation and response for yellow inner leaf development can be detected. Weighted correlation network analysis (WGCNA) resulted in identification of gene co-expression modules that differentiate between genotypes, between developmental stages or between both. Our study provides new insights into understanding the genetic mechanisms of leafy head formation and the associating development of yellow leaves.

Materials and Methods

Plant materials and growing conditions

Double haploid (DH) lines of heading Chinese cabbage (CC-A03, CC-Z16) and non-heading pak choi (PC-024) were used in this study. The seeds of these three DH lines were germinated on seeding soil for one week and then transplanted into 17cm pots. Pots were placed into three blocks in the greenhouse (Unifarm, Wageningen University & Research, 51°59'11"N latitude, 05°39'52"E longitude) and plants were grown under short day conditions for 8 to 12 weeks.

Observations and sampling

Three individual plants for each DH line per block were observed and numbers of leaves (leaf length > 1cm) were counted once a week during plant growth. The developmental stage, like rosette, folding, heading and also bolting stages were

evaluated each week. Young emerging leaves surrounding the shoot apical meristem, around 2cm long, were harvested each week from three individual plants per block and were combined to one sample. Harvest of the young emerging leaves in the center of the developing head at the heading stage of the Chinese cabbage lines (CC-A03 and CC-Z16) was destructive, as the outer head leaves needed to be removed in order to access the inner central leaves. Leaf samples were harvested starting from week 2 after germination to week 12 for CC-A03 (week 2, week 5, week 8 and week 11) and week 2 to week 8 for CC-Z16 and PC-024 (week 2, week 4, week 6 and week 7). At each time point, three biological replicates (one per block) for each DH line were used for RNA isolation.

Expression analysis

The aim of this analysis was to gain insight into which concepts associated with Chinese cabbage leafy head formation by comparing heading CC with non-heading PC. Total RNA for microarray was isolated from the frozen leaf samples with “RNeasy Plant kit” (Qiagen) and treated with RNase-free DNase I (Invitrogen, Carisbad, CA, USA) to remove DNA contamination.

Microarray hybridization design

The microarray probes for two-color Agilent microarray (Cy3-green/Cy5-red) platform were based on the predicted gene models of the reference *B. rapa* cv. Chiifu (a leafy vegetable inbred line) genome sequence (Wang et al., 2011). This array assembly covers 61,654 probes and covers 40,879 (99.74%) *B. rapa* gene IDs (Bra ID) with 108 (0.26%) scaffold IDs and detailed information refers to Basnet et al., (2013). For microarray hybridization we used developing leaves from three genotypes: two heading Chinese cabbage (CC-Z16 and CC-A03) and one non-heading pak choi (PC-024) at four time points. The array design for this study is given in **Supplemental Table S1**. Two samples from the two consecutive time points of the same genotype were hybridized on one array. Each array has two biological repeats and eight arrays were used for one genotype.

Microarray data analysis

Microarray data were normalized within and between arrays using the limma package in R (Smyth, 2005). “Two Color Separate Channel” method was used to measure the gene transcript abundances. Principal components analysis (PCA) was used to check the dominant modes of variation of all gene transcript abundance data. Then gene transcript abundance differences for three DH lines

(genotype) and between developmental stages (time point) were determined with adjusted p -value ≤ 0.01 and fold change > 1.5 for further analysis.

Weighted gene correlation network analysis (WGCNA) was used to find gene co-expression modules for differential transcript abundance genes according to Pearson's correlation (Horvath & Dong, 2008). Genes with similar co-expression patterns across genotypes, plant developmental stages or their combinations were clustered into a module.

After defining gene modules, analysis of variance (ANOVA) tests were performed to determine the transcript abundance variation according to genotypes, time or both genotype and time at a 0.05 probability level. To cluster the probes at the level of transcript abundance, the hierarchical clustering using Euclidean distance was carried out separately for the probes in genotype effect gene modules, time effect modules or genotype and time effect modules in the statistical software Multi-Experiment Viewer (MeV). The open source software MapMan is used for probes annotation (Usadel et al., 2005) in each category.

For the co-expression network analysis, genes were selected after WGCNA and hierarchical cluster analysis. For each network analysis, gene pairs with at least Pearson correlation coefficient of 0.9 were considered. A parameter “degree of connection” of each node/gene was calculated in the statistical software R, indicating the number of connections (with Pearson correlation coefficient > 0.9) with other genes.

Results

Comparison of morphology between heading Chinese cabbage and non-heading pak choi

In their vegetative growth phase, both Chinese cabbage (CC) and pak choi (PC) go through seedling and rosette stages. In Chinese cabbage this is followed by the folding stage after which the leafy head is formed (**Figure 1**).

After eight weeks both Chinese cabbage CC-Z16 and pak choi PC-024 reached the bolting stage with similar leaf numbers formed. In week one and week two plants were in the seedling stage, followed by the rosette stage, which is characterized by the onset of upward curving of leaves. Younger emerging leaves have increased leaf angles. At week five the leaves of CC-Z16 become incurved with short petioles forming a round head. By contrast, CC-A03 develops slower, with a longer rosette

stage and around weeks 7 till 9 the early heading stage, where leaves curve upwards, and around week 10-11 onset of leafy head formation. At the early heading stage, total leaf numbers of the late CC-A03 forming a head reaches 42, while CC-Z16 counts 25 leaves at this developmental stage, similar to the leaf number of the non-heading pak choi. At heading stage, the two Chinese cabbages have different head shapes: CC-A03 has a cylindrical/round head shape; CC-Z16 has a round head shape.

Figure 1. Morphological characterization of vegetative phases of the early heading Chinese cabbage CC-Z16 with round leafy head and the late heading CC-A03 with cylindrical head and non-heading pak choi (PC-024).

Variation in transcript abundance during plant development

Our aim was to study changes in transcript abundance in the four developmental stages, seedling, rosette, early heading and heading, which occur much later in CC-A03 than in the early CC-Z16. As PC-024 has no-heading stage, and stays in the rosette stage after the seedling stage, but has similar developmental timing from seedling to bolting stage, we isolated leaf tissue for the analysis at identical time points compared to CC-Z16. Biological repeats corresponded very well to each other as visualized by cluster analysis. Principal component analysis (PCA) of transcript abundance of the 61,654 probes on the used customer *B. rapa* array over the three genotypes and four developmental stages showed that plant

developmental stages (seedling, rosette, early heading and heading stages) distribute along the first principal component (PC1) explaining 50.1% of the variation. The early heading (blue symbol) and heading stage (yellow symbol) form a tight group for PC-024 and CC-Z16 especially, while for CC-A03, the early heading stage and heading stage were separated along the x-axis. Major changes in transcript abundance were observed between seedling (red symbol) to rosette (green symbol) and to early heading stages (blue symbol) in all three genotypes. The three genotypes (CC-Z16, CC-A03 and PC-024) separated in the second principal component (PC2), which explained 10.1% of the total variation. The two heading genotypes CC-Z16 and CC-A03 grouped together, separated from non-heading genotype PC-024 (**Figure 2A**).

Figure 2. Sample analysis based on leaf transcript profiles during plant development. (A) Principal component analysis (PCA) of three genotypes (CC-A03, CC-Z16 and PC-024) based on transcript abundance at four developmental stages (seedling, rosette, early heading and heading stages). The orange lines represent heading Chinese cabbages (CC-A03 and CC-Z16), and the green line represents non-heading pak choi (PC-024). Sample labels were colored according to developmental stages: seedling stage-red, rosette stage-green, early heading stage-blue and heading stage-yellow. The samples were clustered based on the overall gene expression. (B) Cluster analysis for the three genotypes and four developmental stages/ time points. The average values of the two biological repeats were used.

The cluster analysis revealed some interesting patterns (**Figure 2B**). Seedling stage (W2) of CC-A03 and CC-Z16 grouped with PC-024 seedling (W2) and rosette stages (W4), which then clustered close to the PC-024 later developmental stages (W6 and W7). A different cluster was formed by the rosette, early heading and heading stages of the two CC's that formed sub-clusters per genotype. Overall, the differences between seedling and later stages explained most of the

clustering, except for the pak choi, as all its developmental stages grouped together with the seedling stages.

Differences in transcript abundance between the early genotypes CC-Z16 and PC-024

First we looked at differences in transcript abundance between developmental stages of heading CC-Z16 and non-heading PC-026 sampled at the same time points (seedling week 2 to rosette week 4, rosette week 4 to early heading week 6 and early heading week 6 to heading week 7). A threshold value of $p\text{-value} \leq 0.01$ and an absolute value of $\log_2\text{Ratio} \geq 1.5$ was chosen, which resulted in a selection of 4372 probes corresponding to 3727 genes with significant differences in transcript abundance. For these selected probes we plotted the numbers belonging to 34 MapMan functional categories indicating developmental stages (**Figure 3**). Differential expressed genes between heading CC-Z16 and non-heading PC-026 were highly represented by the following MapMan categories: development, transport, signalling, protein, RNA, stress, cell, cell_wall, hormone_metabolism and photosynthesis. In addition, a large number of differential expressed genes were not_assigned to functional MapMan categories.

Transcript abundance of these 4372 probes corresponding to 3727 genes was then analyzed by the WGCNA method using both the non-heading PC-024 and the two heading Chinese cabbage data. Seventeen clusters of highly correlated genes were defined. Each module clusters genes with similar transcript abundance patterns either across genotypes (heading CC-Z16, CC-A03 and non-heading PC-024), across different developmental stages (time) or across both. ANOVA tests ($p\text{-value} \leq 0.05$) showed that from all 17 modules, eight (2337 probes) showed a genotype effect, four (736 probes) showed a time effect and five (1299 probes) both a genotype and time effect (**Supplemental Table S2**). As our main interest was to identify genes that are involved in leafy head formation, based on the transcript abundance patterns for each module, nine modules (genotype effect: dark olive green, sienna3, dark magenta, black and grey; time effect: dark turquoise and green yellow; genotype x time effect: steel blue and dark red) with similar transcript abundance between CC-Z16 and CC-A03, but different from PC-024 were selected from 17 modules for further analysis (**Supplemental Figure S1**).

Figure 3. The numbers of genes with differences in transcript abundance between different developmental stages/time points in CC-Z16 or PC-024 belonging to MapMan functional categories. Genes with significant differences in transcript abundance were based on $p\text{-value} \leq 0.01$ and an absolute value of $\log_2\text{Ratio} \geq 1.5$. Week 2 corresponds to seedling stage of both CC-Z16 and PC-024; week 4 to rosette stage, week 6 to early heading stage and week 7 to heading stage in CC-Z16, while weeks 4, 6 and 7 are all rosette stages for PC-024.

Selected modules with genotype, time point or genotype by time effect

The nine modules with contrasting patterns between the two CC's and PC, consisting of five genotype effect modules, two time effect modules and two genotype x time effect modules were analyzed by hierarchical clustering (**Figure 4**).

Figure 4. Hierarchical cluster analysis using the probes from nine WGCNA modules that differentiate the two Chinese cabbages (CC-Z16 and CCA-03) from PC-024. (A) Probes from five genotype (CC-A03, CC-Z16 and PC-024) effect modules. (B) Probes from two development effect gene modules (seedling, rosette, early heading and heading in CC-A03 and CC-Z16 and similar time points for PC-024 as CC-Z16). (C) Probes from two genotype by developmental stage/time effect gene modules.

Hierarchical clustering divided the 1687 genes belonging to genotype effect modules into five clusters (**Figure 4A**). In gene clusters I (186 genes) and II (277 genes) transcript abundance of CC-Z16 and PC-024 was more similar, but different from that of CC-A03. Whereas in gene cluster IV (644 genes) transcript abundance was more similar between CC-A03 and PC-024. The genes in clusters III (239 genes) and V (341 genes) had similar transcript abundance patterns in

the two CC genotypes compared to PC: high transcript abundance in both CC-Z16 and CC-A03 but low in PC-024. The 231 genes from the time effect modules were divided into two clusters (**Figure 4B**). Transcript abundance in these two clusters was significantly different at all time points or developmental stages between all three genotypes. Cluster VI (109 genes) showed increased transcript abundance across the developmental stages, while cluster VII (122 genes) showed decreased transcript abundance. The 581 probes of the two modules displaying both genotype and time effects were divided into three clusters (**Figure 4C**). Transcript abundance of genes from cluster VIII (198 genes) gradually increased across developmental stages, with lower transcript abundance at rosette stage in the two CC genotypes than in PC. Genes in cluster IX (333 genes) showed decreased transcript abundance across developmental stages and a large number of genes showed higher transcript abundance in both CC's compared to PC at rosette stage. Genes in cluster X (50 genes) had high transcript abundance levels in seedling stage of all genotypes, however at later stages transcript abundance in PC remained high, while in both CC's transcript abundance was low after seedling stage.

Enrichment analysis of differentially expressed genes

Cluster III, V, VIII, IX and X were characterized by most pronounced similarity in transcript abundance in the two CC's, different from PC. These genes may have roles in the leafy head formation typical for Chinese cabbages. Except not assigned probes, the numbers of probes belonging to MapMan functional categories under genotype, time and genotype by time effect are presented in **Figure 5**. We focussed on main categories using a cut off value of three percent. The categories with percentages higher than 6% are indicated in the pie chart, while all categories are presented in supplemental **Supplemental Table S3**.

The majority of the differentially expressed genes in the genotype effect clusters (Cluster III and V) were involved in protein (21%), RNA (17%), misc_otherPhosphate (9%), various stimuli (9% signalling and 7% stress), cellular component (4% cell and 4% cell wall, total 8%) and some metabolism components of the hormone, lipid and amino acid metabolism categories. The main functional group of proteins included genes related to ribosomal protein synthesis, including L18, L30, S3 and S11 protein families. In RNA category, genes were represented associated with RNA regulation of transcription factors like MADS-box proteins, MYB proteins, G2-like (GARP) proteins, WRKY domain proteins, basic-leucine zipper (bZIP) proteins and basic helix-loop-helix (bHLH) DNA-binding proteins. In addition, a large number of differently expressed genes

with response to light, calcium, heat and hormone (auxin/cytokinin) stimuli were identified.

Figure 5. Pie charts showing the percentage of genes that belong to MapMan functional categories in selected modules with transcript abundance patterns distinguishing the two heading CC genotypes from the non-heading PC genotype. The genotype effect pie chart includes the combined cluster III and V genes; genotype by time effect modules include cluster VIII, IX and X genes.

Differently expressed genes in genotype x developmental stage effect clusters (VIII, IX and X) have very different transcript abundance patterns and their top functional categories were also not the same. In cluster VIII and X, similar categories were presented: genes associated with functional protein, misc_otherPhosphate, RNA, signalling, stress and DNA are represented with high percentage values (>6%), while the functional categories signalling and cell_wall were highly represented in cluster VIII and transport and DNA in cluster X. Genes in the photosynthesis category ranked first in cluster IX. 41% genes were involved in photosynthesis, which contained light reaction (photosystem I, photosystem II),

calvin cycle and photorespiration. The gene numbers of other top categories were much lower than photosynthesis: misc_otherPhosphate (7%), protein (6%), RNA (6%), stress (6%), hormone and secondary metabolism (6%). Interestingly, some of the genes in these categories were associated with chloroplasts: chloroplast RNA binding (CRB) in RNA category; germin-like proteins (GLP3 and GLP1) in stress category; isoprenoids carotenoids phytoene synthase (PSY), flavonoids dihydroflavonols (ATCHIL) and chloroplast lipxygenase (LOX2) in hormone and secondary metabolism categories.

Top ranking co-expression genes

To further analyze the data we identified genes with high degrees of connectedness within the clusters based on Pearson correlation coefficients. This resulted in a list ranking genes according to their numbers of connections, which in fact illustrates co-regulated networks. These top ranking genes may function as key regulators of biological processes involved in Chinese cabbage or pak choi leaf development.

For the clusters III and V, which include genes that are higher expressed in the two CC's than in PC, the top 20 included eight genes in “protein”, three in “cell” and two in “RNA” categories (Pearson correlation coefficient > 0.9). For cluster VIII, with both a genotype and a developmental stage effect this top 20 also included five genes in the “protein” category, but very interestingly also six genes in the “cell” and “cell wall” classes, but also four in “glycolysis”. Cluster IX like VIII was a genotype x developmental stage effect cluster, but showed opposite expression patterns compared to cluster VIII, with lower expression at later stages, especially in PC. Here the top 20 genes included 11 “photosynthesis” genes and six not assigned genes. The top 20 genes are presented in **Supplemental Table S4**.

Discussion

In this study, we performed transcriptome analysis to get information about the pathways and processes that are important in leafy head formation of Chinese cabbages. Leafy heads form through a series of complex developmental processes and are controlled by multiple internal and external signals. Others and we clearly showed that the leafy head trait is a quantitative trait, regulated by many QTLs with likely small effects. In order to identify pathways that play major roles in governing leafy head formation in Chinese cabbage, we compared transcript abundance in two heading Chinese cabbages and one non-heading pak choi during development.

Comparing Chinese cabbage and pak choi

Although Chinese cabbage and pak choi are closely related (Zhao et al. 2005; Wang et al., 2011; Arias et al., 2014) they have very different leaf shapes and sizes and pak chois do not form a leafy head. We first compared CC-Z16 with PC-024. The commonality between the two is that they have similar growth period, from seedling (week 1) through rosette (week 4) to bolting (week 8) stage. In addition, their leaf surfaces are smooth and not rugose. The differentially expressed genes selected in this group illustrate differences between heading and non-heading as they likely exclude the effect of developmental age. The second comparison is between CC-Z16 and CC-A03. Although both are heading Chinese cabbages, they differ in many aspects: their rosette and heading leaves have different shape and size. CC-Z16 leaves are flat without an obvious mid-vein, while CC-A03 leaves have a rugose surface, as inter-vein cell expansion leads to an irregular surface, with a broad leaf mid vein. Moreover, the rosette and heading stage in CC-A03 develop much later than in CC-Z16 with many more leaves and whereas head shape of CC-Z16 is round, head shape of CC-A03 is cylindrical. As we focus on genes with similar expression patterns between these two CC's, we expect that genes involved in leaf- and head size and shape are excluded from the analysis.

In the Wang et al., (2012) study, they compared the transcriptomes of rosette leaves (RL) and folding leaves (FL) for the inbred CC line Fushanbaotou. They described the stages with leaf numbers: rosette stage with 8–10 expanded leaves, folding stage with 23–25 expanded leaves. We sampled leaves from CC-A03, which is a late heading Chinese cabbage like Fushanbao, at comparable stage (rosette stage with 10–16 leaves, early heading stage with 16–34 leaves), but also looked at both earlier (seedling) and later (heading) stages. Based on our PCA analysis, we conclude that Wang et al. (2012) only sampled a small part of the variation, compared to our study, as a large part of the variation occurs between seedling and rosette stages. As we selected genes that had different transcript abundance in pak choi leaves on the one hand that differed from that of two Chinese cabbages with similar transcript abundance on the other hand at more stages, this resulted in the identification of less differentially expressed genes compared to the Wang et al. (2012) study.

Effect of genotype and time/developmental stage

Leafy head formation, size and shape vary according to the genetic makeup of the plant and are influenced by both internal (development, hormones, age) and

external (environmental) factors. In PCA analysis, the first dimension separated the genotypes according to their developing stages, however the second dimension (explaining 10% of the variation) separated the two CC's from PC. In the cluster analysis, the long rosette stage of PC (week 4 to week 7) formed a subcluster grouping together with seedling samples for all three genotypes in one cluster, which was clearly separated from the other cluster with rosette and heading stages of the two CC's, with sub-clusters dividing the two genotypes. These results indicate that the differences between CC and PC start from the rosette stage.

In addition, hierarchical clustering of genes revealed in WGCNA with significant genotype, time or genotype by time effect assisted in identification of clusters that clearly showed differences between the two CC's on the one hand and the single PC on the other hand. In the time effect clusters VI and VII, there were no obvious differences between genotypes and these most likely do not include genes that play specific roles in leafy head formation. However clusters with clear genotype effects and especially those with genotype and time/developmental stage effect are interesting and can reveal molecular pathways that are important in leafy head formation. Very interestingly, the major MapMan categories to which genes belonged in these clusters differed.

Functional categories of differentially expressed genes involved in leafy head formation

Interestingly, compared to earlier studies of the transcriptome of Chinese cabbages (Wang et al. 2012), we identified two additional categories: cell, cell wall functional categories (genotype clusters III/ V and genotype by time clusters XIII/ X) and photosynthesis category in genotype by time cluster IX. Leaves, especially the rosette leaves, serve as photosynthesizing organs and are thus important for nutrient absorption and plant growth (Kim et al., 2017). Leafy heads increase very fast in volume and weight, by both forming many leaves from the SAM and by increase of the leaf size of the outer heading leaves. During this growth stage, the leafy head becomes very firm with tightly packed leaves. In this stage, the rosette leaves are essential, as they need to generate the energy needed for the growing head, like sugars and nitrogen for amino acids. Rosette leaves indeed increase in size and do not senesce during the growth cycle (van Bueren & Struik, 2017). It would be interesting to measure their photosynthesis efficiency during this growth stage. Compared to the rosette leaves, the inner heading leaves of Chinese cabbage are storage organs that do not photosynthesize as they are shielded from the light. These leaves have different characteristics in terms of

nutritional value, taste and color. In this study, we found significant differences in the expression of genes relating to photosynthesis in the genotype by time effect cluster IX. At rosette stage, the photosynthesis genes have higher levels in the two CC's compared to PC in order to support the head formation by photosynthesis. In early heading and especially heading stage, the inner leaves of CC's become covered by the outer heading leaves, become yellow and transcript abundance of most photosynthesis genes decreased. The PC-024 green central leaves were exposed to the light during all developmental stages, however transcript abundance of photosynthesis genes was lower compared to CC's, likely because plants did not form a head that acts as a strong sink for nutrients. Transcript levels did hardly change after seedling stage. Further investigations of the photosynthesis genes are needed to fully comprehend the roles of the rosette leaves in leafy head formation and may provide leads for breeders to improve head shape and size. Beside the differences in photosynthesis activity between CC's and PC especially at rosette stage, the leaf shapes are also different. Chinese cabbages had large upwards curling smooth or rugose rosette leaves, while pak choi rosette leaves are flat with narrow petioles (this study and Opana, Kuo & Voon, 1988). The leaf shape is defined by cell shape, size and positioning (Kalve et al., 2014). We also observed and quantified cell shape, size and density of both palisade and spongy parenchyma of seedling and rosette leaves of CC-Z16 and PC-024 (chapter 4 of this thesis). This clearly showed that in Chinese cabbage CC-Z16, leaf growth is mainly directed by cell expansion and decreased cell density especially in the spongy parenchyma, while in PC-24 leaf growth was mainly by cell proliferation, resulting in more smaller sized cells that were more densely packed. In Cluster XIII 8% of the genes belong to MapMan categories cell wall and 6% to cell, which confirms that cell growth and division are important processes differentiating PC from CC's during their development. Among the top 20 of genes with highest degrees of connectivity in cluster VIII are the cell category genes Bra014865 (*Arabidopsis* orthologue actin 3) and Bra033737 (tubulin beta chain) involved in cell shape and growth. Therefore, we conclude that differences between CC's and PC during development relate to leaf cell development thereby affecting the final leaf shape and size.

These results provide new insights in leafy head formation and also make evident that besides phenotyping the whole plant development, both morphological observation at cellular level and studies of photosynthetic processes are needed. The choice of two Chinese cabbages with very different phenotypes narrowed down the selection of genes with differences in transcript abundance to better reveal the processes involved in leafy head development of Chinese cabbages.

Supplementary Tables and Figures

Table S1. Array design for the two-color Agilent customer *B. rapa* microarray (green/red) used in this study. (A) Sample information for array materials: tissue used was harvested from central young emergig leaves. (B) Schematic diagram for the two-color array design loop. Cy3 dye is green, Cy5 dye is red.

A. Time point	T1	T2	T3	T4	B.
Stage	Seedling	Rosette	Early Heading	Heading	
CC-A03	Week 2	Week 5	Week 8	Week 11	
CC-Z16	Week 2	Week 4	Week 6	Week 7	
	Seedling	Rosette	---	---	
PC-024	Week 2	Week 4	Week 6	Week 7	

Table S2. ANOVA test association for WGCNA gene clusters with genotype or time or both genotype and time effects.

Cluster	p-value_genotype	p-value_time	Probe NO.
MEblack	2.70E-07	0.652574619	1446
MEdarkmagenta	0.013269828	0.804125755	42
MEdarkolivegreen	0.002644206	0.743355948	48
MEgrey	0.022733117	0.195451571	119
MElightyellow	1.29E-05	0.682068436	104
MEMidnightblue	0.024825112	0.057093745	486
MEsaddlebrown	0.004320065	0.956819425	60
MEsienna3	0.015020576	0.21755285	32
MEdarkturquoise	0.549452006	4.00E-10	85
MEgreenyellow	0.059924347	0	146
MElightgreen	0.16627194	1.43E-06	258
MEsalmon	0.796956832	1.90E-09	247
MEdarkgrey	0	0.003667838	82
MEdarkred	9.71E-05	0.003216904	159
MElightcyan	0.001162931	0.039830504	110
MEsteelblue	0.008535943	7.76E-07	522
MEwhite	0.011703878	0.01100325	426

The association is significant at the 0.05 level in ANOVA analysis. The light green cells indicate genotype effect clusters; green cells indicate time effect clusters; dark green cells indicate genotype and time effect clusters.

Table S3. Number of genes in Mapman categories of hierarchical clusters with genotype (III and V) or genotype by time (VIII, IX and X) effects

Categories	Cluster III & V	Cluster VIII	Cluster IX	Cluster X
protein	98	33	17	8
RNA	81	18	16	5
misc_otherPhosphate	43	22	18	6
signalling	43	17	2	1
stress	34	15	16	4
cell	19	8	14	1
cell_wall	18	14	4	2
transport	15	4	3	5
hormone_metabolism	14	6	9	1
aminoacid_metabolism	13	6	6	-
DNA	13	-	3	3
lipid_metabolism	13	4	3	-
metal_handling	13	2	2	-
secondary_metabolism	8	1	7	-
redox	7	4	5	1
development	6	9	6	1
Photosynthesis	5	1	110	2
ATP_synthesis	4	2	2	-
nucleotide_metabolism	4	-	2	-
N-metabolism	3	-	-	1
OPP	3	-	-	-
C1-metabolism	2	-	-	-
Cofactor_vitamine	2	-	5	-
minorCHOMetabolism	2	-	1	-
fermentation	1	-	-	-
TCA	1	-	4	-
tetrapyrrole_synthesis	1	-	3	-
glyoxylate_cycle	1	1	1	-
majorCHOMetabolism	1	-	3	-
Total number	468	197	262	41

Table S4. The top 20 highest connected genes in cluster III & V VIII & X and IX based on Pearson correlation with coefficient of value 0.9

Genotype effect cluster III & V					TAIR ID
Ranking	All_Nodes	Annotation	Pathways		
1	Bra006775	protein	protein.degradation.ubiquitin.proteasom		AT5G58290
2	Bra038630	not_assigned	not assigned.unknown		AT5G63440
3	Bra003186	nucleotide_metabolism	nucleotide metabolism.synthesis.pyrimidine.orotate phosphoribosyltransferase		AT3G54470
4	Bra018184	cell	cell.organisation		AT5G62700
5	Bra007154	lipid_metabolism	lipid metabolism."exotics" (steroids, squalene etc).trans-2-enoyl-CoA reductase		AT3G55360
6	Bra020572	cell	cell.organisation		AT4G14960
7	Bra034554	protein	protein.synthesis.initiation		AT4G33250
8	Bra036611	protein	protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L18		AT5G27850
9	Bra006338	protein	protein.folding		AT3G02530
10	Bra014334	protein	protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L7		AT2G01250
11	Bra034717	not_assigned	not assigned.unknown		AT5G03495
12	Bra002054	cell	cell.vesicle transport		AT2G17380
13	Bra028864	not_assigned	not assigned.unknown		AT5G02770
14	Bra001788	RNA	RNA.processing.ribonucleases		AT3G20390
15	Bra024481	protein	protein.synthesis.ribosomal protein.prokaryotic.chloroplast.50S subunit.L2		AT4G36130
16	Bra015007	not_assigned	not assigned.unknown		AT3G24160
17	Bra015153	protein	protein.synthesis.initiation		AT2G04520
18	Bra017461	protein	protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L7		AT2G01250
19	Bra001921	amino_acid_metabolism	amino acid metabolism.degradation.aspartate family.methionine		AT3G23810
20	Bra013406	RNA	RNA.regulation of transcription.HSFHeat-shock transcription factor family		AT4G19630

Genotype effect cluster VIII				
Ranking	All_Nodes	Annotation	Pathways	TAIR ID
1	Bra000349	signalling	signalling.calcium	AT2G44310
2	Bra028430	cell_wall	cell wall.precursor synthesis.UGD	AT5G39320
3	Bra014865	cell	cell.organisation	AT3G53750
4	Bra001037	cell_wall	cell wall.cell wall proteins.RGP	AT3G02230
5	Bra028068	signalling	signalling.14-3-3 proteins	AT1G35160
6	Bra008683	cell_wall	cell wall.cell wall proteins.RGP	AT5G15650
7	Bra012376	protein	protein.targeting.secretory pathway.unspecified	AT1G23490
8	Bra016344	protein	protein.targeting.secretory pathway.unspecified	AT2G47170
9	Bra016752	transport	transport.p- and v-ATPases	AT1G12840
10	Bra026938	protein	protein.degradation.ubiquitin.proteasom	AT3G26340
11	Bra040146	glycolysis	glycolysis.cytosolic branch.glyceraldehyde 3-phosphate dehydrogenase (GAP:DH)	AT1G13440
12	Bra040213	glycolysis	glycolysis.cytosolic branch.glyceraldehyde 3-phosphate dehydrogenase (GAP:DH)	AT1G13440
13	Bra009693	protein	protein.degradation.ubiquitin.proteasom	AT5G23540
14	Bra026904	glycolysis	glycolysis.cytosolic branch.glyceraldehyde 3-phosphate dehydrogenase (GAP:DH)	AT3G04120
15	Bra028234	cell_wall	cell wall.precursor synthesis.UGD	AT5G39320
16	Bra033737	cell	cell.organisation	AT5G44340
17	Bra006014	Photosynthesis	PS.lightreaction.ATP synthase.beta subunit	AT5G08680
18	Bra029375	protein	protein.degradation.ubiquitin.proteasom	AT5G23540
19	Bra016729	glycolysis	glycolysis.cytosolic branch.glyceraldehyde 3-phosphate dehydrogenase (GAP:DH)	AT3G04120
20	Bra021292	misc_otherPhosphate	misc.gluco-, galacto- and mannosidases	AT3G18080

Genotype effect cluster IX & X					TAIR ID
Ranking	AIL_Nodes	Annotation	Pathways		
1	Bra009244	Photosynthesis	PS.calvin cycle.RPE		AT5G61410
2	Bra035349	Photosynthesis	PS.photorrespiration.phosphoglycolate phosphatase		AT5G36700
3	Bra020809	not_assigned	not assigned.unknown		AT4G35250
4	Bra008166	not_assigned	not assigned.unknown		AT1G74730
5	Bra021555	Photosynthesis	PS.photorrespiration.glycolate oxydase		AT3G14415
6	Bra031197	not_assigned	not assigned.unknown		AT2G21530
7	Bra015897	not_assigned	not assigned.unknown		AT1G74730
8	Bra011918	not_assigned	not assigned.unknown		AT1G55480
9	Bra013517	Photosynthesis	PS.lightreaction.photosystem II.PSII polypeptide subunits		AT4G21280
10	Bra018955	Photosynthesis	PS.lightreaction.photosystem I.PSI polypeptide subunits		AT1G52230
11	Bra031624	Photosynthesis	PS.lightreaction.photosystem I.PSI polypeptide subunits		AT1G08380
12	Bra037761	Photosynthesis	PS.lightreaction.photosystem I.PSI polypeptide subunits		AT5G64040
13	Bra021556	Photosynthesis	PS.photorrespiration.glycolate oxydase		AT3G14420
14	Bra000663	redox	redox.ascorbate and glutathione.ascorbate		AT4G09010
15	Bra000687	Photosynthesis	PS.lightreaction.ATP synthase.delta chain		AT4G09650
16	Bra005211	not_assigned	not assigned.unknown		AT2G37240
17	Bra016522	Photosynthesis	PS.lightreaction.photosystem II.LHC-II		AT1G19150
18	Bra018415	Photosynthesis	PS.lightreaction.other electron carrier (ox/red).ferredoxin		AT1G10960
19	Bra013013	misc_otherPhosphate	misc.rhodanese		AT5G23060
20	Bra016353	amino_acid_metabolism	amino acid metabolism.synthesis.central amino acid metabolism.alanine		AT1G23310

Figure S1. Barplots of the values of the 17 module eigengenes in CC-Z16, CC-A03 and PC-024. Time points for CC-Z16 and PC-024 were week 2, 4, 6, 7 and for CC-A03 were week 2, 5, 8, 11.

Chapter 4

Morphological differences at cellular level between heading and non-heading *B. rapa* accessions

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Abstract

Brassica rapa includes various leafy vegetable crops divided into two groups: heading and non-heading, with most important representatives the heading Chinese cabbage and non-heading pak choi. In the rosette stage, Chinese cabbage rosette leaves are large and curve upwards, whereas pak choi rosette leaves are relatively small and flat. As leaf shape is defined by cell shape, size and positioning, we studied the differences between Chinese cabbage and pak choi leaves at the cellular level in both leaf primordia and developing seedling and rosette leaves. At rosette stage (after week 4) leaf numbers in Chinese cabbage increased compared to pak choi. This is accompanied by a tighter arrangement of leaf primordia around the shoot apical meristems (SAM) with a larger angle between the developing vascular strands in Chinese cabbage compared to pak choi. During leaf growth, the adaxial palisade and abaxial spongy parenchyma cells of these two morphotypes display differences in cell layers, cell layer thickness, cell numbers, cell area and cell density/air space. Besides differences between Chinese cabbage and pak choi leaves, also differences exist between seedling and rosette leaves and within leaves from tip to the base part. Leaf growth in Chinese cabbage is mainly caused by cell expansion, while in pak choi it is by cell division. Many genes involved in cell proliferation and elongation coordinate leaf morphogenesis. Transcript abundance of selected leaf ad/abxial- and cell division/expansion genes in Chinese cabbage and pak choi SAMs, leaf petioles and leaf tissues point to high cell division rates in the SAM and cell expansion in the growing leaves, but gene expression patterns could not be associated to morphotype or developmental stage specific differences in leaf architecture.

Introduction

Brassica species display rich diversity and single species encompass many morphotypes; interestingly many traits are shared between Brassica species. For example, leafy heading is observed in *B. rapa*, *B. oleracea* and *B. juncea*. The important leafy heading vegetable in *B. oleracea* is cabbage and in *B. rapa* is Chinese cabbage (Cheng et al., 2014). Chinese cabbage (*B. rapa* subs. *pekinensis*) and non-heading pak choi (*B. rapa* subs. *chinensis*) are economically important *B. rapa* leafy vegetable crops. Heading Chinese cabbage has large upwards curving rosette leaves and inwards curving heading leaves, whereas pak choi has relatively small flat rosette leaves and does not form heading leaves. The Chinese cabbage heading leaves lack petioles, are generally rugose with midveins that broaden towards the leaf base, while the pak choi leaves have narrow leaf mid veins and long petioles (Opena, Kuo & Voon, 1988; Moreno et al., 2002).

In plants, leaves are the principal organs for photosynthesis, converting light energy into chemical energy (Byrne, 2006). Light capture by the leaves is affected by leaf shape, size and angle and depends on many aspects, such as genetic predisposition, leaf position and environmental conditions (Falster & Westoby, 2003; Andriankaja et al., 2012). In the model plant *Arabidopsis*, leaf development starts from the shoot apical meristem (SAM) and includes leaf initiation, abaxial/adaxial, differentiation, vascular development, cell division and expansion (Kalve et al., 2014). The differentiation of cells of the SAM into leaf primordia is governed by the local accumulation of the plant hormone auxin (Traas & Monéger, 2010). After acquiring “leaf” identity, leaf growth is sustained by the development of successive polarity gradients. The adaxial epidermis is glossy dark green while the abaxial epidermis is matte, grey green and has less trichomes than the adaxial side (McConnell & Barton, 1998). A certain number of cells are produced by cell division and the mature cell size is determined by cell expansion (Beemster, Fiorani & Inze, 2003). In subepidermal cell layers, the vertically elongated palisade cells closely lie juxtaposed below the adaxial epidermis. Below the palisade layer, larger spongy cells with many air pockets, which allow for the interchange of gases (CO₂, O₂ and H₂O) for photosynthesis, form abaxial internal tissue (Tsukaya, 2004). Also, stomata are more abundant on the abaxial side (Brewer & Smith, 1997; Driscoll et al., 2005).

According to the classical cell theory (Kaplan & Hagemann, 1991) the basic unit of organogenesis, changes in cellular behavior must be responsible for mutant morphology. According to *Neo* cell theory, cell sizes, cell shapes, extents of cell division and orientation of cell division control the leaf shape. Some reports in

Arabidopsis morphogenesis refer to this cell theory (Tsukaya, 2003). For example, palisade cell layers in the *A. thaliana angustifolia* (an) mutant are narrower and thicker than in wild type plants (Col) and this affects the shape and size of the entire leaf, which is narrow and thick (Tsuge et al., 1994). In addition, this theory stipulates the influence of positional cooperation on cell behavior: a decrease in cell division results in an increase in the volume of each cell (Tsukaya, 2002). Overexpression of *Arabidopsis* auxin-binding protein 1 (ABP1) in tobacco leaves resulted in normal sized leaves consisting of larger cells, which is due to increased cell expansion (Jones et al., 1998). The *pointed first leaf 2* (*pfl2*) mutant has narrower leaves than the wild type; at the cellular level, many enlarged cells and intercellular spaces are present and the number of palisade cells is less than that of the wild type (Ito, Kim & Shinozaki, 2000).

Although leaf growth is not yet fully understood at the molecular level, some genes have been identified with roles in the regulatory networks (Gonzalez, Vanhaeren & Inzé, 2012; Kalve, De Vos & Beemster, 2014). The class III homeodomain-leucine zipper (*HD-ZIP III*), *KANADI* (*KAN*), *ARF3/4*, *YABBY*, gene family and *WUS-RELATED HOMEODOMAIN* (*WOX*) genes are involved in determining leaf ad/ab axial polarity. *HD-ZIP III* genes *REVOLUTA* (*REV*), *PHAVOLUTA* (*PHV*) and *PHABULOSA* (*PHB*) are expressed in the leaf adaxial position and are inhibited by *KANs* (*KAN1*, *KAN2* and *KAN3*) that display a similar expression pattern in abaxial position and promote abaxial cell fate (Emery et al., 2003; Eshed et al., 2004). As with the *KANs*, *FILAMENTOUS FLOWER* (*FIL/YAB1*), *YAB2*, *YAB3* and *AUXIN RESPONSE FACTOR3/4* (*ARF3/4*) are expressed on the abaxial side and contribute to the specification of abaxial identity. *KANs* and *YABBY*, *YABBY* and *ARF3/4* establish a positive feedback loop on the adaxial side (Kalve, Vos & Beemster, 2014). In addition, *WOX1* and *PRW/WOX3* as middle leaf domain specific genes are repressed by *KANs* and drive leaf blade outgrowth (Nakata et al., 2012). Besides controlling leaf polarity, some leaf polarity related genes are involved in the leaf growth. Such as *REV* is required to limit cell division in leaves and stems in *Arabidopsis* and *rev-1* mutants caused overgrowth of rosette and cauline leaves with abnormally large and distorted shape (Talbert et al., 1995). The final size and shape of leaves are the result of cell division and cell expansion. New cells are formed by division and expand by increasing in cell size through vacuolization and enlargement of the vacuole (Perrot-Rechenmann, 2010). *GROWTH-REGULATING FACTOR* (*GRF*), *TEOSINTE BRANCHED1/CYCLOIDEA/PCF* (*TCP*), *GRF-INTERACTING FACTOR* (*GIF*) transcription factors promote cell division and *TARGET OF RAPAMYCIN* (*TOR*) pathway, *ARGOS-LIKE* (*ARL*) control the cell expansion (Gonzalez, Vanhaeren & Inzé, 2012; Kalve, Vos & Beemster,

2014). *TEOSINTE BRANCHED 1/CYCLOIDEA/PCF 2 (TCP2)*, *TCP3*, *TCP4*, *TCP10* and *TCP24*, which all contain a *miR319* binding site, control leaf growth. Due to functional redundancy, single loss of function *TCP* mutants display subtle phenotypes (Nag, King & Jack, 2009). Hyper-activation of *TCP4* reduces *Arabidopsis* leaf size and decreases cell proliferation (Sarvepalli & Nath, 2011). *TOR* regulates numerous biological processes, promotes cell proliferation, and is not expressed in differentiated cells (Menand et al., 2002). The expression of *AtTOR* is correlated with the size of rosette leaves, and increasing the expression of *AtTOR* enhances the shoot and the area of rosette leaves (Deprout et al., 2007). Together with the *TOR* pathway, *ARL* plays a role in cell expansion and overexpression of the *AtARL* resulted in larger cotyledons and leaves, whereas the down regulation led to smaller leaves compared to the wild-type Columbia (Hu, Poh & Chua, 2006). Furthermore, *ErbB-3 binding protein1 (EBP1)* and *AUXIN RESPONSE FACTOR2 (ARF2)* control leaf growth by affecting both cell division and cell expansion. *EBP1* expresses high in developing organs and positively regulates leaf growth by promoting cell proliferation in tissues with high division rate, and cell expansion in differentiation cells. Reduced expression or overexpression of *AtEBP1* results in smaller or larger *Arabidopsis* plant size. In potato, *StEBP1* regulates both cell number and cell size in developing leaves and limits cell size in proliferating cells (Horvath et al., 2006). Conversely, *ARF2*, as a repressor of auxin signaling, also represses cell division and organ growth (Schruff et al., 2006).

Here, we characterized the progression of leaf development in two very different leafy *B. rapa* morphotypes. By comparing leaf development in a heading Chinese cabbage and a non-heading pak choi, we first show that there are differences in leaf arrangement around the SAM at the microscopic level between the two types. In addition, the leaf cellular composition data indicates that both cell size and shape vary between leaves of plants during development (seedling and rosette stage) and between pak choi and Chinese cabbage, affecting their phenotypes. Furthermore, we quantify expression of a number of leaf growth related genes during leaf developmental at different positions in the leaf.

Materials and Methods

Plant materials and growth conditions

The heading Chinese cabbage (Chiifu) and non-heading pak choi (PC-024) seeds were sown in June 2016 for leaf arrangement observation. The heading Chinese cabbage (CC-Z16) and non-heading pak choi (PC-024) seeds were sown in July

2017 for leaf structure analysis. Seeds were germinated in seeding soil for one week and then plants were transplanted into Ø17 cm pots in the greenhouse and grown under long day conditions at Unifarm, Wageningen University & Research (51°59'11"N latitude, 05°39'52"E longitude).

Morphological observations and analyses

Shoot apical meristem (SAM) and leaf cellular structures were observed by making micro-sections of both heading Chinese cabbage and non-heading pak choi.

Leaf arrangement around SAM

SAMs were harvested weekly (from week 2 to week 7) per genotype (Chiifu and PC-024, 2016) during all vegetative growth stages from seedling till early heading stage. Of each genotype, three biological replicates per time point were collected. Leaves around the SAM were removed until only a few young leaves were left. Tissues were cut between 0.4 and 1 cm in size and fixed in 4% paraformaldehyde solution (PFA). Samples were kept overnight in fixation solution at 4°C and were embedded in paraffin. The embedding steps were performed according to the Saiga et al. (2008) protocol with some modifications (**Supplemental Table S1**). The embedded tissues were sliced with a microtome at 7 µm and observed using a light microscope (Leica). The angle between SAM and the developing vascular strand was measured by Image J software (**Figure 1A**). Total leaf number was counted from the outside large leaf to the innermost leaf primodium.

Figure 1. Schematic diagrams for shoot apical meristem and leaf measurement methods. (A) Angle between developing vascular strands. (B) Palisade and spongy layers which were used for data measurement and analysis.

Leaf structural observations

Leaf cellular structure observations were carried out using CC-Z16 and PC-024 leaves of plants grown in 2017 by plastic embedding. Samples were cut from three positions: top, middle and bottom of the leaf at seedling stage and rosette stage. Tissue pieces (4-8 mm²) were excised and immediately fixed in 5% glutaraldehyde in 0.1M phosphate buffer, pH7.2, and kept overnight at 4°C. Samples were washed four times for 15min in 0.1mM phosphate buffer and two times for 15min in water. Samples were then dehydrated in increasing concentrations of ethanol (10%, 30%, 50%, 70%, 96% and 100%) for 20min per step. Infiltration and embedding were followed the dehydration step by using the Technovit Glycol Methacrylate Kit 7100 (Heraeus Kulzer). All samples were cut in 9µm transverse sections and were stained with toluidine blue. Sections were observed under a light microscope and photographs were taken using a camera. Three fragments of from three different leaves were examined as biological repetitions.

Mesophyll was characterized by measuring the surface of each cell layer (CL), single cell size (SCS), cell density (CD) and cell number (CN) of both palisade and spongy tissues by Image J software, respectively (**Figure 1B**). More specifically, SCS was the average single area of cells within 8000 µm². CD was the percentage of tissue occupied by total cell areas. The intercellular space was described by the remaining percentage after excluding CD (100%-CD). CN was the average number of cells per 4000 µm² surface unit. The data were subjected to a one-way analysis of variance using the SPSS software 23.0 (SPSS Inc, Chicago, IL, USA). The means of the data were compared by using t-test and the results were considered statistically significant at $P < 0.05$.

Quantitative Real-Time PCR

Transcript abundance of *B. rapa* paralogues of *Arabidopsis* ad/abaxial leaf developmental related genes (*ARF3*, *ARF4*, *KAN1*, *KAN2*, *KAN3*, *YAB1*, *YAB2*, *YAB3*, *YAB5*, *PHV*, *PHB*, *WOX1*, *WOX3* and *REV*) and cell division/expansion genes (*TCP4*, *ORS1*, *TOR*, *ARL*, *EBP1* and *ARF2*) were determined by qRT-PCR. Total RNA was extracted from SAM, leaf petiole and tip, mid and base of seedling and rosette leaves by using the TRIZOL reagent (Invitrogen) and cDNA synthesis was performed with iScript™ kit (BIO-RAD) according to supplier's instructions. qRT-PCR reactions were performed in a 96 position carousel (Light Cycler) with the Light Cycler-RNA amplification kit SYBR Green I (Roche, Mannheim, Germany). Data was collected from three independent biological repeats. *ACTIN*

(*ACT*) was used as reference gene in all expression studies. Information and primer sequences of candidate genes are listed in **Supplemental Table S2**.

Results

Leaf arrangement around SAM

To observe how SAM and surrounding leaves develop over time, a time-series was created of the SAM and surrounding leaves of Chinese cabbage (Chiifu) and pak choi (PC-024) accessions in transverse and longitudinal views (**Figure 2**). Chiifu as a typical Chinese cabbage forms an oblong leafy head with large pale green wrinkled leaves and wide white mid veins. In contrast to this, PC-024 does not form a leafy head and has smooth, dark green leaves with long leaf petioles. Heading Chinese cabbage Chiifu and non-heading PC-024 were at the seedling stage around week 2 and at rosette stage from week 3 to week 4. At week 5, PC-024 started bolting, while Chiifu was at the early heading stage.

As can be seen in the longitudinal sections, the stem beneath the SAM was broader in Chiifu than in PC-024 after the seedling stage (week 3). From week 2 to week 4, the angles of developing vascular strands of PC024 were 100.14°, 100.71° and 102.55°. At week 5, during the transition of the SAM from the vegetative to the generative stage, this angle decreased to 93.32°. For Chiifu, these angles were larger than for PC-024 and obviously increased from week 3 (114.52°) to week 4 (125.25°). At the early heading stage (week 5), the developing vascular strand angle was 118.50° as besides expansion in horizontal direction, now the core also expanded vertically.

The above illustrates that the difference between Chiifu and PC-024 SAMs increases after week 3. In transverse sections, the leaves of Chiifu were more densely arranged around the SAM than in PC-024. Total leaf number (leaf length > 2cm) obviously increased from week 3 (LN: 33) in Chiifu and slightly increased until bolting (LN: 12-17) in PC-024.

Figure 2. Leaf arrangement around the shoot apical meristem. (A) Setions of the SAM and its surrounding leaf initials in heading Chinese cabbage (Chiifu) and non-heading pak choi (PC-024) from week 2 to week 5. In transverse sections, the central black circle is the SAM. Scale bars represent 50um. (B) Angles of developing vascular strands and leaf numbers during development in Chiifu and PC-024.

Cellular structure of leaf tissue

For the comparison of cellular organization of leaves from pak choi and Chinese cabbage, we decided to use the Chinese cabbage line CC-Z16. CC-Z16 leaves, in contrast to leaves of many other Chinese cabbages, have a rather smooth surface. The wrinkled surface of for example Chiifu is not suitable for comparison of leaf cellular structure related to head formation (see **Supplemental Figure S1** for leaves of Chiifu and CC-Z16). The seedling leaves were round with long petioles in CC-Z16 and PC-024. At the rosette stage, the two morphotypes diverge. At rosette stage of Chinese cabbage CC-Z16, leaves differentiate and became large and round, with short petioles and up-wards curling blades. In contrast, at rosette stage PC-024 leaves have long white petioles and blades are flat.

Transverse sections of both seedling and rosette leaves of CC-Z16 and PC-024 illustrated that leaves of both morphotypes clearly displayed the typical dorsiventral structure, with palisade cells at the adaxial side and spongy parenchyma cells at the abaxial side (**Figure 3**). The palisade and spongy mesophyll cells were clearly differentiated at both the tip and middle position of the leaf at seedling and rosette stages in both morphotypes, with more rectangular palisade cells and rounder and irregularly shaped spongy parenchyma cells. This was not the case at the base of the leaves in Chinese cabbage that was not completely adaxialized as palisade parenchyma cells were not formed. Both ad/abaxial cells in the base of the leaves were more spherically shaped than the cylindrical shaped palisade parenchyma cells. The arrangement of cells was evenly with little air space.

Figure 3. Transverse sections of the leaf tip (P1), middle (P2) and base (P3) part of the heading Chinese cabbage (CC-Z16) and non-heading pak choi (PC-024). Scale bars represent 100µm. Rosette leaves used for section are pointed out by black arrows.

Figure 4. Quantified data of leaf mesophyll tissue in both heading Chinese cabbage (CC-Z16) and non-heading pak choi (PC-024). (A) Mean values of number of cell layers (CL), cell layer thickness (CLT), cell density (CD), single cell size (SCS), and cell number (CN) of palisade (green) and spongy (grey) tissues are shown from the tip (P1) and middle (P2) positions of seedling and rosette leaves. Light color indicates seedling; dark color indicates rosette stage.

The number of leaf cell layers (CL) increased from seedling to rosette stage in both morphotypes (**Figure 4**). In CC the middle of the leaves (P2) have more cell layers than the tip (P1); this is mainly due to an increase in cell layers of the spongy parenchyma in seedling stage, while in the rosette stage also the palisade parenchyma cell layers increase. In pak choi P1 and P2 have similar numbers of cell layers. This agrees with the observation that in CC the spongy parenchyma layer is thicker than the palisade cell layer in the rosette stage, which is not the case in PC. In addition, there was a clear decrease in cell number (CN) and increase of single cell area (SCA) in the middle of the leaf (P2) from the seedling to the rosette leaves in CC. Conversely, in PC rosette leaves the CN increased and the SCA decreased from seedling to rosette stage. From seedling to rosette stage, the cell density (CD) decreased, as the intercellular spaces increased during leaf development in both CC and PC. When we compare the mid position of upward curling CC rosette leaves with the flat PC rosette leaves, the difference in cell density (CD) between palisade- and spongy parenchyma tissue is larger in CC than in PC (87% -61% versus 83%-68%, respectively). In both genotypes and leaf positions the spongy mesophyll has larger intercellular spaces than palisade mesophyll. Statistical analysis showed that the mean values of cell layer (CL), cell density (CD) and cell number (CN) of palisade and spongy mesophyll in rosette leaves was significant different between P1 and P2 in CC but not in PC leaves (**Supplemental Table S3**).

Transcript abundance of a selection of genes involved in leaf development was determined at different positions of both CC-Z16 and PC-024 leaves, in the petioles and in SAM enriched tissue (**Figure 5**). Clustering of the expression data revealed that many genes were expressed in SAM-enriched tissue, but not in the leaf petiole and leaf tissues.

Genes with high expression levels in SAM include the cell division/expansion genes *BrEBP1.1*, *BrARL.2*, *BrARL.3*, *BrORS1.1* and *BrARF2.3* and leaf polarity genes *BrYAB1* (1.1 and 1.2), *BrYAB2* (2.1, 2.2 and 2.3), *BrYAB3*, *BrYAB5*, *BrREV1*, *BrPHB2* and *BrKAN2.1*. Among them, *BrEBP1.2* and *BrYAB2.1* were expressed in

both SAM, leaf petiole and leaf tissues, however their transcript abundances were lower in leaf petioles and leaf tissues than in the SAM.

Figure 5. Heat map of transcript abundance of selected genes in different positions of leaves, in petioles or in meristem enriched tissues of the two genotypes. PC/CC indicate pak choi PC-024 /Chinese cabbage CC-Z16e. S/R indicate seedling/rosette stage. P and M indicate leaf petiole and meristem enriched tissue. LP1/2/3 indicate top (LP1), middle (LP2) and base (LP3) of leaves.

Some genes were expressed in specific tissues only: *BrYAB1.1* only showed high expression in SAM enriched-tissue; *BrKAN1* (1.1 and 1.2), *BrREV2*, *BrARF3* (3.1 and 3.2), *BrARF4.1*, *BrYAB1.2*, *BrPHV* and *BrPHB1* also only expressed in the SAM, but their transcript abundances were low; *BrYAB3* expressed in SAM enriched- and leaf tissue, but transcripts were not detected in petioles; the cell division/expansion genes *BrTCP4.1* and *BrTCP4.3* were not detectable in petioles,

transcript abundance was low in the SAM, but higher in leaf tissues at both seedling and rosette stages in CC and PC. In our study, transcript abundance of *YABBY* family genes was generally high in both SAM, leaf petiole and leaf tissues during plant growth. The *B. rapa* genome contains two *YAB1* and three *YAB2* paralogous, while only one *YAB3* and *YAB5* gene. These genes were all expressed in SAM enriched- and leaf tissue, but transcripts for several were not detected in petioles, such as *BrYAB1* (1.1 and 1.2) and *BrYAB3*. Although these genes were expressed in leaf tissue, their transcript abundance differs quantitatively: *BrYAB1* transcript abundance was low; *BrYAB3*, *BrYAB5* and *BrYAB2.2*, *BrYAB2.3* moderate and *BrYAB2.1* high. However *BrYAB1.1* and also *BrYAB2.1* displayed high transcript abundance in SAMs at seedling and rosette stages. In contrast, the *WOX* family was inactive. *BrWOX1.1*, *BrWOX1.2*, *BrWOX3.1* and *BrWOX3.2* were not expressed in SAM, leaf petiole and leaves at seedling and rosette stages in both CC and PC.

Some genes have different expression patterns according to the different genotypes (CC and PC) and different stages (seedling and rosette stage). For example, transcript abundance of *BrTOR.2* was very low in CC and medium in PC. *BrORS1.2*, *BrTOR.1*, *BrTOR.3*, *BrARF2.2* and *BrEBP1.1* were expressed in seedling leaves, but transcripts were not or hardly detectable in rosette leaves in both CC and PC. In contrast, transcript abundance of *BrARL.2* and *BrARL.3* was higher in rosette leaves than in seedling leaves.

Discussion

The shape and size of organs is determined by the size shape and organization of the cells in these organs (Tsukaya, 2003). Thus, understanding the organization and regulation of leaf cells is key to understanding the control of various leaf shapes. We investigated the anatomy of leaves of heading Chinese cabbage (CC) and non-heading pak choi (PC) at different developmental stages to better understand the different phenotypes of their mature plants, with leafy heads for CC and rosettes for PC. The patterning of leaf primordia emergence in SAMs and the anatomy of both seedling and rosette leaves was phenotyped by studying cell numbers, shapes and sizes of the palisade and spongy mesophyll cells. This revealed subtle differences in the arrangement of leaf primordia and composition of palisade and spongy parenchyma layers. To better understand the genetic regulation of leaf development we also profiled transcript abundance of a subset of the genes involved in leaf ad/abaxial polarity formation and cell proliferation in the phenotyped tissue.

Leaf morphogenesis at micro-cellular level

In the vegetative phase leaf primordia continue to be produced from the flanks of the SAM in a regular order. Both CC-Chiifu and PC-024 seedlings show similar curving of leaf primordia over the SAM, however four weeks after sowing differences emerged between these two genotypes, which were then at rosette stage. The leaf primordia in CC were more tightly folded around the SAM than those of PC after week 4 and also more leaves were formed that folded inwards. By counting the numbers and types of leaves in mature CC plants (after leafy head formation), we know the destination of the initiating leaves. At week four, the pre-heading leaves emerge from the CC-Chiifu SAM that will form the heading leaves in heading stage. It is very interesting that exactly after this time point, the angles of developing vascular strands increased in CC, creating space for the formation of many leaves that will form the leafy head. The morphotypic differences between heading CC and non-heading PC clearly showed that the number of leaves decreased in non-heading PC after week 4 and the young leaves were loosely arranged around the SAM with a decrease in the vascular strands angle due to the start of bolting (week 5). According to these morphotypic differences, the arrangements of leaves around the SAM and the angle of the vascular strands correlate with the phenotype of the mature plant, with a leafy head in CC and a rosette in PC.

The final shape and size of leaves is regulated by the extend and the orientation of both cell division and cell growth (Tsuge et al., 1996; Sandalio et al., 2016) and final leaf size is determined by both cell numbers and cell volume, which can compensate each other (Tsukaya, 2002). Mutational studies suggest that a decrease in cell number might trigger an increase in cell volume (Mizukami & Fischer, 2000; Tsukaya et al., 2002). The relationship between cell size and number was also discovered during the CC-Z16 and PC-024 leaf growth process in our study. CC leaves have fewer cells per unit surface area that are larger in rosette leaves than in seedling leaves. By contrast, an increase in cell number and decrease in cell area was observed in rosette leaves compared to seedling leaves in PC. These observations suggest that the increased leaf size is a result of increased cell numbers in PC, and of an increase in single cell area in CC leaves. Leaves are composed of an adaxial layer of palisade parenchymal cells and an abaxial layer of spongy parenchyma cells, flanked by the ad- and abaxial epidermi. Several studies in the model plant *A. thaliana* phenotyped leaf development. Differential growth of the ad- and abaxial epidermis layers caused up or down curvature of leaves (Sandalio et al., 2016). In another study it was found that during leaf

expansion, the spongy parenchyma cells cease division at an earlier stage than palisade cells and compensate for the expansion of the epidermal cells by forming intercellular spaces (Dale, 1988). In our study the intercellular spaces in both the palisade and spongy parenchyma cell layers increased from seedling stage to rosette stage in both CC and PC. However this increase was much stronger in the spongy cell layer. Moreover, the difference between palisade and spongy cell density was higher in upward folding CC rosette leaves than in the flat PC leaves. A similar observation was made in *Arabidopsis tri1-1 auxin resistance (eta1)* mutants with upward curving leaves caused by more densely palisade cells than flat wild type leaves (Esteve-Bruna et al., 2013). Thus, we hypothesize that differences of intercellular space and cell density between palisade and spongy parenchyma layers lead to the upward curvature of Chinese cabbage leaves.

Gene expression controlling leaf shape and size

The establishment of leaf polarity, regulation of cell division and expansion are essential steps for the initiation of marginal meristem activity leading to leaf formation (van der Graaff et al., 2000). As we are interested in the genetic regulation of these processes in leaf initiation and development, we also determined transcript abundance of leaf polarity genes and leaf cell division/expansion genes in heading CC-Z16 and non-heading PC-024 SAM-enriched tissues, leaf petioles and leaves (-top, -mid, -bottom) tissues. Most of the genes were not expressed in leaf petioles, corresponding to the observation that these are composed of very different tissues than leaves.

Adaxial/abaxial polarity establishes in very young leaf primordia that are initiated from the flanks of the SAM (McConnell & Barton, 1998). Thus, we expect that most leaf polarity genes are higher expressed in SAM-enriched tissues (SAM and young leaf primordia), than in the young leaves used in our study. This was indeed the case for most genes, except for the gene *BrYAB2.1*. In *Arabidopsis*, the *YABBY* transcription factors *AtYAB1*, *AtYAB2* and *AtYAB3* have unique expression patterns in the abaxial domains of cotyledons, leaves and floral organs with their expression in leaves confined to the abaxial cell layers that will develop into spongy mesophyll (Siegfried et al., 1999; Watanabe & Okada, 2003; Sarojam et al., 2010; Chen et al., 1999; Watanabe & Okada, 2003). The *B. rapa* genome counts several paralogues of the *YABBY* genes, and their expression differs between enriched SAMs, petioles and leaves with the clearest difference the lack of expression of *BrYAB1.1*, *BrYAB1.2* and *BrYAB3* in petioles and the very high expression of *BrYAB2.1* in both SAMs and leaves in seedling and rosette stages. In *A. thaliana* single or double *YABBY* mutants do not change the leaf phenotype,

however quadruple mutants (*fil-8*, *yab2-1*, *yab3-2* and *yab5-1*) increase the leaf cell layers from 6 to 12 with a lack of palisade/spongy differentiation (Sarojam et al., 2010). We cannot find a correlation between the increase of cell layers in the middle of rosette leaves of CC-Z16 and the lack of a clear ad/ab axial differentiation at the bottom of rosette leaves and transcript abundance of *BrYAB* genes. The polarity genes discussed above affect leaf initiation at early stages, while in later leaf developing stage, the cell division/expansion genes regulate the leaf growth by affecting both cell division and expansion. Compared to leaf polarity genes, cell division/expansion genes were more actively expressed in leaf tissue. The rate of cell division and cell expansion differs between positions in the leaf blade, between genotypes and developmental stages. Different expression patterns for cell division/expansion genes reflect cell behavior during leaf development. As a down regulator for cell proliferation, *TCP4* regulated leaf size by changing cell proliferation during leaf growth and along the axis of leaves (Sarvepalli & Nath, 2011; Mao et al., 2014). We found that *BrTCP4.1* and *BrTCP4.3* have high transcript abundance in leaves, and are not expressed in leaf petiole and SAM-enriched tissue. As a repressor of cell division and expansion (Schruff et al., 2006), *BrARF2.2* and *BrARF2.3* were higher expressed in seedling leaves than rosette leaves. Transcript abundance of *BrARL.2* and *BrARL.3* displays an opposite pattern, low in seedling leaves and high in rosette leaves. *ARL* causes changes in organ size by influencing cell expansion rather than cell proliferation (Hu et al., 2006). These data suggest that the cell division and expansion rate is higher in rosette leaves than seedling leaves, which corresponds to their larger final sizes.

Conclusion

Leaf upward curvature at rosette stage in heading Chinese cabbage, which is the first step in leafy head formation, also represents the first obvious difference in leaf development compared to non-heading pak choi. Comprehensive analysis of cellular composition of both leaf primordia and leaves illustrates the differential growth between the palisade and spongy cell layers that in turn affect leaf curvature. Transcript abundance of selected cell ad/abaxial and cell division/expansion genes differed between cell layers, leaf developmental stages and positions and genotypes, likely influencing leaf shape and growth. To further analyze the relationship between gene expression and leaf development, transcript abundance needs to be determined in specific cell types using in situ hybridization or laser dissection followed by RNAseq analysis in defined cell populations.

Supplementary

Table S1. Fixation and paraffin embedding steps

	Solution	Time	Tm		Solution	Time	Tm
1	4% PFA	Overnight	4 °C	10	100% EtOH + eosin	30 mins	RT
2	1x PBS	30 mins	4 °C	11	100% EtOH + eosin	30 mins	RT
3	1x PBS	30 mins	4 °C	12	50% xylene	2 hrs	RT
4	30% EtOH	2 hrs	4 °C	13	75% xylene	60 mins	RT
5	50% EtOH	2 hrs	4 °C	14	100% xylene	2 hrs	RT
6	70% EtOH	2 hrs	4 °C	15	100% xylene	60 mins	RT
7	85% EtOH	2 hrs	4 °C	16	50% paraffin	3 hrs	63 °C
8	95% EtOH	2 hrs	4 °C	17	75% paraffin	Overnight	63 °C
9	100% EtOH + eosin	Overnight	4 °C	18	100% paraffin	3 days Change twice a day	63 °C

* RT = Room Temperature

Table S2. Primers used for Quantitative Real-Time PCR

<i>B. rapa</i> gene	BRAD ID	Forward Primer 5'—3'	Reverse Primer 5'—3'
<i>BrARF3.1</i>	Bra005465	TGGTGATGCTGTGCTTTTCC	AAGAACTTCGGTGCAGGGA
<i>BrARF3.2</i>	Bra021885	TTGCTTCCCTCCTCTGGAC	GTTCACAAACCCACTCCACC
<i>BrARF4.1</i>	Bra002479	GCTGGTGTAGTGA CTGGAGT	TTTGGGCCCTTGGAGATGACT
<i>BrARF4.2</i>	Bra020243	ACCTGCATCTAACCTGAGCA	ACCATCATGTCGTCTCTACT
<i>BrKAN1.1</i>	Bra008613	CCTTTCCACCAACAACTCTT	AACACCTCTTAGCCTTGGAGAA
<i>BrKAN1.2</i>	Bra023570	GAGGATGCGTTGGAGGAGTA	AGCAGGCTTGTTAGTGGTCT
<i>BrKAN2.1</i>	Bra023254	GAATTACTCGGTGGCCATGAAA	CTTTGTGCGTTGTCTTCACTGT
<i>BrKAN2.2</i>	Bra033844	GAGCAACGCCTAAATCAGTTCT	CTTTGTGCGTTGTCTTCACTGT
<i>BrKAN3.1</i>	Bra040176	TCGGTTCCTACTAAAGGCCAAAG	TTCAATCTCTGGTCTTGTCCTCA
<i>BrKAN3.2</i>	Bra021038	AAAGAGTGAGAGACAAGCCAGA	ATTTGCCTTTAAATGGAGCCGA
<i>BrYAB1.1</i>	Bra040322	AACCCGTGAAGAAAACCAACAT	AATCACACCAACATTAGCAGCA
<i>BrYAB1.2</i>	Bra000378	TTTCTGTGAACATGAGATCGGC	CATCTCTCAGCTCCTCCAGAAT
<i>BrYAB2.1</i>	Bra018624	TCCAGAGATTAGTCACCGTGAG	ACTGACTGGTCTATTGCTTGC
<i>BrYAB2.2</i>	Bra030728	CCCACCTACTCCCATTCATCA	AATGTGGTCGAGAAATGGTTGG
<i>BrYAB2.3</i>	Bra031629	TGATCGTGTGTTGCTATGTCCTC	TGTGAACAACTTGCGTATGGT
<i>BrYAB3</i>	Bra037320	GGCTCATTTCCCTCACATACAC	TCATCACTTCCTCTCCTTCCTG
<i>BrYAB5</i>	Bra000538	AGTACCTTCAGCCTACAACCAA	GAGGAAAGTGTGCCCAATTCTT
<i>BrPHV</i>	Bra032394	GGTTTCTCATCCGTCCTTGTG	CCTCAGGACTTCAGGAACACT
<i>BrPHB1</i>	Bra005398	CATGAAGGTTGCCGAAATCCT	GATAGTTCCACCGTTTCCAGC
<i>BrPHB2</i>	Bra021926	AAACGCTTCACCAATCTTCACA	GGCTCGTCTAAGGTCTTGTCTA
<i>BrREV1</i>	Bra002458	TTGATGAATCTGGTCGAAAGGC	AGTCACATGTCTTCCCATCGTA
<i>BrREV2</i>	Bra020236	GGCACCATTGAGCTTGTGTAT	CCATTGTGCGAGGCTAGTTGTG
<i>BrWOX1.1</i>	Bra022267	GATATGTTTCGGTCGGATGCG	CGGGAAAGCTTTGTCTCTCG
<i>BrWOX1.1</i>	Bra001694	ATCCCTCGCCTCTCTTCTTG	CATTGTTGTCACTACTGCCA
<i>BrWOX3.1</i>	Bra035688	TCTCATCAGGCCATTGGAGT	CGAAGTGTTTTGACCCGTGA
<i>BrWOX3.2</i>	Bra000484	TCAGCCTTCTTCCGTTTGTGTC	TGAGTTTTGGCCCGTGATTG
<i>BrARF2.1</i>	Bra029293	GTGGAGTGTGTTTGTAGCTCA	AATTGTGCCAGTAAACCTCTGC
<i>BrARF2.2</i>	Bra010048	AATTGTGCCAGTAAACCTCTGC	TGAGACCACTAGTATTCCTCGC
<i>BrARF2.3</i>	Bra035910	AGGGAGCATGTAACAAAACGAC	GTAAAGGGTCCATGTTTGCTT
<i>BrEBP1.1</i>	Bra040895	CCCGCAGTATCATTGAAGTTGT	AGGGAGCATGTAACAAAACGAC
<i>BrEBP1.2</i>	Bra012836	TATCAAAGAGCAAACAGGGAGC	CCCGCAGTATCATTGAAGTTGT
<i>BrTCP4.1</i>	Bra027284	TCAGCATATCACCTCTTCCAA	TATCAAAGAGCAAACAGGGAGC
<i>BrTCP4.2</i>	Bra021586	TGGTGATTTTGTGTCAGATT	TCAGCATATCACCTCTTCCAA
<i>BrTCP4.3</i>	Bra001579	CTTCTTTCCACCACCTTCTTC	TGGTGATTTTGTGTCAGATT
<i>BrTOR.1</i>	Bra018828	ATCTCCACCGATGATCTCAACC	CTTCTTTCCACCACCTTCTTC
<i>BrTOR.2</i>	Bra014234	GAAATGGAGGAAGCAGATGACG	ATCTCCACCGATGATCTCAACC
<i>BrTOR.3</i>	Bra039647	TCCGAGTCAAGATCTCCGTTTA	GAAATGGAGGAAGCAGATGACG
<i>BrARL.1</i>	Bra004803	GTCTCTGAATCGACTGTTGCTC	TCCGAGTCAAGATCTCCGTTTA
<i>BrARL.2</i>	Bra037667	TTGCTCCTCCTCCTAATCATCC	GTCTCTGAATCGACTGTTGCTC
<i>BrARL.3</i>	Bra000340	TCGTCAGTGGAGATGGATTGAT	TTGCTCCTCCTCCTAATCATCC
<i>BrORS1.1</i>	Bra025374	CAAGGATGGGGCTAAGAACATG	TCGTCAGTGGAGATGGATTGAT
<i>BrORS1.2</i>	Bra036223	CAACACTCTACTAGCAAGCACC	CAAGGATGGGGCTAAGAACATG

Table S3. Significance analysis for palisade (Pa) and spongy (Sp) mesophyll by comparing leaf tip (P1) to middle (P2) at seedling (S) and rosette (R) stages.

Traits	CC				PC			
	S_Pa	S_Sp	R_Pa	R_Sp	S_Pa	S_Sp	R_Pa	R_Sp
Cell layer	1	0.035**	0.01**	0.03**	0.308	1	0.42	0.506
Cell number	0.016**	0.421	0.094*	0.699	0.550	0.518	0.540	1
Cell area	0.005**	0.042**	0.144	0.262	0.984	0.228	0.774	0.737
Cell density	0.512	0.648	0.015**	0.993	0.09*	0.75	0.885	0.571

p < 0.1*; *p* < 0.05** levels for two samples T-test by comparing leaf tip (P1) to leaf middle (P2)

Figure S1. Leaf structure and form of Chinese cabbage Chiifu and CC-Z16.

Chapter 5

Allelic variation of the Chinese cabbage domestication gene *BrARF3.1* affects leaf phenotypes

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Abstract

Chinese cabbage is a domesticated morphotype within the *B. rapa* species, characterized by its leafy head, which is composed of a number of tightly packed leaves. Several genes involved in leaf ad/abaxial formation were identified as candidate genes for the leafy head traits in both *B. rapa* and *B. oleracea* cabbages, among which *BrARF3.1*. A single nucleotide polymorphism in the C-terminal domain of *BrARF3.1* was associated with the heading (H) phenotype of Chinese cabbage. In our study, over-expression (OE) of the *BrARF3.1_NH* non-heading allele from pak choi in *Arabidopsis* wild type (Col-0) resulted in small plants with thick and downward curling leaves and short roots with many long lateral roots, while Col-0 plants over-expressing the *BrARF3.1_H* allele from Chinese cabbage resembled wild type plants. We tested whether the C-terminal amino acid change (Q to H) differentiated protein-protein interactions of Chinese cabbage *BrARF3.1_H* and pak choi *BrARF3.1_NH* proteins. *BrARF3.1_H* shows similar protein-protein interactions as *AtARF3* with *KANADI*'s and *YABBY*'s, while *BrARF3.1_NH* does not interact with *AtYAB1*, *BrYAB1.1* and *BrYAB1.2* and with *AtKAN4*. Chimeric constructs showed that the single Q to H amino acid change abolishes this interaction only when the other three variable amino acids in the Auxin response factor domain are from the non-heading allele. We also showed that the auxin IAA weakens or abolishes the protein interactions of *BrARF3.1*. These results show that the non-heading *BrARF3.1_NH* allele of pak choi is the mutant allele, which was unexpected as Chinese cabbage with its leafy head is assumed to be domesticated from pak choi.

Introduction

The economically important Brassica genus consists of several species that are cultivated as vegetable, oil, fodder and condiment crops (Nagaharu, 1935). Leafy *Brassica rapa* vegetable crops are grown worldwide and include both heading and non-heading types. Chinese cabbage with its leafy head is one of the most important vegetable crops in China. The leafy head ensures high yields, allows for nutrient storage and tolerance against both biotic and abiotic stresses (Tao et al., 2015; Gao et al., 2017). The Chinese cabbage leafy head is composed of a number of tightly packed heading leaves. Heading leaves are large and round with short petioles and wide mid veins that curve inwards to form a leafy head after the rosette stage (Wang et al., 2012). Pak choi does not form a leafy head, but instead forms a rosette of leaves with light to dark green flat leaf blades and white large fleshy petioles.

The development of leaves in dicotyledonous plants initiates from cells in the shoot apical meristem (SAM), through leaf initiation, adaxial/abaxialization, cell division and expansion to end as the mature leaf (Kalve, De Vos & Beemster, 2014). Polarity of leaves of higher plants is typically achieved by an asymmetric distribution of cell types along the proximo-distal, lateral and adaxial-abaxial axes (Stahle et al., 2009). The ad- and abaxial cell layers of leaves become morphologically distinct (palisade and spongy mesophyll cells respectively) at later developmental stages of the leaf, which is preceded by earlier changes in gene expression patterns.

The molecular mechanisms of leaf and leafy head development in heading Chinese cabbages is still not clear, but in the Cheng et al., 2016 and Liang et al., 2016 studies, *BrARF3.1* (Bra005465), *BrARF4.1* (Bra002479), *BrKAN1* (Bra008613), *BrKAN2.1* (Bra023254) and *BrRDR6* (Bra029957) were identified as candidate genes for domestication signals of Chinese cabbage morphotypes. In these studies whole genome re-sequencing data was compared between heading *B. rapa* and *B. oleracea* cabbages and non-heading accessions to identify selective sweeps and predict candidate genes; many of the candidate genes were related to leaf ad/abaxial development in both species. Association of variation in *BrARF3.1* and *BrKAN2.1* was further validated on a large set of 806 *B. rapa* accessions. A non-synonymous mutation (G/C) was detected in *BrARF3.1* coding exon 9 (2264 in the DNA) and the G genotype strongly associated with the *B. rapa* heading group (322 out of 357 accessions). An insertion (CAG) in *BrKAN2.1* was present in 344 out of 354 heading *B. rapa* accessions. Allelic variation in these genes is associated with leaf-heading morphotypes in *B. rapa* and their orthologues in *A. thaliana* play important roles in the establishment of leaf shape and polarity (Barkoulas et al., 2007; Kalve et al., 2014, Maugarny-CAles and Laufs, 2018).

Adaxial leaf fate is promoted by the *miR166*–*HD-ZIPIII* pathway and there is evidence for feedback communication with the abaxial side by inhibition of *KANADIs* expression in abaxially located cells (Tsukaya, 2013). *YABBYs* up-regulate *KANADIs* and *ARF3/4* on the leaf abaxial side, which establishes a positive feedback loop. However, inversely *KANADIs* inhibit the expression of *HD-ZIPIII* that determines adaxial identity. *miR165/166* genes expressed in the abaxial epidermis produce miRNAs that are mobile and restrict *HD-ZIPIII* gene expression to the adaxial domain while the *TAS3*–*ta-siRNA* produced at the adaxial side migrate to the abaxial side to target the degradation of *ARF3* and *ARF4* transcription factors that promote abaxial cell fate (Chitwood et al., 2009; Bonaccorso et al., 2012). The *miR390*–*TAS3*–*ARF3/4* pathway is also involved in lateral root development and gynoecium formation in *Arabidopsis* (Yoon et al., 2009; Marin et al., 2010; Simonini et al. 2016). *ARF3* and *ARF4* act as sister-pair genes; *ARF4* seems to be redundant in the determination of leaf abaxiality. In *Arabidopsis*, loss of single *ARF3* or *ARF4* gene function do not display clear vegetative phenotypes. This is likely due to overlapping function of the sister pair genes as suggested by Pekker et al, 2005. Conversely, *arf3-arf4* double mutants show more outgrowth on the abaxial leaf surface (Pekker, Alvarez & Eshed, 2005; Hunter et al., 2006; Takahashi et al., 2013). Over-expression of *ARF3* with the 35S promoter results in plants in which only a small percentage displays a weak leaf phenotype (slightly wider and downwards curving leaves). Over-expression of *ARF3* genes mutated in the *TAS3*–*ta-siRNA* target sites (*ARF3mut*), without affecting its amino acid sequence, resulted in plants with small tightly downwardly curled leaves with short stamens and split septa (Hunter et al., 2006; Fahlgren et al., 2006).

A total of 23 *AtARF* genes and 33 *BrARF* genes are identified in the genomes of *Arabidopsis* and *B. rapa* respectively (Mun et al., 2012; Huang et al., 2015). Most ARFs consist of an amino-terminal DNA binding domain (DBD), a middle region (MR) that determines whether the ARF functions as an activator or repressor, and a C-terminal interaction domain III/IV (Guilfoyle & Hagen, 2007; Chandler, 2016). *ARF3* and *ARF4* are classified as one group and contain the repressor domain in their middle region. However, *ARF3* lacks domain III/IV, which interacts with the DBD and may help to stabilize dimers by forming higher order DNA bound ARF complexes (Boer et al., 2014). In the presence of auxin, the Aux/IAA repressor acting on the ARF III/IV domain will be degraded by the Skp1-Cullin-Fbox complex, resulting in activation of the ARF protein (Tiwari, Hagen & Guilfoyle, 2003). The region behind the *ARF3/4* Auxin response factor domain (MR) contains the target sites for the trans-acting short interfering RNAs (*ta-siRNAs*), which target degradation of *ARF3/4* (Williams et al., 2005; Montgomery et al., 2008). siRNA

biogenesis requires the additional activity of *RNA-DEPENDENT RNA POLYMERASE 6* (*RDR6*), which functions in posttranscriptional RNAi of specific endogenous mRNAs that are targeted by ta-siRNAs (Allen et al., 2005; Garcia et al., 2006; Mecchia et al., 2013). Due to the triplication and subsequent gene loss, both *ARF3* and *ARF4* have two copies in *B. rapa*. The two copies of *BrARF3* are expressed and are down-regulated in *B. rapa* seedlings and become up-regulated by auxin treatment (Mun et al., 2012). The expression level of *BrARF3.2* (Bra021885) is lower than *BrARF3.1* (Bra005465), and both *BrARF4.1* (Bra002479) and *BrARF3.1* and *BrARF3.2* have the same expression patterns in the Mun et al. 2012 study. *BrARF4.2* (Bra020243) was not expressed in any tissue and might be a pseudogenized gene copy (Huang et al., 2015).

In this study, we did a comparative analysis between the *BrARF3.1* heading and non-heading alleles of Chinese cabbage and pak choi respectively to shed light on their role in leaf development in different *B. rapa* morphotypes. The two *BrARF3.1* alleles and two chimeric constructs were used to study the effect of especially a C-terminal amino acid change on its function. The effect of *BrARF3.1* was studied by over-expression of the different alleles behind the 35S promoter in *A. thaliana* and phenotyping the plants. Furthermore, we studied the interaction of four different BrARF3.1 proteins and AtARF3 with an array of transcription factors with roles in leaf development. The effects of auxin on the identified protein interactions were explored.

Materials and Methods

cDNA clones

BrARF3.1 and *BrKAN2.1* cDNAs were amplified from both heading Chinese cabbage (CC-A03) and non-heading pak choi (PC-024) by PCR and inserted into pENTR (pDONR221), creating pDR1 (*CCARF3.1*), pDR2 (*PCARF3.1*), pDR5 (*CCKAN2.1*), pDR6 (*PCKAN2.1*). Site-directed mutagenesis based on the constructs (pDR1 and pDR2) was applied to create pDR3 (*CCPCARF3.1*) and pDR4 (*PCCCARF3.1*) as described in **Figure 1**.

Construct	Details
1 D1	N-terminus of Chinese cabbage <i>BrARF3.1</i> including the B3 DNA-binding domain (DBD)
2 D2_CC	MR region of Chinese cabbage <i>BrARF3.1</i> , which contains the Auxin-response super family domain
3 D2_PC	MR region of pak choi <i>BrARF3.1</i> , which contains the Auxin-response super family domain
4 D3_CC	The C-terminus of the <i>BrARF3.1</i> gene originating from Chinese cabbage, with the Q
5 D3_PC	The C-terminus of the <i>BrARF3.1</i> gene originating from pak choi, with the H
6 D1D2_CC	DBD and MR regions of Chinese cabbage <i>BrARF3.1</i>
7 D1D2_PC	DBD and MR regions of pak choi <i>BrARF3.1</i>
8 D2D3_CC	MR region and the C-terminus of the <i>BrARF3.1</i> gene originating from Chinese cabbage
9 D2D3_PC	MR region and the C-terminus of the <i>BrARF3.1</i> gene originating from pak choi
10 D2D3_CCPC	MR region and the C-terminus of the <i>BrARF3.1</i> gene from Chinese cabbage, with the C-terminal SNP from pak choi (H)
11 D2D3_PCCC	MR region and the C-terminus of the <i>BrARF3.1</i> gene from pak choi, with the C-terminal SNP from Chinese cabbage (Q)

Figure 1. Schematic overview of the BrARF3.1_H, BrARF3.1_NH, BrARF3.1H_NH, BrARF3.1NH_H proteins and their domains. Schematic diagram of the amino acid variation in BrARF3.1 (272, 284, 300 and 521aa). The target sites of TAS3 ta-siRNA are indicated in this figure. D1 contains the B3 DNA binding domain (yellow). D2 is the Middle Region of BrARF3.1 with the auxin response factor domain (pink). D3 represents the BrARF3.1 C-terminus (dark grey) with the single amino acid change (H to Q). The red lines indicate the DNA sequences encoding the polymorphic amino acids. The descriptions of all domain constructs are shown in the table.

Plant transformation and phenotypic analysis

For gene expression in plants, pDR1, pDR2 and pDR5 were Gateway cloned into PK7GW2 to create plant expression constructs of genes driven by the strong 35S promoter. *Arabidopsis* over-expression lines were constructed in the Col-0 background as described (Clough and Bent, 1998; modified from Bechtold et al. 1993). Plants (T2 generation) were sown on soil for leaf phenotyping and on half-strength Murashige and Skoog (MS) medium, supplemented with 1.5% w/v sucrose and 0.9% w/v phytoagar, pH 5.7, for root observation.

Yeast Two-Hybrid Analysis

Yeast two-hybrid screening was performed to allow for analysis of the protein-protein interactions of different BrARF3 alleles. In addition, interaction related functional domains of ARF3 in the Y2H were identified.

Yeast two-hybrid system

Arabidopsis AtARF3, the heading and the non-heading cDNA alleles and two chimeric *BrARF3.1* cDNAs were cloned into the PDEST32 “bait” vector (BD) (**Figure 1**). Full-length cDNAs of *BrKAN2.1*, *BrKAN2.2*, *BrYAB1.1* and *BrYAB1.2* were transformed into the PDEST22 “prey” vector (AD). The BD vectors were transformed into yeast strain PJ69-4A and AD vectors into strain PJ69-4a. Yeast transformation was performed according to the Folter and Immink, 2011. In addition, the *Arabidopsis* AD library, provided by the BioScience Department of Wageningen University & Research, was used to screen for interactions in this study. Leaf polarity pathway genes (*YABBY*, *KANADI*, *HD-ZIP* and *WOX* family) were selected from this *Arabidopsis* AD library for interaction screening (**Supplemental Table S1**). Colony growth was scored from a 0 to 5 scale (**Figure 2**). The “0” represents diploid yeast unable to grow on selective medium. The “5” represents a good growing colony and evidence for strong protein-protein interaction.

Figure 2. Scoring scale used to score the diploid yeast clones on selection plates during the Y2H experiment.

Domain analysis of the interaction

For the domain analysis, single domains (D1, D2 and D3) and combinations of domains (D1D2 and D2D3) of the four different BrARF3.1s were amplified by PCR using specific primers and were fused in the PDEST22 “prey” vector (**Figure 1**). These constructs were assayed for interaction with the selected *A. thaliana* and *B. rapa* AD constructs (*BrKAN2.1*, *BrKAN2.2*, *BrYAB1.1* and *BrYAB1.2*). Furthermore, the interactions between the different individual domains were tested.

Protein interaction assays in Y2H and IAA treatments

Before the Y2H interaction experiment, BD constructs with BrARF3.1 and AtARF3 were tested for auto-activation on SD-glu medium lacking -Leu, -Leu/-Ade, -Leu/-His supplemented with different concentrations (0, 1, 5, 10, 15 and 20 mM) of 3-aminotriazole (3-AT). Based on the auto-activation results, the interactions between BDs (BrARF3.1 and AtARF3) and ADs were selected on SD-glu lacking -Leu/-Trp/-His and SD-glu lacking -Leu/-Trp/-Ade supplemented with 3mM 3-AT. Serial dilutions of yeast co-transformed cells were used to measure the strength of the interaction. IAA was dissolved in ethanol and added at concentrations 0, 50 and 100 mM directly to the SD-glu selection medium.

Transcript abundance analysis

Leaf tissues harvested from *BrARF3_H*, *BrARF3_NH* over-expression and Col-0 plants were used for RNA extracted by the TRIZOL reagent (Invitrogen Carlsbad, CA, USA). Genomic DNA was removed using RNase-free DNase I treatment (Invitrogen, and cDNA synthesis was performed with iScript™ kit (BIO-RAD, CA, USA) according to manufacturer's instructions. Transcript abundance was measured by qRT-PCR with the Light Cycler-RNA amplification kit SYBR Green I (Roche, Mannheim, Germany). *ACTIN* (*ACT*) was used as reference gene and six biological replications were used in the study.

Results

Sequence analysis of *BraARF3*

The *B. rapa* genome contains two *ARF3* paralogues, one of which (*BrARF3.1*) has been nominated as candidate gene for the typical leaf-heading trait (Cheng et al., 2016). This gene contains a single nucleotide polymorphism (SNP) near the C-terminus: a G allele in heading accessions and a C allele in non-heading accessions.

We cloned two wild type alleles of *BrARF3.1*, *BrARF3.1_H* (cDNA) with the C-terminal heading allele (G) from Chinese cabbage (CC-A03) and *BrARF3.1_NH* with the C-terminal non-heading allele (C) from pak choi (PC-024) (Figure 1). Besides this C-terminal allelic variation associated with the heading trait, ten SNPs were located in the DBD region, three SNPs in the MR region and two SNPs between these two regions (**Supplemental Figure S1**). As *ARF* translation is regulated by *TAS3* ta-siRNA, the ta-siRNA target sites of *BrARF3.1* were predicted based on its location in *A. thaliana* (Williams et al., 2005; Fahlgren et al., 2006). However, no polymorphisms were present in the *TAS3* ta-siRNA target sites of *BrARF3.1* mRNA. Four of the SNPs were non-synonymous leading to the following amino acid changes: Asparagin (N) to Tyrosin (Y) at 272 aa in the region between DBD and the Auxin response super family domain, Valine (V) to Alanine (A) at 284 aa and Lysine (K) to Arginine (R) at 300 aa in the auxin response superfamily domain and the heading associated SNP at 521 aa in the C-terminus, Glutamine (Q) (heading allele) to Histidine (H) (non-heading allele). To study the effect of the single C-terminal Glutamine (Q) to Histidine (H) aa change (*BrARF3.1_H* and *BrARF3.1_NH* respectively), two chimeric *BrARF3.1* constructs (*BrARF3.1H_NH* and *BrARF3.1NH_H*) were made.

Protein interactions between BraARF3.1 and leaf polarity pathway related genes in *Arabidopsis* and *Brassica*

Bait- and prey-transformed strains were mated and the resulting diploids were cultured in SD-glu/-Leu/-Trp/-Ade/3μM-3AT and SD-glu/-Leu/-Trp/-His/3μM-3AT medium; only the diploids presenting protein interactions were capable of growth. An overview of all the obtained results with scores is shown in **Supplemental Figure S2**.

Y2H screening for *Arabidopsis* prey cDNA library using BrARF3.1 as bait

To compare the functionality of the ARF3 proteins from the different plant species, ARF3 from both *A. thaliana* and *B. rapa* were screened for their interaction with *Arabidopsis* leaf polarity related proteins. In addition, the heading and non-heading alleles *BrARF3_H*, *BrARF3_NH* respectively and their chimeric constructs, *BrARF3_H_NH* and *BrARF3_NH_H* as previously described, were used for Y2H (**Figure 3A**).

Interactions were observed of both At and Br ARF3 with members from the YABBY family (YAB1) and KANADI family (KAN1, KAN2, KAN3, KAN4 / ATS). Interactions of AtARF3 with YAB3, members of the HD-ZIP III (PHV, PHB, AtHB14, AtHB15),

WOX (WOX3), TCP (TCP5), and KNOX (KNAT1 / BP) family were not seen for the BrARF3.1 proteins. No interaction was seen between the AtARF3 and BrARF3.1 proteins with AtYAB2, AtYAB5, AtCCRC, AtINO, AtHB8, AtWOX1, AtWOX4, AtAS2, AtKANT2, AtKANT6, AtSTM nor with members from the TCP family (TCP3, TCP4, TCP9, TCP10, TCP13, TCP17, TCP20 or TCP24).

Figure 3. Yeast two-hybrid interaction overview. (A) Interaction between different ARF3 proteins and various *Arabidopsis* leaf polarity related proteins. ARF3 was in the bait vector and AtYAB1, AtYAB3, AtKAN1, AtKAN2, AtKAN3, AtKAN4, AtPHV and AtPHB were in prey vectors. (B) Interaction between BrARF3 with BrYAB1 and BrKAN2 proteins. (3) Interaction of different BrARF3 proteins with BrYAB1 and BrKAN2 in 50 μ m IAA-SD selective medium. Protein-protein interactions were visualized by differential growth on SD-glu/-Ade/-Leu/-Trp/3 μ M-3AT selective medium.

The four BrARF3.1 proteins show interaction with AtKAN1, AtKAN2 and AtKAN3. Conversely, no interactions with AtYAB3, AtPHV and AtPHB were seen. Interestingly the different BrARF3.1 alleles and their chimeras interacted differently with AtYAB1 and AtKAN4. The heading allele BrARF3.1_H, and both chimeras BrARF3.1H_NH and BrARF3.1NH_H all interacted with AtYAB1 and AtKAN4, however the interaction between BrARF3.1NH_H and AtYAB1 was weak. Interestingly, no interactions of the non-heading pak choi BrARF3.1_NH allele were observed with both AtYAB1 and AtKAN4. The only difference between the chimeric protein BrARF3.1NH_H and the non-interacting pak choi allele BrARF3.1_NH is the single C-terminal change of aa 521 from a Q to an H.

Specific interactions of BrARF3.1 and its domains with BrYAB1, BrKAN2

The interactions between BrARF3.1 and *B. rapa* YAB1 proteins (BrYAB1.1 and BrYAB1.2), KAN2 (BrKAN2.1 and BrKAN2.2) are similar as those between the BrARF3.1 proteins and the *A. thaliana* orthologues (**Figure 3B**). BrARF3.1_NH did also not interact with BrYAB1.1 and BrYAB1.2, while for BrARF3.1_H, BrARF3.1H_NH and BrARF3.1NH_H, strong interactions were perceived for BrYAB1.1 and weaker interactions with BrYAB1.2. Individual domains showed protein-protein interaction related functionality. Single D1 and D2 domains did not interact with AtYAB1, BrYAB1 (YAB1.1 and YAB1.2) nor BrKAN2 (KAN2.1 and KAN2.2), however both D3 alleles did interact with both the At and the two Br YAB1 paralogues. For KAN2, no interaction was observed with single BrARF3.1 domains, however when individual domains were combined, D1D2_NH and D2D3NH_H interacted with BrKAN2.1 and BrKAN2.2. However, D1D2_NH did not result in interaction with AtKAN2 (**Table 1**).

Table 1. Interaction results of BrARF3.1 individual and combination domains with YAB1 and KAN2

Domain	D1	D2 H	D2 NH	D3 H	D3 NH	D1D2 H	D1D2 NH	D2D3 H	D2D3 NH	D2D3 H_NH	D2D3 NH_H
BrYAB1.1	--	--	--	+	+	--	--	--	--	--	--
BrYAB1.2	--	--	--	+	+	--	--	--	--	--	--
BrKAN2.1	--	--	--	--	--	--	+	--	--	--	+
BrKAN2.2	--	--	--	--	--	--	+	--	--	--	+
AtYAB1	--	--	--	+	+	--	--	--	--	--	+
AtKAN2	--	--	--	--	--	--	--	--	--	--	+

“--” indicates negative/no protein-protein interaction; “+” indicates positive protein-protein interaction
D1 contains the B3 DNA binding domain. D2 is the Middle Region of BrARF3.1 with the auxin response factor domain. D3 represents the BrARF3.1 C-terminus with the single amino acid change (H to Q), which associates with heading in Chinese cabbages.

Altogether, auxin was shown to have a uniform effect on the interactions with ARF3. Generally, when auxin (50mM) was applied, the protein-protein interactions of the ARF3 proteins with At and Br proteins were less strong (**Figure 3C**). Auxin treatment also had a dosage-effect with interactions being completely absent at 100mM auxin.

BrARF3.1* over-expression in *Arabidopsis

Both *BrARF3.1_H* and *BrARF3.1_NH* alleles were over-expressed in *A. thaliana* (*Columbia*: Col-0) behind the 35S promoter.

Phenotypes in *BrARF3.1* over-expression plants

Morphological analysis was performed in *BrARF3_H* (named: *BrARF3-H*) and *BrARF3_NH* (named: *BrARF3-NH*) over-expression (OE) lines (T2 generation) (**Figure 4**). The *BrARF3-H* OE plants were indistinguishable from the wild type Col-0 under the growth conditions examined. Remarkably, *BrARF3-NH* OE plants had strong phenotypes as they were smaller with less leaves (**Figure 4A**). The leaves had a pointy end, curled downwards at the long edges and were curved sideways at the tip of the leaf making them often asymmetric from a top down perspective. Moreover, *BrARF3-NH* OE leaf petioles were shorter and more upwards compared to Col-0 (**Figure 4B**). Cytological observations illustrated that *BrARF3-NH* OE leaves were thicker and had less air spaces in their palisade parenchyma layer (15%) and more air space in their spongy (46%) parenchyma layer compared to Col-0 and *BrARF3-H* OE leaves (24-28% and 31-34% respectively) (**Figure 4D**). Other distinct characteristics of over-expression lines were the roots, which were short and displayed more branching in *BrARF3-NH* OE, but not in *BrARF3-H* OE plants (**Figure 4C**).

Figure 4. Morphological characterization of *BrARF3_H* and *BrARF3_NH* over-expression in *A. thaliana* (Columbia: *Col-0*) compared to the wild type *Col-0*. (A) Plants of *BrARF3-H* OE (left), *Col-0* (mid) and *BrARF3-NH* OE (right). (B) Successive rosette and inflorescence leaves from *Col-0* and over-expression plants, arranged from the base (left) to the tip (right) of the stem. (C) Root architecture of 10-day-old seedlings of the *Col-0* (mid), *BrARF3_H* (left) and *BrARF3_NH* (right) OE plants. (D) Histology of transverse sections of leaves for *BrARF3* over-expression plants and *Col-0*. Scale bars were 500 μ m (left) and 100 μ m (mid). Quantified data of leaf mesophyll tissue in air space of palisade (green) and spongy (grey) tissues of *Col-0*, *BrARF3-H* and *BrARF3-NH* OE leaves were arranged from top to bottom.

Transcript abundance of leaf ad/abaxial genes in *Arabidopsis BrARF3.1* over-expression plants

Transcript abundance of *BrARF3.1*, *AtARF3*, *AtARF4*, *AtKAN1*, *AtKAN2*, *AtKAN3* and *AtRDR6* was measured in leaves of *Arabidopsis Col-0*, *BrARF3_H* and *BrARF3_NH* over-expression plants by qRT-PCR (**Figure 5**). Both *BrARF3.1_H* and *BrARF3.2_NH* were highly expressed in leaf tissue of their respective over-expressing plants, but not detected in *Col-0*. *AtARF3* transcripts were present at a similar level in the two over-expression lines and *Col-0*. Remarkably, transcript abundance of *AtARF4*, *AtKAN1*, *AtKAN2* and *AtKAN3* in *BrARF3_H* and *BrARF3_NH* over-expressing leaves

was similar compared to wild type Col-0. *AtKAN2* was higher expressed than *AtARF4*, *AtKAN1* and *AtKAN3*. Interestingly, the *AtRDR6* gene, which is involved in the TAS3–ta-siRNA–ARF3/ARF4 pathway, was more expressed in the *BrARF3.2_NH* OE leaves, that displayed a clear phenotype, than in *BrARF3.2_H* OE and Col-0 wild type leaves.

Figure 5. Transcript abundance measured by qRT-PCR analysis of Arabidopsis leaf polarity related genes in leaf tissue of *BrARF3_H*, *BrARF3_NH* over-expression and Col-0 genotypes. Genes profiled were *BrARF3.1*, *AtARF3*, *AtARF4*, *AtKAN1*, *AtKAN2*, *AtKAN4* and *AtRDR6*. Actin was used as reference gene.

Discussion

Leafy head formation is a quantitative trait, as our group previously identified several selective sweeps related to leafy head formation, and we and others identified several QTLs related to leafy head formation in mapping studies (Ge et al., 2011; Yu et al., 2013; Cheng et al., 2016). As many other ad/abaxial polarity genes are identified in selective sweeps for leafy head formation in both *B. rapa* and *B. oleracea*, including *HD-ZIPIII*, *KAN2*, *ARF3*, *ARF4* and *RDR6*, clearly perturbation ad/abaxial formation plays a role in leafy head formation.

In a leafy head, the leaves fold around each other to form a tightly packed head. For the outer head leaves the abaxial side is exposed to the light, while inner leaves become yellow or white, as they do not receive sunlight. In line with many other domestication events, we assumed that domestication of Chinese cabbage involved mainly loss of function alleles. In this study, we characterized the *ARF3.1* gene for which allelic variation in a C-terminal amino acid (Q to H) associated with leafy

head formation. The heading allele (C-terminal Q), was present in 322 out of 357 heading Chinese cabbages but was also present in 111 out of 439 non-heading *B. rapa* accessions (Cheng et al., 2016), illustrating that variation in this gene alone likely has subtle effects on leaf growth. *ARF3* contributes to abaxial identity of the leaf and the *ARF3* gene plays an essential role in lamina formation by among others promoting *YABBY* transcription. *YABBYs* play a role in both ad/abaxial polarity establishment and promotion of lamina formation (reviewed by Maugarny et al, 2018).

For the above reasons we functionally characterized different *BrARF3.1* alleles, aiming to understand their roles in leafy head formation of Chinese cabbages. We investigated the effects of *BrARF3.1* allelic variation on both the interaction with transcription factors involved in leaf ad/abaxial polarity establishment and blade outgrowth, and by phenotyping over-expression genotypes in *A. thaliana*, as *B. rapa* transformation is still highly inefficient and very genotype dependent. The non-heading associated C-terminal amino acid change abolished or decreased protein interactions with both At- and Br- YAB1 proteins and AtKAN4, suggesting that the non-heading allele represents the mutant allele. Auxin abolishes or weakens the interaction of all *ARF3* constructs with *YAB1's* and *KAN2*. The *A. thaliana* over-expression data illustrate that OE of the non-heading *BrARF3.1* allele results in a clear leaf and root phenotype, with down curving leaf blades composed of less air space in palisade and more air space in spongy parenchyma tissue, and increased lateral root sizes, while the heading allele over-expressors have no or very mild phenotypes. These very interesting findings are further discussed below.

ARF3.1 interactions in Y2H

Interactions of AtARF3 and BrARF3.1 were similar, with all KAN proteins, a subset of the *YABBY* proteins (not with AtYAB 2 and AtYAB5) and the class III HD-ZIP family (AtPHB and AtPHV) proteins, but not identical. Both AtYAB3, and the class III HD-ZIP proteins AtPHB and AtPHV did interact with AtARF3 but not with BrARF3.1 alleles. We do not know what the cause is of this difference, but one possible explanation is that in *Arabidopsis* and in *B. rapa*, genes co-evolved. Testing the interaction between BrARF3.1 with the *B. rapa* orthologous proteins can clarify this. Interactions between KAN1 and ETT (*ARF3*), KAN4 and ETT were found in *Arabidopsis* (Kelly et al., 2012). Thus, it is not surprising that BrARF3.1 interacts with members of the AtKAN family. The *B. rapa* paralogues BrKAN2.1 and BrKAN2.2 showed similar interactions as AtKAN2 with BrARF3.1. Based on these Br protein interaction results, we predict that also the other BrKAN proteins are able to interact physically with BrARF3.1, as translatability of the *A. thaliana* results to *B. rapa* is

suggested. However, definitive conclusions about the interaction of BrARF3.1 with BrKAN1, BrKAN3 and BrKAN4 still need to be confirmed.

Both BrARF3.1 and AtARF3 interacted with AtYAB1, but not with AtYAB2 or AtYAB5. YABBY family members have two conserved domains and three variable regions (Bowman, 2000). YAB1 (FIL), YAB2, YAB3 and YAB5 share some homology in region III, FIL and YAB3 also display similarity in regions II and I (Siegfried et al., 1999; Bowman & Smyth, 1999). Our results suggest that the physical protein-protein interaction of ARF3.1 with AtYAB1 and AtYAB3 may depend on the relatively variable inter-domain regions on YAB region II or I. FIL and YAB3 are reported to act redundantly (Siegfried et al., 1999; Sarojam et al., 2010). In *B. rapa*, there are two homologues of YAB1, BrYAB1.1 and BrYAB1.2. Since, only BrYAB1.1 interacted with BrARF3.1 these two homologues may act redundantly.

Influence of auxin IAA

In the presence of auxin, the Aux/IAA repressor acting on the ARF genes will be degraded by the Skp1-Cullin-Fbox complex, resulting in activation of the ARF genes (Tiwari et al., 2003; Sauer et al., 2006). However ARF3, ARF13 and ARF17 are not like typical ARF genes, as they lack the C-terminal interaction domain (domain III/IV), which interacts with Aux/IAA proteins (Kim et al., 1997; Tiwari et al., 2003). Our studies show however that ARF3 is able to sense auxin as protein-protein interactions do not form or become weaker despite the fact that ARF3 proteins lack the typical auxin sensing domain conserved in most ARFs. The indicative findings that ARF3 is able to respond to auxin are in line with recent articles by Simonini et al., 2016, 2017 and Chandler, 2016, which clearly prove auxin responsiveness of ARF3.

Effect of allelic variants of *BrARF3.1*

Differential interactions of the H and NH alleles of BrARF3.1 were observed with the AtYAB1, AtKAN4 and BrYAB1 proteins. BrARF3.1_H interacted with AtYAB1, AtKAN4 and BrYAB1.1, similar to AtARF3, while BrARF3.1_NH did not interact with YAB1 proteins and only weakly with AtKAN4. The phenotype of *A. thaliana* over-expression *BrARF3.1_NH* plants is severe, with long down curling leaves, reflecting an increase in the growth rate of adaxial tissue relative to the growth rate of abaxial tissue, while over-expression *BrARF3.1_H* plants have no or a weak phenotype only (slight down curving leaves, **Supplemental Figure S3**). Hunter et al., (2006) phenotyped *Arabidopsis* plants over-expressing *ETTIN* (*35S::ETTIN/ARF3*) and plants over-expressing *ETTIN* in which the ta-siARF target sites were mutated

(35S::*ETTINmAB*) without affecting the amino acid sequence. 35S::*ETT* plants produced a lower frequency of morphologically aberrant phenotypes (8%) than did 35S::*ETTmAB* plants (27%). Only 16% of the 35S::*ETTmAB* plants showed elongated, epinastic leaves, very similar to our 35S::*BrARF3_NH* plants. Interestingly Fahlgren et al., (2006) also found that reduced repression of *ARF3mut* (mutated in target sites) by TAS3 ta-siRNA leads to a similar downward curling phenotype as observed for *BrARF3.1_NH OE* plants. These authors conclude that clearly the more severe phenotype was caused by increased transcript level in the *ETTIN-mAB* over-expressors. However they also suggested that plants without phenotype that did have increased *ARF3* transcript abundance, similar to our 35S::*ARF3.1_H* plants, could be explained by the fact that *ARF4* or other targets may contribute to the phenotype. They however also suggested that besides regulation at transcriptional level by ta-siRNA, *ETTIN/ARF3* and *ARF4* activities are also likely regulated at other levels, such as through their interaction with other auxin-related proteins. The 35S::*BrARF3_NH* over-expressing plants also resemble *rdr6* mutant plants that lack ta-siRNA and accumulate increased *ARF3* transcript. Data described above suggests that the *BrARF3_NH* allele causes a more severe phenotype when over-expressed in *A. thaliana* is the mutant allele, as not only transcript abundance of *ARF3* is increased, similar to *ARF3.1_H* over-expression plants, but also its function is affected by altered protein interactions. *BrARF3.1_NH* OE plants with down curving leaves also resembled the leaf phenotypes of 35S:*KAN* lines found by Kerstetter et al., 2001. These results underpin the theory that *KAN* and *ARF* act in concert (Kelley et al., 2012). In addition, the BrARF3.1_NH protein did not interact with AtYAB1, AtKAN4 and BrYAB1.1, unlike AtARF3 and the other BrARF3.1 proteins. AtYAB1 is expressed in leaf primordia and establishes leaf abaxiality in this developmental stage (Siegfried et al., 1999, Sarojam et al., 2010). The loss of interaction of BrARF3.1_NH with YAB1 may effect both leaf polarity but also lamina outgrowth.

The BrARF3.1_H and BrARF3.1_NH allele differed in four amino acids, and we wanted to test whether the C-terminal single amino acid (aa) change (His to Glu) was causal for the changed protein interactions and for the over-expression phenotype. For this purpose chimeric constructs were made and tested for their interactions with other proteins. The BrARF3.1_H, BrARF3.1H_NH and BrARF3.1NH_H interacted in similar ways, whereas only BrARF3_NH behaved differently. The only difference between BrARF3.1_NH and BrARF3.1NH_H is the single aa change at the C-terminus (His to Glu), yet it confers the ability of BrARF3.1NH_H to gain interactions with YAB1 and KAN4 proteins. Surprisingly, this single aa change does not change these protein interactions when present in

combination with the N-terminal region from the Heading allele as BrARF3.1H_NH and BrARF3.1H show identical interactions, like the BrARF3.1NH_H allele. These results imply that the ability of the C-terminal aa change to lose/reduce interactions with YAB1 and KAN4 seems to rely on the presence of at least one of the other aa changes present in the other domains of BrARF3.1. One explanation could be that the D2 variation influences the protein secondary structure resulting in different degrees of access to the C-terminus.

The interactions between BrARF3.1 domains or combinations of domains with YAB1 and KAN2 proteins were also tested to understand the effects of the other AAs changes. While most of the single and double domain combinations did not interact with YAB1 and KAN2 proteins in the Y2H screens, both the BrARF3.1 H and NH alleles of D3 did interact with YAB1. For KAN2, it was different, as certain allelic combinations of both D1D2 and D2D3 domains interacted, however these allelic differences did not match allelic differences of the complete BrARF3.1 protein interactions. The results are hard to understand, as the effect of the interaction of the C-terminal amino acid variation depends on the aa variations in D2. However the constructs of domains represent only part of the BrARF3.1 protein and are thus artificial, which might affect the folding structure of proteins.

Different regulation of ta-siRNA pathway

Besides the four amino acid changes differentiating the heading and the non-heading alleles, several base pairs differ between these alleles (non-synonymous changes). None of those were however detected in the ta-siRNA domains, so we do not think that they influence *BrARF3.1* function via the ta-siRNA pathway. However the *BrARF3.1_NH* and *BrARF3.1_H* SNPs (both synonymous and non-synonymous) outside the target regions might lead to different transcript secondary structures, altering the ta-siRNA repressive ability through influencing accessibility.

Besides the effects of the allelic variation of BrARF3.1 on protein-protein interactions, transcription level changes of other genes that regulate leaf development could also explain the different phenotypes between *BrARF3.1_H* and *BrARF3.1_NH* OE plants. Interestingly, transcript abundance of *AtRDR6*, which mediates ta-siRNA synthesis that regulates *ARF2/ARF3/ARF4* gene transcript abundance, was up-regulated in *BrARF3.1_NH* OE plants and not in *BrARF3.1_H* OE plants. This may lead to reduced expression of the other target ARFs, like *AtARF4*, for which we did not see differences in transcript abundance between the two OE plants and wild type, or *ARF2*, which we did not test.

Conclusion

There are two main hypotheses for the origin of Chinese cabbage: Chinese cabbage originated from hybridization between turnip and pak choi (Li, 1981), or was selected from pak choi (Tan, 1979). Both hypotheses do postulate that pak choi was first domesticated and that Chinese cabbage was selected from that. Arguably, Chinese cabbage *BrARF3.1_H* is the domesticated allele, and based on that we expected that the Heading allele would be the mutated allele. While non-heading pak choi leaves are flat, the Chinese cabbage heading leaves curl upward. The *A. thaliana BrARF3.1_NH* OE plants showed a very clear phenotype and their leaves curled downward. As both protein interactions and transcript regulation by several types of small RNAs together define the ad/abaxial leaf polarity establishment and lamina outgrowth, changes in both protein interactions and transcript levels likely affect this process. As *BrARF3.1_NH* cannot interact with several leaf development related proteins, for example *BrYAB1.1*, and has reduced interaction with the abaxial identity *KAN* proteins, the aberrant phenotype in *Arabidopsis* OE is no surprise. Further comparison between pak choi leaves with *NH BrARF3* alleles and wild type *B. rapa* accessions (turnip and oil types) is needed to understand the role of *BrARF3*. In pak choi domestication as well. As heading is a quantitative trait controlled by multiple genes, allelic variation in *BrARF3.1* likely has a subtle effect on the *B. rapa* leaf phenotypes and cannot induce the leafy head. *YABBY*, *KANADI* and *ARFs* are all considered candidate genes in the domestication of leaf heading (Cheng et al., 2016; Liang et al., 2016), whilst interacting and regulating one another, and thus phenotyping of plants combining allelic variation in several of these genes is needed to fully understand the heading phenotype.

Supplementary Tables and Figures

Table S1. Selection of 33 “prey” constructs in the *Arabidopsis* AD vector library that were screened for interaction with different ARF3 proteins in this study

	Name	TAIR ID	TF family	Coding sequence (bp)
1	YAB1/FIL	AT2G45190	C2C2-YABBY	690
2	YAB2	AT1G08465	C2C2-YABBY	555
3	YAB3	AT4G00180	C2C2-YABBY	732
4	YAB5	AT2G26580	C2C2-YABBY	495
5	CRC	AT1G69180	C2C2-YABBY	546
6	INO	AT1G23420	C2C2-YABBY	696
7	KAN1	AT5G16560	G2-like	1212
8	KAN2	AT1G32240	G2-like	1167
9	KAN3	AT4G17695	G2-like	969
10	KAN4/ATS	AT5G42630	G2-like	831
11	PHV/ATHB9	AT1G30490	HB	2526
12	PHB/ATHB14	AT2G34710	HB	2559
13	ATHB8	AT4G32880	HB	2502
14	ATHB15	AT1G52150	HB	2514
15	WOX1	AT3G18010	HB	1053
16	PRS/WOX3	AT2G28610	HB	735
17	WOX4	AT1G46480	HB	756
18	AS2	AT1G65620	LOB / AS2	600
19	TCP2	AT4G18390	TCP	1098
20	TCP3	AT1G53230	TCP	1176
21	TCP4	AT3G15030	TCP	1263
22	TCP5	AT5G60970	TCP	1083
23	TCP9	AT2G45680	TCP	1071
24	TCP10	AT2G31070	TCP	1086
25	TCP13	AT3G02150	TCP	1068
26	TCP17	AT5G08070	TCP	729
27	TCP20	AT3G27010	TCP	945
28	TCP24	AT1G30210	TCP	975
29	KNAT1/BP	AT4G08150	HB	1197
30	KNAT2	AT1G70510	HB	933
31	KNAT3	AT5G25220	HB / KNOX2	1296
32	KNAT6	AT1G23380	HB	990
33	STM	AT1G62360	HB	1149

CC_A03 M G G L I D L N V T E E Q E E E T T P S S G S G S L S P
 PC_024 ATGGGTGGTTTAATGTAGTCTGAACGTGACGGAGGAGCAAGAAGAAGAACGACACCGTCTTCAGGCTCCGGGTCTCTCTCTCCG

CC_A03 C D S S S S S A S A F G V S G S S S S S S G V C L E L W H A
 PC_024 TGTGATTCTTCGTCTTCAAGTCTTCGGTGTGGTGTGAGTGGGTCTTCTTCTTCGTCTGGGTATGTCTGGAGCTGTGGCATGT

CC_A03 C A G P C C L I S L P K R G S L V L Y F P Q G G H L E Q A P D
 PC_024 TGTGCTGGACCTCATCTCTCCGAAAAGAGGAGCCTCGTGTGTATTTCCTCAGGAGCTTGAACAGGCCCGAT

CC_A03 F S A A I Y G L P P H V F C R I L D V K L H A E T A T D
 PC_024 TTCTCCGCGCGATTTCAGGCTCCCTCCTCAGCGTTCGTGTCTTCTTGTATGTCAAGCTTCACGCAGAGACAGCTACTGT

CC_A03 E V Y A Q V S L L P E S E D V E R K V R E G V I D V D G
 PC_024 GAAGTTTATGCTCAAGTCTCTCTGCTTCTGAGTCTGAGGACGTTGAGAGGAAGGTGCGTGAGGAGTGATAGATGTTGATGCT

CC_A03 G E E D Y E V V K R T N T P H M F C K T L T A S D T S T
 PC_024 GGGGAGGAGATTATGAGGTGTTAAGAGGACTAATACTCCTCATATGTTCTGTAAAACTCACTGCTTCTGTATACAAGTACA

CC_A03 H G G F S V P R R A A E D C F P P L D Y T Q P R P S Q E
 PC_024 CATGGTGGCTTCTGTTCCTCGCGCTGCTGCTGAGGATTGCTTCCTCCTCTGGACTATACCTACGCCAGGCGCTCCAGGAG

CC_A03 L L A R D L H G L E W R F R H I Y R G Q P R R H L L T T
 PC_024 TTCTTGTCTAGGATCTTCATGCGCTGGAGTGGCTTTTCGCCACATATACCGAGGACAACCTAGGAGGCATTGTGCTCACTACT

CC_A03 G W S A F V N K K K L V S G D A V L F L R G D D G K L R
 PC_024 GGAATGGAGTGGCTTTGTGAACAAGAAGAAGCTTGTCTCTGGTGATGCTGTGCTTTTCTCAGAGGTGATGATGGCAAAGTGGCG

CC_A03 L G V R R A S Q I E G A S A F S S Q Y N / Y Q N M N H N N F
 PC_024 TTGGGAGTGAGAGAGCTTCTCAAAATCGAAGGTGCTTCTGCTTTCTCCAGTCAGTATATCAGAACATGAACCAATAATTTTC

CC_A03 A E V V / A H A I S T N S A F N I Y Y N P K / R A S W S N F I I
 PC_024 GCTGAACTAGTTTCATGCCATATCCACCAATAGTGTCTTCAACATCTACTACAACCCCAAGGCAAGCTGGTCAAACCTTCATAATC

CC_A03 P A P K F L K T V D Y P F C I G M R F K A R V E S E D A
 PC_024 CCTGCACCGAAGTTCTTGAAGACTGTGTACTATCCATTGTTATCGGTATGAGATTCAAAGCAAGGGTTGAGTCTGAAGATGCC

CC_A03 S E R R S P G I I T G I N D L D P I R W P G S K W R C L
 PC_024 TCTGAGAGAAGATCCCTCGGGATAATAACTGGAATCAACGACTTGGATCCTATCAGGTGGCCTGGTTCATAATGAGATGCCTC

CC_A03 L V R W D D T D A N G H H Q Q R I S P W E I E P S G S I
 PC_024 TTGGTAAGGTGCGACGACACTGATGCAATGGGCATCATCAACAGCGCATCTCACCATGGGAGATAGAACCATCTGGTTCAATC

CC_A03 S S S T S G S F I T T G P K R S R I G F S S G K P D I P V S
 PC_024 TCCAGTTCAGGCAGCTTCATAACAACCTGGTCCCAAGAGAAGCAGGATTGGCTTTTCTCAGGAAAGCCTGACATCCCTGTCTCT

CC_A03 G S G D V D P Q T L R N L * R V L Q G Q E I F H G F I N
 PC_024 GGTTCGTGGTCCACAGACTTTGAGGAATCTCTAAGATTCCAGAGGCTTTCGAAGGTCAAGAAATTTTCACGGTTTCATCAAC

CC_A03 T S S D G G A G A R R G R F K G T E F G D S Y G F H K V
 PC_024 ACCTCTTCGGATGGTGGTGTGCTGGTGCAGGAGAGCGGTTCAAAGGAACGGAGTTTGGTGACTCTTATGGGTTCCATAAGGTC

CC_A03 L Q A G G T C A G E A T V P A Y P M M T D Q R H Q G M S Q R N I W C
 PC_024 TTGCAAGGTCAAGAACTGTTCCCGCTTACCCAATGATGACCGATCAACGGCAGCAGGGGATGAGCCAGGAACATTGTTGTT

CC_A03 G P F Q N F S T R I L P P S S V Q / H V S S P L P S S G P N
 PC_024 GGTCCGTTCAGAACTTTAGCACAGTATCTCCCGCCATCTTCGGTCCAGGTGTCTTCACTACTACCGTCAAGCGGTCTTAAC

CC_A03 V R M E E H H G G S G R C R L F G F P L R D E T T A V G
 PC_024 GTGCGGATGGAAGAGCACCGAGGTTTCGGGCAGGTGACGGCTGTTTGGTTTCCCGTTGAGGGATGAGACCAGCGGTTGGG

CC_A03 S G M V V G G P C V E G Q N G S S I R G G V S A V Q S S H
 PC_024 TCTGGGATGGTTGTTGGCCCTTGTGTGGAAGGGCAAAATGGGAGTTCCATAAGAGGTGGTGTTCGTGTTTCAGAGCAGTCAT

CC_A03 H G R D I Y G M R D M L L D I A L *
 PC_024 CATGGAAGGCATATATGGGATGAGAGATATGTTGCTAGACATTGCTCTCTAG

Domain 1 (D1): B3 DNA binding domain Domain 2 (D2): Auxin response super family domain
 Reverse D1 primer (overlapping with D1) Reverse D1 primer (non-overlapping with D1) Forward D2 primer (right non-synonymous SNP)
 Reverse D2 primer (overlapping with D2) Reverse D2 primer (non-overlapping with D2) Forward C-terminus primer

Figure S1. BrARF3.1 (Bra005465) CDS and amino acid sequences in heading Chinese cabbage (CC-A03) and non-heading pak choi (PC-024). Yellow color indicates the ARF amino-terminal DNA binding domain (DBD). Green color indicates the BrARF3.1 repression domain in middle region (MR). Red characters refer to allelic or amino acid variation between BrARF3.1_H and BrARF3.1_{NH}.

Figure S3. Overview of phenotypes *BrARF3.1_NH*, *BrARF3.1_H* over-expression lines and *Col-0* at 36 days after sowing.

Chapter 6

General Discussion

The *Brassica* genus consists of 38 species, including several species with great economical value that display extreme morphological diversity as a result of selection during domestication (Cheng et al., 2013). What makes *Brassica*'s unique is that they diverged into many different morphotypes, ranging from oil crops, turnips and kohlrabi's to cauliflowers. The whole genome triplication shared by all *Brassica* species facilitated this convergent selection of morphotypes (Cheng et al. 2016). Leafy heads, which represent the harvested organs of cabbages and Chinese cabbages, were domesticated independently (Cheng et al. 2016). However leafy head formation is not restricted to *Brassica*'s, as lettuce (*Lactuca sativa* L.) and radicchio of Treviso (*Cichorium intibus* L.) also form heads. In nature however leafy head formation is not found, likely because it is a disadvantage for the plant as propagation by seeds is impeded. While domestication traits in seed crops like grains have been studied extensively, this is not the case for leafy vegetables (Doebley et al., 2006; Ishimaru et al., 2013). Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) with large leaves and heads of different shapes was likely domesticated from pak choi (*B. rapa* subsp. *chinensis*) (Tan, 1979; Li, 1981), as the leafy head trait of Chinese cabbage has clear advantages for both farmers and consumers, related to yield, stress resistance and storability. In recent years, scientists started to study this domestication trait, but the developmental and genetic mechanisms underlying leafy head formation remain unclear. This thesis aims to better understand leafy head formation in Chinese cabbage.

In this thesis, we studied leafy head formation in Chinese cabbage at many different levels, from macro (population level) to micro (cell, gene and protein-interactions). We first analyzed the head formation related traits in field grown plants to gain insight into genetic regulation and phenotypic variation of the plant architecture. We then analyzed transcript abundance during development in two Chinese cabbage and one pak choi genotype to reveal molecular pathways involved in their developmental processes. Furthermore, by comparing heading Chinese cabbage with non-heading pak choi, the differences in leaf cellular structure were investigated. Finally the auxin response factor gene *BrARF3*, with allelic variation associated with leafy head formation in Chinese cabbage, was investigated for its effect on leaf cellular structure. In this general discussion chapter, I will explain my research processes combined with the findings of this thesis and those of other researchers. As auxin plays a prominent role in leaf initiation and growth and *BrARF3* is a leafy head domestication candidate gene in Chinese cabbage, pointing again to roles for auxin, the effects of auxin on leafy

head formation are discussed and my recommendations for further research on this subject are presented.

Better understanding of traits that contribute to Chinese cabbage leafy head formation

The Chinese cabbage leafy head is composed of many heading leaves tightly arranged around each other. Most phenotypic and genetic studies focus on head traits, such as head size, head shape, head weight, head length, head width and number of heading leaves. In several studies, many quantitative trait loci (QTLs) are detected for these head traits by phenotyping segregating populations derived from crosses between heading and non-heading *B. rapa* morphotypes (Kubo et al., 2010; Ge et al., 2011; Inoue et al., 2015). These QTL explain variation in head traits, but do not explain why leafy heads are formed and which traits contribute to leafy head formation. Some studies have shown that the initial stages of leafy head development, formation of rosettes and folding leaves play an important role in leafy head development in Chinese cabbage (Xiao et al., 2013; Wang et al., 2012). Other studies described that the rosette and upward folding leaves accept and transmit external environmental signals into morphological responses, which also affect the leafy head (Yu et al., 2000; Wang et al., 2012). However, all of these studies did not systematically analyze which rosette leaf traits relate to plant development, thereby affecting head formation in Chinese cabbage at heading stage. In chapter 2, we phenotyped 152 Chinese cabbage cultivars and identified the correlation between rosette leaf characteristics during development and leafy head traits at heading stage. This clearly demonstrated the effect of rosette leaf traits on heading -degree, -size and -shape. In addition, a number of co-locations of QTL for rosette leaf (leaf width) with heading degree, rosette leaf (leaf length and leaf petiole length) with head traits (head height and head weight) were found. These findings made us decide to study several rosette leaf traits in more detail aiming to better understand the process of leafy head formation in Chinese cabbage. The phenotypic observations also revealed how difficult it is to phenotype the development of Chinese cabbage plants, as especially the organization of the leaves around the main stem (phyllotaxis), the angles of petioles and leaf blades and the leaf symmetry and curvature together with the leaf sizes all contribute to the size and shape of the final leafy head. 3D imaging together with data analysis tools and mathematical modeling are needed to be able to quantify plant development.

As mentioned above, variation of leaves at rosette stage related to the later leafy head formation. In chapter 3, we describe the phenotypes of two Chinese cabbages and one pak choi from seedling to heading stage at the whole plant level and profile transcript abundance in emerging leaves at different developmental stages in a non-heading pak choi and in two very different Chinese cabbages. In addition, anatomical studies of leaf morphology described in chapter 4 increased our understanding of the cellular mechanism that controls seedling and rosette leaf organ shape and size. The current understanding of leaf curvature (down and upward curvature) is that it is caused by the differential growth on the adaxial and abaxial surface (epidermal cells) of leaves. Increased growth in abaxial surface cells, as well as a reduction of adaxial surface cells induces upward curvature of leaves (Sandalio et al., 2016). If abaxial epidermal cells display increased growth, the spongy parenchyma layer needs to follow either by increasing cell size, cell numbers or cell packing/organization. In our study, compared to the flat leaf blades in non-heading pak choi, the leaf upward curvature in heading Chinese cabbage is a consequence of differences in size, numbers and positioning of adaxial (palisade) and abaxial (spongy) cells. Differences also exist between organization of cells in different parts of the leaf, like the tip, mid section and base of the leaf. In order to curve in both directions (length and width), to form a round leafy head, cell growth needs to differ depending on the position in the leaf. Chinese cabbage leafy heads consists of incurved leaves and the incurving leaves must have different cell growth patterns than the rosette leaves. In a recent review paper describing fruit shapes, it was also concluded that the final size and shape of organs are controlled by the rate, duration and plane of cell divisions and the direction and degree of cell expansion (van der Knaap & Østergaard, 2017). Thus, mathematical modeling of cell growth by analyzing 2D and 3D images at different developmental stages could help to describe the growth process and to identify the essential processes during leafy head formation.

Co-expression of many genes and microRNA's regulate cell division and cell expansion that all define leaf developmental stages (Maugarny-Cales and Laufs, 2018). By profiling only a subset of these genes involved in ad/abaxial formation and leaf outgrowth could not explain the observed differences in cell organization of the leaves and positions within leaves at different developmental stages, however it did indicate that the incurving process of the leaves is regulated by differential regulation of leaf genes at different positions of the leaf.

Genetic control of leaf development

Leaf development is the result of coordination of cell division/expansion and differentiation and is affected by both genetic (internal) and environmental (external) regulatory factors (Tsukaya, 2004; Cookson et al., 2005; Gonzalez et al., 2012). At the molecular level, the transcriptome of rosette and folding leaves in Chinese cabbage was studied (Wang et al., 2012; Gao et al., 2017). This genome-wide analysis of gene transcript abundances suggested that environmental stimuli, like temperature, light, day length and carbohydrate levels, and the regulation of transcription factors, calcium and protein kinases are involved in regulating the leafy head formation. Multiple pathways and differentially expressed genes were marked for rosette leaf growth regulation. In their study transcript abundance of many regulators involved in normal leaf growth were identified which could not be separated from the genes that initiated the leafy head development. In chapter 3, non-heading pak choi (PC-024) rosette leaves were used as a control for transcriptome analysis of Chinese cabbage leaves using a *B. rapa* Agilent 60-mer microarray. Two Chinese cabbage genotypes (CC-A03 and CC-Z16) were used for this study, an early heading genotype (CC-Z16) that forms spherical heads and a late heading genotype forming cylindrical heads. Leaf samples were collected at defined developmental stages, from seedling, rosette to early heading and heading stages. Transcript abundance of a subset of the genes (40,879) showed differences in transcript abundance among genotypes or developmental stages. Weighted correlation network analysis (WGCNA) for differential transcript abundance according to Pearson's correlation resulted in the identification of 17 modules of co-regulated genes and analysis of variance (ANOVA) revealed the modules that were differentially expressed across genotype, developmental stage or genotype by developmental stage. For the modules with genes having differences in transcript abundance between the single pak choi genotype and the two Chinese cabbage genotypes, hierarchical clustering was performed. And correlation analysis resulted in a list of genes with most connections to other genes (the so called hub genes). Very interestingly, genes in certain annotation classes (protein, categories photosynthesis, cell and cell wall) were overrepresented in the top 20.

Over the last few years, the action of microRNAs (miRNAs) that regulate gene expression of several biological processes is the subject of active research (Eulalio et al., 2018). In the model plant *Arabidopsis*, miRNAs play a key role in leaf development (Pérez-Morales et al., 2017). In polarity establishment, the *TAS3-ta-siRNA-ARF3/ARF4* pathway plays an important role, in which the trans-acting

short-interfering RNA3 (*TAS3*) down-regulates the expression of auxin response factors 3 and 4 (*ARF3/ARF4*) to promote abaxial cell fate determination (Montgomery et al., 2008; Rodriguez et al., 2014). The *miR166-HD-ZIPIII* pathway, with *micro-RNA166* down-regulating homeodomain-leucine zipper III (*HD-ZIPIII*), specifies adaxial cell fate (McConnell et al., 2001). Furthermore, in the leaf maturation process, the *miR319*-regulated *TCP4* pathway plays a role, in which *micro-RNA319* down-regulates *TEOCINTE BRANCHED1/CYCLOIDEA/PCF4* (*TCP4*) to determine the final leaf size through control of cell proliferation (Schommer et al., 2014). In *B. rapa*, the roles of miRNAs in Chinese cabbage leafy head development are reported in many studies (Wang et al., 2013; Wang et al., 2014). The miRNAs target genes in *B. rapa* belong to the same families as in *Arabidopsis*, including many transcription factors, such as *APETALA2* (*AP2*), *Teosinte branched1/Cycloidea/PCF* (*TCP*), *Myeloblastosis* (*MYB*), *Basic region/leucine zipper motif* (*bZIP*), *Auxin response factors* (*ARF*), and *Auxin/indole-3-acetic acid* (*AUX/IAA*) genes (Wang et al., 2011; Wang et al., 2013). Among them, *miRNA390a-regulated TCP4* has been reported to modulate Chinese cabbage leafy head shape through control of cell division in leaves (Mao et al., 2014). In a recent paper, association between leaf curvature and miRNA expression was investigated (Ren et al., 2018). *miR166* expression was associated with downward curving of leaves and *miR319a* with wavy margins. Very interestingly the authors also showed that overexpression of *Brp-MIR166g-1* caused rosette leaves to change from horizontal to downward curving, while folding leaves changed from upward curving to flat which resulted in a decrease of the head size. *BrARF3.1*, an AUXIN RESPONSE FACTOR that is identified as a domestication gene for head formation, is also regulated by small RNAs (Ta-siRNA). This gene was studied in chapter 5.

How does allelic variation in the domestication gene Auxin Response Factor 3 (*ARF3*) affect the leafy head trait?

Disturbance of ad/abaxial leaf development seems to be associated to leafy head formation, as many of the genes involved are candidate genes in domestication signals for leafy head formation in *B. rapa* or *B. oleracea*, such as *KAN*, *HD-ZIP*, *ARF3/4* and *RDR6* (Cheng et al., 2016; Liang et al., 2016). Allelic variation of *BrARF3.1* was associated with heading Chinese cabbage when analyzing *B. rapa* re-sequence data of a large collection of heading (H) and non-heading (NH) accessions and this association was further confirmed in a larger collection of *B. rapa* accessions (Cheng et al., 2016).

In our study, over-expression (OE) of the non-heading *BrARF3.1* allele (*BrARF3.1_NH*) in *Arabidopsis Col-0* caused leaf downward curvature, upwards curving of the petioles and increased the lateral root lengths, while plants overexpressing the heading allele of *BrARF3.1* (*BrARF3.1_H*) resembled the non-transformed control. It is worth mentioning that under certain environmental conditions, the *BrARF3.1_H* over-expression plants showed mild phenotypes, intermediate between wild type and OE non-heading *BrARF3.1* plants. Heading and non-heading alleles differed in only few amino acids, however the C-terminal amino acid change by itself caused this phenotype when the other amino acid changes resembled the non-heading allele. We cloned the *BrARF3.1_NH* from pak choi and *BrARF3.1_H* from Chinese cabbage. As Chinese cabbage is domesticated from non-heading *B. rapa* forms, likely from pak choi, we assumed that the *BrARF3.1_H* is the domesticated gene. During plant domestication, most plants gain traits through loss of function of the genes during selection (Doebley et al., 2006; Purugganan & Fuller, 2009; Ishimaru et al., 2013) and as a consequence most domestication events are recessive mutations. We thus hypothesized that the *BrARF3.1* heading allele was recessive, which was supported by the absence of a phenotype for the *H-ARF3.1* over-expression plants and the clear phenotype of the non-heading *NH-BrARF3.1* allele OE plants. However, the Y2H protein-protein results in this study showed that the domesticated *BrARF3.1_H* allele retains interaction with BrYAB1 and AtKAN4, while this interaction is absent for the *NH* allele. This suggests that the *BrARF3.1* heading allele is functional. Lack of a phenotype in OE *Col-0* plants can be due to feedback regulation by the Ta-si-RNA system (Hunter et al., 2006; Fahlgren et al., 2006). The clear phenotype by the OE *NH-BrARF3.1* allele is likely caused by disturbed interactions with other ad/abaxial proteins that together define leaf ad/abaxial polarity. To know which allele is dominant, we need to cross the two OE lines and phenotype the resulting F1 plants.

In *Arabidopsis*, *KAN*, *YAB* and *ARF3/4* form a feedback loop controlling ad/abaxial leaf development. *BrKAN2.1* is another candidate domestication gene highly associated with leafy head formation in *B. rapa*, with a “CAG” insertion (exon) in the heading allele of Chinese cabbage (Cheng et al., 2016). We checked the effect of this allelic variation on interactions of other proteins with *BrKAN2.1*. We examined interactions of both *BrKAN2.1* alleles (*BrKAN2.1_H*, *BrKAN2.1_NH*) and its paralogue *BrKAN2.2* by cloning them into the Y2H AD vector with the *BrARF3.1* alleles in the BD vector (*BrARF3.1_H* and *BrARF3.1_NH* in BD) and found no differential interaction. As the BD-*BrKAN2* genes alone showed very strong auto-activation in the used yeast system, we could not check the

BrKAN2.1_H and BrKAN2.1_NH interaction in BD with the library of other proteins in AD. However, we still find it important to screen interactions of the different BrKAN2.1 alleles to improve understanding of their influence in plant domestication.

Another possible explanation for the different phenotypes between *BrARF3.1_NH* and *BrARF3.1_H* over-expression plants is the regulation of micro RNAs. As a siRNAs target, *ARF3* transcript abundance is affected by both the mi390 and *TAS3* tasi-RNA pathways that regulate leaf development and lateral root growth in *Arabidopsis* (Fahlgren et al., 2006; Marin et al., 2010). *TAS3* transcripts are cleaved by the *miR390*-*ARGONAUTE7* (*AGO7*) complex and double stranded RNA is made by *RNA-DEPENDENT RNA POLYMERASE6* (*RDR6*) and *SUPPRESSOR OF GENE SILENCING3* (*SGS3*). Then *DICERLIKE4* (*DCL4*) and *DOUBLE-STRANDED RNA BINDING PROTEIN 4* (*DRB4*) dice the double stranded RNA into *ta-siARFs* (Figure 1).

Figure 1. *TAS3*–*ta*-siRNA–*ARFs* pathway in plant. (A) Schematic diagram of the *TAS3* pathway. *miRNA390* loaded *AGO7* cleaves the *TAS3* precursor RNA. Then *RDR6* and *SGS3* convert the cleavage product into a double-stranded RNA. The double stranded RNA diced into *tasiARFs* by *DCL4* and *DRB4*. *tasiARFs* inhibit *ARF2*, *ARF3*, and *ARF4* mRNA expression. (B) Mobile patterns for *ta*-siRNA–*ARF* and *ARF3*. *ta*-siRNA–*ARF* biogenesis at the two adaxial cell layers (dark brown) and form a gradient across the leaf. *ARF3* expresses on leaf abaxial side and is reduced by *ta*-siRNA–*ARF*.

The *ta-siARFs* biogenesis takes place only at the two upper adaxial cell layers of developing leaves and *ta-siARFs* move to the abaxial cell layers to form a gradient

across the leaf (in maize) (Benkovics & Timmermans, 2014). The accumulation of *ta-siARFs* reduces the activity of *ARF2*, *ARF3* and *ARF4* through degradation of their transcripts (Williams et al., 2005). Interestingly 35S::*TAS3* plants had longer lateral roots, whereas mutations in *miRNA390*, *RDR6* and *DCL4* caused plants to have shorter roots than the wild type (Marin et al., 2010). Interestingly, transcript abundance of *AtRDR6* in our *BrARF3.1_NH* overexpression Col-0 *Arabidopsis* plants with longer lateral roots was higher than in control or *BrARF-3_H* OE plants with wild type root morphology. These results suggest that the interplay between *TAS3* ta-siRNAs and their target *BrARF3.1* are changed between the plants overexpressing heading or non-heading alleles. However, we did not check the transcript levels for the other genes and microRNAs, which belong to *TAS3-ta-siRNA-ARFs* pathway. This should be done in future studies.

ARF3 response to auxin

Many factors influence leafy head formation, such as temperature, light intensity, and auxin (Ito, 1957; He et al., 2000). Among them, auxin is the most important component: auxin controls plant cell division and elongation and its distribution within leaves results in curling of leaves (Sandalio et al., 2016); regulates the balance between adaxial and abaxial leaf cell growth (Braun et al., 2008); explains the epinasty/hypnasty by AUX/IAA-ARF signaling pathways and auxin signaling is regulated by miRNA (Khraiwesh et al., 2012).

ARFs transduce auxin signals during organ growth and development. At low auxin concentration, ARFs are repressed through interaction with Aux/IAA family genes via domains III and IV. At high auxin concentrations, ARFs are released from inhibition by degradation of Aux/IAA proteins (Weijers et al., 2005). Unlike most ARFs, *ARF3* lacks the C-terminal domains III and IV and it was suggested that it likely cannot directly respond to auxin (Finet et al., 2012). However, the finding that *ARF3* is able to respond to auxin was recently reported by Simonini et al., (2016). In their study, *ARF3*-TF interactions were directly affected by IAA. Similar results were found in *B. rapa* (chapter 5): BrYAB1 and BrKAN2 interactions with BrARF3.1 decrease when auxin concentrations are increased. Remarkably, *ARF3*, *YABBY*'s and *KANADI*'s form a feedback loop regulating leaf growth. Our results demonstrate that Auxin affects the interaction between leaf ad/adaxial developmental proteins. In addition, ta-siRNA is involved in auxin signaling. Therefore, we propose to study how phytohormones influence *ARF3* functioning in planta and to study the distribution of auxin and the role of auxin genes in leafy head formation.

Conclusions and final remarks

How leafy heads form is a new research topic in recent years. Better understanding of the leafy head associated traits is a prerequisite for genetic studies. Our phenotypic investigations revealed that leafy head formation is related to the phenotype of leaves during earlier developmental stages. I propose to quantify both leaf and whole plant phenotypes during Chinese cabbage plant development and to use these parameters in genetic association studies to reveal causal genes. I showed clear effects of allelic variation of *BrARF3.1* in both Y2H and OE studies, which convinced us of its role in leafy head formation. However, the protein interactions need to be confirmed *in planta* using bimolecular fluorescence complementation (BiFC) assays in tobacco leaves. In addition, I propose to tag the *BrARF3.1* alleles with GFP to be able to isolate intact protein complexes by an immunoprecipitation assay followed by LCMS (Liquid Chromatography Mass Spectrometry) to identify unknown members of the complex (interacting proteins). At this moment the GFP fusion proteins did not show a clear phenotype when over expressed in *A. thaliana*, so I did not yet conduct these experiments. Besides *BrARF3.1*, several other candidate domestication genes were identified. Allelic profiling of their haplotypes in a large collection of accessions with different heading phenotypes combined with mathematical parameters for leaf and leafy head traits can result in identification of genes involved in the leafy head trait. To study the gene expression network in developing *B. rapa* leaves, I isolated mRNA from leaf adaxial/abaxial cells obtained by laser dissection. The mRNA is now being sequenced and analysis will shed light on the *B. rapa* leaf primordia transcriptome. As miRNAs play important roles in leaf development, also their abundance in time and place needs to be analyzed. Functional studies of candidate genes in *B. rapa* are difficult as the production of transgenic plants is extremely ineffective. Improvement of the transformation system is thus needed for this research. An alternative is the use of CRISPR-Cas by direct RNA transfection to protoplasts, which is a system that now works in *B. oleracea* and should be tested for *B. rapa*.

Overall, studying the complicated leafy head trait in *B. rapa* Chinese cabbages with their complex genomes is challenging, however increasing genomic data sets (genome, transcriptome but also proteome and metabolome) together with improved phenotyping platforms and plant biotechnological tools will definitely advance our knowledge in this interesting domestication trait.

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Summary

The leafy head is the harvested organ of Chinese cabbage and is composed of a large number of tightly wrapped heading leaves surrounding the shoot apex. For both farmers and consumers, the Chinese cabbage leafy head trait has clear advantages, related to yield, stress resistance and storability. The leafy head is an obvious domestication trait, also found in cababges (*B. oleracea* ssp. *Capitata*), lettuce (*Lactuca sativa* L.) and radicchio of Treviso (*Cychorium intibus* L.). The formation of the leafy head is genetically determined, however affected by both internal and external factors. The developmental and genetic mechanisms underlying leafy head formation are however poorly understood. In recent years, scientists started to study this domestication trait, which resulted in identification of domestication signals and QTL for leafy head formation. In this thesis I link morphological observations (plant, tissue and cell level) to genetic regulation (by population genetic analysis, transcriptome analysis) and gene functional experiments to explain how leafy heads form in Chinese cabbage.

In chapter 2, we determined the relationship between variation in rosette-leaves and leafy heads by phenotyping 152 Chinese cabbage accessions. The rosette leaf width clearly was associated with heading -degree, -size and -shape. However, the rosette leaf petiole- and leaf blade length were not correlated with final heading degree in this collection. Moreover, the leaf number of the mature head is not correlated to heading degree nor head shape. Hereafter, to link genotypic data (molecular markers) and phenotypic data (head formation related trait measurements), QTL explaining heading and heading related traits were identified. In QTL analysis, two parental populations from a cross between non-heading pak choi and Chinese cabbage (Doubled haploid population and F2 population) were used. We found that QTLs for rosette leaf area length, and petiole length co-localized on LG A06 in both populations. And QTLs for rosette leaf width all co-located with heading degree QTL on LG A03 in the DH population and LGA04 in the F2 population. These results provided additional evidence for the correlation between rosette leaf width and heading degree. Candidate genes could be identified from the co-located QTL region. This relationship between the size and shape of rosette leaves that affect heading-degree, -size and -shape, is relevant for breeders that select for optimal quality of Chinese cabbage leafy heads.

In chapter 3, genome wide transcriptome analysis was performed in Chinese cabbages and pak choi young emerging central leaves during development. We compared two Chinese cabbages, which differed in earliness, leaf size, shape and

numbers and leafy head shapes, with one single non-heading pak choi. Cluster analysis of transcript abundance clearly showed that the cluster with transcript abundance of leaves from seedling stages of all genotypes together with all pak choi rosette time points is separated from the later developmental stages (rosette, early heading and heading) of both Chinese cabbages. By Weighted correlation network analysis (WGCNA) and hierarchical clustering using Euclidean distance, gene clusters with transcript abundance patterns distinguishing the two Chinese cabbages from the single pak choi were identified. We found that genes in the MapMan categories photosynthesis, Cell and Cell wall were highly enriched in these selected clusters. Genes in photosynthesis category were highly expressed during rosette and early heading in Chinese cabbages while low expressed in the heading inner leaves that are not exposed to light. Genes in Cell and Cell wall categories related to the differences in leaf blade growth between Chinese cabbage and pak choi, especially at the rosette stage. Overall, comparison of the transcriptome between leaves of two very different Chinese cabbages with pak choi during plant development allowed the identifications of specific gene categories associated with leafy head formation. We concluded that both leaf cellular structures in rosette leaf stages and photosynthesis during development should be studied to help us explain how Chinese cabbage heads form.

In order to better understand how seedling and rosette leaf anatomy relates to Chinese cabbage leafy head formation, we study the morphological differences at cellular level between heading and non-heading *B. rapa* pak choi and Chinese cabbage morphotypes in **chapter 4**. We phenotyped Chinese cabbage and pak choi seedling and rosette leaves at the cellular level to associate leaf morphologies to cell shapes, sizes, density and arrangements. We found that the tightly arrangement of young leaf primordia around the shoot apical meristems forming a larger angle between the developing vascular strands in Chinese cabbage differed from the arrangement in non-heading pak choi. We also concluded that the adaxial palisade and abaxial spongy parenchyma cell growth is closely related to leaf curvature. In Chinese cabbage, leaf growth is mainly caused by cell expansion, while in pak choi it is by cell division. Combined with these cellular level phenotypic analyses, the transcript abundance of cell division/expansion genes were profiled. The transcript abundance of *BrRCP4*, *BrARF2*, *BrARL.2* and *BrARL.3*, which relates to both cell division and expansion, differed between seedling and rosette leaves. Their transcript abundance also differed within leaves, between tip middle and base part in Chinese cabbage leaves only. The latter observation is likely associated with the leaf curvature of Chinese cabbage

leaves, while pak choi leaves are flat, which requires similar growth rates in all leaf positions.

In chapter 5, we study the function of leafy head formation associated gene, *BrARF3.1*. A single nucleotide polymorphism in the C-terminal domain of *BrARF3.1* associated with heading (G) and non-heading (C) phenotypes in *Brassica rapa* was investigated. Besides this single SNP we identified another ten SNPs located in the DNA binding domain, three SNPs in the Auxin response factor domain and two SNPs between these two regions when comparing the Chinese cabbage (CC-A03) *BrARF3.1_H* and pak choi (PC-024) *BrARF3.1_NH* alleles. This resulted in four amino acids (aa) changes in the *BrARF3.1* protein. Overexpression (OE) of the *BrARF3.1_NH* non-heading allele from pak choi in *Arabidopsis* wild type (Col-0) resulted in a strong phenotype, with small plants having thick and downward curling leaves and short roots with many long lateral roots. In contrast Col-0 plants overexpressing the *BrARF3.1_H* allele from Chinese cabbage resembled wild type plants. We tested whether the C-terminal amino acid change (Q to H) differentiated protein-protein interactions of Chinese cabbage and pak choi heading and non-heading *BrARF3.1* proteins respectively. *BrARF3.1_H* showed similar protein-protein interactions compared to *AtARF3*, with *KANADI*'s and *YABBY*'s, while *BrARF3.1_NH* did not interact with *AtYAB1*, *BrYAB1.1* and *BrYAB1.2* and weaker with *AtKAN4*. Chimeric constructs showed that the single Q to H amino acid change abolishes this interaction only when the other three variable amino acids in the Auxin response factor domain are from the non-heading allele. We also showed that the auxin IAA weakens or abolishes the protein interactions of *BrARF3*. These results show that the non-heading *BrARF3.1_NH* allele of pak choi is the mutant allele, which was unexpected as Chinese cabbage with its leafy head is assumed to be domesticated from pak choi.

Taken together, research described in this thesis links the morphological observations on leaf and leafy head development to molecular genetic results, both at the population and cellular levels. I summarize and discuss the most important results from this thesis in relation to published studies **in chapter 6** and provide suggestions for future studies on leafy heading in Chinese cabbage.

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XiaoXue Sun

孙晓雪

19 November 2018, Wageningen

About the author

XiaoXue Sun was born on December 7, 1988 in Liaoning, China. In 2011, she obtained her bachelor degree at the Academy of Agricultural Sciences at Northwest A&F University, Yangling Agricultural Hi-tech Industries Demonstration Zone, Shanxi, China. After receiving her BSc degree, she continued her master study specializing in Plant Breeding and Genetic Resources in Wageningen University & Research, the Netherlands. She graduated in 2013. In 2014, her PhD project started as a cooperation between Plant Breeding at Wageningen UR and Institute of Vegetables and Flowers (IVF) at the Chinese Academy of Agricultural Sciences (CAAS). This thesis presents the outcomes of her PhD research on “*Morphological and genetic characterization of the leafy head of Chinese cabbage (Brassica rapa)*”.

Sun, X., Bucher, J., Ji, Y., van Dijk, A. D., Immink, R. G., & Bonnema, G. (2018). Effect of ambient temperature fluctuation on the timing of the transition to the generative stage in cauliflower. *Environmental and Experimental Botany*.

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Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: XiaoXue Sun
Date: 19 November 2018
Group: Laboratory of Plant Breeding
University: Wageningen University & Research

1) Start-Up Phase	<i>date</i>
► First presentation of your project Functional Studies of Heading in <i>Brassica rapa</i>	30 Mar 2015
► Writing or rewriting a project proposal Functional studies of the Auxin Response Factor 3 (ARF3) to dissect its role in the heading phenotype of Chinese cabbages	Apr-Jun 2014
► Writing a review or book chapter	
► MSc courses	
► Laboratory use of isotopes	
<i>Subtotal Start-Up Phase</i>	
	7.5 *
2) Scientific Exposure	<i>date</i>
► EPS PhD student days EPS PhD Student Days 'Get2Gether', Soest, NL	29-30 Jan 2015
EPS PhD Student Days 'Get2Gether', Soest, NL	10-11 Apr 2017
► EPS theme symposia EPS Theme 4 Symposium 'Genome Biology', Wageningen, NL	03 Dec 2014
EPS Theme 1 Symposium 'Developmental Biology of Plants', Leiden, NL	08 Jan 2015
EPS Theme 3 Symposium 'Metabolism and Adaptation', Wageningen, NL	10 Feb 2015
EPS Theme 1 Symposium 'Developmental Biology of Plants', Leiden, NL	28 Feb 2017
► National meetings (e.g. Lunteren days) and other National Platforms Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	14-15 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	10-11 Apr 2017
► Seminars (series), workshops and symposia <i>Symposium:</i> Plant Breeding Research Day 2014, Wageningen, NL	30 Sep 2014
<i>Symposium:</i> EPS symposium 'Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding', Wageningen, NL	11 Dec 2014
<i>Symposium:</i> 'Wageningen Young Academy: Rewriting our genes?', Wageningen, NL	30 Sep 2016
<i>Symposium:</i> 1st 'WUromics: Technology- Driven Innovation for Plant Breeding', Wageningen, NL	15 Dec 2016
<i>Seminar:</i> Orgrun Mittelsten Scheidr, 'Genetics and epigenetics: a complex relationship'	19 Nov 2014
<i>Seminar:</i> Yves van de Peer, 'The evolutionary significance of gene and genome duplications'	03 Feb 2015
<i>Seminar:</i> Florian Schiestl, 'Evolution of floral signals in plants: mechanisms and consequence'	12 Mar 2015
<i>Seminar:</i> Jiming Jiang, 'Structure and evolution of centromeres: lessons learned from plants'	01 Apr 2015
<i>Seminar:</i> Xiaowu Wang, 'Subgenome Parallel Selection Drives Diversification and Convergent Morphotype Evolution in Brassica crops'	21 Oct 2015

<ul style="list-style-type: none"> ► Seminar plus ► International symposia and congresses BRASSICA 2016 (20th Crucifer Genetics Conference & 19th Australian Research Assembly on Brassicas), Melbourne, Australia BRASSICA 2018, St. Malo, France ► Presentations <i>Poster:</i> 'Effect of ambient temperature variation on curd initiation in cauliflower' - Annual Meeting 'Experimental Plant Sciences' <i>Talk:</i> 'Auxin Response Factor 3.1 plays a major role in the heading phenotype of Chinese cabbage (<i>B. rapa</i>)' - EPS Theme 1 Symposium <i>Talk:</i> 'A single amino acid change in Auxin Response Factors 3-1 and 4-1b play a major role in heading of Chinese cabbage in <i>B. rapa</i>' - BRASSICA 2016 <i>Talk:</i> 'How does allelic variation of the Brassica rapa domestication gene ARF3-1 affect leaf development' - BRASSICA 2018 ► IAB interview ► Excursions Company visit to Tomato world, Honselersdijk, NL Company visit to Bejo Zaden B.V., Warmenhuizen, NL 	03-06 Oct 2016 01-03 Jul 2018 13-14 Apr 2015 28 Feb 2017 05 Oct 2016 02 Jul 2018 24 Oct 2016 26 Sep 2018
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Subtotal Scientific Exposure

13.2 *

3) In-Depth Studies <ul style="list-style-type: none"> ► EPS courses or other PhD courses Course 'Systems Biology: Statistical Analysis of ~Omics Data', Wageningen, NL Course 'Transcription Factors and Transcriptional Regulation', Wageningen, NL Course 'JoinMap', Wageningen, NL ► Journal club ► Individual research training Training for 2 months in Hebei Agriculture University, China 	<u>date</u> 15-19 Dec 2014 12-14 Dec 2016 18 May 2016 Nov-Dec 2017
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Subtotal In-Depth Studies

5.8 *

4) Personal Development <ul style="list-style-type: none"> ► Skill training courses Second KNAW NWO PhD Event, Amsterdam, NL EPS Introduction Course, Wageningen, NL Course 'Scientific Writing', Wageningen, NL Course 'Information Literacy PhD including EndNote Introduction', Wageningen, NL Duch language course, Wageningen, NL Third KNAW NWO PhD event for Chinese PhDs, Amsterdam, NL ► Organisation of PhD students day, course or conference ► Membership of Board, Committee or PhD council 	<u>date</u> 05 Jun 2014 20 Jan 2015 Jan-Mar 2015 27-28 Oct 2015 Sep-Dec 2016 24 Nov 2016
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Subtotal Personal Development

4.4 *

TOTAL NUMBER OF CREDIT POINTS	30.9 *
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Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits.

* A credit represents a normative study load of 28 hours of study.

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Propositions

1. Sequencing of individual plants from populations to generate data to construct high density genetic maps should replace molecular markers like SNPs or SSRs. (this thesis)
2. Study of complex domestication traits to optimize crop yield needs a multidisciplinary approach. (this thesis)
3. Scientific research of topics for which few references are available challenges the researcher to be more creative.
4. Analysis of large datasets will only lead to new breakthrough insights when very specific questions are asked.
5. The replacement of paper books and journals by digital books, articles and webpages forms because of the sheer volume of them, a challenge to separate the wheat from the chaff.
6. Continuously working in the same environment might be good for your career, but not for science.
7. The value of a foreign diploma depreciates when domestic development rises.

Propositions belonging to the thesis entitled

“Morphological and genetic characterization of the leafy head of Chinese cabbage (*Brassica rapa*)”

Xiaoxue Sun
Wageningen, 19 November 2018