(In)Sensitivity towards High Ambient Temperature in Cauliflower: Confirmation and Continuation

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(In)Sensitivity towards High Ambient Temperature in Cauliflower: Confirmation and Continuation

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Contents

Abstract

Flowering time control has been a much focused topic in the recent years and much has been described in model plants like *Arabidopsis.* With the close relationship to the model plant, however, the *Brassica* crops are less studied in this topic. Furthermore, it is of great scientific and economic importance to investigate the floral behaviour of *Brassica* crops like cauliflower under different stress conditions. It has been reported that increased ambient temperature may cause delayed curd formation in cauliflower and such delay may cause economical loss of the growers. In this thesis we investigated the effect of a 7-day heat treatment (27/22 ^{o}C, day/night) on the growth of cauliflower variety Lindurian and Fremont in the 5th week followed by expression analysis of key floral regulating genes like ***FLC SOC1*** etc. to understand the molecular mechanism behind the delay. We found that the heat treated plants from both cultivars showed delayed switch of meristem growth. Further gene expression analysis showed that ***FLC, SOC1*** and ***LFY*** were not affected by the heat treatment, while ***FUL*** and ***AGL24*** appeared to be repressed and delayed in their expression in meristems of the heat treated group. These results may suggest that the increased ambient temperature delays cauliflower curd formation not by directly affecting integrator genes like ***FLC*** and ***SOC1***, but (partly) by negatively regulate those floral promoting genes. This study also provided information for further research to identify putative genes involved in the heat response or the ambient temperature pathway of flowering time control. In this study we found that the 24-hour and the 7-day time point were not optimum to evaluate the effect of heat treatment on gene expressions. Recommendations on further investigations were also discussed in this thesis.

Introduction

Brassicaceae

The Brassicaceae, also known as the mustard family or the crucifers family, is a widely distributed family of angiosperm plant kingdom and it consists more than 330 genera and 37000 species (Price et al., 1994). The Brassicaceae family is of agricultural and scientific importance. Contributing the most to the agricultural importance, genera *Brassica* consists some of the worlds' most important vegetables like cabbage, cauliflower, broccoli,. Also it includes economic crops like oilseed rape. *Arabidopsis thaliana*, which is also a member of the Brassicaceae family, is a model plant used in almost every aspect of plant-related researches.

Arabidopsis

The genus Arabidopsis contains nice species and eight subspecies. Among these species, A. thaliana is the first plant and third multicellular organism to be completely sequenced and is being widely used as model plant in almost every discipline of biological studies (Koch et al., 2008). Arabidopsis is being used as a model plant for its relatively small genome size and chromosome number (~157mb, n=5), short generation time and ease of conducting physiological and functional studies coupled with simple transformation methods (Buell and Last, 2010).

Brassica

Brassica is a genus of plants in the Brassicaceae and it consists of several important vegetables like head cabbage (*B. oleracea* var. *capitata*), cauliflower (*B. oleracea* var. *botrytis*), Brussels sprout (*B. oleracea* var. *gemmifera*), and Chinese white cabbage (*B. rapa* ssp. *pekinensis*). Apart from the vegetable varieties, the *Brassica* genus also contains economic and industrial varieties. Canola (*B. napus*) is used to produce edible oil and Ethiopian mustard (*B. carinata*) is used as a possible source of biofuels (Cardone et al., 2003). According to Food and Agriculture Organization of the United Nations (FAO), the total production of *Brassica* crops (Chinese/mustard cabbage, pak-choi, white/red/savoy cabbage, Brussels sprouts, collards, kale, kohlrabi, cauliflower and broccoli) in 2012 was 91.4 million tonnes worldwide and 12.3 million tons in the Europe (FAO, 2014).

The *Brassica* genus consists of three diploid species: *B. rapa* (genome composition AA, 2n = 20), *B. nigra* (BB, 2n = 16) and B. oleracea (CC, 2n = 18). The interspecific hybridization and polyploidization across each pair of the diploid species led to the evolution of another three amphidiploid species*: B. juncea* (AABB, 2n = 36), *B. carinata* (BBCC, 2n=34) and *B. oleracea* (AACC, 2n = 38). The genetic relationship was described by Nagaharu U and thus named as the well-known "Triangle of U" (Nagaharu, 1935).

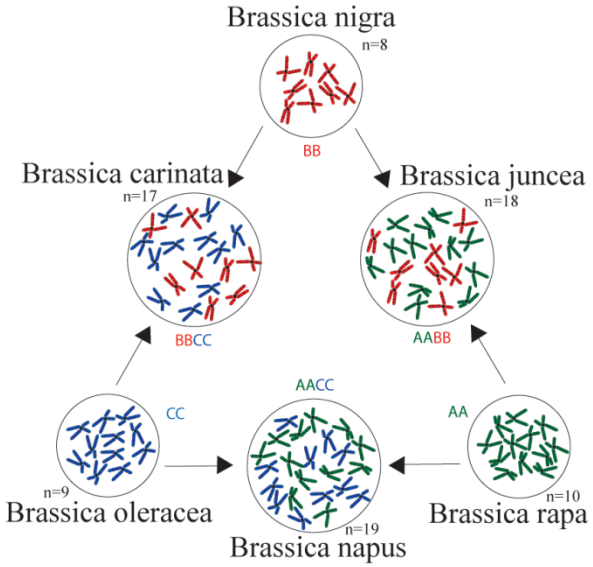

Figure 1 Triangle of U described the genetic relationships between the three amphidiploid species (*B. carinata, B.napus and B. juncea*) and their diploid ancestral species (*B. nigra, B. oleracea and B. rapa*). Different colours and capital letters (A, B and C) represent the chromosome sets originated from each of the three diploid species.

It is believed that the *Brassica* genus is closely related to Arabidopsis during evolution and they diverged about 20 million years ago (Koch et al., 2000). Such close relationship makes genus *Brassica* an excellent system to study genome structure and gene expression in the *Brassica* plants (Lukens et al., 2003).

Brassica oleracea ssp. *Botrytis* (Cauliflower)

B.oleracea, known as wild cabbage in its uncultivated form, is a biennial species that includes many important cole vegetables like cabbage, broccoli and cauliflower. Vegetables of this species are categorized into 7 cultivar groups (Guusje Bonnema et al., 2011) (Figure 2). *B.oleracea* crops contain many nutrients including essential vitamins, amino acids, and minerals and they are largely accepted worldwide as important vegetables.

Cultivar Groups	Acephala	Alboglabra	Botrytis	Capitata	Gemmifera	Gongylodes	Italica
Examples	Kale	Chinese broccoli	Cauliflower	Cabbage	Brussels sprouts	Kohlrabi	Broccoli
Phenotypes							

Figure 2 The *B.olercea* species consists of seven cultivar groups: Acephala, Alboglabra, Botrytis, Capitata, Gemmifera, Gongylodes and Italica. Representative crops of each group are listed.

*B.oleracea.*ssp *Botrytis* (Cauliflower) is an important *Brassica* vegetable. It has a large immature inflorescence known as the curd, which is generally considered as an early arrested stage of indeterminate inflorescence development (Anthony et al., 1996). It is generally recognized that five developmental stages exists between vegetative growth and flowering: (1) the vegetative stage; (2) initiation of inflorescence resulting in formation of secondary meristems in axils of bracts; (3) curd development by the reiteration of meristems, each new meristem giving rise to a higher order; (4) curd maturity with no flower initials; (5) floral differentiation and elongation of some of the inflorescence branches (Anthony et al., 1996). Like many other *Brassica* crops, Cauliflower plants go through a juvenile stage during which plant is unable to a promotory stimulus from the environment (Booij and Struik, 1990). In the case of Cauliflower, curd formation can only occur or be initiated after the end of juvenile stage. Other factors like genetic background and ambient temperature affects the development of curd and thus it is important to understand the effect of such environmental factors on properties such as flowering time during the growth of cauliflower.

Flowering time

Flowering time is a complex trait under the regulation of multiple genes and signal pathways as well as different environmental factors. These environmental factors include photoperiod, nutrient level, ambient temperature etc. and temperature is regarded as one of the most important factors. Change in ambient temperature may lead to uncertainties in the growth of plants and thus affecting the production of crops.

Pathways controlling flowering time have been intensively studied in model plants like *Arabidopsis*. Some over 300 genes have been identified to be involved in flowering time control in Arabidopsis (Fornara et al., 2010). With the help of mutant analysis under different environmental conditions and genetic and molecular interaction studies, many of these genes are generally referred to by the exogenous or endogenous cues they respond to, i.e. the vernalisation, photoperiod, gibberellin and autonomous pathways (Jung and Müller, 2009). Other pathways like Ageing and Ambient temperature also contribute to the whole network of flowering time control (Fornara et al., 2010). Photoperiod is integrated via the control of transcription activation of ***FLOWERING LOCUS T (FT)*** by ***CONSTANS (CO)*** (Turck et al., 2008). The FT protein is transported to meristem through the vascular tissues and activates the transcription of floral meristem identity genes such as ***APETALA (AP1)*** (Wigge et al., 2005). Other studies also indicated that the mRNA of ***FT*** is able to move independently to meristem and to be directly involved in long distance florigenic signalling (Li et al., 2011; Li et al., 2009). Also the photoperiod pathway is closely regulated by circadian clock via controlling the diurnal oscillation in in ***CO*** expression (Suárez-López et al., 2001).

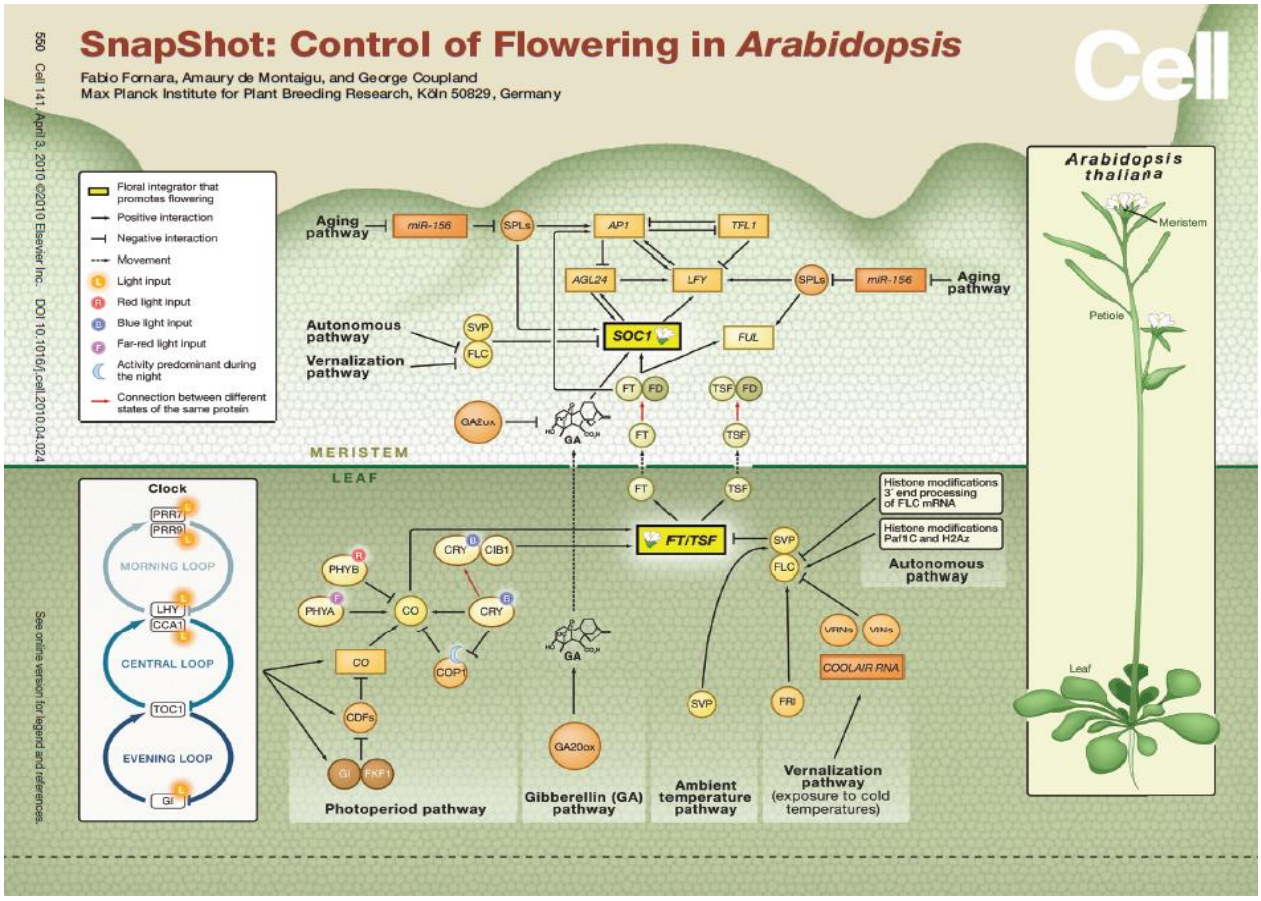

Figure 3 Pathways controlling flowering time in Arabidopsis (Fornara et al., 2010).

Vernalisation is a process developed in plants to ensure the synchronization between flowering time and their favourable environments. ***FLOWERING LOCUS C (FLC)*** is one of the key genes that regulate vernalisation requirements and plants' responses in Arabidopsis. This MADS-box transcription factor itself acts as a repressor of floral transition and its expression is controlled both positively and negatively by various regulators (Figure 3)Figure 3 Pathways controlling flowering time in Arabidopsis (Fornara et al., 2010).. For example, ***FRIGIDA (FRI)*** is a major activator of ***FLC*** explaining much of natural variation in vernalisation requirements in Arabidopsis (Johanson et al., 2000). The activation of ***FLC*** by ***FRI*** and ***FRI***-like genes is dominant over ***FLC*** repression by autonomous pathway genes (Bäurle and Dean, 2006) but can be overcome by vernalisation (Schmitz and Amasino, 2007).

Other pathways are also influential over flowering time control in *Arabidopsis*. For example, Ambient temperature pathway can influence the expression of ***FLC*** through a floral repressor gene ***SHORT VEGETATIVE PHASE (SVP)***. Also genes involved in Gibberellin (GA) pathway have effect on the flowering time. Different sources of input in the control network are integrated by floral integrator genes. For example, ***SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1), FT*** and its

homolog ***TWIN SISTER OF FT (TSF)*** are strong flowering promoters that integrate vernalisation and photoperiod pathways in *Arabidopsis* (Turck et al., 2008). A set of floral meristem identity genes are required for the actual formation of flowers, for example, ***AP1 LEAFY (LFY)*** and ***FRUITFUL (FUL)***.

In cauliflower, the importance of flowering time control is valued in the formation of curd, which is an inflorescence and is the marketable part of the crop. One of the main issues in cauliflower production is that some varieties show a delayed curd formation after exposure to higher ambient temperature. Studies like Duclos and Björkman (2008) and Anthony et al. (1996) proposed that similar to *Arabidopsis*, the genes such as ***LFY, AP1***, ***FUL*** etc. played important role in controlling flower formation in cauliflower. However, the study in cauliflower is less intensive compared to that in *Arabidopsis***.**

Research goals

There are two major parts of this project. One is to assess the effect of high ambient temperature on curd formation of cauliflower. This goal will be achieved by a continuous observation of growth of two cauliflower genotypes (one is sensitive to ambient temperature and the other one not) under both control and heat-treated conditions.

The other goal of this minor thesis is to assess expression levels of genes that are found important in earlier studies in cauliflower. This part serves as a confirmation of former results and it also provides additional information with the analysis from leaf materials

In addition, an RNA-Seq analysis was performed in the research group of Prof. Richard Immink to identify differentially expressed genes in both *Arabidopsis* and cauliflower under different temperatures. An addition goal of this minor thesis also includes sorting the results from this experiment and identifies potential candidates for future studies.

Material and methods

Plant materials

Two common hybrid cauliflower cultivars, i.e. cv. Fremont and cv. Lindurian were used in this study. Both cultivars were suitable for growth in early spring and late summer. Fremont was known for its stable performance under heat stress while Lindurian, in contrast, was more sensitive to heat and may show delayed curd formation. An extra cultivar Skywalker was used as protection lines.

Experiment set-up

Plants from the two cultivars are placed into two greenhouse compartments located in WUR's Unifarm (Wageningen, NL). In each compartment, four blocks (A-D) were divided and 70 plants from the two cultivars were placed randomly in each block (Figure 4). Plants were sown in February 2014. Identical temperature condition (21 °C in the day and 16 °C in the night, 21/16 °C) was applied to both compartment except that a 7-day heat treatment of 27/22 °C was applied in one compartment in Week 5 (i.e. the SWAP group) while the other one remained unchanged (i.e. the Control group). The other environmental factors such as water, fertilization, light, etc. were identical for both compartments.

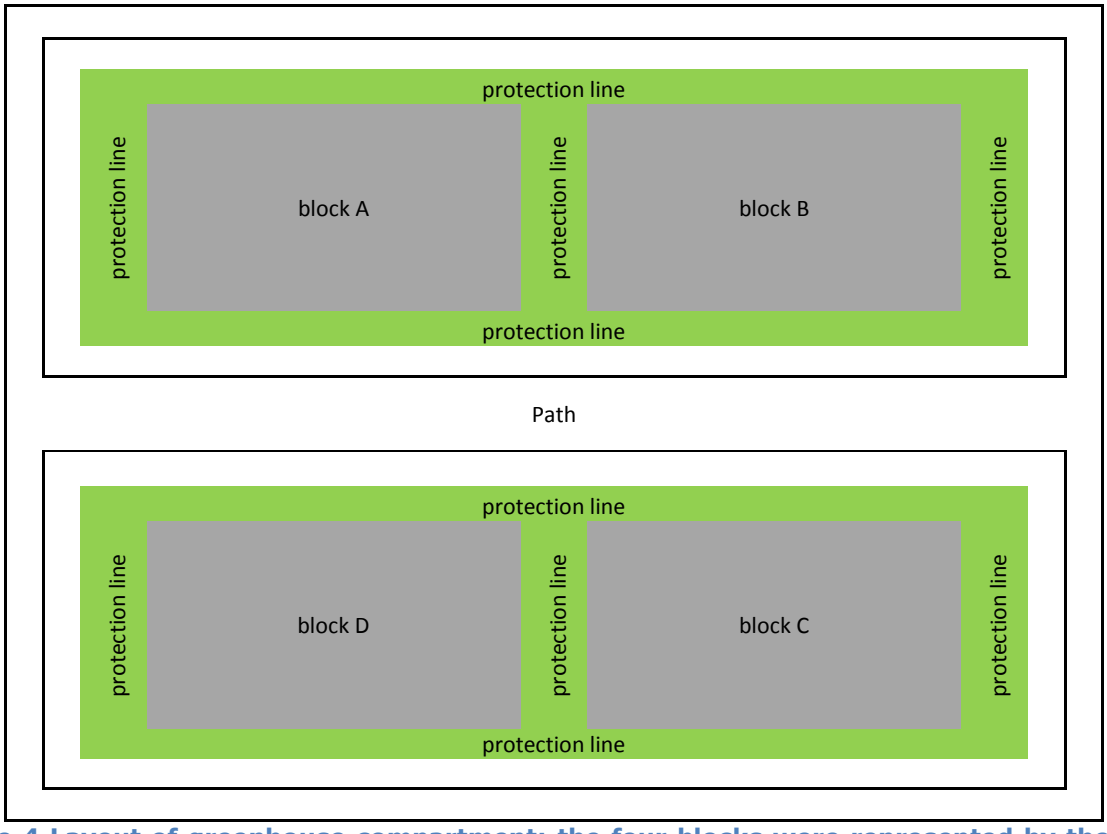

Figure 4 Layout of greenhouse compartment: the four blocks were represented by the grey box, in which Lindurian and Fremont plants were mixed and placed randomly. A row of cultivar Skywalker was placed around each block as protection line. The same layout was applied for both control and SWAP groups.

Meristem observation

The developmental stage of the apical meristem was identified by stereomicroscope observation every Tuesday at 11:00 am until week 16. During observation, leaves covering meristem were carefully removed and growth stages of the meristem were determined according to the pre-set references (Figure 5).

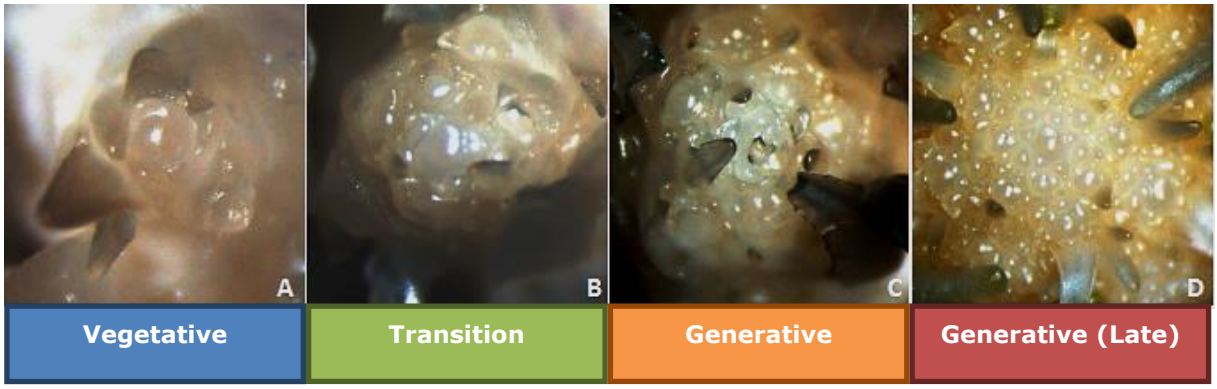

Figure 5 Three stages of the cauliflower shoot apical meristem development. A. In the vegetative stage, the apex is surrounded by a triangle shape. B. During the transition the triangle shape is broken. C and D: in the generative stage the multiple domes are formed resulting in the typical curd formation ((Lubbers, 2013))

Tissue harvest

During the weekly meristem observation, the tip of meristem (meristem enriched tissue) were cut off and immediately frozen in liquid nitrogen in a labelled 1.5 ml screw-cap tube. The first fully formed leave from the same plant was also sampled and stored in a corresponding tube in liquid nitrogen. For each leaf, a mixture of tissues from tip, middle and stem part of the leaf were taken. These samples were stored at -80°C and will were later used for gene expression analysis.

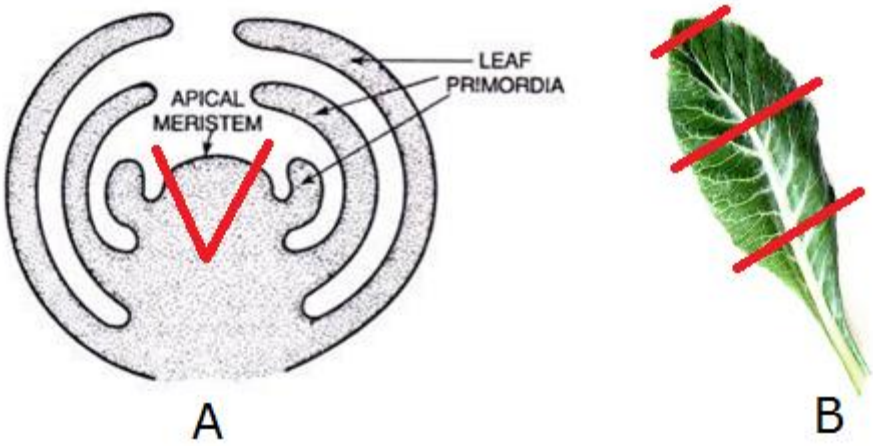

Figure 6 Illustration of sampling methods for meristem (A) and leaf(B) materials. Red line indicates the cutting position.(Image source: yourarticlelibrary.com and letzcook.blogspot.nl).

RNA isolation and cDNA synthesis

Frozen meristem tissues were grinded in the tube using a special grinding tool and the frozen leaf materials were grinded in the tube with 2 stainless steel balls using TissueLyser II (Qiagen) at 30 Hz for 2 minutes.

Total RNA was isolated from the grinded tissues using "RNeasy kit" (Qiagen), following the "Plants and Fungi" protocol described by the manufacturer. DNA contamination was removed using RNase-free DNase I treatment (Invitrogen) following manufacturer's instructions. After DNase treatment, the samples were purified with the "RNeasy kit" (Qiagen) using the 'RNA clean-up' protocol described by manufacturer. The concentration and quality of RNA samples were quantified using Nanodrop 1000 spectrophotometer (Thermo Scientific) where an absorption ratio of ~2.0 at 260/280 is considered as pure for RNA samples. RNA quality were also checked using gel electrophorese. The presence of DNA in the RNA samples was also checked using qRT-PCR.

The cleaned RNA samples were diluted with nuclease free water (Qiagen) to 100 ng/µl in a total volume of 10 µl for cDNA synthesis. cDNA synthesis was performed using "iScript™ cDNA Synthesis Kit" (Biorad) following manufacturer's instructions. A final volume of 20 µl per sample was obtained and it was diluted with MQ water at 1:40 ratio to reach ~30 ng/µl for later works.

Primer design

Due to the limited time of this thesis, only Block B, C and D of Lindurian is included in the expression analysis.

Specific primers were designed for a set of reference genes (***GAPDH, EF-1,TIPS-41,CAC,ELF-A,UBQ-2, TUB-6***) and candidate genes (***PA200, COL-5, PRR-1, PRR-3, CRN-1***). Full cDNA sequences were retrieved from *Arabidopsis* database (https://www.arabidopsis.org) and were blasted against *B. oleracea* database (http://www.ocri-genomics.org/bolbase/). Sequence comparisons were conducted using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) for those with multiple orthologous genes and specific primers were designed using Primer3Plus (http://primer3plus.com). Primers from previous studies were also used and these primers includes: ***ACTIN*** (Reference gene), ***SOC1, LFY, AGL24, FUL-a, FUL-c, FLC-1a, FLC-1b, FLC-2, FLC-3, FLC-4.*** Among these genes, the primer of ***ACTIN*** was designed by Johan Bucher and the rest were designed by Xiaoxue Sun.

Quantitative Real-Time PCR

qRT-PCR reactions were performed on 96-well plates using the standard protocol (Figure 7) on CFX96 Thermal Cycler (BioRad). Each well contains a 10 µl system with 5 µl of SYBR Green Supermix, 3.4 µl of MQ water, 0.3 µl of forward primer, 0.3

µl of reverse primer and 1 µl of cDNA. Cq values of each sample were recorded by the machine and was exported into Microsoft Excel for calculation.

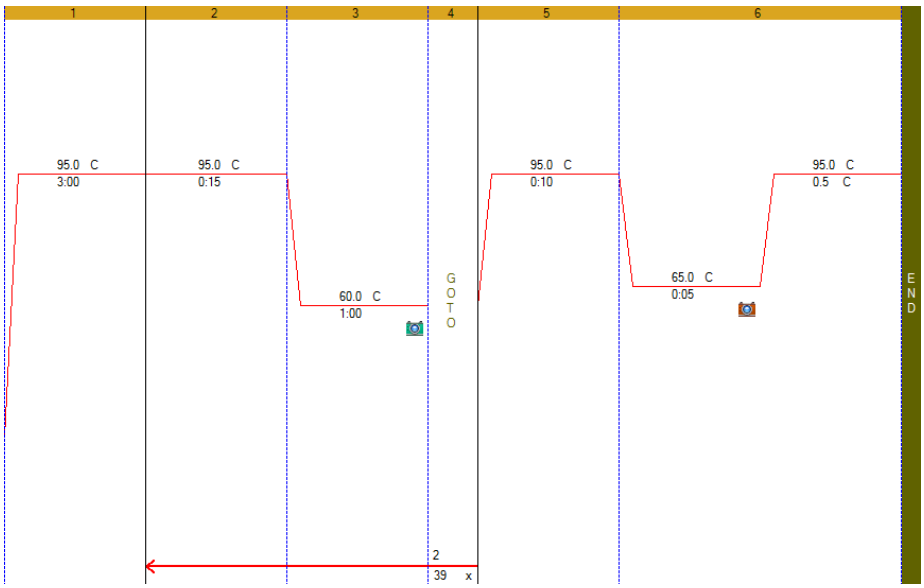

Figure 7 qRP-PCR protocol used in the study: 95°C 3 min, 39 cycles of 95°C 15 sec and 60°C 1 min, 95°C 10 sec, 65°C 5 sec and 95°C.

For the analysis of candidate genes, three biological repeats were included for each time point and reference gene was placed on the same plate every time to avoid errors.

Xiaoxue Sun offered help in the qRT-PCR analysis by running plates for ***SOC1, LFY*** and ***AGL24***.

Data analysis

The difference of expression between the candidate genes and the reference genes were calculated as the following:

$$\Delta Cq = Cq\ (Candidate\ Gene) - Cq\ (Reference\ Gene)$$

And the relative expression level of candidate compared to the reference gene is calculated as

$$Relative\ Expression = 2^{-\Delta Cq}$$

So a lower value stands for a lower expression level compared to that of the reference gene.

Results

Heat treatment led to delayed curd formation in cauliflower

The cauliflower cultivars Lindurian and Fremont were treated with a heat period of 27°C/22°C day/night for 7 days in the 5th week after sowing to study the effect of increased ambient temperature on the two cultivars. The growth stages were recorded weekly by destructively measurement of the meristem of one randomly selected plant from each block in both Control and SWAP compartments.

The two cultivars behaved differently in the experiment (Figure 8). Lindurian is expected to be a sensitive variety towards heat treatment. In control group, plants remained in vegetative stages until week 9. In week 10, 2 plants from block C were grouped in to transition stage and 1 plant was identified as generative in block A. In week 11, only half of the sampled plants were still in vegetative stage. Among the 8 plants sampled in week 12, only 1 plant in block A remained vegetative while the other ones have entered either transition or generative stage, indicating most of the Lindurian plants in the control group had switched away from vegetative stage. This was confirmed by the fact that from this week on, every sampled plant was identified as generative.

Sampled plants remained vegetative until week 11 in the SWAP group. A rapid switch was observed in week 12 with only one vegetative plant observed. From week 14 on, all sampled plants were at generative stage. There was a difference of 2 weeks between the Control and SWAP groups of Lindurian in terms of the length of vegetative stage and 1 week in terms of the week when all sampled plants were generative. The switch from vegetative into lather growth stages were delayed in the SWAP group, to which the heat treatment was applied.

A similar result was observed in Fremont. It remained vegetative until week 7 in the control group and week 9 in the SWAP group. The first transition plants were recorded in Block A and B of control group at the time of week 8. One week later, only two plants in block A of control group remained vegetative while the others were all at transition stage. All sampled plants from control group entered generative stage from week 10 on. Vegetative stage lasted 2 more weeks in the SWAP group and generative stage was reached 1 week later when compared to that in the control group.

From the record of this experiment, it may be concluded that the one-week heat treatment led to a similar delay in meristem growth in both Lindurian and Fremont, regardless the difference in their tolerance against heat stress. However, Lindurian did appear to be more affected by the heat treatment as it showed more instability in the sampling result between vegetative and generative stages. In Fremont, however, the shift appeared more uniform and quicker than that in Lindurian. Another

interesting result is that the heat treatment seemed to accelerate the process of meristem growth in both cultivars. It took Lindurian 3 weeks to completely switch into generative stage in control group while it was 2 weeks in SWAP group. In Fremont it was 2 weeks and 1 week, respectively.

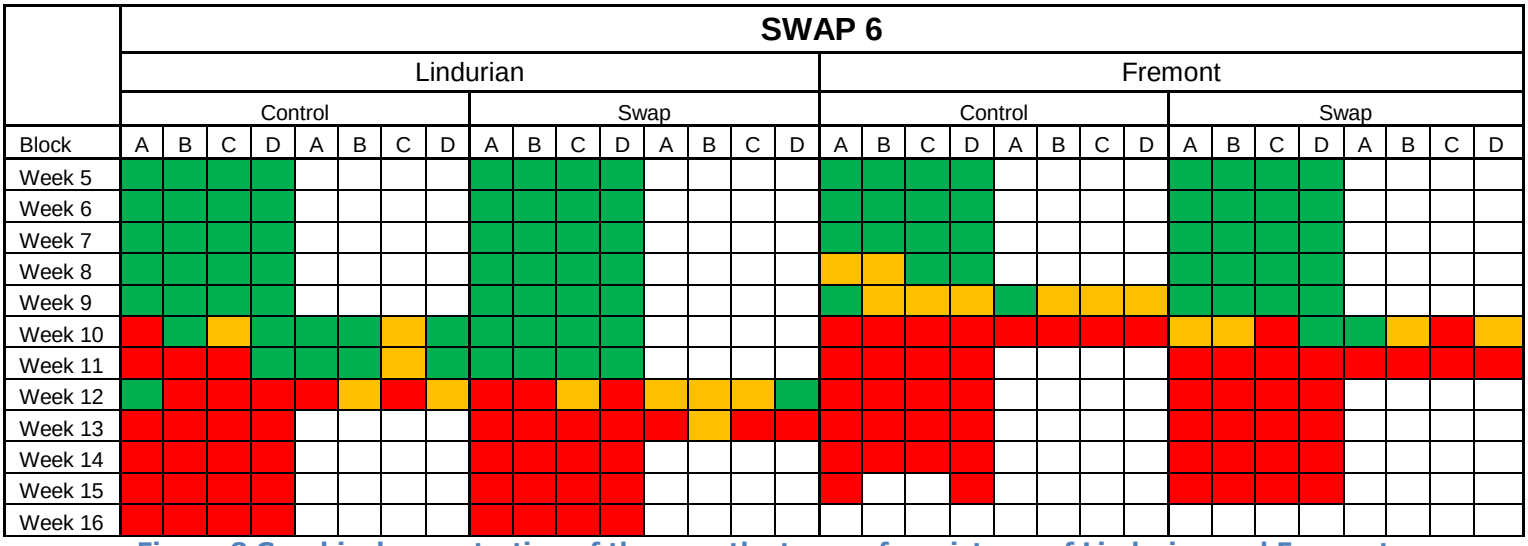

Figure 8 Graphic demonstration of the growth stages of meristems of Lindurian and Fremont during the scale of this study. Green indicates vegetative stage, yellow transition and red indicates generative stage. Double amount of samples were harvested for week 10~ 12 and 12~13 respectively in Control and Swap groups of Lindurian and that of week 9~10 and 10~11 respectively in Fremont.

In previous experiments, block effect was found when analysing growth of the plants. In this study, it was observed that sampled plants from block A and D tended to be delayed when compared to block B and C. For example, in week 11 of Lindurian control group, 3 out of 4 plants from block A and D remained vegetative while only 1 in block B and C. Also a similar situation was found in week 9 of Fremont control group and week 10 of Fremont swap group. Taking the structure of the greenhouse into consideration, such difference may come from the difference in shading as block B and C had slightly more even light condition and less shading from the frame of the building.

Many flowering time genes were found unaffected in the RNA-Seq research

The RNA-Seq study performed in corporation with the research group of Richard Immink generated a list of ~19000 entries of genes detected in both *Arabidopsis* and cauliflower before and after 24 hours of heat treatment with an extra cold treatment in *Arabidopsis*. Bioinformatics processing of these data resulted in a list of genes with their TAIR gene number, fold change and P-value for their expression before and after treatment, together with their annotation information. This list was cross compared with other lists of genes that were summarized from publications and were proven to be involved in flowering time control in *Arabidopsis*.

Eventually, 112 flowering time related genes were found in the RNA-Seq list. These genes included many of the important flowering time regulators such as ***SOC1, FLC, CO, FT, FRI, LFY*** etc. However, only 5 genes were found to be significantly differentially expressed before and after the heat treatment in cauliflower and 21 in *Arabidopsis*. 26 genes were found significantly differentially expressed in the cold-treatment in *Arabidopsis*, among which 14 were also significant in the heat test. Interestingly, among these 14 genes, 5 are involved in photoperiod/circadian clock regulation. For example, expressions of ***CCA1, PRR9*** were both found highly significant in both cold and heat treatment. Both genes were down regulated after heat treatment and were up regulated after exposed to cold. Nevertheless, neither of these genes were found significantly differentially expressed in the experiment of cauliflower.

What's more, we did not find significant change in expression for the major flowering time regulators like ***SOC1, CO, AP1, LFY, FUL, FD, FLC*** etc. before and after the temperature switch in either cauliflower or *Arabidopsis*. Such result suggested that in order to evaluate effect of temperature on the expression levels of these floral genes, a longer interval may be needed as these genes did not tend to change in the early stages of growth.

Expression analysis

As was mentioned before, only samples from Lindurian Block B C and D were used in the expression analysis. Hereon the control and SWAP group mentioned in the report, if not specifically indicated, refers to those from Lindurian.

Selection of reference genes

References are used to normalize expression levels of candidate genes in qRT-PCR experiments. At the beginning of the study, ***Actin*** was tested on the cDNA samples from this experiment. Instead of giving relatively stable Cq values across tissues, it gave a largely fluctuating reading with Cq values ranging from 20~26 in control group and 20~27 in SWAP group. Such instability will cause difficulties for the interpretation of the final results. The quality absorbance ration from Nanodrop indicates that the RNA samples and the corresponding cDNA samples were mostly pure. Also the gel electrophoresis test for a set of randomly selected RNA samples confirmed their qualities. Furthermore, test runs of RNA samples on qRT-PCR with universal reference gene gave no amplification reading, indicating DNA contaminations/debris were removed from the RNA samples.

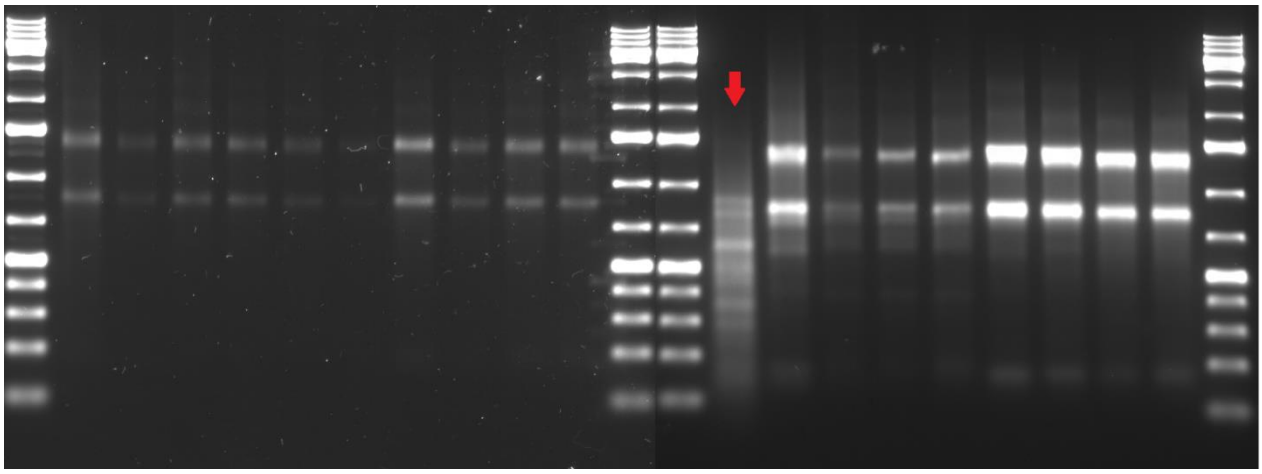

Figure 9 The two bands from agarose gel electrophoresis of a set of randomly picked RNA samples indicated that the isolation and clean-up of RNA samples were successful. The red arrow pointed to a blurred band where a pipetting error occurred during the preparation of gel.

To better understand the instability occurred in the samples, new primers were designed for ***GAPDH, EF-1, TIPS-41, CAC, ELF-A, UBQ-2, TUB-6*** using the *B.oleracea* database *Bolbase.* ***GAPDH, EF-1*** were tested in earlier experiments to be possible candidate references genes in *B. rapa* and ***TIPS-41, CAC, ELF-A, UBQ-2, TUB-6*** were suggested by studies like Brulle et al. (2014) to be suitable for expression analysis of *B. oleracea* materials. ***Actin*** designed for *B. oleracea* was also included in this test.

In most SWAP group samples, Cq values fell within the range expected for each gene (Figure 10). It was obvious that in some of the samples Cq values formed certain patterns. For example, several peaks were observed for both meristem and leaf samples, as was indicated by red arrows in Figure 10. However, the graph clearly showed that these abnormal values were sample-dependent as they were mostly observed for the same set of samples. Similar results were also found for the control group, where the Cq values were also highly sample-dependent (see appendix).

This round of reference gene test provided extra information on the sample quality in the experiment. Ideally, though, it was expected that a reference gene should give stable (±0.5) Cq values, the fact that multiple reference genes behaved in a parallel fashion provided an argument to continue the expression analysis. We assume that the samples that give high/low Cq values in reference genes will give similar change to those in the candidate genes.

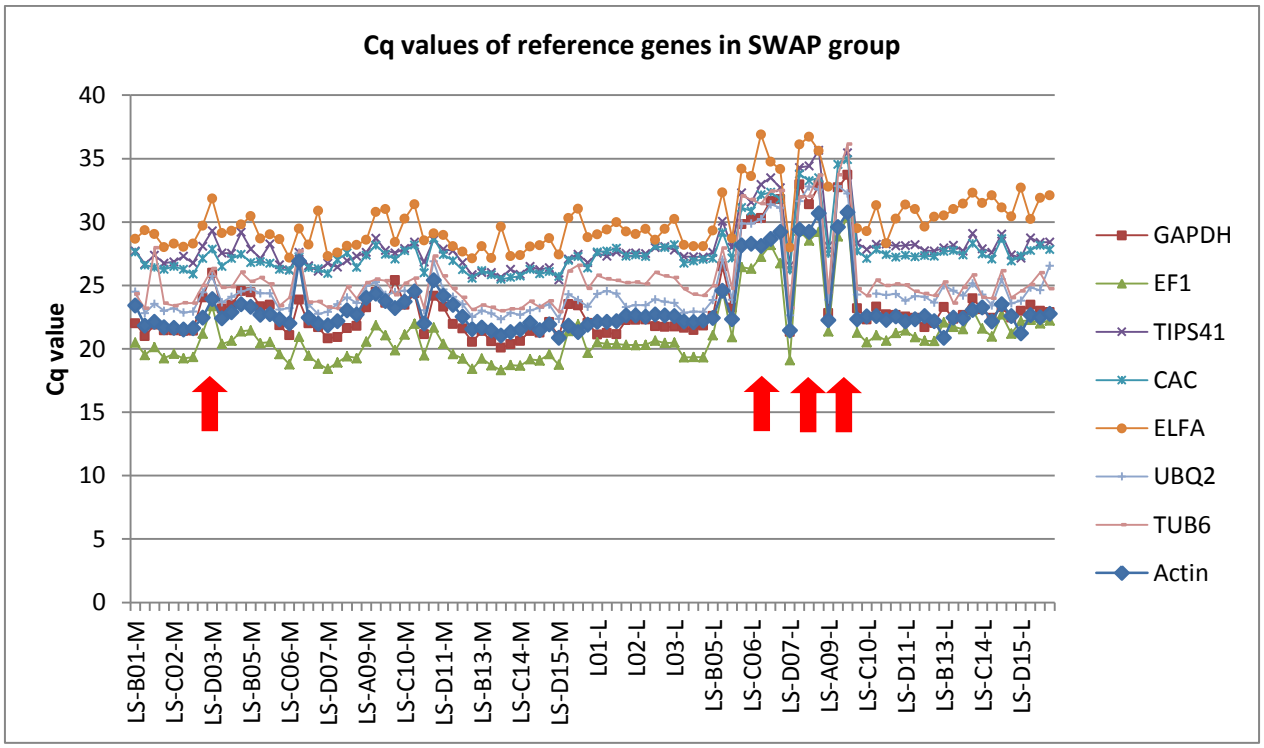

Figure 10 Cq values of 8 candidate reference genes

Having tested the reference genes in all samples, we made the decision of using ***Actin*** as the reference gene in the coming experiment. ***Actin*** gave an expected Cq value at around 21 and the highest "peak" was around 30, providing enough space to adjust for the candidate genes whose expression levels were expected to be lower than that of ***Actin***. Those samples which gave extreme Cq values were marked in the later sections by grey colour (e.g. Figure 13). We decided to keep the data from these samples in the analysis; however, we have to keep in mind when interpenetrating expression results that the samples which gave extreme Cq values may still be normalized in a different scale when compared to an optimum situation with stable reference gene and high quality samples.

Expression of candidate genes were not different within 1-week period

A 7-day heat treatment was applied to SWAP group in the 5th week after sowing. We would like to investigate if the expression levels of the candidate genes were different at the end of the heat treatment.

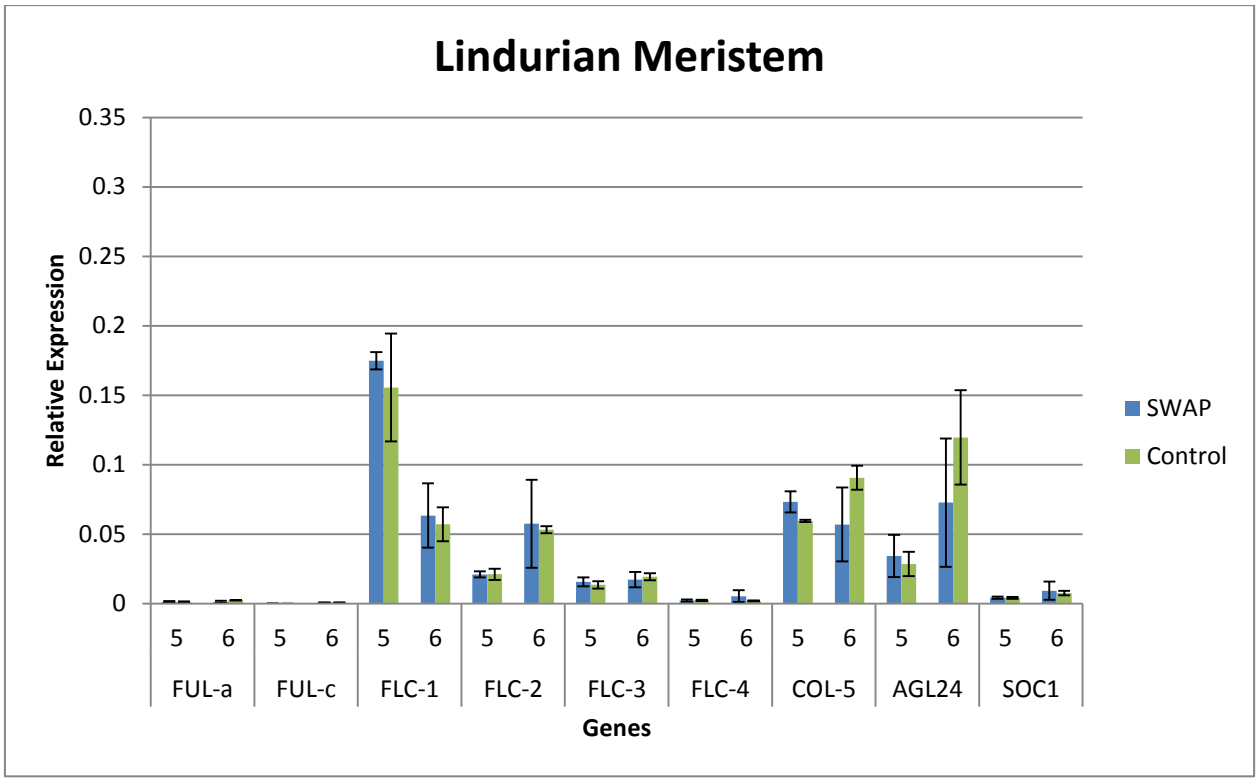

Figure 11 Relative expression levels of candidate genes in week 5 and 6 in meristem sample of Lindurian.

Figure 11 showed the expression level of tested candidate genes in the meristem samples of Lindurian. ***FUL-a, FUL-c, FLC-4, COL-5 and SOC1*** were lowly expressed in the two weeks while the rest genes showed higher expression levels. For all the tested genes, we did not observe a different pattern between SWAP group and Control group. Expression levels of ***FLC-1*** , for example, were significantly lower in both groups after the heat treatment, indicating that the difference was not likely caused by the treatment, but rather the result of an internal fluctuation. ***FLC-2*** showed a reverse pattern as its expression increased in week 6, but no difference was observed between control and SWAP group. ***FLC-3***, unlike the other paralogues, showed no significant difference between the two groups in the two weeks. For ***COL-5***, the expression seemed to differ as it increased in control group while decreasing in SWAP group. However, the difference between week 5 and 6 in SWAP group was not significant. ***AGL24*** showed increased in both control and SWAP group, but the scale of increase was larger in control group than that in SWAP group.

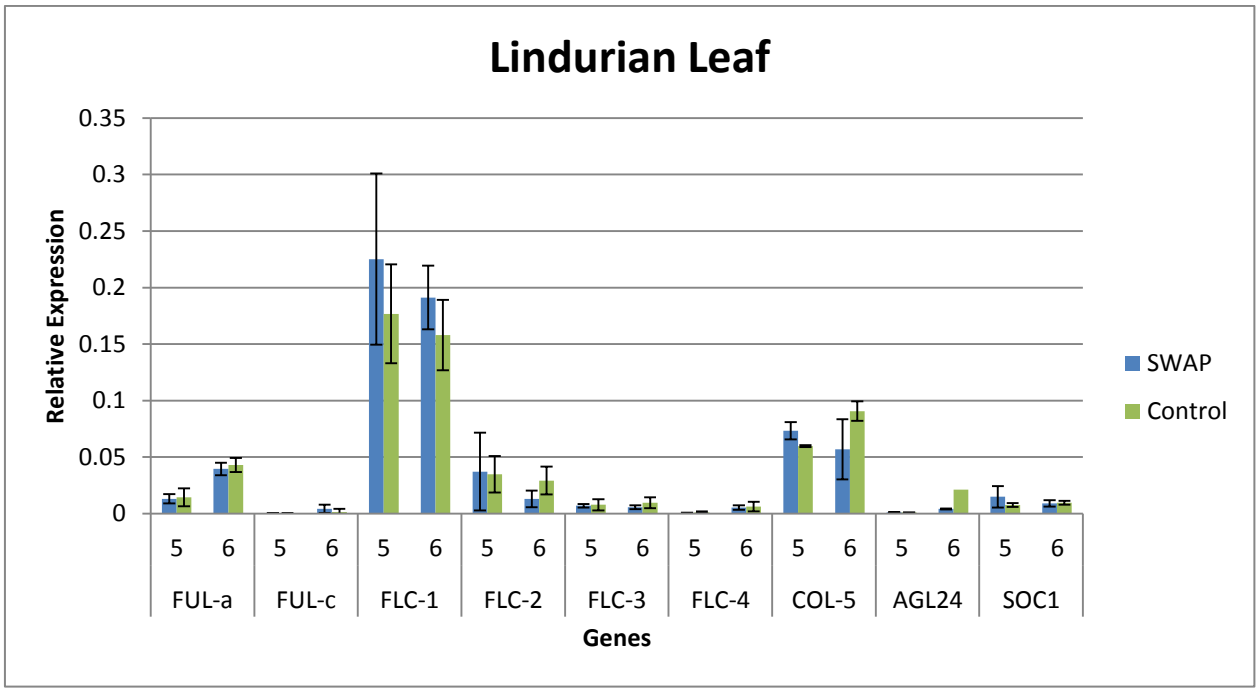

Figure 12 Relative expression levels of candidate genes in week 5 and 6 in leaf sample of Lindurian.

Figure 12 showed the expression of candidate genes in leaf materials in week 5 and 6 of Lindurian. Similarly, no significant difference was observed between the two groups. However, the expression level of ***FLC-1*** and ***COL-5*** appeared to be higher in SWAP groups than that in control groups.

FUL genes showed expression difference between growth stages and were affected by heat

FUL (FRUITFUL) is a floral integrator and flower meristem identity gene that encodes a MADS-box transcription factor. In our earlier studies, it was found that ***FUL*** showed a stage-determined increase in its expression in meristem materials from Fremont ((Sun, 2013). Here we would like to investigate its expression in the more sensitive cultivar Lindurian and examine the expression in both leaf and meristem.

FUL-a and ***FUL-c*** were tested in the experiment. The relative expression of ***FUL-a*** was low, yet still within detectable range, in both meristem and leaf of Lindurian (Figure 13 A and B). In meristem, the expression of ***FUL-a*** stayed low in control group until week 9 when a sharp increase was observed. In the meristems from SWAP group, the expression of ***FUL-a*** remained low until week 10.This was also the beginning of transition stage. It was noticed that the expression of ***FUL-a*** was higher in the control group than that in the SWAP group. So it appeared that the heat treatment influenced both the expression of ***FUL-a*** in meristem tissues in both expression level and expression time. The Expression levels of ***FUL-a*** in leaves were higher than that in meristems. ***FUL-a*** showed a gradually increasing expression from week 1 to week 12 and then the expression was relatively higher in the weeks

of 13 till 16. The differences between control and swap group was not significant in terms of expression levels or the stages. It appeared that the heat mainly had an effect in the expression of ***FUL-a*** in meristem materials.

FUL-c showed a more contrasting result between the two groups and the two tissues compared to its paralogue ***FUL-a*** (Figure 13 C and D). Relative expression of ***FUL-c*** remained undetected until week 8 in meristems of both SWAP and control groups. In control group, the expression level increased slightly in week 8 and 9, followed by a sharp increase in week 10 and maintained high since then. Similarly, the expression increased slightly from week 8 to week 10 and then remained in a higher level since week 12, which was the transition point for the sampled plants from SWAP groups. Meanwhile, the relative expression of ***FUL-c*** in meristem was significantly lower in SWAP group than that in control group. ***FUL-c*** expression remained low in the leaves through the 16 weeks. Regardless of the low expression level in leaves, ***FUL-c*** also showed a similar pattern as that in meristem tissues as its expression went higher after entering the generative stage.

Expression levels of both ***FUL-a*** and ***FUL-c*** increased as the plants entered generative stage. For both genes, the expression in meristems from SWAP group was lower than in those from control group. Meanwhile, a delayed increase was observed for both genes in SWAP meristems. These results indicated that FUL paralogues may be used as indicator genes of meristematic growth in cauliflower. Also the expression analysis indicated that the heat treatment may result in the repression of FUL genes' expression and cause delayed activation of FUL genes, thus eventually causing the delay in curd formation.

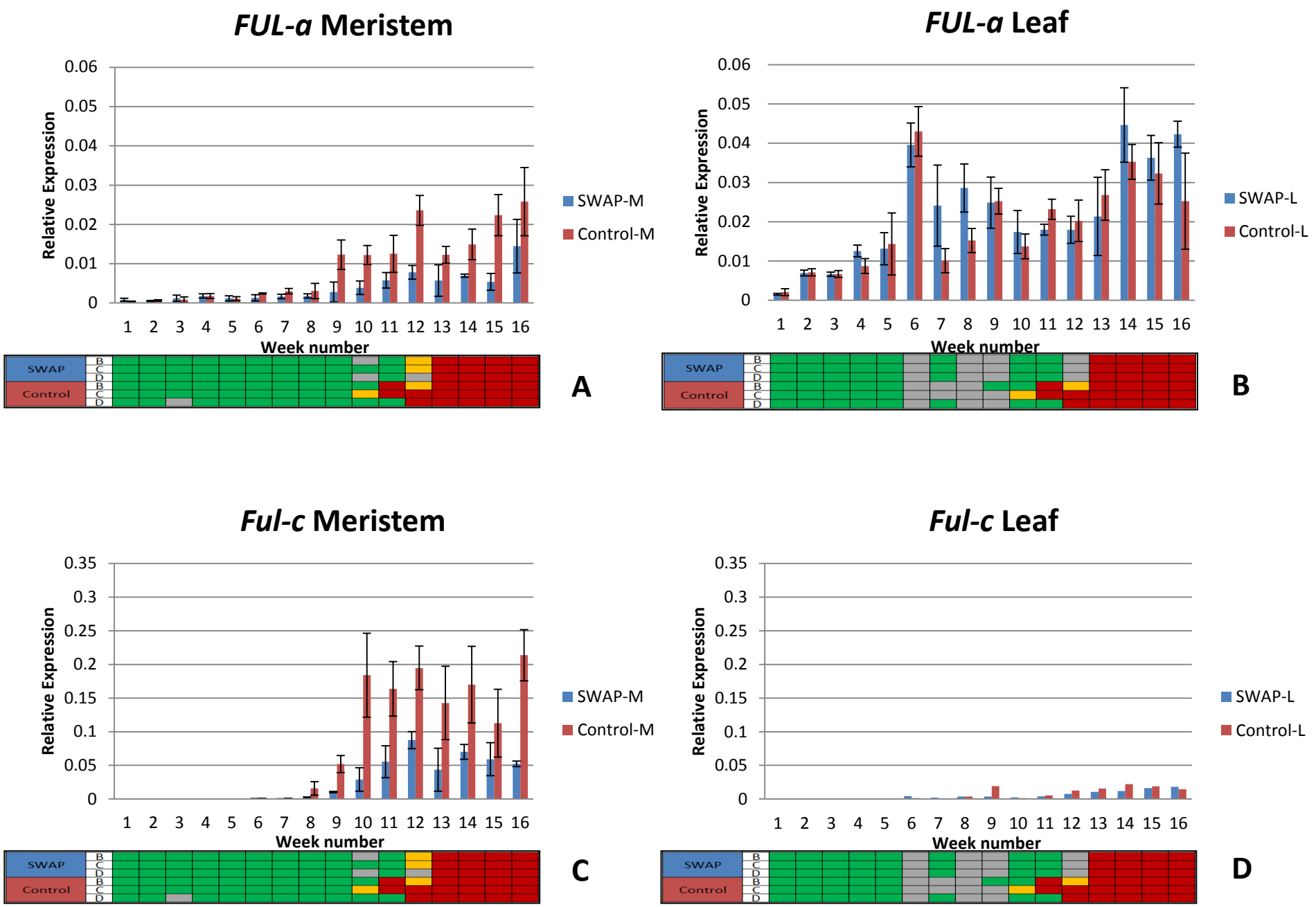

Figure 13 Relative expression levels of *FUL-a* and *FUL-c* in meristem and leaf of Lindurian during the experimental period (week 1 – week 16). The coloured cells under week number indicate the growth stage of the sample: green=vegetative, yellow=transition and red=generative. Grey cell indicates that the sample gave an extremely high/low Cq value of reference gene.

FLC paralogues expressed differentially in cauliflower tissues

FLC is an important MADS box transcription factor that represses flowering. ***FLC*** inhibits the expression of floral initiation genes such as ***FT***, ***FD*** and ***SOC1*** and such suppression can be broken by regulation pathways like vernalisation pathway and autonomous pathway. In the present study, we analysed the expression of four ***FLC*** paralogues in cauliflower with an intention of understand the regulation of ***FLC*** under as affected by the heat treatment.

FLC-1 was abundant in both meristem and leaf tissues (Figure 14 A and B). In meristem tissues, ***FLC-1*** was highly expressed early from week 1 to week 5, prior to a dramatic decrease in week 6 after which the expression stayed low. In leaf materials, ***FLC-1*** was also highly expressed at the beginning. However, it maintained highly expressed until week 8 and week 9 which was 3-4 weeks later than that in meristem. After the gradual decrease of in week 8-9, expression level of ***FLC-1*** was greatly lowered to almost background level after week 10 and remained low since then. Such significant changes in expression took place simultaneously in control and SWAP group in meristem tissues and were delayed by a similar period of 3 weeks in leaf materials. These results indicated that the expression of ***FLC-1*** was not directly affected by the heat treatment in the presented study.

FLC-2 displayed an different expression pattern compared to its paralogue ***FLC-1*** (Figure 14 C and D). Its expression in meristem maintained at a low level in the first 5 weeks after sowing and then stayed at a slightly higher level after week 6. A similar increasing fashion was also observed in leaves, where the expression was higher in generative stage than that in vegetative stage. Again we did not observe clear effect of the heat treatment on the expression of ***FLC-2*** in terms of either expression level or expression time. The increase of ***FLC-2*** expression in leaves of SWAP group did appear to be 1-2 weeks earlier than that in control group, but this remained unclear due to the sample quality issues.

FLC-3 was expressed in a much lower level than ***FLC-1*** and ***FLC-2*** in meristem (Figure 14 E) and as low as around background level in leaves (Figure 14 F). In meristem, expression of ***FLC-3*** in both control and SWAP showed a similar low-high-low pattern. In control group, expression of ***FLC-3*** continued to increase gradually until week 8 and increased dramatically in week 9 and started to decrease in later weeks. Although the pattern was similar to that in control group, the expression level was lower than that in control group. These results may indicate that the heat treatment had a negative effect in repressing the expression of ***FLC-3*** in meristem. ***FLC-4*** did not show expression level higher than the detectable range and was thus considered as not expressed.

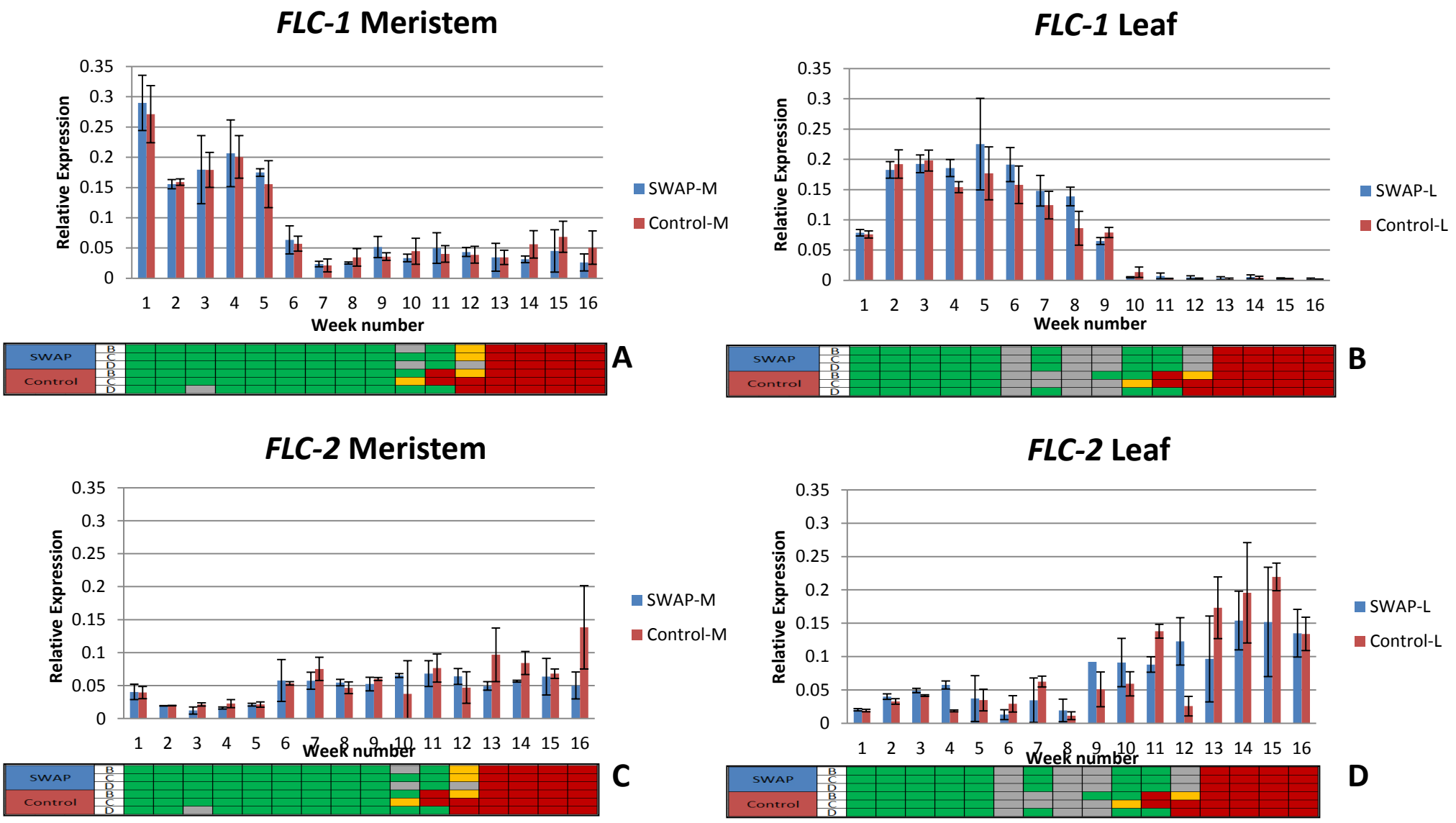

Figure 14 Relative expression levels of FLC-1(A-B) and FLC-2(C-D) in meristem and leaf of Lindurian during the experimental period (week 1 – week 16). The coloured cells under week number indicate the growth stage of the sample: green=vegetative, yellow=transition and red=generative. Grey cell indicates that the sample gave an extremely high/low Cq value of reference gene.

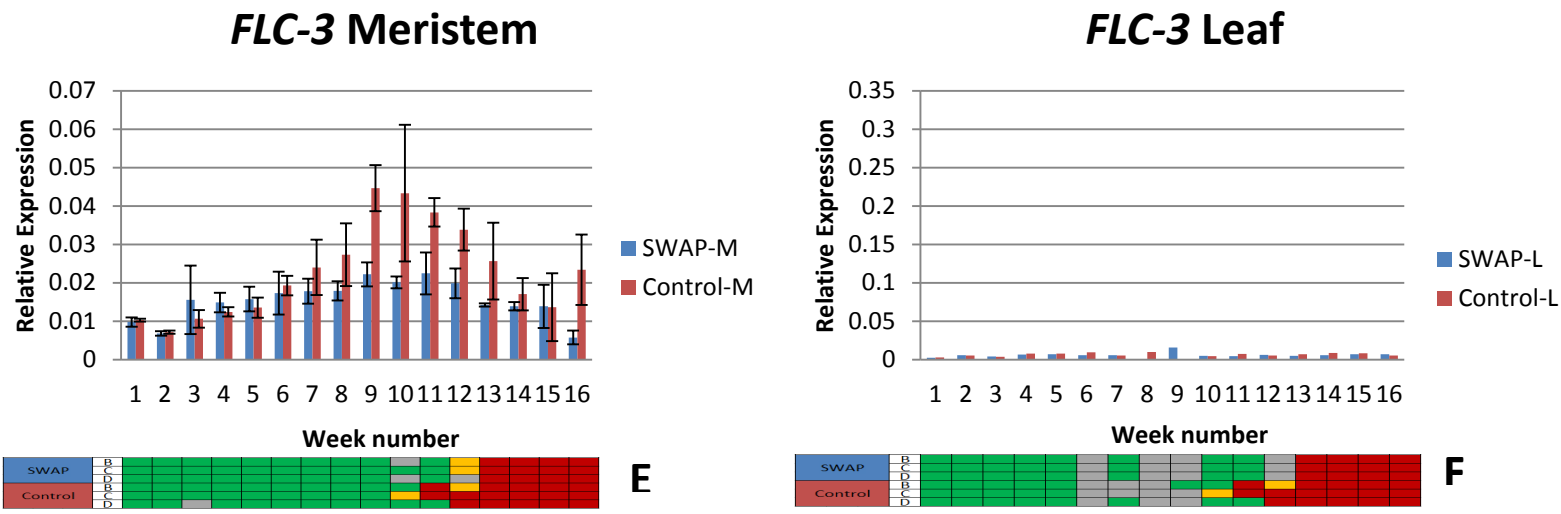

Figure 13 (continued) Relative expression levels of FLC-3 in meristem (E) and leaf (F) of Lindurian during the experimental period (week 1 – week 16). The coloured cells under week number indicate the growth stage of the sample: green=vegetative, yellow=transition and red=generative. Grey cell indicates that the sample gave an extremely high/low Cq value of reference gene.

SOC-1, *AGL-24* and *LFY* showed corresponding expression patterns

SOC1 is another important MADS-box transcription factor that functions as floral integrator in the meristem. This floral promoting gene can be negatively regulated by ***FLC*** and positively regulated by ***AGL24*** (***AGAMOUS-LIKE 24***). Meanwhile ***SOC1*** also up-regulates ***AGL24*** and ***FUL*** in meristem. In this study we also examined the expression of ***SOC1***, ***AGL24*** and ***LFY*** in our materials.

Relative expression of ***SOC1*** was low in both meristem and leaf (Figure 15 A and B). In meristem tissues, ***SOC1*** remained lowly expressed in the control group until week 13, which was 2-3 weeks after the first observed switched meristem. The expression stayed relatively high after week 13 in control group. Interestingly, an increase in expression level was also observed in SWAP group with a similar 2-week delay from first switched meristem. In leaves, however, the expression of ***SOC1*** remained low and no obvious patterns were observed.

AGL24 also showed an increasing pattern in meristem tissues (Figure 15 C). The relative expression increased from week 4 in both groups and remained high from week 6. The expression level of ***AGL24*** appeared to be higher in control group as compared to that in SWAP group. A similar increasing pattern was also found for ***AGL24*** in the leaf materials where expression increased in both groups from week 5 and 6. Again the heat treatment showed an putative effect as expression of ***AGL24*** was slightly lower in SWAP group than that in control group.

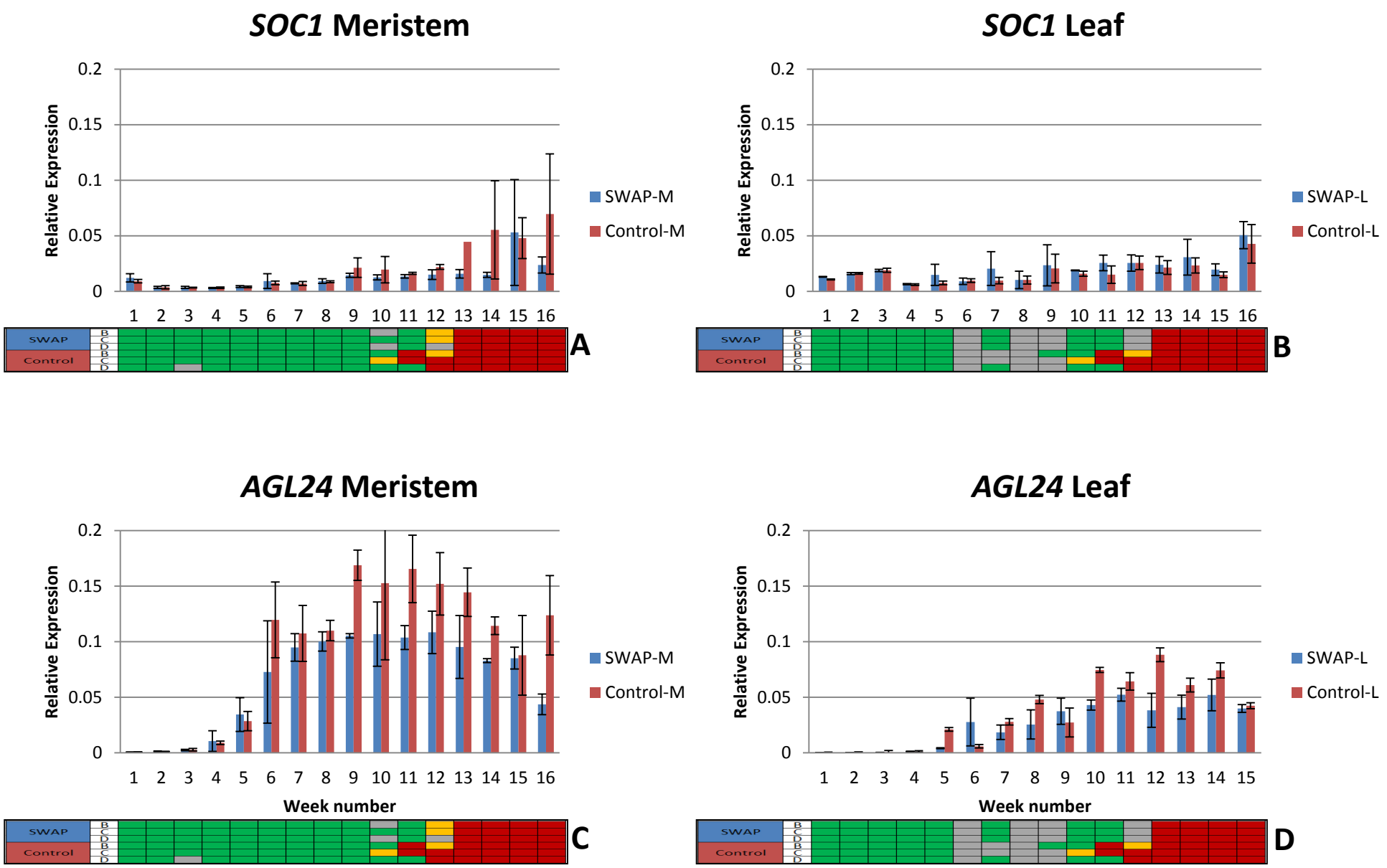

Figure 15 Relative expression levels of *SOC1(*A and B) and *AGL24* (C and D) in meristem and leaf of Lindurian during the experimental period (week 1 – week 16). The coloured cells under week number indicate the growth stage of the sample: green=vegetative, yellow=transition and red=generative. Grey cell indicates that the sample gave an extremely high/low Cq value of reference gene.

LFY was found to express only in meristem. In our study, we found ***LFY*** to be relatively lowly expressed also in meristem (Figure 16). The relative expression remained lower than 0.01 until week 13 and week 10, respectively, in control and SWAP group.

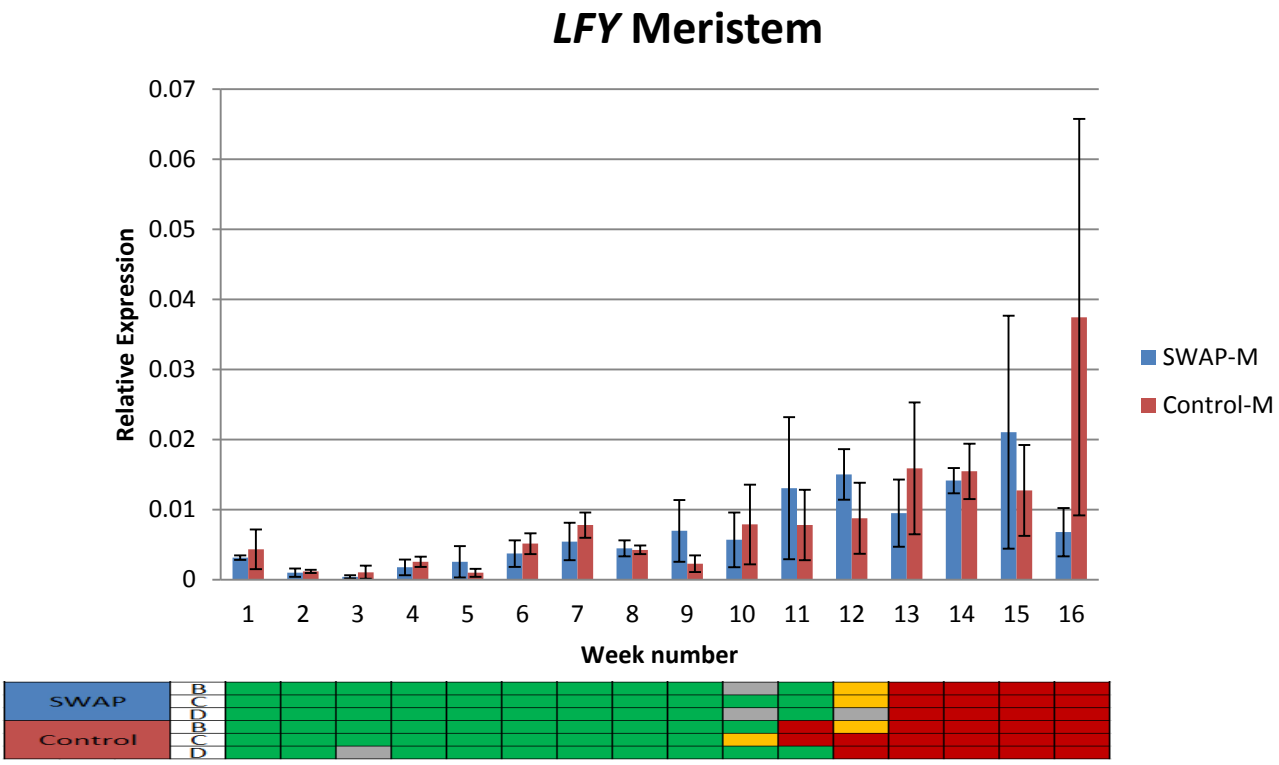

Figure 16 Relative expression levels of *LFY* in meristem of Lindurian during the experimental period (week 1 – week 16). The coloured cells under week number indicate the growth stage of the sample: green=vegetative, yellow=transition and red=generative. Grey cell indicates that the sample gave an extremely high/low Cq value of reference gene.

Discussion:

Higher ambient temperature delays the development of meristem in cauliflower

It was known that an increased ambient temperature would result in earlier flowering in *Arabidopsis*. In cauliflower, however, the higher temperature usually leads to the opposite, i.e. a delayed growth. In the presented study we applied one week of heat treatment (27 ^{o}C in day time and 22 ^{o}C in the night) to two cauliflower cultivars, Lindurian and Fremont. Apart from the heat treated week for the SWAP group, all plants were grown under the same condition and with a suitable growth temperature of (21/16^{o}C) until 16 weeks after sowing.

In this experiment, we found that the increased ambient temperature caused delay in the meristematic switch from vegetative stage into generative stage in both tested cultivars (Figure 8). Lindurian showed a 2-week delay in terms of the first appearance of switched plant and a 1-week delay to reach a fully switched stage. Fremont was expected to be more stable under higher temperature than Lindurian. However, we observed a similar extend of delay in Fremont as that in Lindurian. In Fremont, the first switched plant appeared 2 weeks later in the SWAP group, and it also took Fremont 1 more week to reach a fully switched stage in the SWAP group.

This experiment (SWAP6) was the 6th experiment of the whole project. In earlier experiments, we have observed delayed switch of 2~5 weeks in Fremont under different growth seasons (see appendix). As for Fremont, it showed 1 week of delay in SWAP2 and showed almost no delay of switch in SWAP3. These earlier experiments were in line with the conclusion that the 1-week heat temperature would cause delay in curd formation of cauliflower varieties. This conclusion was also confirmed by other cultivars tested in our earlier experiments, for example, Skywalker, Steady in SWAP4 and Amerigo, Bejo2803 in SWAP6. What was not consistent through the series of experiment was that the number of delayed weeks varied between and within tested cultivars. Obviously the growth conditions were not identical in all years and the growth can be affected by other environmental factors such as light intensity, day length, soil structure etc.. These factors affects the growth of crops individually and also in interaction with each other, thus making the prediction of harvest time, yield and quality more difficult.

Modern vegetable production required growers to ensure the best harvest time as well as the quality of marketable part of crops. With such standard increasing as the development of the production and marketing system, being unable to control or predict the growth of crops may lead to loss of growers. For these reasons, it has become more important to understand timing of flowering and floral development in crops like cauliflower and to understand their requirement for and responsiveness to

temperature change, which is a major factor in adaptation of elite germplasm in regional climate (Jung and Müller, 2009).

1 day or 1 week may be insufficient to expose the effect of heat treatment

In the RNA-Seq study an interval of 24 hours was set to evaluate expression difference of genes after heat/cold treatment. More than 100 genes that have been reported to be involved in flowering time control was detected in the RNA-Seq experiment. Earlier analysis also found high correlation between the RNA-Seq data and qRT-PCR results, proving that RNA-Seq provided high-throughput and accurate tool to evaluate gene expression changes (Lubbers, 2013). However, we noticed that except for photoperiodic/circadian clock genes like ***CCA1*** and ***PRR9***, no major floral integrators or other floral genes were found to be significantly differentially expressed within the in 24 hour. In addition, we further tested ***FUL-a FUL-c FLC-1~4 COL-5 AGL24*** and ***SOC1*** at an interval of 7 days (Figure 11and Figure 12). Again we did not observe different expression patterns between control and SWAP groups. These results may indicate that the expression of these genes, affected by temperature or not, may not be exposed shortly after the treatment.

In the study of Sun (2013), one of the two ***CRY2*** paralogues was found to be down regulated after 24 hours of heat treatment in Lindurian but not in Fremont. Other genes tested in the study did not show significant difference in expression level within 24H or 1 week. In fact, we noticed in the expression levels of some genes to be different after entering the generative stage (discussed in the later section). For these reasons, we may conclude that in order to investigate the effect of temperature in gene expression, especially in those floral genes, samples from later growth stages should also be included.

Heat treatment may affect the switch of meristem by affecting floral genes

In the expression analysis, we focused on ***FLC SOC1 AGL24 FUL*** and ***LFY*** to study their expression through the whole experimental period, which is in total 16 weeks from sowing. These five genes were closely related in the regulation of flowering time.

Encoding a MADS-box transcription factor, ***FLC*** is a major repressor of flowering in *Arabidopsis* (Deng et al., 2011) and many *Brassica* crops like *B.rapa* (Xiao et al., 2013). In our study, we found that ***FLC*** was in not affected by the temperature swap (Figure 14). 4 putative ***FLC*** paralogues was identified in earlier studies and we numbered them as ***FLC-1~FLC-4***. These four paralogues behaved differently in the presented study. ***FLC-1*** showed a very contrasting expression between the vegetative stage and generative stage in both meristem and leaf of Lindurian, where it started with a high expression and was later repressed to an very low level. Interestingly, the transcription of***FLC-1*** was repressed around week 6 in meristem and week 8 in leaf. Neither the timing nor the expression level appeared to be affected

by the temperature SWAP, which was in accordance with earlier experiment of the research (Lubbers, 2013). This expression pattern matches the suggested function of ***FLC***. ***FLC-1*** was repressed earlier in meristem than that in leaf, but the remaining expression level was higher in meristem than that in leaf. This may indicate that ***FLC-1*** could be the major floral repressor in cauliflower. It has been reported that ***FLC*** repress flowering in a dosage-dependent manner in *Arabidopsis* (Michaels and Amasino, 1999) and it appeared that its homologs in *Brassica* species behaves in a similar role in repressing flowering (Xiao et al., 2013). ***FLC-1*** was repressed earlier in leaves than that in meristem. This may be explained by its interactions with other genes. For example, ***FLC*** represses ***FT*** in leaves where ***FT*** is expressed and the ***FT*** protein was transported to the meristem (Corbesier et al., 2007). The lower expression of ***FLC*** in meristem allows other floral promoting factors like ***FT*** to be effective in inducing flowering. It was also noticed that ***FLC-1*** remained at a low but detectable level in meristem while showing almost no expression in leaves after switching into generative growth. This may suggest that ***FLC-1*** have other direct or indirect function in regulating other pathways. For example, it has been reported that ***FLC*** showed flowering-dependent and flowering-independent effects on leaf development in *Arabidopsis* (Willmann and Poethig, 2011).

FLC-2 and ***FLC-3*** showed a contrasting expression pattern when compared to that of ***FLC-1***. Both ***FLC-2*** and ***FLC-3*** showed an increased expression as the plant entered generative stage. Such results may suggest that these two ***FLC*** genes are either true ***FLC*** homolog that does not function in repression of cauliflower meristematic switch; or they are not real ***FLC*** homologs in cauliflower. It has been suggested that different ***FLC*** paralogues may function differently in different genetic backgrounds (Xiao et al., 2013). ***FLC-4*** did not show expression in our experiment. It may be a result of the timing of gene expression as some genes may be switched off either due to the time point of sampling, which is around noon in our case; or the fact that ***FLC-4*** is non-functional.

SOC1 is a major floral pathway integrator that was directly supressed by ***FLC*** (Hepworth et al., 2002). In our study we found that in meristem tissues, ***SOC1*** was repressed in early weeks of growth (week 1-5) and started to increase after week 6. Similar trend was observed in leaf materials with less scale of increase and a delay in the increase (Figure 15 A and B). Interestingly, the timing of change in ***SOC1*** was in line with that in ***FLC-1***, thus confirming the regulative interaction between the two. ***FLC*** expression was higher in the early weeks and such high expression of ***FLC*** repressed ***SOC1***'s expression. As the growth continues the expression of ***FLC*** was lowered, allowing ***SOC1*** to express and promote the switch from vegetative to generative growth. However, we did not find a significant difference between control and SWAP groups in the expression of ***SOC1*** either, indicating that heat treatment did not cause a delay of meristem switch by directly affecting ***SOC1***. A possible explanation may be that the increased temperature affects the switch by influencing

other flowering time genes. For example, it has been reported that higher temperatures promotes flowering in *Arabidopsis* by elevating ***FT*** expression and such temperature-induced morphological changes was found to be related to ***PIF4*** (Kumar et al., 2012). In the RNA-Seq data, we found slightly reduced but insignificant difference within the 24-hour treatment of heat in cauliflower while it was significantly lower in *Arabidopsis.* The result supported our hypothesis that the heat treatment may decrease the expression of floral promoters like ***PIF4*** in cauliflower and thus leading to a delayed switch into generative stage. However, it was in contrast against earlier findings that heat treatment increased ***PIF4*** (Kumar et al., 2012). Such difference may be a result of different day length and also the timing of sampling, as was discussed earlier, the 24-hour interval may not be a suitable length to detect such expression changes.

AGL24 was another MADS transcription factor that promotes generative growth in *Arabidopsis* (Michaels et al., 2003). In our study we found that in both meristem and leaf materials, expression levels of ***AGL24*** started to increase at around week 6 (Figure 15 C and D). Interestingly, the expression levels were higher in the control group than that in the SWAP group, indicating a possible effect of heat treatment. Meanwhile the increase of ***AGL24*** expression seemed to be delayed in the swap group by 1-2 weeks. These results may suggest that the heat treatment supressed the expression of ***AGL24*** in cauliflower by lowering its expression level and delaying the expression. This may explain the delay in meristem switch between the SWAP and control group. Since the expression of ***FLC*** was not likely to be affected by temperature in our study, the change in ***AGL24*** expression was then less likely to be the result of ***FLC*** regulation. This is in accordance with the study of Michaels et al. (2003) in *Arabidopsis* which confirmed that ***AGL24*** is either acting downstream of ***FLC*** like ***SOC1***, or affecting plants' phenotype in a ***FLC*** dependent fashion.

FUL was also described to be promoting floral growth in *Arabidopsis* as mutation lines of ***FUL*** in *Arabidopsis* failed to produce floral meristem (Melzer et al., 2008). In our study, both genes showed increased expression after plants' switch into generative stage, thus confirming their positive relationship with the growth of cauliflower. For ***FUL*** family, a stage-dependent expression was found in meristem. Similar result was also described in earlier study of Sun (2013). Hence, ***FUL*** genes may be proposed as marker genes that marks vegetative, transition and generative stages in curd formation of cauliflower. In addition, expression of ***FUL*** genes in meristem was lower in SWAP group than that in control group. Considering the positive effect of ***FUL*** in promoting generative switch, it suggests that the heat treatment may also delay the meristem growth by repressing the expression of ***FUL*** genes.

Conclusion

In this thesis we investigated the effect of a 7-day high ambient temperature (27/22 °C, day/night) on the growth of cauliflower variety Lindurian and Fremont in the 5th week after the sowing in February 2014. Except the heat treatment, all plants were grown in 21/16°C condition. We found delayed meristem switch in both cultivars by a similar scale. To be specific, both cultivars showed a 2-week delay in the first appearance of switched plant, and a 1-week delay after the control group had fully switched into generative stage. The length of delay, however, varied for different varieties under different conditions. It can be concluded that the increased ambient temperature would delay the meristematic switch of cauliflower and such delay, to our current knowledge, is difficult to predict.

We further investigated the expression of some flowering-time related genes across the whole experimental period, which was in total 16 weeks. We did not found significant differences between control and SWAP group in the expression levels of the tested genes in 1-week-time after the heat treatment. Considering the results in our earlier RNA-Seq study, it can be concluded that the expression change of the major floral genes may not be detected shortly after the heat treatment.

It was confirmed that ***FUL*** family genes may be used to mark the growth stages from vegetative to generative stages as they showed stage-dependent increase in their expression.

FLC was down-regulated in both leaf and meristem of cauliflower after week 6 and 8, respectively. Other tested floral genes confirmed there function of promoting floral switch by showing an increasing expression after the generative switch of meristem.

Heat treatment did not affect the expression of ***FLC*** or ***SOC1***, but seemed to repress the expression of ***FUL*** and ***AGL24*** in meristems. Also the elevation of ***FUL*** and ***AGL***'s expression appeared to be delayed. Hence we conclude that in the scope of our experiment, heat treatment did not delay the meristematic switch by directly influencing ***FLC*** or ***SOC1***, but more likely by repressing and delaying the expression of floral promoting genes like ***FUL*** and ***AGL24***.

Further study

In this experiment, the same plant was evaluated for meristem growth stage and immediately sampled destructively. Such procedure ensures that the meristematic stage is known for every plant sampled. It is relatively slower and the sample quality may depend on the experience of the sampler. Also the reference used to identify growth stage is not clear enough, especially for the inexperienced. In order to better identify the growth stage, it may be suggested that an experienced personnel to be responsible for the identification through the whole experiment. Also more morphological and/or molecular markers should be used when possible to make the phenotypic data more reliable.

In our expression analysis, we were confronted with a problem of sample quality. There may be several sources of such problems. Firstly, sampling was inconsistent in the experiment. Sampling was firstly conducted by Johan Bucher and then by Yongran Ji after week 9. Similar to the stage identification, such step should be done by the same person for the whole experiment. Secondly, sample quality may be affected by grinding prior to RNA isolation. In this thesis we used a special grinder to grind the meristem materials. This tool, although very handy, may cause loss from the already-limited meristem materials, thus leading to insufficient RNA concentration. Leaf veins made the leaf materials very difficult to be evenly grinded. An solution to this firstly grind the whole leaf roughly with mortar and pestle, then scoop the same amount of tissue into the collection tube to be further grinded, either with the tool or with grinding balls, into even and fine powders. Lastly, the RNA isolation step may also bring sample quality problems. Although the isolation kit is highly efficient and the protocol is handy, mistakes may happen. Thus it is not recommended to isolate too many samples per day as it may affect the consistency in conducting the protocol.

Based on this experiment and the earlier results, a refined list of genes may be concluded for future study. These genes may be tested on more contrasting cultivars under different treatments in order to draw a clear response network towards the increased ambient temperature in order to better understand the mechanism of cauliflower's delayed growth under heat conditions. This study provided indication of the major floral genes' expression in cauliflower. Further studies may be conducted in a better designed way with more repeats to better quantify and qualify the roles of these genes in controlling cauliflowers' responses to heat. In addition, a field trial may also be included the test the behaviour of cauliflower under field conditions.

Due to the limited time of the minor thesis, only the samples form the sensitive cultivar Lindurian was used in the expression analysis. Should further resources permit, it would be interesting to look into the expression of this same set of genes in the more stable cultivar Fremont.

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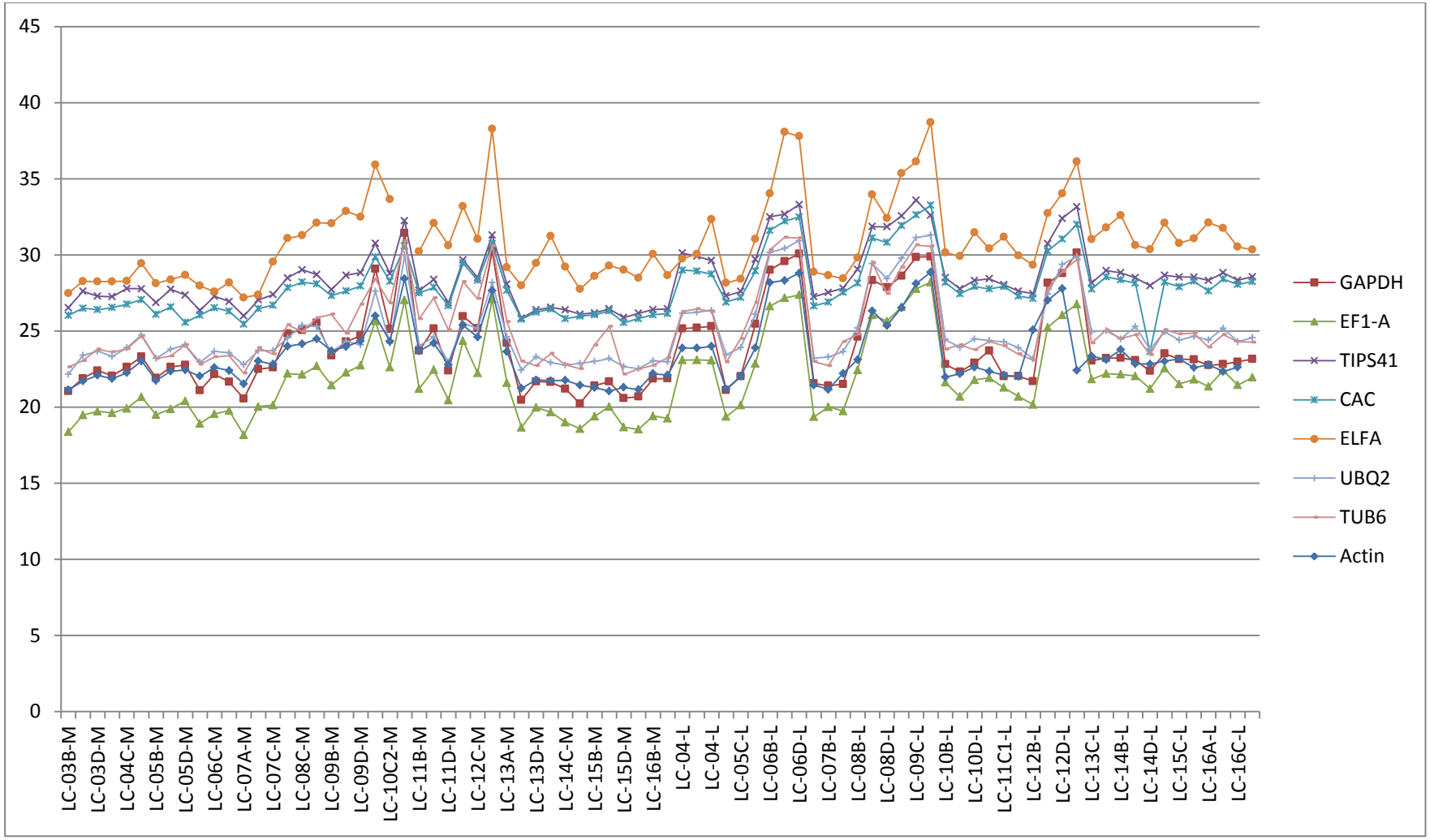
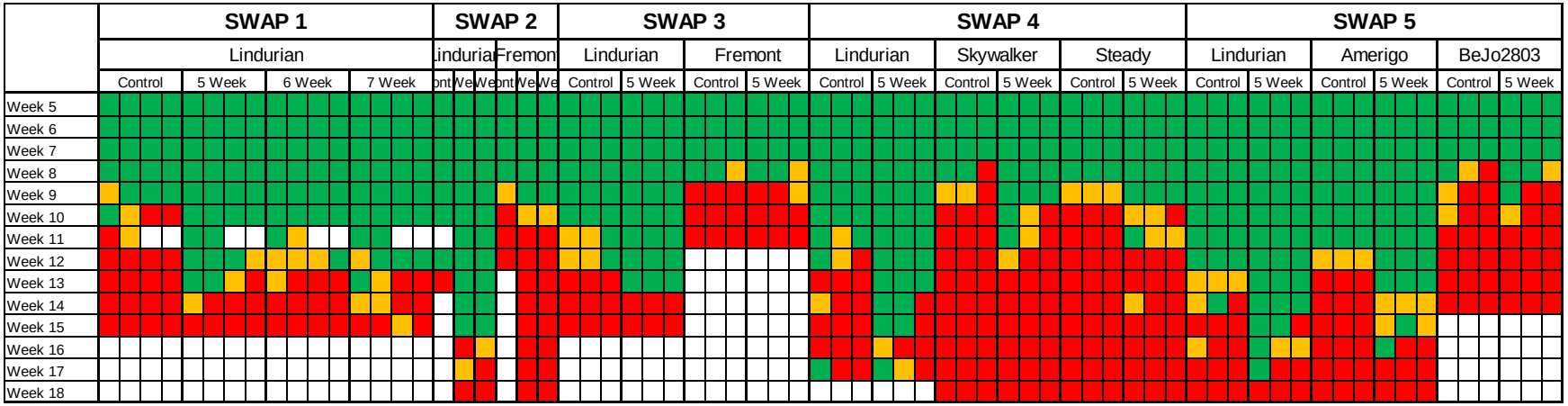
Appendix

Appendix 1 Primers sets used in the experiment

Gene		5'-3' sequence	Gene		5'-3' sequence
ACTIN	F	CGTGGACATCAGGAAGGAC	FUL-a	F	AGAGGGAGAAGAAAACGGGTC
	R	GAACCACCGATCCAGACACT		R	GTCAATGACGATGCTCCACC
AGL24	F	GGCTGGAGAAATCGCTTGAATC	FUL-c	F	GAGAGGGAGAAGAACACGGT
	R	TAGCCATTTCCAACGTCACTAG		R	TTCCGCCAGTGATGATGCTA
CAC	F	CAATCGATTGCTTGGTTTGG	GAPDH	F	CACTGACAAGGACAAGGCTGCTGCT
	R	CAAGTCCAAGATTTCTTCTCTCC		R	CGGCTCTTCCACCTCTCCAGTCCTTC
COL5-a	F	CACCTCCGTTCCGTTTAACC	LFY	F	TGGAATGCGACTAGGTGGTT
	R	CGAAACGGCCTTTGATCCTT		R	TTTGATGCCGTACCGTTCAC
COL5-b	F	GAACAGGGAGGCTAGGGTTT	PA200-a	F	ACCTGGAAGTTCGGCACA
	R	TGAAGCGTAAACATGGCTCG		R	AACACCACCTCGACAAAAGC
CRN1-a	F	CGAGTGGAAGCCTAACCTCA	PA200-b	F	ATGTCCCATTCCTCAGCACA
	R	AAAATCAACCGCCCACACTC		R	CGTAACAGCTTCAGACGCTC
CRN1-b	F	GAGATCCCCAACGCTCCTTA	PRR1-a	F	TGGGAAAAGCTAACCGGAAA
	R	GGACTCCTCCCAAGCTTCTT		R	TCCGGATAGAGACTTGACGC
CRN1-c	F	ACTATCTGATGCGAGGGTGG	PRR1-b	F	TCAGACCTCAGGACAACGTT
	R	CTTCAACTCCACGCCAGAAC		R	CCTATCCCTGCTTGATGTGGA
EF1	F	TTTCGAGGGTGACAACATGA	PRR3-a	F	GTCCTTGAAGCCTGGAGAGT
	R	CCGTTCCAATACCACCAATC		R	GAGGAAATCAACAGCGCCAT
ELF-A	F	CCCGTTATGTAAGCCGGTTG	PRR3-b	F	AACAACGGTGCTACTTCTGC
	R	TTCAACGCAGATATGGCAGC		R	AACGGGACTGTCTTGAGGAG
FLC-1a	F	TCGTCAGCTTTCCGTTCTCT	SOC1-a	F	TGAAACATGAGGCAGCAAAC
	R	TGACTGACGATCCAAGGCTT		R	TGCAGCTCCTCTATCGAACA
FLC-1b	F	TTCCCTGGTTCAGCTGGAG	SOC1-b	F	TGGGAGAAGGCATAGGAACA
	R	CGGCTCGCACAAGATTACT		R	GCTTCTCGTTTTCTGCAGCTA
FLC-2	F	GCTTCTCGTTGTCTCCTCCT	TIPS41	F	TGAAGAGCAGATTGATTTGGCT
	R	CGCTTACACCACCGACATTT		R	ACACTCCATTGTCAGCCAGTT
FLC-3	F	GCCCTCTCCGTAACTAGAGC	TUB6	F	GGAATGGATACCGAACAACG
	R	GTGGGAGAGTTACCGGACAA		R	CAACGCTAGTCTCAGCAGCA
FLC-4	F	TGATGATCTCAATGCCCTGGA	UBQ2	F	ATATTCGTGAAGACGCTG
	R	GGGCAGTCTCAAGGTGATCT		R	CTCAACTGGTTGCTGTG



Appendix 2 Cq values for reference genes in control group of Lindurian. Similar to that in SWAP group, the abnormal Cq values overlaps across the samples, indicating that the high/low Cq values were caused by sample quality.



Appendix 3 Graphic demonstration of meristem growth stages of Lindurian and Fremont in previous 5 SWAP experiments. Green colour indicates vegetative stage, yellow transition and red indicates generative stage.