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WAGENINGEN, THE NETHERLANDS

Morphological and molecular characterization of heading traits in Chinese cabbage (Brassica rapa)

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2015/6/4

Morphological and molecular characterization of heading traits in Chinese cabbage (*Brassica rapa*).

Thesis for Master of Plant Sciences (MPS)

With specialization

In

Plant Breeding

By:

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Submitted to Wageningen UR Plant Breeding of Wageningen University and Research Centre (WUP)

Morphological and molecular characterization of heading traits in Chinese cabbage (*Brassica rapa*).

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May, 2015

Acknowledgements

This report was prepared as my Major Master Thesis of Plant Breeding in Plant Science of Wageningen University from September 2014 to March 2015. There have been a lot of people helping me during the whole project, without them this thesis might not have been finished. I greatly appreciate their help.

I would like to sincerely thank my supervisor Dr. Ir. Guusje Bonnema. Her intelligence, patience, passion, diligence and devotion to research and especially to innovative technologies are merits that I deeply admire. It has been a great honour to be her student. I am really thankful for her suggestions and encouragements. The patient guidance she always does helps me complete this thesis report and gives me lots of good comments on my writing skills.

I also want to show my big appreciation to my daily study advisor, Xiaoxue Sun. She is a very nice advisor. She was always there to support me technically and materially. Thanks for her help especially at the start of my project. Whether in the greenhouse or in the lab, she had taught me a lot of academic knowledge and experimental skills. Thanks for her inspiring me and providing such an interesting project for me to work on. I feel quite lucky to have worked with her.

In particular, a thank you to Johan Bucher who helped me takes care of our plant materials in the greenhouse, and also who has shown me great experimental skills in the lab. Your high academic attitude and humorous life style are very impressive. It is also happy and lucky for me to have worked with you. And I would like to express a heartfelt appreciation to all the people who in one way or another supported my master thesis in University.

Most of all, here I want to express my gratitude to my families, my dear mother, father and my wife. Without their supports and unconditional love, I cannot live gratefully or work happily.

Contents

Abstract

The project was performed to describe plant development in Chinese Cabbage (CC) and Pak Choi (PC). Special focus was on the leaf development and CC`s heading trait in comparison with PC`s non-heading trait. In addition, we investigated the expression levels of heading related genes through the plants` developmental stages. Three CC genotypes (Z16, A003 and CC168) and four PC genotypes (Glu024, PC175, PC184 and PC101) were planted in three blocks in the greenhouse. We defined three specific growth stages (rosette, folding and heading stage) in CC genotypes mainly by leaf angle and leaf edge curls. Heading in Z16 and A003 were characterized by erect growth of leaves and also leaf edge curling that form a head shape. The head shape of Z16 was round and for A003, head shape was cylindrical. We analysed the gene expression profiling from all seven genotypes using central leaf samples harvested weekly and also from expanded rosette, folding and heading leaves during the heading stage in CC genotypes. All the eight candidate genes (***Br-Arf3.1, Br-Arf4.1, Br-Kan2.1, Br-Axr1, Br-Brx.1, Br-Arr15, Br-Ga20ox1, Br-Rcd1.2***), which were identified in selected sweeps in Chinese cabbage, did not show an expression pattern related to the *B. rapa* heading sub species. Three Auxin related genes (***Br-Arf3.1, Br-Arf4.1, Br-Axr1***) were sequenced in the genotypes studied, and their expression patterns also did not relate to the allele compositions. Based on this study, we propose more detailed observation of leaf development, and the data in gene expression profiling can be used to select samples for further experiments.

Key words: *Brassica rapa*, Chinese cabbage, growth stages, leafy heading, ARF-gene expression

1 Introduction

1.1 Brassicaceae

The Brassicaceae is a medium-sized and economically important family of flowering plants (Angiosperms), which includes approximately 340 genera and more than 3350 species (Hall, Fiebig et al. 2002). This family includes the genus Brassica, which contains many important crop vegetables, such as *Brassica rapa* (Chinese cabbage, Pak Choi and turnip), *Brassica oleracea* (broccoli, cabbage, cauliflower), oilseed crops (*Brassica napus*, *Brassica rapa*, *Brassica juncea* and *Brassica carinata*) and condiments (Fahey and Talalay 1995).

Cultivated Brassicas are represented by six *Brassica* species. The genetic relationship between six major species is described by the well know triangle of U (Nagaharu 1935) (Figure 1). The three diploid species *Brassica rapa* (also known as *Brassica campestris*, A genome), *Brassica nigra* (B genome) and *Brassica oleracea* (C genome) formed the amphidiploid species *Brassica juncea* (A and B genomes), *Brassica napus* (A and C genomes) and *Brassica carinata* (B and C genomes) by hybridization (Wang, Wang et al. 2011).

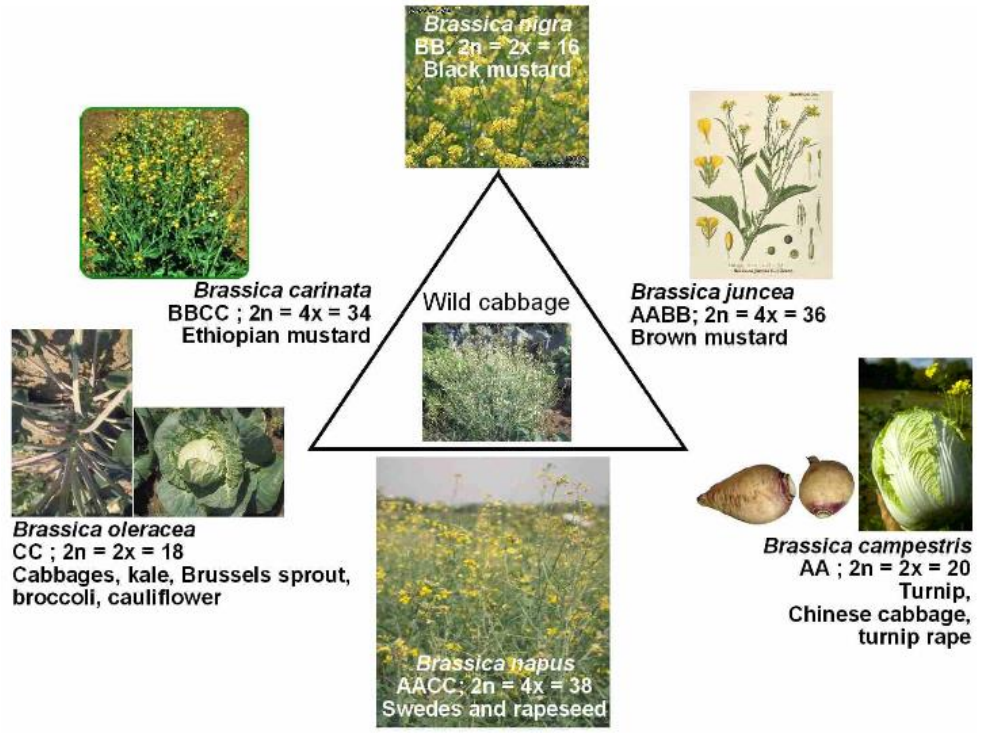


Figure 1 Triangle of U describes the genetic relationships between the three amphidiploid species (*B. carinata*, *B. napus* and *B. juncea*) and their diploid ancestral species (*B. nigra*, *B. oleracea* and *B.rapa*).

1.1.1 *Brassica rapa*

Cultivated subspecies of *B. rapa* likely originated independently in two different centers—Europe and Asia. In the European center, turnip and turnip rape (*oleiferous* forms) are the dominating forms (Reiner, Holzner et al. 1995). Leafy vegetables such as Chinese cabbage, Pak choi and Narinosa probably have been domesticated first in China, East Asia. And China is also the center of origin of Chinese turnip rape (ssp *oleifera*) (Li 1981), which is a unique turnip rape (oil type). These crops

provide collectively 12% of the world's edible vegetable oil production (Labana, Banga et al. 1993). Other accessions of *B. rapa* most likely derived from different morphological types in the two centers of origin and subsequently evolved separately (Zhao, Wang et al. 2005).

Based firstly on the organs used and secondly on their morphological appearance, a number of major species can be distinguished with their sub-species names giving in the past (Diederichsen 2001). These include oil seed types (ssp. *oleifera*), the turnip types (ssp. *rapa*) grown for their swollen stem basis, and a large and diverse group cultivated for their leaves. In these leafy vegetables several subgroups can be clearly distinguished. The Chinese cabbage group (ssp. *pekinensis*) is characterized by large leaves with a wrinkled surface, a pale-green colour, large white midribs and heads of different shapes. Pak choi (ssp. *chinensis*) does not form a head and has darker green and smooth leaves with a pronounced white midrib (Zhao, Wang et al. 2005). Three leafy type *B.rapa* varieties and one turnip type were described by (Van Wyk 2005) shown below (Figure 2).

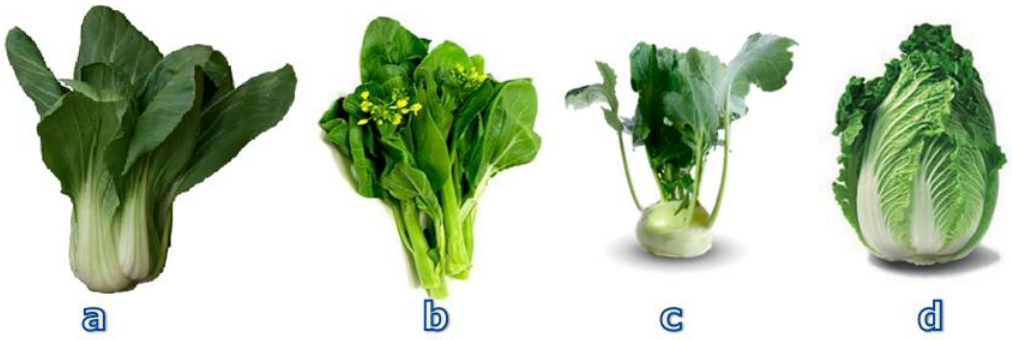


Figure 2 Pictures of leafy *B. rapa* ssp. a: *chinensis (Pak choi)*; b: *parachinensis (Caixin)*; c: *rapa (Turnip);* d: *pekinensis (Chinese cabbage).*

a: ssp. *chinensis*, which includes Pak choi, Bok choi, and Tat soi. These genotypes originated in Southeast Asia and have been cultivated in China and Japan for a long time, where they are also widely naturalized. These genotypes form compact clusters of petioles that are not as densely packed as in cabbage heads. Their fleshy green or white petioles and leafy greens are used from the whole young plants, either cooked in soups, stir-fries or salted and fermented into a can known as Pak choi pickle.

b: ssp. *parachinensis*, Chinese flowering cabbage (Caixin, sometimes called Chinese broccoli, although this name often refers to the related *B. olearacea* var. *alboglabra*), which has elongated flowering shoots and fleshy midribs leaves that do not form into tight clusters or heads. Along with their leaves and buds, the flowering stems are pickled or prepared as a cooked vegetable in some Asian dishes.

c: ssp. *rapa*, turnips, which have a fleshy, globe-shaped root. These genotypes described in Roman accounts dating to 400 B.C. as one of the oldest cultivated root crops. And they are still widely used in Europe and western China, prepared raw or cooked in soups, sautés, and stews. The young leaves are also used as cooked greens.

d: ssp. *pekinensis*, napa or celery cabbage (sometimes known as Peking cabbage, most common as Chinese Cabbage), which may either form simply a loose cluster of fleshy midribs leaves, or may have

a dense head of flat-midrib leaves. These varieties have a long history of cultivation in East Asia, where they are used in soups and as a cooked vegetable.

1.2 Heading Chinese cabbage

The high yield and good storability of Chinese Cabbage makes it as an economically important vegetable worldwide (Liu, Zhang et al. 2014).

1.2.1 Morphological characterization of Chinese cabbage

Growth stages of CC for crop and seed production purposes: (1) emergence stage; (2) seedling stage; (3) rosette stage; (4) heading stage; (5) flowering stage; (6) silique and seed stage (Opena, Kuo et al. 1988). Increasing study focus on the vegetative growth of Chinese Cabbage, which goes through four stages: (1) seedling stage; (2) rosette stage; (3) folding stage; (4) heading stage, characterized respectively with the apparent morphological markers of leaf (Chen 1984).

In the previous research (Opena, Kuo et al. 1988), the leaf development at the seedling stage: two true leaves develop between the fully extended cotyledons and there are normally five leaves in two whorls among early-maturing varieties or eight leaves in three whorls among late-maturing maturing ones. For rosette stage: the leaves are fully expanded into rosette in more or less horizontal position and near the soil surface. There is no folding stage defined inside, next is heading stage: heading starts at about 12^{th} to 13^{th} leaf stage for early-maturing varieties and 24^{th} to 25^{th} for late-maturing ones when the youngest, innermost leaves begin to incurve and touch at their tips.

Folding stage is added into the vegetative growth stages of CC in the later paper (Yu, Peng et al. 2000). At seedling stage, primary and juvenile leaves are differentiated and grow; at rosette stage, plant forms rosette by extending curving-inward leaves; upon entering the folding stage, the folding leaves are differentiated; at heading stage, the heading leaves become bigger and fold into a head, with less space for the inner leaves. Juvenile leaves, rosette leaves, folding leaves and heading leaves are four types of leaves that differ in shape, size, colour and physiological function.

Rosette leaves will form a “frame” which functioned as photosynthetic source organ during plant growth and development. Heading stage is the last vegetative stage of Chinese cabbage, the head leaves surrounding the shoot apexes are tight enough to form heads or hearts thereby becoming storage organ for essential nutrients (Ge, Ramchiary et al. 2011). The initiation and developmental process of the leafy head may be influenced by many factors, including the uneven distribution of *Auxin* levels in the leaves, temperature, weak light, short days and the carbohydrate nutrition level (Ito 1957).

Different Chinese cabbage accessions have different kinds of heading traits. Leafy heads can be divided into four shapes: round, oblong, cylindrical, and cone like (Figure 3). The two most common shapes are round and cylindrical (Mao, Wu et al. 2014).

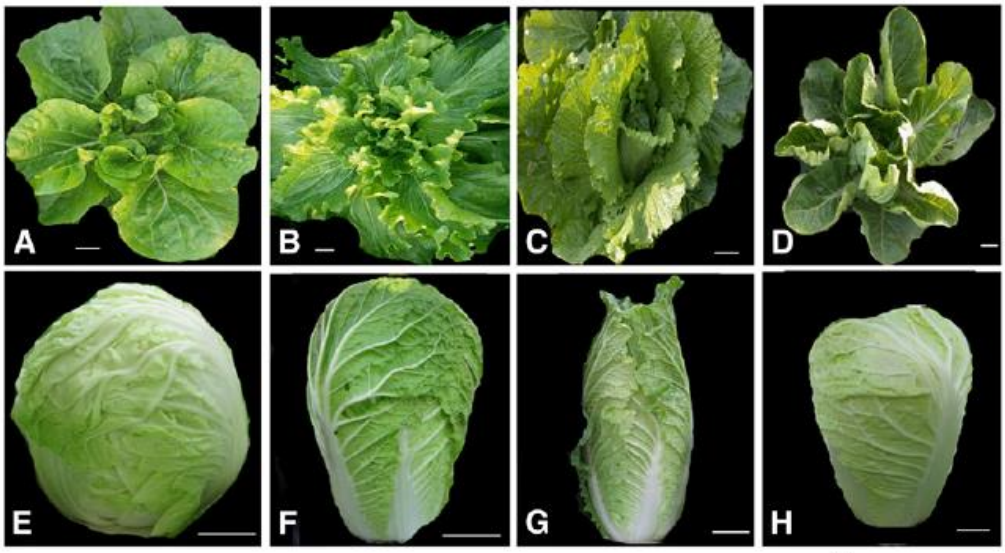


Figure 3 The plants of RILs with round (A), oblong (B), cylindrical (C), and cone-like (D) heads at the rosette stage. E to H, The round (E), oblong (F), cylindrical (G), and cone-like (H) shape heads (Mao, Wu et al. 2014).

1.2.2 Genetic control of heading traits in Chinese cabbage

The understanding of the leafy head development at a molecular level will greatly facilitate the genetic improvement of the Chinese Cabbage yield, its nutritional value and the quality of its appearance (Wang, Li et al. 2012).Along with the high speed of development of sequencing technology, genome sequencing of *Brassica rapa* was initiated as a multinational collaboration between China, the UK, Korea, Canada, Australia, and Japan since 2008 (http://brassica.bbsrc.ac.uk/), and later using Illumina technology accelerated by the group of Wang at IVF-CAAS. It is estimated that the Chinese Cabbage genome contains 46000 genes, whereas 27379 genes are currently believed to exist in the Arabidopsis genome (TAIR9 information: http://www.Arabidopsis.org/) (Hong, Kwon et al. 2008).

There are collaborations between WUR Brassica group and group IVF CAAS. Many *B. rapa* and *B.oleracea* are sequenced. And from the comparison between heading and non-heading accessions in *B.rapa*, identification of selection areas was resulted. Gene content was carefully investigated. Some molecular pathways were selected, like Auxin, Cytokinin (CK), Gibberellic acid (GA) etc. Allelic variations in genes ***Br-ARF3.1***, ***Br-ARF4.1*** *and* ***Br-AXR1*** are speculated that involved in the heading traits formation. From the observation of 806 *B.rapa* accessions (Figure 4), they found the ***ARF3*** gene with the G allele is associated with CC (318/356), while this association is less strong in PC (325 out of 440 have C allele). So there is no strict correlation, and not all CC have the CC alleles for these genes. The heading is a quantitative trait clearly.

Figure 4 Overview of three genes` allele differences in 806 *B.rapa* accessions by IVF. These accessions mainly divide into two categories, heading and non-heading. Same traits accessions can have different genotypes for one gene.

In the model plant Arabidopsis, phytohormones like Auxin, CK, GA, Jasmonic acid (JA) and Salicylic acid (SA) and other molecular networks play important role in controlling the exit from the stem cell maintenance in the shoot apical meristem (SAM), like leaf initiation, polarity, cytoplasmic growth, cell division, endoreduplication, transition phase, cell expansion and differentiation (Kalve, De Vos et al. 2014) (Figure 5). These pathways effects the leaf development, consequently they may also regulate the heading traits in some respects.

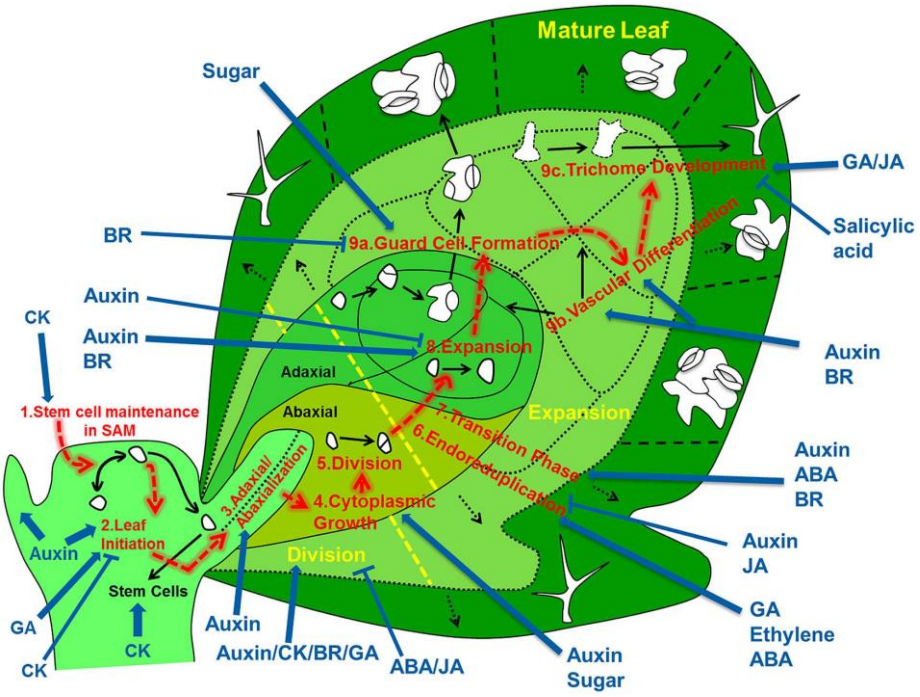


Figure 5 Overview of the regulatory processes that determine the development of a leaf. The developmental path of cells is indicated with red arrows, key regulatory processes are numbered and indicated and regulation of these processes by phytohormones/sugar is shown by blue arrows (pointed and T shaped arrows indicate positive and negative regulation, respectively) (Kalve, De Vos et al. 2014).

a. Auxin mediated signalling pathway related genes

Auxin is one of the plant hormones that affect the expression of genes involved in developmental processes at the cellular, tissue, and organ levels (Mun, Yu et al. 2012). And *Auxin Response Factor* ***(ARF)*** is one of the plant protein families well known for their role in Auxin-mediated responses. It has been reported that the ***ARF*** proteins are encoded by a larger gene family in *Arabidopsis thaliana* (Hagen and Guilfoyle 2002). Mutation of *ARF* genes ***AtARF3*** and ***AtARF4***, resulted in abnormal development of floral organs and leaves (Finet, Fourquin et al. 2010). A similar role in leaf morphology may affect leaf folding and heading in *B. rapa*. Another member of the ***KANADI*** gene family in *A. thaliana* regulate abaxial identity and laminar growth of lateral organs (Pekker, Alvarez et al. 2005). ***KAN2.1***, together with ***KAN1*** are primary determinants of abaxial cell fate (Eshed, Baum et al. 2001). And an early report of *Auxin Resistant 1* (***AXR1***) alleles suggested that ***AXR1*** mutations mainly affect cell numbers in relation to leaf development (Lincoln, Britton et al. 1990). However, another report mentioned that ***AXR1*** mutations affect cell number and cell size, irrespective of cell type as well (Horiguchi, Fujikura et al. 2006).

b. Cytokinin mediated signalling pathway related genes

Cytokinin is a plant hormone that plays positive and negative regulatory roles in many aspects of plant growth and development (Riefler, Novak et al. 2006). And analysis of cytokinins suggested that they are important regulatory factor of plant meristem activity and morphogenesis, with opposing roles in shoots and roots (Werner, Motyka et al. 2001). The result suggested that ***BRX*** promotes Arabidopsis root growth and shoot growth, this gene family also gain-off-function results in epinastic leaf growth (Beuchat, Scacchi et al. 2010). Another report showed that Auxin and cytokinin signalling converge on ***ARR15*** in the central zone of meristem during the development of shoot apical meristem (Su, Liu et al. 2011).

c. Gibberellic acid mediated signalling pathway related genes

Gibberellic acids (GAs) are a class of tetracyclic diterpenoid carboxylic acids, members of which function as hormones in many land plant lineages(Hirano, Nakajima et al. 2007). GAs regulate multiple aspects of plant growth and development, including reproduction (Fleet and Sun 2005). Based on the research result, ***GA20ox1*** has significant effects on floral organ growth (Plackett, Powers et al. 2012) and causes an enlargement of leaves when ectopically expressed in Arabidopsis (Huang, Raman et al. 1998).

d. Jasmonic acid mediated signalling pathway related genes

Recent molecular genetic studies confirmed the involvement of JA both in developmental and defence-related processes (Xie, Feys et al. 1998). It was shown that JA is a class of plant growth regulator that plays pervasive roles in several aspects of plant development, including seed germination, pollen development, responses to mechanical and insect wounding, pathogen infection and drought stress (Schaller 2001). There was also evidence to support a role of JA in Arabidopsis leaf senescence (He, Fukushige et al. 2002). *Radical-induced cell death1 (**RCD1**)* is involved in the jasmonic acid mediated signalling pathway (Overmyer, Tuominen et al. 2000), and another conclusion suggested that ***RCD1*** gene could be an important genetic resource to improve plant function against various stresses (Fujibe, Saji et al. 2004).

1.3 Research goals

This thesis mainly focus on two research questions, the first one is to describe in detail the phenotype differences during plant development between Chinese cabbage and Pak Choi such as leaf growth, heading development stages. The second question is whether and at which developmental stage heading related genes express differently between CC and PC.

In order to answer the first question, 3 CC genotypes and 4 PC genotypes were grown in uniform conditions. Then we made observations at regular intervals. Methods like counting the leaf numbers, checking the leaves` smoothness, leaf growth angle and colours were used in the greenhouse`s experiment. Through this information, research goals for the first question can be: 1. Descriptions of different leaf phenotypes for these genotypes between and within CC and PC; 2. Definitions of different growth stages related to heading in CC genotypes.

The second question in this study can be answered by profiling the expression of heading related genes. Quantitative real time PCR experiments were performed for both heading CC and non-heading PC. Through the candidate genes` relative expression levels, these research goals for the second question can be: 1. Identification of expression patterns in central young leaves at weekly intervals between subspecies CC, PC and related gene alleles; 2. Identification of expression patterns between different growth stages for each genotype in CC and PC; 3. Identification of expression patterns in expanded leaves between leaf types and positions in CC`s heading stage.

2 Materials and methods

2.1 Plant materials and growing conditions

Three heading Chinese cabbage DH (doubled haploid) genotypes (Z16, A003, CC168) and four non-heading Pak choi DH genotypes (glu024, PC101, PC175, PC184) were used in this project.

All the plants of these 7 genotypes were germinated on seeding soil (Lentse zaai en stekgrond) for one week and then transplanted into 17 cm or 21cm pots (soil: Lentse potgrond nummer 4) in Unifarm Nergena, Wageningen UR (51˚59′11″N latitude, 05˚39′52″E longitude) . The temperature was 18 ºC in the day and 16 ºC at night, the humidity was set at 75% in a day time. A dripping system was used for watering and a channel was used as a gutter system in the greenhouse. The plants were grown under long day conditions (16h photoperiod including 8h lighting supplementary) for 8-13 weeks. These plants were fertilized once a week and the rest of the week water was supplied.

The number of seedlings for each CC genotype was 60 including 50 transplanted into big pots (21cm diameter) and 10 into small pots (17cm diameter). The number of seedlings for control group PC is 25 for each genotype, 15 in big pots and 10 in small pots respectively (Table 1).

Table 1 Quantitative distribution of per genotype in CC and PC

	Chinese Cabbage (per genotype)	Pak Choi (per genotype)
The amount of seedlings	60	25
Seedlings transplant into Big pots	50	15
Seedlings transplant into Small pots	10	10

2.2 Greenhouse setting

Due to the sun movement during the day time, there is always a sunshine gradient decreasing from the window side (South) to the door at the corridor side. This asymmetrical sunshine distribution may affect the plants` growth at different positions. Block designs were considered to be able to take this difference into account. The block design included 3 main blocks (1, 2, 3) perpendicular to the sun gradient and 3 subblocks (A, B, C) for each main block in this compartment (Figure 6). All the plant materials, both for small pots (17cm width) and big pots (21cm width), were randomly arranged over each subblock then labelled with their positions (the detail information of labels shown in the appendix). The three main blocks were positioned from the window (south) to the door (north). And every main block was divided into three subblocks arranged from east to west.

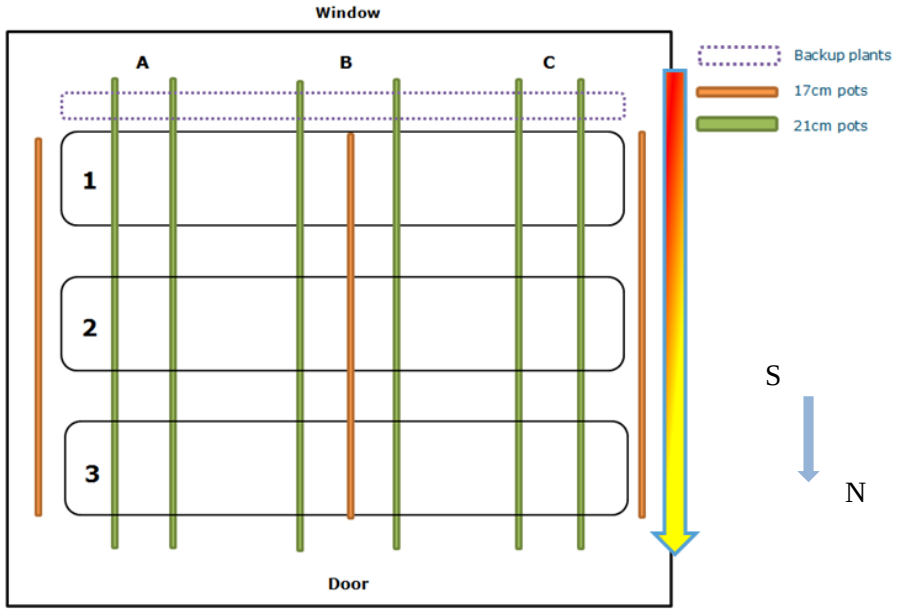


Figure 6 Lay out of the greenhouse compartment: block design included 3 main blocks and 3 subblocks for each main block. the orange lines show the small pots` (17cm width) positions in the greenhouse, and the green lines show the big pots` (21cm width) positions

Table 2 The number of all plants for each genotype in the greenhouse and their quantitative distribution.

		Small pots` block design										Big pots` block design									
Accession	Main blocks	Block 1			Block 2			Block 3			backup	Block 1			Block 2			Block 3			backup
	Subblocks	A	B	C	A	B	C	A	B	C		A	B	C	A	B	C	A	B	C	
CC168		1	1	1	1	1	1	1	1	1	1	5	5	5	5	5	5	5	5	5	1
Z16		1	1	1	1	1	1	1	1	1	1	5	5	5	5	5	5	5	5	5	1
A003		1	1	1	1	1	1	1	1	1	1	5	5	5	5	5	5	5	5	5	1
Glu024		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	6
PC101		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	6
PC175		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	6
PC184		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	6

The plant materials in small pots (17cm width) were placed in the mid and both sides of the compartment. Every subblock contained 7 plants including the 3 CC genotypes and 4 PC genotypes. And these 7 plant materials were randomly placed in subblock (Figure 7).

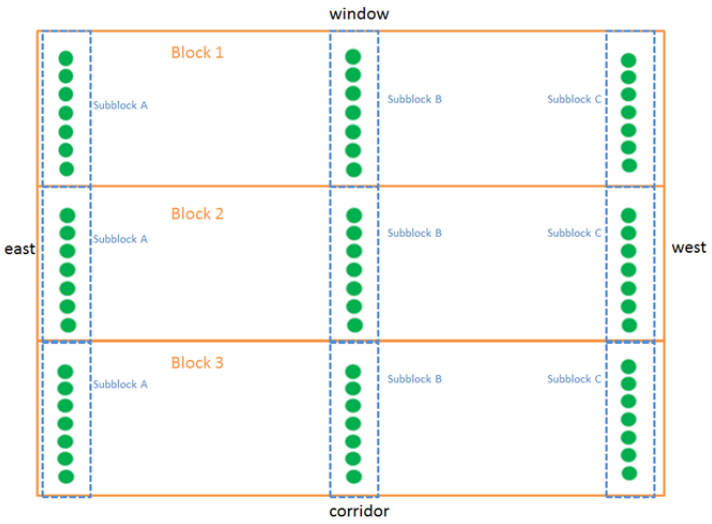


Figure 7 Layout of the small pots` block design in the greenhouse compartment.

As Figure 8 shows, each main block contained three subblock A, B and C. In every subblock, 5 seedlings of each CC accession were planted in the big pots (21cm width) and 1 seedling of each PC accession as well. These 19 plants were randomly placed in this subblock. Totally, there were 19 plants in each subblock and thus 57 in each main block.

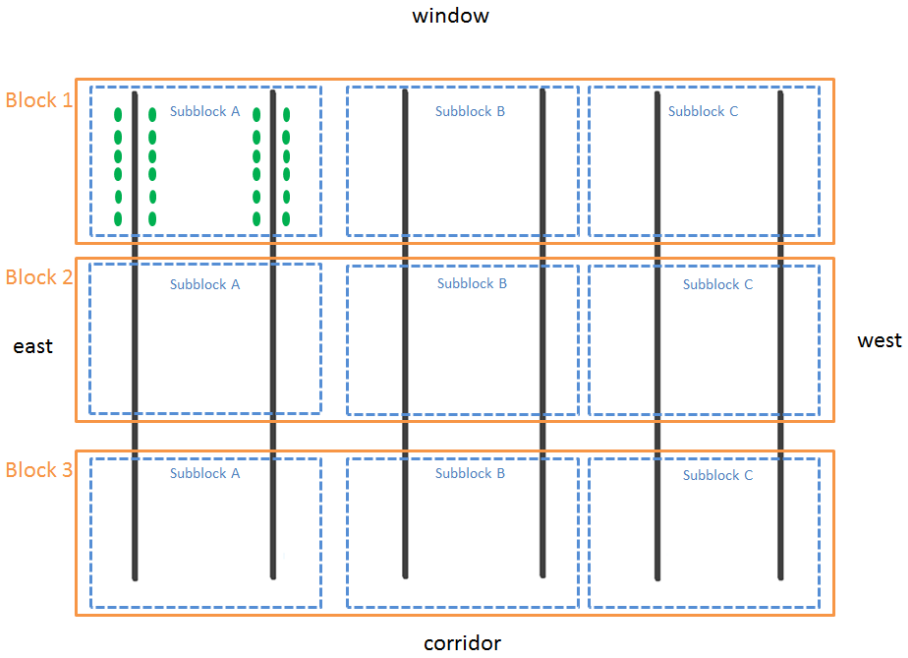


Figure 8 Layout of the big pots` block design in the greenhouse compartment.

2.3 Phenotyping methods

2.3.1 Leaf numbers counting

From the second week on this project in the greenhouse, leaves were counted when sampling plant material. The counting started from the outside oldest leaf to youngest leaf of 4-5 cm in the central part of the plant. As different genotypes had different sampling time length due to their onset of flowering, the number of weeks of counting was different for different genotypes (Table 3). Non-heading PC genotypes Glu024, PC175, PC184 were counted till the 8th week and PC101 till 9th week. The leaves of heading CC genotype A003 were still counted until the 12th week of this project in the greenhouse, Z16 and CC168 were evaluated till 8th week.

Table 3 The number of weeks that leaf numbers were counted for CC and PC genotypes.

The time length of the experiment in the greenhouse	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13
Z16													
CC168													
Glu024													
PC175													
PC184													
A003													
PC101													

2.3.2 Leaf phenotypes observation

During several weeks` experiment in the greenhouse, the leaves` phenotypes in different CC and PC genotypes were carefully observed. The leaf shape, leaf surface, leaf colour and leaf shape were marked. The leaf shape was evaluated like round shape, oval shape or torpedo shape. Leaf surface was evaluated in levels like smooth or wrinkles. And leaf colour was marked from yellow to dark green. The leaf shape was divided into two parts including leaf angle by approximately leaf growth angle comparing with the horizontal line and leaf edge curl by inward curl or outward curl.

2.3.3 Developmental stages observation

Different developmental stages of the plants were recorded by taking photos every Thursday at 9 am. This method started from the second week of the experiment in the greenhouse till the flowering stage of each genotype. As different genotypes had different time length of developmental stages, the time length of observations per genotype differed as well (Table 3). Pictures of one consistent plant/genotype were taken for all the block/subblock combinations (so 9 plants selected per genotype for pictures). These photos were taken from top view and side view respectively, the best one per genotype was taken photos with black background (Figure 9 The best plant material of each phenotype was selected and taken photos weekly both from the top view and side view with black background.). For CC genotypes, three specific growth stages (rosette stage, folding stage and heading stage) were expected to be observed mainly by leaf angles: 0-20° for rosette leaf, 20-60° folding leaf and 60-90° heading leaf.

Figure 9 The best plant material of each phenotype was selected and taken photos weekly both from the top view and side view with black background.

2.4 Harvest leaf samples for RNA isolation

The sampling methods in this experiment were separated into two parts including central leaf harvest and heading stage leaf harvest. For both sampling methods, samples assembled in tubes were marked with their positions in the greenhouse from week 2. For each time point x genotype combination, three biological repeats were taken, which were represented by the three main blocks. And each time point x genotype block sample contained a mixture of leaf samples from each of three plants per subblock.

2.4.1 Central leaf harvest

For the central leaf sampling part, the youngest leaf of 4-5 cm width (Figure 10) was harvested and stored in the 5 ml tube by liquid nitrogen immersion immediately.

The first week`s samples (week 1) were harvested by Xiaoxue Sun, following weeks were samples by myself. At week 1, plants were not yet transplanted and still all in seedling tray. Three biological repeats were taken for each genotype, and each biological repeat was a single plant. After the first week, all other seedlings (not sampled ones) were transplanted into pots and placed according to experimental design in the greenhouse.

For week 2, leaf samples were harvested from 17cm pots. After week 2, leaf samples were harvested from 21cm pot`s plant materials. The protocol for the next weeks' sampling method was the same as the week 2`s. And the time of harvest samples per genotype was different. PC genotypes Glu024, PC175, PC184 were sampled till the 8^{th} week and PC101 till 11^{th} week. The leaves of CC genotype A003 were sampled until the 13^{th} week. Z16 and CC168 were harvested till 8^{th} week. The detail information can be checked in the appendix.

For all the PC genotypes, there was only one plant in each subblock. Before CC genotypes` heading stage, CC genotypes were harvested from one and the same plant of the five plants in each subblock.

Figure 10 Central leaf harvest: the youngest leaf of 4-5 cm was harvested. This photo was taken from PC Glu024 at week 2.

2.4.2 Harvest of leaf samples in heading stage for RNA isolation

Two CC genotypes Z16 and A003 showed obvious heading in this experiment. For these two genotypes, during their heading stages, 3 different types of leaf were harvested including rosette leaf, folding leaf and heading leaf as well. What was more, for each type of leaf, samples were taken from 3 different positions: the top of the leaf, right and left from midvein; the middle, right and left form midvein; the bottom position, right and left from midvein (Figure 11).

During the heading stage, the leaves were also counted. The oldest leaf was number one. And then, the rosette leaf would be counted and we sampled leaf disks (3cm width) from one clear rosette leaf. Continuing to the middle part, the exact border between rosette and folding leaf was hard to be distinguished. The most reliable folding leaf would be counted and sampled. For the heading leaf, the first fully expanded heading leaf was preferred to be harvested. However, in CC A003, the heading leaf was also hard to identify. Therefore the most reliable one with vertical growth angle and yellow colour would be counted and sampled. The detail number of leaf types sampled during the heading stage for these two genotypes are shown in Figure 13. For example, at week 4 in Z16, the 5th leaf was a rosette leaf, the 6th leaf was a folding leaf and the 11th leaf was a heading leaf, and they were sampled.

In order to harvest leaves at heading stage, the leaves needed to be peeled off from outside to inside. This means for each week a new plant was used. The detail harvest materials` information for these two genotypes can be checked in the appendix.

Figure 11 Three types of leaf represent different growth stages, following rosette stage, folding stage and heading stage.

2.5 Primer design

Specific primers were designed for a set of genes that were detected in selective sweeps by comparing heading Chinese cabbages with Non-heading *B. rapa* types (Table 4). Full cDNA sequences were retrieved from Arabidopsis database (http://www.arabidopsis.org/) and then blasted against *Brassica* database (http://brassicadb.org/brad/index.php). Sequence comparisons were conducted using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). And for those with multiple orthologous genes, specific primers were designed using Primer3Plus (http://www.primer3plus.com). Primers from previous studies were also used and these primers included reference gene ***Actin***.

2.6 RNA isolation and cDNA synthesis

Frozen leaf samples (-80°C) were grinded in the mortar using the liquid nitrogen.

All the RNA was isolated from the grinded tissues by using "RNeasy Plant kit" (Qiagen). This method following the plants RNA isolation protocol adapted by Johan Bucher, who is our Brassica group`s technician. After the RNA isolation, the concentration and quality of these RNA samples were quantified by using the NanoDrop 1000 spectrophotometer (Thermo Scintific), where the absorption ratio at 260/280 should be around 2.0 as pure RNA samples. DNA contamination was removed by using RNase-free DNase I treatment (Invitrogen), which followed the manufacturer's protocol inside. And some RNA samples were randomly picked to check their qualities using gel electrophorese.

These cleaned RNA samples were diluted with MQ water to 100 ng/µl in a 10 µl volume cDNA synthesis system. The cDNA synthesis was performed by using "iScript™ cDNA Synthesis Kit" (Biorad) following the manufacturer`s protocol. A final volume of 20 µl cDNA product per simple was obtained and it was diluted with MQ water by the ratio 1:40 reaching a concentration around 30 ng/µl for later experiments.

Table 4 Primers designed for both candidate heading genes and the used reference gene, which included their forward and reverse sequences, primer length, temperature, GC contents and the PCR product size. The annotation information was selected from the gene`s GO Biological Progress (http://www.arabidopsis.org/).

Candidate genes		Brassica ID		5'-3'	Length	TM	GC%	Product size	Annotation
1	rtARF3-a	Bra005465	F	TGGTGATGCTGTGCTTTTCC	20	59.04	50	231	abaxial cell fate specification, auxin metabolic process, auxin-activated signalling pathway, determination of bilateral symmetry, floral meristem determinacy, floral whorl development, flower morphogenesis, organ morphogenesis, pattern specification process, vegetative phase change, virus induced gene silencing, xylem and phloem pattern formation
			R	AAGAACTTCGGTGCAGGGA	19	58.86	52.63		
2	rtARF4	BRA002479	F	GCTGGTGTAGTGACTGGAGT	20	59.03	55	186	abaxial cell fate specification, auxin-activated signalling pathway, vegetative phase change
			R	TTTGGGCCTTGGAGATGACT	20	58.92	50		
3	rtAXR1	Bra015396	F	AGGACGAGTTCAGCAATCCT	20	58.73	50	204	DNA repair, auxin homeostasis, auxin-activated signalling pathway, cullin deneddylation, histone methylation, leaf morphogenesis, photomorphogenesis, positive regulation of transcription, protein deubiquitination, protein neddylation, protein ubiquitination, reciprocal meiotic recombination, response to water deprivation
			R	TCCCCAACATCGGAACAAAC	20	58.39	50		
4	rtKAN2-a	Bra033844	F	CACGGCAACCAACAATAGCT	20	59.12	50	198	carpel development, cell differentiation, embryo development ending in seed dormancy, ovule development, polarity specification of adaxial/abaxial axis, response to abscisic acid, seed dormancy process, seed germination
			R	TGAACAAAACGGGCATGGAG	20	59.04	50		
5	rtBrBRX.1	Bra023219	F	TCAAGTAGAACCCGGTGTCC	20	59.03	55	195	auxin-activated signaling pathway, cytokinin-activated signalling pathway, lateral root development, response to abscisic acid, root development
			R	CTAGCAGGCGTTTGAAGAGC	20	59.28	55		
6	rtBrARR15	Bra003782	F	CGGTGAAGTTAGCAGACGTG	20	58.94	55	179	circadian rhythm, cytokinin-activated signalling pathway, phosphorelay signal transduction system, response to cytokinin
			R	TCGCTTGGAAGATGGAGTGT	20	59.02	50		

Candidate genes		Brassica ID		5'-3'	Length	TM	GC%	Product size	Annotation
7	rtBrGA20OX1	Bra013890	F	CACGTTAGATGCTTCCAGGC	20	58.99	55	175	abscisic acid biosynthetic process, embryo development ending in seed dormancy, flower development, gibberellic acid mediated signalling pathway, gibberellin biosynthetic process, leaf development, oxidation-reduction process, reproductive structure development, seed dormancy process, seed germination, short-day photoperiodism, flowering, unidimensional cell growth
			R	ACTCTCGCCTGCTTTTCTCT	20	59.03	50		
8	rtBrRCD1.2	Bra035511	F	AGTGCAAACAGTGTCCGTTC	20	58.99	50	249	abscisic acid-activated signalling pathway, cell communication, cell death, defence response to bacterium, incompatible interaction, defence response to fungus, embryo development, endoplasmic reticulum unfolded protein response, ethylene-activated signalling pathway, glycolytic process, hyperosmotic response, jasmonic acid mediated signalling pathway, lateral root morphogenesis, viral process, water transport
			R	TCCTGCTAAACTTGCCTGGT	20	59.23	50		
Reference	Actin- *oleracea*		F	ACGTGGACATCAGGAAGGAC	20	54.70	55		
			R	GAACCACCGATCCAGACACT	20	54.70	55		

2.7 Expression profile analysis

2.7.1 Quantitative Real-Time PCR

qRT-PCR reactions were performed on 96-well plates following the standard protocol (Figure 12) by using CFX96 Thermal Cycler (Bio-Rad). Each well contains a 10 µl volume system with 5 µl SYBR Green Supermix, 3.4 µl MQ water, 0.3 µl forward primer, 0.3 µl reverse primer and 1 µl cDNA. As different genotype had different amounts of samples, the plate designs were different. However, the similarities for each plate were that every sample in one well included three biological repeats and there was always one reference gene ***Actin*** to avoid errors and check the quality of these samples. Gel electrophorese was also used for few samples after qRT-PCR reactions, I randomly selected 4 µl products from the plate mixed with 5 µl MQ water and 1 µl loading dye to perform a 10 µl system to test the primer and cDNA quality.

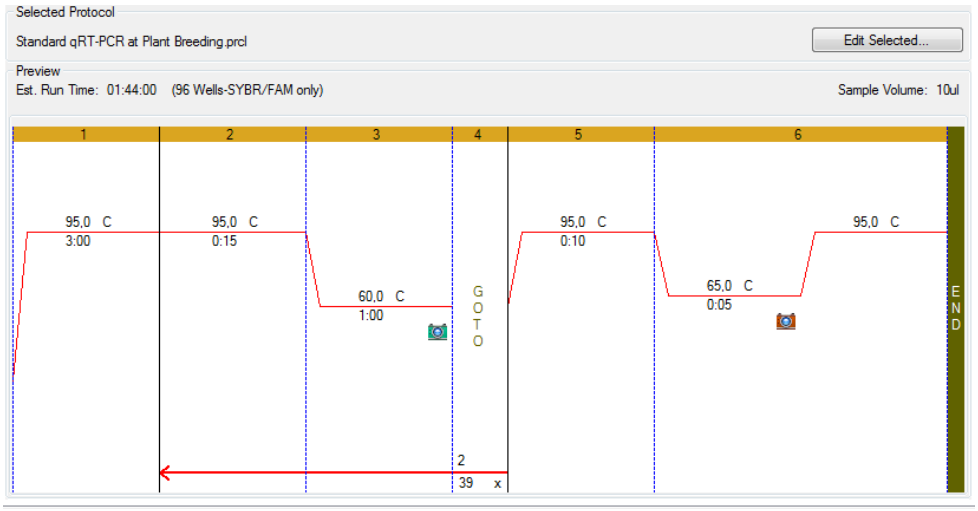


Figure 12 Standard qRT-PCR protocol at Plant Breeding Group: 95 °C 3 min, 39 cycles of 95 °C 15 sec and 60 °C 1 min, 95 °C 10 sec, 65 °C 5sec and 95 °C.

The Cq values of these samples were recorded by the machine automatically. After checking the result through the Bio-Rad program, these data were exported into Microsoft Excel for further calculation.

2.7.2 Data analysis

The expression level differences between the candidate genes and the reference gene were calculated as the following equation:

$$\Delta C_q = C_q(Candidate\ gene) - C_q(\boldsymbol{Actin})$$

And the relative expression level of the candidate genes compared to their expression levels at time point zero was calculated as the following equation (the second week of genotype Z16 was used as comparison/control for all genotypes):

$$\Delta\Delta c_q = \Delta c_q - \Delta c_q(Z16\ week\ 2)$$

$$relative\ expression\ level = 2^{-\Delta\Delta c_q}$$

3 Results

3.1 Morphological observation during development of Chinese cabbage and Pak Choi

In this project, six traits (leaf number, leaf colour, leaf shape, leaf surface, leaf angle and leaf type) were measured during development of CC (Z16, A003, CC168) and PC (Glu024, PC175, PC184, PC101) in the greenhouse.

3.1.1 Leaf numbers

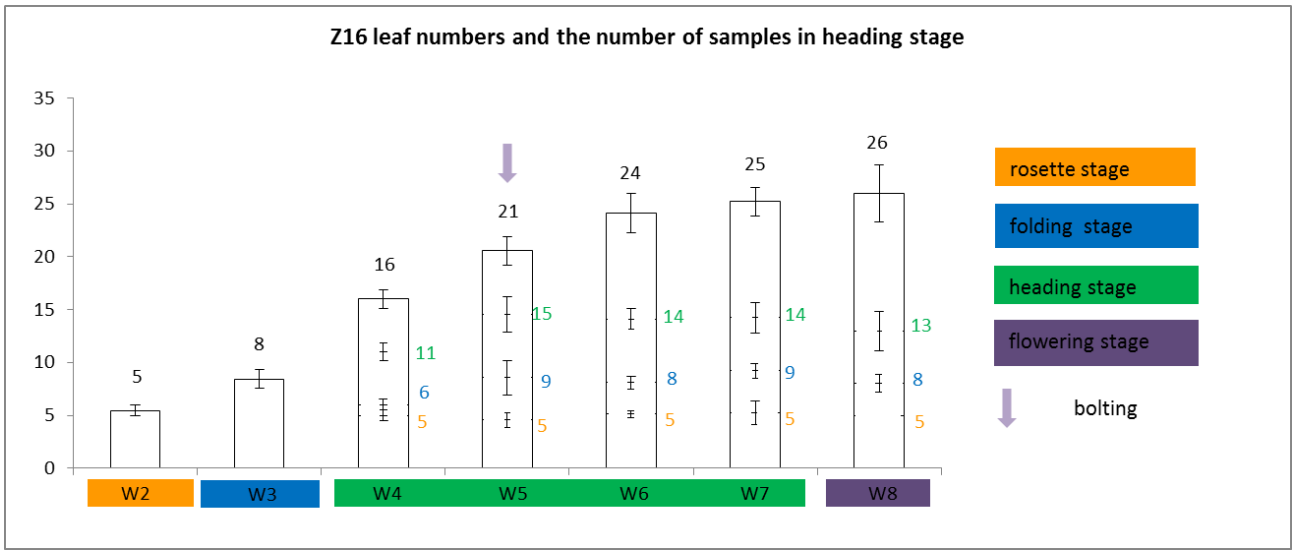
The numbers of leaves were measured weekly from the second week.

For CC Z16 (Figure 13, a), at week 4, the first week of heading stage, the leaf numbers are 16. A dramatic increase from week 3 with eight leaves and this is the strongest increase though Z16`s growth stages. The leaves numbers are stable around 25 in the last three weeks (week 5, 6 and 8).

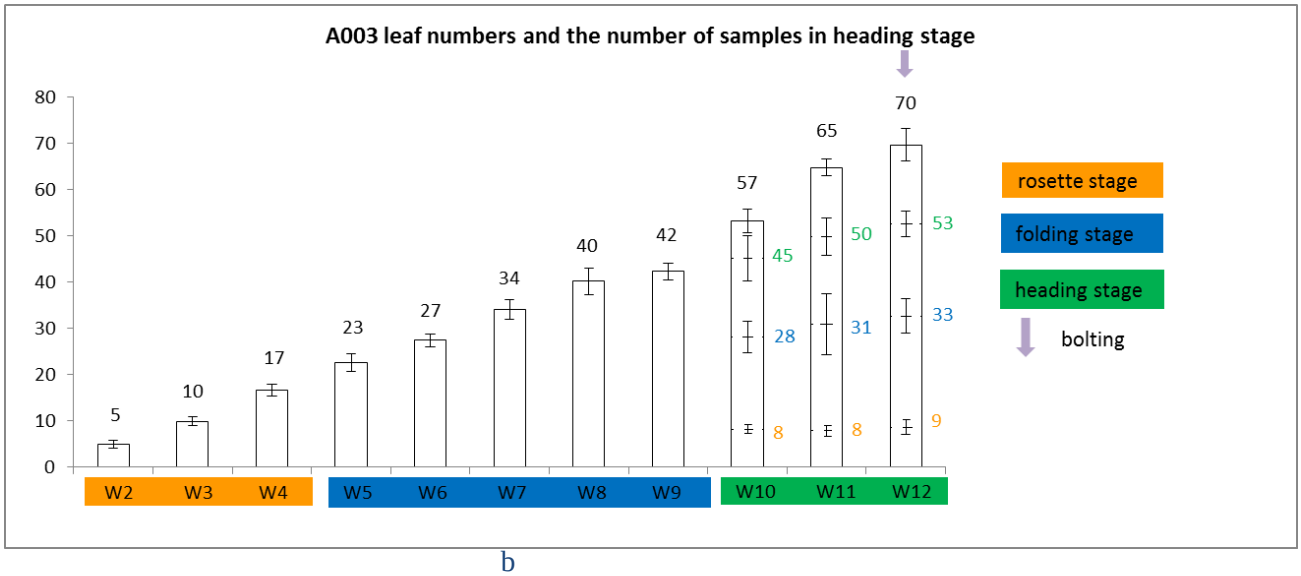
The leaf numbers of A003 (Figure 13, b) increase to 42 at week 9, which is the last week of folding stage. There is a dramatic increase by 15 leaves between the week 9 and week 10, the first week of heading stage. And this is the strongest increase through A003`s growth stages as well. At the last week experiment in the greenhouse, the leaf numbers in A003 are 70.

The first two week`s rosette stage in CC168, leaf numbers increase to ten at week 3 (Figure 13, c). At week 6, the first week of heading stage, leaf numbers increase from 21 at week 5 to 24 at week 6. And during the heading stage, the leaves are stable around 25. There is no dramatic increase from the folding stage to the heading stage for CC168. In addition, the growth situations for all plants of genotype CC168 were bad because leaves started rotting, browning and drying starting from week 5.

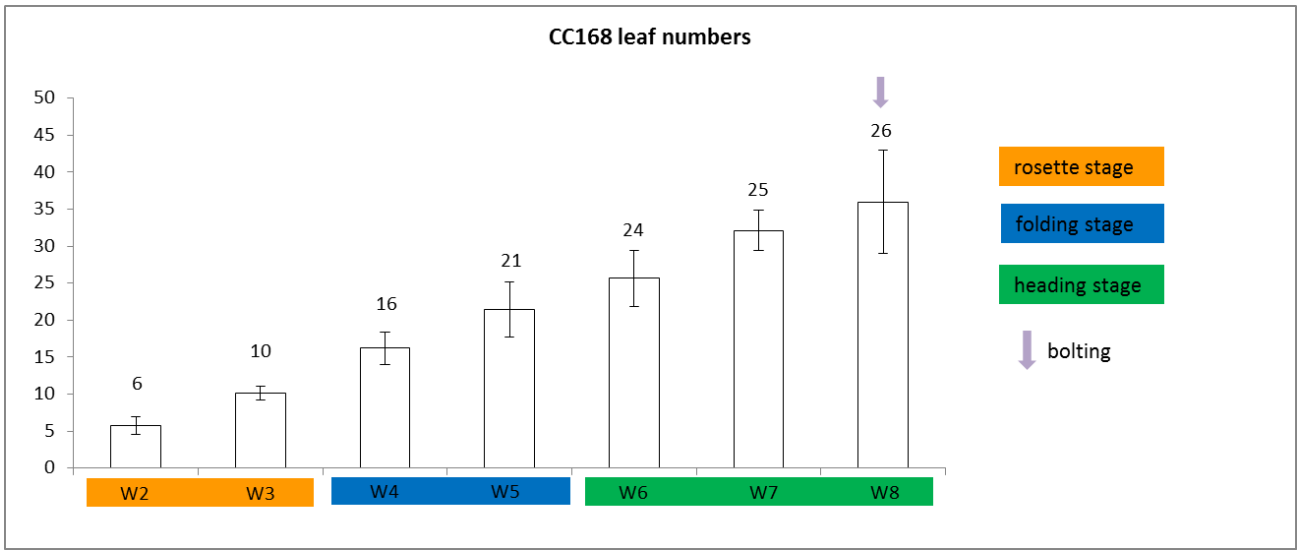
The leaf numbers counted each week are presented for PC genotypes Glu024, PC175, PC184 and PC101 in Figure 14. At week 2, all four genotypes had about five leaves. And the leaf numbers slowly increased along the development of all four PC genotypes`. For PC175, the leaf numbers decrease between week 7 and week 8. The reason may be that new leaves in flowering stage were miscounted as vegetative leaves after week 6, when bolting occurred. And then at week 8, the internode elongated and specific leaves in flowering stage could be easily distinguished from the vegetative leaves, so counting was correct again. For the PC genotypes Glu024, PC175 and PC101, we stopped counting at week 8, while for PC101, last counting was at week 9.



a



b



c

Figure 13 Overview of leaf numbers in three CC genotypes (a) Z16, (b) A003 and (c) CC168. Total leaf numbers are indicated above columns. The number of different leaf type that was sampled for heading leaf harvest is indicated by coloured numbers right to the columns: orange represents rosette leaf type, blue folding leaf type and green heading leaf type. Square colours of weeks indicate growth stages of CC.

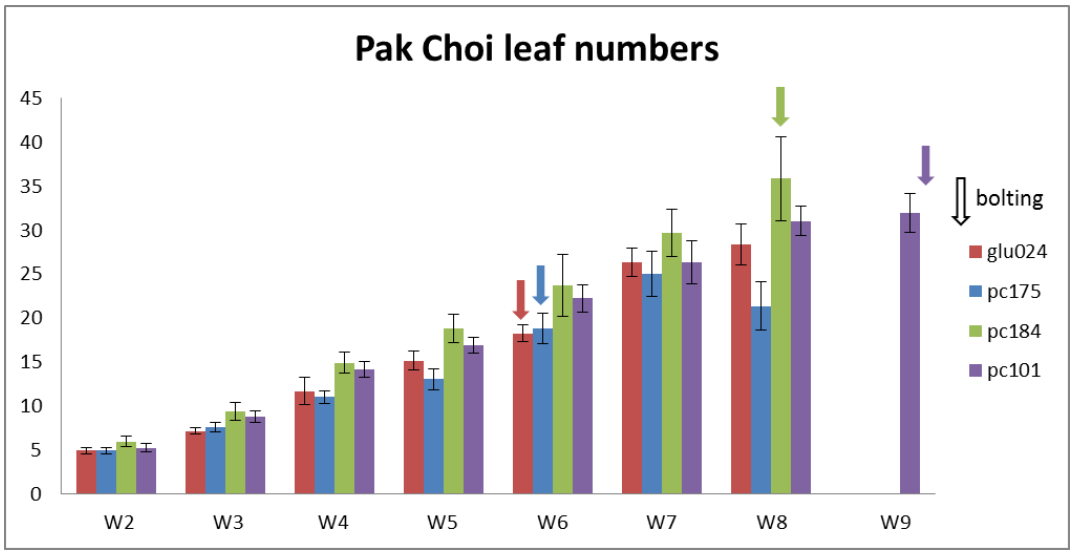


Figure 14 Overview of leaf numbers in four PC genotypes. Different coloured columns indicated different genotypes: red represents PC Glu024, blue PC175, green PC184 and purple PC101.

3.1.2 Leaf phenotypes

During this project, leaf colour, leaf shape, leaf surface were observed and leaf type was defined in the process of CC and PC growth.

Leaf colour

The leaf colours are different between and within these CC and PC genotypes (Figure 15). Z16`s leaf colour is medium green, CC168 is light green and the colour of A003 is between yellow and green. For four PC genotypes, they are all medium green except PC175, which is dark green.

Leaf shape

The leaf shapes at week 2 of these genotypes are indicated (Figure 15). All leaves are oblong shape at leaf young stage. And the fully expended mature leaf shape of these genotypes can be seen from the Figure 19, the continuous photos of developmental stages in CCs and PCs. The mature leaf shapes of Z16 and CC168 are the same as their leaves at young stage, oblong shape. For A003, the young leaf grows and elongates its leaf length and the mature leaf is torpedo shape. The four PC genotypes` leaf shapes remain the same through their development and the mature leaves are still oblong shape.

Leaf surface

Young and mature leaf surfaces of these seven genotypes can be seen in the Figure 15 and Figure 19 respectively. Z16`s leaf at young stage wrinkles heavily especially around the midribs part. The leaf edges curl upwards. These wrinkles will expand though the leaf development, the mature leaf surface is flat and smooth but near the midribs part, there are still wrinkles. For CC168, the young leaf and the mature leaf are similar in leaf surface. All the leaf surfaces are flat and smooth. The young leaf of A003 is similar to its mature leaf. There are trichomes on the top leaf surface. And from the enlarged zoomed picture above the Figure 15, it indicates that those trichomes are on the adaxial side of the leaf, and by the observation in the greenhouse, there are no trichomes on the abaxial leaf surface. PC Glu024 also has trichomes on its adaxial surface. The leaf surface of all these four PC genotypes` is flat and smooth.

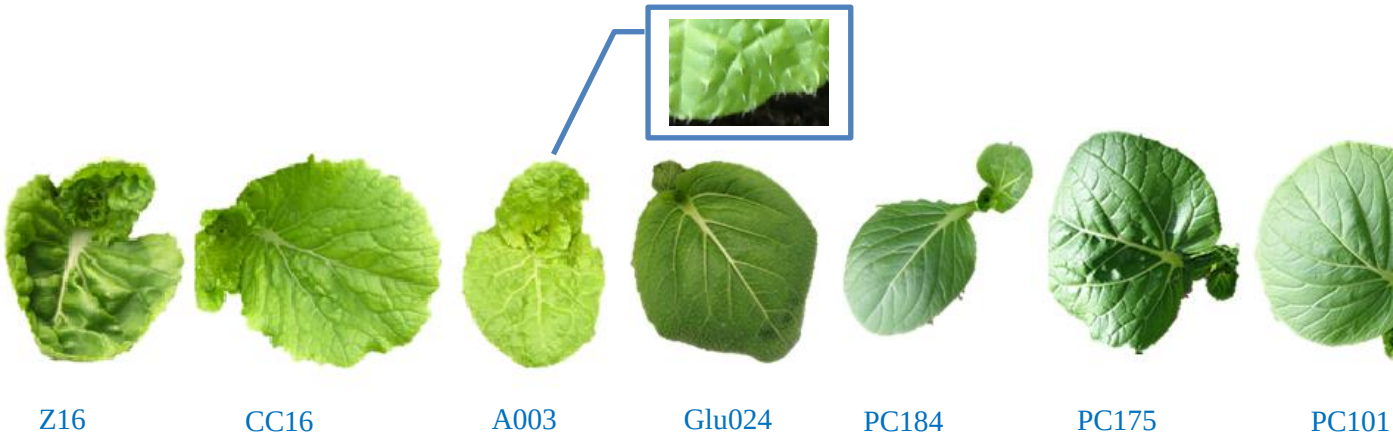


Figure 15 Seven leaf phenotypes at week two from three CC genotypes and four PC genotypes.

Leaf type

In the process of CC and PC growth, the leaf angles and leaf edge curls change. For the CC genotypes, rosette leaf, folding leaf and heading leaf were observed during plant development (Figure 17). For Z16, the rosette leaf angle is around 0-20° parallel to the soil surface. In the folding stage, folding leaf grow upward around 20-60°. And the heading leaves grow upward around 60-90°. For A003, the rosette leaf angle is 0-20° the same as Z16`s. However, the folding leaf angle fluctuates from 20° to 80° or even 90°. And all the heading leaves grow vertically, 90°. The main difference of these two types of leaf in A003 is whether the leaf edge curl enough is overlap with each other at the top position (Figure 16). For both of CC genotypes, the leaf edge curl is different between different leaf types and different genotypes as well. For Z16, rosette leaf edge curls inward especially at the top part. Folding leaf edge curls inward from the middle part to the top part. The whole edge of heading leaf curls inward from the bottom to the top part. For A003, rosette leaf is flat with slight curl at the top part. Some of the folding leaves` edge at the top position curl both inward and outward, some of them grow vertically, but the top part does not overlap with each other. The heading leaf edge curls inward for the whole leaf especially the top part, leaf edge curls heavily and overlaps with each other. CC168`s rosette leaf edge curls outward at the top position, folding leaf edge curls inward and outward, and the heading leaves almost have no green part because of rotting or other symptoms, so the curl situation is unknown (Figure 17).

Figure 16 Top view picture of CC A003 at week ten. Depicted yellow triangle indicates the first three heading leaves that we counted from the outside to the inside of the head.

Leaf growth	Z16		A003		CC168	
	angle	curl	angle	curl	angle	curl
Rosette leaf						
Folding leaf						
Heading leaf						unknown

Figure 17 Overview of leaf growth situations in three CC specific growth stages. These photos were taken third week of heading stage for each genotype. Three coloured arrows indicates three types of leaf, yellow indicates the rosette leaf, blue folding leaf and green heading leaf. In each column the leaf angle is given first, and then the leaf curl is illustrated.

Leaf growth	PC175	PC184
	Glu024	PC101
Seedling leaf		
Week 2-4		
Week 4-6		
After week 6		

Figure 18 Overview of leaf growth situation in four PC growth stages. These photos were taken at week 6. In each column, the petiole angle and leaf angle at different weeks are shown.

The PC leaf types can be divided into two groups concerning the petiole angle and leaf angle in the later developmental stage (Figure 18). The first group includes Glu024 and PC175, the second group includes PC184 and PC101. In the early stage of four PC genotypes (before week 4), the first two seedling leaves grow horizontally to the soil. And for the true leaves, the angle of the petioles fluctuates approximately from 45 to 90^0. The leaf orientation is horizontal or slightly upward. In the middle stage (around week 5 to week 6), the mature leaves expand outward. The angle of the petioles is around 30 to 90^0 for all leaves. And the mature leaves curl downward from the mid part of the leaf. In the later stage (after week 6), leaves of Glu024 and PC175 still grow horizontally and curl downward. However, the leaves of PC184 and PC101 at the central part grow upward.

3.1.3 Development stages observation

Z16 (Figure 19, a)

Z16 has seven weeks` vegetative growth period and this is the shortest among these three CC genotypes. By observing the leaf phenotypes, W2 is defined as its rosette stage, W3 as its folding stage and W4 to W7 as its heading stage.

Rosette stage: W2. From week 2, the second week after transplanting to the greenhouse, the leaf number reaches five per plant. From the picture, the old mature leaves, around three leaves, are the seedling leaves. The two young rosette leaves grow horizontally parallel to the soil and the top position curls inward.

Folding stage: W3. The central young leaves grow upward and curl inward form the middle edge of the leaf. These folding leaves will form a frame circling the heading part.

Heading stage: W4 to W7. At week 4, young erect leaves with large midribs that grow vertically and curls inward heavily for the whole leaf. These leaves will gather tightly and overlap with each other to form a round shape in the next few weeks. There is obvious colour gradient in the leafy heading part, from the inside yellow to outside medium green.

Obvious bolting could be seen inside the leafy head at week 6. Meanwhile, the flowering shoots appeared on the top of bolt and grew upward quickly. At week 8, small flowers were already beyond the whole plant. This week was the start of the flowering stage for Z16.

A003 (Figure 19, c)

A003 has more than 12 weeks` vegetative growth period, formed most leaves among these three CC genotypes. A003 starts rosette stage at week 2. Week 5 is the beginning of folding stage. Week 9 is the end of folding stage. From week 9 heading stage starts.

Rosette stage: W2 to W4. Leaf numbers increase from five to 17. The young central leaves grow horizontally parallel to soil surface. The first two to three long and narrow leaves are seedling leaves. The rosette leaves are shorter than seedling leaves but wider.

Folding stage: W5 to W9. Leaf numbers increase from 23 to 43. At week 5, young folding leaves grow upward with angles from 30 to 80^o. Many folding leaves grow tightly and form a circle like Z16`s folding leaves. Some of the folding leaves in A003 curl inward at the top position and some curl outward at this position.

Heading stage: W10 to W12. Leaf numbers increase from 53 to 70. Week 10 is the first week of heading stage. All the heading leaves in A003 grow vertically. The A003 heading is different with

Z16`s. It is not a round head but a cylindrical head. The midribs of A003 are longer, wider and also thicker than Z16`s. Z16 has round shape leaf and A003 has torpedo shape leaf.

At the 12th week, bolting was visible inside the leafy head. However, no flowers could be seen at week 13, the last week of experiment in the greenhouse. The flowering time for A003 was unknown.

CC168 (Figure 19, b)

CC168 has around 8 weeks` vegetative growth period. The rosette stage is week 2 and week 3, folding stage is week 4 and week 5, heading stage is from week 6 to week 8.

Rosette stage: W2 to W3. Leaf numbers increase from six to ten. The young central leaves grow horizontally parallel to the soil surface. The rosette leaves are flat and curl a little outward around the leaf edge especially at the top position.

Folding stage: W4 to W5. Leaf numbers increase from 16 to 21. At week 4, young folding leaves grow upward with angles from 30 to 80°. Some of the folding leaves curl both inward and outward at the top position.

Heading stage: W6 to W8. Leaf numbers increase from 26 to 37. The plants had symptoms like rotting form week 5. Less and less normal leaves could be observed in the last few weeks except the midribs around the base could be seen.

At Week 8, the last plant of CC168 was abandoned after taking photos and sampling. Meanwhile inside the central parts, early bolting was detected. So the flowering growth time of CC168 is unknown as well.

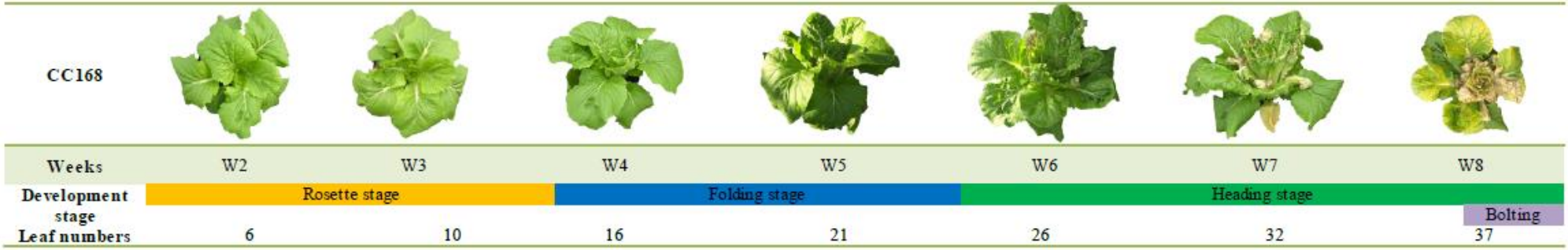
PC genotypes (Figure 19, d-g)

For all four PC genotypes, they did bolt during the course of the experiment. Glu024 and PC175 started bolting at the same time around week 6 and small flowers appeared above the plants at week 8. That means, the time before week 8 is their vegetative stage and after that, they enter generative stage. PC184`s bolting occurred later around week 8. The PC101 bolted latest among these four PC genotypes at week 9.

a


Z16
Weeks
W2
W3
W4
W5
W6
W7
W8
Development stage
Rosette stage
Folding stage
Heading stage
Bolting
Flowering stage
Leaf numbers
5
8
16
21
24
25
26

b


CC168
Weeks
W2
W3
W4
W5
W6
W7
W8
Development stage
Rosette stage
Folding stage
Heading stage
Bolting
Leaf numbers
6
10
16
21
26
32
37

c


A003
Weeks
W2
W3
W4
W5
W6
W7
W8
W9
W10
W11
W12
Development stage
Rosette stage
Folding stage
Heading stage
Bolting
Leaf numbers
5
10
17
23
27
34
40
42
57
65
70

d

Glu024							
Weeks	W2	W3	W4	W5	W6	W7	W8
Leaf numbers	5	7	12	15	18	26	28

e

PC175							
Weeks	W2	W3	W4	W5	W6	W7	W8
Leaf numbers	5	8	11	13	19	25	21

f

PC184							
Weeks	W2	W3	W4	W5	W6	W7	W8
Leaf numbers	6	9	15	19	24	30	35

g

PC101									
Weeks	W2	W3	W4	W5	W6	W7	W8	W9	W10
Leaf numbers	5	9	14	17	22	26	30	32	

Figure 19 Overview of different growth stages of three Chinese cabbage and four Pak Choi genotypes. Photos were taken weekly. From a to g, Z16, CC168, A003, Glu024, PC175, PC184 and PC101. Below the weeks, leaf numbers are indicated and in colour bars, different growth stages for CC are shown.

3.2 Gene expression profiling

3.2.1 Central leaf

The central leaf samples were taken weekly from the first week till the last week of each genotypes` sampling period in the greenhouse. By quantitative real time PCR experiment, the changes of different candidate genes` expression levels are measured. These expression levels are all compared to the expression of the ***Actin*** gene in the same sample and then compared to its expression at week 2 of Z16 as reference control.

a. Chinese Cabbage

Relative expression levels of 8 candidate genes (***Br-Arf3.1***, ***Br-Arf4.1***, ***Br-Kan2.1***, ***Br-Axr1***, ***Br-Brx.1***, ***Br-Arr15***, ***Br-Ga20ox1***, ***Br-Rcd1.2***) in three CC genotypes (A003, Z16 and CC168) through their growth stages are shown as follow (Figure 20):

(*Br-Arf3.1*, a) Its relative expression level fluctuates around 1from W1 to W5 in Z16. A dramatic high expression level appears at W6, The value peaks at 4156, which means gene`s expression increases 4156 folds at week 6. Then the expression level drops sharply to 8 at week 8. In A003, the expression level of ***Br-Arf3.1*** is stable around 0.4-0.6 throughout the 13 weeks except for a small increase in expression (1.34) at week 8. The increase in expression at week 6 for Z16 is very apparent. The expression level of ***Br-Arf3.1*** in CC168 increases from 0.59 to 1.5 between week 1 and week 3. At week 4, it decreases to 0.27 and during the last 4 weeks, the expression level is rather stable.

(*Br-Arf4.1*, b) The gene`s expression level fluctuates between 1.7 to 1 in Z16 before week 4. And this value increases to 1.7 at week 5. It continuously decreases to the lowest value 0.23 at week 8, which means gene`s expression decreases 0.23 folds at the last week. The similar expression pattern can be seen in A003. First 9 weeks, the expression level fluctuates between 2.65 and 1.37. And after a rising to 2.10 at week 11, it decreases dramatically to 0.03 folds less at week 12. In CC168, the expression level of ***Br-Arf4.1*** is around 0.20 at the first four weeks, and then fluctuates around 0.10 during the last four weeks.

(*Br-Kan2.1*, c) This gene`s expression level starts from the minimum value 0.25 at week 1 in Z16. Then it increases to the maximum value 1 at week 2. And during the last 6 weeks till week 8, the expression level slowly decreases to 0.32. This gene doesn`t express in A003. For CC168, ***Br-Kan2.1***`s expression levels increases 0.26 at week 3. Then it drops to the lowest point at 0.04 at week 4, 0.04 folds less than week 3. It stabilizes at 0.09 during week 6 and week 7. Finally it ends with 0.17 at week 8.

(Br-Axr1, d) The expression level increases around 0.3 folds from 0.30 at week 1 to 1 at week 4. Then it declines to 0.07 at week 6. During the last 3 weeks, the value increases again to 0.59 at week 8. Comparing with Z16, the expression of ***Br-Axr1*** in A003 has similar patterns. The expression level steadily increases from 0.42 at week 1 to 3.42 at week 10, which means the gene`s expression increase about 3 folds at week 10. Then it is fluctuated and ending with the value of 3.39 at week 13. The gene`s expression level is very low and fluctuated between 0.01 and 0.09 during the 8 weeks in CC168.

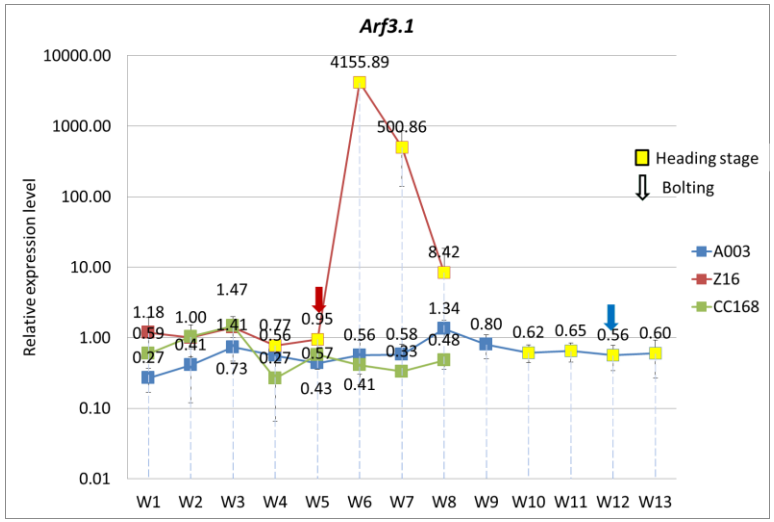
(*Br-Brx.1*, e) This gene only expresses at the first four weeks in Z16. The value increases to 1.70 at week 3 and then decrease to 0.80 at week 4. In A003, it decreases form 0.71 at week 1 to 0.40 at week 2. Then after a growth to 1.39 at week 3, the expression level is fluctuated around 1 till week 10. During the last four weeks from week 10 to week 13, it gradually increases to 2.51 at week 13. And

for CC168, the value remains around 1.00 during the first four weeks. Then it drops to 0.5 at week 5 and keeps stale around 0.3 during the last three weeks.

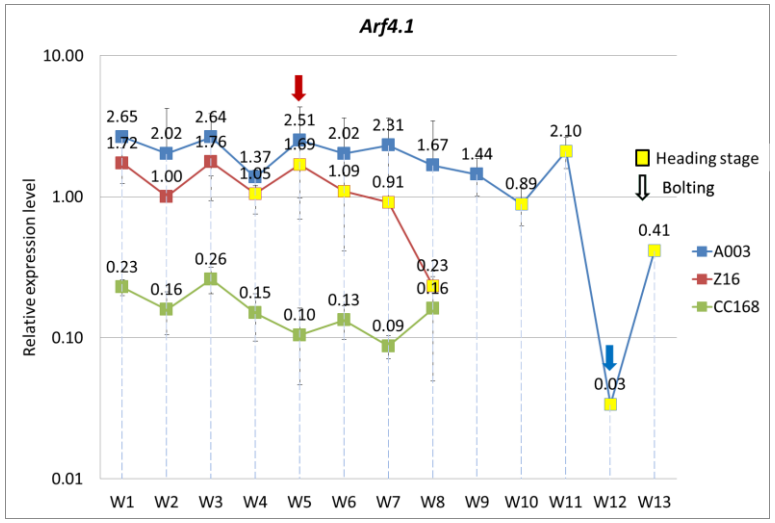
(*Br-Arr15*, f) In Z16, the expression level firstly increases 0.80 folds from 0.20 at week 1 to 1.00 at week 2. Then it drops again 0.76 folds to 0.24 at week 4. And it increases to 0.49 at week 6. After that, it decreases again and ends with 0.08 at week 8. The similar expression pattern of ***Br-Arr15*** can be seen in A003, rising from 0.11 at week 1 to 1.05 at week 4 and decreasing to 0.16 at week 9. And it increases to 0.54 at week 12 then drops to 0.19 at week 13. For CC168, it reaches the peak 1.44 at week 2. Then the expression level declines to 0.26 at week 7 and increases again to 0.41 at week 8.

(Br-Ga20ox1, g) The expression level increases slowly from 0.46 at week 1 to 1.36 at week 6 in Z16. Then it increases sharply to 7.73 at week 8, which means this gene`s expression increase 6.73 folds in two weeks. For A003, the expression level of ***Br-Ga20ox1*** firstly increases from 1.12 at week 1 to 2.39 at week 4. Then it drops to the minimum value 0.34 at week 5. From week 5 to week 10, the expression value stalely grows to the peak of 5.62. The value decreases to 3.23 at week 12 and back to 4.65 at week 13. For CC168, the expression level declines smoothly from 1.97 at week 1 to 0.45 at week 8, around 1.5 folds change.

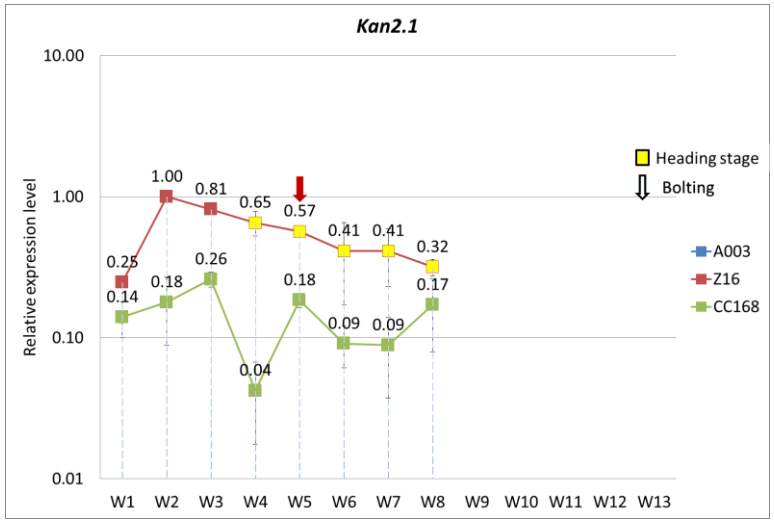
(*Br-Rcd1.2*, h) In Z16, this gene`s expression increases from 0.42 at week 1 to 1 at week 2 and then drops to the lowest point 0.27 at week 4, around 0.73 folds less. The value is fluctuated between 0.78 and 0.26 though the last five weeks from week 3 to week 8. In A003, the expression level of ***Br-Rcd1.2*** is fluctuated between 3.15 and 1.76 before week 9. And then it decreases slowly to 1.20 at week 12, finally peaks at 6.70 at week 13, which means the expression increases about 5.5 folds. For CC168, the value declines from 4.41 at week 1 to 0.45 at week 5 and increases again to 1.29 at week 8.


Arf3.1
Relative expression level
10000.00
1000.00
100.00
10.00
1.00
0.10
0.01
W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 W11 W12 W13
Heading stage
Bolting
A003
Z16
CC168

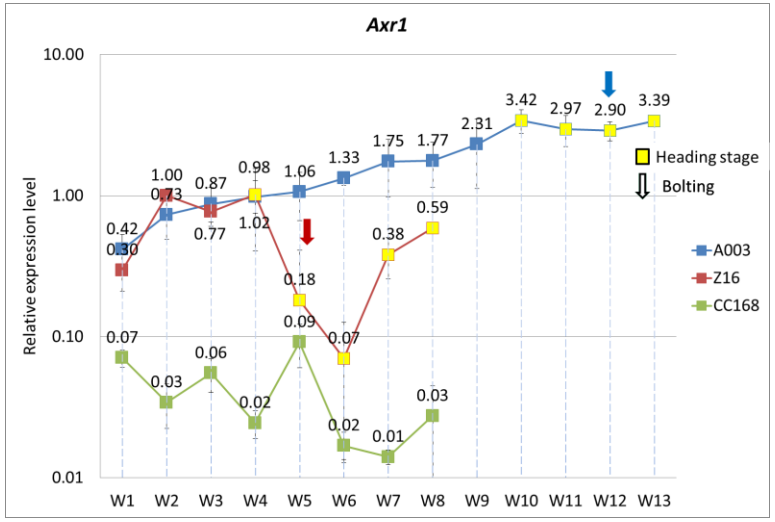
a


Arf4.1
Relative expression level
10.00
1.00
0.10
0.01
W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 W11 W12 W13
Heading stage
Bolting
A003
Z16
CC168

b


Kan2.1
Relative expression level
10.00
1.00
0.10
0.01
W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 W11 W12 W13
Heading stage
Bolting
A003
Z16
CC168

c


Axr1
Relative expression level
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1.00
0.10
0.01
W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 W11 W12 W13
Heading stage
Bolting
A003
Z16
CC168

d

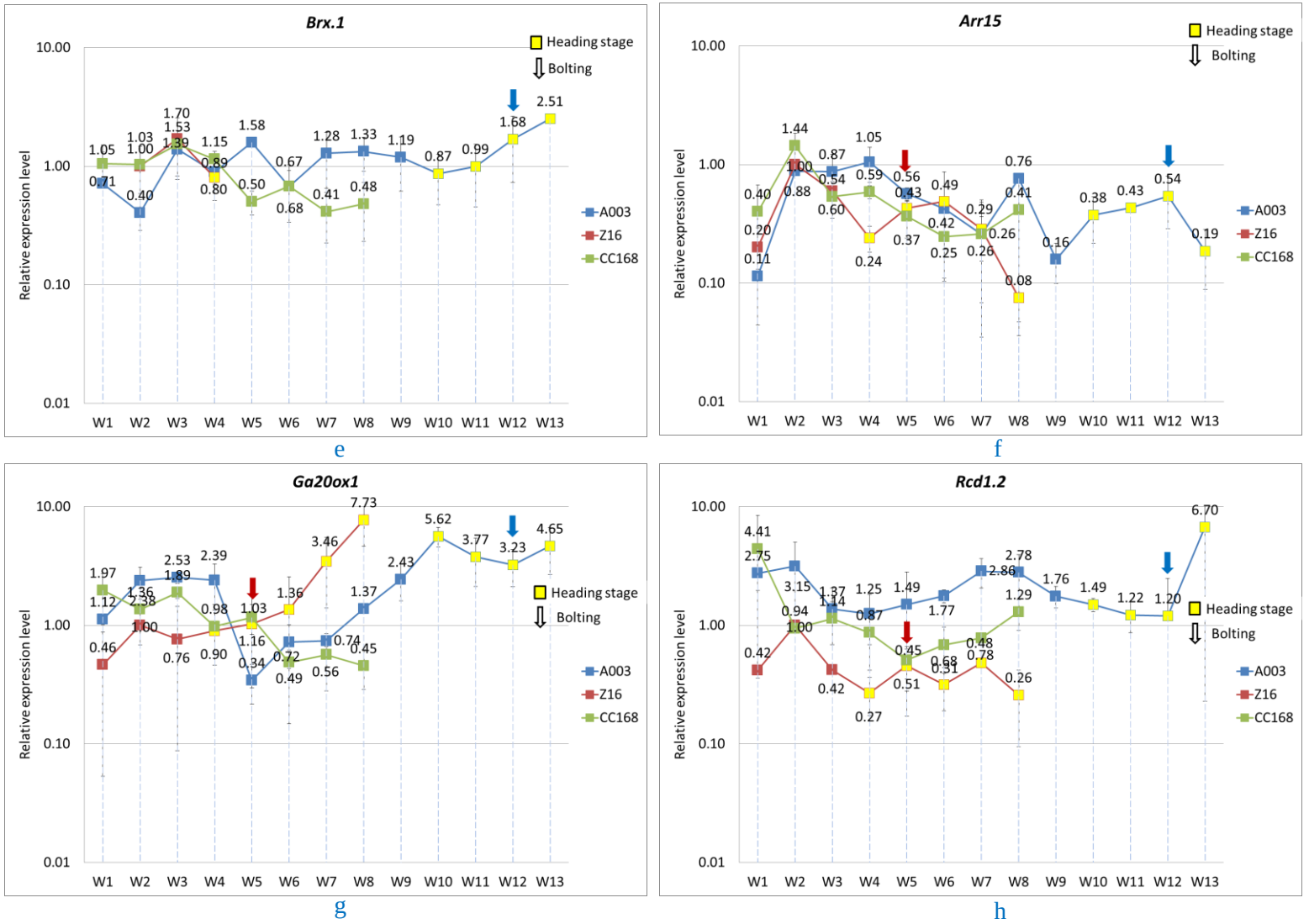


Figure 20 Relative expression levels of eight candidate genes in three CC genotypes through their growth stages. All compares to the reference gene *Actin* and the reference samples taken at week 2 of Z16. (a) *Br-Arf3.1*; (b) *Br-Arf4.1*; (c) *Br-Kan2.1*; (d) *Br-Axr1*; (e) *Br-Brx.1*; (f) *Br-Arr15*; (g) *Br-Ga20ox1*; (h) *Br-Rcd1.2*. The scale of Y-axis had been logarithmic scaled base on 10.

b. Pak Choi

Relative expression levels of 4candidate genes (***Br-Arf3.1***, ***Br-Arf4.1***, ***Br-Axr1***, ***Br-Arr15***) in four PC genotypes (Glu024, PC175, PC184, PC101) through their growth stages are shown below (Figure 21).

(*Br-Arf3.1*, a) For Glu024, the expression level fluctuates around 1 during the first 4 weeks. It increases slowly from 0.64 at week 5 to 2 at week 7 and declines to 0.16 at week 8. PC184 has the similar pattern. It fluctuates between 0.18 and 1 from week 1 to week 4. Then it reaches its peak of 3.81 at week 7 and decreases to 0.25 at week 8. For PC175, it has a fluctuation between 0.06 and 1in the first four weeks as well. At week 6, there is a high value of l193, which means gene`s expression level increases 1193 folds. And then it drops to 0.33 at week 8. The expression level for PC101 continuously increases from 0.74 at week 1 to the peak of 198 at week 6. At week 7, the value declines to 2, and then it remains stable around 1between week 8 and week 11.

(*Br-Arf4.1*, b) Three PC genotypes Glu024, PC175 and PC184 had the same expression pattern through their growth stages. The expression level in Glu024 decreases from 0.19 at week 1 to 0.06 at week 2, and then it increases to 1.96 (1.9 folds more) at week 3 and decreases to 0.93 at week 4. After four weeks increasing form week 4 to 2.04 at week 7, it declines to 0.20 at week 8. For PC175, the expression level reaches the lowest point 0.18 at week 2. Then it increases 2.28 folds to 2.46 at week 3 and decreases to 0.93 at week 4. This value reaches the peak of 3.04 at week 6. And the last two weeks, it decreases to 0.84 at week 8. In PC184, ***Br-Arf4.1*** increases to 2.17 at week 3. This value decreases to 0.73 at week 4 and then increases to 2.09 at week 7. At week 8, it decreases to 0.26, which means the gene`s expression level decreases 1.83 folds. For PC101, the expression level increases from 0.59 at week 1 to 2.17 at week 3. Then it slowly decreases to 0.97 at week 8. Between week 9 and week 11, this value is fluctuated around 1.9.

(*Br-Axr1*, c) This gene expresses stably in the four PC genotypes. In Glu024, it increases to 0.86 at week 4. Then it decreases to 0.64 at week 5 and consciously increases and reaches the peak of 1.65 at week 8. For PC175, the expression level increase during the first three weeks and reaches the highest point of 1.31 at week 3. Then it is fluctuated around 1 till week 8. For PC184, it increases to 1.29 at week 3. And it decreases continuously to 0.60 at week 6. At week 8, the expression level is 1.25. For PC101, the highest point of 1.80 appears at week 3. And the next eight weeks, the expression of ***Br-Axr1*** is fluctuated around 1.

(*Br-Arr15*, d) The expression level in Glu024 increases from 0.03 at week 1 to 0.25 at week 2. Between W2 and W6, this value decreases and reaches the minimum value of 0.03 at week 6. Then it increases to 0.11 at week 8. For PC175, the expression level decreases from 1.03 at week 1 to 0.12 at week 4, this means 0.91 folds less. And it is fluctuated around 0.6 during the last three weeks. For PC184, it decreases from 1.25 at week 2 to 0.08 at week 6. After an increasing to 0.18 at week 7, it ends at 0.12 at week 8. In PC101, the expression of ***Br-Arr15*** increases to 0.98 at week 2. Then it decreases to 0.1 at week 7 and increase to 0.59 at week 11.

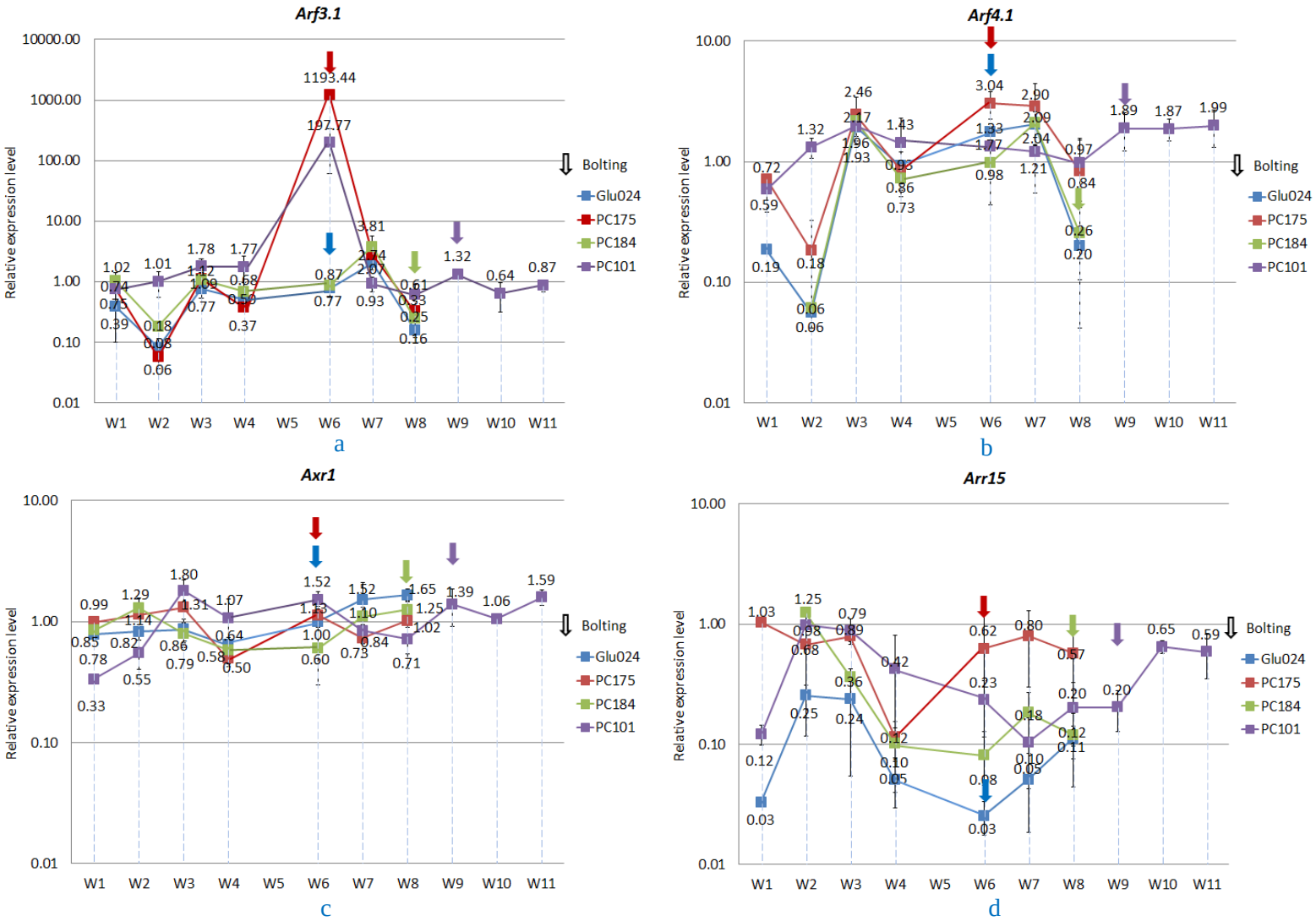


Figure 21 Relative expression levels of four candidate genes in four PC genotypes through their growth stages. All compares to the reference gene *Actin* and the reference samples taken at week 2 of Z16, (a) *Br-Arf3.1*; (b) *Br-Arf4.1*; (c) *Br-Axr1*; (d) *Br-Arr15*. The scale of Y-axis had been logarithmic scaled base on 10 (candidate genes` expression data are missing at week 5 for all PC genotypes).

c. CC & PC

Three Auxin related genes, ***Br-Arf3.1***, ***Br-Arf4.1*** and ***Br-Axr1*** have been sequenced in the three CC and four PC genotypes. Therefore their allelic composition could be determined by Xiaoxue Sun (Table 5). It can be seen that many PC genotypes carry heading alleles for these genes (Figure 4). These genes` expression levels in all seven genotypes are compared, indicating with their allelic composition below (Figure 22).

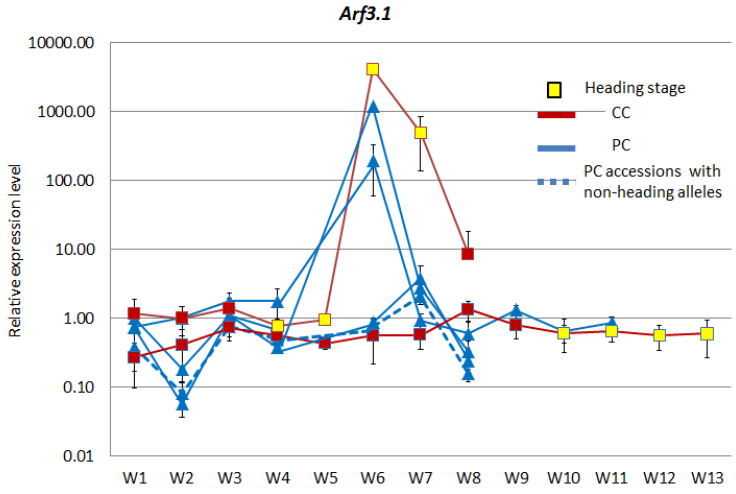
Table 5 Allelic compositions of three Auxin related genes in CC and PC genotypes. Alleles with red colour belong to non-heading allele and blue alleles all belong to heading allele based on selection signal.

	Z16	A003	CC168	Glu024	PC175	PC184	PC101
Br-Arf3.1	G	G	G	C	G	G	G
Br-Arf4.1	AAAA	AAAA	AAAA	Indel	AAAA	Indel	Indel
Br-Axr1	A	A	A	A	A	T	A

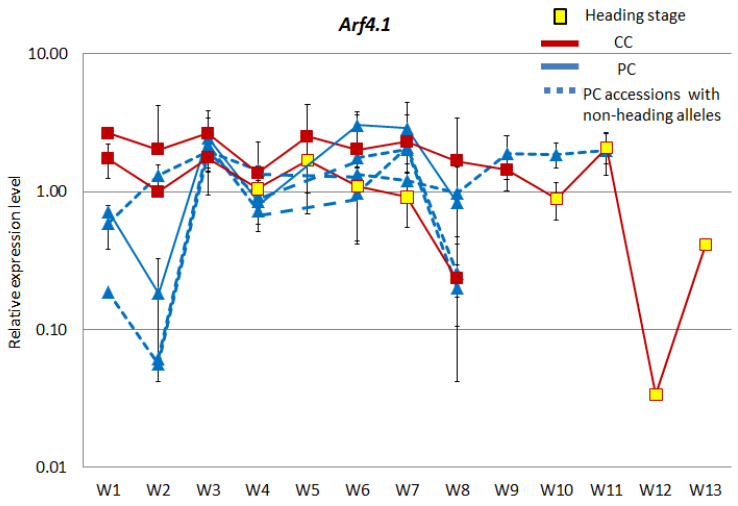
(*Br-Arf3.1*, a) All these genotypes` relative expression levels have low expression levels (around 1.0) at the second week and then they have different expression pattern after week 5. PC175, PC101 and CC Z16 which carry the heading allele have dramatic high values around W6 and decreased in the next weeks. In another expression pattern, ***Br-Arf3.1*** expresses quite stable through these eight weeks in A003, CC168, PC184 and Glu024. For these four genotypes, three carry also heading allele while only Glu024 carries the non-heading allele.

(*Br-Arf4.1*, b) All these genotypes have the similar expression patterns (decrease at the early stage then fluctuate in the middle stage and decrease again at latest stage), except PC101 with non-heading "indel" alleles (the gene`s expression is stable though the growth stage). However, among the similar expression-pattern genotypes, two CCs (Z16, A003) and one PC (PC175) have the heading alleles "AAAA", and two PCs (PC175, PC184) have the non-heading alleles.

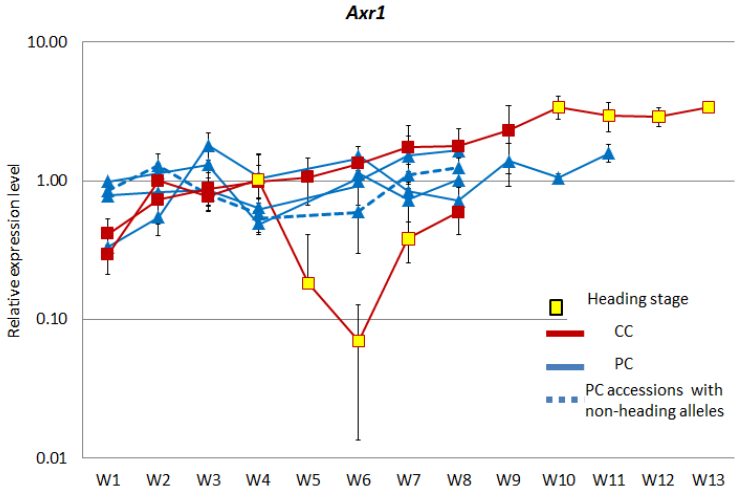
(*Br-Axr1*, c) For this gene, Z16 with heading allele "A" has a strong decrease in the expression levels around week5. However, other six genotypes don't show this decrease pattern. The gene`s expression levels in them are similar that continuously increase. But five of them including one CC and three PC have heading allele "A". Only PC184 has non-heading allele "T".



a



b



c

Figure 22 Relative expression levels of genes all compared to the reference gene Actin and the reference samples taken at week 2 of Z16, (a) *Br-Arf3.1*, (b) *Br-Arf4.1* and (c) *Br-Axr1*. The solid and dash lines indicate genes have allele variations, dashed is non-heading allele.

3.2.2 Gene expression in heading samples of Z16 and A003

Relative expression levels of 5 candidate genes (***Br-Arf3.1***, ***Br-Brx.1***, ***Br-Arr15***, ***Br-Ga20ox1***, ***Br-Rcd1.2***) in different leaf types and both leaf bottom and leaf top positions in CC A03 (week 10, 11, 12) and Z16(week 4, 6, 8) genotypes through their heading stages are shown below (Figure 23). And their expression levels also compare to the reference gene *Actin* and the reference central leaf samples taken at week 2 of Z16.

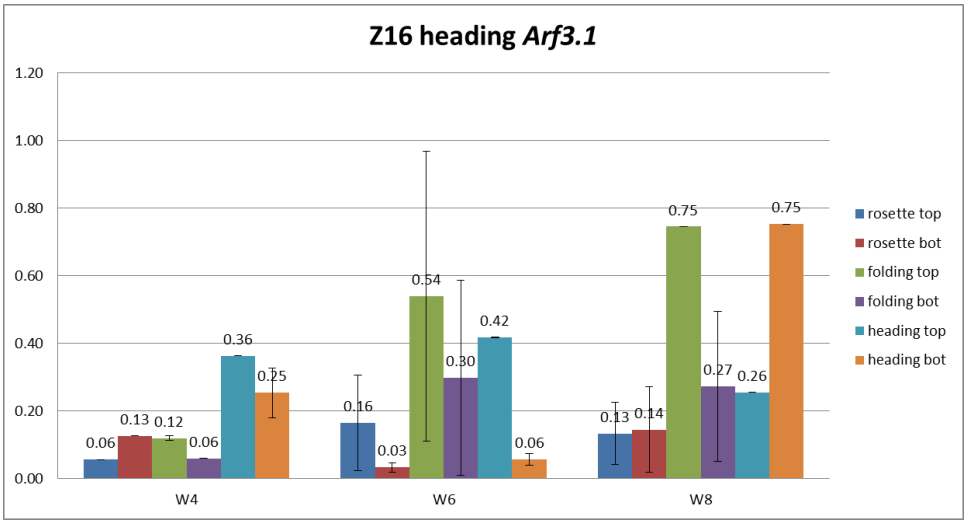
(*Br-Arf3.1*, a) At week 4, the expression levels at top and bottom positions in all 3 leaves (rosette, folding and heading) ranges between 0.36 and 0.06, and the expression level in heading leaf is higher than that in other two types of leaves. At week 6, the expression level at the top position in the folding leaf reaches 0.54 which is the highest value among these six samples. At week 8, expression levels at both top position of folding leaf and bottom position of heading leaf peak at 0.75.

(*Br-Brx.1*, b) From the overview of the data in these three weeks, the expression levels in the rosette and folding leaves increase from week 4 to week 8. The expression levels at top position of the heading leaf are stable around 0.35 in these three weeks. However, the value of bottom position of heading leaf decreases from 0.71 at week 4 to 0.12 at week 6, then it increases to 0.76 at week 8, which means 0.64 folds change in the expression of ***Br-Brx.1***.

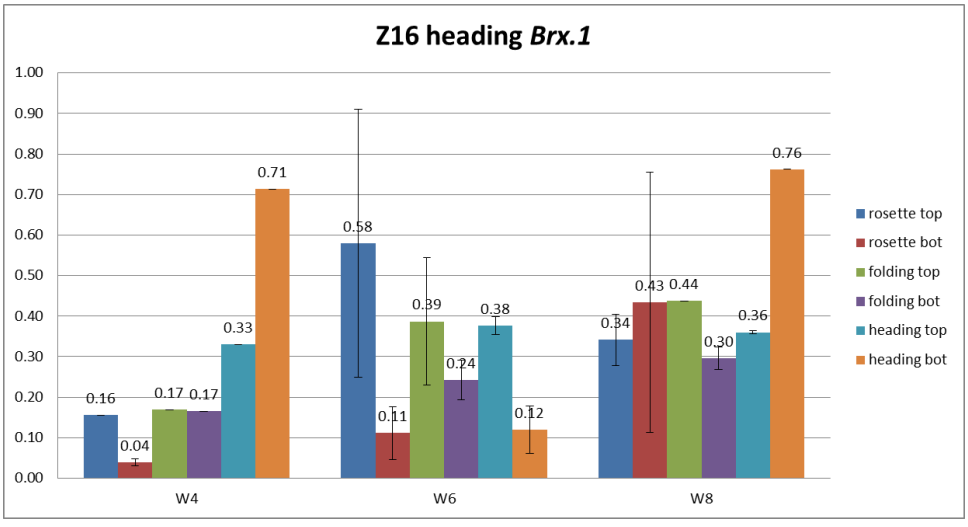
(*Br-Arr15*, c) There is no clear pattern visible. At week 4, the initial heading stage of Z16, the expression levels of ***Br-Arr15*** peak of 1.07 at top position and 1.09 at bottom position both in folding leaf samples. And other expression levels of this gene are all below 0.40, this means 0.6 folds less. And this gene`s expression level doesn't change a lot at top and bottom positions in each leaf type except the folding leaf samples at week 8, the value at top position is 0.30, which is 0.22 more folds than the expression level at bottom position.

(*Br-Ga20ox1*, d) There is no clear pattern visible. The expression levels in all samples at week 4 are higher than the same samples at week 6 and week 8. And the expression levels are very high at the top (6.67) position of folding leaf, which means the expression of ***Br-Ga20ox1*** increases 5.67 folds. At week 6 and week 8, the expression levels are all under 1.00 and the gene expresses higher at bottom position than at top position.

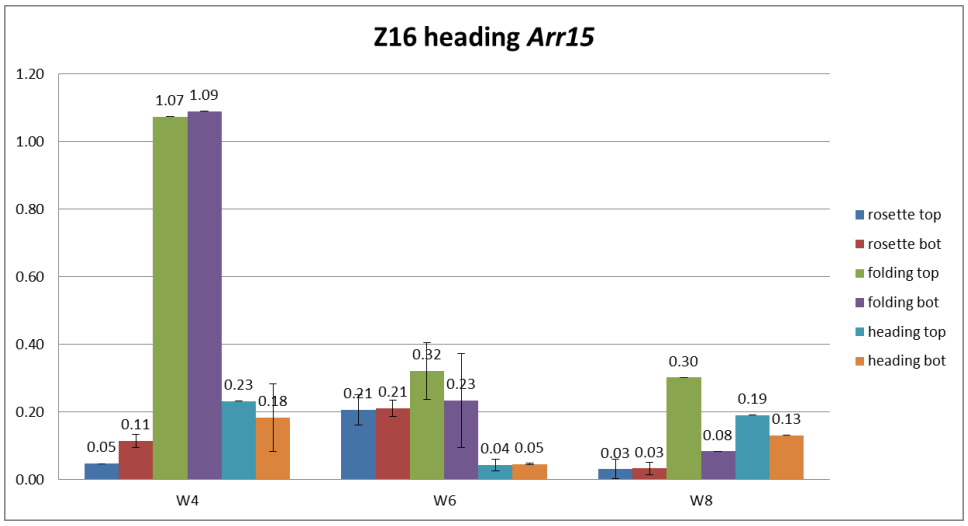
(*Br-Rcd1.2*, e & f) The relative expression levels of ***Br-Rcd1.2*** in Z16` s heading samples at week 4, week 6, week 8 and in A003 at week 10, week 11 and week 12 respectively. Overview these data, this gene has high expression levels both in Z16`s heading parts and A003 heading parts, the average value is 1.86 in Z16 and 3.03 in A003. For Z16, the expression levels at top position are 1.17 folds more than at bottom positions briefly in all three leaf types. However, there is no similar expression pattern at different positions in A003.



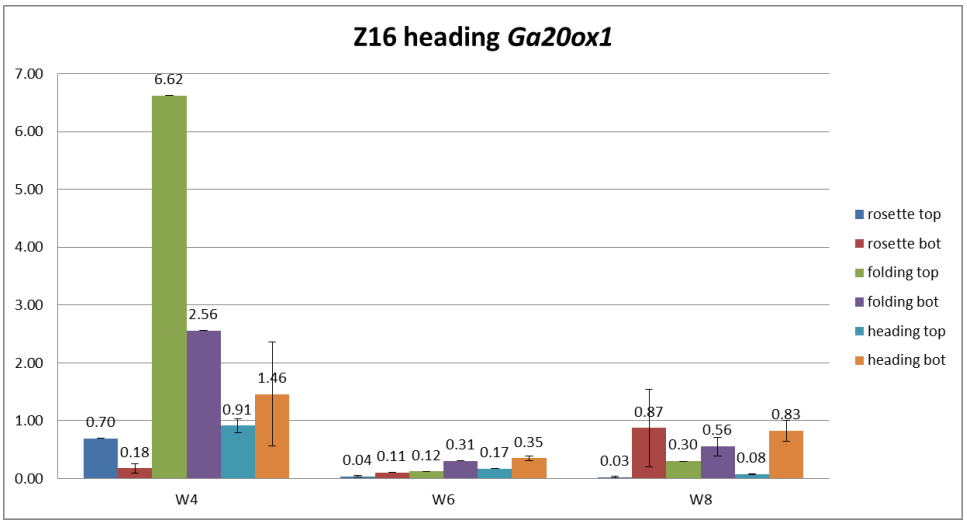
a



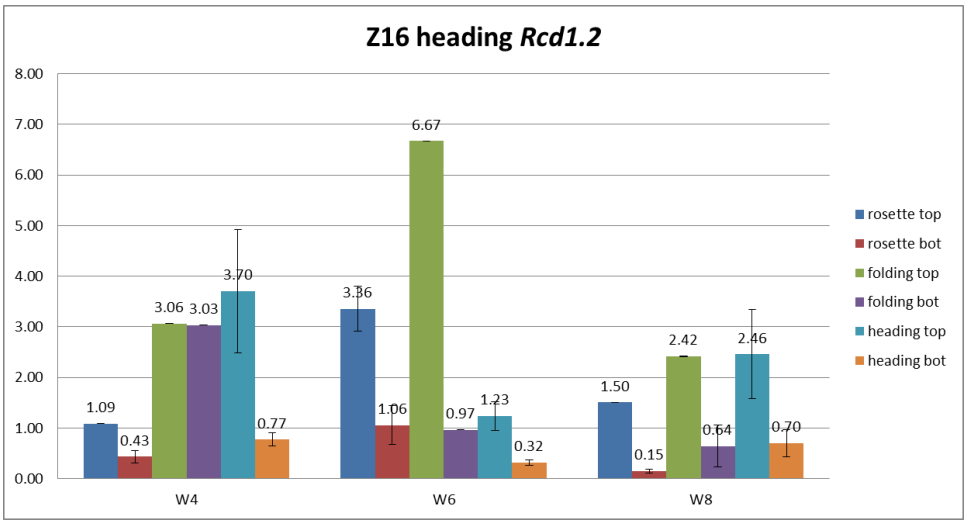
b


Z16 heading *Arr15*
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.05 0.11 1.07 1.09 0.23 0.18
0.21 0.21 0.32 0.23 0.04 0.05
0.03 0.03 0.30 0.08 0.19 0.13
W4
W6
W8
rosette top
rosette bot
folding top
folding bot
heading top
heading bot

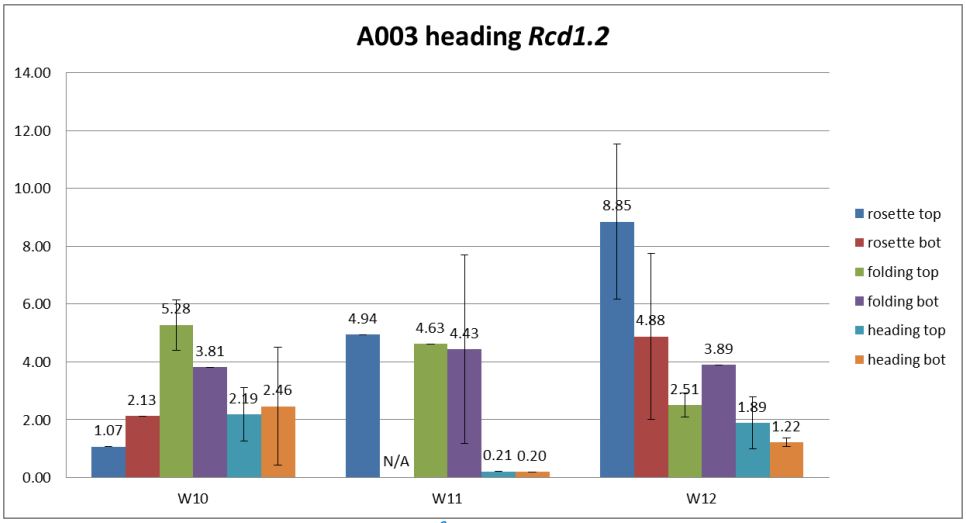
c


Z16 heading *Ga20ox1*
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0.70 0.18 6.62 2.56 0.91 1.46
0.04 0.11 0.12 0.31 0.17 0.35
0.03 0.87 0.30 0.56 0.08 0.83
W4
W6
W8
rosette top
rosette bot
folding top
folding bot
heading top
heading bot

d


Z16 heading *Rcd1.2*
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1.09 0.43 3.06 3.03 3.70 0.77
3.36 1.06 6.67 0.97 1.23 0.32
1.50 0.15 2.42 0.64 2.46 0.70
W4
W6
W8
rosette top
rosette bot
folding top
folding bot
heading top
heading bot

e



f

Figure 23 Relative expression levels of candidate genes in the heading leaf samples for Z16 and A003. All compared to the reference gene *Actin* and the reference samples taken at week 2 of Z16. Different columns'` colours indicate six kinds of heading samples taking from two positions (top and bottom) at three types of leaves (rosette, folding and heading). (a) *Br-Arf3.1*, (b) *Br-Brx.1*, (c) *Br-Arr15*, (d) *Br-Ga20ox1*, (e) *Br-Rcd1.2* in Z16 heading samples; (f) *Br-Rcd1.2* in A003 heading samples.

4 Discussion

4.1 Morphology of leaf development in Chinese cabbage and Pak choi

Leaf angles and leaf edge curls influence the head formation. Comparison of phenotypic traits between heading and non-heading subspecies of *B. rapa* suggests two traits as the major factors influencing head formation: the erect leaf angles and the round-shape leaves without petioles (Ito and Kato 1958). The bend in the basal part of the midrib is the main contribution to the development of erect leaves of heading cultivars (Nishijima and Fukino 2005). In our project, two of three CC genotypes formed the head obviously: round shape for Z16 and cylindrical shape for A003. Compared with other non-heading PCs, these two CCs had no long petioles but the leaf blade flanked the large midribs. And along the midribs, there were erect leaves especially in the head stage. For Z16, the erect leaves were round-shape with short midribs. For A003, the erect leaves were torpedo-shape with long midribs. We believe that the erect leaves formed the basic frame of the head in CCs. The petioles of PC genotypes also grew vertically (Figure 19). The leaves of PC175 and Glu024 did not grow upward, but the leaves of PC184 and PC101 grow upward without curling inward. Comparing with the heading leaves of Z16 and A003 those non-heading leaves were smaller and couldn't curl heavily that overlap with each other to form a close head.

Dramatic leaf numbers increase can be seen from folding stage to heading stage. CC genotypes have distinct developmental stages. This can be easily separated in Z16 as its leaf types in these stages are quite different relating to the leaf number, leaf angle and leaf curl. However, the distinction between rosette, folding and heading stage is not clear in A003. There are more leaf numbers in every stage when comparing A003 with Z16, and the leaf curl is not as obvious as in Z16. The leaf angle of different leaves changes slowly as well. We carefully define the different developmental stages in A003 and combine these stages with the leaf numbers counting weekly (Figure 13 Overview of leaf numbers in three CC genotypes (a) Z16, (b) A003 and (c) CC168. Total leaf numbers are indicated above columns. The number of different leaf type that was sampled for heading leaf harvest is indicated by coloured numbers right to the columns: orange represents rosette leaf type, blue folding leaf type and green heading leaf type. Square colours of weeks indicate growth stages of CC.). It is interesting to see that there is a dramatic increasing from the folding stage to the heading stage in both Z16 (8 leaves more/week relating to 3.5 leaves increasing in average per week) and A003 (15 leaves more/week relating to 6.5 leaves increasing in average per week). We speculate that this may indicate the increasing of growth speed in the heading stage. And there is no such dramatic increasing in the next few weeks of heading stage. Therefore, it seems fast growth only happens in early heading stage.

Different growing conditions lead the Z16`s shorter vegetative growth stage in our project. CC genotype Z16 is early-maturing compared with other two genotypes A003 and CC168. The vegetative growth length for Z16 was 5 weeks, and then it entered the flowering stage in our experiment. From initial bolting to the small flowers formation, it was 35-60 days after sowing. We speculate that this early-maturing phenomenon was caused by the growing situations in the greenhouse. Chinese cabbage would remain vegetative for the longest time at temperatures above 21°C (Lorenz 1946) and daily mean temperature should be above 18°C in order to delay flowering (Guttormsen and Moe 1985). And we specifically check the result in the paper (Zhang et al. 2014), CC genotype Z16 was bolting around 55 days after sowing and 80 days for flowering. Those plant materials were planted on the seedbed during 2012.9 to 2013.2 in IVF-CAAS, Beijing, China. The temperature kept 23-25 ºC in the day and 18-20 ºC at night. The light condition was 16h per day

including supplementary lighting. And the moisture was around 40-50% in the greenhouse. Comparing with our culturing conditions, the experiment season and the light length were the same. However, the temperature in our greenhouse was 18 ℃ in the day and 16 ℃ at night, which was lower than the experiment in China. The moisture was between 70-88%, much higher than in China. As the growth situation of all the CC in the greenhouse were not good after week 5, especially for the genotype CC168. Factors like too much watering, nutrients deficiency may also effect the plants growth. .

4.2 Gene expression profiling during development

There are obvious morphological differences between and within CC and PC genotypes. By detecting the candidate genes` expression levels, we expect to see some patterns that could be dividing into groups like their phenotypes. However, most of the gene expressions profiling results could not be classified according to crop type. So there are no common gene expression regulations, which was against our expectations. Therefore, we prefer to pick some interesting points to check and discuss. And as it was mentioned before, the plant materials CC168 were in bad growth situation in the greenhouse. The samples taken from this genotype were influenced by the disease. So we will not discuss the molecular result of this genotype.

Br-Arf3.1*, *Br-Arf4.1* and *Br-Axr1*`s expression patterns do not relate to sub species or allelic composition.** The morphological result indicates obviously different phenotypes between heading CC and non-heading PC. We expect to see the different genes` expression patterns between CC and PC as well. In the gene expression profiling part, three genes` expression levels are compared between these seven genotypes (Figure 22). For ***Br-Arf3.1***, CC Z16 and PC Glu024, PC101 have similar expression pattern, with dramatic increase in expression at week 5 and 6. For ***Br-Arf4.1***, the gene`s expression levels are quite similar among these genotypes except PC101. For ***Br-Axr1, the gene`s expression level is different between Z16 and other 5 genotypes. Therefore, genes` expression levels seem not relate to subspecies, so not to the heading phenotype as well. And based on the allelic compositions of these three Auxin related genes, we know there are heading alleles and non-heading alleles (Figure 4). Not all PC genotypes carry non-heading alleles for these genes, while most CC genotypes (≥ 90%) carry heading alleles. Relating to our materials, we can see that all three CC genotypes have the CC alleles in these genes respectively, but four PC genotypes carry heading or non-heading alleles for these genes (Table 5). For ***Br-Arf3.1***, PC Glu024 is the only genotype with the PC/non-heading allele “C”. But it has the same gene expression pattern with CC/heading allele genotypes PC101 and CC Z16 (Figure 22). For ***Br-Arf4.1***, Z16, A003 and PC175 have the CC/heading alleles and other three have PC/ non-heading alleles. The gene`s expression levels only shows different pattern in the PC101. For ***Br-Axr1***, PC184 has a non-heading allele “T” instead of heading allele “A” that other six genotypes have. And relating to the expression patterns, the different one is in Z16. Above all, it seems there are no relationships between genes` expression levels and the plant materials` sub species or those genes` allele compositions.

For other candidate genes, we can find that their expression patterns have similarities and also differences between different genotypes. However, these similarities seems still do not relate to sub species heading CC and non-heading PC. Even in heading CC, we cannot find some genes` expressions related with their different growth stages. The nutrients deficiency and unsuitable cultural environments in the greenhouse had influenced the phenotypes of plants and we believe they also had effects on these plants` gene expressions.

Br-Arf3.1* gene expression: is it related to the leaf developmental stage or to initiation of flowering?** The relative expression levels of ***Br-Arf3.1 were different between Z16 and A003 (Figure 20, a). It can be seen that the whole expression levels of this gene in Z16 were higher than in A003. During the vegetative stages, the average expression level for Z16 was 1.06 and this value for A003 was 0.63 (2 folds different). Relating to the difference between these two genotype`s leaf shapes (Figure 15), the young leaf of Z16 had more wrinkles and curved more heavily than A003`s young leaf. The early research reviews that the depression of ***Br-Arf3*** results in acceleration of phase change and severe morphological and patterning defects of leaves and floral organs in *Arabidopsis thaliana* (Fahlgren, Montgomery et al. 2006). And ***Br-Arf3*** is involved in the main pathway regulating the leaf adaxial–abaxial polarity (Adenot, Elmayan et al. 2006) for its expression on the abaxial side is limited by the mature tasiR-ARF (Benkovics and Timmermans 2014). We speculate that ***Br-Arf3.1*** is involved in leaf shape development in CC, and the role is similar compared to it in *A. thaliana*.

Other two papers have shown the role of ***Br-Arf3*** in specifying floral meristem determinacy and pattering floral organs (Sessions, Nemhauser et al. 1997, Liu, Dinh et al. 2014). Focus on the dramatic high expression levels 4155.89 around week 6 in Z16. Even in the next week, the value is still high, 500.86 at week 7. And an impressive increasing the size of bolting appeared around week 6. Taking the morphological change and molecular change into account, we believe that the expression level increasing was related with the initial flowering stage in Z16. And the similar relationship between high gene expression levels and bolting appearance could be seen in PC175 and Glu024. However, for A003, PC184 and PC101, these were no such relationships.

Br-Kan2.1* different gene expression patterns may result from the formation of trichomes between *Arabidopsis* and *B.rapa*.** ***Br-Kan2.1 expresses in Z16 and CC168 but not in A003 (Figure 20, c). Referring to the leaf phenotype of these three genotypes especially the trichomes only exits on A003, we speculate this gene would be involved in blocking the trichomes formation on the leaf surface. This gene also expressed in all PC genotypes including Glu024, which have trichomes on its leaf surface as well. Maybe the trichomes on these A003 and Glu024 were different. However, we had not compared them during the experiment. And relating to the model plant, the difference was obvious in the first two rosette leaves of *Arabidopsis* which possess trichomes (leaf hairs) on their adaxial surface but not their abaxial surface (Telfer, Bollman et al. 1997). And the mutant alleles of ***KAN*** by T-DNA insertion mutagenesis were identified that produced abaxial trichomes on these first two leaves instead of adaxial surface (Kerstetter, Bollman et al. 2001). However, we found the trichomes of A003 were on the adaxial leaf surface (Figure 15). And this ***KANADI*** gene family regulates abaxial identity (Pekker, Alvarez et al. 2005), the leaf edge curl direction may be affected by this gene`s expression. Among these seven genotypes, this gene didn't express in A003. And the torpedo shape leaf of A003 is impressive. We speculate that maybe this gene`s no expression may affect the leaf edge curl.

Br-Brx.1* induces different regulation results on the growth speed between *Arabidopsis* and *B.rapa*.** ***Br-Brx.1 expressed from week 1 to week 13 in A003, but only expressed during first four weeks in Z16 in the central leaf samples (Figure 20, e). Comparing the growth time length between Z16 and A003, Z16 grew faster than A003. We guess that this gene`s cease expression may increase the growth speed in CC. A paper indicates that loss-of-function ***BRX*** can slow down the growth speed and then delay the growth time in *A. thaliana* (Weis, Palm et al. 2015). However, this result is contradictory to our hypothesis. Taking the morphological result in our experiment into account, our plant material Z16 indeed grew faster in our greenhouse even faster than those Z16 in

China. We believe that Z16 stopped expressing ***Brx.1*** gene after week 4 to increase its growth speed to reach the flowering stage.

Br-Ga20ox1* has effect on the floral organ growth.** The expression level of ***Br-Ga20ox1 has a continually increasing pattern starting from the bolting in both CC Z16 and A003 (Figure 20, g). In Z16, the expression level is 1 at week 6 (bolting) and 7.73 at week 8 (flowering stage), which the expression of this gene increase 6 folds. And this gene`s expression level increase as well one week after bolting in A003. We speculate that this gene has effects between the end of vegetative stage and the start of flowering stage. There is one paper indicates that ***GA20ox1*** has significant effect on floral organ growth (Plackett, Powers et al. 2012). And we had observed obvious flowering happened for PC175 at week 8. However, the expression level of ***Br-Ga20ox1*** decreases after its bolting. For Glu024, this gene`s expression level decreases after its bolting as well. For PC101, the value increases.

Further research is needed to investigate the expression of *Br-Rcd1.2* may be related to leaf shape formation. From the genes` expression levels in head leaf samples, it is interesting so see that ***Br-Rcd1.2***`s expression levels at the leaf`s top positions were higher than the bottom positions throughout the three types of leaves in Z16 (Figure 23, e and f). And in A003, it almost had the same pattern except in the rosette and heading leaf samples at week 10, which is the first week of heading stage. However, comparing these two types of leaves at this week with same type of leaves at later weeks, we haven`t found obvious differences. We speculate that this gene may effect on leaf curving especially at the leaf`s top position. But little information can be found that ***Br-Rcd1.2*** is involved in the leaf shape formation. The leaves of Z16 and A003 develop different. They have different sizes, shapes and curls. And the numbers in A003 are higher than other Z16. Further research is needed to investigate whether the ***Rcd1.2*** is involved in leaf shape formation or not.

5 Conclusion

The initial purpose of this study is to research the phenotypic differences between Chinese cabbage and Pak choi, and investigate the expression levels of some heading related genes through their developmental stages.

The CCs have their specific different growth stages included in the development stage. These stages including rosette stage, folding stage and heading stage, are mainly defined by the different leaf angles and leaf edge curls. The timing of the developmental stage is different within CCs, it`s longest in A003 and shortest in Z16. And the leaf numbers corresponding to each growth stages are different within CCs as well. A003 has more leaves in each stage than CC168 and Z16. The leaves within three CC also show different phenotypes including leaf colour, leaf surface and leaf shape. At the later stage of our experiment, Z16 and A003 successfully formed the head, and the head shape in Z16 is round and in A003 is cylindrical. The erect leaves, leaf shape and leaf edge curls all influence the head formation. Comparing with CCs, PCs have no specific growth stages. The leaf numbers have little difference within PCs in each week. And the leaf phenotypes within PCs are similar except in the later stage, the leaves of PC184 and PC101grew upward but the leaves of PC175 and Glu024 still grew horizontally. Comparing with the leaves of CCs, they all have upward midribs or petioles. However, the leaves of CCs curl inward or outward relating to different leaf types especially the heading leaves curl heavily inward and overlap with each other to from a head shape.

The relative expression levels of candidates which compare to the reference gene ***Actin*** and the control sample (central leaf sample taken at week 2 in Z16) show different patterns within seven genotypes. However, all the candidate genes` expression levels seem not relate to the subspecies (CC and PC) in *B.rapa.* And comparing three sequenced Auxin related genes, ***Br-Arf3.1***, ***Br-Arf4.1*** and ***Br-Axr1***, there is no relationship between these genes` expression levels and their allelic compositions in different genotypes. Within CCs, the changes of genes` expression levels are non-informative remarks as these changes are not associated with the growth stages. Especially for the heading stage, none of these eight candidate genes can be recognised as the main factors to the QTL heading traits. For the heading samples, the expression levels of candidate genes seem not relate to leaf types and positions, or to time in weeks.

6 Recommendations:

First of all, it`s necessary for us to figure out what kind of traits we expect to see and when these traits will appear for all genotypes during the experiment. It will be helpful to arrange the time of different methods nicely. In our project, we haven't prepared well enough to see and sample the heading four weeks after we transplanted the seedlings to pots. There were suddenly too much work between week 4 and week 5, and mistake was happened that we haven't sampling central leaves from the PC at week 5. As we known, most of the plants will speed up to produce seeds if the environment was not suitable enough. And relating to our research goal, the long and health vegetative growth of *B. rapa* could offer us more data about leaf growth and head formation. Then it`s important for us to set a suitable environment to cultivate our plants. In our project, all the plants had been influenced somehow by some abiotic factors like nutrients deficiency, too much watering and low temperature. These had affected the plant growth especially the leaves` phenotypes and we believe this abnormal growth had affected candidate genes` expression as well.

For the pheotyping, we didn't take the photos of different leaf types in CC genotypes. And we even didn't take the photos of different head shapes in Z16 and A003. It would be clearer to describe the leaf phenotypes by offering different leaves` photos individually. And we also gave up measuring the leaf angles because the standards in young developmental stage of plants couldn't be used at their middle or later stages. We suggest having a good standard to calculate the leaf angles or even the leaf curls through all the developmental stages of CC and PC genotypes.

For the gene expression profiles, we can see there are no clear patterns at different positions of different leaf types. However, due to the morphological differences between them, we still think that the genes` expression at top position should be quite different with the bot position as well as between leaf types. Our suggestion is that keeps harvesting from the top and bottom positions of three leaf types. Because of time constraints, we only did three weeks heading leaf samples expression level analysis, it would be better to do the expression profiling experiments for all the heading leaf samples.

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sequence provides an important resource for studying the evolution of polyploid genomes and underpins the genetic improvement of Brassica oil and vegetable crops.

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Appendix

Appendix 1: The labelling information of all plants in the greenhouse experiment.

Group	Plot	Description	Position	Plot	Description	Position	Plot	Description	Position	Pedigree Item	Genotype
17cm pot	CEP-0261	CC-168	1-a-1	CEP-0281	Z16	1-a-1	CEP-0291	CC-A003	1-a-1	DH	CC
17cm pot	CEP-0262	CC-168	1-b-1	CEP-0282	Z16	1-b-1	CEP-0292	CC-A003	1-b-1	DH	CC
17cm pot	CEP-0263	CC-168	1-C-1	CEP-0283	Z16	1-C-1	CEP-0293	CC-A003	1-C-1	DH	CC
17cm pot	CEP-0264	CC-168	2-a-1	CEP-0284	Z16	2-a-1	CEP-0294	CC-A003	2-a-1	DH	CC
17cm pot	CEP-0265	CC-168	2-b-1	CEP-0285	Z16	2-b-1	CEP-0295	CC-A003	2-b-1	DH	CC
17cm pot	CEP-0266	CC-168	2-c-1	CEP-0286	Z16	2-c-1	CEP-0296	CC-A003	2-c-1	DH	CC
17cm pot	CEP-0267	CC-168	3-a-1	CEP-0287	Z16	3-a-1	CEP-0297	CC-A003	3-a-1	DH	CC
17cm pot	CEP-0268	CC-168	3-b-1	CEP-0288	Z16	3-b-1	CEP-0298	CC-A003	3-b-1	DH	CC
17cm pot	CEP-0269	CC-168	3-c-1	CEP-0289	Z16	3-c-1	CEP-0299	CC-A003	3-c-1	DH	CC
17cm pot	CEP-0270	CC-168	Backup 1	CEP-0290	Z16	Backup 1	CEP-0300	CC-A003	Backup 1	DH	CC
17cm pot	CEP-0301	PC-101	1-a-1	CEP-0311	PC-184	1-a-1				DH	PC
17cm pot	CEP-0302	PC-101	1-b-1	CEP-0312	PC-184	1-b-1				DH	PC
17cm pot	CEP-0303	PC-101	1-C-1	CEP-0313	PC-184	1-C-1				DH	PC
17cm pot	CEP-0304	PC-101	2-a-1	CEP-0314	PC-184	2-a-1				DH	PC
17cm pot	CEP-0305	PC-101	2-b-1	CEP-0315	PC-184	2-b-1				DH	PC
17cm pot	CEP-0306	PC-101	2-c-1	CEP-0316	PC-184	2-c-1				DH	PC
17cm pot	CEP-0307	PC-101	3-a-1	CEP-0317	PC-184	3-a-1				DH	PC
17cm pot	CEP-0308	PC-101	3-b-1	CEP-0318	PC-184	3-b-1				DH	PC
17cm pot	CEP-0309	PC-101	3-c-1	CEP-0319	PC-184	3-c-1				DH	PC
17cm pot	CEP-0310	PC-101	Backup 1	CEP-0320	PC-184	Backup 1				DH	PC
17cm pot	CEP-0321	PC-175	1-a-1	CEP-0331	PC-glu024	1-a-1				DH	PC
17cm pot	CEP-0322	PC-175	1-b-1	CEP-0332	PC-glu024	1-b-1				DH	PC
17cm pot	CEP-0323	PC-175	1-C-1	CEP-0333	PC-glu024	1-C-1				DH	PC
17cm pot	CEP-0324	PC-175	2-a-1	CEP-0334	PC-glu024	2-a-1				DH	PC
17cm pot	CEP-0325	PC-175	2-b-1	CEP-0335	PC-glu024	2-b-1				DH	PC
17cm pot	CEP-0326	PC-175	2-c-1	CEP-0336	PC-glu024	2-c-1				DH	PC
17cm pot	CEP-0327	PC-175	3-a-1	CEP-0337	PC-glu024	3-a-1				DH	PC
17cm pot	CEP-0328	PC-175	3-b-1	CEP-0338	PC-glu024	3-b-1				DH	PC
17cm pot	CEP-0329	PC-175	3-c-1	CEP-0339	PC-glu024	3-c-1				DH	PC
17cm pot	CEP-0330	PC-175	Backup 1	CEP-0340	PC-glu024	Backup 1				DH	PC

Group	Plot	Description	Position	Plot	Description	Position	Plot	Description	Position	Pedigree Item	Genotype
21cm pot	CEP-0001	CC-168	1-A-1	CEP-0101	Z16	1-A-1	CEP-0151	CC-A003	1-A-1	DH	CC
21cm pot	CEP-0002	CC-168	1-A-2	CEP-0102	Z16	1-A-2	CEP-0152	CC-A003	1-A-2	DH	CC
21cm pot	CEP-0003	CC-168	1-A-3	CEP-0103	Z16	1-A-3	CEP-0153	CC-A003	1-A-3	DH	CC
21cm pot	CEP-0004	CC-168	1-A-4	CEP-0104	Z16	1-A-4	CEP-0154	CC-A003	1-A-4	DH	CC
21cm pot	CEP-0005	CC-168	1-A-5	CEP-0105	Z16	1-A-5	CEP-0155	CC-A003	1-A-5	DH	CC
21cm pot	CEP-0006	CC-168	1-B-1	CEP-0106	Z16	1-B-1	CEP-0156	CC-A003	1-B-1	DH	CC
21cm pot	CEP-0007	CC-168	1-B-2	CEP-0107	Z16	1-B-2	CEP-0157	CC-A003	1-B-2	DH	CC
21cm pot	CEP-0008	CC-168	1-B-3	CEP-0108	Z16	1-B-3	CEP-0158	CC-A003	1-B-3	DH	CC
21cm pot	CEP-0009	CC-168	1-B-4	CEP-0109	Z16	1-B-4	CEP-0159	CC-A003	1-B-4	DH	CC
21cm pot	CEP-0010	CC-168	1-B-5	CEP-0110	Z16	1-B-5	CEP-0160	CC-A003	1-B-5	DH	CC
21cm pot	CEP-0011	CC-168	1-C-1	CEP-0111	Z16	1-C-1	CEP-0161	CC-A003	1-C-1	DH	CC
21cm pot	CEP-0012	CC-168	1-C-2	CEP-0112	Z16	1-C-2	CEP-0162	CC-A003	1-C-2	DH	CC
21cm pot	CEP-0013	CC-168	1-C-3	CEP-0113	Z16	1-C-3	CEP-0163	CC-A003	1-C-3	DH	CC
21cm pot	CEP-0014	CC-168	1-C-4	CEP-0114	Z16	1-C-4	CEP-0164	CC-A003	1-C-4	DH	CC
21cm pot	CEP-0015	CC-168	1-C-5	CEP-0115	Z16	1-C-5	CEP-0165	CC-A003	1-C-5	DH	CC
21cm pot	CEP-0016	CC-168	2-A-1	CEP-0116	Z16	2-A-1	CEP-0166	CC-A003	2-A-1	DH	CC
21cm pot	CEP-0017	CC-168	2-A-2	CEP-0117	Z16	2-A-2	CEP-0167	CC-A003	2-A-2	DH	CC
21cm pot	CEP-0018	CC-168	2-A-3	CEP-0118	Z16	2-A-3	CEP-0168	CC-A003	2-A-3	DH	CC
21cm pot	CEP-0019	CC-168	2-A-4	CEP-0119	Z16	2-A-4	CEP-0169	CC-A003	2-A-4	DH	CC
21cm pot	CEP-0020	CC-168	2-A-5	CEP-0120	Z16	2-A-5	CEP-0170	CC-A003	2-A-5	DH	CC
21cm pot	CEP-0021	CC-168	2-B-1	CEP-0121	Z16	2-B-1	CEP-0171	CC-A003	2-B-1	DH	CC
21cm pot	CEP-0022	CC-168	2-B-2	CEP-0122	Z16	2-B-2	CEP-0172	CC-A003	2-B-2	DH	CC
21cm pot	CEP-0023	CC-168	2-B-3	CEP-0123	Z16	2-B-3	CEP-0173	CC-A003	2-B-3	DH	CC
21cm pot	CEP-0024	CC-168	2-B-4	CEP-0124	Z16	2-B-4	CEP-0174	CC-A003	2-B-4	DH	CC
21cm pot	CEP-0025	CC-168	2-B-5	CEP-0125	Z16	2-B-5	CEP-0175	CC-A003	2-B-5	DH	CC
21cm pot	CEP-0026	CC-168	2-C-1	CEP-0126	Z16	2-C-1	CEP-0176	CC-A003	2-C-1	DH	CC
21cm pot	CEP-0027	CC-168	2-C-2	CEP-0127	Z16	2-C-2	CEP-0177	CC-A003	2-C-2	DH	CC
21cm pot	CEP-0028	CC-168	2-C-3	CEP-0128	Z16	2-C-3	CEP-0178	CC-A003	2-C-3	DH	CC
21cm pot	CEP-0029	CC-168	2-C-4	CEP-0129	Z16	2-C-4	CEP-0179	CC-A003	2-C-4	DH	CC
21cm pot	CEP-0030	CC-168	2-C-5	CEP-0130	Z16	2-C-5	CEP-0180	CC-A003	2-C-5	DH	CC
21cm pot	CEP-0031	CC-168	3-A-1	CEP-0131	Z16	3-A-1	CEP-0181	CC-A003	3-A-1	DH	CC
21cm pot	CEP-0032	CC-168	3-A-2	CEP-0132	Z16	3-A-2	CEP-0182	CC-A003	3-A-2	DH	CC
21cm pot	CEP-0033	CC-168	3-A-3	CEP-0133	Z16	3-A-3	CEP-0183	CC-A003	3-A-3	DH	CC
21cm pot	CEP-0034	CC-168	3-A-4	CEP-0134	Z16	3-A-4	CEP-0184	CC-A003	3-A-4	DH	CC
21cm pot	CEP-0035	CC-168	3-A-5	CEP-0135	Z16	3-A-5	CEP-0185	CC-A003	3-A-5	DH	CC
21cm pot	CEP-0036	CC-168	3-B-1	CEP-0136	Z16	3-B-1	CEP-0186	CC-A003	3-B-1	DH	CC
21cm pot	CEP-0037	CC-168	3-B-2	CEP-0137	Z16	3-B-2	CEP-0187	CC-A003	3-B-2	DH	CC
21cm pot	CEP-0038	CC-168	3-B-3	CEP-0138	Z16	3-B-3	CEP-0188	CC-A003	3-B-3	DH	CC
21cm pot	CEP-0039	CC-168	3-B-4	CEP-0139	Z16	3-B-4	CEP-0189	CC-A003	3-B-4	DH	CC
21cm pot	CEP-0040	CC-168	3-B-5	CEP-0140	Z16	3-B-5	CEP-0190	CC-A003	3-B-5	DH	CC
21cm pot	CEP-0041	CC-168	3-C-1	CEP-0141	Z16	3-C-1	CEP-0191	CC-A003	3-C-1	DH	CC
21cm pot	CEP-0042	CC-168	3-C-2	CEP-0142	Z16	3-C-2	CEP-0192	CC-A003	3-C-2	DH	CC
21cm pot	CEP-0043	CC-168	3-C-3	CEP-0143	Z16	3-C-3	CEP-0193	CC-A003	3-C-3	DH	CC
21cm pot	CEP-0044	CC-168	3-C-4	CEP-0144	Z16	3-C-4	CEP-0194	CC-A003	3-C-4	DH	CC
21cm pot	CEP-0045	CC-168	3-C-5	CEP-0145	Z16	3-C-5	CEP-0195	CC-A003	3-C-5	DH	CC
21cm pot	CEP-0046	CC-168	Backup 1	CEP-0146	Z16	Backup 1	CEP-0196	CC-A003	Backup 1	DH	CC
21cm pot	CEP-0047	CC-168	Backup 2	CEP-0147	Z16	Backup 2	CEP-0197	CC-A003	Backup 2	DH	CC
21cm pot	CEP-0048	CC-168	Backup 3	CEP-0148	Z16	Backup 3	CEP-0198	CC-A003	Backup 3	DH	CC
21cm pot	CEP-0049	CC-168	Backup 4	CEP-0149	Z16	Backup 4	CEP-0199	CC-A003	Backup 4	DH	CC
21cm pot	CEP-0050	CC-168	Backup 5	CEP-0150	Z16	Backup 5	CEP-0200	CC-A003	Backup 5	DH	CC

Group	Plot	Description	Position	Plot	Description	Position	Pedigree Item	Genotype
21cm pot	CEP-0201	PC-101	1-A-1	CEP-0216	PC-184	1-A-1	DH	PC
21cm pot	CEP-0202	PC-101	1-B-1	CEP-0217	PC-184	1-B-1	DH	PC
21cm pot	CEP-0203	PC-101	1-C-1	CEP-0218	PC-184	1-C-1	DH	PC
21cm pot	CEP-0204	PC-101	2-A-1	CEP-0219	PC-184	2-A-1	DH	PC
21cm pot	CEP-0205	PC-101	2-B-1	CEP-0220	PC-184	2-B-1	DH	PC
21cm pot	CEP-0206	PC-101	2-C-1	CEP-0221	PC-184	2-C-1	DH	PC
21cm pot	CEP-0207	PC-101	3-A-1	CEP-0222	PC-184	3-A-1	DH	PC
21cm pot	CEP-0208	PC-101	3-B-1	CEP-0223	PC-184	3-B-1	DH	PC
21cm pot	CEP-0209	PC-101	3-C-1	CEP-0224	PC-184	3-C-1	DH	PC
21cm pot	CEP-0210	PC-101	Backup 1	CEP-0225	PC-184	Backup 1	DH	PC
21cm pot	CEP-0211	PC-101	Backup 2	CEP-0226	PC-184	Backup 2	DH	PC
21cm pot	CEP-0212	PC-101	Backup 3	CEP-0227	PC-184	Backup 3	DH	PC
21cm pot	CEP-0213	PC-101	Backup 4	CEP-0228	PC-184	Backup 4	DH	PC
21cm pot	CEP-0214	PC-101	Backup 5	CEP-0229	PC-184	Backup 5	DH	PC
21cm pot	CEP-0215	PC-101	Backup 6	CEP-0230	PC-184	Backup 6	DH	PC
21cm pot	CEP-0231	PC-175	1-A-1	CEP-0246	PC-glu024	1-A-1	DH	PC
21cm pot	CEP-0232	PC-175	1-B-1	CEP-0247	PC-glu024	1-B-1	DH	PC
21cm pot	CEP-0233	PC-175	1-C-1	CEP-0248	PC-glu024	1-C-1	DH	PC
21cm pot	CEP-0234	PC-175	2-A-1	CEP-0249	PC-glu024	2-A-1	DH	PC
21cm pot	CEP-0235	PC-175	2-B-1	CEP-0250	PC-glu024	2-B-1	DH	PC
21cm pot	CEP-0236	PC-175	2-C-1	CEP-0251	PC-glu024	2-C-1	DH	PC
21cm pot	CEP-0237	PC-175	3-A-1	CEP-0252	PC-glu024	3-A-1	DH	PC
21cm pot	CEP-0238	PC-175	3-B-1	CEP-0253	PC-glu024	3-B-1	DH	PC
21cm pot	CEP-0239	PC-175	3-C-1	CEP-0254	PC-glu024	3-C-1	DH	PC
21cm pot	CEP-0240	PC-175	Backup 1	CEP-0255	PC-glu024	Backup 1	DH	PC
21cm pot	CEP-0241	PC-175	Backup 2	CEP-0256	PC-glu024	Backup 2	DH	PC
21cm pot	CEP-0242	PC-175	Backup 3	CEP-0257	PC-glu024	Backup 3	DH	PC
21cm pot	CEP-0243	PC-175	Backup 4	CEP-0258	PC-glu024	Backup 4	DH	PC
21cm pot	CEP-0244	PC-175	Backup 5	CEP-0259	PC-glu024	Backup 5	DH	PC
21cm pot	CEP-0245	PC-175	Backup 6	CEP-0260	PC-glu024	Backup 6	DH	PC

Appendix 2: The sampling information of all plants in the greenhouse experiment.

Central leaf

Week	Date	CC168	Z16	A003
1	11-9-2014	green box: 1 sample (1 plant)	green box: 1 sample (1 plant)	green box: 1 sample (1 plant)
2	17-9-2014	17cm pots, sp1: 3 samples (3 plants mix/sample)	17cm pots, sp1: 3 samples (3 plants mix/sample)	17cm pots, sp1: 3 samples (3 plants mix/sample)
3	26-9-2014	21cm pots, BP1: 3 samples (3 plants mix/sample)	21cm pots, BP1: 3 samples (3 plants mix/sample)	21cm pots, BP1: 3 samples (3 plants mix/sample)
4	3-10-2014	21cm pots, BP2: 3 samples (3 plants mix/sample)	21cm pots, BP2: 3 samples (3 plants mix/sample)	21cm pots, BP2: 3 samples (3 plants mix/sample)
5	10-10-2014	21cm pots, BP3: 3 samples (3 plants mix/sample)	21cm pots, BP3: 3 samples (3 plants mix/sample)	21cm pots, BP3: 3 samples (3 plants mix/sample)
6	16-10-2014	21cm pots, BP4: 3 samples (3 plants mix/sample)	21cm pots, BP4: 3 samples (3 plants mix/sample)	21cm pots, BP4: 3 samples (3 plants mix/sample)
7	23-10-2014	21cm pots, BP5: 3 samples (3 plants mix/sample)	21cm pots, BP5: 3 samples (3 plants mix/sample)	21cm pots, BP5: 3 samples (3 plants mix/sample)
8	30-10-2014	21cm pots, BP6: 3 samples (3 plants mix/sample)	21cm pots, BP6: 3 samples (3 plants mix/sample)	21cm pots, BP6: 3 samples (3 plants mix/sample)
9	6-11-2014			21cm pots, BP7: 3 samples (3 plants mix/sample)
10	13-11-2014			21cm pots, BP8: 3 samples (3 plants mix/sample)
11	20-11-2014			21cm pots, BP9: 3 samples (3 plants mix/sample)
12	27-11-2014			21cm pots, BP10: 3 samples (3 plants mix/sample)
13	4-12-2014			21cm pots, BP11: 3 samples (3 plants mix/sample)
Total NO.		22	22	37

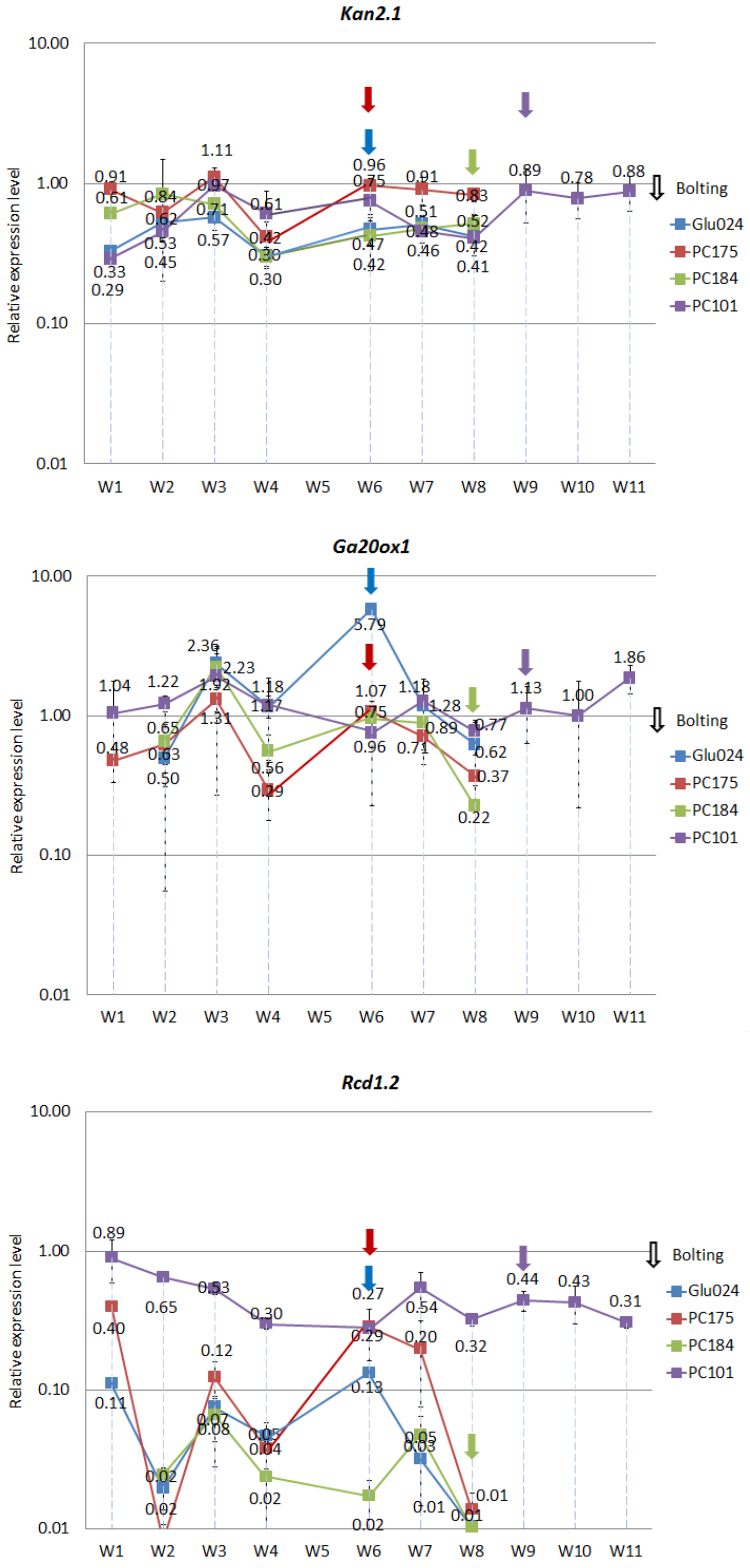
Central leaf

Week	Date	PC024	PC175	PC184	PC101
1	11-9-2014	green box: 1 sample (1 plant)	green box: 1 sample (1 plant)	green box: 1 sample (1 plant)	green box: 1 sample (1 plant)
2	17-9-2014	17cm pots, sp1: 3 samples (3 plants mix/sample)	17cm pots, sp1: 3 samples (3 plants mix/sample)	17cm pots, sp1: 3 samples (3 plants mix/sample)	17cm pots, sp1: 3 samples (3 plants mix/sample)
3	26-9-2014	21cm pots, BP1: 3 samples (3 plants mix/sample)	21cm pots, BP1: 3 samples (3 plants mix/sample)	21cm pots, BP1: 3 samples (3 plants mix/sample)	21cm pots, BP1: 3 samples (3 plants mix/sample)
4	3-10-2014			21cm pots, BP2: 3 samples (3 plants mix/sample)	21cm pots, BP2: 3 samples (3 plants mix/sample)
5	10-10-2014	21cm pots, BP3: 3 samples (3 plants mix/sample)	21cm pots, BP3: 3 samples (3 plants mix/sample)		
6	16-10-2014	21cm pots, BP4: 3 samples (3 plants mix/sample)	21cm pots, BP4: 3 samples (3 plants mix/sample)	21cm pots, BP4: 3 samples (3 plants mix/sample)	21cm pots, BP4: 3 samples (3 plants mix/sample)
7	23-10-2014	21cm pots, BP5: 3 samples (3 plants mix/sample)	21cm pots, BP5: 3 samples (3 plants mix/sample)	21cm pots, BP5: 3 samples (3 plants mix/sample)	21cm pots, BP5: 3 samples (3 plants mix/sample)
8	30-10-2014	21cm pots, BP6: 3 samples (3 plants mix/sample)	21cm pots, BP6: 3 samples (3 plants mix/sample)	21cm pots, BP6: 3 samples (3 plants mix/sample)	21cm pots, BP6: 3 samples (3 plants mix/sample)
9	6-11-2014				21cm pots, BP7: 3 samples (3 plants mix/sample)
10	13-11-2014				21cm pots, BP8: 3 samples (3 plants mix/sample)
11	20-11-2014				21cm pots, BP9: 3 samples (3 plants mix/sample)
12	27-11-2014				
13	4-12-2014				
Total NO.		19	19	19	25

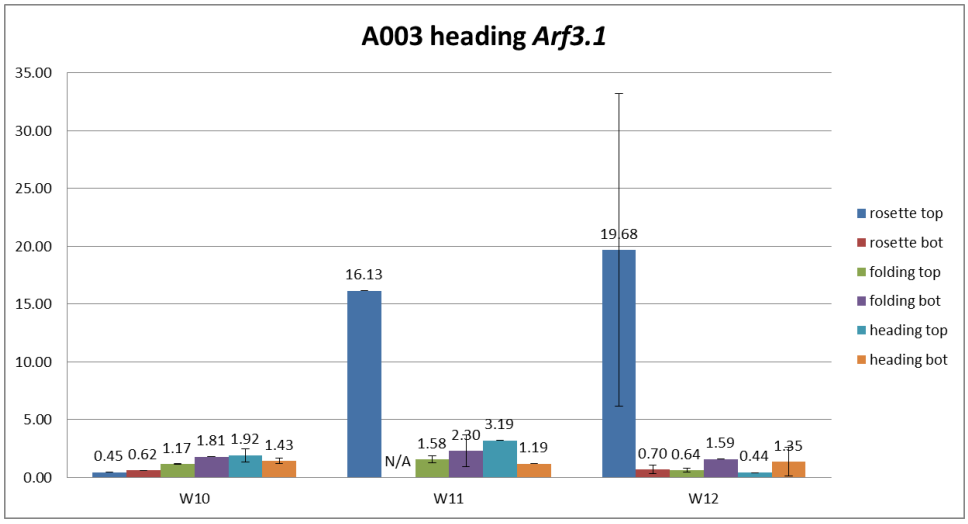
Heading leaf

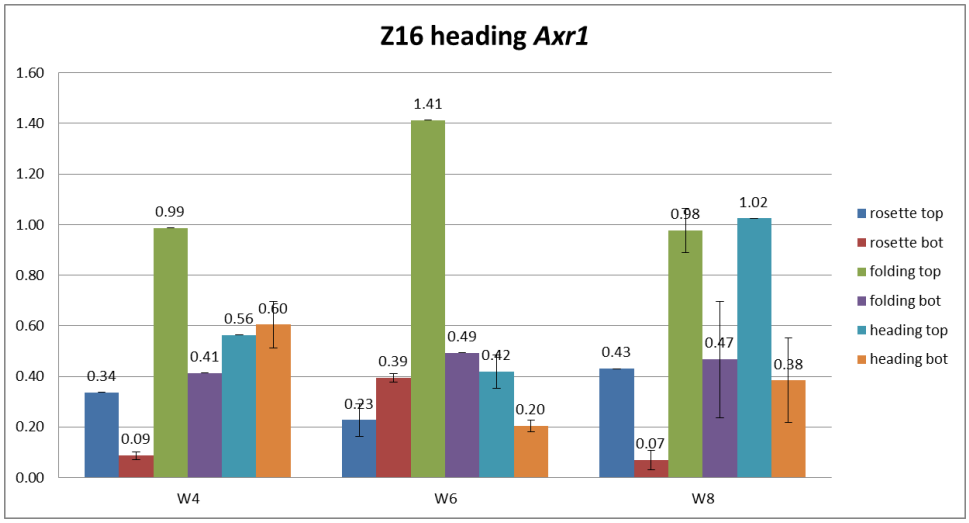
Week	Date	Z16	A003
4	3-10-2014	21cm pots, BP2: 27 samples (3 plants mix/sample)	
5	10-10-2014	21cm pots, BP3: 27 samples (3 plants mix/sample)	
6	16-10-2014	21cm pots, Bp4: 27 samples (3 plants mix/sample)	
7	23-10-2014	21cm pots, BP5: 27 samples (3 plants mix/sample)	
8	30-10-2014	21cm pots, BP6: 27 samples (3 plants mix/sample)	
9	6-11-2014		
10	13-11-2014		21cm pots, BP8: 27 samples (3 plants mix/sample)
11	20-11-2014		21cm pots, BP9: 27 samples (3 plants mix/sample)
12	27-11-2014		21cm pots, BP10: 27 samples (3 plants mix/sample)
13	4-12-2014		BP11: 27 samples (2 17 cm` plants mix/sample) + 1 block x 3 kinds leaves x 3 positions=9 samples (1 21cm` plant)
Total NO.		135	117

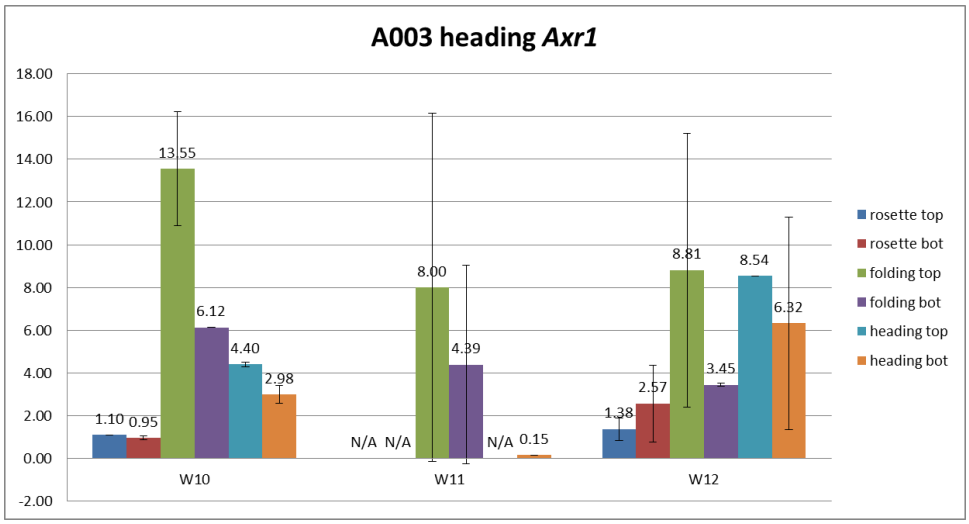
Appendix 3: Relative expression levels of three candidate genes in four PC genotypes through their growth stages. All compares to the reference gene *Actin* and the reference samples taken at week 2 of Z16, *Br-KAN2.1*; *Br-Ga20ox1*; *Br-Rcd1.2*. The scale of Y-axis had been logarithmic scaled base on 10 (candidate genes` expression data are missing at week 5 for all PC genotypes).

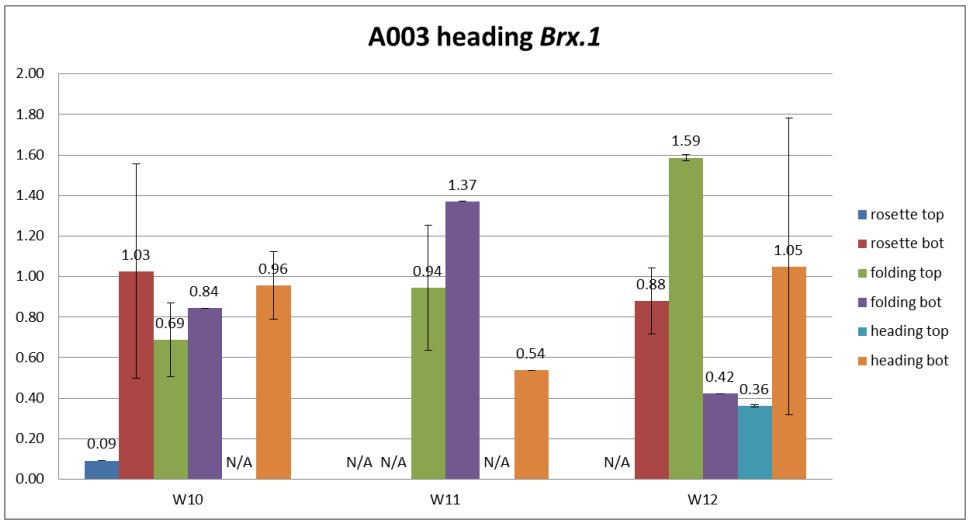


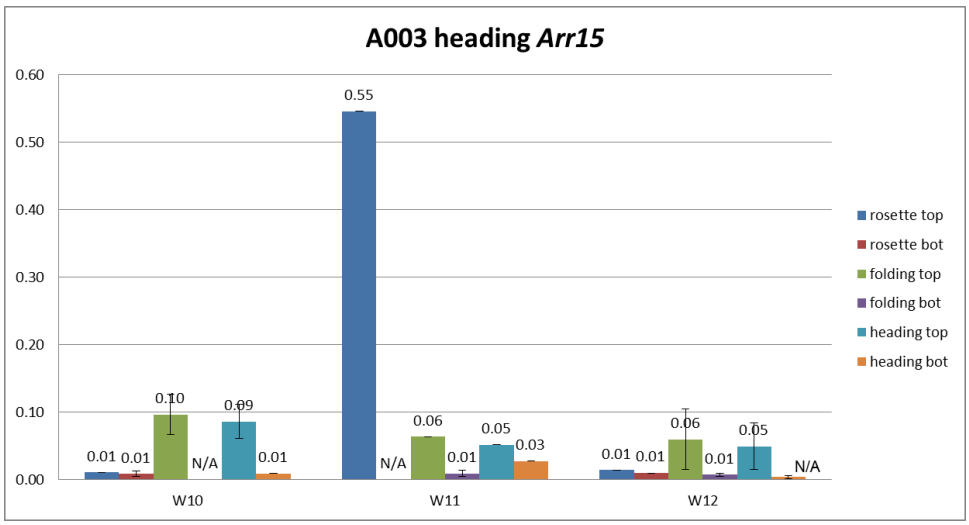
Appendix 4: Relative expression levels of candidate genes in the heading leaf samples for Z16 and A003. All compared to the reference gene *Actin* and the reference samples taken at week 2 of Z16. Different columns'` colours indicate six kinds of heading samples taking from two positions (top and bottom) at three types of leaves (rosette, folding and heading). *Br-Arf3.1*, *Br-Axr1*, *Br-Brx.1*, *Br-Arr15*, *Br-Ga20ox1* in A003 heading samples; *Br-Axr1* in Z16 heading samples.

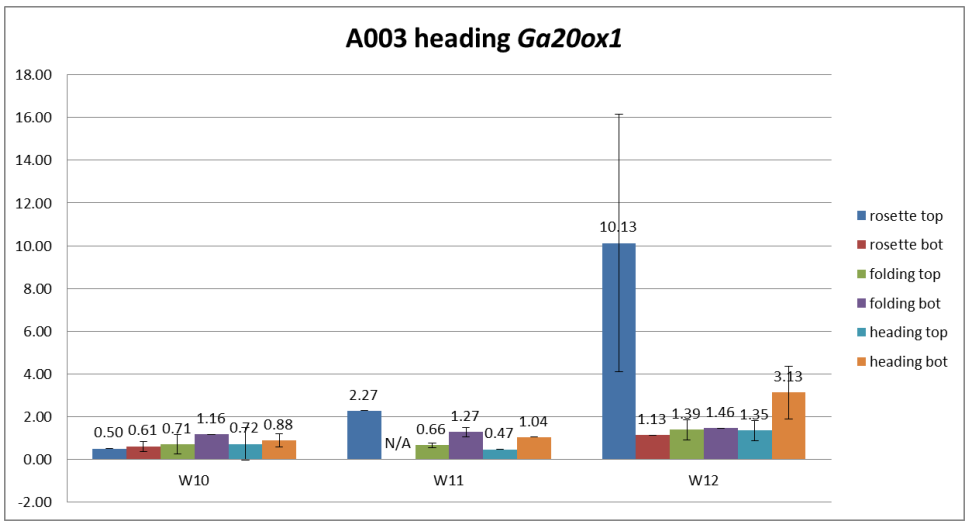







A003 heading *Brx.1*
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.09
1.03
0.69
0.84
N/A
0.96
N/A N/A
0.94
1.37
N/A
0.54
N/A
0.88
1.59
0.42
0.36
1.05
W10
W11
W12
rosette top
rosette bot
folding top
folding bot
heading top
heading bot


A003 heading *Arr15*
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.01 0.01
0.10
N/A
0.09
0.01
0.55
N/A
0.06
0.01
0.05
0.03
0.01 0.01
0.06
0.01
0.05
N/A
W10
W11
W12
rosette top
rosette bot
folding top
folding bot
heading top
heading bot


A003 heading *Ga20ox1*
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
0.50 0.61 0.71 1.16 0.72 0.88
2.27
N/A
0.66
1.27
0.47
1.04
10.13
1.13
1.39
1.46
1.35
3.13
W10
W11
W12
rosette top
rosette bot
folding top
folding bot
heading top
heading bot