The role of ambient temperature in the generative transition of cauliflower

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The role of ambient temperature in the generative transition of cauliflower

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Abstract

Cauliflower (*Brassica oleracea* var. *botrytis*) is a crop that is commonly cultivated for its curd. To have a year-round market supply, most growers grow it continuously throughout the season. To reduce costs and effort of harvesting uniformity and predictability concerning its timing is crucial. Fluctuations in ambient temperature during cauliflower plant development can delay and increase variability in curd initiation time and subsequently harvest time. Although, not all cauliflower cultivars are (equally) sensitive to these fluctuations in ambient temperature, temperature insensitivity is a hard trait to breed for, as the genetic regulation of this trait is still largely unknown. Previous research indicated that alternative splicing of FLC-like MADS-box gene transcripts could play a role in the regulation of flowering by ambient temperature. In this thesis the effect of temperature on curd initiation time is investigated in climatized growth experiments. Expression patterns of curd initiation marker gene *BoFUL-c* and floral regulator *BoFLC* genes in meristem enriched tissue during cauliflower development were analysed. Protein-protein interactions of BoFLC and BoSVP were studied to reveal the effect of differential splicing on protein interaction. Results suggested that the role of photoperiod has a large effect on plant development. However, expression patterns of both *BoFUL-c* and *BoFLC* genes showed almost no resemblance to those measured in previous experiments. Protein-protein interaction patterns of BoFLC and BoSVP revealed that differentially alternative spliced genes show different interaction patterns. Future studies should consider taking measurements to minimize the noise of variation in photoperiod on plant development in their experiments. Furthermore, the effect of alternative differential splicing ratios regarding protein-protein interactions should be studied to elucidate their role in temperature effected curd initiation timing.

Introduction

The shift from vegetative growth to floral development is one of the most important transitions in the life cycle of a plant (Simpson & Dean, 2002). Upon germination plants start a period of vegetative growth during which leaves are formed on the shoot apical meristem (Poethig, 1990). Later in the development of the plant, it experiences a transition from vegetative to generative growth. The timing of this transition depends on the plant species, as well as the individual plant's genotype and its environmental conditions. In order to ensure reproductive success, plants have acquired a sophisticated genetic framework that translates different environmental cues (such as photoperiod, light quality, temperature and abiotic stresses) into genetic pathways that regulate floral timing (Bäurle & Dean, 2006; Simpson & Dean, 2002).

The plant's system of genetic pathways to flower and reproduce under optimal environmental and developmental conditions can cause undesirable effects in agricultural settings. Exemplary are crops of which immature generative parts are harvested such as cauliflower and broccoli. In vegetable crops with a short shelf life, this dependency on environmental conditions can heavily influence the harvest timing and quality. Consequences are a fluctuation in the market supply and price, and hence increased financial risks for the growers (J.E. Olesen & Grevsen, 2000a; Wheeler & Salter, 1974; H.-J. Wiebe, 1990).

Curd crops as broccoli (*B. oleracea* var. *italica*) and cauliflower (*B. oleracea* var. *botrytis*) are dependent on ambient temperature as one of the main environmental cues in curd initiation, curd development and subsequently harvest timing. Regarding the changing climate from moderate to more extreme weather conditions, harvest planning gets increasingly difficult for these species that are so dependent on environmental conditions. Therefore developing cultivars that have predictable harvest time under diverse environmental conditions has become a major breeding goal in *B. oleracea* (var. *italica* and *botrytis*) (R. Uptmoor, Li, Schrag, & Stützel, 2011; Ralf Uptmoor, Schrag, Stützel, & Esch, 2008).

Cauliflower development

The development of cauliflower can be divided into three phases; the juvenile phase, the curd induction phase and the curd growth phase (J.E. Olesen & Grevsen, 2000b; Wurr, Fellows, & Fuller, 2004). The juvenile phase is regarded as a phase of vegetative growth during which no induction is possible. In this phase temperature is positively correlated with growth (Wurr et al., 2004). In the curd induction phase plants respond to ambient temperature (vernalisation) to allow curd induction. In the curd growth phase the curd grows with a declining relative rate compared to increasing temperatures, as assimilation does not increase with temperatures above 14C (J.E. Olesen & Grevsen, 2000b; Pearson, Hadley, & Wheldon, 1994).

The main source of variation in the time and length of the harvest period can be attributed to the duration of the curd induction phase (Booij, 1990; H. J. Wiebe, 1975; Wurr et al., 2004). Under optimal temperatures curd induction is highly homogeneous in developmental time (number of initiated leaves) until transition and takes relatively short (H. J. Wiebe, 1975). However, the number of initiated leaves until transition and the duration of the transition (under optimal conditions) are different for each genotype. Early Summer (E.S.) cultivars need an induction period of only 6 days under optimal temperatures, while Winter (W.) cultivars need an induction period of 40 days at the optimum temperature (Table 1) (Wurr & Fellows, 2000). When counted from sowing date Booij (1990) found the optimal chronological age to start curd induction to be at 35 days after sowing for E.S. cultivar Delira.

Table 1 Curd initiation time characteristics per maturity type; Early Summer (E.S.), Summer Autumn (S.A.), Winter (W.) (Wurr & Fellows, 2000)

Maturity type	Final leaf number	Minimum leaf number at end of juvenility	Days after (trans)planting when induction began	Cardinal temperatures of induction (C)			Duration of induction at optimal temperature
				Minimum	Optimum	Maximum	
E.S.	14-57	10	5-41	2-3	9–13	23-24	6d
S.A.	21-50	13	10–20	9	10	21	8d
W.	32-122	22	19-109	5	13-14	23	40d

Genetic Background

Although a lot of research has been done on the phenotypic effects of ambient temperature regulated curd induction, underlying genetic pathways are still largely unknown. Up until now, most research on the underlying genetic pathways to ambient temperature regulated floral timing has been carried out in the model species *Arabidopsis thaliana.* Like cauliflower, the flowering time of *A. thaliana* can be heavily influenced by ambient temperature. While high temperatures can delay curd initiation in cauliflower, flowering in most *A. thaliana* accessions is known to be accelerated by higher ambient temperatures (Verhage, Angenent, & Immink, 2014).

Floral timing by ambient temperature in *A. thaliana*

There are multiple mechanisms that control the ambient temperature dependent flowering response in *A. thaliana*, which could give insight in the ambient temperature dependent flowering curd initiation response mechanisms in *B. oleracea*. Some of these mechanisms can be directly linked to flowering time control, while other mechanisms are indirect and are also dependent upon other environmental signals (e.g. day-length and age) (Verhage et al., 2014). A small overview of the main floral inducing pathways and key genes involved is depicted in Figure 1.

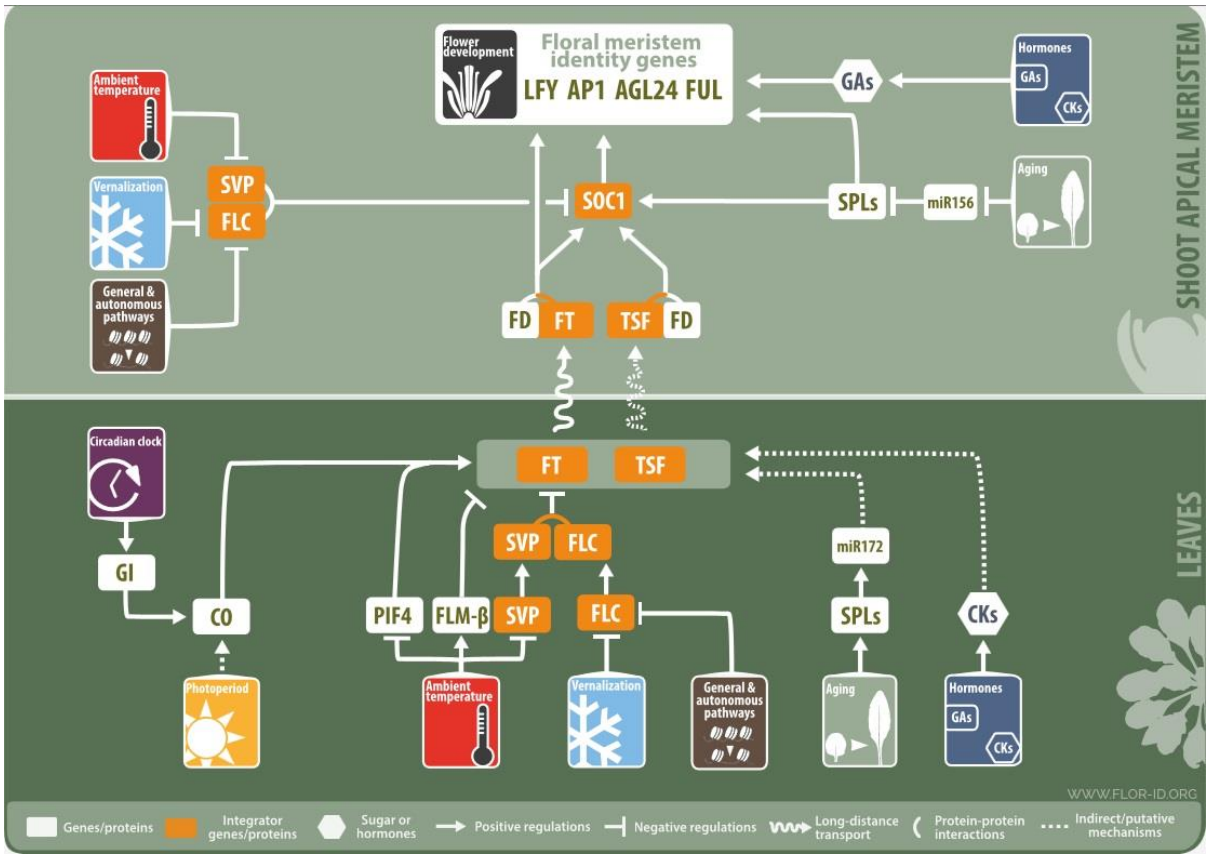


Figure 1 Floral inducing pathways in A. thaliana. Multiple endogenous and environmental cues regulate floral timing through various floral inducing or repressing pathways. Different pathways might overlap and often converge to a few key regulators (Bouché, Lobet, Tocquin, & Périlleux, 2016).

One of the basic changes in *A. thaliana* upon elevated temperatures is chromatin remodelling. Chromatin remodelling is a mechanism by which the DNA is either expanded or condensed to allow the DNA to fit in the nucleus. In the condensed form DNA is tightly wrapped around the histones (that confer the nucleosome), hindering the DNA from binding to other proteins. Upon elevated temperatures H2A.Z containing nucleosomes promote eviction of this histone, resulting in better accessible DNA (Kumar & Wigge, 2010). With regard to flowering, the eviction of H2A.Z facilitates PHYTOCHROME INTERACTING FACTOR 4 (PIF4) to bind to the promotor of floral regulator *FLOWERING LOCUS T (FT)* to upregulate its expression (Figure 1A)(Capovilla, Schmid, & Posé, 2015).

Another histone modification that is known to affect flowering time is the position and deposition of the trimethylation of the lysines of histone H3 (Angel, Song, Dean, & Howard, 2011). Histone modification on H3K27me3, H3K4me3 and H3K36me3 affect *FLC* expression and subsequent floral repression. Upon elevated temperatures H3K27me3 Jumonji demethylase JMJ30 is more abundant in *A. thaliana* resulting in an increase of FLC, preventing precocious flowering (Gan et al., 2014). H3K4me3 Jumonji demethylase JMJ14, which represses *FT* and *TSF* independently of FLC (Jeong et al., 2009; F. Lu, Cui, Zhang, Liu, & Cao, 2010; Yang, Jiang, Jiang, & He, 2010), was found to be more abundant at increased temperatures in *B. oleracea* (unpublished data RNA-Seq).

Moreover, histone modifications are also able to affect the splicing machinery. As of now two models (kinetic and adaptor) have been proposed. In the kinetic model, the speed of transcription is the main factor affecting the splicing. Upon entering a condensed region, DNA Polymerase II slows down, leading to an increased inclusion of nearby alternative exons. In the adaptor model, mRNA is alternatively spliced due to splicing affecting proteins that bind to the histone (Figure 1B) (Zhou, Luo, Wise, & Lou, 2013). Either way, the splicing machinery itself is subjected to alternative splicing as well, that can subsequently result in differential alternative splicing of other genes. Altogether, alternative splicing affects 42-61% of the plants transcriptome (in *A. thaliana*) (Shang, Cao, & Ma, 2017).

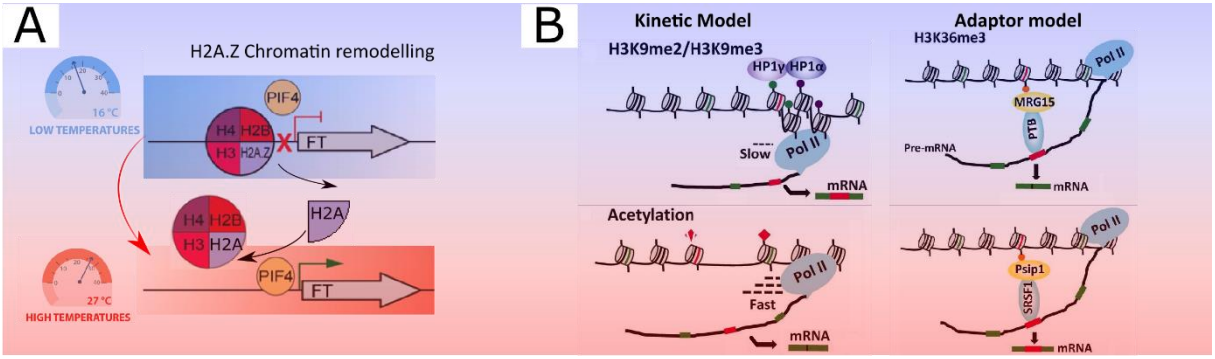


Figure 2. Various mechanisms influencing floral timing by ambient temperature. (**A**) At high ambient temperatures H2A.Z is evicted from the nucleosome allowing for PIF4 to bind to the promotor region of floral activator *FT* (Kumar & Wigge, 2010). Adapted from (Capovilla et al., 2015) (**B**) The effect of chromatin-composition on alternative splicing. Two models have been posed. In the kinetic model, the speed of transcription is the main factor affecting the splicing. Upon entering a condensed region, DNA Polymerase II slows down, leading to an increased inclusion of nearby alternative exons. In the adaptor model, mRNA is alternatively spliced due to splicing affecting proteins that bind to the histone. Adapted from (Zhou et al., 2013).

Alternative splicing is an additional layer of control by which plants can adapt to changing environmental conditions (Shang et al., 2017). Both ambient temperature and day-length dependent flowering pathways are known to confer proteins with an antagonistic function that originate in differentially alternative spliced mRNA from the same gene.

In the ambient temperature dependent pathway, *FLC*-like MADS-box transcription factor gene *FLOWERING LOCUS M (FLM)* is differentially alternative spliced upon elevated ambient temperatures (Posé et al., 2013). The proteins derived from the two main splice forms of *FLM* (*FLMβ* and *FLMδ*) function antagonistically through interaction with SHORT VEGETATIVE PHASE (SVP). In low ambient temperatures, FLMβ, which is the predominant splice form, forms a protein-protein complex with SVP that represses flowering by downregulating *SOC1* expression and putatively binding to *FT*. In higher ambient temperatures, FLMδ becomes the predominant splice form. However, the SVP-FLMδ complex shows no DNA-binding ability and does not repress flowering (Capovilla et al., 2015; Verhage et al., 2014).

In the day-length dependant pathway *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* is known to be alternatively spliced based on both temperature and light-intensity (Filichkin et al., 2010; Ling, Zhou, Baldwin, & Xu, 2015). Of the two main splice variants (*CCA1α* and *CCA1β*) only CCA1α shows DNA binding ability (Seo et al., 2012). This splice variant, that is predominantly present in low temperatures binds to and represses *JMJ30*, a flowering time related histone demethylase. As about half of the transcriptome is affected by alternative splicing, very likely more genes in the flowering pathway will have divergent functions upon differential splicing events.

Next to expression and differential alternative splicing, ambient temperature also affects protein stability. Whereas some proteins are degraded faster upon higher ambient temperatures, other proteins are more stable at higher temperatures, prolonging their activity. JMJ30 shows activity for over twice as long in high ambient temperatures (29°C compared to 22°C). In vitro tests showed that this was not only due to enhanced expression or mRNA stability, but also due to impaired proteasome mediated degradation (Gan et al., 2014). On the other hand, SVP is actively degraded by the 26S proteasome under rising temperatures, reducing its potential to repress flowering (J. H. Lee et al., 2013).

Furthermore, changes in ambient temperature also affects the aging related flowering pathway on a different level. Micro-RNAs 156 and 172, regulate flowering by transcriptional inhibition of several *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (SPL) transcription factors (miR156) and *APETALA 2* (*AP2*)-like transcription factors (miR172) that directly affect expression of floral regulator *FT* (Aukerman & Sakai, 2003). Expression of miR156 and miR172 are both age and temperature dependent; miR156 decreases over time and has elevated expression under low temperature conditions, while miR172 increases over time and has elevated expression under high temperature conditions(Kim et al., 2012; H. Lee et al., 2010; Mathieu, Yant, Mü, Kü, & Schmid, 2009).

To grasp the interconnection between the different pathways and their link to flowering time regulation an overview has been made of the abovementioned processes in a gene-protein interaction network. In Figure 3, a clear overview of the main proteins and miRNA involved in the temperature dependent flowering time pathway (in *A. thaliana*) are presented by means of a flowchart.

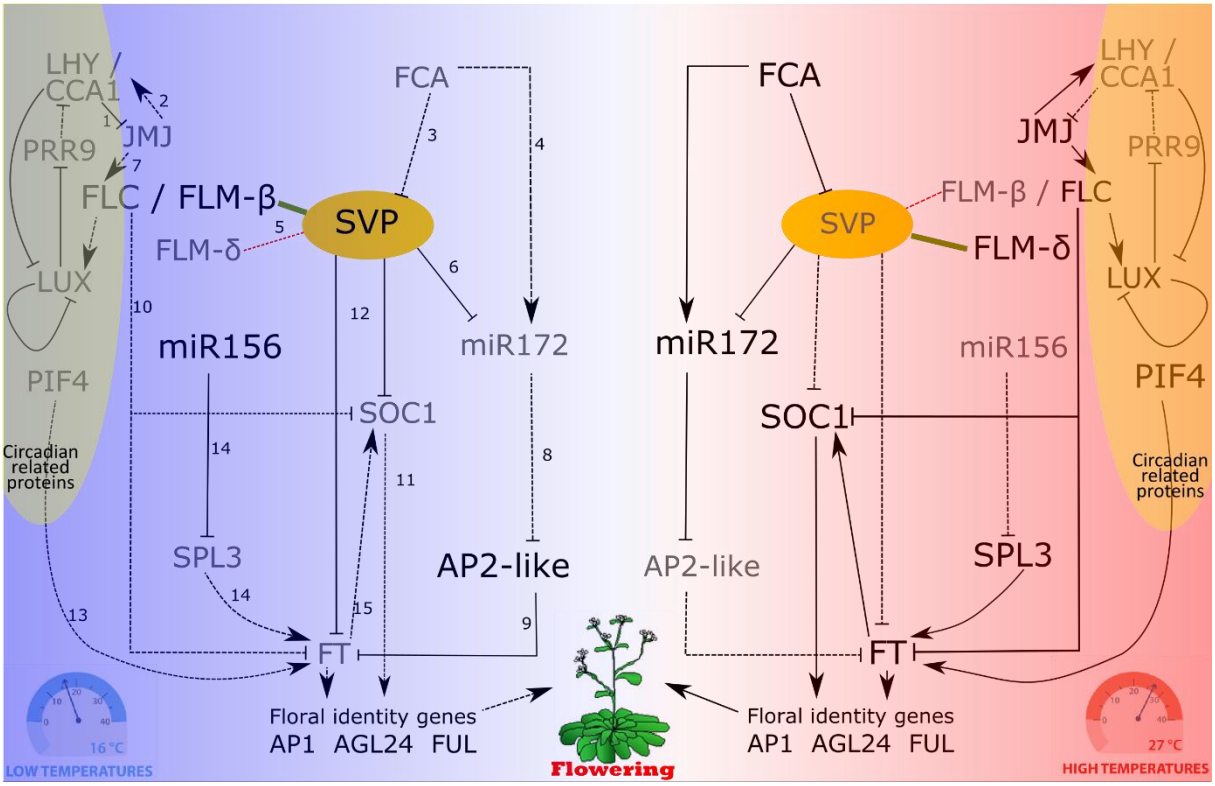


Figure 3. The regulatory network of floral timing by ambient temperature in *A. thaliana*. Arrow lines indicate the type of interaction; solid=active; dashed=inactive. Interaction between SVP and FLM take place at protein-protein level. Other interactions generally take place at gene expression level. References: 1(S. X. Lu et al., 2011). 2(Jones et al., 2010). 3(Capovilla et al., 2015). 4(Capovilla et al., 2015; Jung, Seo, Ahn, & Park, 2012). 5(Posé et al., 2013). 6(Capovilla et al., 2015). 7(S. X. Lu et al., 2011). 8,9(Aukerman & Sakai, 2003). 10(Searle et al., 2006). 11(Patapoutian, Peier, & Story, 2003). 13(Posé et al., 2013). 14(Kim et al., 2012). 15(Verhage et al., 2014).

Translatability of *A. thaliana* developmental pathways to *B. oleracea* var. *botrytis*

Arabidopsis and *Brassica* are both part of the *Brassicaceae* family. The *Brassica* genus consists of three diploid species *B. rapa* (AA), *B. nigra* (BB) and *B. oleracea* (CC) that have undergone a whole genome duplications (named α and β) and a triplication (γ), that was followed by another triplication event after their divergence of the *Arabidopsis* lineage (Jiao et al., 2011; Liu et al., 2014; Lysak, Koch, Pecinka, & Schubert, 2005). In a diploidization that followed substantial parts of the genome were reshuffled and many genes were lost (Town, 2006). 24 relatively complete triplicated regions have been found to cover 58% (26,485) of the genes in *B. oleracea* and occupy 72% (19,628) of the genes in *A.thaliana* (Liu et al., 2014; SCHRANZ, Lysak, Mitchell-Olds, & MITCHELLOLDS, 2006). Therefore, many genes are conserved between *A. thaliana* and *B. oleracea.* Exemplary are the flowering time regulating MADS-box genes, of which even the characteristic *B. oleracea* var. *botrytis* curd gene *CAULIFLOWER* (*CAL*) is also present in *A. thaliana* (Arthur, 2002). However, because of the WGD and WGT events at the origin of the *Brassica* species, one should be aware of increased copy numbers. Paralogues can be redundant, but can also have obtained new functions (neofunctionalization) or have pseudogenized.

Although the general flowering pathway and the main flowering time regulating genes are conserved between *A. thaliana* and cauliflower (*B. oleracea* var. botrytis), there is a major difference in the way they flower. *A. thaliana* has a relatively short inflorescence stage compared to cauliflower, which shows an arrested stage of indeterminate inflorescence development; known as curd formation (Anthony, James, & Jordan, 1996). Because of the short inflorescence stage in *A. thaliana,* inflorescence and floral initiation are hard to separate and are sometimes even referred to as the same event (Henderson & Dean, 2004).

Moreover, one of the key floral regulators by ambient temperature in *A. thaliana; FLM* has no functional orthologue in cauliflower. The fact that it is unknown if the function of this gene could have been taken over by another *FLC*-like MADS-box transcription factor like the *MAF* genes advocates for in-depth research in the genetic (and epigenetic) basis of floral transition in *B. oleracea* (var. *botrytis*). In a recent study (Matschegewski et al., 2015) a GWAS has been performed to zoom in on the genomic regions conferring temperature dependent curd initiation regulatory genes. 18 QTLs were identified and a heritability of 0.93 was observed for temperature-related curd induction. In a more recent study (Hasan et al., 2016) 31 significant QTLs for ambient temperature-related curd induction were mapped on eight linkage groups. Candidate genes in the QTLs regions from both studies were *FRIGADA*-like (*FRL*) vernalisation pathway genes as well as *FLC* genes*, SPL*-like ambient temperature pathway genes and the *AP1* meristem floral identity gene. As QTL analysis only pointed to the loci of temperature responsive floral regulators Matschegewiski et al. (2015) pointed out that candidate gene research is needed elucidate genetic variation in temperature response and floral transition in cauliflower.

To elucidate the underlying genetic mechanism to time ambient temperature responsive curd initiation in *B. oleracea* an NWO granted program (Thermoflow II) was started. To achieve this goal, gene expression and gene function studies were carried out. The aim of the gene expression studies was to investigate the effect of ambient temperature on gene expression patterns in cauliflower, by measuring meristematic gene expression in cauliflower plants that were raised under differential climatized temperature conditions. The aim of the gene function studies, was to investigate the effect of differential alternative splicing on protein function in protein-protein interactions.

In this thesis the effect of ambient temperature on the timing of the generative switch of cauliflower and the role of *BoFLC* genes in this process was investigated. Meristem switch timing data of cauliflower plants grown in a climatized greenhouse and material from Syngenta and Rijk Zwaan, grown in a non-climatized greenhouse was generated and analysed to investigate the effect of high temperatures on generative switch timing. From the plants grown in a climatized greenhouse meristem enriched tissue was harvested for gene expression analyses. *BoFUL-c* meristem expression was determined and used as additional molecular marker for the generative switch. From this data timepoints for subsequent RNA-Seq analyses were selected.

Furthermore, the harvested meristem enriched tissue was also used to study meristem expression patterns of the *BoFLC* genes, to investigate their role in ambient temperature affected timing of the generative switch. A gene function study was carried out on available BoFLC splice variants to check for the differential interaction patterns.

In the gene function study, yeast two-hybrid protein-protein interaction assays were carried out to check for the interaction of candidate genes with floral regulators. By searching for homologues to the temperature dependent flowering network in *A. thaliana*, a narrow selection of genes had been identified as possible causal agent for temperature dependent curd initiation. Based on a previous gene expression study in 6 cauliflower varieties (Appendix 1), BoFLC, BoSVP and BoJMJ14 were selected for yeast two-hybrid protein-protein interaction screens, to identify their role in the temperature responsive curd initiation pathway.

In the gene expression study cauliflower varieties were grown under different temperature conditions to investigate the effect of temperature in the expression pattern of the curd initiation pathway in *B. oleracea* var. *botrytis*. Previous temperature assays were used to determine optimal climate conditions and phenotyping moments for observation of differential expression of temperature effected curd initiation related pathways. In this thesis an experiment was conducted where plants were treated for 7 days with high temperatures at 35 days after sowing. From the start of the heat treatment (35 days after sowing) every 3 days plant meristem tissue was harvested for phenotyping and gene expression analyses, to obtain precise data on switch timing and temporal gene expression.

Materials & Methods

Expression analysis approach

Starting materials

Two (E.S.) hybrid cauliflower cultivars (*Brassica oleracea* var. botrytis) were used in this study: the temperature sensitive cv. Skywalker and the temperature insensitive cv. Steady. Seeds were obtained from Bejo and Syngenta. Seeds were sown on 02-06-2017 in nursery sow trays in rows with an interspacing of ±3cm. Six trays with a total of 600 planted seeds were put in a climatized greenhouse compartment (21/16°C ± 1°C day/night) to germinate. 14 days after sowing 560 of the most uniform seedling plants were transplanted to ø15 cm pots.

Growing conditions

Optimal growing conditions for the gene expression measurements and analyses were determined by analysing the effect of the climate conditions from previous experiments on the variation in curd induction time and expression of its molecular marker *BoFUL-c*. Experimental conditions (referred to as SWAP) in which plants received a one-week high temperature treatment (27/22°C day/night) starting at 35 days after sowing were found to show the optimal variation in curd initiation timing. Increasing phenotyping moments from once a week (like in previous experiments) to once per three days would give a higher resolution of the gene expression pattern that is associated with curd formation.

To allow testing for the effect of elevated temperatures on curd initiation time, the plants were put into two climate controlled greenhouse compartments (Radix Serre 2.9 and 2.11) at 21/16°C (day/night) under long day conditions. 35 days after sowing the temperatures in the one of the compartments was raised to 27/22°C (day/night) for seven days. To be able to correct for (micro)environmental effects, plants were distributed according to a random block design, with 3 blocks per greenhouse compartment (Figure 4).

Table 2. Plant phenotyping overview. Phenotyping and plant biomass determination moments in days after sowing (DAS). As phenotyping was destructive the amount of phenotyping moments was limited.

			Treatment	Control	Control	SWAP	SWAP
			Cultivar	Steady	Skywalker	Steady	Skywalker
			Total plants available	140	140	140	140
Wk 4	Test harvest			8	8	8	8
	28 DAS	Fri (30-06-2017)		8	8	8	8
Wk 5	35 DAS (0H)	Fri (7-7)		8	8	8	8
	36 DAS (24H)	Sat (8-7)		8	8	8	8
	38 DAS	Mon (10-7)		8	8	8	8
Wk 6	41 DAS	Thy (13-7)		8	8	8	8
	44 DAS	Sun (16-7)		8	8	8	8
	47 DAS	Wed (19-7)		8	8	8	8
Wk 7	50 DAS	Sat (22-7)		8	8	8	8
	53 DAS	Tue (25-7)		8	8	8	8
Wk 8	56 DAS	Fri (28-7)		8	8	8	8
	59 DAS	Mon (31-7)		8	8	8	8
	62 DAS	Thy (3-8)		8	8	8	8
Wk 9	65 DAS	Sat (6-8)		8	8	8	8
	68 DAS	Wed (9-8)		8	8	8	8
Wk 10	71 DAS	Sat (12-8)		8	8	8	8
	74 DAS	Tue (15-8)		8	8	8	8
		Count		**136**	**136**	**136**	**136**

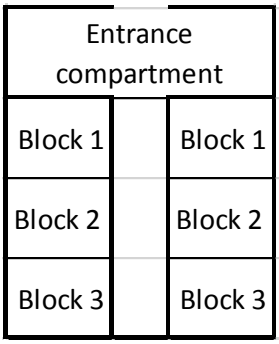


Figure 4. Overview random block design per greenhouse compartment. 280 cauliflower plants were grown in each of the two greenhouse compartments. In the experimental compartment the plants received a high temperature treatment for 7 days (27/22°C ± 1°C day/night). In the control compartment plants were kept at 21/16°C ± 1°C day/night. Both treatments were carried out under long day conditions

Phenotyping

Phenotyping was destructive; At each phenotyping (harvest) moment 8 plants per cultivar were harvested for each treatment: 2 plants from block 1; 3 plants from block 2 and 3 plants from block 3 (Table 2, Figure 4). Per block the fresh and dry matter weight of the plants was measured, for further analyses[1]. Furthermore, each plants meristem developmental stage (see Figure 5) was visually assessed under a binocular and noted for comparative analysis. Directly after the assessment, meristem enriched tissue was cut out, put in an Eppendorf screwcap tube and frozen in liquid nitrogen. Tubes were labelled

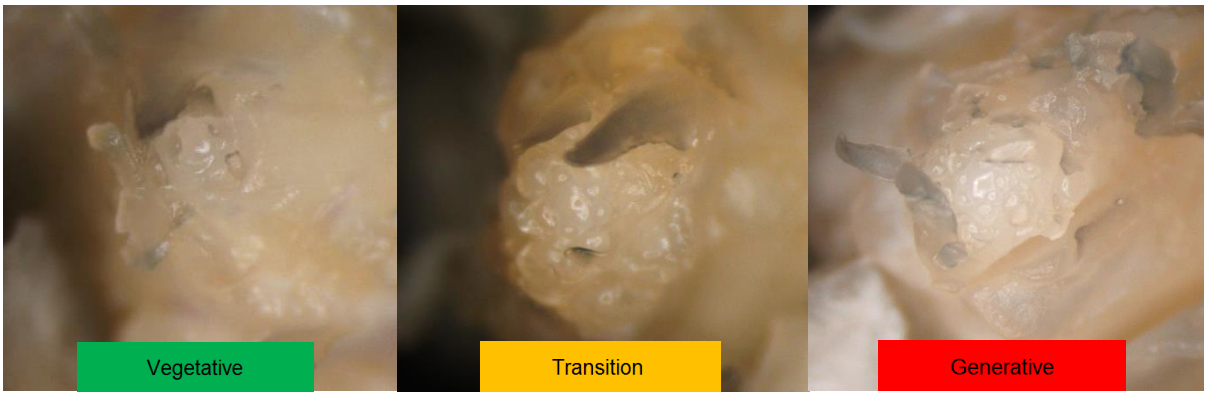


Figure 5 Developmental stages from vegetative to generative visualise by SEM. Left: a vegetative meristem (single dome, only leaf primordia) Middle: meristem transition (main meristem arrests, secondary meristems are formed). Right: generative meristem (meristem developmental arrest of secondary meristems, iterative meristems are formed. (Bucher, n.d)

and stored in the -80°C freezer. Upon completion of the phenotyping experiment, frozen meristems were pooled by block and grinded for RNA isolation.

Comparative analysis phenotyping

Results of the visually assessed meristems were compared to visually assessed meristems from a similar previous experiment. In this experiment (SWAP4) that started in March (sowing date: 01-03-2017), the same cultivars were used and the control treatment had the exact same temperature conditions (21/16°C ± 1°C (day/night)).

Furthermore, results were also compared with a simultaneously run experiment (SWAP Nergena) that was carried out in a non-climatized greenhouse compartment. In this experiment the temperature effect of non-climatized greenhouse conditions were tested on cauliflower plants that were sown and raised for ± 5 weeks at a breeding company. Transplanting at different moments gave the possibility to check for the effect of differential temperature conditions in the plants development on curd initiation time.

RNA Isolation

From the frozen meristem enriched tissue total RNA was isolated according to the RNeasy® RNA Isolation protocol (QIAGEN). An additional DNase I treatment (INVITROGEN) was carried out to break down any remaining genomic DNA. This was followed up by an RNA Cleanup treatment according to the RNeasy® RNA Cleanup protocol (QIAGEN). RNA Cleanup product was used for real-time PCR expression analyses and RNA-Seq analyses (beyond this thesis). For expression analyses in quantitative real-time PCR 50 ng/µL of RNA in a total volume of 20µL sample was reverse transcribed to cDNA using the BIO-RAD iScript™ cDNA Synthesis Kit. Upon reverse transcription working dilutions (20x diluted) were made with filter sterile MQ water.

Real-time PCR

Due to its price not all the RNA samples could be analysed by RNA-Seq gene expression analysis; therefore, a selection of samples comprising the moment of the generative meristem switch on molecular level was to be made. To that end real-time PCR expression analysis was carried out on all samples to determine expression of the curd initiation marker gene *FUL-c*. By measuring fluorescence of the (SYBR Green fluorescent amplicon containing) reaction mix after every PCR cycle, the plotted cycle number (Ct)

[1] Only aboveground parts were used

until a set fluorescence threshold could be used as an indication of the initial gene copy number in the sample.

For the executed *FUL-c* real-time PCR, primers were readily available (Table 3). Real-time PCR experiments were carried out using a Biorad CFX96 Touch Real-Time PCR detection system. A 96 wells qPCR setup was made in which each well contained a reaction volume of 10µL: 2,0µL cDNA (1,25 ng/µL); 0,3µL Forward primer (10µM); 0,3µL Reverse primer (10µM); 5,0µL SYBR Green Mastermix and 2,4µL MiliQ water. All samples were tested in duplicate against the reference gene *BoActin*, of which primers were readily available (Table 3). The PCR protocol consisted of a 3 min 95°C Initialization step; then 40 cycles of 15s denaturation at 95°C followed by 60s annealing and extension at 60°C. Upon finishing the PCR protocol, the amplicon melting curve was measured to check the specificity of the amplification.

Real-time PCR Data analysis

Real-time PCR data was exported by CFX Manager to Microsoft Excel for *FUL-c* expression analyses. Samples were distributed over 4 groups corresponding to the cultivar (2) and its treatment (2). Ct (PCR cycle number until threshold) values were used to calculated normalized expression (ΔΔCt) according to the formula: ΔΔCt= ((mean Ct *FUL-c* sample X) – (mean Ct *Actin* sample X)) - ((mean Ct *FUL-c* sample 1) – (mean Ct *Actin* sample 1)) (Livak & Schmittgen, 2001). Subsequently the normalized fold expression (relative expression) was calculated according to the formula: NFE= $2^{\Delta\Delta Ct}$.

Inconclusive results gave rise to another real-time PCR experiment studying the expression of ambient temperature responsive floral regulators *BoFLC1*, *BoFLC3* and *BoFLC5*. The expression of these genes was tested and analysed using abovementioned methods for *FUL-c* expression. *BoFLC1* and *BoFLC3* primers were readily available (Table 3). For *BoFLC5* primers still had to be designed, as this gene had only recently been found to be present in *B. oleracea* var. botrytis.

Primer design

BoFLC5 primers were designed using the Primer3Plus website and were ordered via BioLego®. Primers were tested for specificity by NCBI Primer BLASTN against *B. oleracea*. A total of 2 primer pairs with Tm 60°C (±1°C), free of dimer and hairpin structures, with an amplicon length >150bp were designed (Table 3). Primers pairs were tested by PCR. PCR results were visualized by electrophoresis on a 1% agarose gel. Only if a single band of product corresponding to the expected product size was found, the primer pair was used in real-time PCR experiments.

Table 3 Primers real-time PCR experiments. *BoActin, BoFUL-c, BoFLC1* and *BoFLC3* primers were readily available and were designed over an intron[2]. *BoFLC5* could not be designed over an intron as it was a truncated gene and no *BoFLC5* specific primers could be designed over an intron. Tm and GC content were retrieved from NCBI primer BLAST, for *BoFUL-c, BoFLC1, BoFLC3* and *BoActin*, primer length was calculated using a BLAST against the TO 1000 PANgenome database. *BoFLC5* data was retrieved from Primer3Plus while designing the primers.

Gene	TO 1000 no.	Primer	Sequence	Tm (°C)	GC%	cDNA amplicon length
BoFUL-c	Bo7g098190	Fw	GAGAGGGAGAAGAACACGGT	58.74	55.00	121bp
		Rev	TTCCGCCAGTGATGATGCTA	59.17	50.00	
BoFLC1	Bo9g173370 Bo9g173400	Fw	TCGTCAGCTTTCCGTTCTCT	59.04	50.00	141bp
		Rev	TGACTGACGATCCAAGGCTT	59.02	50.00	
BoFLC3	Bo3g005470	Fw	GCTTCTCGTTGTCTCCTCCT	59.11	55.00	190bp
		Rev	CGCTTACACCACCGACATTT	58.85	50.00	
BoFLC5	n.a. (gene= BOLEPAN _00005424)	Fw1	CTGATGATCTTAAGGCCCTGGAT	59.67	47.83	137bp
		Rev1	CTCTAGCTCAACGAGGGAATCC	59.96	54.55	
		Fw2	TGCTGATGATCTTAAGGCCCTG	60.16	50.00	146bp
		Rev2	GGTGATCCTCTAGCTCAACGAG	59.97	54.55	
BoActin	Bo5g117040	Fw	ACGTGGACATCAGGAAGGAC	59.39	55.00	175bp
		Rev	GAACCACCGATCCAGACACT	59.39	55.00	

[2] Designing a primer pair over an intron makes it possible to visualize whether the amplicon is from gDNA or cDNA origin.

Gene function approach

In the gene function approach, the function of different candidate gene variants is tested by yeast two-hybrid protein-protein interaction assays. In the GAL4 yeast two-hybrid system, which was used for this thesis, two interacting fusion proteins (a hybrid) activate a transcriptional factor that result in activation of reporter genes (Figure 6). In this study the reporter gene was an auxotrophic marker, which enables only interacting yeast colonies to grow on the selective media.

For the gene function approach, a cauliflower gene expression library was available. The used expression library consisted of reverse transcribed RNA (cDNA) from meristem enriched tissue of three temperature sensitive cultivars (Amerigo, Lindurian and Skywalker) (Appendix 1A). cDNA was cloned into the AD vector system to allow for yeast two-hybrid library screening. The titre of the library was $2E^5$ (colonies/mL).

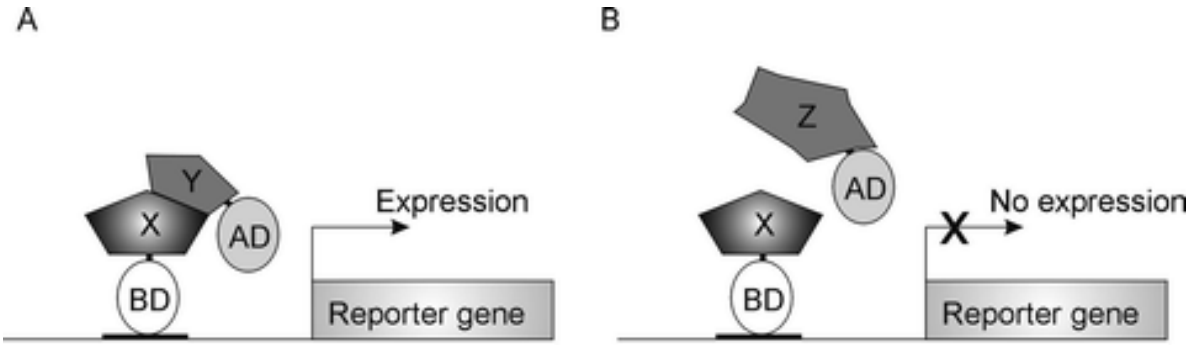


Figure 6 Outline of the yeast two-hybrid (Y2H) GAL4 system. (A) In the GAL4 yeast two-hybrid system, two interacting fusion proteins (a hybrid) activate a transcriptional factor resulting in the activation of a reporter gene. The used yeast GAL4 transcription factor consists of two functional domains, a DNA binding domain (BD) and a transcription activation domain (AD), that upon fusion establish the GAL4 transcriptional activator resulting in the activation of a reporter gene. (B) The reporter gene does not get activated when no hybrid is formed (de Folter & Immink, 2011).

Yeast two-hybrid screens and assays

To test protein-protein interaction of the candidate genes *FLC, JMJ14* and *SVP,* yeast two-hybrid interaction screens and assays were carried out. *BoFLC1* ((Bo9g173370, Bo9g173400), *BoFLC3* (Bo3g005470) and *BoJMJ14* (Bo1g022410) were readily available in PJ69-4α (BD) yeast cells. *BoSVP1* (Bo4g149800) was cloned from the cDNA expression library into DH5α electrocompetent (*E. coli*) cells using the Gateway® cloning system (Invitrogen, 2013). Primers used for cloning *BoSVP1* are noted in Table 4.

Table 4 *BoSVP1* PCR primers for Gateway® cloning. PCR primers for *BoSVP1* to clone the gene into the Gateway® cloning system. Primers include attB sites (marked red) needed in the Gateway® cloning process.

Gene	TO 100 no.	Primer	Sequence	Tm (°C)	GC%	cDNA amplicon length
BoSVP1	Bo4g149800	Fw	GGGGACAAGTTTGTACAAAAA AGCAGGCTCTATGGCGAGAGA GAAGATTCAG	59	46.15	726bp
		Rev	GGGGACCACTTTGTACAAGAA AGCTGGGTACTAACCACCATA CGGTAAGC	61	50.00	

Library screens

Both *BoFLC3* and *BoJMJ14* yeast colonies were transformed with the cDNA expression library of temperature sensitive cauliflower cultivars. Upon transformation the yeast solution was plated on 24 ø15cm selective media bacto agar plates (500µL per plate) containing SD glu -LWH + 3mM 3-AT (dextrose (D-glucose) and yeast nitrogen base lacking Leucine Tryptophan and Histidine + 3mM 3-amino-1,2,4-triazole), to check protein-protein interactions. A small part of the yeast solution (5µL) was plated on a ø10cm selective media bacto agar plate containing SD glu -LW and incubated for 3 days at

30°C to determine transformation frequency. Selective plates for protein-protein interaction testing were kept at 20°C for 10 days. Transformation and selection was carried out according to the yeast two-hybrid protocol as described by de Folter & Immink (2011) and a colony PCR (cPCR) was carried out on the positive clones.

Matrix based screen

To check for the reliability of the yeast two-hybrid library screen system, a targeted yeast two-hybrid matrix based screen was carried out according to the protocol described by de Folter & Immink (2011). After cloning *BoSVP1* into PJ69-4α (BD) yeast cells all protein-protein interactions between available *FLC* variants and *SVP* variants from *A. thaliana* and *B. oleracea* were tested. Interaction between *AtFLC* and *AtSVP* was set as a positive control.

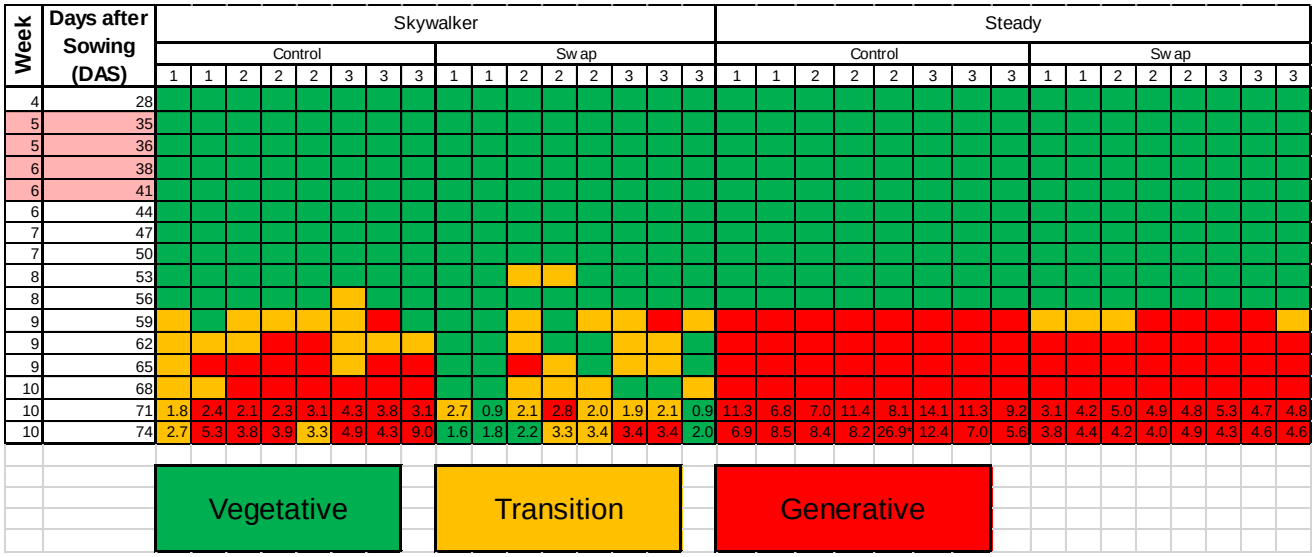
Results

Phenotypic results

At the moment of harvest the cauliflower meristems were evaluated on their developmental stage (Figure 5):

- Vegetative (single dome shaped meristem)
- Transition / early generative stage (main meristem arrests; secondary meristems are visible)
- Generative stage (meristem developmental arrest of secondary meristems, iterative meristems are formed)

For the two cultivars transition timing from vegetative to generative was significantly different under the varying temperature treatments. While transition for cv. Steady was almost simultaneous in the control and high temperature treatment, cv. Skywalker's transition was very variable and arguably delayed in the high temperature treatment (Figure 7). Although transition in cv. Steady was quite homogenous the curd diameter at both 71 and 74 days after sowing (DAS) is significantly different between the two treatments ($p>0.001$). Furthermore, one plant of cv. Steady riciness was found during phenotyping at 74 DAS.



Week	Days after Sowing (DAS)	Skywalker Control								Skywalker Swap								Steady Control								Steady Swap							
		1	1	2	2	2	3	3	3	1	1	2	2	2	3	3	3	1	1	2	2	2	3	3	3	1	1	2	2	2	3	3	3
4	28																																
5	35																																
5	36																																
6	38																																
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9	59																																
9	62																																
9	65																																
10	68																																
10	71	1.8	2.4	2.1	2.3	3.1	4.3	3.8	3.1	2.7	0.9	2.1	2.8	2.0	1.9	2.1	0.9	11.3	6.8	7.0	11.4	8.1	14.1	11.3	9.2	3.1	4.2	5.0	4.9	4.8	5.3	4.7	4.8
10	74	2.7	5.3	3.8	3.9	3.3	4.9	4.3	9.0	1.6	1.8	2.2	3.3	3.4	3.4	3.4	2.0	6.9	8.5	8.4	8.2	26.9*	12.4	7.0	5.6	3.8	4.4	4.2	4.0	4.9	4.3	4.6	4.6

Figure 7 Cauliflower meristem development in different temperature conditions: control 21/16°C (day/night); SWAP elevated temperatures (27/22°C) from 35 – 42 days after sowing (marked pink). At the last two phenotyping moments the diameter of the meristem (mm) was also measured. *One plant of cv. Steady showed riciness.

Validation phenotypic results

Abovementioned results were compared to similar experiments to validate the effectiveness of the used method for testing the effect of changes in ambient temperature on meristem development. In the SWAP4 experiment (started 3 months before our experiment) the meristem switch from vegetative to generative in the control treatment showed a difference in timing of the switch. In the SWAP4 experiment (Figure 8) Skywalker showed a clear moment of transition in the control experiments (all at 63DAS), while in our experiment this timing was variable (56-74DAS). On the contrary, Steady showed a clear transition in our experiment (control) experiment (59DAS) while having a more variable transition in SWAP4 (56-63DAS).

Furthermore, the comparison of our experimental results with a simultaneously performed non-climatized cauliflower meristem transition experiment, showed some interesting similarities. The group of cultivars that was transplanted on June 20th, a few days before a period of high temperatures (Figure 10) showed a similar transition period to our experiment's high temperature treatment (both 21 days). The group of cultivars that was transplanted one week later (after the period of high temperatures) showed a similar transition period to our experiment's control treatment (Figure 9 vs. Figure 7). Though the specific temperatures were different between the experiments, the day-length, light intensity and quality were similar.

Week. Nr.	DAS	SWAP 4																					
		Skywalker												Steady									
		Control						Swap						Control						Swap			
		1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3
Week 5	35																						
Week 6	42																						
Week 7	49																						
Week 8	56																						
Week 9	63																						
Week 9	66																						
Week 10	70																						
Week 10	73																						
Week 11	77																						

Figure 8 Cauliflower meristem development in different temperature conditions: control; 21/16°C (day/night); SWAP; elevated temperatures (27/22°C) from 35 – 42 days after sowing.

Weeks after transplant	Skywalker									Steady								
	Syngenta			Rijk Zwaan 1			Rijk Zwaan 2			Syngenta			Rijk Zwaan 1			Rijk Zwaan 2		
1	n.d	n.d	n.d	1	1	1	1	1	1	n.d	n.d	n.d	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	2	2	2
6	1	1	1	2	1	2	1	1	1	3	2	2	3	3	3	2	2	2
7	1	2	1	1	2	3	2	3	3	3	3	3	3	3	3	3	3	3
8	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
9	2	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
10	2	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
11	3	3	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3	3	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Figure 9 Cauliflower meristem development under non-climatized greenhouse conditions. Developmental stage: vegetative (1), transition (2) or generative (3) are noted for each of the harvested meristems (n.d.= no data). Chronological age is measured from the moment of transplant as plants appeared to be in developmental arrest until transplanting. Syngenta plants were transplanted at 20-06-2017 (37DAS); Rijk Zwaan 1 plants were transplanted on 26-06-2017 (43DAS); Rijk Zwaan 2 plants were transplanted on 13-07-2017 (50DAS). Mean temperature in the greenhouse from transplant until full transition to generative stage for Skywalker was resp. 22,61°C; 22,39°C and 22,18°C.

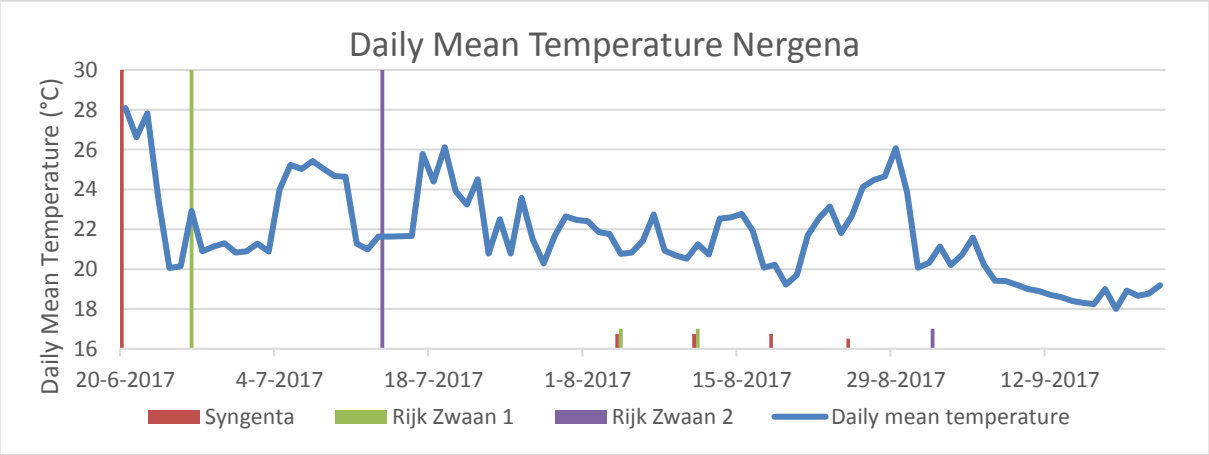

Figure 10 Mean daily temperatures greenhouse compartment Nergena. Vertical lines indicate transplant moment of cauliflower plants from companies to the greenhouse. Syngenta plants were transplanted at 20-06-2017 (37DAS); Rijk Zwaan 1 plants were transplanted on 26-06-2017 (43DAS); Rijk Zwaan 2 plants were transplanted on 13-07-2017 (50DAS). The harvest moments on which Skywalker plants were found to be in transition from vegetative to generative stage are marked with a tick per experiment. Mean temperature in the greenhouse until full transition to generative stage for Skywalker was resp. 22,61°C; 22,39°C and 22,18°C.

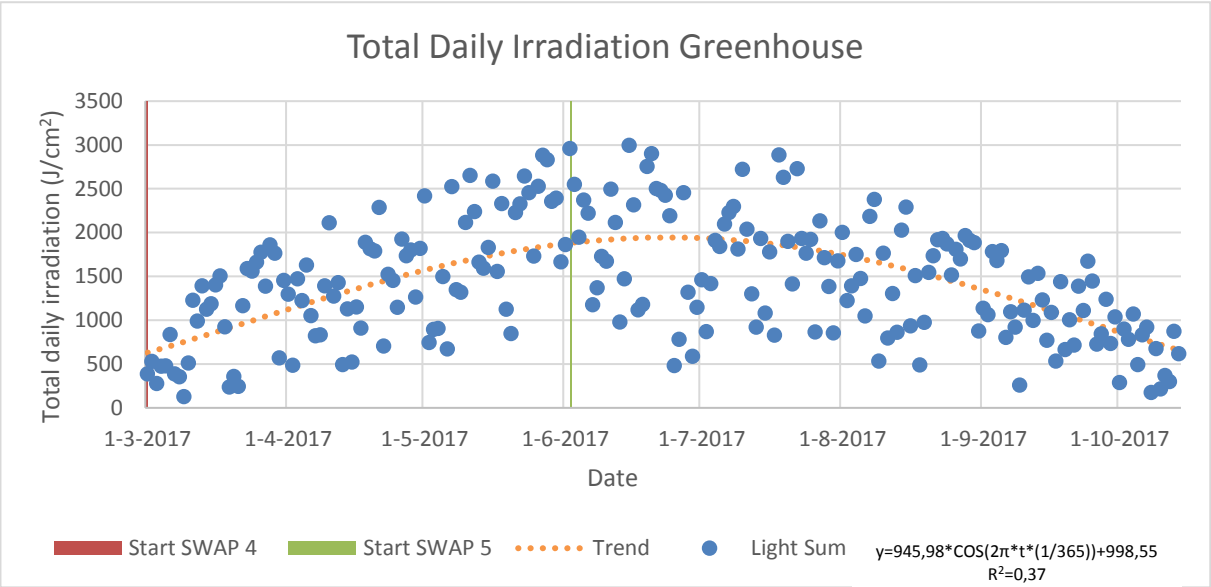

Figure 11 Daily light sum (J/cm²) in the period from March 1st until October 14th measured above Radix Serre (Wageningen). Although irradiation values differ largely from day to day, mean irradiation was significantly higher during the SWAP5 experiment than during the SWAP 4 experiment (p>0.001)

Weight analysis

Upon harvesting the plants, their fresh and dry matter (g) was measured per block from the experimental setup (2-3 plants). Average dry matter accumulation of the plants can be approached exponentially at $f(x)=0.168e^{0.0637x}$ ($R^2=0.97$) in which x is harvest moment in days after sowing. However, during the transition of the plants from vegetative to generative the plant's dry matter accumulation halts temporarily in the temperature insensitive cultivar (Steady control no weight accumulation from 56-62 DAS; Steady SWAP no weight accumulation from 59-62 DAS).

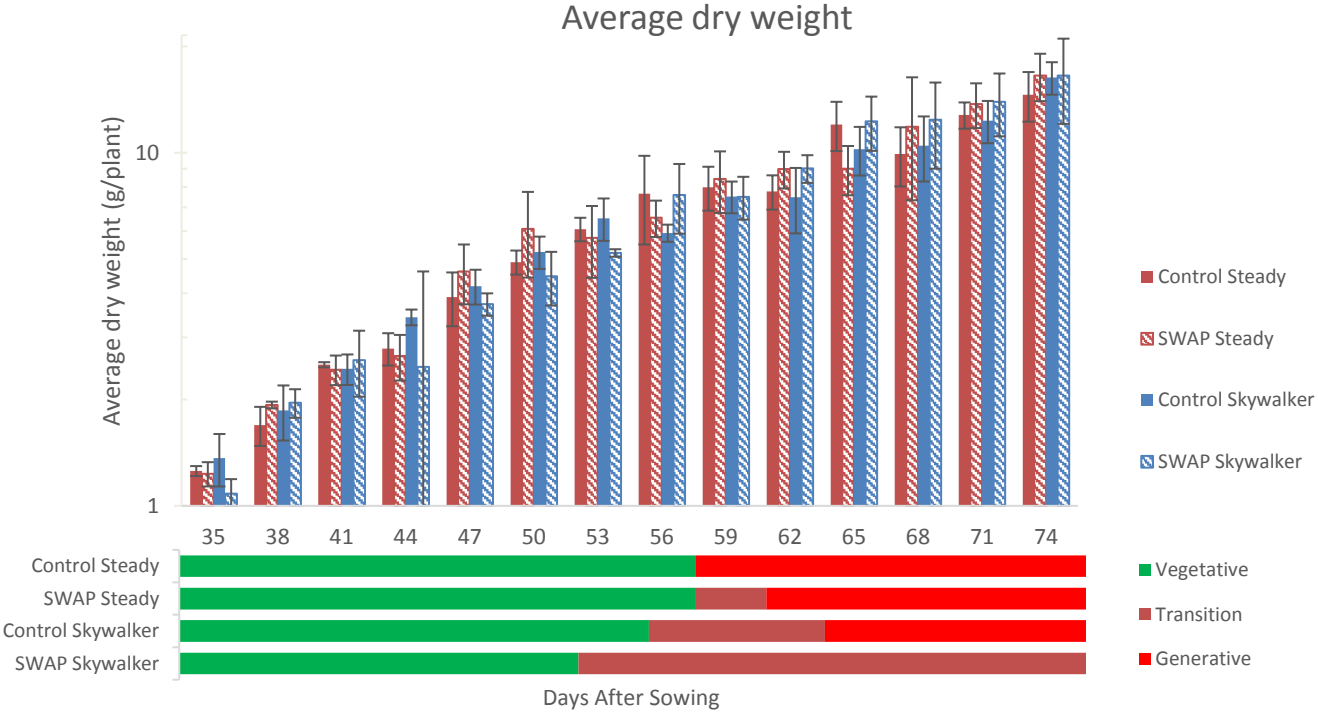

Figure 12 Average dry matter accumulation (g/plant), measured per harvest moment. Error bars indicate the standard deviation between the biological replicates.

Expression analyses

FUL-c expression analyses was carried out on all samples harvested from blocks 2 and 3 (pooled per block as biological replicates). For each biological replicate, the mean value of 2 technical replicates

(SD>0.5) was used in the real-time PCR data analyses. Relative *FUL-c* expression was calculated by plotting the normalized fold expression against the meristem developmental stage (Figure 13). Although there was no clear visible point in time that could generally indicate the start of the developmental transition (vegetative to generative), all samples containing generative meristems showed a normalized fold expression of >40x the first vegetative samples of that treatment. As the variation between some of the biological replicates was rather high, an electrophoresis agarose (1%) gel was run with real-time PCR product to check for possible gDNA contaminations.

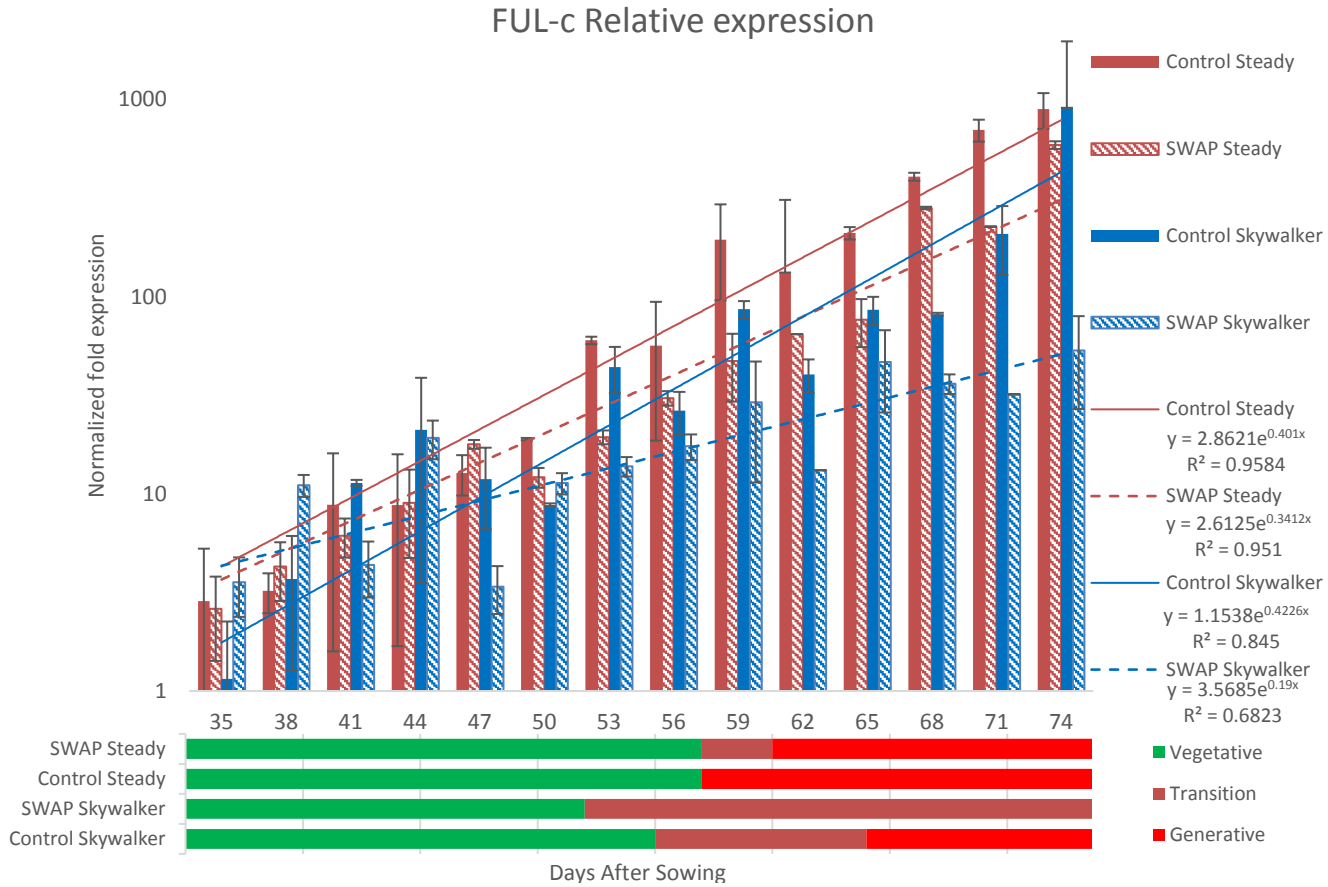

Figure 13 FUL-c normalized fold expression (log scale). Below the graph an indication of the phenotypic developmental stage is given per treatment. Error bars indicate the standard deviation between the 2 blocks. Standard deviation does not differ significantly between the treatments

RNA purity tests

A selection of 20 real-time PCR product samples were run on a 1% agarose gel. 5 samples showed multiple bands, revealing a non-specific amplification. *BoFUL-c* primers were designed over an intron so it was assumed that the longer fragment (observed in some of the samples), would be due to gDNA contamination. Upon running PCR with both purified RNA samples and their cDNA complements as templates, the presence of genomic DNA in the cDNA and RNA samples was confirmed (Figure 14). However, no significant correlation between gDNA contamination and *BoFUL-c* expression appeared. Therefore, comparative analyses were made between the dry matter accumulation of the plants and their *FUL-c* expression pattern, as the assumption was made that variation in dry matter could have been a more specific indicator of developmental age than chronological age was.

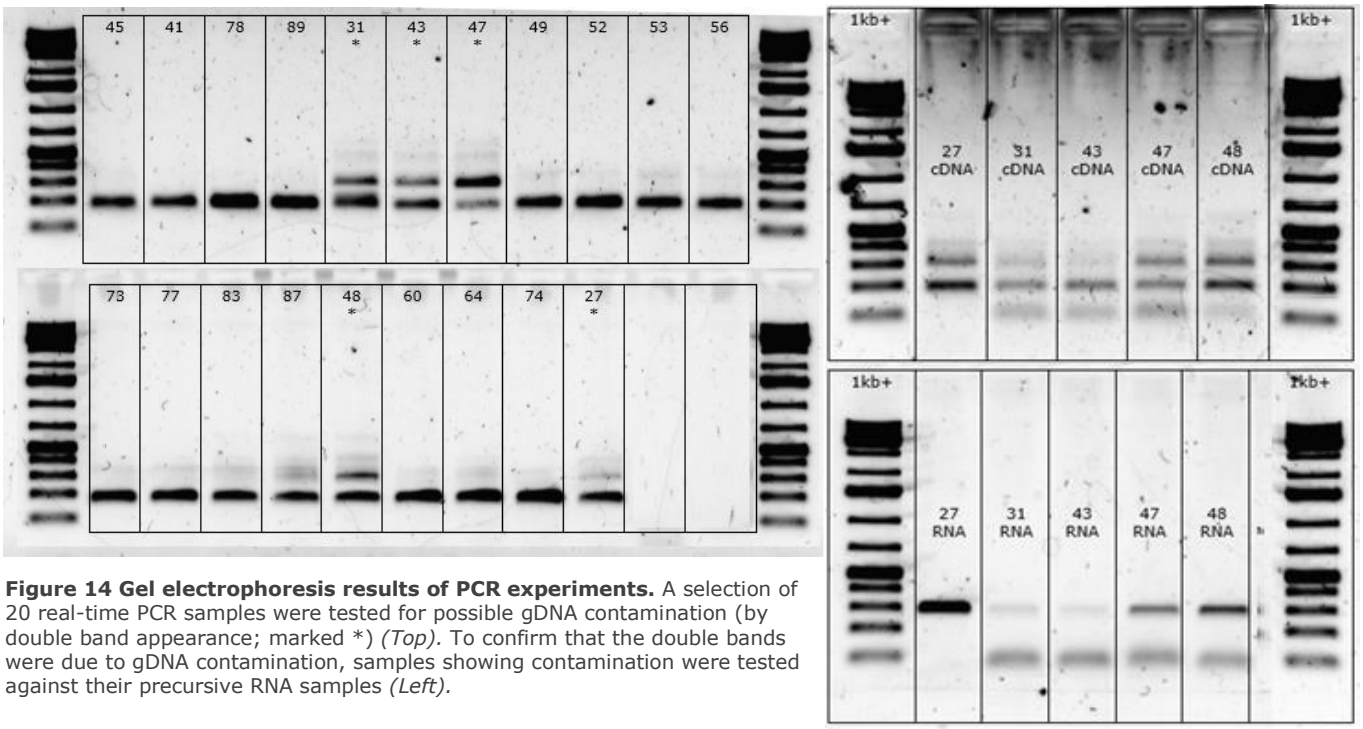

Figure 14 Gel electrophoresis results of PCR experiments. A selection of 20 real-time PCR samples were tested for possible gDNA contamination (by double band appearance; marked *) *(Top).* To confirm that the double bands were due to gDNA contamination, samples showing contamination were tested against their precursive RNA samples *(Left).*

Comparative analyses

Multiple regression analyses between the dry weight accumulation and *BoFUL-c* expression revealed that the effect of dry weight accumulation was more significant than the effect of the plant's age on *BoFUL-c* expression in Skywalker, while Steady showed an opposite pattern (Table 5). As dry weight accumulation data were exponential, the logarithmic transformation Ln(W) was performed on the dry weight values prior to the regression analyses. For *BoFUL-c* expression, the Normalized fold expression values were used.

Pairwise comparison on the dry weight difference (ΔLn(W)) between the biological replicates against the *BoFUL-c* expression difference (Δ(ΔΔCt)) between the biological replicates, indicated there was no significant difference between both variables (Skywalker p=0.12 Steady p=0.91). Regression analyses of the effect of ΔLn(W) on Δ(ΔΔCt) revealed a significant effect of weight difference on expression difference for cv. Steady (F. pr.=0.02). No significant effect was found for cv. Skywalker (F. pr.=0.21).

Table 5 Multiple regression analyses between the dry weight accumulation and *BoFUL-c* meristem expression of cauliflower plants (cv. Steady and Skywalker) grown under different temperature conditions. For Steady the effect of time (harvest moment) was more significant than the effect of weight on *BoFUL-c* expression, for Skywalker the opposite was true. However only p-values of time (harvest moment) in Steady were found to be significant. Regression prediction was significant for every treatment.

Treatment	***Regression prediction***	***P-value plant age (t)***	***P-value weight Ln(W)***	***P-value intercept***	***Regression significance***
Control Steady	*y=.15t+1.10W-4.73*	*0.02*	*0.24*	*0.02*	*2.25E-15*
SWAP Steady	*y=.17t+.0025W-4.39*	*2.39E-03*	*1.00*	*0.01*	*7.08E-14*
Control Skywalker	*y=.074t+1.66W-2.08*	*0.23*	*0.09*	*0.24*	*2.95E-13*
SWAP Skywalker	*y=-.028t+1.99W+2.05*	*0.70*	*0.06*	*0.36*	*2.34E-08*

Real-time PCR experiment FLC's

To make a more accurate indication of the meristem transition at molecular level, the meristem expression levels of *BoFLC1, BoFLC3* and *BoFLC5* were tested by real-time PCR. Due to very low expression of *BoFLC3,* no normalized fold expression pattern could be plotted. When testing the *BoFLC3* primers against *BoFLC3* variants from the cDNA expression library (see Appendix 1A) it was found to not specifically anneal to any of the available *BoFLC* variants.

Real-time PCR expression data of *BoFLC1* and *BoFLC5* were used to calculate relative expression using the ΔΔCt method. In the *BoFLC1* expression pattern, the treatment effect on normalized fold expression is significant on the three harvest moments prior to transition. *BoFLC5* did not show significant differential expression during the experiment (Figure 15).

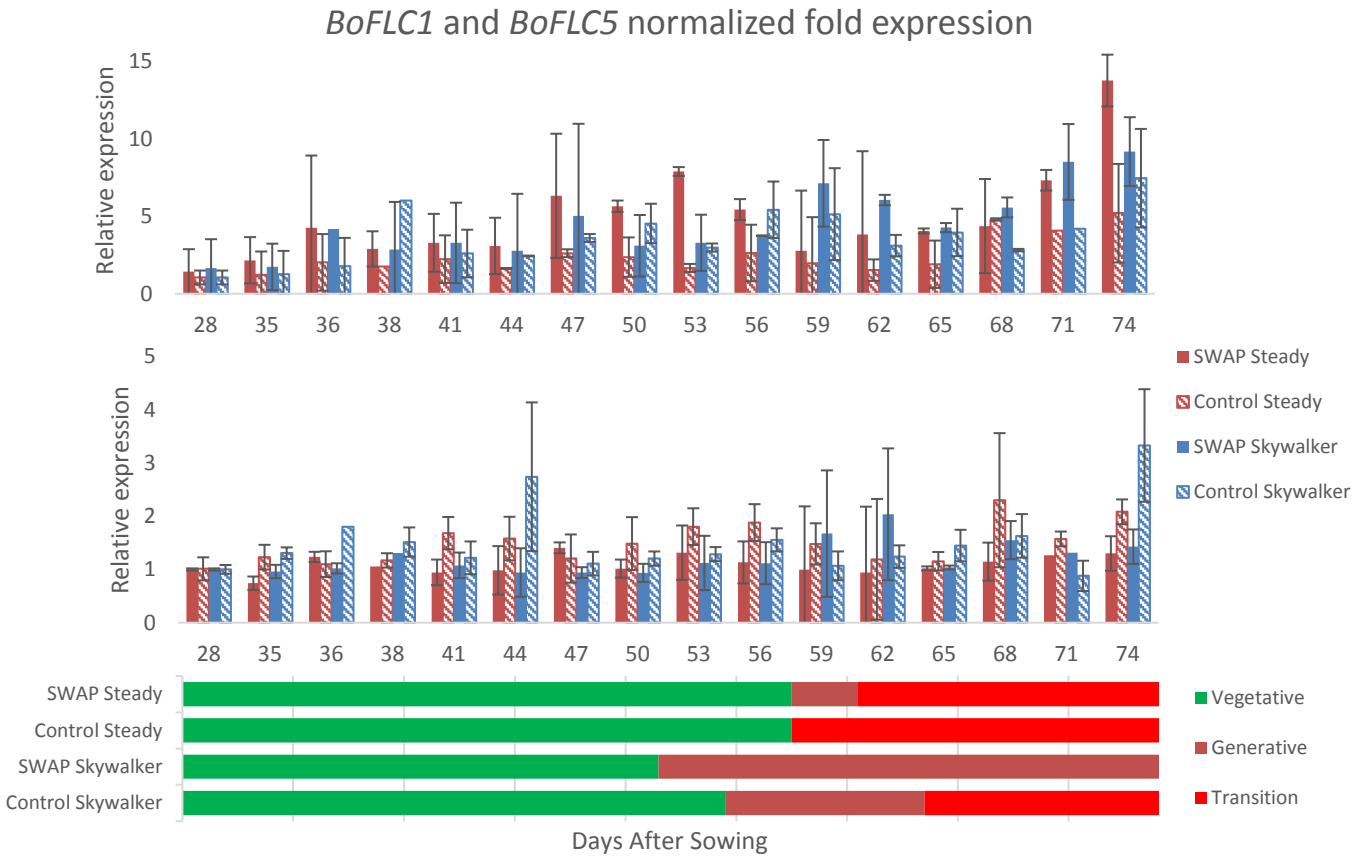

Figure 15 *BoFLC1* (top) **and *BoFLC 5*** (bottom) **normalized fold expression ($2^{\Delta\Delta Ct}$)**. Below the graph an indication of the phenotypic developmental stage is given per treatment. Error bars indicate the standard deviation between the biological repeats.

Yeast two-hybrid test

Both BoFLC3 and BoJMJ14 were screened in a yeast two-hybrid library screen against the cDNA meristem expression library of three temperature sensitive cauliflower cultivars (from plants of 35 days). Normal transformation frequencies (according to protocol) were found ($\pm 10^6$ for both BoFLC3 and BoJMJ14) within 3 days past transformation (normal appearance time). In both screens no sign of interaction was present between the proteins of interest (in BD vector) and cDNA library (in AD vector) proteins on the selective media plates in the period of 5-7 days past transformation and plating (normal appearance time of interaction colonies).

Around 9 days past transformation the first protein-protein interaction colonies appeared on the selective media plates of BoFLC3 and BoJMJ14. One day later a few new interaction colonies appeared. All colonies were transferred to new selective media plates (-LWH dropout medium and 1mM 3AT). On the regrown colonies, a cPCR was performed. cPCR product of colonies of the cPCR products that showed a clear band upon running it on an electrophoresis gel were send out for sequencing.

Analyses of the BoFLC3 interaction colony sequence data using the BLASTN to the Brassica sequence database, revealed COP9 signalosome complex subunit 5b (protein breakdown related protein) and some unidentified proteins as interactors (Appendix 2). The expected interaction with floral regulator SVP (known FLC interactor in *A.* thaliana) was not found. It was therefore assumed that the library screen results might not have correctly reflected the interactome of BoFLC3. Analyses of the BoJMJ14 interaction colony sequence data using BLASTN alignment to the Brassica sequence database, revealed that protein-protein interactions were appearing with filament-like plant protein 4 (microtubule binding protein), COP9 signalosome complex subunit 5b (protein breakdown related protein and some unidentified proteins (Appendix 2).

As none of the expected BoFLC interactions were found, a targeted yeast two-hybrid matrix screen was carried out. In this screen protein-protein interactions between *FLC's* and *SVP's* from *A. thaliana* and *B. oleracea* were screened. To do so *BoSVP1* was cloned from the temperature sensitive cauliflower expression library by PCR. Upon cloning *BoSVP1* into electro competent DH5α cells the presence of the *BoSVP1* insert was checked by cPCR product electrophoresis and consecutive sequencing. Sequencing revealed 4 full length variants of the *BoSVP1* gene and one truncated variant (Figure 16, Appendix 3).

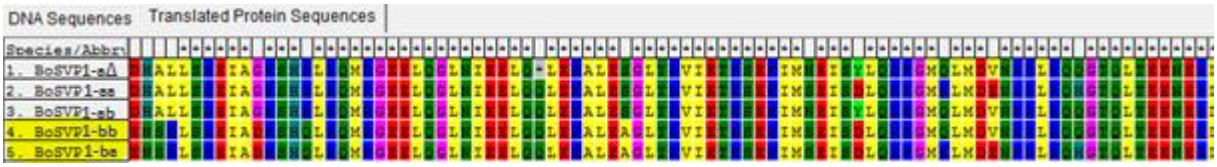

Figure 16 Difference in protein sequence BoSVP1 variants. BoSVP1 variants show variation between the first and second half of the protein. In the first half of the aa sequence BoSVP1-aΔ, BoSVP1-aa and BoSVP1-ab are the same, just like BoSVP1-bb and BoSVP1-ba. In the second half BoSVP1-ab and BoSVP1-bb are similar, just like BoSVP1-aa and BoSVP1-ba. BoSVP1-aΔ shows a stop codon (*) halfway the aa sequence.

All the *BoSVP1* gene variants were tested in the targeted yeast two-hybrid matrix screen against available variants of BoFLC1 and BoFLC3. AtSVP variants and AtFLC were used as positive control. A strong protein-protein interaction was found between BoFLC1-c and BoSVP1-ba. Protein-protein interactions were found between BoFLC3 and BoSVP1 variants, however these interactions were weak. Strong interaction between BoFLC3 and BoSVP1(ab) appeared only on the selective media testing plates containing SD glu –LWA (Figure 17).

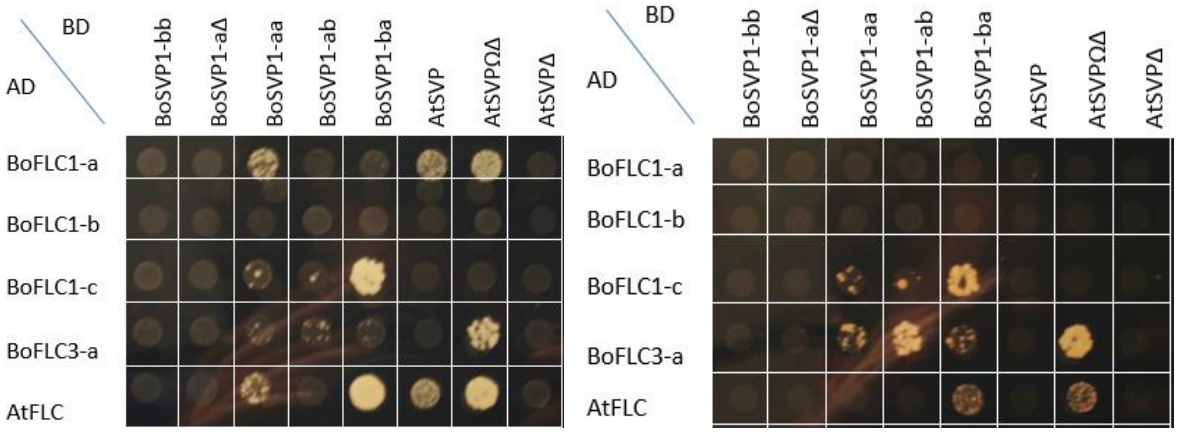

Figure 17 Protein-protein interaction in a Yeast two-hybrid matrix based screen. Interactions between FLC variants and SVP1 variants of both *B. oleracea* and *A. thaliana* on selective media. Left: all protein-protein interactions (selective media: SD glu –LWH + 5mM 3AT in bacto agar) Right: strong protein-protein interactions (selective media: SD glu –LWA in bacto agar). Spot intensity represents the intensity of the interaction.

Discussion

Growth conditions

Cauliflower development from germination until harvest can be divided into three developmental phases: juvenility, curd induction and curd growth; which all start at phenotypically distinctive moments. Developmental age measured by leaf number has been used and validated as a marker for the start of the curd induction phase (Table 1) (Booij, 1990; Booij & Struik, 1990; H. J. Wiebe, 1975; Wurr & Fellows, 2000; Wurr et al., 2004), as it explained more of the variation in the start of the induction phase than any temporal or environmental variable (Booij, 1987). Though, in an experiment carried out under climatized conditions (growth chamber) chronological age could be used as an indication of the start of the induction phase (Booij & Struik, 1990). As the cauliflower plants in the SWAP experiments (4&5) were grown in climatized greenhouse compartments, it was assumed that chronological age could be a good indicator of the start of the induction phase.

However, a distinctive difference was found in the transition timing between the cauliflower plants of the SWAP4 (start: 01-03-2017) and SWAP5 (start: 02-06-2017) experiment. Growth and development of cauliflower are (next to temperature) also strongly influenced by solar radiation (J.E. Olesen & Grevsen, 2000c), this is therefore most likely to be the cause of variation between the experiments. Solar radiation was relatively low in the first 5 weeks of the SWAP4 experiment (12 days under 500 J/cm^2; see Figure 11), it is assumed that this might cause a delay in the start of the curd induction phase. and should therefore also delay the start of the high temperature treatment. To prevent this the start of high temperature treatments should be started at a certain developmental age instead of chronological age. To do so the developmental timing of the curd induction phase should first be determined for each tested cultivar.

Dry weight accumulation

Olesen and Grevsen (1997), found intercepted solar radiation to be linearly related to dry matter accumulation in cauliflower (vegetative stage). Since (intercepted) solar radiation is a strong influencer of cauliflower development, dry weight was expected to be the explanatory variable for developmental age (and thus transition timing) in this experiment. Previous experiments also pointed to *BoFUL-c* meristem expression as a marker (explanatory variable) for transition timing). However, multiple regression analyses for the effect of time and dry weight accumulation on normalized fold *BoFUL-c* expression, revealed that in none of the treatments the effect of dry weight was a significant factor on transition timing. Nonetheless, regression analyses for the effect of intraday variation in dry weight (ΔLn(W)) on intraday variation in normalized fold *BoFUL-c* expression revealed significant correlation for cv. Steady. More biological replicates would be needed to allow for calculation of a significant correlation factor of intraday dry weight variation on intraday variation in *FUL-c* expression. Since no significant correlation was found for Skywalker, it was not possible to make a significantly corrected *BoFUL-c* expression analyses, in which intraday expression variation was corrected by intraday dry weight variation.

To reduce variation in *BoFUL-c* expression within the samples of the same age, it was considered to pool the samples per harvest moment by meristem developmental stage (instead of block). As this pooling strategy turned out not to be feasible due to the small-scale setup of this experiment (no biological replicates for several timepoints), the choice was made to stick to the pooling per harvest moment per treatment per block. However, no significant difference in standard deviation in *BoFUL-c* expression was found between the different treatments, although there was a significant difference in variation between the period of visual meristem transition of the different treatments. Therefore, pooling per meristem developmental stage would probably not have decreased variation within the samples.

Expression analyses validation and RNA purity tests

To validate *FUL-c* expression results for specificity, real-time PCR products can be analysed by melting curve and gel electrophoresis (Bustin & Nolan, 2004; Ririe, Rasmussen, & Wittwer, 1997). In normal real-time PCR with one amplicon, only a single melting curve can be observed and a single product would be visible upon gel electrophoresis. If amplicons of different sizes are present multiple peaks should be observable in the melting curve. Even though on gel two bands (products) of different sizes were visible only a single melting peak was observed for several samples. Moreover, some *BoFUL-c* real-time PCR melting curves that showed a double peak did not show multiple bands on the electrophoresis gel.

BoFLC expression

The thesis report data from Sun (2013), revealed different expression patterns for both *BoFLC1* and *BoFLC3* (*BoFLC1* decreasing abundance *BoFLC3* increasing abundance towards the generative switch) to what I found. Results of my experiments (*BoFLC1* no significant increase/decrease over time and *BoFLC3* non-measurable) were expected to be in resembling with those of Sun (2013), as the plant growth conditions were similar and the same primers were used in the real-time PCR experiments. However, in the previous experiments different cultivars (Lindurian and Fremont vs. Skywalker and Steady in my thesis) were used for real-time PCR as well as for RNA-seq. Variation in measured gene expression could be an effect of allelic variation or differential alternative splicing, in which the gene transcript variants that were measured are more predominant in one cultivar than in the other, resulting in biased measurements. To check whether this has happened all *BoFLC1* and *BoFLC3* variants should be sequenced and the primers that were used should be checked against the sequences.

Repeatability

The results that were obtained by real-time PCR, might raise some concerns regarding repeatability of the experiment. Common guidelines for increasing result significance in real-time PCR experiments are to use multiple biological replicates and three technical replicates for each biological replicate (Edwards, Logan, & Saunders, 2004; Taylor, Wakem, Dijkman, Alsarraj, & Nguyen, 2010). However, due to a lack of time only two technical replicates were used for both biological replicates in the *BoFUL-c* real-time expression experiment and no technical replicates were used in the *BoFLC* genes real time expression experiment. Furthermore, the technical replicates of *BoFUL-c* samples with expression values above 30 Ct often showed a large standard deviation >0.5 Ct. Although variance in Ct value between technical replicates is known to increase with Ct value (Stowers, Haselton, & Boczko, 2010), this makes it hard to make a significant estimation of the relative amount of gene transcript and to observe the effect of other possible sources of error, such as gDNA contamination.

Yeast two-hybrid library screens

Yeast two-hybrid library screens are an efficient method to identify a protein's interactome (de Folter & Immink, 2011). Therefore, results of both BoFLC3 and BoJMJ14 yeast two-hybrid protein-protein interaction library screens were not meeting the expectations, as known interactors did not appear. Although the transformation efficiency was good and visible within 3 days past transformation (within the margins of the protocol described by de Folter & Immink (2011)), hybrid interaction colonies with protein-protein interactions also appeared much later than the expected 5-7 days past transformation.

However, the expected BoFLC3 interactor BoSVP1 also showed only weak interaction in the matrix based yeast two-hybrid screen. Furthermore, the titre of the used cDNA library was lower compared to previously used cDNA libraries ($2*10^5$ compared to $\pm 10^7$) (van der Wal, personal communication). Therefore, results obtained from the yeast two-hybrid library screens were marked as inconclusive.

Yeast two-hybrid matrix based screen

A remarkable result of the matrix based screen is the appearance difference in interaction strength between BoFLC3-a and BoSVP1-ab on the different selective media. Selective media lacking adenine (-LWA) is known to have a stronger selective pressure on the yeast colonies than selective media lacking histidine (-LWH). However, the BoFLC3-a – BoSVP1-ab interaction hybrid shows more vigorous growth on the adenine lacking media than on the histidine lacking media. However, the positive control interaction AtSVP – AtFLC only appears on histidine lacking media. As the AtSVP – AtFLC is a well-studied interaction in planta (Li et al., 2008; Mateos et al., 2011), the histidine lacking media (-LWH) is thought to give a more realistic representation of the protein-protein interactions in planta than the adenine lacking media (-LWA).

Conclusions and recommendations

Plant growth and development is affected by multiple environmental factors, such as temperature, irradiation and CO_2. Combined with crop specific properties, these environmental factors determine the potential plant's dry matter accumulation (Poorter, Nagel, Co, & Water, 2000; Rabbinge, 1993) Plant specific development is also affected by these factors, giving rise to variation in developmental timing (Booij & Struik, 1990; Sultan, 2000). Comparative data analysis revealed that the effect of solar irradiation on developmental timing in our experiments were probably very large, as plants of the SWAP 4 experiment might still have been in their juvenile stage during the high temperature treatment. To make experiments carried out during different seasons of the year more comparable it is recommended to start the high temperature treatment at a fixed plant developmental time point (e.g. leaf number) instead of a fixed chronological time point.

In the real-time PCR gene expression analyses the choice was made to pool meristems from different developmental stages per time point into two biological replicates. This led to a large variation in measured *BoFUL-c* expression between the biological replicates. Furthermore, *BoFUL-c* expression per developmental stage for temperature sensitive cv. Skywalker could not be calculated due to admixture of meristems from different developmental ages. To decrease variation between biological replicates in future experiments it is recommended not to pool (different) meristems into one biological replicate. Since harvested RNA was far more than needed[3], one meristem per biological replicate could suffice. By using one meristem per biological replicate more biological replicates could be used per time point reducing the standard error of the mean in expression analyses. Furthermore, the effect of developmental stage per time point on gene expression could be analysed.

Results of the real-time PCR expression analyses were quite different from those done in previous experiments. To identify the source of this difference (experimental or (epi-)genetic) it is recommended to do a similar experiment with multiple cultivars (e.g. Skywalker, Lindurian, Steady and Fremont). If the expression patterns are similar to those analysed in previous experiments and in this thesis, expression patterns may be cultivar dependent. If not the experiment in this thesis might have been compromised. To find out what the effect of the gDNA contaminations was on the expression analyses it is recommended to redo the RNA Cleanup experiment with a DNase treatment, (preferably) using DNase from a different batch or manufacturer, as multiple gDNA contaminations were found after previous treatments. Another option to test the accuracy and significance of the real-time PCR experiments and to get a comprehensive overview of differential gene expression during the experiment is to do RNA Seq analysis. By RNA Seq analysis temperature effected differential gene expression can be identified for further testing in protein-protein or protein-transcription factor interactions.

Although yeast two-hybrid library screening is a nice tool to identify the interactome of proteins, screening results in this thesis were inconclusive. To find out what caused the unexpected results it is recommended to do another yeast-two hybrid library screen with BoFLC1-c; a BoFLC1 splice variant that showed strong interaction with BoSVP1-ba in the matrix based screen. If interaction with BoSVP1-ba is found in this library screen; the hypotheses that a proper BoFLC3 library screen should identify BoFLC3 interaction with BoSVP might have been wrong. However, if a BoFLC1-c library screen does not reveal any interaction between BoFLC1-c and BoSVP1-ba, it can be concluded that the BoFLC3 (and probably also BoJMJ14) library screens failed. If so, it is recommended to optimize the library titre and redo the library screen with BoFLC1-c.

Once the library screens with the cauliflower cDNA expression library have been verified to function properly, it is recommended to screen for the interactors of different BoCCA1 variants. In *A. thaliana CCA1* is a floral regulator gene that has been shown to have a temperature affected alternative splicing ratio (Seo et al., 2012), forming a bridge between the temperature- and circadian/photoperiod effected floral timing pathway. If BoCCA1 shows similar interactions with genes from the floral timing pathway expression patterns might even be used in gene expression models to correct for the effect day length in the meristem enriched gene expression experiments.

Matrix based screens between BoFLC variants and BoSVP1 variants established that differentially spliced genes show differential interaction; differential splicing forms of BoFLC1 showed differential interaction with different alternative splicing forms of BoSVP1. This is underlines the importance of studying post-

[3] Varying per replicate 5-15x more RNA than needed was harvested

translational RNA and protein modifications, as these differentially interacting proteins originated from the same gene. These differential interactions could be a link in plants sensitivity to ambient temperature for curd initiation. By comparing the sensitive cDNA cauliflower library to an insensitive cDNA cauliflower library for abundance of these splice variants, one could check whether this protein-protein interaction pattern could be a key in the plants temperature sensitivity regarding curd initiation time.

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Appendices

Appendix 1

Temperature sensitive (A) and insensitive (B) cauliflower expression library contents.

Total RNA meristem enriched tissue from samples below was isolated and reverse transcribed into cDNA. Samples were harvested just before a high temperature treatment (0H) at 35 days after sowing, or 24H after the start of the heat treatment (36 days after sowing). Plants were grown in a (partly)climatized greenhouse either in Wageningen or Marne.

Temperature sensitive cauliflower expression library contents.

Sample ID	Cultivar	Harvest time	Location	RNA content (ng/µL)
K01	Skywalker	0H	Marne	252
K02	Skywalker	0H	Marne	224
K241	Skywalker	24H	Marne	421
K242	Skywalker	24H	Marne	262
A01	Amerigo	0H	Marne	259
A02	Amerigo	0H	Marne	260
A241	Amerigo	24H	Marne	282
A242	Amerigo	24H	Marne	192
L1S	Lindurian	0H	Marne	318
L2S	Lindurian	0H	Marne	335
L3S	Lindurian	24H	Marne	265
L4S	Lindurian	24H	Marne	190
L51	Lindurian	0H	Wageningen	308
L52	Lindurian	0H	Wageningen	272
L55	Lindurian	24H	Wageningen	197
L56	Lindurian	24H	Wageningen	404

Temperature insensitive cauliflower expression library contents.

Sample ID	Cultivar	Harvest time	Location	RNA content (ng/µL)
S01	Steady	0H	Marne	330
S02	Steady	0H	Marne	196
S241	Steady	24H	Marne	247
S242	Steady	24H	Marne	321
F1S	Fremont	0H	Marne	234
F2S	Fremont	0H	Marne	101
F3S	Fremont	24H	Marne	205
F4S	Fremont	24H	Marne	80
B01	BeJo2903	0H	Marne	307
B02	BeJo2903	0H	Marne	214
B241	BeJo2903	24H	Marne	269
B242	BeJo2903	24H	Marne	383
B51	BeJo2903	0H	Wageningen	136
B52	BeJo2903	0H	Wageningen	228
B55	BeJo2903	24H	Wageningen	163
B56	BeJo2903	24H	Wageningen	274

Appendix 2

Blast results cPCR yeast two-hybrid library screen *BoFLC3*. A colony PCR (cPCR) with vector specific primers was performed on the colonies that were regrown on selective media (LB agar –LWA dropout + 5mM 3AT). Colonies that showed a clear band in the gel electrophoresis were send out for sequencing. Hits found by comparing sequences to the brassica database using the BLAST are noted below.

Colony no.	Gene name	Genomic location	Gene size(bp)
1	Brassica oleracea var. oleracea COP9 signalosome complex subunit 5b, mRNA	LOC106295030	1383
12	Brassica oleracea var. oleracea COP9 signalosome complex subunit 5b, mRNA	LOC106295030	1383
24	Brassica oleracea var. oleracea uncharacterized LOC106301109, transcript variant X2, mRNA	LOC106301109	2544
34	Brassica oleracea var. oleracea COP9 signalosome complex subunit 5b, mRNA	LOC106295030	1383
41	Brassica rapa uncharacterized LOC108869770, mRNA	LOC108869770	1317

Blast results cPCR yeast two-hybrid library screen *BoJMJ14(variant 1)*. A colony PCR (cPCR) with vector specific primers was performed on the colonies that were regrown on selective media (LB agar –LWA dropout + 5mM 3AT). Colonies that showed a clear band in the gel electrophoresis were send out for sequencing. Hits found by comparing sequences to the brassica database using the BLAST are noted below.

Colony no.	Gene name	Genomic location
1	Brassica oleracea var. oleracea filament-like plant protein 4, transcript variant X4, mRNA	LOC106294736
6	Brassica oleracea var. oleracea filament-like plant protein 4, transcript variant X4, mRNA	LOC106294736
8	Brassica oleracea var. oleracea uncharacterized LOC106301109, transcript variant X2, mRNA	LOC106301109
	Brassica rapa AUGMIN subunit 6	LOC103850194
11	Brassica oleracea var. oleracea filament-like plant protein 4, transcript variant X4, mRNA	LOC106294736
20	Brassica oleracea var. oleracea filament-like plant protein 4, transcript variant X4, mRNA	LOC106294736
26	Brassica oleracea var. oleracea 26S proteasome regulatory subunit 4 homolog A-like, mRNA	LOC106312310
27	Brassica oleracea var. oleracea filament-like plant protein 4, transcript variant X4, mRNA	LOC106294736
28	Brassica oleracea var. oleracea COP9 signalosome complex subunit 5b, mRNA	LOC106295030
31	Brassica oleracea var. oleracea uncharacterized LOC106301109, transcript variant X2, mRNA	LOC106301109
34	Brassica oleracea var. oleracea filament-like plant protein 4, transcript variant X4, mRNA	LOC106294736
37	Brassica oleracea var. oleracea filament-like plant protein 4, transcript variant X4, mRNA	LOC106294736
38	Brassica oleracea var. oleracea COP9 signalosome complex subunit 5b, mRNA	LOC106295030

Appendix 3

Genetic and protein sequences *BoSVP1* variants cloned from the temperature sensitive cauliflower expression library.

> BoSVP1-aΔ

ATGGCGAGAGAGAAGATTCAGATCAGGAAAATCGACAACGCGACGGCGAGACAAGTAACGTTCTCAAAACGAAGA
AGAGGTCTTTTCAAAAAAGCTGAAGAGCTATCCGTTCTCTGCGACGCTGATGTTGCTCTCATCATCTTCTCTTCCACT
GGAAAGCTCTTTGAGTTTTGTAGCTCCAGCATGAGGGAAGTGTTAGAGAGGCATAACTTGCAGTCAAAGAACTTGG
AGAAGCTAGATCAACCATCTCTTGAGTTACAGCTGGTCGAGAACAGCGATCACGCCCTGTTGAGCAAAGAGATTGC
GGGAAAGAGCCACCGATTAAGGCAAATGAGAGGAGAGGAACTTCAAGGACTTAACATTGAAGAACTTCAACAGCTG
GAAAAGGCCCTTGAATCCGGTTTGACTCGCGTAATTGAAACCAAGAGTGAAAAGATTATGAGTGAGATCAGTGACC
TTCAAAGAAAGGGAATGAAATTGATGGATGAGAACAAGCGGCTAAGGCAGCATGGAACACAACTAACAGAAGAGA
ACGAGCGACTAGGCAAGCAAATATATAATAATATGCATGAAAGATACGGTGGTGTTGAGTCGGAGAAGACCGCCGT
GTACGAGGAAGGGCACTCGTCAGAGTCCATTACTAACGCCGGAAACTCCACCGGCGCTCCTGTTGACTCCGAGAG
CTCCGATACCTCTCTAAGGCTCGGCTTACCGTATGGTGGTTAG

> BoSVP1-aa

ATGGCGAGAGAGAAGATTCAGATCAGGAAAATCGACAACGCGACGGCGAGACAAGTAACGTTCTCAAAACGAAGA
AGAGGTCTTTTCAAAAAAGCTGAAGAGCTATCCGTTCTCTGCGACGCTGATGTTGCTCTCATCATCTTCTCTTCCACT
GGAAAGCTCTTTGAGTTTTGTAGCTCCAGCATGAGGGAAGTGTTAGAGAGGCATAACTTGCAGTCAAAGAACTTGG
AGAAGCTAGATCAACCATCTCTTGAGTTACAGCTGGTCGAGAACAGCGATCACGCCCTGTTGAGCAAAGAGATTGC
GGGAAAGAGCCACCGATTAAGGCAAATGAGAGGAGAGGAACTTCAAGGACTTAACATTGAAGAACTTCAATAGCTG
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TCAAAGAAAGGGAATGCAACTGATGGACGTGAACAAGCGGCTAAGGCAGCAAGGGACACAGTTAACGGAAGAGAA
CGAGCGACTAGGTCAGCAGATATACAATAATGTGCATGAAAGATACAGTGGAGGTGAATCAGAGAACACTGCCGTG
TACGAAGAAGGACACTCATCGGAGTCCATTACTAACGCCGGAAACTCCACAGGCGCTCCTGTTGACTCCCAGAGCT
CCGATATCTCCCTTAGGCTCGGCTTACCGTATGGTGGTTAGTACCCAGCTTTCTTGTACAAAGTGGTTTGATGGCCG
CTCACTAAGTAAGACGTCGAGCTCTGAGTAAGTAACGGCCGCCACCGCGATGGAGCTTTGGACTTCTCCCCGAGAG
TGGGGGGTGAAAAAGAAAAAAAAAAAAAAGACTCCTGGGGAAAAAATATAGGGGGTTTAAAAGACGCCCAAAAAAG
CCCGTGGGGGGGAAGAGATGGGGTGTTGACGACGCCGAA

> BoSVP1-ab

ATGGCGAGAGAGAAGATTCAGATCAGGAAAATCGACAACGCGACGGCGAGACAAGTAACTTTCTCTAAACGAAGAA
GAGGTCTTTTCAAAAAAGCTGAAGAGCTCTCCGTTCTCTGCGACGCTGATGTTGCCCTCATCGTCTTCTCTTCCACC
GGAAAGCTCTTCGAGTTTTGTAGCTCCAGCATGAGGGAAGTACTAGAGAGGCATAATTTGCAGTCAAAGAACTTGG
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GGGAAAGAGCCACCGATTAAGGCAAATGAGAGGAGAGGAACTTCAAGGACTTAACATTGAAGAACTTCAACAGCTG
GAAAAGGCCCTTGAATCCGGTTTGACTCGCGTAATTGAAACCAAGAGTGAAAAGATTATGAATGAGATCAGTTATCT
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CGAGCGACTAGGTCAGCAGATATACAATAATGTGCATGAAAGATACGGTGGAGGTGAATCAGAGAACACTGCCGTG
TACGAGGAAGGACACTCATCGGAGTCCATTACTAACGCCGGAAACTCCACAGGCGCTCCTGTTGACTCCGAGAGCT
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AAGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTGCCATCCAGCTGCA
GCTCTGGCCCTTTCTCAAATCTCTTATGTCAAAAAAAAATAAAAATATCCCTAAAAAAGTTGGGTGTGAAAGAGAAG
GGAAGAGAGGGAGGGGAAGAGGAGGGAGAGGGGTGGGCGGGAGAGGGAGAGAGGAG

> BoSVP1-bb

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TTCTTCGCCCGAAAGTGGGGGGGGTTGAAAAAAGGGAAGAGAATTCAAAACCTCTGGGCGAAGAAAAAAGAGGGG
GTTTTAGAAGATCCCCTAAACAAAAGGGGGTCGCGTTGAATGAAAAGGGGGGGGGGGGA

> BoSVP1-ba

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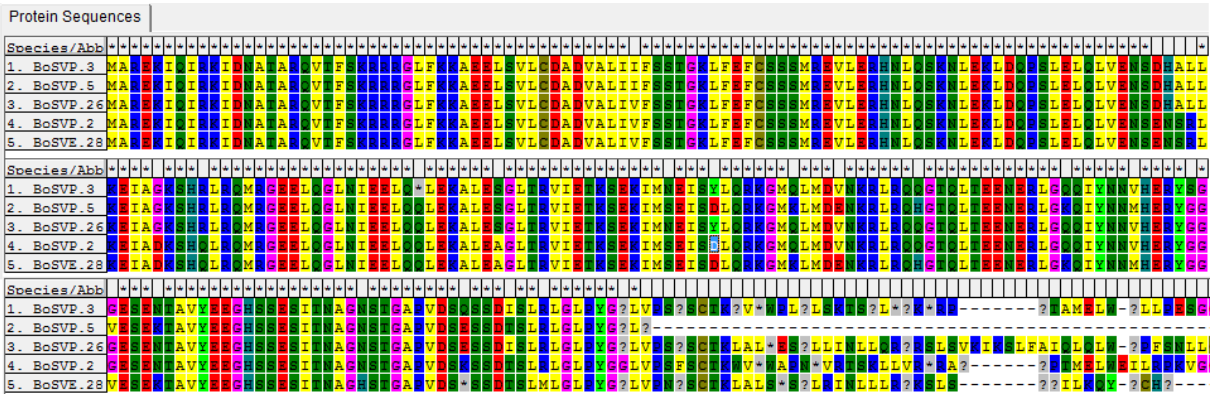

Protein sequence alignment BoSVP1 variants. Working titles are used in the description (BoSVP.3 = BoSVP1-aΔ; BoSVP.5 = BoSVP1-aa; BoSVP.26 = BoSVP1-ab; BoSVP.2 = BoSVP1-bb; BoSVP.28 = BoSVP1-ba)