Investigating Vernalization in *Brassica oleracea*

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Abstract

Flowering in Purple Sprouting Broccoli (PSB, *Brassica oleracea italica*) is accelerated by prolonged exposure to cold temperatures, a process called vernalization. However, PSB plants cannot be vernalized until they have grown through an insensitive "juvenile phase". I studied juvenility and vernalization in four genotypes of PSB. The chronological duration of the juvenile phase varied among genotypes and with environmental conditions. I propose measuring the duration of the juvenile phase in terms of leaf number in order to account for differences in development rate under different environmental conditions. The four genotypes also varied in both their qualitative vernalization requirements and quantitative vernalization responses.

The Brassica genus is closely related to the model plant *Arabidopsis thaliana*, in which *FLOWERING LOCUS C* (*FLC*) plays a central role in controlling the vernalization response. In Arabidopsis *FLC* encodes a floral repressor and is strongly expressed before vernalization in winter annual accessions but stably down-regulated during vernalization. Studies in *Brassica napus* and *B. rapa* have suggested that the role of *FLC* has been conserved among the Brassica species. I measured the expression of three of the four *B. oleracea* homologues of *FLC* in response to vernalization. There was no clear correlation between *BoFLC1* expression level and vernalization response. *BoFLC3* was transiently repressed during vernalization. *BoFLC4* was also repressed during vernalization, and the stability of this repression appeared to correlate with the strength of the vernalization response. This resembles the situation in Arabidopsis, in which the stability of *FLC* down-regulation during cold-treatment is associated with natural variation in vernalization response.

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1 General introduction to flowering time, vernalization, and the Brassica genus

1.1 Flowering time is controlled by both endogenous and environmental cues including vernalization

The timing of the transition from vegetative growth to flowering is critical to the reproductive success of plants (Simpson & Dean 2002; Shindo et al. 2005) and is important in agriculture and breeding (Lagercrantz et al. 1996; Bohuon et al. 1998; Kole et al. 2001; Putterill et al. 2004; Kim et al. 2007; Razi et al. 2008). Flowering time is controlled by both endogenous and environmental cues including photoperiod, gibberellic acid, ambient temperature, light quality and quantity, nutrient and water supply, and vernalization (Levy & Dean 1998; Simpson & Dean 2002).

Vernalization is "the acquisition or acceleration of the ability to flower by a chilling treatment" (Chouard 1960). Thus vernalization does not involve the actual initiation of flowering, but promotes the competence to flower. Some plants, known as "biennials", have an obligate requirement for vernalization in order to flower. Others, sometimes referred to as "winter annuals", show a facultative response to vernalization (in which the competence to flower is accelerated) but do not have an obligate requirement for vernalization in order to flower (Chouard 1960; Michaels & Amasino 2000). Vernalization generally requires a long period of exposure to low temperature (Henderson et al. 2003). The vernalization response is quantitative: increasing the duration of cold exposure results in a greater response, until a saturation point is reached and no further response occurs (Henderson et al. 2003). In the wild a vernalization response can help to ensure that plants flower in spring when conditions are favourable (Levy & Dean 1998; Simpson & Dean 2002).

1.2 Flowering time in Arabidopsis thaliana is controlled by a network of integrated pathways that converge upon the floral pathway integrator genes

The small, dicotyledonous plant *Arabidopsis thaliana* (family Brassicaceae), henceforth referred to as "Arabidopsis", is an ideal model plant in which the molecular bases of flowering time has been extensively studied. A summary of the pathways that control flowering in Arabidopsis is shown in Figure 1. Arabidopsis has a fast generation time, and produces large numbers of seed (Dean 1993). The Arabidopsis genome is small (approximately 125 MB), has been completely sequenced (Initiative 2000), and contains relatively little repetitive DNA (Dean 1993). Arabidopsis is a facultative long-day plant (Levy & Dean 1998; Yoo et al. 2005; Turck et al. 2008). Most Arabidopsis ecotypes are winter-annuals, and are late-flowering unless vernalized (Levy & Dean 1998). Arabidopsis also has a wide geographical distribution. Natural accessions of Arabidopsis show a wide range of variation in flowering time and response to vernalization (Lempe et al. 2005; Shindo et al. 2005; Shindo et al. 2006).

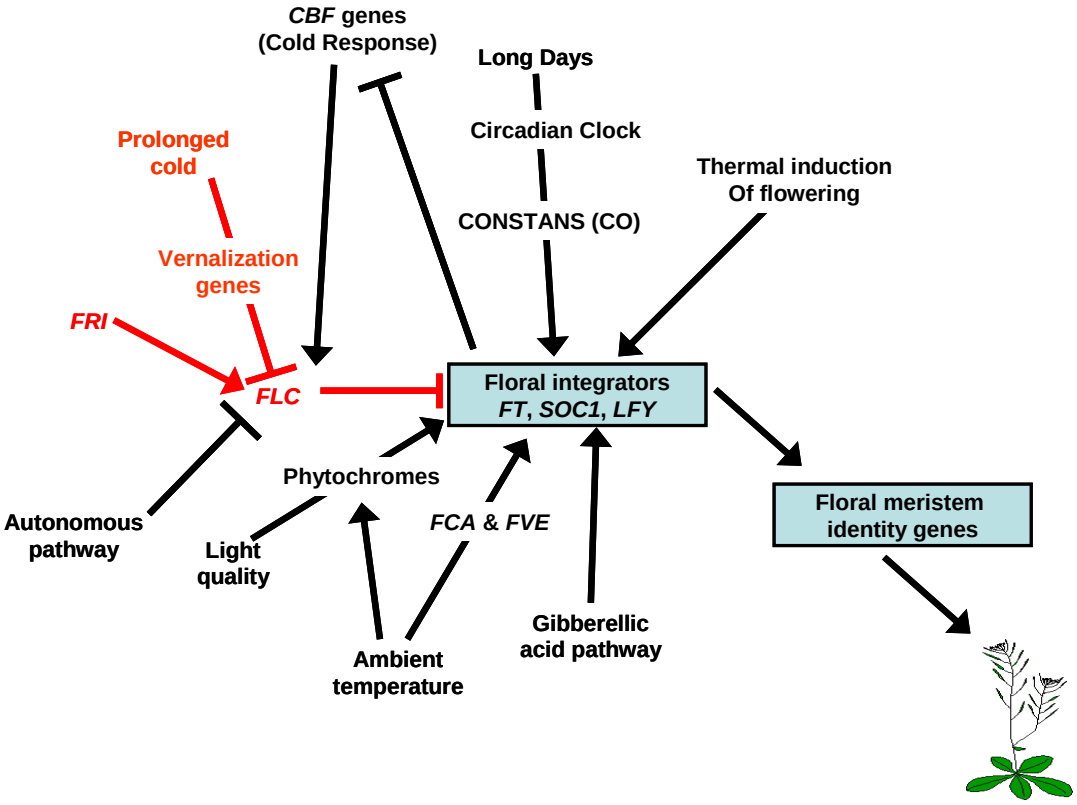


Figure 1: A summary of the pathways that influence flowering time in *Arabidopsis thaliana* described in this chapter (adapted from Henderson & Dean (2004)). The components of the vernalization pathway are highlighted in red. There are four major flowering-time pathways (the photoperiod/long-day, gibberellic acid, autonomous, and vernalization pathways) and several "secondary" pathways such as the light quality and ambient temperature pathways. All of these pathways converge upon a common set of targets: the floral integrator genes: *FLOWERING LOCUS T* (*FT*), *AGAMOUS-LIKE 20/SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*AGL20*/*SOC1*), and *LEAFY* (*LFY*). These in turn activate the floral meristem identity genes, which cause the apex to switch from a vegetative to a reproductive mode and produce flowers.

The transition to flowering in Arabidopsis is controlled by a network of pathways that converge upon a common set of targets known as the floral pathway integrators: *FLOWERING LOCUS T* (*FT*), *AGAMOUS-LIKE 20*/*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*AGL20*/*SOC1*), and *LEAFY* (*LFY*) (Figure 1) (Henderson & Dean 2004). These include the photoperiod, vernalization, autonomous, gibberellic acid (GA), ambient temperature, elevated growth temperature, light quality, and cold stress pathways (Simpson & Dean 2002; Blazquez et al. 2003; Cerdan & Chory 2003; Halliday et al. 2003; Halliday &

Whitelam 2003; Henderson & Dean 2004; Kim et al. 2004; Franklin & Whitelam 2005; Balasubramanian et al. 2006; Franklin 2009; Seo et al. 2009). The floral pathway integrators activate the floral meristem identity genes *APETALA1* (*AP1*), *APETALA2* (*AP2*), *FRUITFULL* (*FUL*), *CAULIFLOWER* (*CAL*), and *LFY*, which control the transition of the meristem from vegetative growth to flowering (Simpson & Dean 2002; Henderson & Dean 2004).

Long photoperiods promote flowering in Arabidopsis via CONSTANS (CO). *CO* expression is regulated by the circadian clock (Suarez-Lopez et al. 2001; Valverde et al. 2004). Nuclear CO protein is degraded in the darkness and in the morning but in the evening it is stabilized by light (Valverde et al. 2004). As a result CO protein only accumulates at the end of a long day (Valverde et al. 2004). CO promotes flowering (in long days) by activating the expression of the floral pathway integrator genes *FT* and *SOC1* (Samach et al. 2000; Suarez-Lopez et al. 2001), although *FT* appears to be the primary target of CO in the leaves (Wigge et al. 2005; Yoo et al. 2005). The up-regulation of *SOC1* by CO may occur via *FT* (Yoo et al. 2005) and possibly also by activation by CO (Michaels et al. 2005). *FT* is activated in the phloem companion cells of the leaves by CO in response to long days. FT protein acts as a mobile signal, travelling through the vasculature from the leaves to the apex (Jaeger & Wigge 2007; Notaguchi et al. 2008). In the apex *FT* interacts with the bZIP protein FD, which is expressed in the apex but not the vasculature. FT and FD interact at the apex to activate floral meristem identity gene(s) including *AP1* (Abe et al. 2005; Wigge et al. 2005; Jaeger & Wigge 2007).

Gibberellic acid promotes flowering by positively regulating the expression of *LFY* (Blázquez et al. 1998; Blazquez & Weigel 2000) and *SOC1* (Moon et al. 2003). Gibberellic acid is required for flowering under short days (Wilson et al. 1992). Gibberellic acid appears to promote *SOC1* expression under both long and short days whereas the CO only promotes *SOC1* expression under long days. So gibberellic acid and CO redundantly activate *SOC1* under long days but under short days only the gibberellic acid pathway activates *SOC1*. Therefore the gibberellic acid biosynthesis mutant *ga1-3* does not activate *SOC1* expression under short days (Moon et al. 2003).

The vernalization-responsive late flowering of winter-annual Arabidopsis accessions is controlled by two loci that act synergistically to delay flowering: *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*[1]) (Michaels & Amasino 1999; Michaels & Amasino 2000; Michaels & Amasino 2001; Michaels et al. 2003). FLC acts as a dosage-dependent repressor of flowering (Michaels & Amasino 1999; Sheldon et al. 1999; Sheldon et al. 2000b) by repressing the expression of the floral pathway integrators *FT* and *SOC1* (Lee et al. 2000; Michaels et al. 2005; Helliwell et al. 2006). *FRI* up-regulates *FLC* expression (Michaels & Amasino 1999; Sheldon et al. 1999; Johanson et al. 2000; Michaels & Amasino 2001; Shindo et al. 2005) by a mechanism that requires the *FRI*-related gene *FRIGIDA LIKE 1* (*FRL1*) (Michaels et al. 2004), *FRIGIDA-ESSENTIAL 1* (*FES1*) (Schmitz et al. 2005), and *SUPPRESSOR OF FRIGIDA* (*SUF4*) (Kim & Michaels 2006). During vernalization *FLC* is down-regulated (Michaels & Amasino 1999; Sheldon et al. 2000b), and the extent of this down-regulation is proportional to the duration of the cold exposure (Sheldon et al. 2000b). This vernalization-induced repression of *FLC* is mitoticaly stable but reset in each generation (Michaels & Amasino 1999; Sheldon et al. 2000b). Therefore vernalization promotes the competence to flower by releasing the *FLC*-mediated block on flowering.

The genes of the autonomous pathway, *FCA*, *FY*, *FPA*, *FVE*, *LD*, *FLD* and *FLK*, promote flowering by repressing the expression of *FLC* (Henderson et al. 2003; Baurle & Dean 2006). Mutants of autonomous-pathway genes are late-flowering under both long-and short days, but their late-flowering phenotype can be overcome by vernalization (Simpson & Dean 2002; Henderson et al. 2003). However, Henderson et al. (2003) reported that naturally occurring loss-of-function mutations in autonomous genes that contribute to natural variation in the vernalization requirement of Arabidopsis had not been found.

[1] *FLC* has also previously been known as *FLOWERING LOCUS F* or *FLF*. See SHELDON, C. C. BURN, J. E. PEREZ, P. P. METZGER, J. EDWARDS, J. A. PEACOCK, W. J. & DENNIS, E. S. (1999). The FLF MADS box gene: A repressor of flowering in Arabidopsis regulated by vernalization and methylation. *Plant Cell* **11**(3), 445-458, SHELDON, C. C. ROUSE, D. T. FINNEGAN, E. J. PEACOCK, W. J. & DENNIS, E. S. (2000b). The molecular basis of vernalization: The central role of FLOWERING LOCUS C (FLC). *Proceedings of the National Academy of Sciences of the United States of America* **97**(7), 3753-3758.

Flowering in Arabidopsis is delayed at low ambient temperatures (Thingnaes et al. 2003; Samach & Wigge 2005). For example Landsberg *erecta* flowers with 10 leaves when grown at 23°C but 15 when grown at 16°C (Blazquez et al. 2003). Both the average daily temperature and the difference between daytime and night-time temperature affect the flowering time (measured as the number of rosette leaves at the time of visible bud) in Arabidopsis (Thingnaes et al. 2003). Two genes of the autonomous pathway, *FCA* and *FVE*, are believed to be involved in mediating flowering time in response to ambient temperature (Blazquez et al. 2003). As well as acting redundantly in a temperature-independent mechanism to repress *FLC* expression, *FCA* and *FVE* also appear to act together in a temperature-dependent manner to regulate flowering time in response to ambient temperature. This temperature-dependent role of *FCA* and *FVE* appears to be at least partially independent of *FLC* but involve the regulation of the floral-integrator *FT* (Blazquez et al. 2003). The functions of *FCA* and *FVE* in the thermosensory pathway are mediated by the MADS-box gene *SHORT VEGETATIVE PHASE* (*SVP*). *SVP* appears to act downstream of *FCA* and *FVE* in the thermosensory pathway and negatively regulate *FT* expression (Lee et al. 2007).

Small increases in ambient growth temperature above 23°C can strongly induce flowering in non-inductive photoperiods. For example the Arabidopsis ecotypes Ler and Col both flowered considerably earlier at 25 and 27°C than at 23°C under short days. There is substantial variation among natural Arabidopsis accessions and mutants in this thermal response. This thermal induction of flowering is suppressed by *FLC*, e.g. in autonomous pathway mutants (in which *FLC* is strongly expressed). Whereas a closely-related gene, *FLM*, appears to be involved in modulating thermosensitivity. This thermal induction of flowering acts upstream of *FT* and does not require *CO* but does depend upon giberellin (Balasubramanian et al. 2006).

Intermittent cold-treatment delays flowering in Arabidopsis. Intermittent cold-treatment is believed to be sensed by *FVE*, leading to increased *FLC* expression and hence delayed flowering time (Kim et al. 2004). *FVE* is also involved in regulating the cold response. The mutant *acg1*, which is a null allele of *FVE*, has altered cold-responsive gene expression and did not show delayed flowering in response to intermittent cold-treatment like wild-type plants. In Arabidopsis *CBF/DREB1* are

induced by cold exposure. The CBF/DREB1 proteins regulate the expression of cold-induced genes that increase freezing-tolerance (Thomashow 1999). In *acg1* mutants expression of *CBF* genes occurred earlier in response to cold than in wild-type plants, although similarly increased transcript levels were observed in both mutants and wild-types. Freezing tolerance was greater in cold-acclimated *acg1* plants than in wild-type plants (Kim et al. 2004).

Feedback also occurs between the cold response and the regulation of flowering time. Increased expression of the *CBF* genes due to cold during vegetative growth leads to activation of *FLC* expression, delaying flowering. But *SOC1* represses the expression of the *CBF* genes. Therefore the cold response is suppressed when flowering is induced and *SOC1* expression increases (Seo et al. 2009).

GIGANTEA (*GI*) appears to positively regulate freezing tolerance. *gi-3* mutants have reduced constitutive freezing tolerance and cold-acclimation ability compared to wild-type plants. But *GI* appears to regulate freezing tolerance independently of the *CBF* genes. Intermittent cold also delays flowering more strongly in *gi-3* mutants than in wild-type plants. This suggests that there may be a link between flowering time and cold response through *GI* in Arabidopsis (Cao et al. 2005).

Flowering in Arabidopsis is accelerated as part of the shade-avoidance response of plants to a low ratio of Red:Far Red (R:FR) light. Low R:FR indicates the presence of neighbouring vegetation or over-shading by other vegetation (Halliday et al. 1994; Halliday et al. 2003; Franklin & Whitelam 2005). The acceleration of flowering in response to low R:FR is evident in both the time taken to initiate flowering and the leaf number at flowering (Halliday et al. 1994). The shade-avoidance response also includes increased stem and petiole elongation (Halliday et al. 2003). Variations in the ratio of R:FR light are detected by a group of photoreceptors called the phytochromes (Halliday et al. 2003; Franklin & Whitelam 2005). These are synthesized in an inactive red-light-absorbing form, Phytochrome Red or "Pr" and undergo conversion into an active far-red-light-absorbing form, Phytochrome Far Red or "Pfr". The conversion of Pr to Pfr is optimized in red light and the reverse reaction is optimized in far-red light (Franklin & Whitelam 2004; Franklin & Whitelam 2005; Franklin 2009). Hence in most irradiation conditions the two forms exist in a dynamic photoequilibrium, with the relative concentration of the active

form, Pfr, depending upon the ratio of R:FR light (Franklin & Whitelam 2005; Franklin 2009).

PhyB plays the major role in the perception of low R:FR and mediating the shade-avoidance response (Halliday et al. 1994; Halliday et al. 2003; Adams et al. 2009) but the relative contributions of the different phytochromes depends upon the environmental conditions e.g. ambient temperature (Halliday et al. 2003; Halliday & Whitelam 2003; Adams et al. 2009). At 22°C phyB appears to be the main regulator in the induction of flowering by low R:FR light ratios (Halliday et al. 2003). *phyB* mutants grown at 22°C show constitutively elongated petioles and early flowering compared to the wild-type (Halliday et al. 2003). But when *phyB* plants are grown at 16°C the early flowering response of *phyB* mutants is abolished (Halliday et al. 2003; Halliday & Whitelam 2003; Franklin & Whitelam 2005; Franklin 2009). *phyB* mutants grown at 16°C did still show the elongated petiole phenotype as in those grown at 22°C though (Halliday et al. 2003). At this cooler temperature phyE and phyD appear to play a greater role in regulating flowering (Halliday et al. 2003; Halliday & Whitelam 2003; Franklin & Whitelam 2005; Franklin 2009). But phyB still appears to play a role in the control of flowering at this temperature through interactions with other phytochromes (Halliday & Whitelam 2003).

The temperature-dependent control of flowering by phytochromes appears to be correlated with the expression of the floral integrator gene *FT* (Halliday et al. 2003). *FT* was up-regulated compared to wild-type in *phyB* and *phyAphyBphyD* mutants grown at 22°C. This correlates with the early flowering phenotypes displayed by these mutants when grown at this temperature. But *FT* expression was lower and more constant among the genotypes when plants were grown at 16°C. At this temperature these mutants did not flower early compared to wild-type (Halliday et al. 2003). This suggests that phyB (and also phyA and phyD to a lesser extent) may repress flowering in warm temperatures and that they achieve this at least partially by repressing *FT* (Halliday et al. 2003). Another gene, *PHYTOCHROME AND FLOWERING TIME 1*, or *PFT1*, appears to act downstream of phyB to regulate *FT* expression (Cerdan & Chory 2003; Boss et al. 2004). The repression of *FT* at 16°C in *phyAphyBphyD* may be due to the action of phyE. The loss of phyE in addition to phyA, phyB, and PhyD led to accelerated flowering and increased *FT* expression (at

16°C) (Halliday et al. 2003). In one accession of Arabidopsis, Bla-6, the early-flowering response to low R:FR light is blocked by high levels of *FLC*, which inhibit *FT* expression. But Bla-6 still showed an elongation response to low R:FR. These high levels of *FLC* result from a "weak" *FY* allele combined with functional *FRI* & *FLC* (Adams et al. 2009). Therefore there is interaction between the light-quality pathway and the regulation of flowering by ambient temperature and components of the autonomous and vernalization pathways which all appear to converge upon *FT*.

1.3 The stable epigenetic repression of FLOWERING LOCUS C *(*FLC*) expression is central to vernalization in Arabidopsis*

FLC, though not a floral integrator gene, plays a key role in the control of flowering by multiple pathways (Figure 1). *FLC* is a target for the autonomous and vernalization pathways, is also regulated by cold stress via the CBF genes, and affects plant responses to photoperiod, light quality etc by repressing the expression of the floral pathway integrator *FT*. Therefore vernalization, by stably repressing *FLC* expression, plays a critical role in controlling flowering time.

1.3.1 High *FLC* expression in non-vernalized plants requires chromatin modifications that are associated with actively transcribed genes

The high *FLC* expression caused by *FRI* in winter-annual Arabidopsis accessions is associated with high levels of H3K4me3 in *FLC* chromatin, which appear to be brought about by an Arabidopsis PAF1-like complex (He et al. 2004; He & Amasino 2005; Kim et al. 2005; He 2009; Jiang et al. 2009). In *Saccharomyces cerevisiae* (yeast) the PAF1 complex (RNA Polymerase II Associatd Factor 1 Complex) is involved in recruiting a complex containing the H3K4 methyltransferase SET1 to the chromatin of target genes, which catalyzes the trimethylation of H3K4. H3K4 trimethylation is a mark associated with actively transcribed genes (He 2009). The Arabidopsis PAF1-like complex contains homologues of components of the yeast PAF-1 complex: EARLY FLOWERING 7 (ELF7), EARLY FLOWERING 8 (ELF8)/VERNALIZATION INDEPENDENCE 6 (VIP6), and VERNALIZATION INDEPENDENCE 4 (VIP4). ELF7 is a homologue of yeast PAF1, ELF8/VIP6 is a homologue of yeast CTR9, and VIP4 is a homologue of yeast LEO1 (Zhang & Van

Nocker 2002; He et al. 2004; He & Amasino 2005; Kim & Michaels 2006; He 2009). This complex may also include VERNALIZATION INDEPENDENCE 5 (VIP5, a homologue of yeast RTF1) (Oh et al. 2004; Kim et al. 2005; Kim & Michaels 2006; He 2009). A homologue of the human PAF1 complex component HSK18, VERNALIZATION INDEPENDENCE 3 (VIP3), has also been identified in Arabidopsis (He 2009). A putative histone methyl transferase EFS (EARLY FLOWERING IN SHORT DAYS, which contains a SET domain) also appears to be involved in the regulation of *FLC* expression. *EFS* is required for the increased H3K4me3 in *FLC* chromatin and increased *FLC* expression that occurs in lines containing an active *FRI* allele and in autonomous pathway mutants. EFS may be recruited to *FLC* by the PAF1-like complex (Kim et al. 2005). *SUF4* and other genes of the *FRI* pathway (which includes *FRI*, *SUF4*, *FES*, and *FRL1*) may be required to recruit the EFS/PAF1-like complex to the *FLC* locus (Kim & Michaels 2006).

Two Arabidopsis *trithorax* genes, *ARABIDOPSIS TRITHORAX 1* (*ATX1*, a homologue of *Drosophila trithorax*) and its closest homologue *ARABIDOPSIS TRITHORAX 2* (*ATX2*) are required to activate *FLC* expression (Pien et al. 2008; He 2009). *ATX1*and *ATX2* appear to act in a partially redundant manner to activate *FLC*. *ATX1* is required for H3K4me3 deposition in *FLC* chromatin, although loss of *ATX1* activity did not suppress H3K4 dimethylation in *FLC* chromatin (Pien et al. 2008). ATX1 and ATX2 may be recruited to the *FLC* locus by AtPAF1c, leading to increased H3K4me3 in *FLC* chromatin, upregulating *FLC* expression and repressing flowering (He 2009).

An Arabidopsis homologue of the human COMPASS-like H3K4 methyltransferase complex component WDR5, named WDR5a, has also been shown to promote *FLC* expression and repress the floral transition in Arabidopsis. WDR5a is enriched at the *FLC* locus by *FRI*, leading to increased trimethylation of H3K4 and upregulation of *FLC* expression. The winter-annual growth habit may result from the recruitment of a WDR5a-containing COMPASS-like complex to *FLC* by *FRI* (Jiang et al. 2009).

Full activation of *FLC* also appears to require the deposition of histone variant H2A.Z in *FLC* chromatin by an Arabidopsis SWR1c-like complex. This complex contains homologues of the components of the yeast SWR1 complex that deposits

H2A.Z in chromatin including PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1 (PIE1), an Arabidopsis homologue of yeast SWR1 (Choi et al. 2007; Deal et al. 2007; He 2009).

FLC expression also requires monoubiquination of Histone H2B (H2Bub1) (He 2009). H2Bub1 in Arabidopsis is mediated by HISTONE MONOUBIQUINATION 1 &2 (HUB1 and HUB2) and ubiquitin-conjugating enzyme 1 and 2 (UBC1 and UBC2). H2Bub1 is also believed to promote H3K4me3 in Arabidopsis (He 2009). Actively transcribed *FLC* chromatin is also marked by H3K36 di- and tri-methylation, and this may also involve the Arabidopsis PAF1 complex (He 2009).

1.3.2 During vernalization levels of "active" chromatin modifications in *FLC* chromatin are reduced and levels of "repressive" ones increase, leading to the stable down-regulation of *FLC* expression

The stable epigenetic repression of *FLC* during vernalization involves modifications to histones in *FLC* chromatin. Levels of H3K9 & H3K14 acetylation and H3K4me3 ("active" marks) are reduced and levels of histone 3 lysine 9 and 27 di- and tri-methylation (H3K9me2/me3 and H3K27me2/me3, marks associated with repressed chromatin) are increased (Bastow et al. 2004; Sung & Amasino 2004b; Sung et al. 2006a; Sung et al. 2006b; Wood et al. 2006; Finnegan & Dennis 2007; Greb et al. 2007; Schmitz & Amasino 2007; He 2009).

The repression of *FLC* during vernalization (and thus the vernalization response) requires the PHD (plant homeodomain) protein VERNALIZATION-INSENSITIVE 3 (VIN3), which is expressed only during cold exposure (Sung & Amasino 2004b; Wood et al. 2006). VIN3 appears to be required for the H3 deacetylation and increase in methylation of H3K9 and H3K27 associated with vernalization, as these modifications do not occur if VIN3 function is lost (Sung & Amasino 2004b; Wood et al. 2006). *VIN3* expression is not induced until after a sufficient duration of cold exposure to be effective for vernalization. *VIN3* expression then increases with the duration of cold but decreases upon a return to warmer conditions. The repression of *FLC* only begins after *VIN3* expression is induced, and the extent of this repression correlates with the level of *VIN3* expression. Thus *VIN3* may be involved in

distinguishing long and short periods of cold and ensuring that flowering is not induced by short periods of cold (Sung & Amasino 2004b; Wood et al. 2006). *VIN3* interacts with another PHD protein called VERNALIZATION 5/ VIN3-LIKE 1 (VRN5/VIL1) to form a heterodimer and both of these are required for the H3 deacetylation and H3K27me3 in *FLC* chromatin associated with its stable repression during vernalization (Sung et al. 2006b; Greb et al. 2007; Schmitz & Amasino 2007; He 2009).

The vernalization-induced increase in H3K27 methylation in *FLC* involves a polycomb-like protein complex that contains homologues of components of the *Drosophila* Polycomb Repressive Complex 2 (PRC2). These are SWINGER (SWN, a homologue of E(z)), FERTILIZATION INDEPENDENT ENDOSPERM (FIE, a homologue of Esc), VERNALIZATION 2 (VRN2, a homologue of Su(z)12), and MULTICOPY SUPPRESSOR OF IRA 1 (MSI1, a homologue of p55) (de Lucia et al. 2008). During vernalization VIN3 and VRN5 interact with these core PRC2 proteins and another PHD protein, VERNALIZATION5/VIN3 LIKE 1 (VEL1), to form a vernalization-specific PHD-PRC2 complex that increases H3K27me3 throughout *FLC*, resulting in stable silencing of *FLC* (de Lucia et al. 2008). Whereas VRN2 associates constitutively across the *FLC* locus independently of cold, VRN5 was not found to be associated with *FLC* in non-vernalized plants. But during vernalization VRN5 associates with a specific region of *FLC* intron 1. This association is dependent upon VIN3 and triggers the silencing of *FLC*. When plants are returned to warm conditions, VRN5 associates more broadly across the FLC locus, and this is coincident with increases in H3K27me3, which maintains the silencing of *FLC* (de Lucia et al. 2008).

The vernalization-induced increase in dimethylation of H3K9 in *FLC* also requires VIN3 and VRN2, and also a DNA binding protein called VERNALIZATION 1 (VRN1) (Levy et al. 2002; Sung & Amasino 2004b). Although VRN1 is not required for H3K27 dimethylation in *FLC* during vernalization (Sung & Amasino 2004b). Both *VRN1* and *VRN2* are constitutively expressed (although levels of VRN2 protein increase during vernalization) and are required for the maintenance rather than the establishment of *FLC* repression (Gendall et al. 2001; Levy et al. 2002; Bastow et al. 2004; Sung & Amasino 2004b; Wood et al. 2006).

The maintenance of the epigenetically repressed state of *FLC* following vernalization also requires LIKE HETEROCHROMATIN PROTEIN 1/TERMINAL FLOWER 2 (LHP1/TFL2), the Arabidopsis homologue of HETEROCHROMATIN PROTEIN 1 (Mylne et al. 2006; Sung et al. 2006a). Vernalization leads to an increased association of LHP1 with the *FLC* locus, which is maintained following a return to warmer conditions. LHP1 is not required for the vernalization-induced dimethylation of H3K9 in *FLC* but is required to maintain this increased H3K9 dimethylation after vernalization when plants are returned to warmer conditions (Sung et al. 2006a). One study has found that while LHP1 binds to H3K9me2, H3K9me3, and H3K27me3 in vitro it associates almost exclusively with H3K27me3 in vivo. But LHP1 is not involved in the production of this mark in *FLC* chromatin during vernalization (Dennis & Peacock 2007; Turck et al. 2007). LHP1 is also required to maintain the decreased H3K4 trimethylation of *FLC* following vernalization, though it is not required for this decrease to occur (Sung et al. 2006a).

The epigenetic silencing of *FLC* during vernalization also requires histone arginine methylation (Schmitz et al. 2008). Prolonged cold-exposure leads to an increase in levels of symmetric dimethylation of histone 4 arginine 3 (H4R3sme2) in *FLC* chromatin. In *atprmt5* mutants of Col-*FRI* the repression of *FLC* during vernalization is incomplete and unstable and H3K9 and H3K27 methylation does not increase after cold-treatment. So H4R3sme2 appears to be necessary for increased H3K9 and H3K27 methylation and epigenetic silencing of *FLC* during vernalization. Flowering is also delayed both with and without vernalization compared to Col-*FRI* in these mutants. However, these mutants do still show a reduced response to vernalization. This reduced vernalization response may be due to repression of other members of the *FLC* clade (Schmitz et al. 2008).

The down-regulation of *FLC* expression during vernalization appears to involve an increase in the production of antisense RNAs from a region 3’ of the poly-adenylation site of *FLC*. Antisense transcripts produced from this region of *FLC* are known as COOLAIR. The induction of COOLAIR during cold-treatment occurs before induction of *VIN3* and is independent of *VIN3*. This increase in antisense transcription during cold-treatment may repress sense transcription from *FLC* before

the addition of repressive histone marks to *FLC* chromatin by the PHD-PRC2 complex which ensure that *FLC* is stably repressed (Swiezewski et al. 2009).

Therefore in Arabidopsis the stable repression of *FLC* is critical to the vernalization response, and this stable repression depends upon both chromatin modifications and antisense RNA. These chromatin modifications involve many genes/complexes homologous to genes/complexes involved in chromatin modifications in animals/yeast.

1.4 There is extensive natural variation in the vernalization response of Arabidopsis due to allelic variation at both the FRI *and* FLC *loci*

Arabidopsis has a near-worldwide distribution (Shindo et al. 2005), with considerable variation in flowering time and responsiveness to vernalization amongst natural accessions (Strange et al.; Lempe et al. 2005; Shindo et al. 2005; Shindo et al. 2006). The rapid-cycling habit (i.e. no vernalization requirement) has arisen independently several times during evolution , due to both loss-of-function *fri* mutations (Johanson et al. 2000; Shindo et al. 2005) and weak *flc* alleles (Michaels et al. 2003; Shindo et al. 2005). This suggests that, by conferring a rapid-cycling habit, these mutations improve fitness in some environments (Shindo et al. 2005). So far, rapid-cycling accessions with mutations in *FRL1* (which is required for the up-regulation of *FLC* by *FRI*) have not been identified (Michaels et al. 2004).

Variation in the degree to which *FLC* is stably repressed by different durations of cold, but not variation in rate of *FLC* suppression or of VIN3 induction, also contributes to natural variation in vernalization response amongst Arabidopsis accessions (Shindo et al. 2006). In accessions that are slow to respond to vernalization (and require a long period of cold to saturate the response), *FLC* expression is repressed during shorter periods of cold as in fast-responding accessions but this repression is unstable. *FLC* expression increases again when they are returned to warmer conditions. This could be related to the differential accumulation of the histone modification H3K27me3, possibly due to variation in *cis*-elements of *FLC* (Shindo et al. 2006).

1.5 Vernalization in Arabidopsis also involves FLC-independent mechanisms, and at least some of these involve other MADS-box genes

Although the epigenetic repression of *FLC* is a major component of the vernalization response in Arabidopsis, vernalization can still accelerate flowering in an *flc* null mutant (Michaels & Amasino 2001; Simpson & Dean 2002). Therefore, vernalization involves both *FLC*-dependent and *FLC*-independent mechanisms (Michaels & Amasino 2001). *SOC1* and *FT* are also up-regulated by vernalization in an *flc* null mutant, suggesting that they are targets of both *FLC*-dependent and *FLC*-independent components of vernalization. But both an *ft soc1* double mutant and an *flc ft soc1* triple mutant showed a vernalization response, so both the *FLC*-dependent and *FLC*-independent pathways must also have other targets (Moon et al. 2005).

Two *FLC*-independent branches of the vernalization pathway involve the MADS-box genes *AGAMOUS-LIKE 24* (*AGL24*) and *AGAMOUS-LIKE 19* (*AGL19*). These both act as flowering promoters and both are activated following long periods of cold exposure (Alexandre & Hennig 2008).

Five other Arabidopsis MADS-box genes that are known to function in the control of flowering time have been designated as *MADS AFFECTING FLOWERING 1–5* (*MAF1–5*). MAF1 (also known as FLOWERING LOCUS M/FLM) acts as a repressor of flowering (Scortecci et al. 2003). *MAF1* acts together with another MADS-box gene, *SHORT VEGETATIVE PHASE* (*SVP*), to delay flowering, and this delay is overcome by inductive-photoperiods (Scortecci et al. 2003). MAF2 acts as a floral repressor, and is believed to act independently of *FLC* to prevent the promotion of flowering by short periods of cold-exposure (Ratcliffe et al. 2003). *MAF2* mRNA level is only reduced by long durations of cold. And in a *maf2* mutant flowering is accelerated by shorter periods of cold than are required in the wild-type, despite similar *FLC* expression (Ratcliffe et al. 2003). *MAF2* represses *SOC1*, even when repression of *SOC1* by *FLC* is reduced by cold-treatments (Ratcliffe et al. 2003). Ratcliffe et al. (2003) also suggested that MAF3 and MAF4 act as repressors of flowering, and the expression of these genes is reduced by cold-treatments. In contrast, MAF5 may function as a promoter of flowering. Vernalized seedlings have

higher levels of *MAF5* transcript than non-vernalized seedlings. But over-expression suggests that MAF5 acts as a weak floral repressor (Ratcliffe et al. 2003). However, MAF5 could still act as a floral repressor in some genetic backgrounds by competing with other FLC-like proteins that are more potent repressors of flowering (Ratcliffe et al. 2003).

In this study I will focus upon the role of *FLC* in vernalization rather than the *FLC*-independent pathways. However, it is important to consider that there are *FLC*-independent pathways involved in the vernalization response.

1.6 Vernalization depends upon both the temperature and duration of a cold-treatment

Biological processes, such as vernalization, have three cardinal temperatures: the base or minimum temperature, T_{MIN}; the optimum temperature, T_{OPT}; and the maximum temperature, T_{MAX} (Yan & Hunt 1999a; Streck 2002; Streck 2003). The rate of the process is zero or negligible below T_{MIN} (Porter & Gawith 1999) and increases from T_{MIN} to reach a maximal rate (R_{MAX}) at T_{OPT} (Porter & Gawith 1999; Yan & Hunt 1999a). The rate then decreases with further increases in temperature until it reaches zero at and above T_{MAX} (Porter & Gawith 1999; Streck 2003). Therefore, the response of a plant to a vernalization treatment depends upon both the treatment temperature (which determines the rate of progress towards saturation) and its duration (Craigon et al. 1995; Streck 2002; Streck 2003; Streck & Schuh 2005).

Some authors have represented the vernalization rate as a bi-linear function of temperature. I.e. the rate of vernalization increases linearly from T_{MIN} to T_{OPT} and then declines linearly from T_{OPT} to T_{MAX}. This kind of bi-linear model has been used to model vernalization in carrot (Atherton et al. 1990; Craigon et al. 1990) and in Winter Wheat (Craigon et al. 1995). Other studies have used a three-stage linear function in which vernalization rate is maximal across a range of temperatures (from T_{OPT1} to T_{OPT2}) and declines linearly to T_{MIN} and T_{MAX} either side of this plateau. Wurr et al. (1993) used this approach to model vernalization as a function in cauliflower, although the plateau between T_{OPT1} and T_{OPT2} was found to be very narrow (9.0–9.5°C). Both of these models assume linear relationships between rate and temperature and abrupt changes in rate. But the response of plant development

rate to temperature is not actually linear. As temperature rises above T_{MIN} development rate initially increases only gradually. The increase in rate with temperature then becomes approximately linear before levelling out and reaching a maximum at T_{OPT} . Above T_{OPT} development rate decreases with further increases in temperature (Shaykewich 1995). Non-linear functions such as the beta function (or a modified version of it) have been used to model vernalization in several species e.g. wheat (Yan & Hunt 1999b; Yan & Hunt 1999a), the "Snow Queen" lily (Streck & Schuh 2005), carrot (Yan & Hunt 1999b; Yan & Hunt 1999a), and onion (Streck 2003).

In order to quantify a vernalization requirement a unit is required that takes into account the effects of both the temperature and the time spent at that temperature. If the rate of vernalization increases linearly from T_{MIN} to T_{OPT} and then decreases linearly from T_{OPT} to T_{MAX} then vernalizing degree days (V°CD) can be used (Atherton et al. 1990; Craigon et al. 1990). The number of V°CD within a certain period is given by calculating the number of growing degree days (GDD) above T_{MIN}. One GDD occurs when the observed temperature is 1°C above T_{MIN} (sometimes referred to as T_{BASE}) for 24 hours (McMaster & Wilhelm 1997; Snyder et al. 1999). In an environment with fluctuating temperature the average of the maximum and minimum daily temperatures is often used to calculate GDD (McMaster & Wilhelm 1997). Often it is assumed that the rate of the process remains constant at temperatures above T_{OPT} (McMaster & Wilhelm 1997; Snyder et al. 1999). But this is unrealistic as it does not account for the detrimental effect of increasing temperatures above T_{OPT} (Shaykewich 1995; Yan & Hunt 1999a). Hence supra-optimal temperatures are converted to the equivalent sub-optimal temperatures using Equation 1 before calculating V°CD (Atherton et al. 1990; Craigon et al. 1990). No V°CD accumulate at temperatures below T_{MIN} or above T_{MAX}.

$$T_{EFF} = T_{OPT} - \left[\left(\frac{T_{OPT} - T_{MIN}}{T_{MAX} - T_{OPT}}\right) \times (T - T_{OPT})\right]$$

Equation 1: Equation for calculating the sub-optimal temperature (T_{EFF}) that gives the same rate of vernalization as a given supra-optimal temperature, T. This model assumes that vernalization rate increases linearly from T_{MIN} to T_{OPT} the decreases linearly to T_{MAX}. From Craigon et al. (1990).

Effective vernalization days (VD) have been used to quantify vernalization when the temperature response function for vernalization rate is non-linear. One VD occurs when the temperature is at the optimum temperature for vernalization for one day. At temperatures above and below T_{OPT}, only a fraction of a VD occurs per 24 hours. No VD accumulate at temperatures greater than T_{MIN} or less than T_{MAX} (Streck 2002; Streck 2003; Streck et al. 2003; Streck & Schuh 2005).

Table 1 gives estimated cardinal temperatures for vernalization in a range of crops, including examples estimated using data from both field and controlled environment room experiments. Wurr et al. (1993; 1995) estimated cardinal temperatures for vernalization and constructed models of the process in summer/autumn cauliflower (*Brassica oleracea botrytis*) and calabrese (*Brassica oleracea italica*) respectively (Table 1). But both sets of cardinal temperatures must be treated with caution. Wurr et al. (1993) used field data to estimate the cardinal temperatures, and acknowledged that the rare occurrence of temperatures below 9°C meant that lower values of T_{MIN} were unlikely to be estimated. Another study showed that treating the same cultivar at 5°C reduced the number of leaves at curd initiation (Hand & Atherton 1987; Wurr et al. 1993). The minimum and maximum temperatures for vernalization in calabrese given by Wurr et al. (1995) were found by extrapolating outside the range of their data (which came from controlled-environment cabinets at 7 to 23°C) (Tan 1999). When modelling vernalization, it may also be necessary to account for the de-vernalizing effects of high temperatures (Chujo 1966; Wurr et al. 1993; Cao & Moss 1997).

Species, variety, and cultivar	T_{MIN} °C	T_{OPT}	T_{MAX}	**Study**	**Notes on these estimates**
Brassica oleracea italic cv. Shogun (Calabrese)	-2.8	15.8	23.6	Wurr et al. (1995)	T_{MIN} and T_{MAX} estimated by extrapolation beyond experimental temperature range (Tan 1999)
Brassica oleracea botrytis cv. White Fox (Summer/autumn Cauliflower)	9	9–9.5*	21	Wurr et al. (1993)	Based upon field data. T_{MIN} is likely to be an overestimate (see text)
Winter wheat	-4.8	5.2	26.6	Craigon et al. (1995)	Estimated using extrapolation of a bilinear function
Carrot cv. Chantenay Red Cored	-1.0	6.5	16.0	Atherton et al. (1990)	Estimated using extrapolation of a bi-linear function
Carrot cv. Chantenay Red Cored	0.0 (assumed)	5.7±0.5	21.3±1.4	Yan and Hunt (1999b)	Estimated from the data of Atherton et al. (1990) using the beta function
Wheat	-1	2	15	Wang and Engel (1998)	Estimated/determined based upon other literature sources
Wheat	-1.3±1.5	4.9±1.1	15.7±2.6	Porter and Gawith (1999)	Means ± standard error, based upon several literature sources

Table 1: Selected examples of cardinal temperatures for vernalization in different plants reported in scientific literature. *These are the upper and lower optimum temperatures for vernalization (T_{OPT1} and T_{OPT2}), vernalization rate is maximal between these temperatures.

1.7 The response to a vernalization-treatment may depend upon other factors such as developmental phase and photoperiod

Following germination the shoot apical meristem (SAM) of plants passes through three phases: a juvenile-vegetative phase, during which it is not competent to flower; an adult-vegetative phase, during which flowering can be induced by appropriate cues; and finally the reproductive phase, during which flowers are produced (Poethig 1990; Lawson & Poethig 1995; Kerstetter & Poethig 1998; Baurle & Dean 2006; Amasino 2010). Some plants can be vernalized as germinating seeds. These are known as "seed-vernalization responsive", and include winter-annual accessions of Arabidopsis (Sheldon et al. 1999; Lin et al. 2005). Many other plants are "plant-vernalization responsive" and cannot be vernalized until they reach the adult-vegetative phase; a cold-treatment applied during the juvenile phase will not lead to vernalization and so flowering will not be accelerated (Lin et al. 2005). E.g. the perennial relative of Arabidopsis *Arabis alpina* (Wang 2007) and many Brassicas are plant-vernalization responsive (Thomas 1980; Hand & Atherton 1987; Wurr et al. 1993; Guo et al. 2004; Lin et al. 2005; Baurle & Dean 2006).

Vernalization promotes/establishes the competence to flower in response to appropriate cues rather than leading to flowering per se (Chouard 1960; Michaels & Amasino 2000). For example in *Sinapis alba* (family Brassicaceae) vernalization accelerates flowering but only long vernalization treatments accelerate flowering in short days. Shorter vernalization treatments accelerate flowering in long days but not in short days. Following a short vernalization treatment *S. alba* will flower if immediately exposed to long days but not if kept under short days at 20°C for two weeks before being exposed to long days. During vernalization *SaFLC* (a homologue of Arabidopsis *FLC*) is down-regulated but this down-regulation is unstable following short periods of cold. The rise in *SaFLC* expression following short periods of cold may explain why long days are needed immediately after short cold-treatments for flowering to be induced (D'Aloia et al. 2008).

The effect of a vernalization treatment may also depend upon the photoperiod during vernalization. A cold-treatment may be more effective under short photoperiods

(Roberts et al. 1988; Brooking & Jamieson 2002), although this is not always the case (Mahfoozi et al. 2001). A period of short photoperiods may also be able to partly substitute for a low-temperature vernalization treatment, or be required in addition to one. This phenomenon is known as "short-day vernalization" (Roberts et al. 1988; Brooking & Jamieson 2002; Liu 2007).

1.8 Studies of vernalization are often confounded by the invisible nature of the process and by concurrent vegetative development

The process of vernalization itself is invisible, so the vernalization response must be evaluated based upon its after-effects (Brooking 1996; Yan & Hunt 1999b). The calendar or thermal time to a specified developmental stage (Brooking 1996)—such as flowering (Streck 2002), bolting (Atherton et al. 1990; Craigon et al. 1990), or final leaf appearance (Craigon et al. 1995)—and the final leaf number at flowering (FLN) (Brooking 1996; Robertson et al. 1996; Koornneef et al. 1998) are often used to measure the acceleration of flowering by vernalization. But the appearance of the after-effects of vernalization can depend upon the post-treatment photoperiod and temperature (Roberts et al. 1988; Wang et al. 1995; Yan & Hunt 1999b; D'Aloia et al. 2008).

Measurements of vernalization by its after effects can also be confounded by continued vegetative development during vernalization (Brooking 1996). If vegetative development continues during vernalization then the effect of the vernalization treatment upon final leaf number/time to flowering will depend upon both the effect of vernalization and the response of vegetative development to temperature (Wang et al. 1995; Brooking 1996; Robertson et al. 1996). Plants vernalized at low temperatures may have a lower final leaf number than those treated at higher temperatures due to differences in the rate of leaf production at the different temperatures, rather than the effect of the vernalization treatment (Chujo 1966). Restricting moisture level during vernalization treatment reduced the growth that occurred during vernalization, but vernalization was less effective too (Hoogendoorn 1984). Another approach in wheat is to define the point of saturation of vernalization as when the number of leaves and primordia initiated at the end of a

vernalization treatment is equal to the final number of leaves produced (Brooking 1996; Brooking & Jamieson 2002).

1.9 The Brassica genus is closely related to Arabidopsis and contains many important crop species

The Brassica genus (family Brassicaceae) shares a recent common ancestor with Arabidopsis 14.5–20.4 million years ago (Yang et al. 1999; Rana et al. 2004). *Brassica* species are grown as oilseed, vegetable, and condiment crops and as animal fodder (Bohuon et al. 1998; Paterson et al. 2001), and may have anti-cancer properties (Verhoeven et al. 1996; Verhoeven et al. 1997; Verkerk et al. 2009). *Brassica* species include both diploids and amphidiploids. The genomes of three diploid *Brassica* species—*B. nigra* (the A genome), *B. rapa* (the B genome), and *B. oleracea* (the C genome)—have undergone hybridization to form the amphidiploids *B. juncea* (AABB), *B. napus* (AACC), and *B. carinata* (BBCC) (Figure 2) (U 1935; Lan et al. 2000; Lukens et al. 2004).

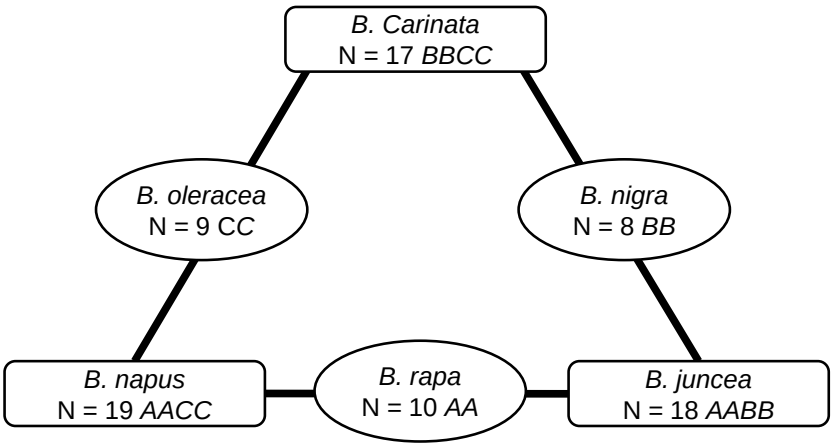


Figure 2: U's triangle (U 1935) shows the relationships between some of the most commercially important diploid and amphidiploid *Brassica* species. The diploid genomes of *B. rapa*, *B. nigra*, and *B. oleracea* are designated as the A, B, and C genomes respectively. Hybridization amongst these species has formed the amphidiploids *B. juncea*, *B. napus*, and *B. carinata* (U 1935; Lan et al. 2000).

Lagercrantz & Lydiate (1996) proposed that the diploid genomes of *Brassica nigra*, *B. rapa*, and *B. oleracea* evolved through a hexaploid ancestor, in which an ancestral unit genome was triplicated. They also suggested that the Arabidopsis genome (which is approximately one third of the size of the *B. nigra* genome) may be similar

in content and possibly organization to the diploid progenitor genomes of this hexaploid ancestor (Lagercrantz & Lydiate 1996; Lagercrantz et al. 1996). These hypotheses were based upon maps of each of the three species constructed with a common set of RFLP probes. They were supported by subsequent comparative mapping between the same *B. nigra* mapping population and Arabidopsis using *A. thaliana* probes (Lagercrantz 1998).

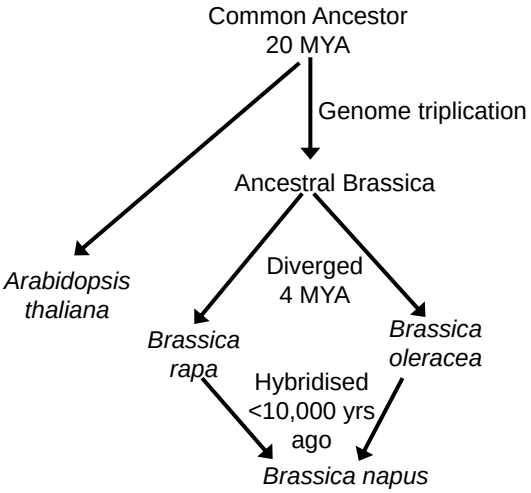


Figure 3: A whole-genome triplication is believed to have occurred during the evolution of the diploid Brassicas (including *B. rapa* and *B. oleracea*) from a common ancestor with Arabidopsis. *B. rapa* and *B. oleracea* have also hybridized to form the amphidiploids *B. napus*. Diagram adapted from Rana et al. (2004).

Other authors have questioned the strength of the evidence that the diploid *Brassica* genomes evolved through a hexaploid ancestor. Lukens et al. (2003) compared the *A. thaliana* genome with a *B. oleracea* genetic map using a test for collinearity and found little evidence for triplication. Another study using direct transcriptome mapping found that Arabidopsis chromosomes were unevenly represented amongst the duplicated segments of the *B. oleracea* genome, conflicting with the hypothesis of genome triplication in an ancestral hexaploid (Li et al. 2003). But a recent comprehensive comparison of the Arabidopsis and *Brassica napus* genomes strongly supported the hypothesis that the diploid *Brassica* genomes evolved through a hexaploid ancestor (Parkin et al. 2005).

There has been a high rate of chromosomal rearrangements since the divergence of the lineages leading to Arabidopsis and *Brassica* (Lagercrantz & Lydiate 1996;

Lagercrantz 1998; Lukens et al. 2003; Lukens et al. 2004; Ziolkowski et al. 2006). Although the overall genetic content of the A, B, and C genomes is approximately equivalent, chromosomal fusions/fissions mean that they have different numbers of chromosome (Lagercrantz & Lydiate 1996). But there is still substantial collinearity and conservation between the Arabidopsis and *Brassica* genomes (Lagercrantz et al. 1996; Lagercrantz 1998; Parkin et al. 2005). So studies in *Brassica* may benefit greatly from the extensive research carried out in Arabidopsis (Lagercrantz et al. 1996; Lagercrantz 1998; Parkin et al. 2005) e.g. for the identification of candidate genes for traits in *Brassica* species (Paterson et al. 2001; Parkin et al. 2005).

1.10 Many Brassica crop species are (plant-)vernalization-responsive and their flowering time has important consequences for agriculture

The *Brassica* species are facultative long-day plants (Axelsson et al. 2001) and exhibit substantial variation in flowering time (Axelsson et al. 2001) and vernalization requirement (Guo et al. 2004). Many Brassica crops, such as cabbage, winter oilseed rape, and many types of Cauliflower are biennial and require vernalization in order to flower (Wurr et al. 1988; Wurr et al. 1993; Teutonico & Osborn 1995; Osborn et al. 1997; Lin et al. 2005).

The vernalization responses of Brassica species have considerable impacts upon their breeding and agriculture. Premature flowering caused by low temperatures can reduce the harvest yield and quality in *B. rapa* (Yuan et al. 2009) And the effect of weather conditions upon curd initiation makes it difficult to achieve a continuous supply of Cauliflower in the UK (Wurr et al. 1988). Poor predictability of flowering-time is also a major difficulty in growing many Brassica crops, leading to considerable wastage due to unscheduled harvests and "gluts" in production (DEFRA, poster for project HL0186). Climate change may also bring warmer winters in temperate areas, which could have significant effects upon crops that require low temperatures for the initiation of flowering e.g. cauliflower (DEFRA, poster for project HL0186; Wurr et al. 2004). Therefore it is important to understand how Brassica crops respond to different temperatures and to different durations of cold.

1.11 The extensive knowledge of the molecular mechanisms underlying flowering-time and vernalization in Arabidopsis is being used to guide studies in Brassica species

The central role of *FLC* in the vernalization response appears to have been widely conserved among the Brassicaceae. The repression of *FLC* homologues has been associated with vernalization in the perennial species *Arabis alpina* (Wang 2007; Wang et al. 2009b) and *Arabidopsis halleri* (Aikawa et al. 2010) and also in *Sinapis alba* (D'Aloia et al. 2008). Because a whole-genome triplication is believed to have occurred during the evolution of Brassica species from a common ancestor with Arabidopsis (Lagercrantz & Lydiate 1996; Lagercrantz et al. 1996; Lagercrantz 1998; Rana et al. 2004; Parkin et al. 2005) they would be expected to have multiple homologues of *FLC*. Multiple homologues of *FLC* have now been isolated from *B. napus* (Tadege et al. 2001), *B. rapa* (Schranz et al. 2002; Kim et al. 2007), and *B. oleracea* (Schranz et al. 2002; Lin et al. 2005; Okazaki et al. 2007; Razi et al. 2008). Several of these have been linked to QTL for flowering time/vernalization response or with natural variation in flowering time (Kole et al. 2001; Schranz et al. 2002; Okazaki et al. 2007; Yuan et al. 2009). At least some of the *FLC* homologues in each of these species are also down-regulated during vernalization (Tadege et al. 2001; Li et al. 2005; Lin et al. 2005; Kim et al. 2007). *FLC* homologues from *B. rapa* (Kim et al. 2007) and *B. napus* (Tadege et al. 2001) also delay flowering when over-expressed in Arabidopsis.

1.12 The importance of juvenility and vernalization in Purple Sprouting Broccoli (Brassica oleracea italica)

Purple sprouting broccoli (*Brassica oleracea italica*) is a winter-annual/biennial sub-species that requires vernalization to flower, but must grown through an insensitive "juvenile phase" before it can respond to vernalization (Irwin, personal communication). The current market of Purple Sprouting Broccoli in the UK has an estimated value of £4M, and UK growers meet approximately 85% of consumption (DEFRA, poster for project HL0186).

At present the Purple Sprouting Broccoli (PSB) season is limited. Extending the season could potentially increase the value of the market to £20M. The harvest date of Purple Sprouting Broccoli can also be difficult to predict (DEFRA, poster for project HL0186). This is also a problem with cauliflower (*Brassica oleracea botrytis*), for which the strong influence of weather conditions on curd initiation makes achieving a continuous supply in the UK is difficult (Wurr et al. 1988). Predicted increases in winter temperatures due to global warming may have important implications for the growing of *Brassica* crops that require vernalization to flower, such as PSB (DEFRA, poster for project HL0186).

The different vernalization requirements of PSB are important in controlling maturation. Understanding the molecular basis of the control of flowering time and vernalization response in PSB may help to plan production. This could also assist the development of varieties in which development is less sensitive to temperature, or with more robust vernalization requirements. This may improve the predictability of harvest dates and continuity of supply. The knowledge gained should also be applicable to other *Brassica* crops, such as cauliflower.

1.13 Hypotheses and aims

Four genotypes of Purple Sprouting Broccoli (PSB) with different vernalization responses—E1, E5, E8, and E9—were provided by Elsoms Seeds. Previous field studies have suggested that E1 has a facultative vernalization requirement whereas E5, E8, and E9 have obligate vernalization requirements. PSB is also considered to have a juvenile phase during which plants are not competent to be vernalized. These genotypes had not previously been grown and vernalized in controlled environment conditions.

Hypothesis 1: The chronological duration of the juvenile phase will vary with environmental conditions

Hypothesis 2: The chronological duration of the juvenile phase will vary among the four genotypes of PSB

Aim 1: Test hypotheses 1 and 2 and estimate the number of leaves at which the juvenile phase ends in each genotype

Hypothesis 3: E1 has a facultative vernalization requirement

Hypothesis 4: E5, E8, and E9 have obligate vernalization requirements

Hypothesis 5: All four genotypes can be vernalized at 5, 10, and 15°C

Hypothesis 6: All four genotypes respond to both 6 and 10 weeks of cold-treatment

Aim 2: Test hypotheses 3–6 and establish a vernalization treatment that is effective for vernalization in all four genotypes

Hypothesis 7: The expression of each of the homologues *BoFLC1*, *BoFLC3*, and *BoFLC4* is stably down-reguled during vernalization in each genotype

Hypothesis 8: Each of the homologues *BoFLC1*, *BoFLC3*, and *BoFLC4* show a similar pattern of expression in the young leaves and apex before and following vernalization in each genotype.

Aim 3: Develop a quantitative real-time reverse-transcriptase-PCR (Q-RTPCR) assay to measure the expression of each of the *BoFLC* homologues individually in these genotypes of PSB and test hypotheses 7 and 8

2 Determination of the juvenile phase of Purple Sprouting Broccoli based upon leaf number

2.1 Introduction

In higher plants the shoot apical meristem (SAM) passes through three phases during post-embryonic development: the juvenile- and adult-vegetative phases and the reproductive phase (Poethig 1990; Lawson & Poethig 1995). Following germination the SAM enters the juvenile-vegetative phase, during which it is not competent to flower even if exposed to appropriate environmental cues (Kerstetter & Poethig 1998; Baurle & Dean 2006; Amasino 2010). This phase varies in duration from just a few days in some herbaceous species to many years in some perennials (Poethig 1990; Amasino 2010). The SAM then enters the adult-vegetative phase (Poethig 1990; Kerstetter & Poethig 1998; Baurle & Dean 2006). During the adult-vegetative phase the SAM can be induced (by appropriate cues) to enter into the reproductive phase i.e. to undergo the transition from vegetative growth to flowering (Poethig 1990; Kerstetter & Poethig 1998; Baurle & Dean 2006).

The structures/organs produced by the SAM change during these three phases of development. Leaves produced during the juvenile phase are often smaller and simpler than adult leaves. Juvenile and adult leaves may also differ in their phyllotaxy, pattern of epidermal differentiation, and ability to produce adventitious roots (Poethig 1990; Kerstetter & Poethig 1998; Poethig 2003; Baurle & Dean 2006). Leaves with both juvenile and adult characteristics are often produced during the transition from the juvenile to the adult phase (the vegetative phase change) (Poethig 1990; Kerstetter & Poethig 1998; Baurle & Dean 2006). When the SAM enters the reproductive phase it begins to form bracts and flowers (or cones) (Poethig 1990; Lawson & Poethig 1995; Telfer & Poethig 1998). Because of the polar nature of plant growth (i.e. the fact that plants grow by the addition of nodes by the SAM) these phases are separated spatially in the different types of leaves/organs on the plant (produced by the SAM) as well as temporally in the life of the plant (Lawson & Poethig 1995).

Arabidopsis thaliana is a facultative (or "quantitative") long-day plant (i.e. it flowers earlier under long days than short days) (Gregory & Hussey 1953; Jackson 2009; Amasino 2010) but must pass through the juvenile phase before it is competent to respond to inductive long photoperiods (Schwarz et al. 2008). The juvenile leaves of Arabidopsis have smooth margins and are relatively round whereas adult leaves are ovate with serrated margins. Adult leaves also have a higher blade-to-petiole ratio than juvenile leaves and tend to curl down at the edges (Chuck & Hake 2005; Willmann & Poethig 2005). Leaf shape changes gradually in Arabidopsis though and is not ideal as a marker of the vegetative phase change (Telfer et al. 1997). The best available marker for developmental phase in Arabidopsis is the distribution of trichomes on the leaves. Trichomes are present on the adaxial surface of both juvenile- and adult-vegetative leaves. But adult leaves also have trichomes on their abaxial surface whereas juvenile leaves do not. During the vegetative phase change abaxial trichomes first appear on the midrib. Their distribution then increases till they cover the entire abaxial surface of the adult leaves (Telfer et al. 1997). However, winter-annual accessions of Arabidopsis can be vernalized as imbibed/germinating seeds (before the end of the juvenile phase) i.e. they are seed-vernalization responsive (Lin et al. 2005; Baurle & Dean 2006). So Arabidopsis shows juvenility in leaf development and photoperiod response but not in vernalization response.

Many Brassicas do show juvenility in their vernalization response i.e. they are plant-vernalization-responsive. They are not competent to respond to vernalization until they have grown through an insensitive juvenile phase and reached a certain developmental stage (Hand & Atherton 1987; Lin et al. 2005; Baurle & Dean 2006). Brussels sprouts (*Brassica oleracea*) (Thomas 1980), cabbage (*B. oleracea capitata*) (Hossain et al. 1990; Lin et al. 2005), cauliflower (*B. oleracea botrytis*) (Hand & Atherton 1987; Wurr et al. 1993; Wurr et al. 1994; Guo et al. 2004), green broccoli (calabrese) (*B. oleracea italica*) (Tan 1999), and Purple Sprouting Broccoli (*B. oleracea italica*) (Irwin & Kennedy, personal communication) are all generally considered to have a juvenile phase during which they are insensitive to vernalization. A cold-treatment applied during the juvenile phase will not result in vernalization i.e. flowering will not be accelerated.

Juvenility can act as a confounding factor in vernalization experiments. Plants may not flower either because the cold-treatment is not effective for vernalization (e.g. the temperature is inappropriate or the cold-treatment was not continued for long enough) or because the treatment was applied before the end of the juvenile phase, when the plant was not competent to respond to it. If the length of the juvenile phase is not known then it may not be possible to distinguish between these two causes. As a result incorrect conclusions may be drawn about the vernalization response or, in QTL experiments, QTL for juvenility rather than vernalization may be detected. It would be useful to be able to determine when plants have reached the adult-vegetative phase, so that the confounding effect of juvenility can be eliminated from vernalization experiments.

Juvenility has been extensively studied in cauliflower, and the length of the juvenile phase is often defined by a number of initiated leaves (Hand & Atherton 1987; Booij & Struik 1990; Wurr et al. 1993; Guo et al. 2004). The leaf number at the phase change in cauliflower has been found to be stable under different light conditions (irradiance and photoperiod), whereas the duration of the juvenile phase in chronological time can vary (Hand & Atherton 1987). The length of the juvenile phase (defined by leaf number) varies amongst different genotypes of cauliflower (Hand & Atherton 1987; Wurr et al. 1988; Wurr et al. 1993; Wurr et al. 1994). Some studies have also found a slight effect of cold-treatment of seeds upon flowering time, but it is still accepted that cauliflowers have a juvenile phase at least from the point of expansion of the cotyledons (Hand & Atherton 1987). The length of the juvenile phase has also been defined in terms of leaf number in Brussels sprouts (Thomas 1980) and Calabrese (green broccoli) (Tan 1999).

Some studies have found that the leaf number at which the juvenile phase ends in cauliflower can vary with environmental conditions. Wurr et al. (1994) found that the leaf number at the end of the juvenile phase could vary with environmental conditions. Fellows et al. (1999) found that juvenility ended at a considerably higher leaf number in cauliflower plants that were sown in October and kept over winter in modular trays (in a ventilated polythene tunnel/frost-protected glasshouse) before transplanting to the field in spring than in plants raised in controlled environment cabinets at approximately 15°C. They suggested that the apex diameter might

provide a more consistent marker for the end of juvenility. But for controlled environment experiments it appears adequate to measure the juvenile phase in terms of leaf number, and this provides a convenient and non-destructive measure.

I wanted to establish how long was required for each of four genotypes of Purple Sprouting Broccoli (PSB) to reach the adult-vegetative phase. These genotypes are designated as E1, E5, E8, and E9. I also wanted to establish whether the duration of the juvenile phase of PSB varies amongst genotypes and with environmental conditions, as in cauliflower, and whether leaf number could provide a means of estimating the end of the juvenile phase to aid the design and analysis of vernalization-experiments. In order to achieve this I analyzed the responses of flowering time in these four genotypes to cold-treatment at 5°C for 10 weeks following different periods of pre-growth, and with a constant period of pre-growth given under different conditions.

2.2 Materials and methods

2.2.1 Plant material

All of the experiments described in this thesis involved four genotypes of Purple Sprouting Broccoli provided by Elsoms Seeds: E1, E5, E8, and E9. Previous field experiments suggested that E1 responds to vernalization by flowering early, but does not have an obligate requirement for vernalization in order to flower. This genotype is usually sown in March/April, planted out in the summer, and heads in November. E5, E8, and E9 all appeared to require vernalization in order to flower and were progressively later flowering (Irwin & Kennedy, personal communication). I grew plants of these genotypes in two separate experiments: one in 2008–2009, referred to as "experiment I", and another in 2009–2010, referred to as "experiment II". In experiment I plants were treated at 5, 10, and 15°C for 6 and 10 weeks after 50 days pre-growth. In experiment II plants were treated at 5 and 10°C for 6 and 10 weeks after 50, 60, and 70 days pre-growth. Throughout both experiments the plants were grown in compost and were watered daily and fed as required. The full results of these experiments are analyzed in chapter 3. For the purposes of investigating the juvenile phase in this chapter I wanted to exclude the effects of the temperature and duration of cold-treatment upon vernalization response. Therefore only the plants

treated for 10 weeks at 5°C (a treatment that was effective for vernalization in all four genotypes, discussed in chapter 3) are included in the analyses in this chapter.

2.2.2 Experiment I: The responses of the Purple Sprouting Broccoli genotypes E1, E5, and E8 to 10 weeks cold-treatment at 5°C with 50 days pre-growth

Plants of E1, E5, and E8 were "pre-grown" for 50 days from sowing in a warm glasshouse in Norwich, UK, in October–December 2008. The plants were germinated in compost then pricked out into modular trays. They were then transferred to 0.5-litre pots when they were 48 days old. The natural light in the glasshouse was supplemented with 16 hours per day of artificial light (from 0000–1600 hours). The glasshouse was set to maintain a minimum temperature of 18°C during the artificial "day" and 16°C during the artificial "night". 50 days after sowing, two plants of each genotype were transferred to a Huurre cold-room at 5°C with 8 hours per day of artificial light for 10 weeks. After the 10 weeks cold-treatment the plants were returned to the glasshouse. At this time they were also re-potted into 2-litre pots. The plants were arranged randomly by hand on one bench of the glasshouse. The natural light in the glasshouse was supplemented by 16 hours per day (from 00:00–16:00) of artificial light until 1st April 2009. On this date the artificial lighting was stopped and the plants received only natural light from then on. The schedule of treatments is given in Table 2.

The number of visible leaves on each plant was recorded the day before transfer to the CERs and at the end of the cold-treatment. For each genotype two–three plants were kept in the glasshouse throughout the experiment as non-vernalized controls (with supplementary artificial lighting until 1st April as described above). The plants were scored for the number of days from the end of cold-treatment until inflorescence buds were first visible at the apex (DTI) and until the first flower opened (DTF).

Pre-growth (days from sowing)	Duration of cold	Sown	Into cold	Out of cold
50 (Exp I)	10 weeks	22nd Oct 2008	12th Dec 2008	20th Feb 2009
70 (Exp II)	10 weeks	8th May 2009	17th Jul 2009	25th Sep 2009
60 (Exp II)	10 weeks	18th May 2009	17th Jul 2009	25th Sep 2009
50 (Exp II)	10 weeks	28th May 2009	17th Jul 2009	25th Sep 2009

Table 2: The dates of sowing and transfer to and from the 5°C cold room in experiments I & II. In experiment I there were two plants of each genotype for E1, E5, and E8 but no E9 plants. In experiment II there were five plants of each genotype for E1, E5, and E8 and three plants for E9

2.2.3 Experiment II: The responses of the Purple Sprouting Broccoli genotypes E1, E5, E8, and E9 to 10 weeks cold-treatment at 5°C with 50, 60, and 70 days pre-growth

Plants of E1, E5, E8, and E9 were "pre-grown" for 50, 60, and 70 days from sowing in a warm glasshouse under natural light in Norwich, UK, in May–August 2009. The plants were germinated in compost then pricked out into P15 trays. They were then transferred to 1-litre pots when they were 35-50 days old. The glasshouse was set to maintain a minimum temperature of 18°C during the day and 16°C during the night. The plants were then transferred to a cold-room at 5°C with 8-hours per day of artificial light for 10 weeks. (This was the same cold-room was used as in experiment I). The sowings were timed such that all cold-treatments ended on the same day (25th September) as detailed in Table 2. On this day all of the plants were transferred to a warm glasshouse with 16 hours per day of artificial light (from 0000–1600 hrs) plus natural light. This glasshouse was set to maintain a minimum temperature of 18°C during the artificial "day" and 16°C during the artificial "night". The plants were re-potted into 2-litre pots 47 days after the end of cold-treatment.

The plants were split between three adjacent benches in the glasshouse according to the pre-growth period. I.e. the plants pre-grown for 50 days before cold-treatment all on one bench, those pre-grown for 60 days on another, and those pre-grown for 70 days on a third. This was done to avoid the plants that had been pre-grown for longer (hence were larger) over-shading those that had been pre-grown for less time. The benches for each pre-growth period were assigned randomly. The plants were arranged randomly by hand within each bench. They were subsequently rearranged

randomly by hand as necessary to fill gaps left by the removal of plants that had flowered and been disposed of.

The number of visible leaves on each plant was recorded the day before transfer to the CERs and at the end of the cold-treatment period. Non-vernalized control plants were sown 70 days before the end of the cold-treatments. The plants were scored for the number of days from the end of the cold-treatments until inflorescence buds were first visible at the apex (DTI), until "buttoning" i.e. when the main inflorescence head reached 10 mm diameter (DTB), and until the first flower opened (DTF), and for the number of nodes on the main stem at buttoning (FLN at buttoning).

2.2.4 Statistical analysis

The results of experiment II (DTI only) were analyzed using two-way ANOVA (with no blocking) in Genstat (11^{th} edition) for the main effects of pre-growth period and genotype and the interaction effect of pre-growth period*genotype. I also calculated the expected means squares (EMS) for each of these effects and for the residual error based upon the results of the ANOVA. I used these to estimate the proportion of variation accounted for by each of these effects as described in Appendix 3. I also used the standard errors of the differences of the means calculated by the ANOVA to carry out t-tests to compare the DTI of each genotype and the average DTI of all four genotypes with 50 and 60, 50 and 70, and 60 and 70 days pre-growth.

2.3 Results and discussions

2.3.1 The time taken to reach competence to flower varies amongst the genotypes and with environmental conditions

The percentages of plants of each genotype flowering within 100 days of the end of cold-treatment with each pre-growth period in experiments I and II are given in Table 3. The E1 NV controls in experiment I also formed visible inflorescence buds, on average 231 days after sowing (111 days after the end of the 10-week cold-treatment period for the other plants). None of the other NV controls formed visible inflorescence buds in either experiment (discussed further in chapter 3).

E9 has the longest juvenile phase of the four genotypes tested in terms of chronological time. This was the only genotype in which no plants formed inflorescences when cold-treated for 10 weeks after 50 days pre-growth in experiment II (Table 3). With 60 days pre-growth two of the three E9 plants flowered, and the one that did not had two fewer leaves before cold-treatment than the two that did (14 leaves compared to 16 for each of the other two, Appendix 2). So the 60 days pre-growth appears to have been just sufficient for E9 to reach the adult-vegetative phase at least in these two plants. All of the E1 plants at each pre-growth period in both experiments formed visible inflorescences (Table 3). None of the E5 or E8 plants formed visible inflorescence buds with 50 days pre-growth in experiment I. And only four out of five of the E5 plants and three out of five of the E8 plants formed visible inflorescences with 50 days pre-growth in experiment II. This suggests that E1 has the shortest juvenile phase.

Experiment	**pre-growth period**	**% plants formed visible inflorescence buds within 100 days from end of cold-treatment (no. reps in brackets)**			
		E1	**E5**	**E8**	**E9**
Exp I	50 days	100 (2)	0 (2)	0 (2)	n/a
Exp II	50 days	100 (5)	80 (5)	60 (5)	0 (3)
	60 days	100 (5)	100 (5)	80 (5)	67 (3)
	70 days	100 (5)	100 (5)	100 (5)	100 (3)

Table 3: The percentage of plants of the PSB genotypes E1, E5, E8, and E9 forming visible inflorescence buds within 100 days of the end of cold-treatment for 10 weeks at 5°C following different pre-growth periods in experiment I and experiment II.

The chronological duration of the juvenile phase varied between the two experiments, probably due to differences in the conditions in the glasshouse during pre-growth. Four out of the five E5 plants and three of the five E8 plants formed inflorescences when cold-treated for 10 weeks after 50 days pre-growth in Experiment II. But none of the plants from either of these genotypes formed inflorescences when given the same cold-treatment after 50 days pre-growth in Experiment I (Table 3). In experiment II the pre-growth was carried out during the spring/summer rather than the autumn/winter as for experiment I (Table 2). The plants were pre-grown in the same glasshouse in both experiments and the plants in experiment I were given 16 hours per day of artificial light. But in experiment I the natural day-length (from sunrise to sunset) was only 10 hours 10 minutes–7 hours 43

minutes. During the 50-day pre-growth period in experiment II the natural day-length was 16 hours 16 minutes–16 hours 51 minutes (see sowing and transfer dates in Table 2[2]). So although the photoperiod was only slightly greater in experiment II, the plants received much longer hours of natural light. The natural daylight is likely to have been much more intense than the artificial light and the quality of the natural light may have been more favourable for plant growth. The glasshouse was also warmer during pre-growth in experiment II than in experiment I. Therefore it is likely that the reduced chronological duration of the juvenile phase in E5 and E8 in experiment II compared to experiment I is due to faster vegetative development driven by increased photoperiod, light intensity (and possibly quality), and temperature. This result is unsurprising as the chronological duration of the juvenile phase in cauliflower has been shown to vary with irradiance and photoperiod (Hand & Atherton 1987).

The number of days from the end of cold-treatment till the first appearance of visible inflorescence buds at the apex (DTI) for the plants in experiment II is shown in Figure 4. The DTI is used as a measure of flowering time because the DTB and DTF were both noticeably affected by bud abortion (discussed in detail in chapter 3). The full data for DTI for all plants from experiments I and II are given in Appendix 1 and Appendix 2 respectively. In experiment I the E1 plants formed visible inflorescence buds after an average of 10.5 days from the end of cold-treatment (95% confidence interval +/- 0.98). This data is not included in statistical analyses though, due to the confounding effect of the different pre-growth conditions between the experiments. Because none of the E9 plants cold-treated after only 50 days pre-growth formed visible inflorescences they were given an arbitrary value of 100 DTI to allow comparisons to the other genotypes and pre-growth periods. The other plants that did not form visible inflorescence buds within 100 days of the end of cold-treatment in experiment II were excluded from the graph and from the statistical analyses. This was done because their failure to do so appeared to be anomalous or due to

[2] The natural day-length (i.e. the time between sunrise and sunset) was calculated using the "sun calculator" at http://www.timeanddate.com/worldclock/sunrise.html, accessed 26th April 2011

them having a low leaf number at the start of cold treatment compared to the other plants given the same pre-growth period (discussed in chapter 3 and Appendix 2).

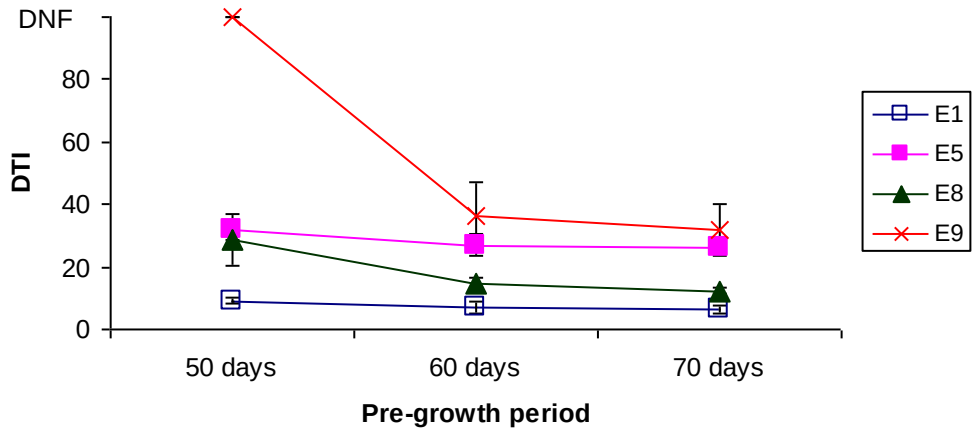


Figure 4: The average number of days taken to form visible inflorescence buds (DTI) following cold-treatment at 5°C for 10 weeks with different pre-growth periods by each of the four PSB genotypes E1, E5, E8, and E9 (experiment II). None of the E9 plants pre-grown for only 50 days formed visible inflorescences within 100 days of the end of cold treatment. These plants were given an arbitrary value of 100 DTI (shown as "DNF"). The E5 and E8 plants that did not form visible inflorescence buds within 100 days of the end of cold-treatment are excluded from the dataset because their failure to do so appeared to be anomalous or due to an abnormally low leaf number at the start of cold-treatment (Appendix 2).. The error bars are 95% confidence intervals. There are 2–5 individuals of each genotype for each pre-growth period (not counting the DNFs that were excluded).

Within each genotype the DTI decreased with increasing pre-growth period, but the pattern of this decrease varied among the genotypes (Figure 4). In E1 and E5 the DTI decreased gradually and relatively steadily from 50 to 70 days pre-growth. In E8 and E9 there was a much sharper decline in DTI between 50 and 60 days pre-growth. This was followed by a more gradual decline from 60 to 70 days pre-growth. This difference resulted in considerable variation in the magnitude of the differences in DTI among the genotypes at the different pre-growth periods. But the order in which the genotypes formed inflorescence buds remained the same: E1 was the fastest genotype to form visible inflorescence buds at each pre-growth period, followed by E8, E5, and E9. With 50 days pre-growth E5 formed visible inflorescence buds at a similar time to E8, after an average of 31.75 days (95%

confidence interval +/-2.81) compared to 28.67 days for E8 (95% confidence interval +/-8.57). This was much quicker than even the arbitrary value of 100 days assigned to E9. With 70 days pre-growth E8 formed visible inflorescence buds much sooner than E5, after an average of 12.20 days compared to 26.40 for E5 (95% confidence intervals +/-0.96 and 2.81 respectively). But with this pre-growth period E9 formed visible inflorescence buds at a similar time to E5, on average after 32.00 days (95% confidence interval +/-7.84). E1 was the quickest genotype to form visible inflorescence buds with each pre-growth period.

A degree of caution is necessary in interpreting the results of the ANOVA and t-tests carried out because the E9 plants given 50 days pre-growth (which did not form visible inflorescences) were assigned an arbitrary value of 100 DTI. This is only an arbitrary quantitative measure of flowering time. The use of a constant arbitrary value also means that the standard error of the mean and standard deviation for these plants will be zero, which is unrealistic and violates the assumption of homogenous variances and normality of data. This could affect the estimation of the significance of effects in ANOVA by reducing the average standard error. These plants had not formed visible inflorescence buds when discarded after 100 days from the end of cold-treatment. Therefore 100 DTI is the lowest value that could be reasonably assigned to these plants. This means that the likelihood of obtaining "false positive" significant results is minimized. Due to the low number of replciates in each group tests for homogeneity of variances and normality of data within each group (assumptions upon which ANOVA is based) are unreliable. Therefore caution must be exercised when interpreting the results of these tests.

ANOVA confirmed that the effects of genotype, pre-growth, and the interaction of genotype*pre-growth (i.e. the difference in how the genotypes responded to pre-growth period) were all significant ($p<0.001$, Table 4). I also calculated the expected means squares (EMS) based upon the ANOVA and estimated the proportion of variance accounted for by each of these effects as described in Appendix 3. The effect of the genotype accounted for approximately 50% of the total variation and the effect of the pre-growth period for approximately 8%. The interaction effect of genotype*pre-growth period accounted for approximately 40% of the total variation, and the residual error for approximately 2%. The low residual

error suggests that this model explains the observed data well. These should not be regarded as genuinely quantitative estimates of the proportion of variation accounted for by each effect because arbitrary values were assigned to the E9 plants pre-grown for only 50 days. But this analysis does strongly suggest that the primary factors affecting DTI in this experiment were the genotype and the interaction of the genotype with the pre-growth period, whereas the effect of the pre-growth period was relatively small.

Factor	**ANOVA for effect upon DTI in experiment II**	
	P-value	**Approximate % variation accounted for**
Genotype	<0.001	50
Pre-growth	<0.001	8
Genotype*pre-growth	<0.001	40
Error (residual)	n/a	2

Table 4: The effects of genotype, pre-growth period, and genotype*pre-growth period on the days taken from the end of cold treatment to form visible inflorescence buds (DTI) in the four PSB genotypes E1, E5, E8, and E9 in experiment II. All of the plants were cold-treated for 10 weeks at 5°C, with pre-growth periods of 50, 60, and 70 days. None of the E9 plants given 50 days pre-growth formed visible inflorescences within 100 days of the end of cold-treatment. These plants were given an arbitrary value of 100 DTI. The E5 and E8 plants that did not form visible inflorescence buds within 100 days of the end of cold-treatment are excluded from the dataset because their failure to do so appeared to be anomalous or due to an abnormally low leaf number at the start of cold-treatment (Appendix 2). The full ANOVA table is shown in Appendix 4 and the method used to calculate the percentage of variation accounted for by each effect is shown in Appendix 3.

I performed T-tests (using the standard errors of the differences calculated by the ANOVA) to compare the DTI at each pre-growth period both for each genotype individually and for the experiment as a whole. The p values obtained are given in Table 5.

Genotype	P value of null hypothesis (that the difference in DTI is due to chance)			SED (from ANOVA)	Degrees of freedom
	50d vs 70d	50d vs 60d	60d vs 70d		
E1	>0.050	>0.050	>0.050	2.204	9
E5	>0.050	>0.050	>0.050	2.204	9
E8	<0.001	<0.010	>0.050	2.204	9
E9	<0.001	<0.001	>0.050	2.846	5
All genotypes	<0.001	<0.001	>0.050	1.162	35

Table 5: The results of t-tests for the differences in DTI among plants of the PSB genotypes E1, E5, E8, and E9 cold-treated for 10 weeks at 5°C after different periods of pre-growth in experiment II. The t-tests were performed using the standard errors of the difference between the means calculated in the ANOVA in Table 4, which are given in this table.

There was no significant difference in DTI between 60 and 70 days pre-growth in any of the genotypes or for the whole data set (i.e. all of the genotypes together) (Table 5). For E1 and E5 there was also no significant difference in DTI between the 50 and 70 day or 50 and 60 day pre-growth periods. For E8 and E9 and for the whole data set (i.e. all of the genotypes together) the decrease in DTI between the 50 and 60 day and 50 and 70 day pre-growth periods was significant ($p<0.010$). This suggests that the large and significant interaction affect of genotype*pre-growth found by the ANOVA is due to a significant decrease in DTI between the 50 and 60 day pre-growth periods that occurs in E8 and E9 but not in E1 and E5. The pre-growth period and interaction of genotype*pre-growth period do not appear to have a significant effect upon DTI with 60–70d pre-growth.

The more rapid decline in DTI between 50 and 60 days in E8 may reflect the plants still being in the vegetative phase change after 50 days pre-growth. The percentage of plants of E8 that formed inflorescences with 60 days pre-growth was greater than with 50 days pre-growth but only by one plant (Table 3), so no real meaning can be attached to this difference. However, the E8 plants in experiment I did not respond to vernalization after 50 days pre-growth in less favourable conditions (Table 3). So it seems likely that the 50 days pre-growth period in experiment II was near to the minimum at which E8 would respond to cold treatment. This suggests that the level of competence to respond to vernalization increases progressively during the vegetative phase change resulting in an increase in the percentage of plants flowering

and a reduction in DTI, but this effect is only seen clearly in E8. All of the pre-growth periods in both experiments were sufficient for the E1 plants to reach the adult phase. And in E5 and E9 the pre-growth periods were all either sufficient for the plants to have made the transition completely (50 days plus for E5 and 60 days plus for E9 in experiment II) or not sufficient for them to have started the transition (50 days in experiment I for E5 and 50 days in experiment II for E9).

The hypothesis that the transition from juvenile to adult vegetative phase is gradual rather than abrupt is in agreement with published reports (Poethig 1990; Baurle & Dean 2006). In wheat the duration of cold required to reach a stage of vernalization insensitivity declines as plants age, suggesting that sensitivity to vernalization increases with plant age (Wang et al. 1995). But in cauliflower Hand & Atherton (1987) found no effect of the plant age beyond the transition upon competence to respond to vernalization. This is in agreement with my finding that the decrease in DTI with plant age after completion of the vegetative phase change was only very gradual and was not significant (Figure 4 & Table 5).

2.3.2 The leaf number at the end of the juvenile phase is approximately equal in E1, E5, and E8 but greater in E9

The average numbers of visible leaves for each genotype at the end of each pre-growth period are given in Figure 5. These are the averages of all of the plants at each pre-growth period in each experiment. That is, all of the plants in experiment I and all of the plants from the sowings on the 8th, 18th, and 28th May 2009 in experiment II (see chapter 3). A randomly selected subset of these plants were assigned to the 10-week-5°C cold-treatment in each experiment, and thus included in the analyses in this chapter. The average numbers of visible leaves at the end of pre-growth ("NV") and at the end of cold treatment ("T0") for only the plants from experiment II that were included in the analyses in this chapter (i.e. only the plants treated for 10 weeks at 5°C) are given in Figure 6.

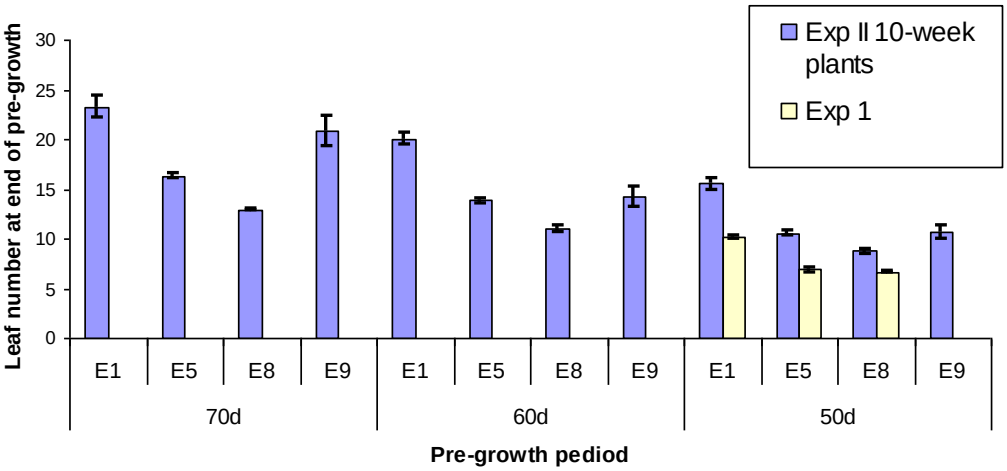


Figure 5: The average number of visible leaves produced by each of the PSB genotypes E1, E5, E8, and E9 at the end of different pre-growth periods in experiments I and II. The pre-growth was carried out in a warm glasshouse under natural light, with 16 hours per day of artificial light in experiment I only. The error bars are 95% confidence intervals. There are 9–20 plants of each genotype for each treatment. (All of the plants from experiment I and from the 8th, 18th, and 28th May 2009 sowings in experiment II are included, of which a randomly selected subset were given the 5°C cold-treatment described in this chapter).

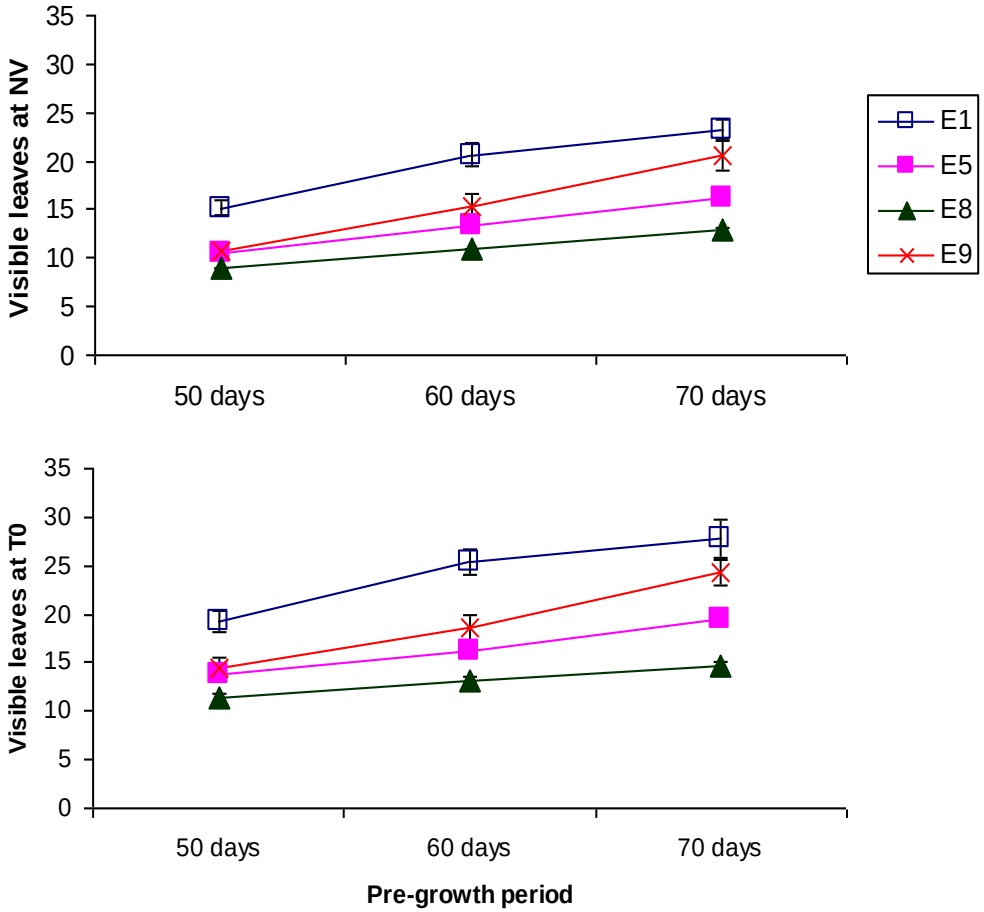


Figure 6: The average number of visible leaves produced by each of the PSB genotypes E1, E5, E8, and E9 at the end of different 50, 60, and 70 days pre-growth ("NV") and at the end of 10 weeks cold-treatment at 5°C following these periods of pre-growth ("T0"). The error bars are 95% confidence intervals. There are 3-5 replicates of each genotype for each pre-growth period.

In cauliflower the chronological duration of the juvenile phase varies with irradiance and photoperiod whereas the leaf number at phase change has been shown to remain constant (Hand & Atherton 1987). Therefore I calculated the minimum number of leaves at which each of the PSB genotypes in this study responded to vernalization (by forming visible inflorescence buds within 100 days of the end of a 10-week-5°C cold-treatment) based upon the data in Figure 5 and the flowering-time data discussed above. This is approximately 10.2 leaves in E1 (50d, exp I), 10.6 leaves in

E5 (50d, exp II), 8.7–11.1[3] leaves in E8 (50–60d, exp II), and 15[4] leaves in E9 (60d, exp II). Leaf number was found to be a stable measure of the end of juvenility in cauliflower in the above study. So it seems reasonable to assume that plants of these genotypes pre-grown under similar conditions in future experiments will respond to cold-treatments given after they have reached these numbers of emerged leaves. But these are only estimates of a leaf number at which the plants are competent to be vernalized rather than of the end of the juvenile phase *per se*. It is possible that these genotypes may respond to cold treatment at a slightly lower leaf number, and in E1 it is not possible to give a leaf number at which plants will not be competent to respond. Juvenility ended at a considerably higher leaf number in cauliflower plants that were sown in October and kept over winter in modular trays (in a ventilated polythene tunnel/frost-protected glasshouse) before transplanting to the field in spring than in plants raised in controlled environment cabinets at approximately 15°C (Fellows et al. 1999). So these estimates of the leaf number at the end of juvenility should only be applied to glasshouse-grown plants.

ANOVA showed that genotype, pre-growth period, and the interaction of genotype*pre-growth all significantly affected the number of leaves both at the end of pre-growth ("NV") and at the end of cold-treatment ("T0") ($p<0.001$ in each case, Appendix 4). This shows that there are significant differences among the genotypes in both leaf initiation rate and the change in leaf initiation rate with time (between 0 and 70 days from sowing). At each time-point and with each pre-growth period E1 had the most leaves followed by E9, E5, and E8. But E5 and E9 had very similar leaf numbers at 50d pre-growth. As the pre-growth period increased, E9 began to produce more leaves relative to the other genotypes. By 70 days from sowing E9

[3] The lower value of 8.7 for E8 is based upon the average number of leaves at the end of the 50 day pre-growth period in experiment II, when the vegetative phase change appears to have started but not yet to be complete (see text). The higher value of 11.1 leaves is based upon the average leaf number at the end of the 60 day pre-growth period when the vegetative phase change appears to have been completed.

[4] This value is estimated based upon the fact that, although the average number of leaves for E9 with 60 days pre-growth was 14.3, a plant with only 14 leaves did not form visible inflorescence buds whereas the two with 16 leaves did (Appendix 2).

had several more leaves than E5 and nearly as many as E1. This is likely to account for the significant interaction effect of pre-growth*genotype. The pattern of variation in leaf number amongst the pre-growth periods and genotypes was very similar for the leaf number at NV and T0 (Figure 6). This suggests that the genotypes all produced leaves at a similar (and much retarded compared to during pre-growth) rate during cold-treatment.

The juvenile phase appears to last for a similar number of leaves in E1, E5, and E8. The vegetative phase change appears to have been completed at a leaf number of around 10–11 in both E5 and E8. This is also the minimum number of leaves at which E1 has been shown to respond to cold-treatment, although it may still respond at a lower leaf number. But these genotypes take different periods of time to produce this number of leaves. The time taken to produce this minimum number of leaves also correlates with the flowering times of these genotypes when they are grown in the field (E1 is earlier than E5 which is earlier than E8). This suggests that variation in the duration of the juvenile phase (possibly caused by a reduction of leaf initiation rate in E8 compared to E5) may contribute to this variation in flowering time.

E9 does not fit this pattern, as it produces approximately five more leaves by the phase change than the other genotypes but takes only 60 days from sowing to do so. The chronological duration of the juvenile phase in E9 is also greater than in E5 and E8. But E9 has more leaves than either of these other genotypes at 60d pre-growth and above (Figure 5 and Figure 6). This may be due to a difference in the relationship between leaf initiation rate and developmental time in E9. Mutations have been found in Arabidopsis that affect the leaf number of the first leaf with abaxial trichomes (a marker for the adult-vegetative phase) but not the timing of the first production of abaxial trichomes (Telfer et al. 1997). As the leaf number at the end of the juvenile phase varies among different genotypes of cauliflower (Hand & Atherton 1987; Wurr et al. 1988; Wurr et al. 1993; Wurr et al. 1994) it is not surprising that duration of the juvenile phase, in terms of both leaf number and chronological duration, varies among different genotypes of PSB.

2.3.3 The role of micro RNAs in controlling the vegetative phase change in Arabidopsis is likely to be conserved in PSB but this may or may not be linked to juvenility in vernalization response

In Arabidopsis the micro RNA *miR156* plays a key role in coordinating the vegetative phase change by repressing the expression of ten of the S*QUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes (Willmann & Poethig 2007; Fornara & Coupland 2009; Wu et al. 2009; Poethig 2010). These *miR156*-repressed *SPL* genes form four major caldes *SPL3*/*SPL4*/*SPL5*, *SPL2*/*SPL10*/*SPL11*, *SPL9*/*SPL15*, and *SPL6*/*SPL13* (Wu et al. 2009). *MiR156* is expressed strongly in juvenile plants, and its expression decreases as plants age, whereas the expression of the *miR156*-repressed *SPL* genes increases as plants age (Wu & Poethig 2006; Willmann & Poethig 2007; Schwarz et al. 2008; Wang et al. 2009a; Wu et al. 2009; Yamaguchi et al. 2009; Poethig 2010).

The miR156-repressed *SPL* genes act to promote flowering both directly and by up-regulating the expression of another micro RNA, *miR172*. *SPL3* promotes the expression of the floral meristem identity genes *LFY*, *AP1*, and the flowering time/floral meristem identity gene *FRUITFULL* (*FUL*) (Ferrándiz et al. 2000; Yamaguchi et al. 2009; Poethig 2010). *SPL4* and *SPL5* also appear to act redundantly with SPL3 in up-regulating these three genes (Yamaguchi et al. 2009). *SPL9* promotes the expression of *FUL* as well as the floral integrator gene *SOC1* and the *SOC1* paralogue *AGAMOUS-LIKE 42* (*AGL42*) (Wang et al. 2009a; Poethig 2010). *SPL9* and *SPL10* act redundantly to promote the transcription of *miR172* (Wu et al. 2009; Poethig 2010). Other *SPL* genes such as *SPL15* and *SPL11* (the closest homologues of *SPL9* and *SPL10* respectively) probably also act redundantly in this (Wu et al. 2009). *MiR172* has the opposite pattern of expression to *miR156* i.e. its expression increases as plants age (Aukerman & Sakai 2003; Willmann & Poethig 2007; Poethig 2009; Wu et al. 2009). *MiR172* represses *AP2* and the *AP2*-like transcription factors *TOE1*, *TOE2*, *TOE3 SMZ*, and *SNZ* (Aukerman & Sakai 2003; Schwab et al. 2005; Mathieu et al. 2009; Wu et al. 2009). TOE1, TOE2, SMZ, and SNZ have been shown to repress flowering and TOE3 and AP2 may also act as floral repressors. Therefore miR172 promotes flowering by repressing these genes (Aukerman & Sakai 2003; Mathieu et al. 2009). *TOE1* and *TOE2* act redundantly

with *SMZ* and *SNZ* to repress flowering specifically in long days (Mathieu et al. 2009). SMZ has been shown to repress the transcription of the floral integrator gene *FT* in the leaves (Mathieu et al. 2009). This prevents up-regulation of *FT* expression in response to long photoperiods. FT protein acts as a mobile signal that is induced in the leaves in response to long days and travels to the apex, where it interacts with FD to promote flowering (Abe et al. 2005; Wigge et al. 2005; Jaeger & Wigge 2007; Notaguchi et al. 2008). So repression of *FT* induction by the *AP2*-like genes represses the induction of flowering by long days and high *miR172* expression in the adult phase releases this repression. SMZ also appears to repress the expression of *SOC1* and *AP1* at the apex (Mathieu et al. 2009).

The *SPL* genes also promote the vegetative aspects of the adult phase, such as the formation of abaxial trichomes on leaves, both directly and via the up-regulation of *miR172* expression. The *miR172*-repressed transcription factors *TOE1* and *TOE2* promote juvenile epidermal identity (Wu et al. 2009). Abaxial trichome production (a feature of adult leaves that marks the transition to the adult phase) is accelerated in plants that over-express *miR172b* under the 35S promoter or that are homozygous for *toe1-2* or *toe2-1* mutations. Constitutive expression of *TOE1* or *TOE2* delays abaxial trichome production (Wu et al. 2009). *SPL3*, *SPL4*, and *SPL5* also promote adult patterns of epidermal differentiation (that is, abaxial trichome production) in the leaves (Wu & Poethig 2006; Poethig 2009; Wu et al. 2009). *SPL9* also appears to regulate epidermal identity independently of its role in promoting *miR172b* transcription (Wu et al. 2009). *SPL9* and *SPL10* also promote adult leaf shape (Fornara & Coupland 2009).

The sequential action of *miR156* and *miR172* appears to control the transition of Arabidopsis from the juvenile to the adult phase (Figure 7) (Wu et al. 2009). The transition from high levels of *miR156* to high levels of *miR172* appears to be mediated by *SPL9* and *SPL10*, and this in turn promotes the transition to the adult vegetative phase (Fornara & Coupland 2009). Each of these micro RNAs appears to be regulated by its own target genes in a negative feedback loop. *SPL9* and *SPL10* are both repressed by *miR156* but also promote the transcription of a *miR156* precursor, *miR156a*. *TOE1* and *TOE2* also promote the transcription of a precursor of *miR172*, *miR172b*. These negative feedback loops may contribute to the stability

of the juvenile and adult phases of development (Fornara & Coupland 2009; Wu et al. 2009).

The *SPL* genes have recently been described as forming a new flowering-time pathway in Arabidopsis (Wang et al. 2009a). The low expression of the *SPL* genes during the juvenile phase (due to high levels of *miR156*) may limit competence to flower (Wang et al. 2009a; Poethig 2010). The (day-length-independent) decrease in *miR156* expression and increase in *SPL* gene expression with age allows flowering in response to inductive photoperiods during the adult phase (Wang et al. 2009a). This may result from the release of repression of *FT* induction (as *miR172* expression is induced by the increased expression of the *SPL* genes) as described above. The induction of flowering by exposure to long photoperiods is reduced in Arabidopsis plants over-expressing *miR156b* and in *spl9 spl15* double mutants (Schwarz et al. 2008). *SPL* genes may also act downstream of *FT* in the apex during the induction of flowering, so their low expression levels during the juvenile phase could limit the ability of *FT* to induce flowering (Wang et al. 2009a). *SPL* gene expression can also be induced strongly and rapidly in the apex by long photoperiods (Wang et al. 2009a). This may contribute to photoperiodic induction of flowering. The increase in SPL gene expression with plant age may also eventually allow flowering without FT/FD activity (Wang et al. 2009a) which could explain why Arabidopsis can still flower (late) without exposure to long days (Gregory & Hussey 1953; Amasino 2010).

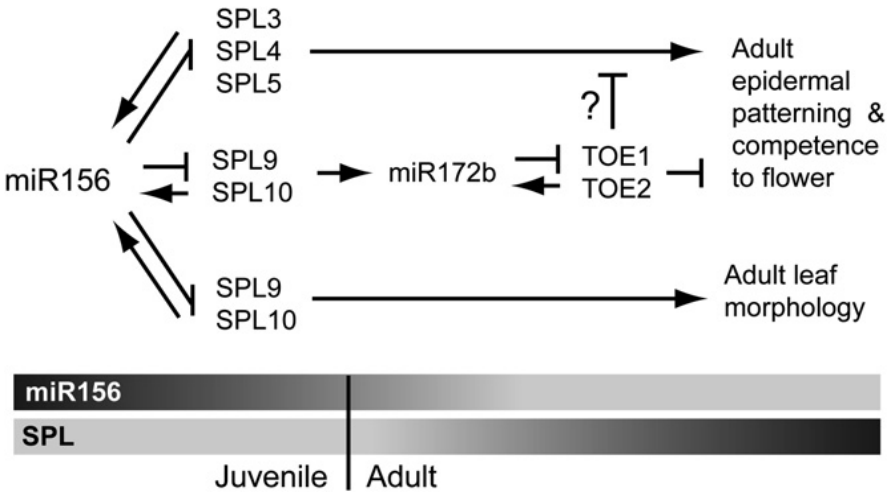


Figure 7: A model for how the sequential expression of *miR156* and *miR172* may regulate the vegetative phase change and reproductive competence in Arabidopsis, reproduced from Wu et al. (2009) figure 6. *Mir156* is strongly expressed during the juvenile phase and its expression decreases with age. *MiR156* represses the expression of *SPL* genes, which promote the expression of adult leaf morphology and epidermal patterning and flowering. Some *SPL* genes also promote the expression of *miR172*, which promotes adult epidermal patterning and the competence to flower by repressing the expression of the *AP2*-like transcription factors such as *TOE1* and *TOE2*. *MiR172* expression follows an opposite pattern to that of *miR156*, increasing with plant age. Both *miR156* and *miR172* are regulated by negative feedback from genes that they repress (*SPL9&10* and *TOE1&2* respectively).

MiR156 and *miR172* also have a reciprocal expression pattern and appear to play a similar role in regulating the vegetative phase change in maize (Willmann & Poethig 2007; Chuck et al. 2009; Poethig 2009). This suggests that this mechanism has been conserved amongst both dicots and monocots. So a very similar mechanism may regulate the vegetative phase change in PSB, which is much more closely related to Arabidopsis than maize is. Both the vernalization and photoperiod pathways in Arabidopsis converge on the floral pathway integrator genes *FT* and *SOC1* (as discussed in chapter 1). The de-repression of *FT* by action of the *SPL* genes (via *miR172* and the *AP2*-like transcription factors) may explain juvenility in photoperiod response in Arabidopsis. But winter-annual accessions of Arabidopsis do not show juvenility in vernalization response whereas PSB and many other Brassicas do (Lin et al. 2005; Baurle & Dean 2006). So it is uncertain whether juvenility in vernalization response in PSB may be controlled by micro RNAs in a similar way.

2.3.4 The role of *TFL1* in controlling juvenility in vernalization response in *Arabis alpina* may be conserved in PSB

Arabis alpina (family Brassicaceae) is a perennial, polycarpic species and is closely related to both Brassica and Arabidopsis. But *A. alpina* lacks the genome triplication that occurred in a common ancestor of the Brassica species. Flowering in *A. alpina Bonn* accession is promoted by long days but this accession does not require vernalization. *A. alpina Bonn* shows juvenility in photoperiod response; long-day treatment can only accelerate flowering in adult and not juvenile plants. *A. alpina Pajares* accession requires vernalization in order to flower and like PSB, shows juvenility in vernalization response. A vernalization treatment that is started during the juvenile phase, which lasts for 3–4 weeks, will not promote flowering in *A. alpina Pajares* (Wang 2007).

In Arabidopsis the repression of the floral repressor *FLC* is central to the vernalization response. High levels of *FLC* prevent flowering before vernalization, but during vernalization *FLC* expression is stably repressed, rendering the plant competent to flower (as discussed in chapter 1). Wang (2007) isolated an orthologue of *FLC*, named *AaFLC*, from *Arabis alpina*. Like *FLC* in Arabidopsis, *AaFLC* encodes a strong floral repressor. *AaFLC* expression was down-regulated in the leaves and apexes of both juvenile and adult *A. alpina Pajares* plants during cold-treatment for 12 weeks. Therefore the inability of juvenile *A. alpina* to respond to vernalization is not due to a failure to repress *AaFLC* (Wang 2007). Homologues of Arabidopsis *FLC* appear to act as floral repressors and play a similar role in controlling vernalization response in several Brassica species, including PSB (discussed fully in chapter 4). So it seems unlikely that the insensitivity of juvenile Brassica plants to vernalization is related to a failure to repress *BoFLC* homologues.

Wang (2007) also isolated orthologues of the Arabidopsis floral pathway integrator genes *SOC1* and *FT* from *A. alpina*, named *AaSOC1* and *AaFT*. Both of these appeared to promote flowering and to be repressed by *AaFLC*, similar to *SOC1*and *FT* in Arabidopsis. *AaSOC1* expression increased during vernalization in the leaves and apexes of both adult and juvenile plants. But only adult plants flowered following vernalization. *AaFT* expression increased in the apexes of adult but not juvenile plants 8 weeks after the start of cold-treatment. But some adult *A. alpina*

Pajares plants formed reproductive leaves and even flowers in some cases if returned to warm conditions after only 5 weeks of cold-treatment whereas juvenile plants did not. At this point *AaFT* expression was not increased in the apexes of adult plants. This lack of correlation between *AaFT* and *AaSOC1* expression patterns during cold-treatment in juvenile and adult plants and the flowering responses of these plants to cold-treatment suggests that neither of these genes mediate juvenility in vernalization response in *A. alpina Pajares*.

The incompetence of juvenile *A. alpina* plants to respond to vernalization appears to involve a failure to repress *AaTFL1*, an orthologue of Arabidopsis *TERMINAL FLOWER 1* (*TFL1*), during vernaliation (Wang 2007). In Arabidopsis *TFL1* acts to repress flowering and maintain inflorescence indeterminacy (Wang 2007). *AaTFL1* also appears to act as a floral repressor. *AaTFL1* is highly expressed in the sub-domain of the shoot apexes and the provasculature in apexes in both juvenile and adult plants before vernalization treatment. During vernalization treatment (starting after 5 weeks of cold-treatment) *AaTFL1* expression is restricted to a more limited region in apexes of adult plants but not in the apexes of juvenile plants. This allows floral meristems to be initiated on the flanks of the shoot apical meristem in adult but not juvenile plants. The differential competence of side branches to respond to vernalization in *A. alpina Pajares* may also involve different expression patterns of *AaTFL1* (Wang 2007).

TFL1 homologues also appear to be involved in controlling juvenility in vernalization response in citrus. *CsTFL1*, a citrus *TFL1* homologue, was found at higher levels in the stems of juvenile citrus plants than adult plants both before and during vernalization, suggesting that it may be involved in preventing flowering in juvenile plants (Pillitteri et al. 2004; Wang 2007). A homologue of *TFL1* isolated from apple, *MdFTL1*, also appears to be involved in controlling juvenility (Kotoda & Wada 2005; Wang 2007). Reducing *MdTFL1*expression by DsRNAi led to much earlier flowering in apple trees (Wang 2007). Suppressing *MdTFL1* using antisense RNA has also reduced the juvenile phase in apple trees and led to earlier flowering (Kotoda et al. 2002). This suggests that the role of *TFL1* in controlling juvenility in vernalization response has been conserved in such distantly related species as apple, *A. alpina* and Citrus. So it seems likely that homologues of *TFL1* may play a similar

role in controlling juvenility in vernalization response in Brassica species, which are closely related to *A. alpina*.

This suggests that juvenility in vernalization response in *A. alpina*, and possibly PSB, may be controlled differently to juvenility in photoperiod response and the morphological aspects of the vegetative phase change in Arabidopsis. In Arabidopsis *miR156* appears to regulate both morphological aspects of the vegetative phase change (such as the appearance of abaxial trichomes) and the competence to respond to photoperiod (via releasing the repression of *FT* induction) as described above. But Arabidopsis can be vernalized as imbibed seeds whereas many Brassicas and *A. alpina* show juvenility in vernalization response (Lin et al. 2005; Baurle & Dean 2006; Wang 2007). In *A. alpina* juvenility in vernalization response may be controlled through the differential expression patterns of *AaTFL1*, and a similar mechanism could be involved in the closely related Brassica species.

2.3.5 An unambiguous marker for the vegetative phase change would aid studies in Brassica

It would be ideal to identify a clear morphological marker for the adult vegetative phase of PSB, as leaf number does not always provide an accurate means of estimating the end of the juvenile phase in cauliflower (Wurr et al. 1993; Wurr et al. 1994; Wurr & Fellows 1998; Fellows et al. 1999). Mutations have also been found in Arabidopsis that affect the leaf number of the first leaf with abaxial trichomes (a marker for the adult-vegetative phase) but not the timing of the first production of abaxial trichomes (Telfer et al. 1997). In many plants leaves produced during the juvenile phase are smaller and simpler than adult leaves (Poethig 1990). I did not examine leaf morphology in detail in this study, but general observations suggested that this is the case in PSB. The cotyledons were simple, round, and flat. The first true leaves were often serrated, but were thin and fairly flat, and not noticeably waxy. As the plants aged the leaves became tougher and often "crinkly" in shape and obviously serrated, especially in E8. In Experiment I the E1 plants kept for the longest also began to produce very waxy leaves: the upper epidermises of these leaves were silvery in colour due to the amount of wax on the surface. Further studies of leaf development during the juvenile phase may identify features that change clearly at the vegetative phase change and could be used as markers for this

transition. But in Arabidopsis the features of the leaves that change throughout vegetative development do so gradually, so are not ideal as markers for the vegetative phase change (Telfer et al. 1997). Leaf morphology and growth can also be affected by environmental conditions as well as developmental phase (Kerstetter & Poethig 1998). The appearance of abaxial trichomes on the leaves is often used as a marker for the end of the vegetative phase change in Arabidopsis (Telfer et al. 1997). Future studies will be needed to determine whether the distribution of trichomes also changes during development in PSB, and whether this may also provide an appropriate marker for the vegetative phase change as it does in Arabidopsis. *MiR156* may also provide a molecular marker for the vegetative phase change (Poethig 2010). However, even if a suitable marker for the vegetative phase change in PSB is found then its use may be complicated if the vegetative phase change and juvenility in vernalization response are controlled differently.

2.4 Conclusions

The duration of the juvenile phase varies among the four genotypes of PSB, resulting in a significant interaction effect of genotype*pre-growth upon flowering time. It will be important in future vernalization experiments involving these genotypes and/or populations derived from them to ensure that a sufficient pre-growth period is allowed for all of the genotypes to complete the vegetative phase change. This must be measured in terms of developmental time rather than chronological time, as the chronological duration of the juvenile phase varies with environmental conditions. At present the best measurement available for this is the number of emerged leaves. To ensure that the effect of juvenility is excluded the plants should be grown until they have at least as many leaves as the plants grown for 60d before cold-treatment in experiment II, as there was no significant effect of pre-growth or genotype*pre-growth beyond this stage.

3 The effect of temperature and treatment duration upon vernalization in Purple Sprouting Broccoli

3.1 Introduction

The effectiveness of a cold-treatment for vernalization depends upon both its temperature (which determines the rate of progress towards saturation of the vernalization response) and its duration. It is widely accepted that vernalization has three cardinal temperatures. The rate of vernalization increases from zero below a minimum effective temperature (T_{MIN}) to a maximum rate at the optimum temperature for vernalization (T_{OPT}). (Some models of vernalization response have considered there to be minimum and maximum "optimum" temperatures, between which vernalization rate is maximal). The rate of vernalization then declines with further increases in temperature above T_{OPT}, reaching zero above the maximum effective temperature (T_{MAX}) (Atherton et al. 1990; Craigon et al. 1990; Wurr et al. 1993; Streck 2003). The combined effect of the temperature and duration of a cold-treatment upon vernalization can be given in vernalizing degree days (V°CD) (Atherton et al. 1990; Craigon et al. 1990) or effective vernalization days (VD) (Streck 2002; Streck 2003; Streck & Schuh 2005). V°CD can be used where the relationships between vernalization rate and temperature are linear. One V°CD occurs when the temperature is 1°C above the T_{MIN} for vernalization but below T_{OPT} for 24 hours. Temperatures above T_{OPT} are converted to the equivalent temperatures below T_{OPT} before calculating the V°CD that have occurred over a period (Atherton et al. 1990; Craigon et al. 1990). One VD occurs when the temperature is at T_{OPT} for vernalization for 24 hours, but less than one VD occurs in 24 hours if the temperature is above or below T_{OPT} (Streck 2002; Streck 2003; Streck et al. 2003; Streck & Schuh 2005). The further away the treatment temperature is from T_{OPT} the longer the treatment will need to be prolonged to accumulate sufficient V°CD or VD to saturate the vernalization response.

Cardinal temperatures for vernalization reported in *Brassica oleracea* crops vary considerably. The optimum temperature(s) for vernalization has often been reported as being between 4 and 10°C (Thomas 1980; Wurr et al. 1993; Guo et al. 2004). Wurr et al. (1993) estimated the optimum temperature for vernalization in

cauliflower, cultivar White Fox, to be 9–9.5°C. But the optimum temperature for "curd initiation" in Cauliflower has been estimated to be over 10°C in other studies and as high as 15.8°C (Pearson et al. 1994; Wheeler et al. 1995). Although Pearson et al. (1994) questioned whether this constituted a true "vernalization response". One study also reported an optimum temperature for vernalization of 15.8°C in calabrese (Wurr et al. 1995). T_{MAX} for vernalization has been estimated to be greater than 20°C in Cauliflower (Wurr et al. 1993) and Calabrese (Wurr et al. 1995). Estimates of T_{MIN} for vernalization have ranged from -2.8°C in Calabrese (Wurr et al. 1995) to 9°C in Cauliflower (Wurr et al. 1993).

In chapter 2 I showed that the duration of the juvenile phase of Purple Sprouting Broccoli varied among the four genotypes E1, E5, E8, and E9. This conclusion was based upon a common cold-treatment of 10 weeks at 5°C under 8-hour photoperiods. The plants used in chapter 2 came from two experiments that I carried out: experiment I and experiment II. In experiment I there was only one pre-growth period but there were three treatment temperatures—5, 10, and 15°C—and two treatment durations—6 and 10 weeks (except in E9 where there were no 10-week treatments). This experiment also included NV controls for each genotype. In experiment II plants of each genotype were treated for both 6 and 10 weeks at both 5 and 10°C after each of the pre-growth periods—50, 60, and 70 days. (There was no 50d-10-week-10°C treatment for E9 in this experiment though as insufficient plants of this genotype germinated). Only the 10-week-5°C treatments were included in chapter 2 to ensure that the effects of temperature and duration of cold-treatment were excluded from the analysis.

I wanted to establish what temperatures and durations of cold-treatment were effective for vernalization and how this varied among these genotypes. In this chapter I have extended my analyses to cover all of the combinations of pre-growth period (50, 60, and 70d), treatment duration (6 and 10 weeks), and treatment temperature (5, 10, and 15°C and NV) for each genotype in experiments I and II.

3.2 Materials and methods

3.2.1 Experiment I: The responses of the Purple Sprouting Broccoli genotypes E1, E5, E8, and E9 to 6 and 10 weeks cold-treatment at 5–15°C with 50 days pre-growth

The plants were grown and treated as described in chapter 2 except that plants were treated at 10 and 15°C as well as 5°C and for 6 weeks as well as 10 weeks. The 10 and 15°C treatments were given in Gallenkamp controlled environment rooms (CERs) with 8 hours per day of artificial light. The 6-week treated plants were sown and transferred to the CERs at the same time as the 10-weeks treated plants described in chapter 2, but were removed four weeks earlier. For each genotype two–three plants were kept in the glasshouse throughout the experiment as non-vernalized (NV) controls. The NV controls were re-potted into 2-litre pots when they were 75 days old (25 days into the cold-treatment period for the other plants). All of the cold-treated plants were re-potted into 2-litre pots upon transfer back to the glasshouse. The plants were assigned to the different treatment temperatures and durations randomly. During post-vernalization growth in the glasshouse the plants were arranged randomly by hand on two benches. The plants cold-treated for 10 weeks were put on one bench and those cold-treated for 6 weeks on the other to avoid the larger plants that had been cold-treated for 6 weeks over-shading those that had been treated for 10 weeks. The schedule of plantings and transfers between CERs and glasshouses for this experiment is given in Table 6.

The plants were scored as described in chapter 2: for the number of days from sowing until the first appearance of visible inflorescence buds at the apex (DTI) and until the opening of the first flower (DTF). The number of visible leaves on each plant was also counted at the end of the pre-growth period and at the end of the cold-treatments.

Pre-growth (days)	Cold-treatment Duration (weeks)	Sown	Into cold	Out of cold
50	6 weeks	22nd/30th Oct 2008	12th/19th Dec 2008	22nd/30th Jan 2009
50	10 weeks	22nd Oct 2008	12th Dec 2008	20th Feb 2009
n/a	NV	22nd/30th Oct 2008	n/a	n/a

Table 6: The dates of sowing and transfer to and from the CERs in experiment I. Three plants of each genotype were cold-treated at 5, 10, and 15°C for 6-weeks in CERs. Two plants of each genotype were treated at each temperature for 10-weeks (none for E9). Three plants of E1, E5, and E8 and two for E9 were kept in the glasshouse as non-vernalized (NV) controls. All plants were sown on the same day except for some of the E9s which were sown on the 30th October due to poor germination among those sown on the 22nd October. The E9 plants sown in 30th October were transferred to and from the CERs one week later accordingly.

3.2.2 Experiment II: The responses of the Purple Sprouting Broccoli genotypes E1, E5, E8, and E9 to 6 and 10 weeks cold-treatment at 5 and 10°C with 50, 60, and 70 days pre-growth

The plants were grown and treated as described in chapter 2 except that plants were treated at 10°C as well as at 5°C and for 6 weeks as well as 10 weeks. The same cold-room and CER were used as for the 5 and 10°C treatments in Experiment I. The plants were sown in six batches on different dates such that all of the cold-treatments ended on the same day (25th September 2009). The plants from each of these six sowings were assigned to the two treatment temperatures randomly. Five plants of each genotype were randomly selected from each batch to be treated at each temperature. The remaining plants from each batch were treated in the same way as the 5°C plants but destructively sampled at different time-points throughout the experiment for examination of *BoFLC* expression in the young leaves and apexes (see chapter 4). Due to poor germination in E9 there were only 3–4 plants for each treatment, with none for 10°C with 50 days pre-growth and 10-weeks cold treatment. Nor were any E9 plants destructively sampled for use in chapter 4. "Non-vernalized control" plants were sown on the 17th July so that they were 70 days old when the other plants were returned to the glasshouse following cold-treatment. These non-vernalized controls remained in the glasshouse throughout the experiment and were

not cold-treated at any point. The schedule of plantings and transfers between CERs and glasshouses for this experiment is given in Table 7.

For post-cold-treatment growth in the glasshouse the plants were divided between three benches in the glasshouse according to pre-growth period: one bench for the plants that received 50 days pre-growth, one for those given 60 days, and one for those given 70 days. This was done to avoid the plants that had been pre-grown for longer (hence were larger) over-shading those that had been pre-grown for less time. The benches for each pre-growth period were assigned randomly. The plants were arranged randomly by hand within each bench. They were rearranged randomly by hand as necessary to fill gaps left by the removal of plants that had flowered and been disposed of. The non-vernalized control plants were dispersed randomly among the three benches. The plants were re-potted into 2-litre pots 47 days after the end of cold-treatment.

All of the plants were kept for 100 days from the end of cold-treatment or until they had flowered (whichever was sooner). A few plants that showed signs of forming inflorescences were kept till 150 days from the end of cold treatment but the rest were discarded after 100 days from the end of cold-treament.

The plants were scored as described in chapter 2: for the number of days from the end of the cold-treatments until the first appearance of visible inflorescence buds at the apex (DTI), until "buttoning" i.e. when the main inflorescence head reached 10 mm diameter (DTB), and until the opening of the first flower (DTF). The plants were also scored for the number of nodes on the main stem at buttoning (FLN at buttoning). The number of visible leaves on each plant was also counted at the beginning and end of the cold-treatments.

Pre-growth (days)	Vern duration	Sown	Into cold	Out of cold
70	10 weeks	8th May 2009	17th Jul 2009	25th Sep 2009
60	10 weeks	18th May 2009	17th Jul 2009	25th Sep 2009
50	10 weeks	28th May 2009	17th Jul 2009	25th Sep 2009
70	6 weeks	5th Jun 2009	14th Aug 2009	25th Sep 2009
60	6 weeks	15th Jun 2009	14th Aug 2009	25th Sep 2009
50	6 weeks	25th Jun 2009	14th Aug 2009	25th Sep 2009
NV	None	17th Jul 2009	n/a	n/a

Table 7: The dates of sowing and transfer to and from the CERs in experiment II. Three–five plants of each genotype were treated at 5°C and five at 10°C for 6 and 10 weeks with an 8-hour photoperiod (artificial light) then transferred to a warm glasshouse. At least 10 plants of each genotype were grown in the glasshouse and not cold-treated to act as non-vernalized controls. (There were no E9 plants in the 10-week 10°C cold-treatment with 50 days pre-growth due to poor germination).

3.2.3 Statistical analysis

The results were analyzed using ANOVA (with no blocking) and t-tests in Genstat (11th edition). A mixture on one-way ANOVAs for a single main effect and two way ANOVAs for two main effects and the interaction of these effects was used. For one of these two-way ANOVAs I also calculated the expected means squares for each of these effects and for the residual error based upon the results of the ANOVA and used these to estimate the proportion of variation accounted for by each effect as described in Appendix 3.

3.3 Results and discussions

3.3.1 Flowering-time measurement

In Arabidopsis the total number of leaves at flowering is often used as a measure of flowering time, as this is closely correlated with the time to flowering (Koornneef et al. 1991; Koornneef et al. 1998; Lempe et al. 2005). No more vegetative nodes form once the shoot apical meristem has made the transition to flowering, therefore the total number of leaves depends upon the time taken to make this transition. In PSB side-shoots form in the axils so the number of nodes on the main stem should be counted instead of the actual number of leaves (as side shoots will also form leaves).

The transition from adult-vegetative leaves to bracts is gradual in PSB though, so it is difficult to distinguish nodes formed before and after the plant committed to flowering. The morphology of the inflorescence can also make counting the number of nodes on the main stem difficult. A lot of nodes form very close together at the apex as the inflorescence forms, then become distinct later as the internodes elongate. In experiment II the number of nodes on the main stem (FLN) was counted at the time when the inflorescence head at the apex first reached 10 mm diameter. But even this was very difficult (especially in E5) due to the morphology of the apex. It would also not be possible to compare flowering time in terms of the number of nodes on the main stem between the different genotypes used in this study as they produced leaves/nodes at different rates (chapter 2).

The DTB and DTF were both affected by bud abortion due to poor light quality in the later stages of experiment II. The plants were given 16 hours of artificial light per day in the glasshouse from the end of cold-treatment (on 25th September 2009) until the end of the experiment. But the duration of natural light decreased greatly during this time. This appeared to result in large amounts of bud abortion. On some plants no flowers opened even long after the inflorescence buds had formed. In at least one plant visible inflorescence buds formed but all of the buds then died before the inflorescence reached 10 mm diameter. The time taken to form visible inflorescence buds (DTI) is likely to have been less adversely affected by bud abortion. Therefore the data for DTB, DTF, and FLN at buttoning are not shown. The leaf numbers of each plant before and at the end of cold-treatment are also given in Appendix 2. The flowering time is given as the number of days from sowing (for Experiment I) or the end of cold-treatment (experiment II) until the first appearance of visible inflorescence buds at the apex (DTI).

3.3.2 Percentages of plants flowering within each treatment

The percentages of plants of each genotype that formed visible inflorescence buds within 100 days of the end of each cold treatment are shown in Table 8 (experiment I) and Table 9 (experiment II). Two of the E8 plants treated at 10°C for 6 weeks with 70 days pre-growth and one of those given this treatment after 50 days pre-growth in experiment II also formed visible inflorescence buds 120–150 days after the end of cold-treatment. In experiment I the non-vernalized plants of E1 also

formed visible inflorescence buds 220–253 days from sowing (average 231.00 days from sowing). The E1 plants treated at 15°C for 6 weeks formed visible inflorescence buds on average 46.33 days after this (229–311 days from sowing, average 277.33 days from sowing or 185.33 days from the end of cold-treatment). The E1 plants treated at 15°C for 10 weeks did not form visible inflorescence buds even 340 days after sowing (after which time they were discarded). None of the E5, E8, or E9 plants formed visible inflorescence buds in experiment I because the pre-growth period was too short (see chapter 2 for details). These plants were discarded after 237 days from sowing (229 days from sowing for the E9 plants sown on 30th April 2011, see Table 6) and only the E1 plants kept for longer.

Weeks cold-treat-ment	**% plants formed visible inflorescence buds within 100 days with each treatment temperature (°C) (no replicates in brackets)**											
	E1			**E5**			**E8**			**E9**		
	5	**10**	**15**	**5**	**10**	**15**	**5**	**10**	**15**	**5**	**10**	**15**
6	100 (3)	100 (3)	0* (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)
10	100 (2)	100 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	n/a	n/a	n/a
NV	0 (3)**			0 (3)			0 (4)			0 (2)		

Table 8: The percentage of plants of the PSB genotypes E1, E5, E8, and E9 that formed visible inflorescence buds within 100 days after 6 and 10 weeks of cold-treatment at 5, 10, and 15°C with 50 days pre-growth (experiment I). *All of the E1 plants treated for 6 weeks at 15°C also formed visible inflorescence buds but not until an average of 185.33 days from the end of cold-treatment. **All of the E1 NV plants also formed visible inflorescence buds after an average of 231.00 days from sowing.

Pre-growth period	Weeks cold-treatment	Percentage flowering (number of replicates in brackets)							
		E1		**E5**		**E8**		**E9**	
		5°C	10°C	5°C	10°C	5°C	10°C	5°C	10°C
50 days	6	100 (5)	100 (4)	0 (5)	0 (5)	0 (5)	20* (5)	0 (3)	0 (3)
	10	100 (5)	100 (5)	80 (5)	80 (5)	60 (5)	80 (5)	0 (3)	n/a
60 days	6	100 (3)	100 (4)	0 (5)	0 (5)	0 (5)	80 (5)	0 (3)	0 (3)
	10	100 (5)	100 (4)	100 (5)	100 (5)	80 (5)	80 (5)	67 (3)	0 (3)
70 days	6	100 (4)	100 (5)	0 (5)	0 (5)	40 (5)	60* (5)	0 (3)	0 (3)
	10	100 (5)	100 (5)	100 (5)	100 (5)	100 (5)	100 (5)	100 (3)	0 (3)
	NV	0 (10)		0 (10)		0 (10)		0 (5)	

Table 9: The percentage of plants of the PSB genotypes E1, E5, E8, and E9 that formed visible inflorescence buds within 100 days after 6 and 10 weeks of cold-treatment at 5 and 10°C with different pre-growth periods (experiment II). *Two E8 plants treated for 6 weeks at 10°C after 70 days pre-growth and one given this treatment after 50 days pre-growth also formed visible inflorescence buds 120–150 days after the end of cold treatment (not included in the percentage flowering here).

3.3.3 6–10 weeks cold-treatment is sufficient for vernalization in PSB depending upon the genotype and the pre-growth period

All of the E1 plants cold-treated at 5 or 10°C for either 6 or 10 weeks formed visible inflorescence buds in each experiment, regardless of the pre-growth period (Table 8 and Table 9). Given sufficient pre-growth (see chapter 2 and above) E5 and E8 also formed visible inflorescences with 10 weeks of cold-treatment at 5°C or 10°C (except for some anomalous individuals, discussed below). E9 only formed visible inflorescence buds following 10 weeks of cold-treatment at 5°C, and not 10 weeks at 10°C. E8 also responded to cold-treatment lasting only 6 weeks. But none of the E8

plants treated for 6 weeks at 5°C with 50 and 60 days pre-growth formed visible inflorescence buds. The percentage of E8 plants that formed visible inflorescence buds following 6 and 10 weeks of cold-treatment at 10°C given after 60 days pre-growth was the same. But fewer E8 plants formed visible inflorescence buds following 6 weeks cold-treatment than did following 10 weeks cold-treatment at 5°C with 50 and 70 days pre-growth. Fewer E8 plants formed visible inflorescence buds following 6 weeks cold-treatment at 5°C than did following 10 weeks cold-treatment at 5°C with 70 days pre-growth. None of the E5 and E9 plants given only 6 weeks cold-treatment formed visible inflorescence buds within 100 days of the end of cold-treatment. Some E5 plants treated for 6 weeks did begin to show signs of forming inflorescences (the apex becoming more bulbous and surrounded in many tightly clasped leaves/bracts). But the apexes of these plants did not go on to develop any further towards inflorescence formation even after several more weeks (data not shown). This shows that 6 weeks of cold treatment is sufficient for vernalization in the early-flowering genotype E1 but only just sufficient for vernalization in E8 and not sufficient in E5 and E9.

The effect of pre-growth upon the percentage of E8 plants flowering in experiment II was greater with 6 weeks cold treatment than 10 weeks. This may be because the 10-week treatments were near to saturating, so could induce flowering even in many of the plants that were still in the transition to the adult phase and not fully competent to respond to cold treatment. The 6-week cold-treatment may have only been sufficient to induce flowering in the most sensitive plants i.e. those that had completed the vegetative phase change and were fully competent to respond to cold-treatment.

3.3.4 Presentation of flowering-time data and treatment of plants that did not form visible inflorescence buds

The mean number of days from sowing to the first appearance of visible inflorescence buds at the apex (DTI) for the E1 plants in experiment I is shown in Figure 8. The mean number of days from the end of cold-treatment to the first appearance of visible inflorescence buds at the apex (DTI) for E1, E5, E8, and E9 in experiment II is shown in Figure 9.

Some E1 plants in experiment II that were treated for 10 weeks at 10°C formed visible inflorescence buds whilst still in cold-treatment. These were given a value of 1 DTI in figure 9 and in all statistical analyses.

Plants that did not form visible inflorescence buds within 100d of the end of cold treatment (henceforth referred to as "DNFs" for "did not flower") were either excluded from the data set or given an arbitrary value of 100 DTI in Figure 8 and Figure 9 and in statistical analyses. The full scoring data and details of how all DNFs were treated are given in Appendix 2.

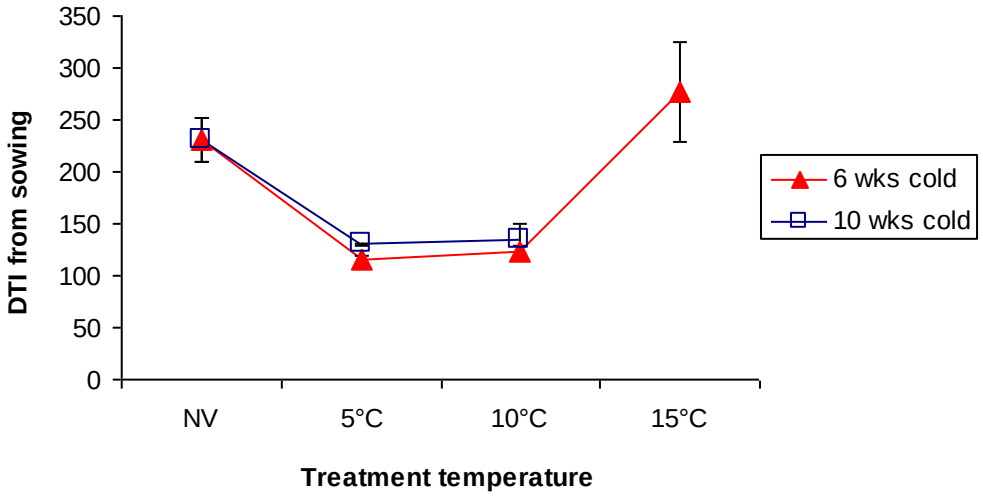


Figure 8: The average number of days taken from sowing to form visible inflorescence buds (DTI) by the PSB genotype E1 with different cold-treatments given after 50 days pre-growth (experiment I). There were three plants for each of the 6-week treatments, two plants for each of the 10-week treatments, and three "NV" plants (kept in the glasshouse throughout the experiment). The error bars are 95% confidence intervals.

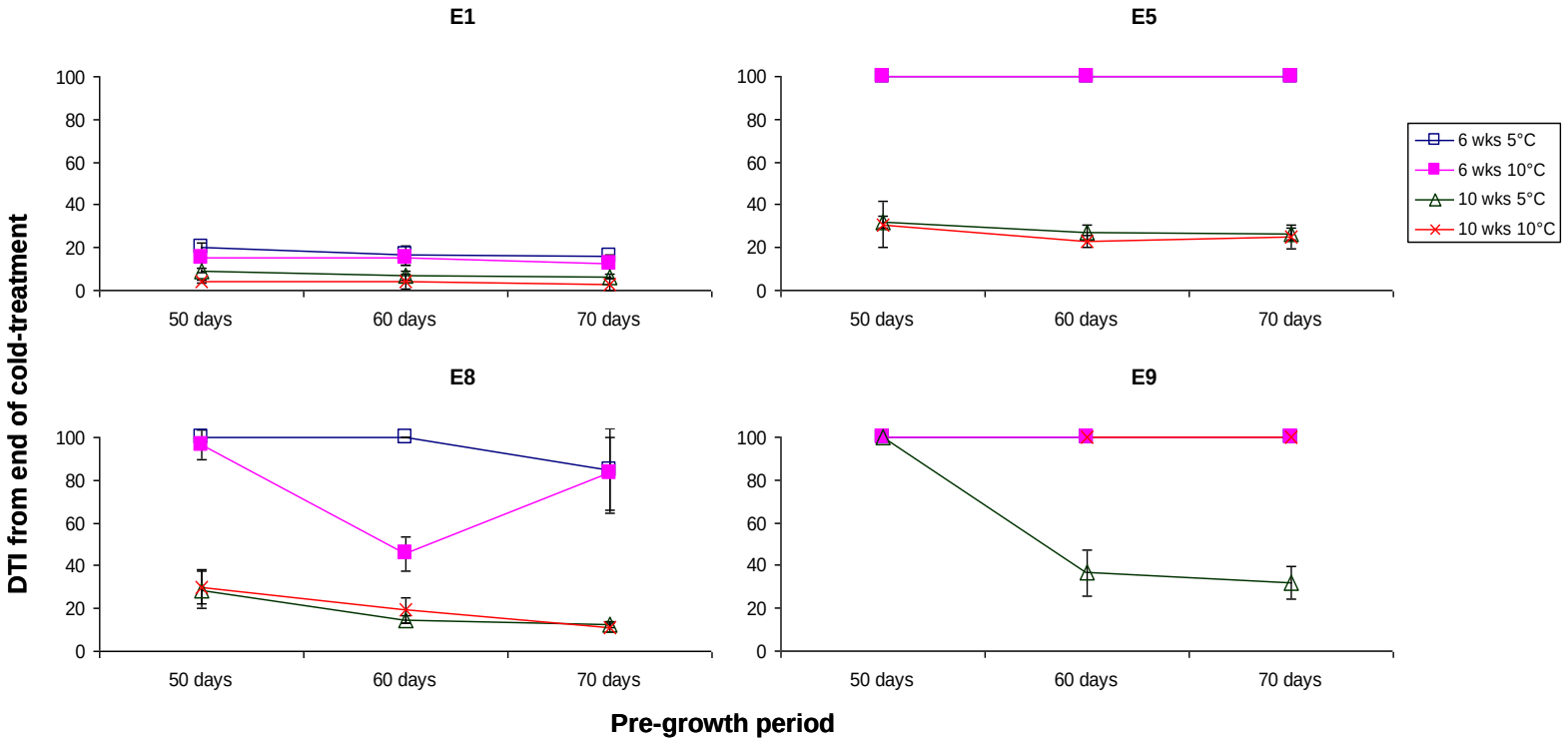


Figure 9: The average number of days taken to form visible inflorescence buds (DTI) by each of the four PSB genotypes E1, E5, E8, and E9 following cold-treatment at 5 and 10°C for 6 and 10 weeks with different pre-growth periods (experiment II). Plants that formed visible inflorescence buds whilst still in cold-treatment were given a value of 1 DTI. Plants that did not form visible inflorescence buds within 100 days of the end of cold-treatment were either excluded from the data or were given an arbitrary value of 100 DTI (see text and Appendix 2 for details). The error bars are 95% confidence intervals. There are 2–5 plants of each genotype included in the data for each cold-treatment with each pre-growth period (except for E9, 10-weeks, 10°C where there were no plants).

DNFs were excluded where the pre-growth period appeared to be just on the cusp of the phase change, based upon their leaf number at the end of pre-growth and the data in chapter 2. In these cases the failure to form inflorescences was likely to be due to the pre-growth being slightly insufficient. The three E8 plants that did not form visible inflorescence buds with 100 days of the end of 10 weeks cold-treatment (one at 10°C, two at 5°C) after 50 days pre-growth were excluded because the results of chapter 2 suggest that these plants were still within the phase transition. One E8 and one E9 plant given 10 weeks cold-treatment (at 10 and 5°C respectively) after 60 days pre-growth were also excluded on this basis. These plants had a particularly low leaf number at the end of pre-growth compared to the other plants sown on the same date and, based upon the leaf number and the results of chapter 2, may still have been in the juvenile phase or the vegetative phase change.

DNFs were also excluded if their failure to flower appeared to be anomalous i.e. if all of the other plants of that genotype at that treatment formed inflorescences soon after the end of cold-treatment. This was the case for the two E5 plants cold-treated for 10 weeks after 50 days pre-growth that did not form visible inflorescence buds. Two E8 plants were also excluded from the data set on this basis: the ones that did not form visible inflorescences with 10 weeks cold-treatment at 5°C following 60 days pre-growth and 6 weeks cold-treatment at 10°C with 60 days pre-growth. These plants are therefore not represented in Figure 9 or in the statistical analyses performed in this chapter.

DNFs that appeared not to have formed inflorescences due to the cold-treatment being insufficient for vernalization or the pre-growth period clearly too short to reach the phase change were given an arbitrary value of 100 DTI to allow them to be represented in Figure 9 and included in the statistical analyses. This was done in each case where none of the plants from one genotype given a certain pre-growth period and cold-treatment formed visible inflorescence buds within 100 days of the end of cold-treatment i.e. where the percentage flowering was zero (Table 9). This arbitrary value was also used where only a minority of the other plants formed visible inflorescence buds and/or they were formed very late. This was the case for all of the 6-week cold-treatments of E8 except for 6-weeks at 10°C after 60 days pre-growth.

The arbitrary value of "100" as the DTI for plants that did not form inflorescences was chosen because all of the plants were kept for 100 days from the end of cold-treatment (unless they flowered sooner, in which case they were discarded after flowering). Using this minimal value minimizes any skewing of the data and hence the risk of obtaining "false positive" results in statistical tests. But it must be considered that this is an arbitrary value to represent no visible inflorescence bud formation and is not a quantitative measure of flowering time or vernalization response.

3.3.5 E1 has a facultative vernalization response whereas E5, E8, and E9 have obligate vernalization requirements

In experiment I the average DTI was 97.00–116.33 days greater in the E1 plants grown in the glasshouse under continuous long-days and warm temperatures than for those that were cold-treated for 6 and 10 weeks at 5 or 10°C (Figure 8). (The glasshouse was set to maintain a minimum temperature of 16°C during the "night" and 18°C during the "day"). None of the E5, E8, and E9 plants formed visible inflorescence buds without vernalization in this experiment (Table 8). The E5, E8, and E9 plants were discarded 237 days from sowing (229 days from sowing for the E9 plants sown on 30th April 2011, see Table 6). This was approximately two weeks after the first of the non-vernalized E1 plants formed visible inflorescence buds. So it is not possible to say for certain that none of the E5, E8, or E9 plants would ever have flowered without vernalization. However, none of them showed any signs of forming inflorescences when they were discarded. None of the NV plants in experiment II formed visible inflorescence buds (Table 9). This result was expected as these plants were only kept for approximately 170 days from sowing and the E1 plants in experiment I took an average of 231 days from sowing to form visible inflorescence buds (Figure 8). All of the field experiments that were done with these genotypes prior to this project had included a period of winter. But in another glasshouse experiment carried out at the John Innes Centre, Norwich, E1 flowered without vernalization whereas E5 and E9 did not (Irwin & Soumpourou personal communication). This suggests that E5, E8, and E9 have an obligate requirement for vernalization in order to flower so are "biennials" (Michaels & Amasino 2000) whereas E1 only has a facultative requirement for vernalization.

Arabidopsis accessions from different habitats show similar variation in both qualitative vernalization requirement and quantitative response to vernalization to these PSB genotypes. Shindo et al. (2006) found that some winter-annual accessions such as Edi-0 (from Edinburgh, UK, 56.0 degrees latitude) have a facultative vernalization requirement. That is they flower without vernalization but flower considerably earlier when vernalized. Other accessions such as Lov-1 (from Lovvik, northern Sweden, 62.5 degrees latitude) and Ull-2-5 (from Ullstorp, southern Sweden, 55.3 degrees latitude) have a much stronger vernalization requirement. Lov-1 and Ull-2-5 did not flower within 5 months/120 leaves of sowing unless vernalized. In contrast the un-vernalized Edi-0 plants flowered with approximately 110 leaves. Edi-0 also shows a clear decrease in flowering time following even short periods of cold-treatment (2 weeks) whereas Lov-1 and Ull-2-5 require longer periods of cold-treatment (6 and 4 weeks respectively) to induce flowering (Shindo et al. 2006). This is similar to the variation in vernalization requirement and response seen among the four PSB genotypes in this study. Like Edi-0, E1 did not require vernalization to flower but responded strongly (i.e. all plants flower early) to a relatively short period of cold-treatment (6 weeks). In contrast 6 weeks of cold-treatment did not induce a flowering response in E5 and E9 and only led to a weak response in E8. E5, E8, and E9 all have an obligate vernalization requirement, which was fulfilled by 10 weeks of cold-treatment at an appropriate temperature with sufficient pre-growth (Table 8, Table 9, Figure 8, & Figure 9). This resembles the situation in Lov-1 and Ull-2-5 (Shindo et al. 2006). So the variation in both qualitative vernalization requirement and quantitative vernalization response (i.e. the requirement for long versus short periods of cold) that exists in Arabidopsis accessions from different habits is also found among different genotypes of PSB.

3.3.6 10 weeks cold-treatment is sufficient to saturate the vernalization response in E1 and possibly in E8

Three of the E1 plants that were cold-treated for 10 weeks at 10°C with 70 days pre-growth and one that was cold-treated for 10 weeks at 10°C with 60 days pre-growth formed visible inflorescence buds whilst still in cold-treatment despite the 8-hour photoperiod in the CER (Appendix 2). These plants were given a value of 1 DTI in Figure 9 and in all calculations and statistical analyses. This shows that these cold-

treatments had saturated the vernalization response of these plants as flowering had been initiated during cold-treatment. The other E1 plants from these treatments formed visible inflorescence buds within 9 days of transfer back to the glasshouse (Appendix 2). It also quite possible that the 10-week-5°C treatment was saturating in E1, but that the plants took longer to form visible inflorescences because of the lower temperature during cold-treatment reducing the rate of development of the inflorescence after the initiation of flowering. The vernalization responses of E5 and E8 may also have been saturated in one or both of the 10-week treatments with 70 days pre-growth, as all plants formed visible inflorescence buds within 34 and 14 days (respectively) of the end of cold treatment (Figure 9 & Appendix 2). But it is not possible to say for certain whether these treatments were saturating as it is possible that flowering would occur even earlier with a longer cold-treatment. E5 and E8 may have flowered later than E1 with the 70-day-10-weeks cold-treatments due to either less complete (non-saturated) vernalization or simply taking longer to develop visible inflorescence buds after the initiation of flowering had occurred.

Vernalization is defined as the "acquisition or acceleration" of competence to flower by cold-treatment (Chouard 1960). Therefore, the cold-treatment makes a plant competent to flower but does not actually initiate flowering. Flowering can then occur later, under inductive conditions (Chouard 1960; Michaels & Amasino 2000). For example in *Sinapis alba* (Brassicaceae) relatively short periods of cold only promote flowering if they are immediately followed by long days (D'Aloia et al. 2008). The fact that four E1 plants formed inflorescences whilst still in the 10°C CER under 8-hour photoperiods shows that E1 does not require long days to induce flowering following vernalization. This is unsurprising because E1 is usually sown in April (in the UK) and planted out into the field in the summer to head in November (Irwin personal communication). Other cues (such as internal developmental cues) may act to induce flowering after vernalization in E1.

It is not possible to establish whether flowering could be induced by vernalization in the absence of subsequent long days in E5, E8, and E9 because none of the plants from these genotypes formed visible inflorescence buds until several days after transfer back to the glasshouse (with long photoperiods of at least16 hours, Figure 9 & Appendix 2). If any of these genotypes do have an obligate requirement for long

days to induce flowering after vernalization then they would never initiate flowers before the end of cold-treatment under short-photoperiods. This seems unlikely though because Brassicas are facultative long-day plants (Axelsson et al. 2001). E5, E8, and E9 also generally head in March–May in the field (Irwin, personal communication). Several winter Brassica crops (e.g. winter cauliflower) are also cropped in late winter/early spring when day-length is still fairly short, though longer than the 8-hour photoperiods used during cold-treatment in this study (Elsoms Vegetable Seed Catalogue 2011/2012).

3.3.7 The maximum temperature for vernalization of Purple Sprouting Broccoli varies among genotypes

The E1 plants that were cold-treatment at 15°C for 6 weeks formed visible inflorescence buds on average 46.33 days later than the NV control plants that were kept in the glasshouse throughout the experiment (Table 8 & Figure 8). The E1 plants that were cold-treated at 15°C for 10 weeks did not form visible inflorescence buds at all, even after 340 days from sowing (119 days after the average time at which the NV plants formed visible inflorescence buds). This shows that the T_{MAX} for vernalization in E1 is below 15°C, but it is above 10°C, as all plants formed visible inflorescence buds within 100 days after cold-treatment at 10°C for 6 and 10 weeks (Table 8 & Table 9). This would mean that the plants grown at 15°C received no vernalizing degree days (V°C D) or effective vernalization days (VD), because the temperature was above the T_{MAX} for vernalization. So their flowering was not accelerated by vernalization compared to the NV control plants. While in the 15°C CERs these plants would also have received fewer growing degree days (GDD, see chapter 1) than the NV plants because the glasshouse was warmer than 15°C. (The glasshouse was set to maintain a minimum temperature of 16°C during the "night" and 18°C during the "day"). Low ambient temperatures delay flowering in Arabidopsis e.g. Arabidopsis flowers later when grown at 16°C that at 23°C (Blazquez et al. 2003; Thingnaes et al. 2003; Samach & Wigge 2005). The plants in the 15°C CER also received fewer hours of light during the cold treatment (because the CERs had only an 8-hour photoperiod). The light in the CERs (artificial light only) is also likely to have been less intense than the light in the glasshouse during daylight hours (which was also supplemented with 16 hours artificial light). These

less favourable conditions in the 15°C CER could account for the delay in forming inflorescence buds compared to NV, despite both treatments providing no vernalization.

The delay in the formation of visible inflorescence buds compared to NV caused by treating E1 plants for 6 weeks at 15°C in short-day CERs was approximately equivalent to the length of the cold-treatment (on average 46.33 days). This suggests that the plants did not progress towards flowering at all whilst in the CER. But growth was still occurring during this period as these plants produced on average 8 more leaves during the 6-weeks that they were in the CER (data not shown). On this basis, I would not have expected such a long delay in forming visible inflorescence buds. The stress of transfer to and from the CER may have further retarded development towards flowering. The NV plants were re-potted into 2-litre pots 25 days into the cold-treatment period. But the cold-treated plants were not re-potted until after they were returned to the glasshouse. However, the NV E1 plants had on average 19.3 leaves before they were re-potted, whereas the E1 plants treated at 15°C for 6 weeks had on average 18.0 leaves at the end of cold-treatment. Therefore these two sets of plants had a similar number of leaves when re-potted and so this factor should not have had much effect upon development.

The plants treated at 15°C for 10 weeks did not form inflorescences even 100 days after the NV plants. Even a complete suspension of their development during the 10 weeks in the CER could not account for this. However, these plants remained in 0.5 litre pots for even longer than those treated at 15°C for 6-weeks (until after the end of the 10 weeks cold-treatment). They had on average 24.5 leaves by the end of cold-treatment. This is more than either the NV plants or those treated at 15°C for 6 weeks. The NV and 6-week-15°C treated plants formed inflorescences on average on the 10th June and 26th July respectively, when natural day-length was at around its greatest. This is likely to have promoted flowering as Brassicas are facultative long-day plants (Axelsson et al. 2001). The 6-week 15°C treatment delayed visible inflorescence bud formation by approximately 6 weeks. So I would not have expected the plants treated at 15°C for 10 weeks to form visible inflorescence buds until 4 weeks later still. The day length would have declined by this time. When grown in the field E1 generally flowers in November (under much shorter days).

But in the absence of vernalization long days may be needed in order to promote flowering. The plants were also extremely large by this point, but still in 2-litre pots. And the temperature in the glasshouse is likely to have been high. Hence these plants were likely to be experiencing considerable stress (in spite of regular feeding and watering) which could have affected their flowering behaviour.

E9 plants formed visible inflorescences when treated at 5°C for 10 weeks, with 60 and 70 days pre-growth, but never when treated at 10°C (Table 8, Table 9, & Figure 9). All of the E1, E5, and E8 plants treated for 10 weeks of cold at either 5 or 10°C with 70 days pre-growth formed visible inflorescence buds within 100 days of the end of cold-treatment. All of the E1 and E5 and 80% of the E8 plants given the same cold-treatments after 60 days pre-growth also formed visible inflorescence buds within 100 days of the end of cold-treatment (Table 9). This suggests that T_{MAX} is between 5 and 10°C in E9 but is greater than 10°C in E1, E5, and E8. As discussed above, the results of experiment I show that T_{MAX} in E1 is between 10 and 15°C. However, there was no 15°C treatment in experiment II and the pre-growth period in experiment I was insufficient for E5 and E8 to become competent to respond to vernalization (see chapter 2). So it is not possible to determine T_{MAX} in E5 and E8 definitively.

In a subsequent QTL experiment carried out at the John Innes Centre E9 flowered following cold-treatment at both 5 and 10°C (under 8-hour photoperiods) (Irwin & Soumpourou, personal communication). The difference in the response of E9 to cold treatment at 10°C in these two experiments may be due to differences in the light spectra of the CERs used. The 5°C cold room used in experiments I and II in this study and the 10°C CER used in the QTL experiment had similar spectra. The Gallenkamp CER used for the 10°C treatment in experiment II had a different spectrum of light even though the total light levels were the same. The plants in the QTL experiment were also potted out into 5-litre pots at the end of cold-treatment. The plants in experiment II were kept in 1-litre pots until 47 days after the end of cold-treatment then transferred to 2-litre pots. This could have caused "pot-stress" in the experiment II plants, which may have affected flowering time.

3.3.8 There is significant variation in the quantitative vernalization response to both the temperature and duration of cold-treatment among PSB genotypes

In chapter 2 I showed that the timing of inflorescence formation following vernalization in PSB depends strongly upon the genotype, and is also affected significantly by the pre-growth period and the interaction of the genotype and the pre-growth period (within the range of genotypes and pre-growth periods used in experiment II). The results presented above show that the time taken to form inflorescence buds also varies with the temperature and duration of cold-treatment. The effects of these factors also appear to depend upon the genotype and the pre-growth period (Table 9 & Figure 9). I performed ANOVAs (using Genstat) to test for the significance of the effects of these factors and the interactions among them upon DTI in experiment II. The outputs of these ANOVAs are given in full in Appendix 5.

In chapter 2 I showed that the juvenile phase ends by approximately 10–11 leaves in E1, E5, and E8 and 15 leaves in E9. In experiment I only the E1 plants were competent to respond to vernalization. In Experiment II the E1 plants were competent to respond to vernalization with all three pre-growth periods but the E9 plants were not competent to respond to vernalization with only 50 days pre-growth. With 60 days pre-growth in E9 and 50 and 60 days pre-growth in E5 and E8 some plants may also have been within the vegetative phase change and not fully competent to respond to vernalization. Therefore, there may be a confounding effect of juvenility when plants given different pre-growth periods are analysed together.

I carried out ANOVAs for the effects of genotype, temperature of cold-treatment, duration of cold-treatment, genotype*temperature, and temperature*duration of cold-treatment. This has the benefit of drawing data from as many plants as possible. E.g. for an ANOVA for the effects of temperature upon DTI in one genotype with 10-weeks cold-treatment there are 15 plants treated at 5°C and 15 treated at 10°C that are compared (5 at each temperature for each pre-growth period). This also allows the investigation of the possibility that the effects of temperature, genotype, and duration of cold-treatment vary with pre-growth period. However, it is important to consider the possible confounding effects of juvenility. In particular, a

significant difference in DTI in E8 and E9 between 50 and 60 days pre-growth was found in chapter 2. For this reason I have excluded some individuals that did not flower apparently because they were still in the juvenile-adult phase change from the statistical analysis. I have also carried out some analyses separately for different pre-growth periods and with E9 (for which the effect of pre-growth identified in chapter 2 appeared to be greatest) excluded to eliminate the significant effect of pre-growth upon DTI between 50 and 60 days pre-growth.

ANOVA relies upon the assumptions that the data are approximately normally distributed and the variances within each group are homogenous. Due to the low number of replicates in each treatment tests for normality or homogeneity of the variances were unreliable. There were also several cases where not all of the plants in a treatment flowered. In these cases any calculation of the variance would be based upon arbitrary assumptions. However, apparent variation in the heterogeneity of response was observed among the different genotypes, pre-growth periods and treatments (Figure 9 & Appendix 2). When the treatment was only barely sufficient for vernalization (e.g. with 50 days pre-growth period and/or 6 weeks cold-treatment in E8) the DTI appears to have been more variable than when the treatment was at or near to saturating (see the full data in Appendix 2). Therefore caution must be exercised in interpreting the outputs of these tests.

It is not practical to compare the data from all treatments of all genotypes together due to the large number of plants that did not form visible inflorescence buds (DNFs). The DNFs were excluded from the data or given an arbitrary value of 100 DTI as described above. This means that every combination of treatment and genotype for which no plants formed inflorescences had a mean DTI of 100 and a standard error of 0. This will affect the results of statistical tests. The estimations of significance may be affected because the average standard error of the data is reduced. Assigning all DNFs the same arbitrary value of 100 DTI may also reduce the apparent significance of the differences between treatments and genotypes where there were plants that did not flower. For example all of the E5 and E9 plants treated at 5 and 10°C for 6 weeks were given an identical arbitrary value of 100 DTI. This would affect the estimation of the significance of the differences in DTI that were seen between these two treatment temperatures in E1 and with 10 weeks cold-

treatment in E9 (Table 9 & Figure 9). The 100 DTI assigned to plants that did not flower is also an arbitrary quantitative measure of flowering time. Therefore caution must be exercised in making any quantitative conclusions from the statistical analysis. However, this is the minimum DTI value that could be assigned to the DNFs so the likelihood of a "false-positive" significant difference occurring is minimized. Therefore although it is reasonable to draw qualitative conclusions from ANOVAs performed on data including these arbitrary values (e.g. whether an effect is significant or not significant) it is not possible to draw firm quantitative conclusions (precisely how significant or how large an effect is) where DNFs are involved.

Because the effect of the duration of cold-treatment depends upon the genotype (Table 9 & Figure 9) I performed ANOVAs for the effects of genotype, temperature of cold treatment, and the interaction effect of genotype*temperature separately for the 10-week and 6-week treated plants. The P-values obtained are shown in Table 10. The effects of genotype, temperature, and the interaction of genotype*temperature were all significant with both durations of cold-treatment. So the DTI varies significantly among the genotypes, the temperature of the cold-treatment has a significant effect upon DTI, and this effect varies significantly among the genotypes (although it is possible that there is a confounding effect of juvenility).

I also performed ANOVAs for the effects of genotype, temperature, and genotype*temperature upon DTI for the data from each pre-growth period and duration of cold-treatment separately. The P values obtained are shown in Table 10. Because all plants pre-grown for 70 days had completed the juvenile-adult phase change (chapter 2) it can be assumed that there is no confounding effect of juvenility in the ANOVAs carried out on these plants. However, among the plants pre-grown for 50 days there will be a confounding effect of juvenility because at least the E9 plants did not flower due to the pre-growth period being too short (chapter 2) regardless of the treatment temperature, and so were all assigned an arbitrary value of 100 DTI. The effect of the genotype remained significant in each case ($p<0.001$). With 10-weeks cold treatment the effect of temperature and the interaction of genotype*temperature were significant with 60 and 70 days pre-growth ($p<0.001$)

but neither was significant with 50 days pre-growth ($p>=0.05$). At 50 days there was no difference in the DTI for E9 with 5°C and 10°C (both given arbitrary values of 100 DTI) but at 60 and 70 days the E9 plants that were treated at 5°C formed visible inflorescences whereas those treated at 10°C did not. Therefore the temperature may have a significant effect in E9 with 60 and 70 days pre-growth but not in the other genotypes and not in E9 with 50 days pre-growth. I repeated these ANOVAs for the 10-week cold-treatments with the E9 data excluded. The P values obtained are shown in Table 10. The effect of the genotype upon DTI was still significant ($p<0.001$) at each pre-growth period (among E1, E5, and E8 only). But the effect of temperature was not significant ($p>0.05$) at any of the pre-growth periods. The interaction effect of genotype*temperature was not significant with 50 or 70 days pre-growth ($p>0.05$) and was only just significant with 60 days pre-growth ($p=0.040$). Therefore the main significant effect of temperature and the interaction of genotype*temperature appears to be that E9 responds to 10 weeks of cold-treatment at 5°C but not at 10°C, whereas E1, E5, and E8 respond strongly at both temperatures. With 6-weeks cold treatment the effect of temperature and the interaction of genotype*temperature was only significant with 60 days pre-growth. This is likely to be due to the fact that four of the five E8 plants cold-treated for 6 weeks at 10°C with 60 days pre-growth formed visible inflorescences within 100 days, compared to none of those treated for 6 weeks at 5°C (Table 9 & Figure 9). As a result, all of the E8 plants cold-treated at 5°C for 6 weeks with 60 days pre-growth were given an arbitrary value of 100 DTI (Appendix 2).

Treatment durations included (weeks)	Pre-growth periods included (days)	Genotypes included	P values for effects (ANOVA)		
			Genotype	Temp	Genotype*Temp
10	All	All	<0.001	0.037	<0.001
10	50	All	<0.001	0.468	0.475
10	60	All	<0.001	<0.001	<0.001
10	70	All	<0.001	<0.001	<0.001
10	50	E1, E5, E8	<0.001	0.491	0.509
10	60	E1, E5, E8	<0.001	0.625	0.040
10	70	E1, E5, E8	<0.001	0.119	0.657
6	All	All	<0.001	0.006	0.006
6	50	All	<0.001	0.066	0.363
6	60	All	<0.001	<0.001	<0.001
6	70	All	<0.001	0.737	0.988

Table 10: The P values obtained from ANOVAs for the effects of genotype, temperature of cold-treatment, and genotype*temperature upon DTI among the four PSB genotypes (E1, E5, E8, and E9) in experiment II. Plants were cold-treated for 6 and 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth in a warm glasshouse. The DTI (days until the first appearance of visible inflorescence buds at the apex) was measured from the end of cold-treatment. Although the exact p values are displayed, these should not be regarded as quantitative measures of significance because they are calculated based upon data that includes the plants that did not form visible inflorescence buds (DNFs). These plants were either excluded or given an arbitrary value of 100 DTI (see text and Appendix 2), neither of which provides a quantitative representation of flowering time. The full ANOVA tables are given in Appendix 5.

To minimise the confounding effects of any non-homogeneity of variances among the genotypes and treatments and of the cases where no plants flowered I also performed ANOVAs separately for the different genotypes. In each genotype I performed an ANOVA for the effects of the temperature of cold-treatment, the duration of cold-treatment, and the interaction of temperature*duration of cold-treatment upon DTI for the whole set of data from both the 6 and 10-week-cold-treatments. I then performed separate ANOVAs for the effect of temperature only upon DTI within just the 10-week treated and (where applicable) just the 6-week treated plants of each genotype. The P values obtained are shown in Table 11.

Genotype	Treatment durations included (weeks)	P values for effects (ANOVA)		
		Temp	duration	Temp*duration
E1	6 & 10	<0.001	<0.001	0.780
	6	0.006	n/a	n/a
	10	<0.001	n/a	n/a
E5	6 & 10	0.297	<0.001	0.297
	6	n/a	n/a	Na
	10	0.311	n/a	n/a
E8	6 & 10	0.064	<0.001	0.018
	6	0.022	n/a	n/a
	10	0.484	n/a	n/a
E9	6 & 10	0.001	<0.001	0.002
	6	n/a	n/a	n/a`
	10	0.012	n/a	n/a

Table 11: The P values obtained from ANOVAs for the effect of temperature of cold-treatment, the duration of cold treatment, and the interaction of temperature*duration of cold treatment upon DTI in each of the PSB genotypes, and for the effect of temperature upon DTI within each genotype for each duration of cold-treatment in experiment II. Plants were cold-treated for 6 and 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth in a warm glasshouse. The DTI (days until the first appearance of visible inflorescence buds at the apex) was measured from the end of cold-treatment. Although the exact p values are displayed, these should not be regarded as quantitative measures of significance in E5, E8, and E9 because they are calculated based upon data that includes plants that did not form visible inflorescence buds (DNFs). These plants were either excluded or given an arbitrary value (see text and Appendix 2), neither of which provides a quantitative representation of flowering time. The P values can be regarded as quantitative in E1 as there were no DNFs for this genotype. The full ANOVA tables are given in Appendix 5.

The effect of the duration of cold-treatment upon DTI was significant ($P<0.001$) in each genotype (Table 11). The effect of the temperature of the cold-treatment was significant in E1 and E9 ($P<0.001$) but not significant in E8 or E5 ($P>0.05$). There was also a significant interaction effect of temperature*duration of cold-treatment in E8 ($P<0.05$) and E9 ($P<0.05$) but not in E1 or E5. The significant interaction effect

of temperature*duration in E9 should be ignored because all of the plants given 6 weeks cold-treatment were assigned an arbitrary value of 100 DTI. (Therefore this apparent significant interaction effect is due to the fact that there was a difference in DTI among the plants treated for 10 weeks (when some of those treated at 5°C formed visible inflorescence buds) but not among those treated for 6 weeks (when none did) because all DNFs were given the same arbitrary value regardless of temperature). When the data for each genotype were split by the duration of cold-treatment there was a significant effect of temperature upon DTI in E1 with both 6 and 10 weeks cold ($P<0.05$ and $P<0.001$ respectively), in E8 with 6 weeks cold ($P<0.05$), and in E9 with 10 weeks cold ($P<0.05$). As expected from the previous ANOVAs there were no significant effects of temperature in E5 with 10 weeks cold or in E8 with 10 weeks cold. (None of the E5 or E9 plants formed inflorescences with the 6-week treatments so these data were not tested).

This suggests that 10°C is a significantly more effective temperature for vernalization in E1 and may also be in E8. But the difference in effectiveness between 5 and 10°C in E8 is only apparent when the treatment is far from saturating (e.g. 6 weeks of cold-treatment). 10 weeks of cold-treatment may saturate the vernalization response at either temperature, masking the difference in their effectiveness. There was no significant difference in the response of E5 to cold treatment at both 5 and 10 °C and E9 only responded to cold-treatment at 5°C.

I also performed individual t-tests for the difference in DTI between the E1 plants treated at 5 and 10°C at each pre-growth period and treatment duration. The results of these tests are shown in Table 12. The E1 plants treated at 10°C formed inflorescences significantly earlier than those treated at 5°C with both 6 and 10 weeks cold-treatment after 50 days pre-growth and with 6 weeks cold-treatment after 70 days pre-growth ($P<0.05$). The difference between the plants treated for 10 weeks after 70 days pre-growth was not quite significant ($p = 0.060$). But this is likely to be an underestimate of the significance of this difference because the three plants that formed visible inflorescence buds whilst in cold-treatment at 10°C were given a value of 1 DTI. There was no significant difference between the two treatment temperatures when either 6 or 10 weeks cold-treatment were given after 60

days pre-growth. In each case the Levene's test confirmed the null hypothesis that the variances were approximately homogenous (p>=0.05).

Genotype, duration of cold-treatment, and pre-growth period	P values (for DTI with 5°C vs 10°C cold-treatment)	
	Levene's test of Variances	T-test of difference between means
E1, 6 wks, 50 days	0.69	0.025
E1, 6 wks, 60 days	0.59	0.632
E1, 6 wks, 70 days	0.57	0.017
E1, 10 wks, 50 days	0.41	<0.001
E1, 10 wks, 60 days	0.36	0.186
E1, 10 wks, 70 days	0.05	0.060

Table 12: The results of t-tests for the significance of the differences in DTI between the E1 plants cold-treated at 5 and 10°C for 6 and 10 weeks with different pre-growth periods in experiment II.

For the ANOVA performed upon the entire set of data for E1 I also calculated the expected means squares (EMS) for the effects of the temperature of cold-treatment, the duration of cold-treatment, and the interaction of temperature*duration of cold-treatment and used these to calculate the proportion of variation accounted for by these effects as described in Appendix 3. (I did not do this quantitative analysis of the effect sizes in E5, E8, or E9 because there were missing and arbitrarily assigned DTI values in these genotypes so quantitative analyses should be avoided). The effect of the duration of cold-treatment accounted for 82% of the variation in DTI. The effect of temperature only accounted for 8% of the variation in DTI. The residual error accounted for 10% of the variation. (The non-significant interaction of temperature*duration of cold-treatment did not account for any of the variation in DTI). So in E1 the main factor affecting flowering time (measured by DTI) was the duration (6 versus 10 weeks) rather than the temperature (between 5 and 10°C) of cold-treatment although both effects were significant.

These analyses and the results of chapter 2 suggest that the main factors affecting the timing of inflorescence formation in this study were the pre-growth period, the genotype, the duration of cold-treatment, and the interaction of the genotype*pre-growth genotype*duration of cold-treatment. The temperature of cold-treatment (5°C versus 10°C) also had a significant effect in some cases. However, the effect of the temperature appears to have generally been small compared to the effect of the

treatment duration. This suggests that the optimum temperature for vernalization is unlikely to be sharply defined. A graph of vernalization rate against temperature would probably have a plateau (as shown in Wurr et al. (1993) fig 2 a & d) or smooth curve (as shown in Streck (2003), Streck et al. (2002; 2003), and Streck & Schuh (2005)) at around 5–10°C rather than a sharp peak at Topt (as shown in Wurr et al. (1993) fig 2 b, c, & e and in Atherton et al. (1990) & Craigon et al. (1990)). This makes sense because the plant development rate responds to temperature in a non-linear manner (Shaykewich 1995). However, it is also possible that the lack of any significant effect of 5 versus 10°C in some cases is due to the fact that these temperatures are actually either side of the optimum. If the optimum temperature for vernalization in E5 was 7.5°C then even if there was a sharp decline in vernalization rate either side of this temperature there may not be any difference in vernalization rate between 5 and 10°C.

The E1 plants treated at 5°C for 6 and 10 weeks in experiment I formed visible inflorescence buds slightly sooner on average than those treated at 10°C but the difference was farily small compared to the 95% confidence intervals (Figure 8 & Appendix 1). In experiment I there were only 3 plants for each 6-week treatment and 2 for each 10-week treatment. In experiment II there were 3–5 replicates at each of the three pre-growth periods with both 6-and 10-weeks cold treatment and in each case the 10°C-treated plants formed visible inflorescence buds sooner (on average) than the 5°C plants (Figure 9). This difference was significant or nearly so at two of the three pre-growth periods with each of the durations of cold-treatment (Table 12). Therefore it is likely that 10°C is actually more effective at accelerating inflorescence formation in E1, but this effect is only small.

3.3.9 The measurement of vernalization response may be confounded by variation in the post-vernalization conditions and continued vegetative development during cold-treatment

The number of days taken to form visible inflorescence buds (DTI) depends upon both the timing of the initiation of flowering (which in turn depends upon vernalization) and the rate of inflorescence development following initiation. All of the plants in experiment II were grown in the same glasshouse after vernalization to minimize the confounding effect of post-vernalization conditions. But there will still

have been some differences in conditions between the benches. (The plants were split between benches based upon pre-growth period to minimize shading caused by differing plant size, but were randomly arranged within each bench). The light and possibly temperature in the glasshouse also varied throughout the experiment due to external conditions. Artificial lighting (16 hours per day) and heating were used to minimize the effects of external conditions. But in general the light intensity and temperature in the glasshouse are likely to have declined with time after the end of cold-treatment (on 25th September). This may have retarded inflorescence development among the plants that initiated flowering later. To minimize this effect I used the DTI as a measure of flowering time rather than the DTB or DTF. The formation of visible inflorescence buds was the first stage at which a plant could be seen to have committed to flowering. So the DTI should be less affected by the rate of inflorescence development than the DTB and DTF. Most of the plants cold-treated for 10 weeks in experiment II that formed visible inflorescence buds did so within 50 days of the end of cold-treatment, over which time the conditions are not likely to have varied too greatly.

As shown in experiment I, the temperature of a cold-treatment affects both the number of vernalizing degree days (V°CD)/effective vernalization days (VD) and growing degree days (GDD) received by a plant. Plants treated at 10°C may receive fewer or a similar number of V°CD/VD than those treated at 5°C (if T_{OPT} is close to 5°C) but could still form inflorescences sooner due to receiving more GDD (resulting in greater vegetative development occurring during cold-treatment). In the E1 plants cold-treated at 10°C for 10 weeks with 70 days pre-growth inflorescence formation was clearly initiated before the end of cold-treatment (as the plants had formed visible inflorescences already when transferred to the glasshouse). But if plants treated at both 5 and 10°C initiated flowering at the same time, those at 10°C would be expected to form visible inflorescence buds sooner due to the higher temperature allowing faster inflorescence development. The E1 plants at 10°C may have also been progressing towards flowering independently of the vernalization response more rapidly than those at 5°C because they were receiving more GDD. (As shown in experiment I and discussed above, low temperatures delay progress towards flowering in E1 when there is no effect of vernalization). This could affect the timing of inflorescence formation independently of the vernalization response.

Therefore, it is not possible to determine conclusively whether 10°C was a more effective temperature for vernalization *per se* than 5°C in E1 (or in any case where inflorescence formation occurs soon after the end of cold-treatment or the genotype involved can flower without vernalization).

Brooking (1996) & Brooking & Jamieson (2002) suggested that in wheat saturation of the vernalization response could be defined as occurring when the total number of leaves and primordia is the same as the final leaf number. This definition could potentially be applied to PSB, using the number of vegetative nodes rather than leaves. The strength of the response to vernalization would be inversely correlated with the number of vegetative nodes appearing between the end of cold-treatment and inflorescence formation. In the case of E1 in experiment II, plants that formed inflorescences whilst still in cold-treatment would not form any more vegetative nodes after transfer to warm conditions. Plants in which flowering had been initiated during cold-treatment may form a few more vegetative nodes that were initiated before the transition of the apex. Plants that had not initiated flowering whilst in cold-treatment would continue forming vegetative nodes once transferred to warm conditions until flowering was initiated. However, there are three problems to using this approach to measure vernalization response in PSB. Firstly, it is difficult to distinguish nodes formed in the vegetative phase from those formed in the reproductive phase (i.e. after the initiation of flowering), as the transition from leaves to bracts is gradual. Secondly, the final number of nodes could only be used to compare the response to different vernalization treatments within a genotype rather than among the genotypes because the different genotypes produce leaves/nodes at different rates (discussed in chapter 2). Thirdly, if plants are cold-treated under short days but require long-days following vernalization to initiate flowering then they could not initiate flowering (so would continue forming vegetative nodes) until returned to the glasshouse (with long-days) regardless of whether the vernalization response was saturated. At present it seems that the most practical and reliable means of measuring vernalization response in PSB is by the time taken to form visible inflorescence buds (the DTI).

3.3.10 Cardinal temperatures for vernalization in PSB are lower than those estimated in Calabrese and Cauliflower

The T_{MAX} for vernalization is considerably lower in E1 (10–15°C) and E9 (5–10°C) than the T_{MAX} of 20°C in cauliflower and 23.6°C in Calabrese (and similar to the T_{OPT} of 15.8°C in calabrese) reported by Wurr et al. (1993; 1995) and the T_{OPT} of up to 15.8°C reported in some studies of cauliflower (Pearson et al. 1994; Wheeler et al. 1995). The fact the 15°C treatment delayed flowering compared to non-vernalized plants provides strong evidence that T_{MAX} in E1 is below 15°C. The strong responses of E1, E5, and E8 to cold-treatment at 10°C are in agreement with the optimum temperature for vernalization of 9–9.5°C estimated in cauliflower by Wurr et al. (Wurr et al. 1993). However, the PSB genotypes used in this experiment were also vernalized effectively by 5°C, and E9 only responded to cold-treatment at this temperature. This is lower than the T_{MIN} of 9°C estimated in cauliflower by Wurr et al. (1993). But this T_{MIN} for cauliflower may have been an overestimate due to the conditions during the field experiments in that study, which made it unlikely that lower cardinal temperatures would have been estimated (Wurr et al. 1993). The fact that the related crops calabrese (Wurr et al. 1995), brussels sprouts (Thomas 1980), swede (Gowers & Gemmell 1988), chinese cabbage (Elers & Wiebe 1984), and Purple Sprouting Broccoli (this study) are all vernalized effectively at temperatures under 9°C suggests that this relatively high value for T_{MIN} may indeed reflect the conditions in the field in that study rather than the actual T_{MIN} of cauliflower.

3.4 Conclusions/summary

The results of this study suggest that genotypes of Purple Sprouting Broccoli vary in both their qualitative requirement for vernalization and qualitative response to vernalization, as do Arabidopsis accessions. The optimum temperature for vernalization in PSB of around 5–10°C (depending upon genotype) suggested by this study is similar to optimum temperatures for vernalization reported in Brussel Sprouts and Cauliflower (Thomas 1980; Wurr et al. 1993; Guo et al. 2004). However, the maximum effective temperature for vernalization in PSB of under 10–15°C (depending upon the genotype) is considerably lower than those reported in cauliflower (Wurr et al. 1993) and calabrese (Wurr et al. 1995). Further work would be necessary to establish the minimum temperature for vernalization in each

genotype and the maximum in E5 and E8 and to more precisely define the cardinal temperatures and minimum duration of cold for vernalization in all of these genotypes. However, for future vernalization experiments it is reasonable to conclude that cold-treatment at 5°C for 10-weeks under short days is an effective vernalization treatment in all of these genotypes provided that an adequate pre-growth period is provided for the plants to complete the vegetative phase change.

4 Analysis of *BoFLC* expression in Purple Sprouting Broccoli

4.1 Introduction

FLOWERING LOCUS C (*FLC*) is central to the vernalization response of winter-annual Arabidopsis accessions (Figure 10). *FLC* encodes a MADS box protein that strongly inhibits flowering by repressing the expression of the floral-pathway integrators *SOC1* and *FT* (Michaels & Amasino 1999; Sheldon et al. 1999; Lee et al. 2000; Michaels et al. 2005; Helliwell et al. 2006). A second gene, *FRI*, is required to promote *FLC* expression to levels that inhibit flowering, and so confer a vernalization response (Michaels & Amasino 1999; Sheldon et al. 1999; Johanson et al. 2000; Michaels & Amasino 2001). During vernalization *FLC* expression is stably repressed, releasing this block on flowering (Michaels & Amasino 1999; Sheldon et al. 2000b).

The stable repression of *FLC* during vernalization involves modifications of histones in *FLC* chromatin, including the trimethylation of Lysine 27 on histone 3 (H3K27me3) (Bastow et al. 2004; Sung & Amasino 2004b; Sung et al. 2006a; Sung et al. 2006b; Wood et al. 2006; Greb et al. 2007; Schmitz & Amasino 2007; He 2009). Trimethylation of H3K27 in *FLC* chromatin during vernalization requires the PHD-gene VIN3, which is induced during long periods of cold (Sung & Amasino 2004b; Sung et al. 2006b; Greb et al. 2007; Schmitz & Amasino 2007). The stable repression of *FLC* makes the plant competent to flower in response to other cues such as long days, which promote the expression of the floral-pathway integrator genes via the photoperiod pathway (see chapter 1). Long days are perceived in the leaves, resulting in the accumulation of *CO* and activation of *FT* in the phloem (Samach et al. 2000; Suarez-Lopez et al. 2001; Valverde et al. 2004). FT protein acts as a mobile signal that travels from the leaves to the apex, where it interacts with *FD* (a bZIP protein expressed in the apex) to promote flowering (Abe et al. 2005; Wigge et al. 2005; Jaeger & Wigge 2007; Notaguchi et al. 2008). The repression of *FLC* by vernalization in Arabidopsis is mitotically stable but reset at meiosis, ensuring that flowering is repressed and the vernalization response restored in the next generation (Michaels & Amasino 1999; Sheldon et al. 2000b).

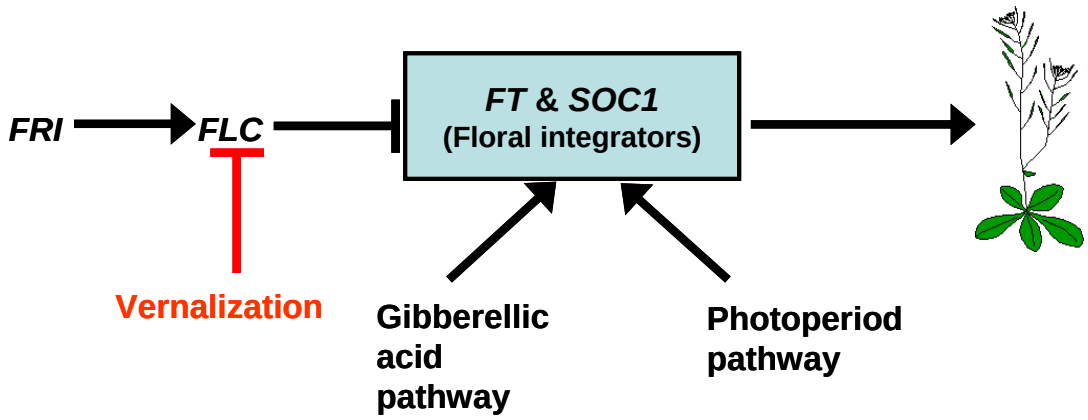


Figure 10: In Arabidopsis FLOWERING LOCUS C (FLC) represses flowering by repressing the expression of the floral integrator genes *FT* and *SOC1*. *FRIGIDA* (*FRI*) up-regulates the expression of *FLC* to levels that inhibit flowering. During vernalization *FLC* expression is stably repressed, releasing this block on flowering. The plant can then flower in response to other cues such as inductive photoperiods.

Arabidopsis accessions show extensive natural variation in vernalization response (Sheldon et al. 2000a; Shindo et al. 2005; Shindo et al. 2006). Some of this variation in associated with variation in the stability of *FLC* repression during vernalization. For example in Edi-0 (from Edinburgh, UK, 56.0 degrees latitude) short periods of cold (e.g. 4-6 weeks at 4°C) lead to stable repression of *FLC* expression and greatly accelerated flowering, indicated by a lower total leaf number at flowering as shown in Figure 11 and Figure 12. Lov-1 (from Lovik, northern Sweden, 62.5 degrees latitude) does not flower following short periods of cold (e.g.4 weeks at 4°C) (Figure 11). *FLC* expression is reduced in Lov-1 during these short periods of cold, but rapidly increases following a return to warmer conditions (Figure 12). Longer periods of cold are needed for stable repression of *FLC* and hence for flowering to be accelerated. The increasing stability of *FLC* repression with longer durations of cold-treatment is correlated with the rate of accumulation of H3K27me3 at *FLC* (Shindo et al. 2006).

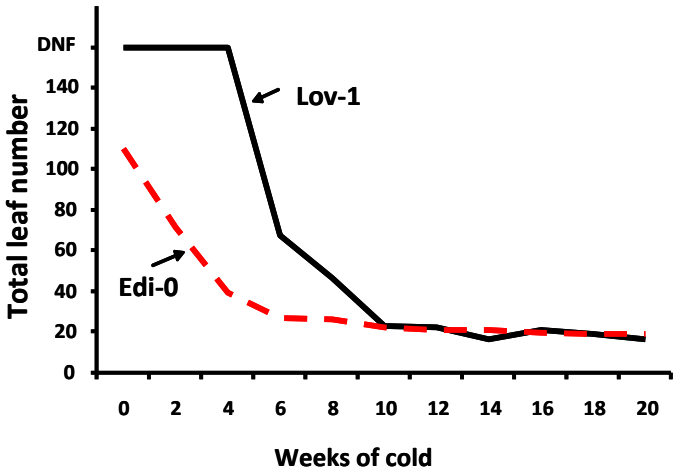


Figure 11: The effect of the duration of cold-treatment at 4°C upon total leaf number in the Arabidopsis accessions Lov-1 and Edi-0. (The total leaf number in Arabidopsis is correlated with the time taken to flower). Lov-1 (from Lovik, Northern Sweden, 62.5 degrees latitude) requires a longer period of cold for vernalization that Edi-0 (from Edinburgh, UK, 56.0 degrees latitude). (From Shindo et al. (2006), the graph was provided by Dr Vincent Coustham).

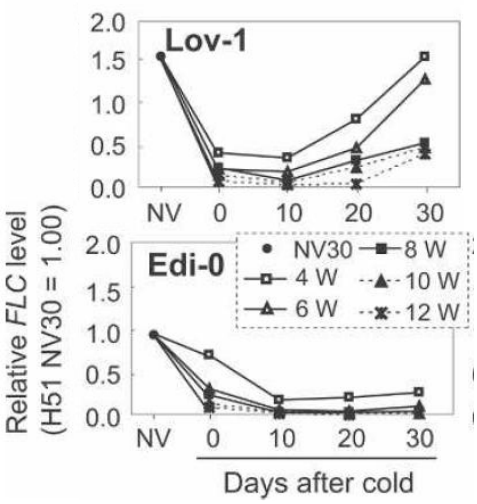


Figure 12: The effect of the duration of cold-treatment at 4°C upon relative *FLC* expression in the Arabidopsis accessions Lov-1 and Edi-0. Lov-1 (from Lovik, Northern Sweden, 62.5 degrees latitude) requires a longer period of cold for vernalization that Edi-0 (from Edinburgh, UK, 56.0 degrees latitude) (Figure 11). *FLC* is down-regulated during short (4–6 week) periods of cold in both accessions but this down-regulation is only stable in Edi-0. In Lov-1 longer periods of cold are necessary for stable repression of *FLC* and hence acceleration of flowering. (Reproduced from Shindo et al. (2006)).

The Arabidopsis *FLC* orthologue of a perennial relative of Arabidopsis, *Arabis alpina* (Brassicaceae) is only transiently down-regulated by vernalization (Wang et al. 2009b). *A. alpina* is a perennial, polycarpic species in which vernalization is required each year in order to promote flowering. Following vernalization flowers were produced from the apexes of the main shoot and axillary shoots that were present before vernalization. But meristems that were either not present or were at an early developmental stage when cold-treatment started formed vegetative shoots after vernalization. These shoots did not flower until the plant was vernalized again. *PEP1*, a floral repressor and the *A. alpina* orthologue of *AtFLC*, is involved in regulating these seasonal cycles of flowering. *PEP1* prevents flowering before vernalization but is repressed during vernalization, permitting flowering. *PEP1* is then reactivated when the plant is returned to warmer conditions. After vernalization *PEP1* limits the duration of flowering and prevents some shoots from flowering until the next season when the plant is vernalized again (Wang et al. 2009b). Studies of a natural population of *A. halleri* also found that an *AtFLC* homologue (*AhgFLC*) is down-regulated during winter but up-regulated as temperatures increase again in spring. Flowering occurs following down-regulation of *AhgFLC*, and the reversion to vegetative growth occurs when *AhgFLC* expression has increased to near pre-winter levels again. This suggests that *AhgFLC* controls the transition to flowering in spring and the later reversion to vegetative growth (Aikawa et al. 2010). So whilst the basic role of *FLC* as a floral repressor is conserved in these species, differences in its expression pattern appear to contribute to the different life histories of these species.

Tadege et al. (2001) isolated five *FLC* genes from a winter cultivar (Columbus) of the amphidiploid *Brassica napus*, using a *cDNA* probe from *AtFLC*. They designated these genes *BnFLC1*, *BnFLC2*, *BnFLC3*, *BnFLC4*, and *BnFLC5* in order of their similarity to *AtFLC* (*BnFLC1* being the most similar). These five *BnFLC* genes formed a clade with *AtFLC* that is distinct from the other *FLC*-like MADS-box proteins in Arabidopsis (Tadege et al. 2001).

The diploid Brassica species *B. oleracea* and *B. rapa* both have four homologues of *FLC*: *BoFLC1*/*BrFLC1*, *BoFLC4*/*BrFLC2*, *BoFLC3*/*BrFLC3*, and *BoFLC5*/*BrFLC5* (Schranz et al. 2002; Okazaki et al. 2007; Razi et al. 2008). Schranz et al. (2002)

cloned four homologues of *AtFLC* from *Brassica rapa*. They designated these *BrFLC1*, *BrFLC2*, *BrFLC3*, and *BrFLC5* based upon their similarity to the cDNA sequences of *BnFLC1–5* isolated by Tadege et al. (2001). *BrFLC1*, *BrFLC2*, and *BrFLC3* mapped to regions collinear with the top of Arabidopsis chromosome 5 (At5) (on chromosomes R10, R2, and R3 respectively). *BrFLC5* mapped to a different region on R3 that is not collinear with this region of At5 (Schranz et al. 2002). Schranz et al. (2002) also cloned three *FLC* homologues from the rapid cycling *Brassica oleracea* TO1000: *BoFLC1*, *BoFLC3*, and *BoFLC5* (named according to their similarity to *BnFLC1–5*). But they were unable to clone a *BoFLC2* sequence. Lin et al. (2005) isolated *BoFLC3-2* and *BoFLC4-1* genes (named based upon the similarity of deduced amino acid sequences to the *BnFLC*s isolated by Tadege et al. (2001)) from cabbage (*B. oleracea capitata*). Okazaki et al. (2007) also isolated *BoFLC1*, *BoFLC3*, and *BoFLC5* genes. They also isolated another *BoFLC* that showed high homology to both *BoFLC4* and *BrFLC2*, and designated it *BoFLC2*. Razi et al. (2008) suggested that this *BoFLC2* was probably the same gene as *BoFLC4*. So, like *B. rapa*, *B. oleracea* has four homologues of *FLC*: *BoFLC1*, *BoFLC3*, *BoFLC4*, and *BoFLC5* (Figure 13). *BoFLC1* and *BoFLC4* have been mapped to linkage groups O9 and O2 respectively and *BoFLC3* and *BoFLC5* have both been mapped to O3 (Razi et al. 2008).

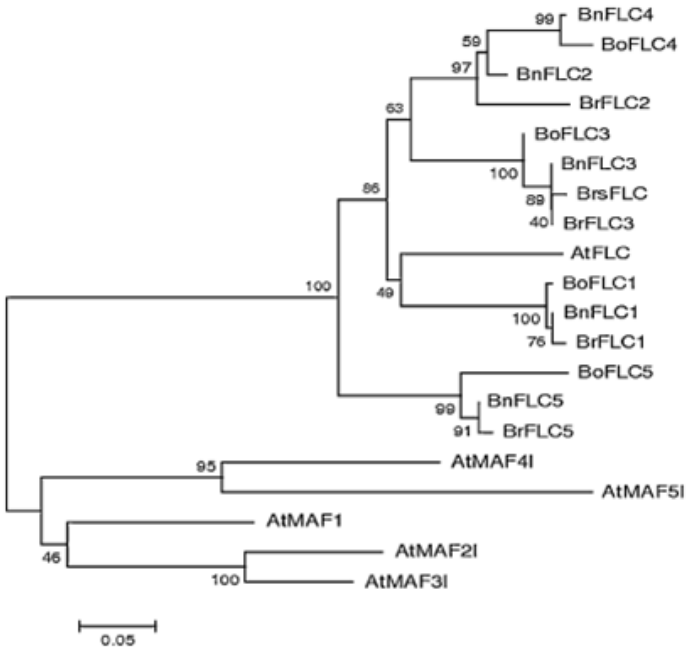


Figure 13: A neighbour-joining phylogenetic analysis of *AtFLC*; *AtMAF1*, *2I*, *3I*, *4I*, and *5I*; *BnFLC1*, *2*, *3*, *4*, and *5*; *BrFLC1*, *2*, *3*, and *5*; *BrsFLC*; and *BoFLC1*, *3*, *4*, and *5* based upon deduced amino acid sequences. The numbers at the nodes are the bootstrap values out of 500 replicates. Reproduced from Razi et al. (2008).

The multiple homologues of *FLC* in Brassica species could act together additively, and this could contribute to the transition from a winter-annual to a biennial (i.e. with an obligate vernalization requirement) life history (Michaels & Amasino 2000; Schranz et al. 2002; Weinig et al. 2007). Transforming winter-annual Arabidopsis with multiple copies of *FLC* can convert it to a true biennial that does not flower without vernalization. So *FLC* appears to control flowering in a rheostat-like manner in Arabidopsis (Michaels & Amasino 2000). The multiple *FLC* genes in Brassica species could increase the number of potential allelic combinations and hence the variation in flowering time conferred by the *FLC* "rheostat" (Schranz et al. 2002; Osborn et al. 2003; Osborn 2004). However, when multiple homologues of a gene are present redundancy among them may allow individual homologues to degenerate into pseudogenes or be completely lost from the genome. This is known as nonfunctionalization (Prince & Pickett 2002). Different homologues of duplicated genes may also take on new functions (neofunctionalization) or different aspects of an ancestral function may be divided between them (subfuntionalization) (Yang et al. 2006).

QTL for flowering time and vernalization-responsive flowering time in *B. rapa*, *B. oleracea*, and *B. napus* have been mapped to regions of the genome collinear with the top of Arabidopsis chromosome 5, where numerous flowering-time genes are located including *CO*¸ *FY*, and *FLC* (Osborn et al. 1997; Bohuon et al. 1998; Axelsson et al. 2001; Kole et al. 2001). This region is represented by three homologous copies in *B. rapa* and *B. oleracea* and six in *B. napus* (an amphidiploid of *B. rapa* and *B. oleracea*) (Lagercrantz et al. 1996; Axelsson et al. 2001). Three *BrFLC* homologues were shown to map to flowering-time QTL in this region: *BrFLC1* to VFR2 (Kole et al. 2001; Schranz et al. 2002) and *BrFLC2* and *BrFLC5* to FR1and FR2 respectively (Schranz et al. 2002). These QTL were identified by Osborn et al. (1997), who found that VFR2 controlled vernalization-responsive flowering time whereas FR1 and FR2 controlled non-vernalization-responsive flowering time. Schranz et al. (2002) showed that this variation was actually reduced by vernalization (Okazaki et al. 2007). No flowering-time QTL were detected close to *BrFLC3* on R3 (Schranz et al. 2002). A splicing-site mutation in *BrFLC1* leading to alternate splicing is also associated with flowering-time variation in *B. rapa* (Yuan et al. 2009). Li et al (2009) also found this splicing site mutation and found that *BrFLC1*and *BrFLC2* were linked to the QTLs for bolting time, budding time, and flowering time. Therefore, multiple *BrFLC* homologues are associated with variation in flowering time/vernalization response.

Expression analysis studies suggest that *Brassica rapa* and *B. napus FLC* homologues act to delay flowering until after vernalization, like *FLC* in Arabidopsis. Each of the five *AtFLC* homologues isolated from *B. napus* (*BnFLC1–5*) delayed flowering when over-expressed in the early-flowering Arabidopsis ecotype *Ler* (Tadege et al. 2001). And the delay in flowering was correlated with the expression level of the transgenic *BnFLC*. Vernalization of *B. napus* also led to a reduction in the expression of these *BnFLC*s, accompanied by an acceleration of flowering. Two studies in Chinese Cabbage (*Brassica rapa pekinensis*) have shown that *BrFLC* expression is reduced during vernalization and that a longer duration of cold-treatment is associated with a greater reduction (Li et al. 2005; Kim et al. 2007). *BrFLC1*, *BrFLC2*, and *BrFLC3* also delay flowering when over-expressed in Arabidopsis, with the degree of the delay being correlated with the expression level (Kim et al. 2007).

Several studies have implicated *BoFLC4* in the vernalization response of *B. oleracea*. Lin et al. (2005) found that *BoFLC* expression is reduced by vernalization of adult cabbage plants but not imbibed seeds. This pattern was likely to be mostly due to *BoFLC4*, and correlates with the acceleration of flowering by vernalization of adult plants but not seeds (Lin et al. 2005). *BoFLC2* (which is equivalent to *BoFLC4*) has been mapped to the peak of the largest QTL for flowering-time in a cross between a vernalization-type and non-vernalization-type *B. oleracea*. Genotyping of the *BoFLC2* locus further supported a role for this gene in controlling flowering time through vernalization (Okazaki et al. 2007). Razi et al. (2008) did not detect a flowering-time QTL in this position, where they also mapped *BoFLC4*. But this discrepancy could be due to the mapping population that they used. This was generated by Bohuon et al. (1998) and neither of the parent genotypes had a functional *BoFLC4* allele. One of the parent genotypes (A12DHd) had no *BoFLC4* allele and the other (GDDH33) had an allele that contained a premature stop codon. One of the parental genotypes used by Okazaki et al. (2007) had a *BoFLC2* allele that did not contain a premature stop codon. Okazaki et al. (2007) did not find flowering-time QTL linked to *BoFLC1*, *BoFLC3*, and *BoFLC5* though. None of the four *BoFLC* homologues constituted strong candidates for any of the flowering-time QTL found by Razi et al (2008); and they found that *BoFLC5* was almost certainly a pseudogene.

In chapter 3 I showed that there is variation in the response of flowering time to both the temperature and duration of cold-treatment among four genotypes of Purple Sprouting Broccoli (PSB, *B. oleracea italica*): E1, E5, E8, and E9. E1 is early flowering and has a facultative requirement for vernalization. E5, E8, and E9 are later flowering had have an obligate requirement for vernalization in order to flower (see chapter 3 for details). Field-experiments carried out at Cornwall and Spalding as part of a HortLINK project have identified a vernalization-responsive flowering-time QTL mapping to the same region as *BoFLC4* in a doubled-haploid population derived from E5 and E9, further supporting a role for this homologue in controlling vernalization-responsive flowering time. (E9 is later flowering than E5). These studies also found QTL mapping to the same region as *BoFLC3* in the same population. However, a lack of data on the expression patterns of these genes during vernalization makes it difficult to assess whether they represent strong candidates for

these QTL. No QTL were found associated with *BoFLC1* or *BoFLC5* in either this population or in two other doubled-haploid population derived from E1 and E5 and E1 and E8 (Irwin, personal communication). At present only a partial sequence of *BoFLC5* has been deduced in these four PSB genotypes but this shows no polymorphisms compared to the reference sequence published by Razi et al. (2008) (Irwin, personal communication). This suggests that this homologue is a pseudogene in our populations as it is in A12DHd and GDH33 (Razi et al. 2008).

Early experiments showed that vernalization is perceived by the shoot apex (Schwabe 1954) but there is evidence that vernalization can also be perceived by other tissues that contain dividing cells. Wellensiek (1961; 1962; 1964) found that young leaves of *Lunaria biennis* plants could be vernalized but mature leaves could not. Cuttings of young leaves from vernalized plants regenerated into plants that flowered, with the percentage flowering increasing with decreasing leaf age at the onset of vernalization. Cuttings of leaves that were fully grown at the start of vernalization regenerated into plants that did not flower. Leaves from unvernalized plants could also be vernalized and then regenerated into flowering plants. This vernalization was shown to be localized to the bottom of the petiole where cell division was occurring. Crosthwaite and Jenkins (1993) suggested that young expanding leaves of sugar beet could respond to vernalization directly by becoming competent to produce a flowering stimulus in response to long days. But their results did not eliminate the possibility that the apex was producing a signal in response to vernalization that travelled to the young leaves, or that the old leaves could also be vernalized but lost their ability to produce a floral stimulus with age (Crosthwaite & Jenkins 1993). In Arabidopsis, root apexes from vernalized plants regenerate into flowering plants whereas those from unvernalized plants regenerate into plants that do not flower unless vernalized (Burn et al. 1993).

In Arabidopsis *FLC* acts in both the leaves and shoot meristem to delay flowering. In the leaves *FLC* represses flowering by repressing the expression of *FT*. This prevents the formation of the systemic signal involving FT. *FLC* expression in the leaves also appears to prevent the formation of a second, *FT*-independent systemic signal that leads to up-regulation of *FD* in the apical meristem. FT and FD are both required for the activation of *SOC1* expression in the apical meristem. In the

meristem *FLC* represses *SOC1* and *FD*, suppressing the ability of the meristem to respond to the FT signal. The repressive effects of *FLC* expression in the leaves and apex upon flowering are additive and expression is required in both the leaves and apex for flowering to be delayed to the extent seen in non-vernalized winter-annual accessions. Vernalization represses *FLC* expression in both the leaves and apex, allowing the leaves to produce these systemic signal(s) and the meristem to respond to them (Searle et al. 2006). This supports the hypothesis that vernalization is perceived in the leaves of Arabidopsis as well as the apex, and that it is necessary in both of these tissues for acceleration of flowering.

Further studies found that *FLC* is repressed in both the young and mature leaves of Arabidopsis during vernalization; but this repression is only maintained in the young leaves. In both young and mature leaves *VIN3* is induced during 4-weeks cold-treatment (though less strongly in mature leaves than in young leaves) and H3K27me3 increases at the transcription-translation start of *FLC*. In young leaves *VIN3* expression decreases when the plant is returned to warm conditions and H3K27me3 spreads across the *FLC* locus. In mature leaves *VIN3* expression remains high after plants are returned to warm conditions but H3K27me3 is removed from the transcription-translation start of *FLC*. These findings support the hypothesis that cell division is necessary for a tissue to sense vernalization in Arabidopsis (Finnegan & Dennis 2007).

I wanted to determine whether *BoFLC* expression was repressed during vernalization in both the apex and young leaves of PSB as *FLC* is in Arabidopsis (Searle et al. 2006; Finnegan & Dennis 2007), and whether differences in *BoFLC* expression levels and the extent/stability of their down-regulation during vernalization correlated with differences in vernalization response/flowering time observed among E1, E5, E8, and E9 in chapter 3. I used quantitative real-time reverse-transcriptase PCR (QRT-PCR) to compare the expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in the young leaves of all four of the genotypes investigated in chapters 2 and 3 (E1, E5, E8, and E9) before and after 10 weeks cold-treatment with 70 days pre-growth. I did not investigate *BoFLC5* expression because this homologue appears to be a pseudogene (above). I also compared the expression of *BoFLC1*, *BoFLC3*, and

BoFLC4 before, at the end of, and two weeks after vernalization in the young leaves and apexes of PSB plants of three genotypes (E1, E5, and E8).

4.2 Materials and methods

4.2.1 Plant growth, sampling, and phenotyping

QRT-PCR analysis of *BoFLC* expression was carried out on samples taken from young leaves of the E1, E5, E8, and E9 plants cold-treated at 5 and 10°C for 10 weeks following 70 days pre-growth in a warm glasshouse in experiment II. These plants were also scored for the number of days from the end of cold-treatment till the first appearance of visible inflorescence buds at the apex. This data along with details of the plant growth methods was presented in chapters 2 and 3. For each of these treatment temperatures there were five plants that were scored for DTI, which were randomly selected from all of the plants sown on 8th May 2009. A young leaf was sampled from each of these plants the day before transfer to the cold-treatment CERs, "NV"; at the end of cold-treatment before the plants were returned to the glasshouse, "T0", and 13–14 and 27–28 days after the plants were returned to the glasshouse, "T14" and "T28". QRT-PCR analysis of *BoFLC* expression was performed upon the young-leaf samples from the quickest two plants of each genotype to form visible inflorescence buds with each cold-treatment temperature (or two randomly selected plants in the case of the E9 plants treated at 10°C).

QRT-PCR analysis of *BoFLC* expression was also carried out on samples of the apex and young leaf of E1, E5, and E8 plants cold-treated for 10 weeks at 5°C following 70 days pre-growth. These plants were also randomly selected from those sown on 8th May 2009. One of these plants was randomly selected to be destructively sampled at each of three time-points: the day that the plants were transferred to cold-treatment, "NV"; the day before the plants were returned to the glasshouse, "T0"; and 15 days after the plants were returned to the glasshouse, "T14". Because the destructive sampling involved removing the apex as well as a young leaf these plants could not be scored for DTI.

An initial RT-PCR test of the primers was carried out using young-leaf samples from one plant of each genotype from experiment I, taken the day before transfer to cold-treatment (after 50 days pre-growth).

All of the samples were immediately frozen in liquid nitrogen and stored at -70°C until RNA extraction.

4.2.2 RNA extraction and cDNA synthesis

Total RNA was extracted using the phenol-chloroform method described in Appendix 6. A 1:10 dilution of the total RNA was then quantified using a Thermo Scientific NanoDrop™ 1000 spectrophotometer, and the quality verified from the A280/260 and A260/230 ratios. For each sample the A280/260 ratio was between 1.80 and 2.10 and the A260/230 ratio was between 2.10 and 3.00. An aliquot of RNA from every sample was also run on a 1.5% agarose gel (stained with ethidium bromide) to check its integrity. For every sample the two bands of rRNA were clearly visible, suggesting that the RNA was not degraded. The RNA was stored at -70°C.

An aliquot of RNA was DNase-treated using an Ambion Turbo DNase kit, according to the manufacturer's instructions (Appendix 7). The DNase-treated RNA was used to synthesise cDNA with the Invitrogen SuperScript™ III First-Strand Synthesis System for RT-PCR kit (Cat. No: 18080-051), following the manufacturer's instructions (Appendix 7). All cDNA synthesis reactions were primed using oligo-dTs. For the samples used for QRT-PCR DNase treatment was performed on 2 µg total RNA from each sample (diluted to 20 µl total volume with autoclaved deionised water). The DNase-treated RNA was quantified using a Nanodrop™ 1000 spectrophotometer (taking the average of two repeat-measurements for each sample) and 300 ng was used to synthesise cDNA. A G-storm G51 thermal cycler was used for all thermal-cycler programs. (For the samples from the experiment I plants, used only for the initial RT-PCR screen of the primers, 12 µg RNA was DNase treated as described above and 1700–1820 ng DNased RNA was used for each cDNA synthesis reaction. All thermal-cycler programs were run on a MWG-Biotech Primus 96 plus thermal cycler).

For the main young-leaf samples (taken at NV, T0, T14, and T28 from the plants scored for inflorescence formation), cDNA was synthesized in two separate plates. Each plate contained one (randomly chosen) biological replicate from each genotype at each time-point to minimize the effect of any inter-run differences. Eight extra cDNA synthesis reactions with the first biological replicate of E8NV were also performed in the plate with the first set of biological replicates to provide sufficient cDNA (when pooled together) to use as a common calibrator sample in all runs (see below). For the destructively taken samples, all cDNAs were synthesized in a single PCR plate. Two control reactions—one with no RNA template and one with no reverse transcriptase—were performed in each of the three plates, and did not yield any product in QRT-PCR with any of the primers used.

4.2.3 Primer design and testing

Primers were designed in Primer 3+ software (Untergasser et al. 2007) for each *BoFLC* homologue based upon the sequences given for the *Brassica oleracea* genotype A12DHd in Razi et al. (2008). Details of these primers and the Genbank numbers of the reference sequences are given in Table 13. The structures of the *AtFLC* and *BoFLC* genes and the exons in which the primers were located are shown in Figure 14. The sizes of the introns and exons in each of these genes are given in Appendix 8. There is substantial variation in the size of the introns among these homologues, particularly for intron 1. However, the exons are similarly or identically sized in all of these homologues. The primers were all located within exons but amplified across at least one intron, to allow cDNA and genomic DNA products to be easily distinguished.

An initial RT-PCR screen of the primers was carried out using the cDNA synthesized from each of the four genotypes from experiment I. A PCR with each set of primers was carried out with cDNA from each genotype. The PCR program consisted of a five-minute 95°C hot-start; then 35 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 30s; followed by a final elongation step of 72°C for 10 min. This was performed using an KJ Research DNA Engine Tetrad PTC-225 thermal cycler for the *BoFLC3*, *BoFLC4*, and *BoFLC5* primers and a G-storm thermal cycler for the *BoFLC1* primers. The products from each PCR were run on an agarose gel. This confirmed that each pair of primers produced only a single band of the size expected

from cDNA from each genotype (with no genomic DNA product formed). The PCR products were sequenced with BigDye V3.1 (Appendix 9). The sequences were aligned with the published reference sequences from Razi et al. (2008) listed in Table 13 using AlignX (Invitrogen). (The aligned cDNA sequences for *BoFLC1*, *BoFLC3*, *BoFLC4*, and *BoFLC5*(from Razi et al. (2008)) are shown in Appendix 10.) This confirmed that each pair of primers amplified only the desired product.

BoFLC homologue (& ref sequence)	Primer sequence, position in reference sequence, exon, and Tm		Expected product size (bp)		Primer efficiency
	Forward primer	Reverse primer	cDNA	g DNA	
BoFLC1 (Genbank AM231517)	GAACGCCCTCTCCGTAACA 4675 bp, Exon 4 65.2 °C	TCGGCTCGCACAAGATTAC 5297 bp, Exon 7 64.0 °C	144	631	1.87
BoFLC3 (Genbank AM231518)	GAGCTATGGTTCACACAATGAGTTAC 3860 bp, Exon 3 63.6 °C	GGCTATCAACAAGCTTCAACATTAG 4175 bp, Exon 5 63.7 °C	173	340	2.02
BoFLC4 (Genbank AY306124)	GTAAGCTTGTGGAATCAAATTCTGA 718 bp, Exon 4 64.1 °C	GGCTATCAACAAGCTTCAACAATAG 886 bp, Exon 5 63.7 °C	128	193	2.00
BoFLC5 (Genbank AM231519)	AACATGCTGATGATCTCAATGC 5462 bp, 2 63.9 °C	ACGAGGGAATCCACGCTTAC 6070 bp, Exon 4 65.2	133	628	n/a
GAPDH (Genbank EF123055)	AGAGCCGCTTCCTTCAACATCATT 339, Exon n/a 69.7 °C	TGGGCACACGGAAGGACATACC 429, Exon n/a 72 °C	112	112	2.00
UBC (Genbank EU593895)	GATCCACCCACCTCGTGTAG 105, Exon n/a 64.4 °C	CTGGAGGGAAGTGAATGGTAAC 213, Exon n/a 63.5 °C	130	130	1.87

Table 13: Details of the *BoFLC* and control gene primers used in this study. All of the *BoFLC* primers were designed using primer 3+ software (Untergasser et al. 2007). The *GAPDH* and *UBC* primers were provided by Dr Judith Irwin. The expected product sizes are based upon the reference sequences. RT-PCR confirmed that all of the primers produced only a single product in the genotypes under study. All of the products from the *BoFLC* primers were sequenced and the sequences compared to the reference sequence. This confirmed that they amplified only the desired product. No genomic DNA products were obtained with any of the cDNAs in either the initial RT-PCR screen of the primers or the subsequent QRT-PCRs (based upon inspection of the melting curves).

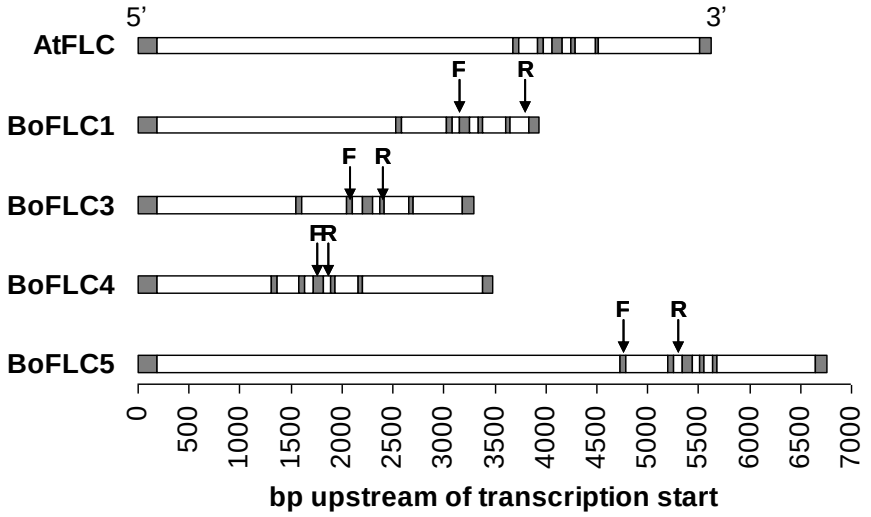


Figure 14: Diagramatic representation of the structures of *AtFLC*, *BoFLC1*, *BoFLC3*, *BoFLC4*, and *BoFLC5* as described by Razi et al. (Razi et al. 2008). (The Genbank accession numbers for these genes are AF116528, AM231517, AM231518, AY306124, and AM231519 respectively). The exons are shaded in grey and the introns are white. The arrows indicate the exons in which the forward (F) and reverse (R) primers for each *BoFLC* homologue used in this study are located. The sizes of the introns and exons in each of these genes are given in Appendix 8. The cDNA sequences for *BoFLC1*, *BoFLC3*, *BoFLC4*, and *BoFLC5* are shown in Appendix 10.

4.2.4 QRT-PCR assay

BoFLC mRNA levels were measured using quantitative real-time RT-PCR (QRT-PCR) with Sigma SYBR® Green JumpStart™ Taq ReadyMix™ with a Bio-Rad Chromo4™ System. All reactions were run using 2.5 µl of cDNA, diluted 10x using autoclaved deionised water. 7.5 µl of a master mix containing 1.9 µl water, 0.3 µl 10 µM forward primer, 0.3 µl 10 µM reverse primer, and 5.0 µl Sigma SYBR® Green JumpStart™ Taq ReadyMix™ was used per reaction. The reactions were carried out in white 96-well non-skirted PCR plates (ABgene thermofast plates), sealed with Bio-Rad optically clear adhesive seals (Microseal "B" films). The same QRT-PCR program was used for all reactions, with a heated lid to prevent sample evaporation. This program consisted of a 2-minute hot-start at 95°C to activate the ReadyMix™, 55 cycles of 95°C for 15s, 60°C for 20s, and 72°C for 30s followed by a fluorescence measurement (at 72°C); then a final elongation step of 72°C for 2 minutes. A melting curve from 50–95°C was performed at the end of every QRT-

PCR run with a fluorescence measurement every 0.5°C. This confirmed that in all cases only the desired product was contributing to the measured fluorescence.

SYBR® Green I dye binds non-specifically to double-stranded DNA (dsDNA). SYBR® Green I fluorescence is minimal when it is free in solution but dramatically increases when it binds to double-stranded DNA (Simpson et al. 2000) so the level of fluorescence is assumed to be proportional to the mass of dsDNA. During PCR the amount of product (hence SYBR® Green I fluorescence) initially increases exponentially. The rate of this increase then slows down, with the increase becoming linear for a period, before ultimately reaching a plateau (Kainz 2000; VanGuilder et al. 2008). The relative quantities of starting template cDNA (hence mRNA in the initial sample) are calculated from the number of PCR cycles required to reach an arbitrary fluorescence threshold (hence quantity of dsDNA) in the exponential phase. The number of cycles taken to reach this fluorescence threshold is called the "threshold cycle" or "Ct" value (Livak & Schmittgen 2001; VanGuilder et al. 2008).

The mRNA level of the gene of interest in each sample was calculated as a "relative expression ratio" (RER). This is the ratio of the expression of the gene of interest (GOI) in a test sample, normalized to one or more reference genes (or another normalization factor such as total RNA), relative to the normalized expression of the GOI in a calibrator sample. The RER of the GOI in the test samples is given as a fold-level relative to the calibrator sample (in which the RER = 1) (Livak & Schmittgen 2001). An E8NV young-leaf sample was used as a calibrator sample because an initial RT-PCR analysis showed that at this time-point E8 had strong expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in the young leaves (data not shown). As described above, eight extra parallel cDNA-synthesis reactions were run with DNase-treated RNA from this sample. (An extra 4 µg total RNA was DNase treated separately from the main samples to provide sufficient RNA for this). QRT-PCRs were run with each sample individually using *GAPDH* and *UBC*. This confirmed that all of these reactions were successful (as all of the Ct values obtained were similar). These eight cDNA samples were then pooled to provide sufficient cDNA for use as a calibrator in all QRT-PCR runs. Because the same calibrator sample was

run on each plate, it also acted as an inter-run calibrator to account for differences between plates.

The Pfaffl (2001) equation (Equation 2) was used to calculate relative expression ratios (RERs) because it includes an average efficiency of each set of primers rather than assuming a perfect doubling of product with each PCR cycle as the Livak (2001) method (also known as the $2^{-\Delta\Delta CT}$ method) does.

$$RER = \frac{EGOI^{\Delta CTGOI(calibrator-test)}}{Eref^{\Delta CTref(calibrator-test)}}$$

Equation 2: The Pfaffl equation (Pfaffl 2001) for calculating relative expression ratios (RERs) using QRT-PCR. *EGOI* and *Eref* are the efficiencies of the primers for the gene of interest and reference gene respectively, calculated from standard curves. In the case of 100% efficiency *E* will be 2 and a perfect doubling of the amount of product will occur with every PCR cycle. *CtGOI* and *Ctref* are the threshold cycle values for the gene of interest and reference gene respectively. These are compared in a *test* sample and a *calibrator* to give the relative expression of each gene.

A standard curve was constructed for each pair of primers using five dilutions of the calibrator sample (E8NV) from 10x–100,000x. Each dilution was run in triplicate. A plot of log(dilution factor) against Ct value gave a linear relationship with each set of primers (Appendix 11) . The relationship between log(dilution factor) and Ct value remained linear down to dilutions of 10,000x. Below this the relationship did not hold for any of the primers except for *GAPDH* (data not shown). Therefore the dynamic range of the primers covers an expression range of at least 1.0–0.001 times the expression level in a 10x dilution of the calibrator sample. The equations of the trendlines (fitted in Microsoft Office Excel 2003) were used to calculate the efficiency of each set of primers (Equation 3). The primer efficiencies are given in Appendix 11.

$$E = 10^{-1/slope}$$

Equation 3: Equation for the calculation of QRT-PCR primer efficiency ("*E*") based upon standard curves (Meijerink et al. 2001; Pfaffl 2001). *E* is 2 where the primers are 100% efficient (i.e. a perfect doubling of the quantity of product occurs with each PCR cycle). *E* is 1 where the primers do not work at all (0% efficiency i.e. no amplification occurs hence the quantity of product does not increase). *Slope* is the gradient of the regression line fitted to a plot of log(dilution factor) versus Ct value for a serial dilution of a single cDNA sample.

Accurate measurement of gene expression by QRT-PCR requires normalization to a suitable reference to control for error/variation introduced through differences in the starting material and during RNA extraction and cDNA synthesis etc (Dheda et al. 2004; Radonic et al. 2004; Huggett et al. 2005). A constant quantity of DNase-treated RNA was used in all cDNA synthesis reactions for QRT-PCR (300 ng, based upon the average of two repeat readings with a Nanodrop™ 1000 spectrophotometer). However, RNA quantification using a spectrophotometer is of limited accuracy (Dheda et al. 2004). Normalization to the starting quantity of RNA alone also does not control for variation in the ratio of mRNA to rRNA (rRNA makes up the majority of total RNA), different reverse-transcription reaction efficiencies, or the possible presence of PCR inhibitors in some samples (Bustin 2002; Dheda et al. 2004; Huggett et al. 2005).

QRT-PCR data is often normalized using "house-keeping genes" (HKGs) that are expressed in all cell types. This has the advantage that the reference gene mRNA has undergone all of the same processing steps (sampling, RNA extraction, DNase treatment, cDNA synthesis etc) as the gene of interest to reach the final measurement, so variation introduced during these steps is controlled for (Radonic et al. 2004; Huggett et al. 2005). Commonly used HKGs include *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPDH*), actins, *UBIQUITIN* (*UBQ*), *UBIQUITIN CONJUGATING ENZYME* (*UBC*), and 18S and 28S rRNA. However, numerous studies have shown that none of these genes are universally stably expressed (Bustin 2002; Dheda et al. 2004; Radonic et al. 2004; Huggett et al. 2005; Jain et al. 2006). The very high expression of *rRNA*s, and the fact that they are only reverse-transcribed with random primers, can also hinder their use as normalisers (Vandesompele et al. 2002; Wong & Medrano 2005; Jain et al.

2006). Using an inappropriate reference gene for normalization can reduce the sensitivity of QRT-PCR and can lead to erroneous results, particularly if it is regulated by the experimental conditions (Dheda et al. 2004; Dheda et al. 2005; Huggett et al. 2005; Jain et al. 2006). This makes it essential to choose and validate reference genes carefully for each experiment (Radonic et al. 2004; Jain et al. 2006).

As QRT-PCR studies of gene expression during vernalization have not previously been reported in *Brassica oleracea italica*, it was necessary to select an appropriate reference for normalization. If a sufficient number of putative reference genes are available, then applications such as GeNorm (Vandesompele et al. 2002) and Normfinder (Andersen et al. 2004) are available to assess their suitability. However, an RT-PCR screen of several HKGs performed by Dr Judith Irwin found only two that gave only a single product in all four of the genotypes under study: *UBIQUITIN CONJUGATING ENZYME* (*UBC*) and *GLYCEREALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPDH*). Therefore their stability was estimated based upon their relative expression ratios in the samples under study normalized to total RNA (based upon the NanoDrop™ measurements) using Equation 4. The relative expression ratios of each HKG across all of the samples under study data are shown in Figure 15 & Figure 16.

$$RER = E^{\Delta CT(calibrator-test)}$$

Equation 4: Equation for calculating the relative expression ratio of a gene in a *test* sample relative to a *calibrator* sample, normalized to total RNA where the total RNA is constant across all samples (Livak & Schmittgen 2001). (In this study 300 ng total RNA was used in all cDNA synthesis reactions used for QRT-PCR, based upon quantification with a NanoDrop™ 1000 Spectrophotometer). *E* is the efficiency of the primers (from 1 for 0% efficiency to 2 for 100% efficiency). *CT* is the threshold cycle value for a gene, which is compared in a "calibrator" and a "test" sample.

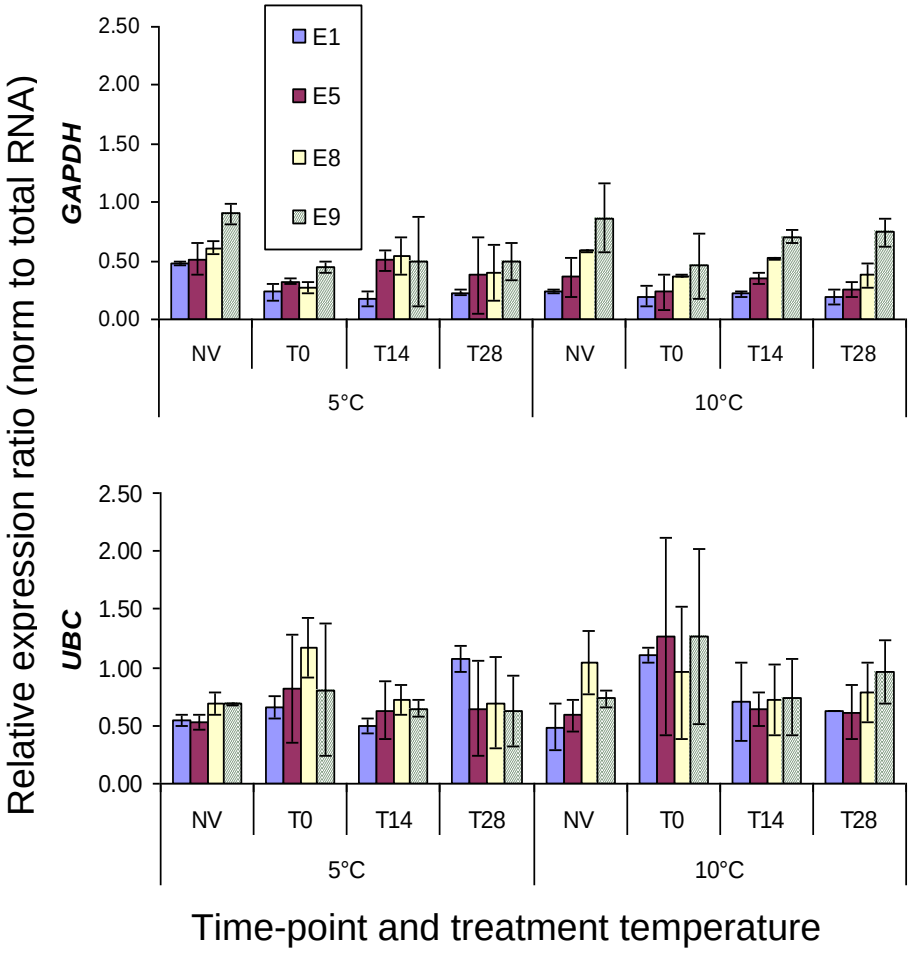


Figure 15: The average relative expression of *GAPDH* & *UBC* in the young leaves of the PSB genotypes E1, E5, E8, and E9 before, at the end of, and after 10 weeks cold-treatment at 5 and 10°C with 70 days pre-growth. The relative expression ratio is calculated relative to an E8NV common calibrator sample using total RNA (as measured with a NanoDrop™ 1000 spectrophotometer) for normalization.

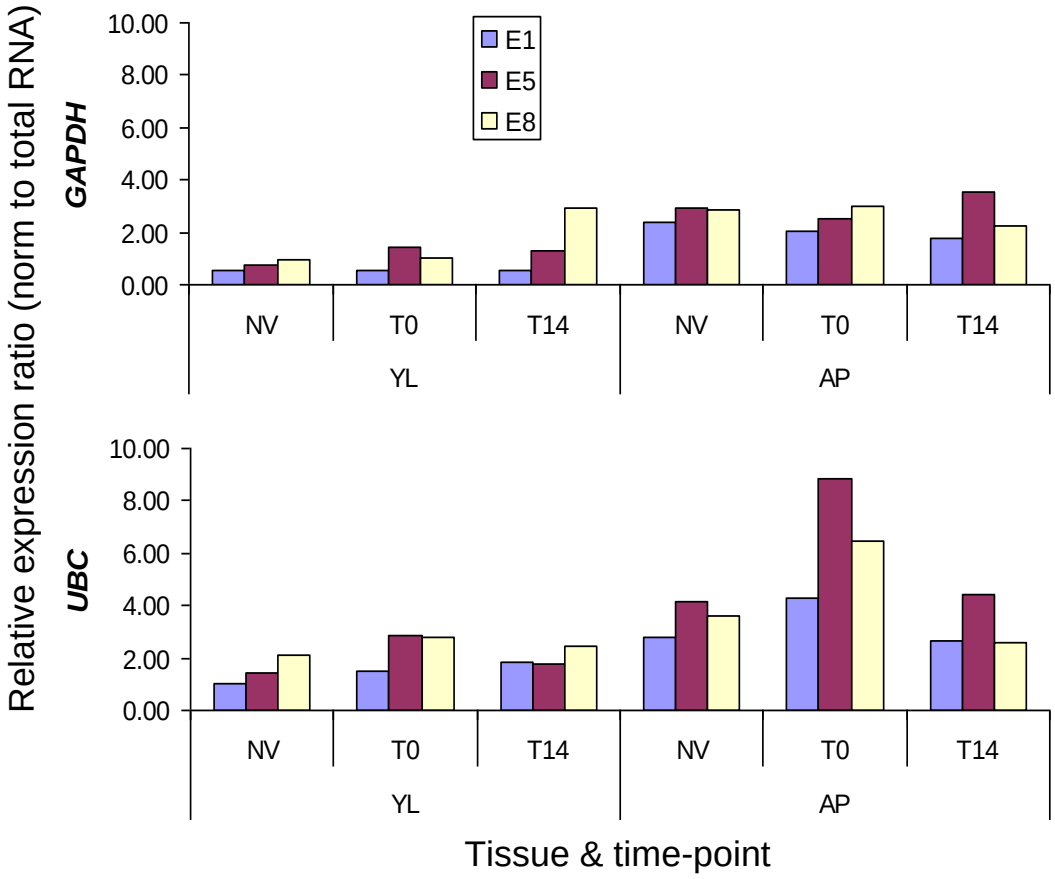


Figure 16: The average relative expression of *GAPDH* & *UBC* in the young leaves and apexes of the PSB genotypes E1, E5, and E8 before, at the end of, and after 10 weeks cold-treatment at 5°C with 70 days pre-growth. The relative expression ratio is calculated relative to an E8NV common calibrator sample using total RNA (as measured with a NanoDrop™ 1000 spectrophotometer) for normalization.

Using the geometric average of multiple reference genes to calculate a "normalization factor" can result in more precise and reliable normalization than using a single control gene (Vandesompele et al. 2002; Hellemans et al. 2007). Therefore the QRT-PCR data were normalized to the geometric average of *GAPDH* and *UBC* expression. To achieve this the denominator of the Pfaffl equation (i.e. $Eref^{\Delta Ctref(calibrator-test)}$) was replaced by the geometric mean of $E^{\Delta Ct(calibrator-test)}$ for *UBC* and *GAPDH*. The data were also normalized to both *GAPDH* and *UBC* expression individually, using the unmodified Pfaffl equation. The data are presented normalized to the geometric average of *GAPDH* and *UBC* in this chapter and the data normalized to *GAPDH* and *UBC* individually are presented in Appendix 12.

Erroneous reactions (i.e. failed reactions those with clearly different Ct values to the other corresponding technical replicates) were excluded manually, based upon examination of the fluorescence plots in Opticon Monitor™ 3.1.32 software. These were likely to be due to pipetting errors and reaction failures. All calculations were then performed in Microsoft Office Excel 2003. There were 2–3 technical replicates for each sample with each gene. The Ct values for the technical replicates of each sample were averaged prior to calculation of the RERs.

A "sample-maximization" strategy was followed, in which the different genes (*BoFLC1*, *BoFLC3*, *BoFLC4*, *UBC*, and *GAPDH*) were assayed in separate QRT-PCR plates. For the destructively taken young-leaf and apex samples, all of the samples from all time-points, tissues, and genotypes were run together in the same plate for each gene (i.e. one plate for each gene). For the main young-leaf samples from NV, T0, T14, and T28 two plates were used for each gene. One for one biological replicate of each genotype, temperature, and time-point was run on one plate and the second biological replicate on another. This approach ensured that all of the direct comparisons (between each individual sample with each gene and the calibrator sample with that gene) were made within the same plate.

4.3 Results and discussions

4.3.1 Flowering time

The average number of days from the end of cold-treatment until the first appearance of visible inflorescence buds at the apex (DTI) for each of the four PSB genotypes (E1, E5, E8, and E9) when cold-treated for 10 weeks at 5 and 10°C with 70 days pre-growth is shown in Figure 17. As discussed in chapter 3, 10 weeks of cold-treatment at 5°C was sufficient for vernalization in E1, E5, E8, and E9. 10 weeks of cold-treatment at 10°C was also sufficient for vernalization in E1, E5 and E8 but not in E9, which did not flower even after 100 days from the end of cold-treatment at 10°C. The 70-day pre-growth period was shown to be sufficient for all four genotypes to reach the adult phase in chapter 2. Therefore there should be no effect of juvenility among the plants used in this chapter. The DTI for the fastest two plants from each genotype that were used for the main (non-destructive) analysis of *BoFLC* expression in the young leaves (at NV, T0, T14, and T28) are shown in Table 14.

The relative expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in the young leaves of the PSB genotypes E1, E5, E8, and E9 at NV, T0, T14, and T28 is shown in Figure 18. The relative expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in the young leaves and apexes of E1, E5, and E8 at NV, T0, and T14 is shown in Figure 19. As these samples came from destructively sampled plants, the exact timing of flowering is not known for these individuals. However, these plants were randomly selected from the same group of plants as those scored for DTI. Therefore it is reasonable to assume that the destructively sampled plants would have formed visible inflorescence buds at a similar time to the average of the plants that were non-destructively sampled and scored for DTI (Figure 17).

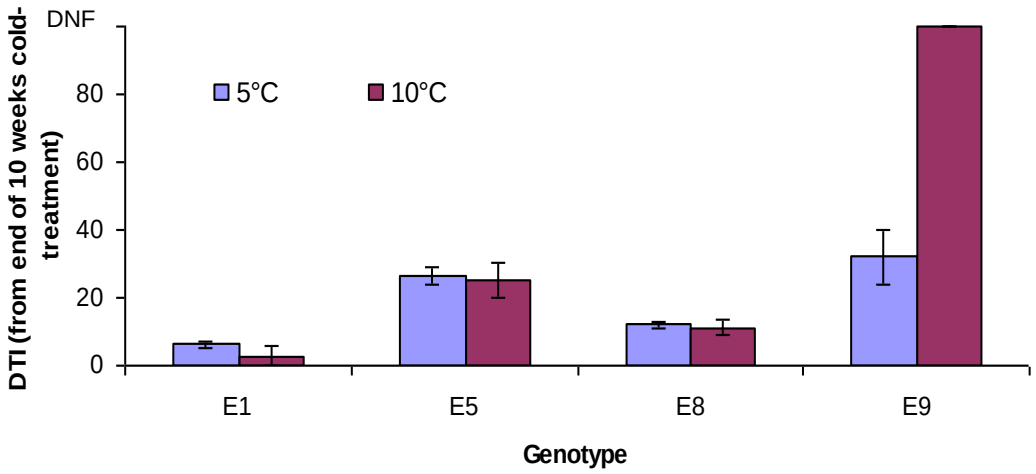


Figure 17: The average number of days taken to form visible inflorescence buds (DTI) by each of the PSB genotypes E1, E5, E8, and E9 following 10 weeks cold-treatment at 5 and 10°C with 70 days pre-growth. (The data for all of the treatment temperatures, durations, and pre-growth periods are shown in Figure 9). There are 3–5 replicates for each genotype at each temperature. The error bars are 95% confidence intervals. Three of the E1 plants treated at 10°C actually formed inflorescence buds while still in cold-treatment. These were given a value of 1 DTI. The E9 plants treated at 10°C did not form visible inflorescence buds within 100 days of the end of cold-treatment (shown as DNF for "did not flower"). The young-leaf samples used for the main *BoFLC* expression analysis in the young leaves of E1, E5, E8, and E9 were taken from the two of these plants that formed visible inflorescence buds the most rapidly (Table 14). The young leaf and apex samples used to compare *BoFLC* expression in these organs in E1, E5, and E8 were taken from other plants that were sown at the same time and grown and cold-treated (at 5°C) alongside these but destructively sampled at NV, T0, and T14.

Genotype	Treatment temperature	DTI (from end of cold-treatment)	
		Replicate 1	Replicate 2
E1	5°C	6	5
	10°C	1	1
E5	5°C	24	25
	10°C	19	21
E8	5°C	12	11
	10°C	10	7
E9	5°C	28	28
	10°C	DNF	DNF

Table 14: The number of days taken to form visible inflorescence buds (DTI) following 10 weeks cold-treatment at 5 and 10°C by each of the plants used for the main *BoFLC* expression analysis in the young leaves at NV, T0, T14, and T28. These are the fastest (or joint fastest) two plants from each genotype to form visible inflorescence buds with each treatment temperature. (For the E9 plants treated at 10°C the two plants were randomly selected because none of the plants formed visible inflorescence buds). All cold-treatments were carried out under 8-hour photoperiods following 70 days pre-growth in a warm glasshouse under long days.

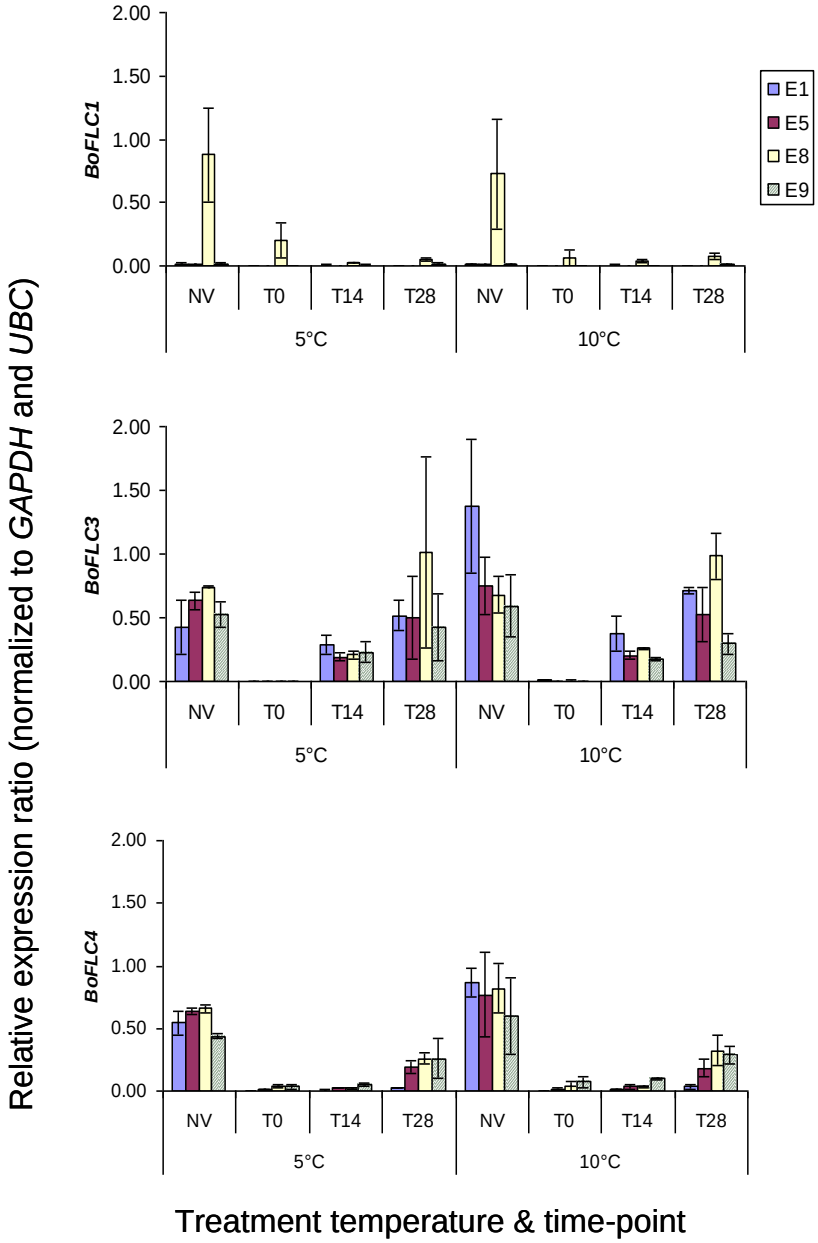


Figure 18: The average relative expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in the young leaves of the PSB genotypes E1, E5, E8, and E9 before and after 10 weeks cold-treatment at 5 and 10°C with 70 days pre-growth. The relative expression ratios are calculated relative to a common E8NV calibrator sample using the Pfaffl (2001) equation. The data are shown normalized to the geometric mean of the relative expression of *GAPDH* and *UBC*. The data are also shown normalized to the relative expression of each of these two genes separately in Appendix 12. There are two biological replicates for each genotype at each time-point and temperature. The error bars represent the 95% confidence intervals.

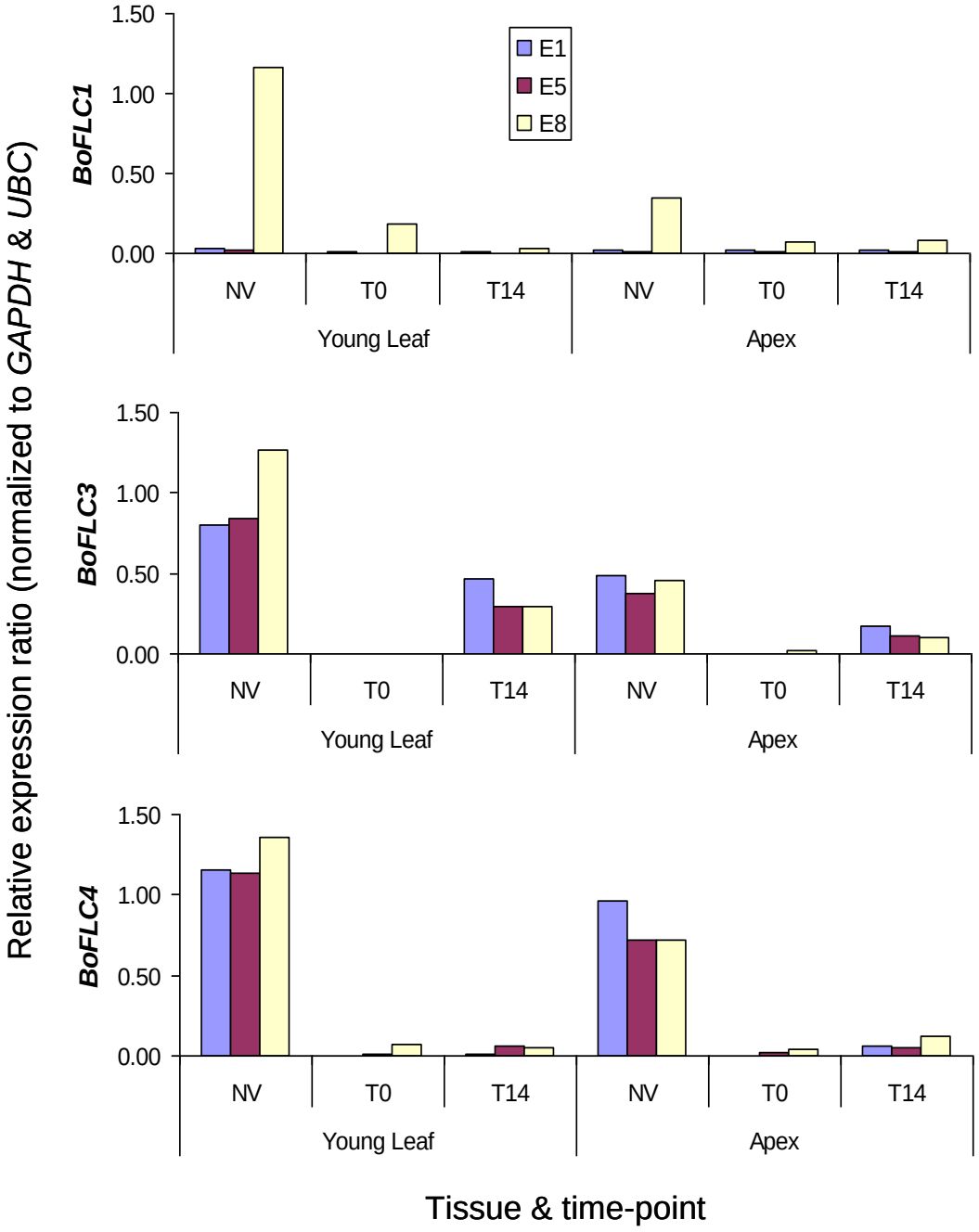


Figure 19: The relative expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in young leaves and apexes of the PSB genotypes E1, E5, and E8 before and after 10 weeks cold-treatment at 5°C with 70 days pre-growth. The relative expression ratios are calculated relative to a common E8NV calibrator sample using the Pfaffl (2001) equation. The data are shown normalized to the geometric mean of the relative expression of *GAPDH* and *UBC*. The data are also shown normalized to the relative expression of each of these two genes separately in Appendix 12. There is one biological replicate of each tissue for each genotype at each time-point and temperature.

4.3.2 *BoFLC1* is expressed much more strongly in E8 than in E1, E5, and E9 but this is not associated with differences in flowering time

BoFLC1 was expressed strongly in the young leaves of E8 before vernalization and then stably down-regulated during vernalization at both 5 and 10°C (Figure 18). But *BoFLC1* was only very weakly expressed in the young leaves of E1, E5, and E9 at all time-points with both treatment temperatures. In some cases no *BoFLC1* fragment was amplified from the young leaves of E5 and E9 at T0 in any of the three technical replicates. In the cases where a *BoFLC1* fragment was amplified at T0 in E5 and E9 the Ct values were very high (34.96–39.27). These Ct values were similar to those at which the standard curves of Ct versus log(dilution factor) broke away from a linear relationship (data not shown). This suggests that the failure to amplify a fragment was due to the extremely low quantity of template. A similar pattern was seen in the apexes and young leaves of the destructively sampled plants: *BoFLC1* was strongly expressed in both the young leaf and apex of E8 before vernalization but stably repressed during vernalization at 5°C, whereas it was only very weakly expressed in the young leaves and apexes of E1 and E5 at all time-points (Figure 19).

The much higher *BoFLC1* mRNA levels in E8 compared to E1, E5, and E9 do not appear to correlate with differences in vernalization response/flowering time (measured by DTI). E8 formed visible inflorescence buds earlier than E5 and E9 with both the 5 and 10°C 10-week cold-treatments (Figure 17). E8 also formed visible inflorescence buds with only 6 weeks of cold-treatment which E5 and E9 did not (chapter 3). This suggests that the much higher *BoFLC1* expression in E8 compared to the other genotypes had little effect upon the time to inflorescence formation. The effect of greater *BoFLC1* expression in E8 may be outweighed by other factors, especially if *BoFLC1* is very readily repressed by even short periods of cold (e.g. 6 weeks). Alternatively, the high levels of *BoFLC1* mRNA in E8 may not be effective in delaying flowering. E.g. the protein may be ineffective at repressing flowering, or the mRNA may not be translated. Previous field experiments carried out at Cornwall and Spalding did not reveal any QTL for flowering time/vernalization response associated with *BoFLC1* in the doubled-haploid population J994 (E1*E8) (Irwin et al. personal communication). However, this only

indicates that *BoFLC1* is not responsible for variation in vernalization response between these genotypes. It does not show that it does not play a role in controlling vernalization response. These results are in agreement with other studies that have found that *BoFLC1* does not constitute a strong candidate for any flowering-time QTL (Okazaki et al. 2007; Razi et al. 2008).

4.3.3 *BoFLC3* is transiently repressed in both the young leaves and apex during vernalization in E1, E5, E8, and E9

BoFLC3 was expressed strongly in the young leaves of E1, E5, E8, and E9 before cold-treatment and transiently down-regulated by 10 weeks of cold-treatment at both 5 and 10°C in each genotype (Figure 18). *BoFLC3* expression was very low in all four genotypes at T0 with both the 5 and 10°C treatments. But *BoFLC3* expression was reactivated to a considerable degree by T14 in all four genotypes following both cold-treatments. By T28 *BoFLC3* expression reached levels similar to or greater than NV in all four genotypes with the 5°C treatment and E5 and E8 with the 10°C treatments. Neither the initial expression level nor the degree of reactivation appears to correlate with the time till inflorescence formation (Figure 17 and Table 14). A similar pattern was seen in both the young leaves and the apexes of the destructively sampled plants. *BoFLC3* was strongly repressed during cold treatment in both the young leaves and apexes of E1, E5, and E8 plants during 10 weeks cold-treatment at 5°C but was then reactivated to a considerable degree in both tissues by T14 in each genotype (Figure 19).

The transient down-regulation of *BoFLC3* during cold-treatment (Figure 18 & Figure 19) resembles the transient repression of *PEP1* in *Arabis alpina*. *PEP1* acts as a floral repressor and is an orthologue of Arabidopsis *FLC*. *PEP1* expression is repressed during vernalization of *A. alpina* in both the shoot apices and leaves. But *PEP1* expression is reactivated following a return to warmer conditions. *PEP1* contributes to the perennial, polycarpic life history or *A. alpina* by preventing flowering until after vernalization and limiting both the duration of flowering and the number of branches that flower following a cold treatment (Wang et al. 2009b). A pattern of seasonal cycles of flowering correlated with down-regulation and re-activation of an *FLC* homologue (*AhgFLC*) is also seen in the perennial *Arabidopsis halleri* (Aikawa et al. 2010).

In E5 and E9 substantial reactivation of *BoFLC3* occurred by T14, but the average time taken to form visible inflorescence buds is 20–30 days. This is not necessarily inconsistent with *BoFLC3* repressing flowering. The apex may have committed to flowering before T14 but taken longer to form visible inflorescence buds. Alternatively it may still be possible for the apex to switch to reproductive mode at or after T14 despite the degree of *BoFLC3* re-activation that has occurred. This seems unlikely though, as *BoFLC3* expression is greater at T28 than NV in E8 whereas other E8 plants grown in Experiment II and given only 6 weeks cold-treatment did not flower until after T28 (see chapters 2 & 3). As increasing durations of cold lead to increasingly stable repression of *FLC* in several vernalization-responsive accessions of Arabidopsis (Shindo et al. 2006), it seems likely that *BoFLC3* would be reactivated more rapidly following 6 weeks cold-treatment than 10 weeks cold-treatment. It seems most likely that the transition of the apex from vegetative to reproductive growth occurred before reactivation of *BoFLC3*.

4.3.4 *BoFLC4* expression is repressed during vernalization in E1, E5, E8, and E9 and the stability of this repression appears to correlate with differences in flowering time among these genotypes

BoFLC4 was strongly expressed in the young leaves of E1, E5, E8, and E9 before cold-treatment and repressed during 10 weeks cold-treatment at both 5 and 10°C but the stability of this repression varied (Figure 18). *BoFLC4* expression at T0 was lowest in E1 (at both 5 and 10°C), and higher in E5, E8, and E9 (at both 5 and 10 °C). *BoFLC4* expression at T0 was highest (though still much lower than at NV) in the E9 plants treated at 10°C. These were the only plants that did not form visible inflorescence buds within 100 days after the end of cold-treatment. The repression of *BoFLC4* expression remained stable in each genotype after cold-treatment at both 5 and 10°C until T14. The repression of *BoFLC4* expression was also fairly stable until T28 in E1. But *BoFLC4* expression increased considerably by T28 in E5 and especially in E8 and E9 at both temperatures. *BoFLC4* expression was also repressed during vernalization in the young leaves and apexes of the destructively sampled plants of E1, E5, and E8 (Figure 19). The repression of *BoFLC4* in the

apex also appears to have been stronger and more stable in E1 and E5 than in E8, although the differences among the genotypes are less clear than in the young leaves.

The variation in the degree and stability of repression of *BoFLC4* by cold-treatment among the genotypes appears to be correlated with the differences in flowering time among the genotypes (chapters 2 & 3, Table 14, and Figure 17). The E1 plants treated at both 5 and 10°C formed inflorescence buds several days before the plants of the other genotypes given the same treatments (Table 14). And E1 had the lowest *BoFLC4* expression at T0 with both cold-treatments. The repression of *BoFLC4* expression was also more stable in E1 than in any of the other genotypes, increasing only slightly by T28 (Figure 18). The E5 and E8 plants treated at 5 and 10°C and the E9 plants treated at 5°C all took longer to form visible inflorescence buds and all had higher *BoFLC4* expression at T0 and T14 than E1 (though still much lower than at NV). *BoFLC4* expression also increased considerably more by T28 in these plants than in E1. The E9 plants treated at 10°C did not form visible inflorescence buds and had the highest *BoFLC4* expression at T0 and T14 and very nearly the highest at T28.

The stable down-regulation of *BoFLC4* during vernalization (Figure 18 & Figure 19) resembles the stable down-regulation of *FLC* during vernalization in winter-annual Arabidopsis accessions (Michaels & Amasino 1999; Sheldon et al. 2000b). This suggests that *BoFLC4* may be playing a similar role to *FLC* in Arabidopsis i.e. repressing flowering until after vernalization. This hypothesis is further supported by the correlation that appears to exist between the stability of *BoFLC4* down-regulation and the time until inflorescence formation amongst the four PSB genotypes. In Arabidopsis accessions that only respond to long periods of cold, such as Lov-1, *FLC* expression is repressed by both long and short periods of cold but reactivated following short (e.g. 4 weeks) periods of cold that do not accelerate flowering. Longer durations of cold-treatment are required for stable *FLC* repression and flowering. In contrast in some Arabidopsis accessions, such as Edi-0, only short periods of cold are required for stable repression of *FLC* and flowering (Shindo et al. 2006). There appears to be some correlation between the extent of *BoFLC4* down-regulation during cold-treatment and the time till inflorescence formation in different PSB genotypes cold-treated for 10 weeks at 5 and 10°C. But the most striking

correlation is between the stability of this repression and the time to inflorescence formation. This suggests that *BoFLC4* may contribute to variation in vernalization response in PSB through differences in the stability of its repression.

Further evidence for the hypothesis that variation in the stability of *BoFLC4* silencing contribute to variation in vernalization response may come from an analysis of its expression in the plants cold-treated for only six weeks (chapters 2 & 3). If the stability of *BoFLC4* repression is important in determining the response to vernalization then I would expect *BoFLC4* to be stably repressed in E1 plants cold-treated for 6 weeks at 5 and 10°C (which formed visible inflorescence buds after cold-treatment, chapter 3). But I would not expect *BoFLC4* to be stably repressed in E5 or E9 plants cold-treated for 6 weeks at 5 and 10°C (which did not form visible inflorescence buds following these treatments). Conversely, the stability of *BoFLC4* down-regulation in these genotypes may be increased (compared to that observed in this study) if cold-treatment is extended beyond 10 weeks.

4.3.5 The initial expression levels of *BoFLC1*, *BoFLC3*, and *BoFLC4* do not appear to be associated with differences in vernalization requirement and response

The initial expression level of *BoFLC1* does not appear to correlate well with vernalization requirement or flowering time in PSB. E8 plants had far higher expression than E5 and E9 but formed inflorescences significantly before either of them (Figure 17, Figure 18, & Figure 19). There is also no clear pattern of NV *BoFLC3* or *BoFLC4* expression amongst the four genotypes under study (Figure 18). *BoFLC4* expression in NV plants is actually lowest in E9, the latest-flowering of the four genotypes (chapters 2 & 3, Figure 17). This suggests that the differences in vernalization requirement and response among these genotypes of PSB are not due to differences in the initial expression level of *BoFLC1*, *BoFLC3*, and *BoFLC4*.

The lack of any apparent correlation between *BoFLC* expression and vernalization requirement and response contrasts to the correlation of flowering time and responsiveness to vernalization with *BnFLC* expression levels among *Brassica napus* genotypes (Tadege et al. 2001). However, three varieties of cabbage with different vernalization requirements were found to have similar initial expression

levels of *BoFLC4-1* (Lin et al. 2005). But these genotypes varied in the degree to which *BoFLC* expression was reduced after vernalization. This supports the hypothesis that it is variation in the repression of *BoFLC* by vernalization rather than the initial expression levels of *BoFLC* that are important in determining vernalization response in *B. oleracea*.

4.3.6 *BoFLC* expression shows a similar pattern in both the apex and the young leaves, suggesting that both organs may be involved in vernalization as in Arabidopsis

The finding that *BoFLC1*, *BoFLC3*, and *BoFLC4* all have similar patterns of expression in the young leaves and apexes of E1, E5, E8, and E9 (Figure 18, & Figure 19) suggests that in studies of vernalization comparisons of *BoFLC* expression can be made based upon young leaf samples. This is advantageous because the young leaves can be repeatedly sampled from the same plants. Sampling the apex is destructive and so requires large numbers of additional plants to be grown so that some can be scored for flowering time. The effects of plant-to-plant variation may also be reduced by using young-leaf samples compared to destructive sampling, because the samples at each time-point come from the same individuals as are then scored for flowering time.

In Arabidopsis *FLC* acts to delay flowering before vernalization in both the leaves and apex, and vernalization represses *FLC* expression in both of these organs (Searle et al. 2006). *FLC* acts in the leaves to repress flowering by preventing the formation of two systemic signals, one involving *FT*, which is activated in the phloem by *CO* under long days (Searle et al. 2006). Both Arabidopsis and Brassicas are facultative long-day plants (Gregory & Hussey 1953; Axelsson et al. 2001; Jackson 2009; Amasino 2010). So *BoFLC*s could potentially act to repress flowering in the leaves by a similar mechanism. The results of this study show that *BoFLC3* and *BoFLC4* are expressed in both the young leaves and apex of four PSB genotypes before vernalization and are repressed in both organs during vernalization (Figure 18, & Figure 19). Therefore *BoFLC*s may also act in both the young leaves and apexes of PSB plants to repress flowering before vernalization.

The down-regulation of *BoFLC3* and *BoFLC4* in a similar manner in both the apex and young leaf (Figure 18, & Figure 19) suggests that vernalization is perceived in both tissues. Therefore the down-regulation of *FLC* in the young leaves of Arabidopsis (Searle et al. 2006; Finnegan & Dennis 2007) appears to have been conserved in PSB. This is in agreement with studies that have suggested young, expanding leaves in which cell division/DNA replication is occurring can be vernalized (Wellensiek 1961; Wellensiek 1962; Wellensiek 1964; Sung & Amasino 2004a; Finnegan & Dennis 2007). But my results do not prove that dividing cells or DNA replication is essential for an organ to perceive vernalization in PSB or for repression of *BoFLC* expression. They only show that the organs in which *BoFLC3* and *BoFLC4* are down-regulated share this feature. In mature leaves of Arabidopsis *FLC* was down-regulated but expression increased upon a return to warmer conditions (Finnegan & Dennis 2007). Further work is necessary to establish whether a similar pattern of down-regulation of *BoFLC* occurs in mature, fully expanded leaves of PSB and whether any such down-regulation is stable.

4.4 Conclusions

BoFLC3 and *BoFLC4* are expressed strongly after 70 days pre-growth in each of the PSB genotypes E1, E5, E8, and E9 and both are repressed during 10 weeks cold-treatment at 5 and 10°C. This repression is only transient for *BoFLC3*. But the extent and stability of down-regulation of *BoFLC4* appear to correlate with the vernalization requirements/responses of the genotypes. QTL for heading date in the field have been found in the *BoFLC4* regions in populations derived from these genotypes (Irwin et al, personal communication) and associated with *BoFLC4* (aka *BoFLC2*) in other *B. oleracea* genotypes (Okazaki et al. 2007). This supports the hypothesis that the observed expression differences are important in determining vernalization response. *BoFLC1* is only strongly expressed in E8 and its expression does not appear to be associated with differences in flowering time or vernalization response among the genotypes. However, this study only examines the expression of *BoFLC* mRNA. This may not always correlate with levels of the actual protein, and gives no information upon the function or potency of the protein. For firm conclusions about the role of these *FLC* homologues to be drawn further work will

be necessary to prove that the proteins formed are still functional floral repressors, for example by over-expression of these genes in Arabidopsis and/or PSB.

5 General discussions and suggestions for future work

5.1 Juvenility

The chronological duration of the juvenile (insensitive to vernalization) phase in PSB depends upon environmental conditions as it does in cauliflower (Hand & Atherton 1987) so hypothesis 1 is accepted. Hypothesis 2 is accepted because the chronological duration of the juvenile phase also varies among the different genotypes of PSB. I have established the minimum numbers of visible leaves by which each of the four PSB genotypes under study reached the adult-vegetative phase when grown in the glasshouse, as stated in aim 1. The leaf number at the phase change varies among genotypes of PSB as it does in cauliflower (Hand & Atherton 1987; Wurr et al. 1988; Wurr et al. 1993; Wurr et al. 1994), being greater in E9 than in E1, E5, and E8. Although the transition to the adult vegetative phase appears to be gradual in PSB there was not a significant change in the response of PSB plants to vernalization (measured in DTI) with plant age after the vegetative phase change. Therefore if plants of these PSB genotypes are pre-grown in warm glasshouses until they have at least these minimum numbers of leaves (beyond which there was no significant effect of pre-growth period upon DTI) then the confounding effects of juvenility can be excluded from vernalization experiments.

Juvenility in vernalization response in *A. alpina* is associated with the failure to restrict *TFL1* expression in the apexes of juvenile plants and not with a failure to repress *AaFLC* (a homologue of Arabidopsis *FLC* that acts as a floral repressor) (Wang 2007). Lin et al. (2005) found that *BoFLC* expression was repressed during vernalization of adult cabbage plants but not imbibed seeds. However, this does not exclude the possibility that *BoFLC* expression would be repressed during cold-treatment of juvenile cabbage plants. The QRT-PCR analyses in chapter 4 show that at least two Brassica homologues of Arabidopsis *FLC*, *BoFLC3* and *BoFLC4*, are down-regulated during vernalization of adult PSB plants. This analysis should be repeated with juvenile plants e.g. the E9 plants pre-grown for only 50 days in experiment II. If juvenility in vernalization response is controlled similarly in PSB and *A. alpina* then I would expect *BoFLC3* and *BoFLC4* expression to be repressed

in the young leaves and apexes of juvenile as well as adult PSB plants. Further studies should also be carried out to test whether *TFL1* homologues in Brassicas such as PSB show different expression patterns during vernalization in juvenile and adult plants, as *AaTFL1* does, and so may be involved in controlling juvenility in vernalization response.

5.2 Vernalization response

The PSB genotypes used in this study show similar variation to Arabidopsis accessions in both their qualitative vernalization requirements and quantitative vernalization responses. Hypothesis 3 is accepted because E1 has a facultative vernalization requirement. That is it flowers without cold-treatment but still shows a strong quantitative response to even short periods of vernalization. This resembles the situation in the Arabidopsis accession Edi-0 (Shindo et al. 2006). Hypothesis 4 is accepted because E5, E8, and E9 all have obligate vernalization requirements. E5, E8, and E9 also all require longer periods of cold-treatment than E1 to be fully vernalized. This resembles the Arabidopsis accession Lov-1, which does not flower within 120 leaves without cold-treatment and requires longer periods of cold-treatment than Edi-0 in order for vernalization to occur (Shindo et al. 2006).

All four genotypes of PSB studied can be vernalized effectively at 5°C and E1, E5, and E8 can be vernalized at 10°C. This is in agreement hypothesis 5 and with reports of an optimum temperature for vernalization of 4–10°C in other *Brassica oleracea* varieties (Thomas 1980; Wurr et al. 1993; Guo et al. 2004). But E1 (and likely E5, E8, and E9) cannot be vernalized at 15°C, contrary to hypothesis 5. This is lower than the maximum temperatures for vernalization of 21°C and 23.6°C that were estimated in cauliflower and calabrese by Wurr et al. (1993; 1995). The maximum temperature for vernalization appears to be between 5 and 10°C in E9, contrary to hypothesis 5, but a more recent study suggests that E9 actually responds to cold-treatment at 10°C. Due to the limited number of treatments used in this study it has not been possible to determine the maximum effective temperature for vernalization in E5 and E8 or the minimum effective temperature in any of the genotypes.

All four genotypes can be vernalized by 10 weeks cold-treatment as stated in hypothesis 6. E1 can also be vernalized by 6 weeks cold-treatment and E8 can be at leasty partially vernalized by 6 weeks cold-treatment. But E5 and E9 showed no flowering response to 6 weeks cold-treatment, contrary to hypothesis 6.

I have established that 10 weeks of cold-treatment at 5°C (under 8-hour photoperiods) is effective for vernalization in E1, E5, E8, and E9, fulfilling aim 2.

5.3 BoFLC *expression*

I have developed a QRT-PCR assay to measure the expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in the PSB genotypes under study, as stated in aim 3. I have shown that these assays are specific to each homologue of *BoFLC*. All three of these homologues are repressed during vernalization for 10 weeks at 5 and 10°C in each genotype studied. But the extent and stability of this repression (and the expression level of *BoFLC1*) varies between homologues and genotypes. So hypothesis 7 is only partially accepted. This variation may reflect divergence or specialization in the functions of these homologues. The pattern of expression of each of these *BoFLC* homologues was similar in both the young leaves and the apexes in E1, E5, and E8 as stated in hypothesis 8, although it was not possible to test this in E9.

The transient repression of *BoFLC3* during vernalization resembles the unstable repression of the *FLC* homologue *PEP1* in the perennial plant *Arabis alpina*. In *A. alpina* the reactivation of *PEP1* expression following vernalization limits both the number of shoots that flower and the duration of flowering, allowing a reversion to vegetative growth (Wang et al. 2009b). This contributes to the perennial, polycarpic life history of *A. alpina*. However, it is not clear what role the reactivation of *BoFLC3* could play in PSB, which is biennial.

The pattern of variation in the stability of *BoFLC4* repression during cold-treatment of these PSB genotypes resembles that seem for *FLC* in the Arabidopsis accessions Edi-0 and Lov-1. In E1 *BoFLC4* is stably repressed in the young leaves during 10 weeks cold-treatment. This is similar to the situation in the Arabidopsis accession Edi-0, which like E1 has a facultative vernalization requirement and responds to short periods of cold. *BoFLC4* is also repressed in E5, E8, and E9 but this repression

is less stable. This resembles the unstable repression of *FLC* in Lov-1, which requires longer periods of cold than Edi-0 for stable *FLC* repression and acceleration of flowering. Similarly, E5, E8, and E9 all appear to require longer periods of cold-treatment than E1 to be vernalized. This suggests that the role of Arabidopsis *FLC* as a floral repressor and in the control of variation in vernalization response has been conserved in *BoFLC4*. This hypothesis is supported by other studies that have found QTL for vernalization response/flowering time associated with *BoFLC4*(/*BoFLC2*) (Irwin, personal communication; Okazaki et al. 2007) and the finding of Lin et al. (2005) that *BoFLC4* was repressed during vernalization of adult cabbage plants. It would be interesting to investigate whether, as in Lov-1, increasing durations of cold-treatment lead to progressively more stable repression of *BoFLC4* in E5, E8, and E9 as the vernalization response becomes saturated. It would also be interesting to investigate whether *BoFLC4* repression is even less stable in these genotypes following shorter periods of cold-treatment, such as the 6-week cold-treatments in experiment II.

BoFLC3 and *BoFLC4* appear to be repressed similarly in both the young leaves and the apex during vernalization of PSB, like Arabidopsis *FLC* (Searle et al. 2006). But further studies will be needed to provide firm evidence for this hypothesis. *BoFLC* expression should be examined during cold-treatment in a greater number of biological replicates and with a range of treatments, ideally including some that are ineffective for vernalization. This will confirm whether it is adequate to monitor *BoFLC* expression in only the young leaves in future experiments as this study suggests. This has the potential to save significant time and resources as well as reducing the effects of plant-to-plant variation associated with destructively sampling multiple plants at different time-points. It would also be interesting to extend the analysis of *BoFLC* expression to the mature leaves of PSB, as in Arabidopsis *FLC* is repressed during vernalization in mature leaves but this repression is unstable upon a return to warmer conditions (Finnegan & Dennis 2007).

QTL for vernalization-responsive flowering time have previously been identified that map to regions of the genome close to or including *BoFLC3* and *BoFLC4* in doubled-haploid populations derived from the PSB genotypes used in this study.

The finding that both of these genes are repressed during vernalization, and that the stability of the repression of *BoFLC4* appears to be associated with variation in vernalization response, provides further evidence that these genes constitute candidates for these QTL. Genotyping these populations for *BoFLC3* and *BoFCL4* may also provide further evidence that these genes are involved in controlling vernalization response e.g. if the flowering times of the population correlated with the segregation of *BoFLC3* or *BoFLC4* alleles from the parents.

The lack of any clear correlation between *BoFLC1* expression and flowering time suggests that this gene does not play a significant role in vernalization. This hypothesis is supported by the lack of reported QTL for flowering time/vernalization response associated with this gene in any study to my knowledge. But this does not prove that *BoFLC1* is not involved in controlling vernalization response. Based upon the partial sequence available for the PSB genotypes used in this study *BoFLC5* appears to be a pseudogene as it is in the *B. oleracea* genotype A12 (Irwin, personal communication(Razi et al. 2008)). But the full sequence for *BoFLC5* should be cloned from the PSB genotypes used in this study to confirm whether it is entirely identical to A12 (and thus can be regarded as a pseudogene) or may play a role in controlling vernalization in PSB.

Although this study has shown an apparent correlation between the repression of *BoFLC3* and *BoFLC4* and vernalization, and the stability of *BoFLC4* repression and vernalization response, further work will be necessary to confirm the involvement of these genes in controlling vernalization response. The QRT-PCR assay in this study is only a preliminary investigation, and should be extended to include a greater number of biological replicates. Ideally, a screen of several putative reference genes should also be carried out using GeNorm (Vandesompele et al. 2002) to ensure that the most reliable normalization strategy possible is adopted. However, QRT-PCR only measures mRNA levels and gives no information on the function or potency of a protein. Each of the *BoFLC* homologues should also be constitutively over-expressed in *Brassica oleracea* and/or Arabidopsis. If a *BoFLC* homologue functions as a floral repressor, then over-expressing it would be expected to delay flowering. However, vernalization may be able to overcome this delay. Therefore it may be ideal to over-express *BoFLC*s in E1 (as this genotype flowers without

vernalization) or in a rapid cycling genotype of *B. oleracea* such as A12. This would allow the effect of over-expression of each *BoFLC* on flowering time in PSB to be assessed both with and without vernalization.

5.4 Links to agriculture

When the PSB genotypes used in this study were grown in field experiments E1 was the earliest to flower, followed by E5, then E8, with E9 taking the longest to flower (Irwin & Kennedy, personal communication). The results of this study suggest that this could be due to variation among these genotypes in three factors: the duration of the juvenile phase, the duration of cold-treatment/number of vernalizing degree days (V°CD) or effective vernalization days (VD) required for vernalization, and the cardinal temperatures for vernalization. E1 has the shortest juvenile phase of the four genotypes (in terms of chronological duration), followed by E5 and E8 with E9 having the longest. Therefore vernalization can start sooner after sowing in E1 than the other genotypes. E1 is also the only genotype that responded significantly more strongly to 10 weeks cold-treatment at 10°C than at 5°C in experiment II. This suggests that E1 has a higher T_{OPT} for vernalization than the other genotypes and especially E9, which did not form visible inflorescence buds following cold-treatment at 10°C. Therefore under the same environmental conditions E1 may accumulate V°C D/VD at a faster rate than the other genotypes. E1 can also be strongly vernalized (i.e. all plants induced to flower) with only 6 weeks cold-treatment. E8 requires 10-weeks of cold-treatment for full vernalization (i.e. for all plants to flower), and E5 and E9 require this long for any flowering to occur. Therefore these genotypes appear to require more V°C D/VD than E1. So the variation in flowering time in the field may be due to the later-flowering genotypes starting to accumulate V°C D later, accumulating them less rapidly, and requiring more in order to be vernalized.

The existence of variation among genotypes in the length of the juvenile phase, the cardinal temperatures for vernalization, and the duration of cold required for vernalization may provide the basis for breeding varieties with a range of flowering times and lines in which development is less sensitive to temperature. This could helpto extend the growing season of PSB in the UK, as well as the regions where it can be grown. The development of varieties that are less sensitive to temperature, as

well as a better knowledge of juvenility and the effect of different temperatures and durations of cold, could also help to better predict harvest date. This could help to reduce waste caused by gluts in production. The finding that E1, E5, and E8 can be vernalized as effectively (if not more so) at 10°C as at 5°C is particularly significant in the context of climate change, which could lead to warmer winters in the UK. A greater understanding of what conditions are effective for vernalization and of juvenility may also help to plan plantings better to achieve a more continuous supply.

Knowing the duration of the juvenile phase and the conditions that are effective for vernalization (10 weeks at 5°C for all genotypes in this study) may also allow plants to be artificially vernalized in controlled environments. E.g. in experiment II the E1, E5, and E8 plants sown in June and cold-treated under short days for 10 weeks at 5°C flowered soon after return to the glasshouse in September. There are already varieties of PSB that are sown in spring to head in late autumn/winter and others that head in February–May. Artificial vernalization in controlled environments could extend the harvest season through the summer (when the daylength in the field would be long enough to promote flowering). PSB plants are already grown in glasshouses and transplanted to the field (Irwin et al. personal communication). Growing them to the end of the juvenile phase in a warm glasshouse and artificially vernalizing them could help to ensure reliable production even out of season if commercially viable.

The knowledge of the duration of the insensitive juvenile phase in terms of leaf number has already been used by Irwin et al. to help ensure that plants for QTL experiments are competent to respond to vernalization before they are transferred to the cold. This has helped to ensure that the variation in flowering time observed (and hence the QTL mapped) is due to variation in vernalization response rather than vernalization in juvenility. The greater understanding of variation in juvenility in vernalization response, the range of effective temperatures, and the durations of cold required could also aid breeding of new PSB varieties, when it is important that both varieties flower at the same time.

The insight into the molecular basis of vernalization in PSB gained in this study may also be applicable to other varieties of *Brassica oleracea* that require vernalization.

For example, in cauliflower unpredictability of supply leads to substantial losses in the UK.

6 Appendixes

Appendix 1: Full data for the time taken to form visible inflorescence buds (DTI, from sowing) for all E1 plants from experiment I

Treatment duration	Treatment temperature	DTI (from sowing)				
		Rep 1	Rep 2	Rep 3	Mean	95% CI
6 weeks	5	110	116	118	114.67	4.71
	10	127	117	124	122.67	5.81
	15	229	292	311	277.33	48.57
10 weeks	5	130	131	n/a	130.50	0.98
	10	126	142	n/a	134.00	15.68
	15	DNF	DNF	n/a	DNF	DNF
NV	n/a	253	220	220	231.00	21.56

Appendix 2: Full data for leaf numbers before and after cold-treatment and the time taken to form visible inflorescence buds (DTI, from end of cold-treatment) for all plants from experiment II

"Plant no." is the reference number assigned to each plant within each sowing for identification purposes, it has no other significance. "Leaves at preV" is the number of visible leaves the day before cold-treatment began. "Weeks cold" and "temp" are the duration and temperature of the cold-treatment. "Leaves at T0" is the number of visible leaves at the end of cold-treatment. "DTI" is the number of days from the end of cold-treatment to the first appearance of visible inflorescence buds at the apex. "DNF" stands for "did not flower" and refers to plants that did not form visible inflorescence buds at any time during the experiment. (All plants were kept for at least 100 days from the end of cold-treatment). DNFs and plants for which DTI was greater than 100 days were either excluded from the data or given an arbitrary value of 100 DTI before the construction of graphs and performance of statistical analyses, as described in the final column.

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons
70	10	10	E1	3	25	36	1	Included (visible buds formed whilst in cold-treatment)
70	10	10	E1	4	25	36	1	Included (visible buds formed whilst in cold-treatment)
70	10	10	E1	5	25	33	2	Included
70	10	10	E1	6	24	34	1	Included (visible buds formed whilst in cold-treatment)
70	10	10	E1	13	19	25	9	Included
70	10	5	E1	1	24	28	8	Included
70	10	5	E1	9	23	27	7	Included
70	10	5	E1	11	24	31	5	Included
70	10	5	E1	12	24	28	6	Included
70	10	5	E1	15	21	25	6	Included
70	10	10	E5	2	17	22	19	Included
70	10	10	E5	3	17	21	21	Included
70	10	10	E5	4	16	20	34	Included
70	10	10	E5	6	16	20	23	Included
70	10	10	E5	7	17	20	28	Included
70	10	5	E5	8	17	19	26	Included
70	10	5	E5	10	16	19	32	Included
70	10	5	E5	12	16	20	25	Included
70	10	5	E5	13	16	19	25	Included
70	10	5	E5	17	16	20	24	Included

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons	
70	10	10	E8	8	13	16	7	Included	
70	10	10	E8	9	13	16	13	Included	
70	10	10	E8	10	13	16	10	Included	
70	10	10	E8	12	13	16	13	Included	
70	10	10	E8	18	13	17	13	Included	
70	10	5	E8	1	13	14	12	Included	
70	10	5	E8	2	13	15	12	Included	
70	10	5	E8	5	12	14	14	Included	
70	10	5	E8	11	13	15	11	Included	
70	10	5	E8	15	13	15	12	Included	
70	10	10	E9	1	18	22	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	10	10	E9	5	22	27	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	10	10	E9	6	23	27	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	10	5	E9	2	19	23	28	Included	
70	10	5	E9	3	21	25	28	Included	
70	10	5	E9	4	22	25	40	Included	

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons
60	10	10	E1	1	20	28	4	Included
60	10	10	E1	2	21	27	1	Included (visible buds formed whilst in cold-treatment)
60	10	10	E1	9	21	27	2	Included
60	10	10	E1	17	21	26	9	Included
60	10	5	E1	4	22	26	6	Included
60	10	5	E1	5	22	27	6	Included
60	10	5	E1	8	20	23	9	Included
60	10	5	E1	12	20	26	4	Included
60	10	5	E1	16	19	25	9	Included
60	10	10	E5	3	14	19	21	Included
60	10	10	E5	8	15	18	21	Included
60	10	10	E5	9	14	19	20	Included
60	10	10	E5	11	14	17	28	Included
60	10	10	E5	17	14	18	25	Included
60	10	5	E5	2	13	16	28	Included
60	10	5	E5	10	13	17	20	Included
60	10	5	E5	15	13	16	28	Included
60	10	5	E5	16	14	16	28	Included
60	10	5	E5	18	14	16	31	Included

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons	
60	10	10	E8	1	11	15	16	Included	
60	10	10	E8	9	11	15	13	Included	
60	10	10	E8	13	11	14	21	Included	
60	10	10	E8	14	9	12	DNF	Excluded	Low preV leaf number
60	10	10	E8	15	11	14	27	Included	
60	10	5	E8	3	11	13	17	Included	
60	10	5	E8	4	11	14	14	Included	
60	10	5	E8	7	11	13	15	Included	
60	10	5	E8	10	11	13	13	Included	
60	10	5	E8	17	11	13	DNF	Excluded	Anomalous
60	10	10	E9	3	13	16	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	10	10	E9	6	12	16	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	10	10	E9	7	15	19	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	10	5	E9	1	16	20	31	Included	
60	10	5	E9	4	14	18	DNF	Excluded	Low preV leaf number
60	10	5	E9	8	16	18	42	Included	

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons	
50	10	10	E1	2	17	25	5	Included	
50	10	10	E1	3	17	24	3	Included	
50	10	10	E1	6	16	23	4	Included	
50	10	10	E1	7	17	25	4	Included	
50	10	10	E1	13	15	23	5	Included	
50	10	5	E1	1	16	19	8	Included	
50	10	5	E1	8	15	20	10	Included	
50	10	5	E1	11	14	18	11	Included	
50	10	5	E1	14	15	18	9	Included	
50	10	5	E1	15	16	21	8	Included	
50	10	10	E5	8	10	16	31	Included	
50	10	10	E5	9	11	16	24	Included	
50	10	10	E5	11	10	16	46	Included	
50	10	10	E5	13	10	14	DNF	Excluded	Anomalous
50	10	10	E5	17	11	16	22	Included	
50	10	5	E5	1	10	13	DNF	Excluded	Anomalous
50	10	5	E5	4	10	14	32	Included	
50	10	5	E5	5	11	14	35	Included	
50	10	5	E5	10	11	14	32	Included	
50	10	5	E5	16	11	*	28	Included	

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons	
50	10	10	E8	2	9	12	20	Included	
50	10	10	E8	4	9	12	37	Included	
50	10	10	E8	6	9	13	36	Included	
50	10	10	E8	8	9	13	27	Included	
50	10	10	E8	9	8	11	DNF	Excluded	Low preV leaf number
50	10	5	E8	1	9	11	DNF	Excluded	Low preV leaf number
50	10	5	E8	3	9	12	DNF	Excluded	Low preV leaf number
50	10	5	E8	5	9	11	34	Included	
50	10	5	E8	10	9	11	20	Included	
50	10	5	E8	11	9	12	32	Included	
50	10	5	E9	1	10	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	10	5	E9	2	11	15	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	10	5	E9	3	11	15	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons	
70	6	10	E1	2	23	26	11	Included	
70	6	10	E1	10	21	26	14	Included	
70	6	10	E1	11	25	28	13	Included	
70	6	10	E1	14	23	27	10	Included	
70	6	10	E1	15	22	25	14	Included	
70	6	5	E1	3	24	25	16	Included	
70	6	5	E1	7	23	25	17	Included	
70	6	5	E1	8	23	26	16	Included	
70	6	5	E1	12	25	28	14	Included	
70	6	10	E5	1	18	20	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	10	E5	5	18	21	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	10	E5	6	17	17	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	10	E5	11	17	19	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	10	E5	17	17	19	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E5	2	18	19	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E5	10	17	18	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E5	12	17	17	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E5	13	17	18	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E5	19	17	18	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons	
70	6	10	E8	2	15	17	62	Included	
70	6	10	E8	4	14	17	63	Included	
70	6	10	E8	11	14	17	127	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	10	E8	14	14	17	90	Included	
70	6	10	E8	16	14	16	143	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E8	9	14	15	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E8	10	14	15	73	Included	
70	6	5	E8	15	13	15	50	Included	
70	6	5	E8	17	14	16	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E8	18	14	15	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	10	E9	1	20	23	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	10	E9	4	16	19	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	10	E9	5	20	23	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E9	2	20	21	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E9	3	19	21	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
70	6	5	E9	6	17	18	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons	
60	6	10	E1	1	21	22	15	Included	
60	6	10	E1	5	20	22	19	Included	
60	6	10	E1	6	19	22	14	Included	
60	6	10	E1	17	20	22	12	Included	
60	6	5	E1	7	20	22	14	Included	
60	6	5	E1	9	20	21	14	Included	
60	6	5	E1	13	20	21	21	Included	
60	6	10	E5	1	14	15	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	10	E5	6	14	*	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	10	E5	7	14	16	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	10	E5	9	15	16	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	10	E5	15	15	17	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E5	4	14	15	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E5	8	15	15	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E5	12	11	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E5	14	14	14	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E5	17	13	14	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons	
60	6	10	E8	4	11	13	37	Included	
60	6	10	E8	7	10	11	DNF	Excluded	Anomalous
60	6	10	E8	10	11	13	43	Included	
60	6	10	E8	14	12	14	56	Included	
60	6	10	E8	15	12	14	46	Included	
60	6	5	E8	5	11	12	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E8	6	11	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E8	8	11	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E8	16	11	12	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E8	18	12	14	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	10	E9	1	14	17	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	10	E9	4	15	18	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	10	E9	9	17	20	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E9	2	16	18	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E9	3	17	18	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
60	6	5	E9	7	14	16	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons	
50	6	10	E1	3	16	19	14	Included	
50	6	10	E1	5	13	*	14	Included	
50	6	10	E1	8	13	15	18	Included	
50	6	10	E1	13	14	17	16	Included	
50	6	5	E1	1	16	18	20	Included	
50	6	5	E1	4	15	17	19	Included	
50	6	5	E1	7	15	17	18	Included	
50	6	5	E1	9	13	16	18	Included	
50	6	5	E1	10	14	16	24	Included	
50	6	10	E5	3	12	14	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E5	4	10	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E5	7	10	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E5	8	10	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E5	9	11	14	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E5	5	11	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E5	6	11	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E5	10	11	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E5	14	11	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E5	15	11	12	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective

Appendix 2 cont

Days pre-growth	Weeks cold	Temp (°C)	Genotype	Plant No.	Leaves at preV	Leaves at T0	DTI	DTI included/excluded/arbitrary value and reasons	
50	6	10	E8	2	8	10	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E8	6	9	10	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E8	18	9	11	82	Included	
50	6	10	E8	19	9	10	134	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E8	20	8	10	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E8	8	8	9	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E8	9	8	10	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E8	10	8	10	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E8	16	9	11	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E8	17	8	9	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E9	1	12	14	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E9	2	11	15	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E9	4	11	14	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	10	E9	6	11	13	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E9	3	12	*	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E9	5	13	15	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective
50	6	5	E9	8	10	12	DNF	Arbitrary value of 100 DTI	Treatment appears ineffective

Appendix 3: Calculation of the proportion of variation accounted for by each factor in ANOVA

For a two way ANOVA for the main effects A and B and the interaction effect of A*B the expected means squares (EMS, σ^2_A, σ^2_B, σ^2_{AB}, and σ^2 respectively) can be calculated from the equations:

$MS_A = \sigma^2 + n\sigma^2_{AB} + nb\sigma^2_A$

$MS_B = \sigma^2 + n\sigma^2_{AB} + na\sigma^2_B$

$MS_{AB} = \sigma^2 + n\sigma^2_{AB}$

$Error = \sigma^2$

Where *n* is the harmonic mean of the number of replicates in each treatment, a is the number of treatments for effect A, b is the number of treatments for effect B, MS_A = the mean squares of A from the ANOVA table, MS_B = the mean squares of B from the ANOVA table, MS_{AB} = the mean squares of A*B from the ANOVA table, and Error = residual mean squares from the ANOVA table. These equations can be rearranged to:

$\sigma^2_A = (MS_A - \sigma^2 - n\sigma^2_{AB})/ nb = (MS_A - \sigma^2 - (MS_{AB} - \sigma^2))/ nb$

$\sigma^2_B = (MS_B - \sigma^2 - n\sigma^2_{AB})/ na = (MS_B - \sigma^2 - (MS_{AB} - \sigma^2))/ na$

$\sigma^2_{AB} = (MS_{AB} - \sigma^2)/n$

$\sigma^2_{ERROR} = \sigma^2$

The proportion of variation accounted for by each effect and by the residual error can then be estimated by dividing the EMS for that effect by the sum of the EMS for both main effects, the interaction effect, and the error and multiplying the result by 100%.

Appendix 4: Output tables from the ANOVAs described in chapter 2 for the effects of genotype, pre-growth period, and the interaction of genotype and pre-growth period upon the number of days taken to form visible inflorescence buds (DTI) following 10 weeks cold-treatment at 5°C with 50, 60, and 70 days pre-growth and upon the number of visible leaves before and at the end of cold-treatment (experiment II)

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Genotype	3		14136.16	4712.05	387.90	<.001
Pregrowth	2		3619.43	1809.71	148.98	<.001
Genotype.Pregrowth	6		5951.16	991.86	81.65	<.001
Residual	37	(5)	449.47	12.15		
Total	48	(5)	23822.49			

Appendix 4 a: ANOVA for the effects of genotype, pre-growth period, and the interaction of these factors (genotype.pregrowth) upon DTI following 10 weeks cold-treatment at 5°C after 50, 60, and 70 days pre-growth among E1, E5, E8, and E9 (experiment II).

Sourceofvariation	d.f.	s.s.	m.s.	v.r.	Fpr.
Genotype	3	614.0593	204.6864	298.50	<.001
Pregrowth	2	381.8148	190.9074	278.41	<.001
Genotype.Pregrowth	6	49.4741	8.2457	12.02	<.001
Residual	42	28.8000	0.6857		
Total	53	1074.1481			

Appendix 4 b: ANOVA for the effects of genotype, pre-growth period, and the interaction of these factors (genotype.pregrowth) upon leaf number at the end of the pre-growth period among E1, E5, E8, and E9 plants pre-grown for 50, 60, and 70 days (experiment II).

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Genotype	3		976.064	325.355	273.63	<.001
Pregrowth	2		383.503	191.752	161.27	<.001
Genotype.Pregrowth	6		70.328	11.721	9.86	<.001
Residual	41	(1)	48.750	1.189		
Total	52	(1)	1459.472			

Appendix 4 c: ANOVA for the effects of genotype, pre-growth period, and the interaction of these factors (genotype.pregrowth) upon leaf number at the end of 10 weeks cold treatment at 5°C among E1, E5, E8, and E9 plants pre-grown for 50, 60, and 70 days (experiment II).

Appendix 5: Output tables from the ANOVAs described in chapter 3 for the effects of temperature, genotype, duration of cold-treatment, and the interactions among these effects upon the number of days taken to form visible inflorescence buds (DTI) following cold-treatment for 6 and 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth (experiment II)

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Genotype	3		50093.3	16697.8	130.80	<.001
Temp	1		574.4	574.4	4.50	0.037
Genotype.Temp	3		5770.4	1923.5	15.07	<.001
Residual	88	(8)	11234.4	127.7		
Total	95	(8)	66288.0			

Appendix 5 a: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, E8, and E9 plants cold-treated for 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Genotype	3		20140.03	6713.34	202.04	<.001
Temp	1		18.18	18.18	0.55	0.468
Genotype.Temp	2		51.24	25.62	0.77	0.475
Residual	21	(5)	697.77	33.23		
Total	27	(5)	20898.68			

Appendix 5 b: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, E8, and E9 plants cold-treated for 10 weeks at 5 and 10°C with 50 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Genotype	3		15265.24	5088.41	346.15	<.001
Temp	1		934.44	934.44	63.57	<.001
Genotype.Temp	3		5222.22	1740.74	118.42	<.001
Residual	24	(3)	352.80	14.70		
Total	31	(3)	21502.47			

Appendix 5 c: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, E8, and E9 plants cold-treated for 10 weeks at 5 and 10°C with 60 days pre-growth.

Sourceofvariation	d.f.	s.s.	m.s.	v.r.	Fpr.
Genotype	3	15825.40	5275.13	398.34	<.001
Temp	1	841.00	841.00	63.51	<.001
Genotype.Temp	3	6134.80	2044.93	154.42	<.001
Residual	28	370.80	13.24		
Total	35	23172.00			

Appendix 5 d: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, E8, and E9 plants cold-treated for 10 weeks at 5 and 10°C with 70 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Genotype	2		3728.60	1864.30	50.76	<.001
Temp	1		18.12	18.12	0.49	0.491
Genotype.Temp	2		51.34	25.67	0.70	0.509
Residual	19	(5)	697.77	36.72		
Total	24	(5)	4147.84			

Appendix 5 e: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, and E8 plants cold-treated for 10 weeks at 5 and 10°C with 50 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Genotype	2		1800.54	900.27	64.68	<.001
Temp	1		3.42	3.42	0.25	0.625
Genotype.Temp	2		104.62	52.31	3.76	0.040
Residual	21	(2)	292.30	13.92		
Total	26	(2)	2189.41			

Appendix 5 f: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, and E8 plants cold-treated for 10 weeks at 5 and 10°C with 60 days pre-growth.

Sourceofvariation	d.f.	s.s.	m.s.	v.r.	Fpr.
Genotype	2	2305.40	1152.70	100.67	<.001
Temp	1	30.00	30.00	2.62	0.119
Genotype.Temp	2	9.80	4.90	0.43	0.657
Residual	24	274.80	11.45		
Total	29	2620.00			

Appendix 5 g: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, and E8 plants cold-treated for 10 weeks at 5 and 10°C with 70 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Genotype	3		121797.8	40599.3	351.87	<.001
Temp	1		922.8	922.8	8.00	0.006
Genotype.Temp	3		1525.7	508.6	4.41	0.006
Residual	95	(1)	10961.3	115.4		
Total	102	(1)	135205.7			

Appendix 5 h: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, E8, and E9 plants cold-treated for 6 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	s.s.	m.s.	v.r.	Fpr.
Genotype	3	44794.48	14931.49	1417.23	<.001
Temp	1	38.53	38.53	3.66	0.066
Genotype.Temp	3	34.96	11.65	1.11	0.363
Residual	28	295.00	10.54		
Total	35	45162.97			

Appendix 5 i: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, E8, and E9 plants cold-treated for 6 weeks at 5 and 10°C with 50 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Genotype	3		34727.99	11576.00	1121.77	<.001
Temp	1		2336.43	2336.43	226.41	<.001
Genotype.Temp	3		5092.23	1697.41	164.49	<.001
Residual	24	(1)	247.67	10.32		
Total	31	(1)	41576.47			

Appendix 5 j: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, E8, and E9 plants cold-treated for 6 weeks at 5 and 10°C with 60 days pre-growth.

Sourceofvariation	d.f.	s.s.	m.s.	v.r.	Fpr.
Genotype	3	44275.7	14758.6	112.91	<.001
Temp	1	15.0	15.0	0.11	0.737
Genotype.Temp	3	16.3	5.4	0.04	0.988
Residual	27	3529.1	130.7		
Total	34	47836.2			

Appendix 5 k: ANOVA for the effects of genotype, temperature, and the interaction of these factors (genotype.temp) upon DTI among the E1, E5, E8, and E9 plants cold-treated for 6 weeks at 5 and 10°C with 70 days pre-growth.

Sourceofvariation	d.f.	s.s.	m.s.	v.r.	Fpr.
Temp	1	143.407	143.407	21.76	<.001
Wks_vern	1	1426.515	1426.515	216.43	<.001
Temp.Wks_vern	1	0.521	0.521	0.08	0.780
Residual	50	329.557	6.591		
Total	53	1900.000			

Appendix 5 l: ANOVA for the effects of temperature, duration of cold-treatment (wks_vern), and the interaction of these factors (temp.wks_vern) upon DTI among the E1 plants cold-treated for 6 and 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Temp	1		18.39	18.39	1.11	0.297
Wks_vern	1		79856.80	79856.80	4820.10	<.001
Temp.Wks_vern	1		18.39	18.39	1.11	0.297
Residual	54	(2)	894.64	16.57		
Total	57	(2)	78032.09			

Appendix 5 m: ANOVA for the effects of temperature, duration of cold-treatment (wks_vern), and the interaction of these factors (temp.wks_vern) upon DTI among the E5 plants cold-treated for 6 and 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Temp	1		901.3	901.3	3.58	0.064
Wks_vern	1		68654.6	68654.6	273.01	<.001
Temp.Wks_vern	1		1513.1	1513.1	6.02	0.018
Residual	50	(6)	12573.6	251.5		
Total	53	(6)	76827.5			

Appendix 5 n: ANOVA for the effects of temperature, duration of cold-treatment (wks_vern), and the interaction of these factors (temp.wks_vern) upon DTI among the E8 plants cold-treated for 6 and 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Temp	1		3625.1	3625.1	12.52	0.001
Wks_vern	1		4201.9	4201.9	14.51	<.001
Temp.Wks_vern	1		3501.6	3501.6	12.09	0.002
Residual	29	(1)	8397.9	289.6		
Total	32	(1)	18773.0			

Appendix 5 o: ANOVA for the effects of temperature, duration of cold-treatment (wks_vern), and the interaction of these factors (temp.wks_vern) upon DTI among the E9 plants cold-treated for 6 and 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	s.s.	m.s.	v.r.	Fpr.
Temp	1	73.391	73.391	9.35	0.006
Residual	23	180.609	7.853		
Total	24	254.000			

Appendix 5 p: ANOVA for the effect of temperature upon DTI among the E1 plants cold-treated for 6 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	s.s.	m.s.	v.r.	Fpr.
Temp	1	105.880	105.880	19.19	<.001
Residual	27	148.948	5.517		
Total	28	254.828			

Appendix 5 q: ANOVA for the effect of temperature upon DTI among the E1 plants cold-treated for 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Temp	1		36.77	36.77	1.07	0.311
Residual	26	(2)	894.64	34.41		
Total	27	(2)	928.96			

Appendix 5 r: ANOVA for the effect of temperature upon DTI among the E5 plants cold-treated for 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Temp	1		2375.0	2375.0	5.95	0.022
Residual	27	(1)	10780.7	399.3		
Total	28	(1)	13073.8			

Appendix 5 s: ANOVA for the effect of temperature upon DTI among the E8 plants cold-treated for 6 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Temp	1		39.47	39.47	0.51	0.484
Residual	23	(5)	1792.90	77.95		
Total	24	(5)	1825.76			

Appendix 5 t: ANOVA for the effect of temperature upon DTI among the E8 plants cold-treated for 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Sourceofvariation	d.f.	(m.v.)	s.s.	m.s.	v.r.	Fpr.
Temp	1		6162.8	6162.8	8.81	0.012
Residual	12	(1)	8397.9	699.8		
Total	13	(1)	14267.2			

Appendix 5 u: ANOVA for the effect of temperature upon DTI among the E9 plants cold-treated for 10 weeks at 5 and 10°C with 50, 60, and 70 days pre-growth.

Appendix 6: Protocol for hot-phenol RNA extractions for Brassica leaf and apex tissue

Precautions to avoid RNase contamination:

Wear gloves throughout this procedure and change them regularly. Ensure that all pipette tips etc are certified RNase free. Wipe bench and pipettes with ethanol using blue roll before starting.

When preparing solutions for use in this procedure use deionised water, wear gloves and change them regularly, ensure all glassware (bottles, measuring cylinders etc) is thoroughly cleaned, and autoclave solutions where possible (not for SDS, though autoclaved water can be used to prepare this).

Homogenization buffer recipe

Composition	**Volume of stock solutions for:**			
	100 ml	**200 ml**	**400 ml**	
100 mM Tris pH 8–9	10 ml	20 ml	40 ml	of 1 M
5 mM EDTA pH 8	1 ml	2 ml	4 ml	of 0.5 M
100 mM NaCl	2 ml	4 ml	8 ml	of 5 M
0.5 % SDS	5 ml	10 ml	20 ml	of 10%

Notes

This protocol is a modified version of the standard Dean-lab protocol for phenol-chloroform RNA extractions from Arabidopsis. This version includes an additional phenol-chloroform wash, which helps to ensure that the RNA obtained is as pure as possible, as well as additional information about quantification and checking for degradation.

Preparation of samples

Use approximately 0.4 ml of tissue per sample. Grind leaf samples to a fine powder in 1.5 ml microcentrifuge tubes using polypropylene “pellet pestles”. Pre-cool the pestles with liquid nitrogen and ensure that the tissue does not thaw at any point. Use a clean, autoclaved pestle for each sample. For tough tissues such as apexes and large samples, use baked porcelain pestles and mortars to grind the tissue then

transfer approximately 0.4 ml tissue to a 1.5 ml microcentrifuge tube. (Fill the mortar with liquid nitrogen to cool it then add the tissue and grind.) The grinding can be done in advance and samples stored at -80 °C. Samples must be kept frozen until the homogenization buffer is added.

Day 1

For each sample to be processed, prepare a 1.5 ml microcentrifuge tube containing 500 µl homogenization buffer (see recipe above), 250 µl saturated phenol solution (pH8), and 5 µl β-mercaptoethanol. Place these in a heating block at 60 °C.*

Add the hot-phenol mixture to the ground samples, without letting the samples thaw first, and vortex thoroughly. It may be necessary to use a 200 µl pipette tip to dislodge sample from the bottom of the eppendorf tube to ensure thorough mixing. Shake for 15 minutes.*

Add 250 µl chloroform and shake for 10 minutes then spin at top speed in a microcentrifuge (room temperature) for 10 minutes.*

Transfer 550 µl** of the aqueous layer (upper layer) to fresh tubes and add 550 µl phenol-chloroform. Shake for 10 min and spin for 10 min in a microcentrifuge (top speed, room temperature).*

Transfer 500 µl** of the aqueous layer (upper layer) to a fresh tube with 500 µl phenol-chloroform. Shake for 10 min and spin for 10 min in a microcentrifuge (top speed, room temperature).*

Transfer 500** µl of the aqueous layer to fresh tubes with 400 µl isopropanol and 50 µl 3M sodium acetate (pH5.5). Invert the tubes to ensure mixing.*

Incubate at -80 °C for 15 minutes then spin at 4 °C for 30 minutes at top speed in a cooled microcentrifuge.

Remove all supernatant and the leave tubes open on (clean) blue roll to dry for approximately 10 minutes.

Re-suspend pellet in 500 µl autoclaved deionized water then add 500 µl 4M lithium chloride. Invert the tubes to ensure mixing.

Incubate overnight at 4 °C

* - This step **must** be carried out in a fume cupboard.
** - It will often not be possible to transfer this volume of aqueous layer. If necessary leave some of the aqueous layer behind and transfer slightly less than the stated volume to avoid transferring solid material or organic layer.

Day 2

Spin tubes at 4°C for 30 minutes in a cooled microcentrifuge (top speed).

Remove the supernatant and wash the pellet in 900 µl 80% ethanol (pipette up and down to ensure that the pellet is washed thoroughly).

Spin at 4 °C for 5 minutes in a cooled microcentrifuge (top speed) then use a P1000 (or equivalent) pipette to remove most of the ethanol. (The pellet is fragile and may be easily dislodged – tipping the tubes to remove the ethanol wash is best avoided). Spin briefly to get all of the ethanol wash to the bottom of the tube then remove with a P100 (or equivalent) pipette.

Leave tubes open on blue roll for approximately 10 minutes to allow the pellet to dry

Re-suspend pellet in 60 µl autoclaved deionized water (less if the pellet looks particularly small).

Differences to protocol used for initial primer test samples

For the samples used for the initial RT-PCR screen of the *FLC* primers and sequencing of the products the second phenol-chloroform wash was not included but an additional precipitation step was used. Following the addition of sodium acetate/isopropanol, incubation at -80°C, and removal of the supernatant the pellet was re-dissolved in 500 µl autoclaved deionized water and another 50 µl of 3 M sodium acetate and 400 µl of isopropanol were added. The mixtures were then incubated at -80°C for another 15 minutes and spun at 4°C for 30 minutes at top speed in a microcentrifuge. The supernatants were then removed and the pellet dissolved in 500 µl sterile water before adding 500 µl 4M lithium chloride and proceeding as described above.

Quantification and test for degradation

Aliquot 2 µl of the re-suspended RNA solution from the above step into fresh tubes and add 18 µl of autoclaved deionized water. Use a NanoDrop™ spectrophotometer to quantify 1.5 µl this solution (multiply the concentrations up by 10 to get the concentration of the main solution). The report (containing the 280/260 and 280/230 values) can be saved as a tab-delimited text file suitable for import into Microsoft Excel.

Add 2 µl bromothemol blue loading buffer to the remainder of each aliquot and run on a 1.2% agarose in 1xTBE gel (stained with ethidium bromide) with 1kb DNA ladder as markers to check that the RNA is not degraded. (Adjust the amount of solution loaded according to the concentration, to load 0.7–1.8 µg RNA per well. This will usually mean loading 5–10 µl). The gel tray, comb, and tank should be cleaned with detergent (e.g. SDS) and rinsed with deionised/RNase free water before use to avoid RNase contamination.

Store RNA at -80 °C

Appendix 7: Protocol for DNase treatment of total RNA and cDNA synthesis using Ambion TURBO™ DNase and Invitrogen Supserscript™ III kits

DNase treatment protocol

Dilute an aliquot of RNA in autoclaved deionized water to give 20 µl of 1000 ng/µl total RNA

Add 2 µl DNase buffer (0.1 vol) and 0.5 µl DNase to 20 µl diluted total RNA and mix gently

Incubate for 30 min at 37°C

Add 2 µl (0.1 vol) DNase inactivation reagent (resuspended by flicking).

Incubate for 5 min at room temp with occasional mixing by flicking tubes

Spin at 10,0000g for 1.5 min and transfer RNA to fresh tubes.

Transfer supernatant (containing DNased RNA) to fresh tubes and discard original tube (containing pellet of DNase inactivation reagent).

Spin the fresh tubes and check that no pellet forms (to confirm that no inactivation reagent has been carried over)

cDNA synthesis protocol

Re-quantify DNased RNA using Nanodrop™ 1000 spectrophotometer, taking the average of two repeat readings

Use 300 ng DNased RNA per reaction and make up to 8 µl with autoclaved deionized water

Add 1 µl of 50 uM oligo dT and 1 µl of 10 mM dNTP MIX per reaction

Incubate for 5 min at 65 deg C then put on ice for at least 1 min

Add 10 µl cDNA synthesis master mix to each reaction: (2 µl 10*RT buffer, 4 µl 25mM MgCl2, 2 µl 0.1 M DTT, 1 µl RNaseout (40 u/µl), and 1 µl superscript™ III RT (200 U/µl) per reaction)

Incubate for 50 min at 50 deg C, then 5 min at 85 deg C to terminate reactions and chill on ice

Add 1µl RNase H to each reaction

Incubate 20 min at 37 deg C

Store in -20 freezer

Differences to protocol used for initial primer test samples

For the RNA samples from experiment I that were used for the initial RT-PCR screen of the primers 12 µg total RNA was used per DNase reaction, diluted to 18 µl. The DNase treatment was otherwise as described above. The cDNA synthesis was performed as described above using 1700-1820 ng DNase treated total RNA (based upon quantification with a Nanodrop™ spectrophotometer).

Appendix 8: Intron and Exon sizes in AtFLC, BoFLC1, BoFLC3, BoFLC4, *and* BoFLC5, *from Razi et al. (2008)*

	Exon 1	**Intron 1**	**Exon 2**	**Intron 2**	**Exon 3**	**Intron 3**	**Exon 4**	**Intron 4**	**Exon 5**	**Intron 5**	**Exon 6**	**Intron 6**	**Exon 7**	**Total**
AtFLC **(Genbank AF116528)**	185	3493	58	178	62	90	100	78	42	194	42	992	102	4757
BoFLC1 **(Genbank AM231517)**	185	2342	58	433	62	78	100	85	42	225	42	177	105	3934
BoFLC3 **(Genbank AM231518)**	185	1364	58	438	62	87	100	80	42	234	42	493	105	3290
BoFLC4 **(Genbank AY306124)**	185	1123	61	208	62	85	97	65	42	236	42	1171	105	3482
BoFLC5 **(Genbank AM231519)**	185	4537	59	410	62	85	97	81	42	77	42	970	106	6753

Appendix 9: Sequencing protocol

PCR products should be cleaned up if necessary.

Load 1 µl each PCR product to be sequenced into a 96-well plate or PCR strip

Add 9 µl of master mix to each well (master mix recipe per reaction: 1µl Big Dye v3.1 – Applied Biosystems (Cat. No. 100 reactions 4337455), 1.5µl 5x Sequencing reaction Buffer (supplied with BigDye – store at 4ºC), 1µl Primer (3.2pmol/µl), and 5.5µl dH_20)

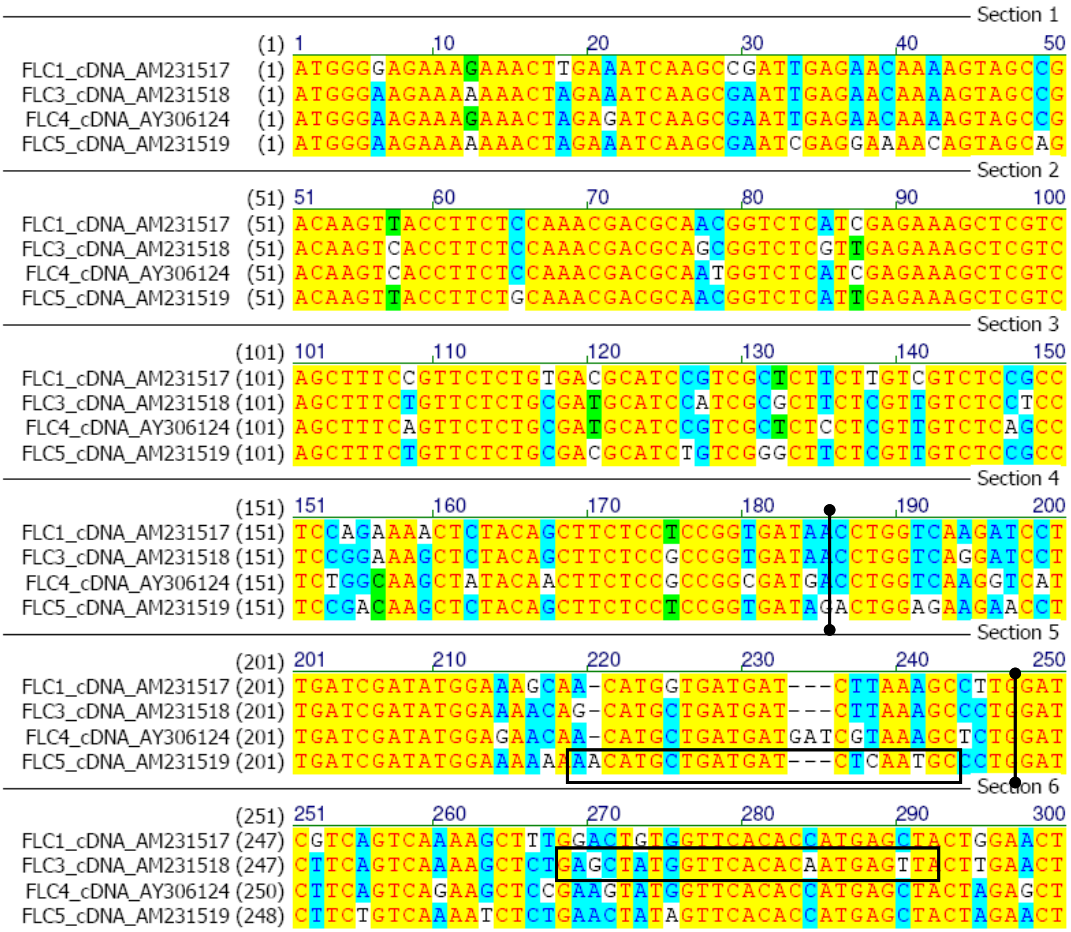
Run on the following PCR program:

Initial denaturation step:	96°C for 1 min
25 cycles:	Denature 96°C for 10 sec ramp 1°C/sec
	Anneal 50°C for 5 sec ramp 1°C/sec
	Extend 60°C for 4min ramp 1°C/sec
	Cool 4°C for 10mins
Hold:	8°C forever (until ready to precipitate)

Freeze samples and submit to The Genome Analysis Centre to be run on the sequencer

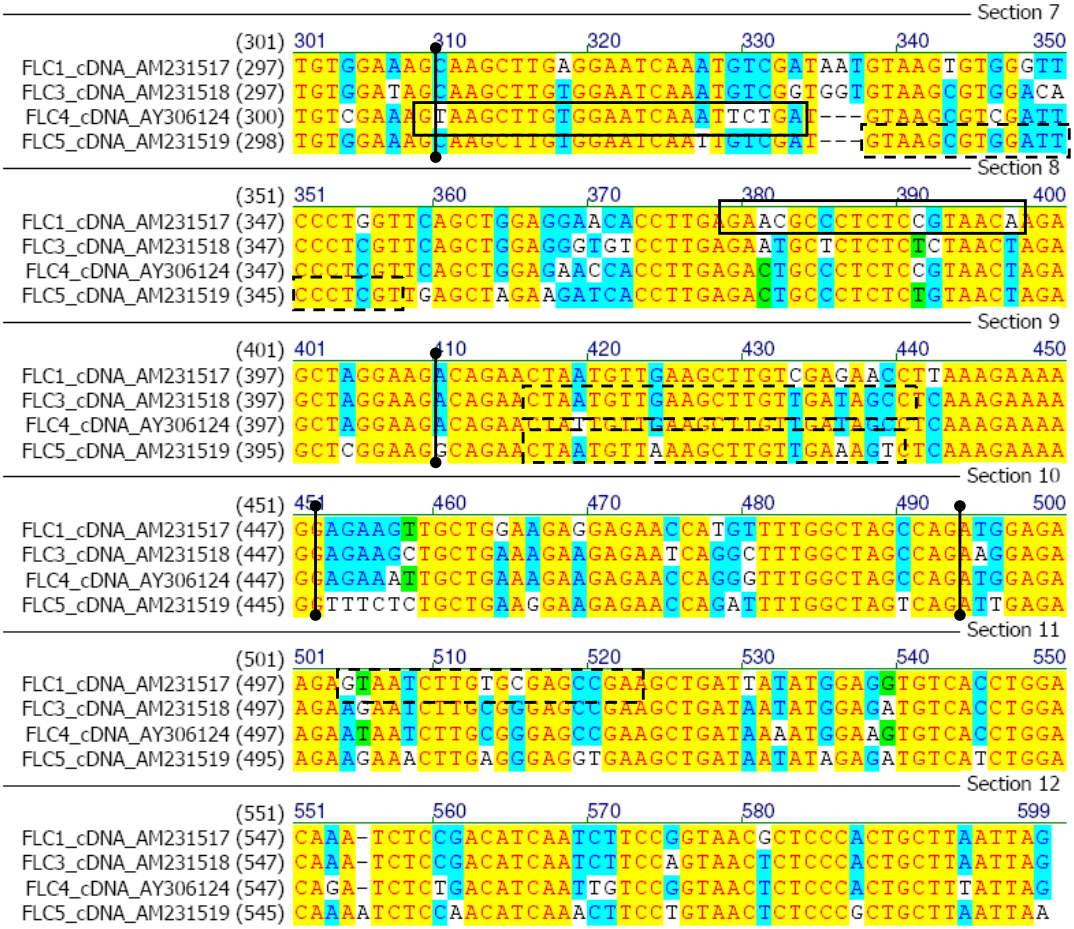
Appendix 10: Aligned cDNAs of BoFLC1, BoFLC3, BoFLC4, *and* BoFLC5 *reference sequences, from Razi et al. (2008)*

The numbers beginning "AM" and "AY" are the Genbank accession numbers for each gene. Bases that are identical in all four homologues are highlighted in yellow, those identical in three homologues in turquoise, and those identical in two homologues in green. The binding sites for the forward primers (listed in Table 13) are shown by boxes with solid edges. The binding sites for the reverse primers (listed in Table 13) are shown by boxes with dashed edges. The exon boundaries are shown by vertical black lines.

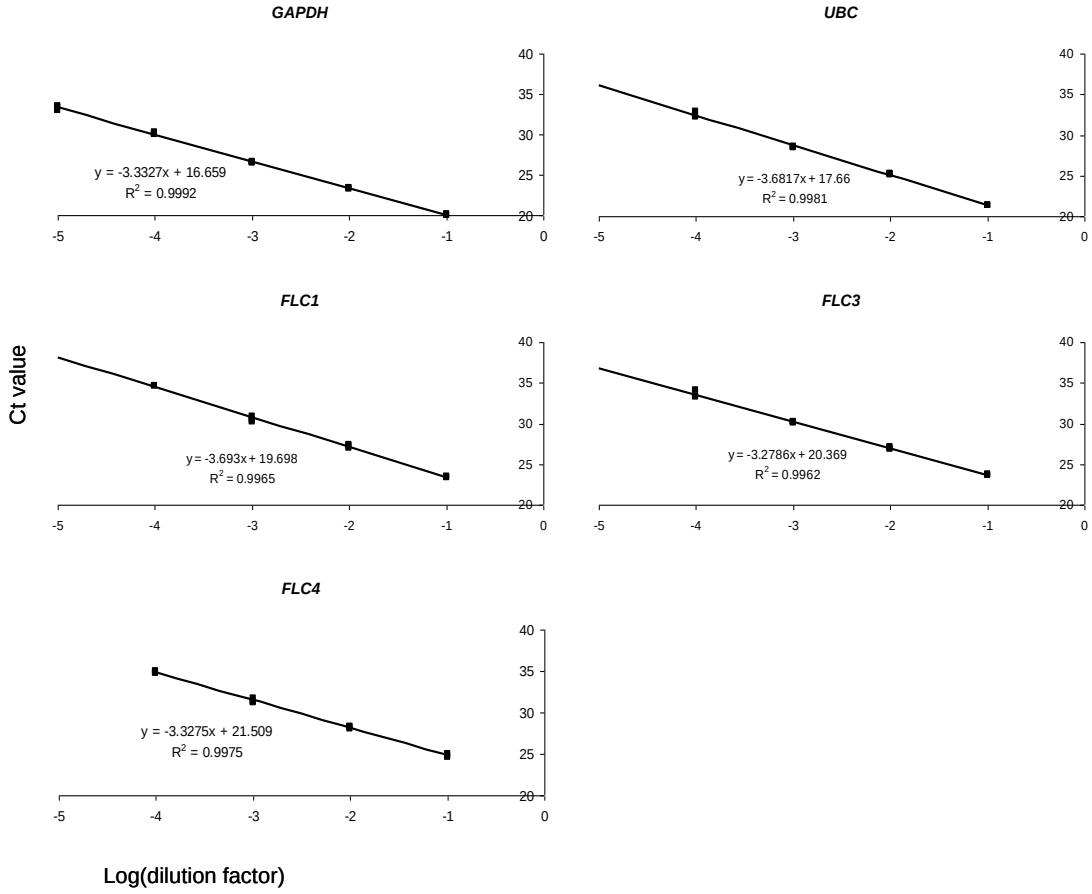


Appendix 10 cont.: Aligned cDNAs of BoFLC1, BoFLC3, BoFLC4, *and* BoFLC5 *reference sequences, from Razi et al. (2008)*

The numbers beginning "AM" and "AY" are the Genbank accession numbers for each gene. Bases that are identical in all four homologues are highlighted in yellow, those identical in three homologues in turquoise, and those identical in two homologues in green. The binding sites for the forward primers (listed in Table 13) are shown by boxes with solid edges. The binding sites for the reverse primers (listed in Table 13) are shown by boxes with dashed edges. The exon boundaries are shown by vertical black lines.



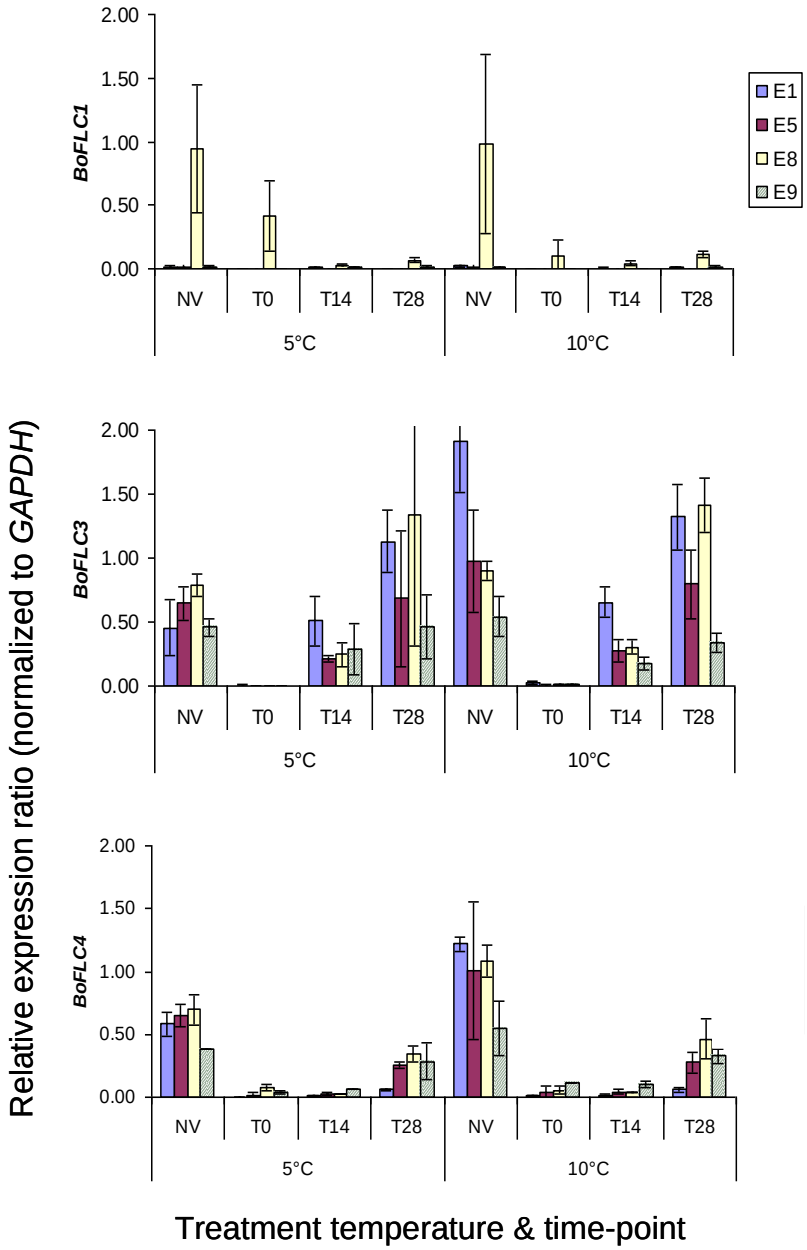
Appendix 11: Calculation of QRT-PCR primer efficiencies from standard curves



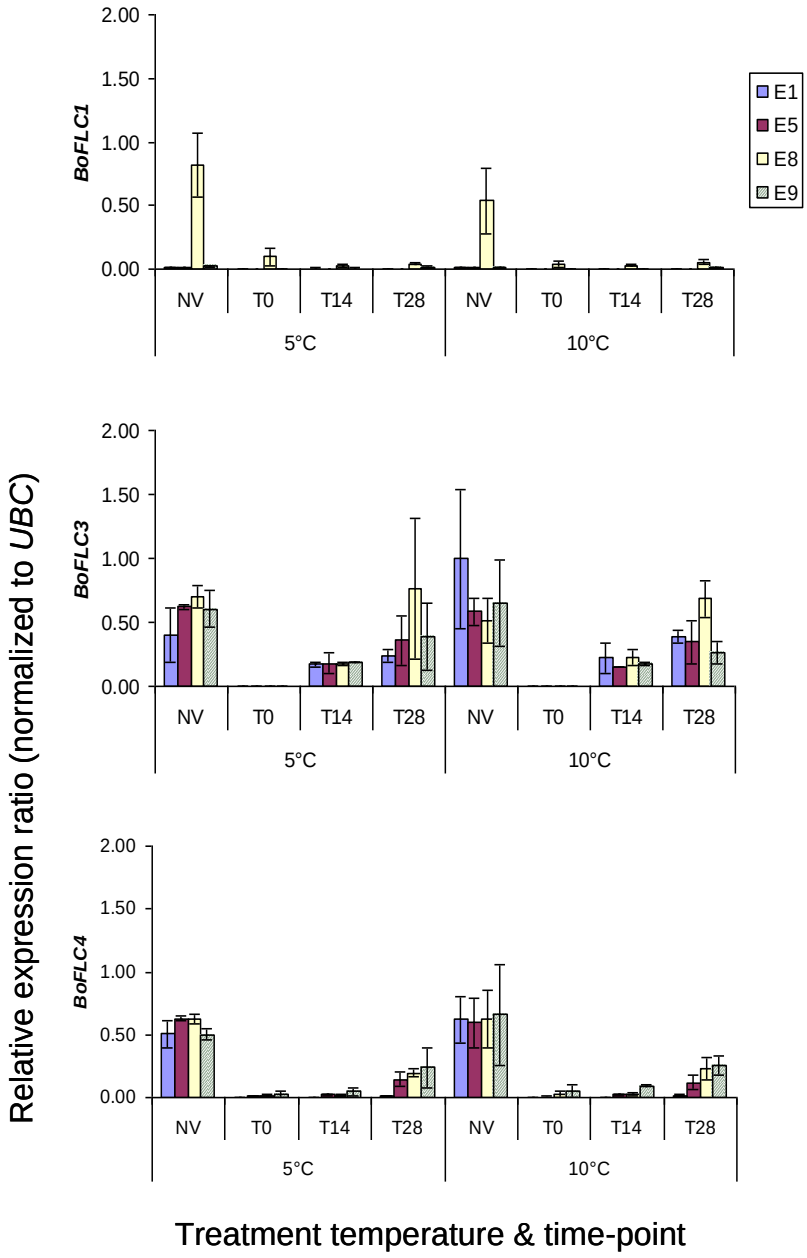
The efficiency of each pair of primers was calculated from the slope of the standard curve of log(dilution factor) vs Ct value using Equation 3. This gives a value for the efficiency of the primers from 1 (no amplification) to 2 (perfect doubling of product with each PCR cycle)

Primers	Slope	R2	Efficiency
GAPDH	-3.3327	0.9992	2.00
UBC	-3.6817	0.9981	1.87
FLC1	-3.6930	0.9965	1.87
FLC3	-3.2786	0.9962	2.02
FLC4	-3.3275	0.9975	2.00

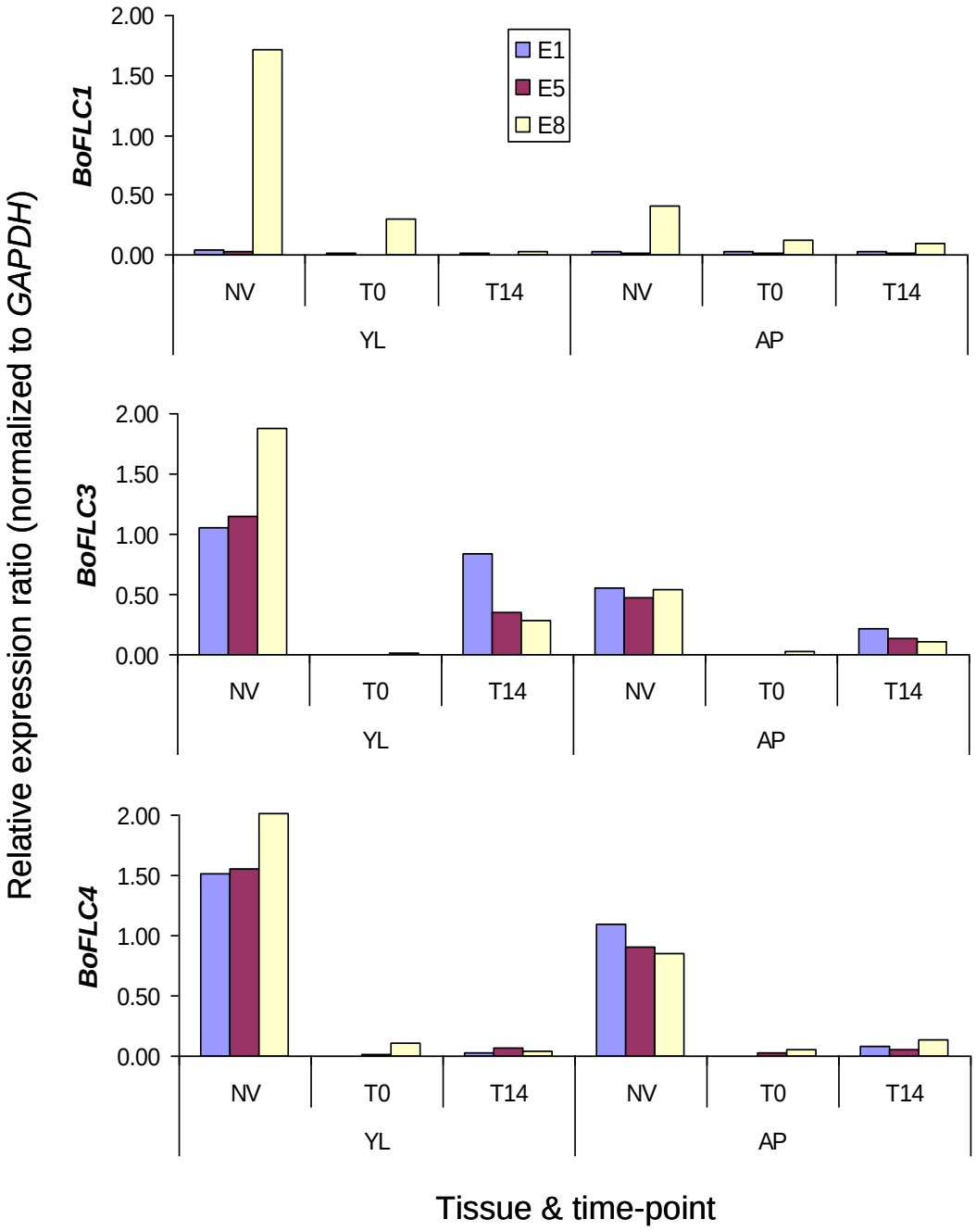
Appendix 12: BoFLC *expression data normalized to* UBC *and* GAPDH *individually*



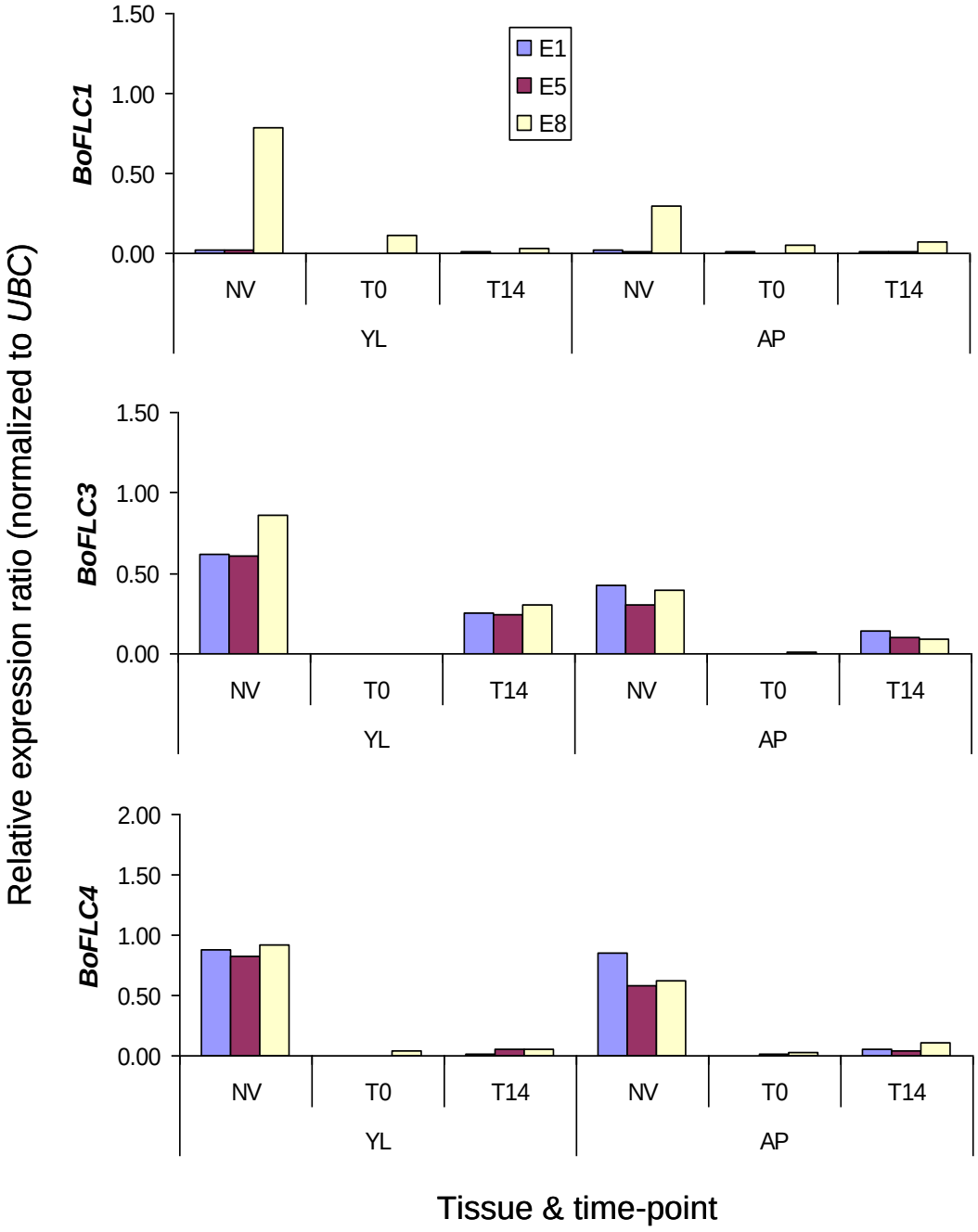
Appendix 10 a: The average relative expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in the young leaves of the PSB genotypes E1, E5, E8, and E9 before and after 10 weeks cold-treatment at 5 and 10°C with 70 days pre-growth. The relative expression ratios are calculated relative to a common E8NV calibrator sample using the Pfaffl (2001) equation. The data are shown normalized to *GAPDH*. There are two biological replicates for each genotype at each time-point and temperature. The error bars represent the 95% confidence intervals.



Appendix 10 b: The average relative expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in the young leaves of the PSB genotypes E1, E5, E8, and E9 before and after 10 weeks cold-treatment at 5 and 10°C with 70 days pre-growth. The relative expression ratios are calculated relative to a common E8NV calibrator sample using the Pfaffl (2001) equation. The data are shown normalized to *UBC*. There are two biological replicates for each genotype at each time-point and temperature. The error bars represent the 95% confidence intervals.



Appendix 10 c: The relative expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in young leaves and apexes of the PSB genotypes E1, E5, and E8 before and after 10 weeks cold-treatment at 5°C with 70 days pre-growth. The relative expression ratios are calculated relative to a common E8NV calibrator sample using the Pfaffl (2001) equation. The data are shown normalized to *GAPDH*. There is one biological replicate of each tissue for each genotype at each time-point and temperature.



Appendix 10 d: The relative expression of *BoFLC1*, *BoFLC3*, and *BoFLC4* in young leaves and apexes of the PSB genotypes E1, E5, and E8 before and after 10 weeks cold-treatment at 5°C with 70 days pre-growth. The relative expression ratios are calculated relative to a common E8NV calibrator sample using the Pfaffl (2001) equation. The data are shown normalized to *UBC*. There is one biological replicate of each tissue for each genotype at each time-point and temperature.

7 List of Abbreviations

95% CI:	95% Confidence Interval
A230/260/280:	Absorption at 230/260/280 nm wavelength
ANOVA:	Analysis of Variance
AP:	Apex
CER:	Controlled-Environment Room
COMPASS:	Complex of Proteins Associated with Set 1
Ct:	Threshold Cycle value (in QRT-PCR)
DTB:	"Days to buttoning" i.e. the number of days taken for an inflorescence head of 10 mm diameter to form
DTF:	"Days to floerwing" i.e. the number of days taken for the first flower to open
DTI:	"Days to inflorescence" i.e. the number of days taken for inflorescence buds to first become visible at the apex
E:	The efficiency (from 1–2) of a set of primers in QRT-PCR
EMS:	Expected means squares
FLN:	Final leaf number
GDD:	Growing Degree Days
gDNA:	Genomic DNA
GOI:	Gene of interest
H2A.Z:	Histone variant H2A.Z
H2Bub1:	Histone H2B monoubiquitination
H3K27me2/3:	Histone 3 lysine 27 di/trimethylation
H3K36me2/3:	Histone 3 lysine 36 di/trimethylation
H3K4me3:	Histone 3 lysine 4 triemthylation
H3K9me2/3:	Histone 3 lysine 9 di/trimethylation
H4R3sme2:	Histone 4 arginine 2 symmetrical dimethylation
miRNA:	Micro RNA
mRNA:	Messenger RNA
MYA:	Millions of years ago
NV:	Non-Vernalized (either a control plant kept in the glasshouse throughout an experiment or the time-point immediately before the beginning of cold-treatment)

PAF1c:	RNA Polymerase II Associated Factor I complex
PCR:	Polymerase Chain Reaction
PHD:	Plant homeo-domain
PRC:	Polycob Repressor Complex
PRC2:	Polycomb Repressor Complex 2
PSB:	Purple Sprouting Broccoli
QRT-PCR:	Quantitative (real time) Reverse Transcriptase Polymerase Chain Reaction
QTL:	Quantitative trait loci
RER:	Relative expression ratio
RFLP:	Restriction fragment length polymorphism
rRNA:	Ribosomal RNA
RT-PCR:	Reverse Transcriptase Polymerase Chain Reaction
SED:	Standard Error(s) of the Difference(s)
SWR1c:	The yeast SWR1 complex
T0:	The timepoint at which a cold-treatment ends
T14:	14 days after the end of a cold-treatment
T28:	28 days after the end of a cold-treatment
T_{MAX}:	The maximum temperature at which a biological process (e.g vernalization) occurs
$T_{MIN}/T_{BASE:}$	The minimum temperature at which a biological process (e.g vernalization) occurs i.e. the "base" temperature for that process
T_{OPT}:	The temperature at which a biological process (e.g vernalization) occurs at its maximum rate (R_{max})
V°CD:	Vernalizing Degree Days
VD:	Effective vernalization days
YL:	Young leaf

8 List of gene and protein names

AaFLC/*PEP1*:	*Arabis alpina FLOWERING LOCUS C*/*PERPETUAL FLOWERIN 1*
AaFT:	*Arabis alpina FLOWERING LOCUS T*
AaSOC1:	*Arabis alpina SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
AaTFL1:	*Arabis alpina TERMINAL FLOWER 1*
AGL42:	*AGAMOUS-LIKE 42*
AhgFLC:	*Arabidopsis halleri FLOWERING LOCUS C*
AP1:	*APETALLA 1*
AP2:	*APETALLA 2*
ATX:	*ARABIDOPSIS TRITHORAX*
ATX1/2:	*ARABIDOPSIS TRITHORAX 1/2*
BnFLC:	*Brassica napus FLOWERING LOCUS C*
BoFLC:	*Brassica oleracea FLOWERING LOCUS C*
BrFLC:	*Brassica rapa FLOWERING LOCUS C*
CAL:	*CAULIFLOWER*
CBF/*DREB1*:	*CRT/DRE binding factor/DRE-binding factor 1*
CO:	*CONSTANS*
CsTFL1:	*Citrus sinensis TERMINAL FLOWER 1*
CTR9:	A component of the yeast Paf-1 complex
E(z):	*Enhancer of Zeste*
EFS:	*EARLY FLOWERING IN SHORT DAYS*
ELF7	*EARLY FLOWERING 7*
ELF8/*VIP6*:	*EARLY FLOWERING 8*/*VERNALIZATION INDEPENDENCE 6*
FCA:	A member of the Arabidopsis autonomous flowering pathway
FD:	*FLOWERING LOCUS D*
FES1:	*FRIGIDA ESSENTIAL 1*
FIE:	*FERTILIZATION-INDEPENDENT ENDOPSERM*
FLC:	*FLOWERING LOCUS C*
FLD:	*FLOWERING LOCUS D*
FLK:	*FLOWERING LATE KH MOTIF*

FLM:	*FLOWERING LOCUS M*
FPA:	A member of the Arabidopsis autonomous flowering pathway
FRI:	*FRIGIDA*
FRL1:	*FRIGIDA-LIKE 1*
FT:	*FLOWERING LOCUS T*
FUL:	*FRUITFULL*
FVE:	A member of the Arabidopsis autonomous flowering pathway
FY:	A member of the Arabidopsis autonomous flowering pathway
GAPDH:	*GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*
GI:	*GIGANTEA*
HP1:	*HETEROCHROMATIN PROTEIN 1*
HSK18:	A component of the human Paf1 complex
HUB1/2:	*HISTONE MONOUBIQUINATION 1/2*
LD:	*LUMINIDEPENDENS*
LEO1:	A component of the yeast Paf-1 complex
LFY:	*LEAFY*
LHP1/*TFL2*:	*LIKE HETEROCHROMATIN PROTEIN 1*/*TERMINAL FLOWER 2*
MAF1/2/3/4/5:	*MADS AFFECTING FLOWERING 1/2/3/4/5*
MdTFL1:	*Malus domestica TERMINAL FLOWER 1*
miR156:	*Micro RNA 156*
miR172:	*Micro RNA 172*
MSI1:	*MULTICOPY SUPPRESSOR OF IRA 1*
P55:	A component of the *Drosophila* Polycomb Repressive Complex 2 (PRC2)
PHYA/B/D/E:	*PHYTOCHROME A/B/D/E*
PIE1:	*PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1*
RTF1:	A component of the yeast Paf-1 complex
SaFLC:	*Sinapis alba FLOWERING LOCUS C*
SET1:	A yeast H3K4 methyltransferase that contains a SET domain (a domain that was first recognized as conserved among the genes *SUPPRESSOR OF VARIEGATION 3-9* (*SU(VAR)3-9*), *ENHANCER OF ZESTE* (*E(Z)*), and *TRITHORAX* (*TRX*) (Dillon et al. 2005))

SMZ:	*SCHLAFMÜTZE*
SNZ:	*SCHNARCHZAPFEN*
SOC1/AGL20:	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1/AGAMOUS-LIKE 20*
SPL:	*SQUAMOSA BINDING PROTEIN LIKE*
Su(z)12:	*Suppressor of zeste 12*
SUF4:	*SUPPRESSOR OF*
SVP:	*SHORT VEGETATIVE PHASE*
SWN:	*SWINGER*
TFL1:	*TERMINAL FLOWER 1*
TOE1/2/3:	*TARGET OF EAT 1/2/3*
UBC:	*UBIQUITIN CONJUGATING ENZYME*
VIN3:	*VERNALIZATION INSENSITIVE 3*
VIP3–5:	*VERNALIZATION INDEPENDENCE 3–5*
VRN1:	*VERNALIZATION 1*
VRN2:	*VERNALIZATION 2*
VRN5/*VIL1*:	*VERNALIZATION 5*/*VIN3-LIKE 1*
WDR5:	A component of the human COMPASS-like H3K4 methyltransferase complex
WDR5a:	An Arabidopsis homologue of human WDR5

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