The role of winter bud dormancy in *Brassica napus* floral development and its genetic control

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Abstract

Winter annuals are grown for their high yield and require vernalisation to promote spring flowering. However, winter type *Brassica napus* undergoes the floral transition during autumn before flowering in spring. Here, I show that following autumnal floral transition winter-type *B. napus* requires chilling to promote floral development and optimal yield formation. Winter warming applied to emerging floral buds is associated with delayed budding, flowering and lower thousand grain weight of winter-type *B. napus*. However, the extent of this delay is dependent on genotype and crop type of *B. napus*. Some crop types, e.g. semi-winter and Swedes show accelerated floral development in response to winter bud warming. Here, I show that during winter warming in winter-type *B. napus*, genes associated with bud dormancy are upregulated in developing inflorescences, including abscisic acid signalling genes through the BRANCHED-1 dormancy module. This provides evidence that in winter type *B. napus* the floral delay induced by winter warming is a bud dormancy response. Using a diversity set of *B. napus*, I uncovered genetic variation in the control of warm winter bud dormancy and present evidence that the activation of bud dormancy is mediated through control of *FLOWERING LOCUS C (FLC)* genes that remain active after the floral transition. I also show that the control of flowering time both before and after the floral transition involves the *FLC* regulator *PLANT HOMOLOGOUS TO PARAFIBROMIN (PHP)* and genetic variation in *PHP* is associated with control of flowering time and development during chilling. As warming during winter floral development is associated with yield loss, the work here shows the importance of bud dormancy in the context of climate change and presents a genetic target for breeding climate resilient rapeseed.

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This thesis includes material from the published work below:

Lu, X., O'Neill, C.M., Warner, S., Xiong, Q., Chen, X., Wells, R. and Penfield, S., 2022. Winter warming post floral initiation delays flowering via bud dormancy activation and affects yield in a winter annual crop. Proceedings of the National Academy of Sciences, 119(39), p.e2204355119.

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Chapter 1. Introduction

1.1 Climate change threatens global crop productivity

Globally, climate change is predicted to reduce arable land area and cause crop yield losses of 5.6% - 8.6% per degree (°C) of warming (Asseng et al., 2011, 2015; Liu et al., 2016; Zhao et al., 2017). Even mild temperature increases caused by fluctuations in the El-Niño-Southern Oscillation (ENSO) and North Atlantic Oscillation (NAO), have historically reduced South American maize yields and European wheat yields, highlighting the significance of even moderate temperature anomalies for temperature-related reductions in crop yield (Kettlewell et al., 1999; Cane *et al.,* 1994). The effects of climate change will however vary depending on region and crop, but overall, it is expected that warmer temperatures will reduce final yield. For instance, climate change will directly lower crop yields through increased drought stress (MacDonald and Glen, 2010), heat stress (Teixeira *et al.,* 2013), flooding and frost stress (Barlow *et al.,* 2015) and indirectly through changes to soil fertility and structure (St.Clair *et al.,* 2010), increased disease and pest burden (Chakraborty and Newton, 2011; Singh *et al.,* 2023) and timing of flowering (Roberts *et al.,* 1993). Whilst climate change is expected to negatively impact the productivity of many crops globally, there are some exceptions to this trend. For instance, in some regions of the world, including Northern Europe, crop yields of major winter annual crops, such as wheat, barley and oilseed rape, are expected to increase due to milder winters and longer growing seasons (Olesen *et al.,* 2002; Olesen *et al.,* 2011; Faye *et al.,* 2023). However, due to the complexity underpinning the relationship between crop yields and arable environmental conditions, like temperature, such models should not detract from the severe threat climate change poses to global crop production. Therefore, , understanding how climate change will affect crop yields could be used to develop targeted breeding strategies to ensure future food security.

1.1.1 Climate change will reduce the reliability and intensity of winter chill

While climate change may benefit northern European crop yields overall, several winter annual and perennial crops grown in this region require a period of cold over winter months (hereafter described as ‘winter chill’) to facilitate optimal fruit development and seed set. Such crops include winter oilseed rape (WOSR), winter wheat, apples and stone fruits. (Atkinson *et al.,* 2013; Fernandez *et al.,* 2023). Climate change threatens these overwintering crops as the intensity and predictability of winter chill will decrease (Luedeling, 2012; Luedling, 2009). For

instance, in some perennial crops like apples and various stone fruits, insufficient chilling results in delayed bud break which consequently delays anthesis. This can influence a variety of key yield-related ecological and physiological processes, like reproductive alignment with pollinator emergence, flower fertilisation, and rate of vegetative growth (Atkinson *et al.,* 2013). This delay to bud break and anthesis often also results in enhanced floral bud abscission, resulting in smaller or aborted buds, reduced flower size and pedicel lengths, reduced fruit set, abnormal size and shape of fruits and lowered final harvest quality (Atkinson et al., 2013). This highlights the importance of sufficient winter chill to ensure optimal timing of key developmental events in overwintering crops which impacts final yield. The duration and intensity of cold needed before warmth can cause bud break is referred to as the chilling requirement (CR). This chilling requirement determines the subtropical cultivation limits of many temperate crops, and as such, climate change related warming is reducing the area of arable land where such crops can be grown. In response, breeding efforts are now underway to produce 'low chill' crop varieties in an attempt to safeguard food security under a future warmer climate (Atkinson *et al.,* 2013; Campoy *et al.,* 2018; Fadón *et al.,* 2021).

1.1.2 Inaccuracies of predicting crop responses to climate change

Crop models are used to anticipate the impacts of climate change on agricultural yields and inform the potential impact of adaptation strategies. However, often these models do not accurately reflect what is seen in field experiments (Wang *et al.,* 2020), which means they can underestimate crop losses (Campoy *et al.,* 2019; Fernandes *et al.,* 2020; Wang *et al.,* 2020; Wang *et al.,* 2022). These inaccuracies often arise from insufficiencies or gaps in input data. For instance, many models rely on statistical derivations of key phenological or climatic data due to a lack of sufficient high quality historical data (e.g., rainfall, soil water content, and sowing dates), this introduces uncertainties and errors into the models (Wang *et al.,* 2022; Kephe *et al.,* 2021; Chapagain *et al.,* 2022; Pasley *et al.,* 2023). The stringency to which potential errors in input data are categorised also impacts the model output (Wang *et al.,* 2022). Another source of error arises from inaccurate phenological data. For example, the Agricultural Productions Systems sIMulator (APSIM) prediction for *Brassica napus* previously relied on inaccurate physiological information about the critical photoperiod needed for rapeseed development (He *et al.,* 2017a). While most models rely on outdated estimates of the biological limitations of pest, disease and weed damage (Silva and Giller, 2020; Pasley *et al.,* 2023). Even where there is sufficient data, predicting the interplay between different factors *in silico*, such as Nitrogen stress and soil water balance, can be challenging and can result in model uncertainty (Wang *et al.,* 2022).

One of the greatest sources of model variation and uncertainty is predicting the response of plant growth to temperature (Asseng *et al.,* 2013; Bassu *et al.,* 2014; Li *et al.,* 2015), which can account for >50% of the uncertainty in predicted grain yields (Wang *et al.,* 2017). This is in part because crops do not show a consistent response to temperature stimulus throughout development. In winter annuals for instance, warming during the winter chilling period delays development, but in spring the same warming will accelerate growth. Therefore, to adapt and understand how a changing climate will impact crop production, we need to better understand and document the processes by which crop physiology and behaviour is affected by warming conditions. This will improve agricultural models.

1.2 The effects of climate change on Oilseed Rape

1.2.1 WOSR *Brassica napus* is threatened by warm winter temperatures

One key crop at risk from climate change is *Brassica napus*, commonly called oilseed rape. Winter Oilseed Rape (WOSR) suffers from yield instability: in the UK rapeseed shows year to year yield fluctuations of 0.8 tonnes ha^{-1}, which can cost up to £150 million in lost yields for each degree (°C) of winter warming (Brown *et al.,* 2019). The inter-annual variation in rapeseed yield is predominantly caused by weather variation, including precipitation and temperature. Despite breeding efforts over the past 40 years, this inter-annual yield variation has remained a problem for rapeseed producers (Rondanini *et al.,* 2011). Differences between genotypes, and their interaction with the environment, only plays a small role in inter-annual yield variation (Sidalaukas and Bernotas, 2003; Nowosad *et al.,* 2016*;* He *et al.,* 2017; Robertson *et al.,* 2015; Rondanini *et al.,* 2012).

Compared to other crop species like wheat, limited attention has been paid to quantify the global yield potential and constraints of *B. napus* (He *et al.,* 2017b; Wang *et al.,* 2022). It is predicted that *B, napus* will face yield decline in most growing regions due to reduced rainfall, high temperature stress, increased disease prevalence, hastened development causing reduced time for grain filling, and drought (Evans *et al.,* 2009; Anwar *et al.,* 2015; Pullens *et al.,* 2019). This will reduce the area where rapeseed can be reliably cultivated (Jaime *et al.,* 2018),

It was previously assumed that, unlike warmer regions of the world, rapeseed yield would increase in cooler regions like northern Europe under climate change as harsh winter temperatures reduce (Pullens *et al.,* 2019). However, more recently, statistical and modelling approaches showed that warmer winter temperatures are actually associated with reduced rapeseed yield (He *et al.,* 2017; Shariff *et al.,* 2017; Brown *et al.,* 2019). Specifically, temperature between the end of November and the beginning of December is highly predictive

of high or low yield (Brown *et al*., 2019). Intriguingly, December temperatures correlate with the North Atlantic Oscillation (NAO) index (Brown *et al.,* 2019), an atmospheric weather phenomenon that influences temperatures in the UK (Hurrell, 1995; Wilby *et al.,* 1997). The NAO also influences wheat grain yield and quality (Kettlewell *et al.,* 1999), implying warmer winters may impact other winter annual crops too. This is contrary to the expectation that warmer conditions in northern Europe will increase yields of major winter annual crops, such as wheat and barley, with climate change (Olsen *et al.,* 2011).

1.2.2 Importance and evolutionary history of *Brassica napus*

B. napus is a diverse crop and the third largest source of vegetable oil globally, it is also grown for animal feed, biodiesel, or tubers from swedes and leafy greens from Siberian kale (Bušić et al., 2018; Wang *et al.,* 2018). In the UK alone, *B. napus* is worth £600-702 million to the economy annually (*Defra*, UK, 2015-2020). Therefore, understanding how *B. napus* responds to climate change is of huge agronomic importance.

B. napus is a neo-allotetraploid (AACC, 2*n* = 38) crop from the Brassicaceae family. It originated in the contact zone of its two progenitors, in northern Europe or the Mediterranean, around 1910-7180 years ago following hybridisation of *B. rapa* (AA, 2*n* = 20) and *B. oleracea* (CC, 2*n* = 18) (Arias *et al.,* 2014; Chalhoub *et al.,* 2014; Sun *et al.,* 2017; Lu *et al.,* 2019). The original *B. napus* was a winter oilseed rape (WOSR) requiring a period of winter cold for floral promotion, with subsequent crop-types, like spring varieties and non-oilseed varieties, such as Swedes, selectively bred afterwards.

1.2.3 Breeding oilseed rape

B. napus has been domesticated across the world in a range of environmental conditions (Zou *et al.,* 2019). Different *B. napus* 'crop types' form distinct breeding pools grouped based on flowering time and requirement for winter cold to induce spring flowering (vernalisation). Winter oilseed rape (WOSR) is still the predominant high-yielding crop type grown in Europe. WOSR is late flowering, requiring a prolonged period of vernalisation to flower. By contrast, spring-type oilseed rape (SOSR) is early flowering as it bypasses the vernalisation requirement to flower. SOSR are predominantly grown in North America, South-East Asia and Australia (Friedt and Snowdon, 2009). Semi-winter oilseed rape (SWOSR) varieties were bred in the 1940s-50s (Qian *et al.,* 2006) and have a reduced vernalisation requirement that is fulfilled in mild winters common in the Yangtze River basin, China's largest rapeseed growing region (Werner *et al.,* 2018). Flowering time differences between these crop type groupings has been

associated with allelic variation around key flowering time genes including *Bna.FRI, Bna.FLC* and *Bna.FT,* which will be discussed later (Schiessl *et al.,* 2015; Wang *et al.,* 2011). Most breeding effort has focussed on flowering time, oil content, disease resistance and seed quality while the impact of winter temperature has been largely overlooked (Friedt and Snowdon, 2010).

1.2.4 The phenology of WOSR

Winter annual crops, including oilseed rape, are cultivated in temperate climates due to their high yield potential. Farmers sow winter annuals in late summer or autumn they then grow vegetatively before overwintering, which is followed by bolting and flowering in the spring and subsequently fruit development and seed set in early summer. To ensure winter annuals flower in spring, not winter, they require a prolonged period of chilling before they can flower under warmer and longer days of spring, this is known as 'vernalisation' (Chouard 1960; Figure 1.1A). Laboratory studies in *A. thaliana* suggested that vernalisation took one to three months of temperatures between 1 and 10 °C, after which floral development will occur in warm spring conditions (Simpson and Dean, 2002; Figure 1.1A). However, more recently it was shown that *B. napus* will undergo the floral transition in late autumn, not spring as expected (O'Neill *et al.,* 2019; Figure 1.1B). This phenology seems general to the Brassicaceae family, as biennial brussel sprouts (*B. oleraceae var. gemmifera*), cabbage (*B. oleraceae* var. capitata) and the perennials *A. alpina* and *A. lyrata* all proceed through the floral transition during chilling conditions (Stokes and Verkerk, 1951; Wang *et al.,* 2009; Kemi *et al.,* 2019). Critically for WOSR, warmer temperatures after the floral transition in early winter are associated with yield loss (Brown *et al.,* 2019; Figure 1.1B), in a process that appears independent of the control of the floral transition. While the mechanism behind this remains unknown, understanding it will be important for adapting *B. napus* to warming environments, considering that warmer temperatures after the floral transition are associated with yield loss.

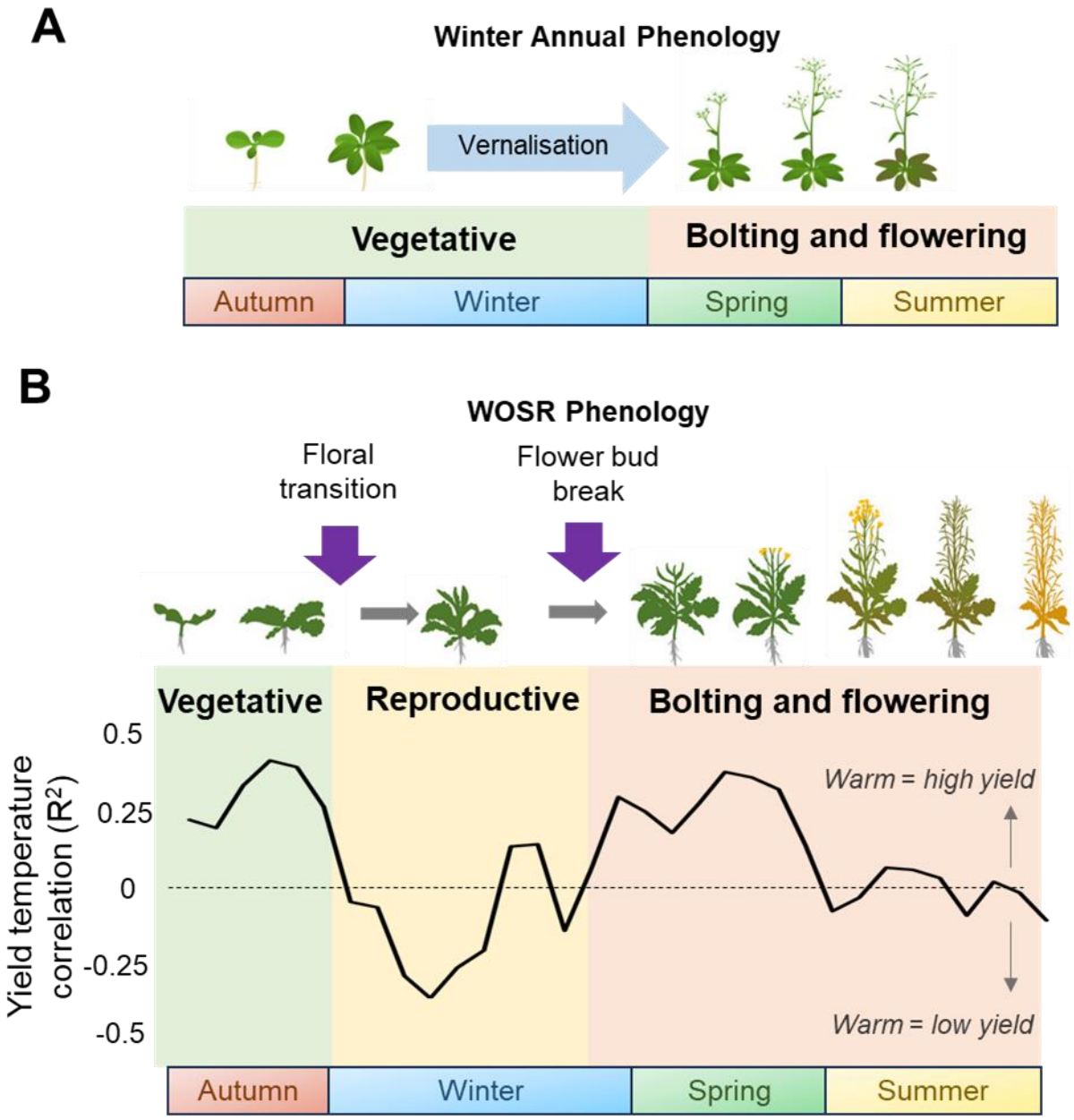


Figure 1.1 Phenology of winter annual and WOSR over a typical growing season, key floral stages marked in diagrams and purple arrows. A) Typical phenology of a winter annual based on laboratory studies on *A. thaliana*. Winter annuals require prolonged cold to facilitate floral development in spring but remain vegetative under winter conditions until warm long days in spring. B) *B. napus* florally transitions in late autumn (O'Neill et al., 2019) before inflorescent buds overwinter and then flower in spring. The yield temperature correlation is from Brown et al., (2019) and indicates when warmer temperatures are associated with low yield, whenever the solid line is above the dashed line it indicates warmer temperatures are associated with higher yield but whenever the solid line is below the dashed line it indicates warmer temperatures are associated with lower yield (indicated in grey text).

1.2.5 Transferring knowledge between *B. napus* and *A. thaliana*

Many genetic studies on the control of vernalisation and the floral transition have been carried out in the model species *Arabidopsis thaliana.* Fortunately, these findings can be more easily transferred into *B. napus* that other crops because B*. napus* and the model species *A. thaliana* diverged recently in evolutionary history, around 14.5 – 20.4 million years ago and are both members of the Brassicaceae family (Chalhoub *et al.,* 2014).

However, there are still complications to inferring gene function in *B. napus* from *A. thaliana* homologs. This is because whole genome triplication and hybridisation events in *B. napus* have resulted in large gene number copy variation (Cheng *et al.,* 2014), with an average gene copy number of 4.4 for each *A. thaliana* homolog in *B. napus* (Parkin *et al.,* 2010). However, some genes including key flowering time regulators, have as many as twelve gene copies which varies from variety to variety (Schiessl *et al.,* 2014; Schiessl *et al.,* 2017b).

During the evolutionary divergence of *B. napus* mutations have accumulated across these duplicated gene copies. This resulted in genes acquiring novel functions (neofunctionalization), losing some of their original function and specialising in one area (subfunctionalisation) or losing their ancestral function entirely, sometimes referred to as 'pseudogenes' (Conant and Wolfe, 2008). Indeed, across the *Brassica* genus there have also been a significant number of chromosomal rearrangements and fusions (Lagercrantz, 1998; Lukens *et al.,* 2003), which can further complicate copy number variation and gene functionalisation.

Despite this, *B. napus* still shows remarkable synteny with *A. thaliana* and shares up to 85% coding similarity (Parkin *et al.,* 2005; Trick *et al.,* 2009). That means many homologs share function between *B. napus* and *A. thaliana* and knowledge transfer between the two species can inform, although not fully account for, genetic control of *B. napus* homologs. Studies on *A. thaliana* are therefore useful for inferring gene function in *B. napus.*

1.3 Molecular control of floral transition in winter annuals

1.3.1 Why flowering time is important.

The timing of flowering is critically important to the reproductive success of a plant. The timing of flowering is influenced by endogenous and environmental cues such as day-length, ambient temperature, nutrient status and hormones. In perennial trees that require winter chill, winter temperature before bud break influences timing of flowering (Atkinson *et al.,* 2013).

Early flowering can expose plants to damaging frosts, shorten the vegetative phase, and cause ecological mismatch where flowering occurs before pollinator emergence.(Thomson,

2010; Kudo and Ida, 2013; Franks, 2015). While late flowering will push seed maturation into late summer, exposing vulnerable reproductive structures to unfavourable seed maturation temperatures (Yang and Zhang, 2010). In *B. napus*, timing of flowering correlates with seed yield, nitrogen use efficiency, oil quality, plant vigour and disease resistance (Berry *et al.,* 2010; Raman *et al.,* 2016*;* Raman *et al.,* 2019). Furthermore, in rice and barley, flowering time genes are either directly implicated in grain yield or closely associated with it (Xue *et al.,* 2008; Zhang *et al.,* 2012; Cuesta-Marcos *et al.,* 2009). This underlies the importance of understanding the molecular control of flowering in crop species.

1.3.1.1 The molecular pathways that control flowering

Extensive work in the model species *A. thaliana* has discovered the mechanism of flowering time control. Flowering time is controlled by integration of a range of environmental and endogenous signals. In *A. thaliana,* flowering time is controlled by at least four pathways: ageing, vernalisation, photoperiod and hormonal (gibberellin pathway) pathways. The overall timing of flowering depends on the combined effects of these pathways, which primarily act on the core floral integrator genes *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF CONSTANS 1* (*SOC1*) and *LEAFY* (*LFY*) (Samach *et al.,* 2000; Moon *et al.,* 2003; Lee and Lee, 2010; Kardailsky *et al.,* 1999).

The ageing pathway is regulated by opposing action of two microRNAs, miR156 and miR172, which act on *SQUAMOSA PROMOTOR BINDING PROTEIN LIKE* (*SPL*) genes and *APETALA2* (*AP2*)-like floral repressors (Wu and Poethig, 2006; Aukerman and Sakai, 2003; Figure 1.2). The hormone pathway, predominantly involving gibberellin (GA), promotes flowering through the balance of GA levels and is essential for flowering of Arabidopsis in short days (Wilson, Heckman and Somerville, 1992; Jacobsen and Olszewski, 1993; Figure 1.2). The photoperiod pathway enables flowering during long days by activating *CONSTANS* (*CO*) which in turn promotes transcription of *FT* (Samach *et al.,* 2000; Sawa *et al.,* 2007; Figure 1.2).

Lastly, vernalisation describes the promotion of flowering following prolonged exposure to winter cold necessary for silencing of the floral repressor *FLOWERING LOCUS C (FLC)* promoting flowering regardless of day length (Figure 1.2). This is described in detail below.

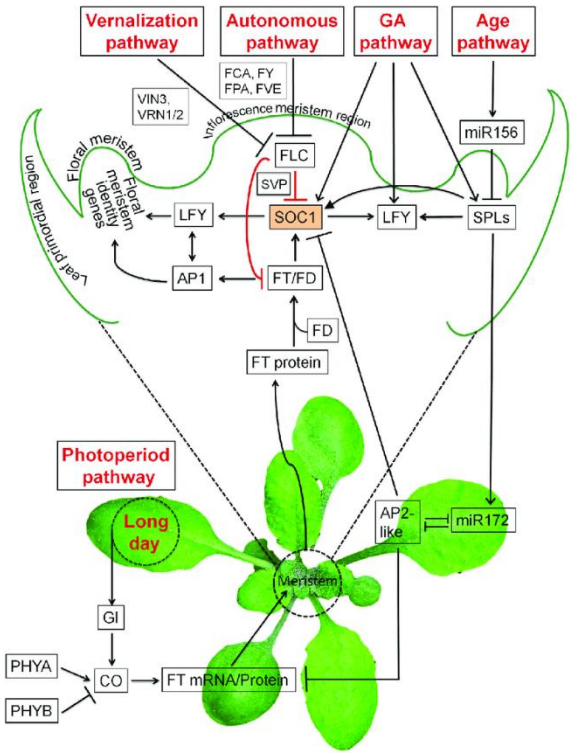


Figure 1.2 Overview of the major flowering pathways in *A. thaliana*. Reproduced from Teotia and Tang (2015). Here the general RNA processing factors contributing to *FLC* expression is referred to as the 'autonomous pathway'.

1.3.2 Molecular control of vernalisation in the laboratory

In winter annuals vernalisation response is crucial for preventing autumn flowering (Michaels *et al*., 2005). There are two major genes that influence vernalisation in Arabidopsis: *FRIGIDA (FRI*) and *FLOWERING LOCUS C* (*FLC*), together they control up to 70% of natural variation in flowering time and vernalisation requirement (Napp-Zinn, 1961; Burn et al., 1993; Koornneef et al., 1994; Clarke and Dean 1994; Johanson et al., 2000, Lempe *et al.,* 2005; Shindo *et al.,* 2005).

The downregulation of *FLC* in response to winter cold enables spring flowering. This feature is shared across many eudicots and other species, e.g. cereals (Reeves *et al.,* 2007). In *A. thaliana, FLC* acts as a floral repressor, preventing transcription of *FT*, *SOC1* and *FD*, thus preventing flowering during autumn (Searle *et al.,* 2006; Helliwell *et al.,* 2006). During

vernalisation, colder temperatures lead to a decrease in *FLC* expression allowing *FT* activation and subsequently enabling flowering during the following spring (Searle *et al.,* 2006). A complex network of environmentally and internally regulated genes influences the precise level of *FLC* expression.

In addition to *FLC*, there are five other *FLC* clade members that also influence flowering time and are involved in vernalisation. These include *MADS AFFECTING FLOWERING* (*MAF1*) (commonly known as *FLOWERING LOCUS M (FLM)),* and *MAF2-5* which are located in a 22-kb tandem repeat region of the *Arabidopsis* genome*,* and all act as floral repressors (Ratcliffe *et al.,* 2003; Scortecci *et al.,* 2001; Parenicova *et al.,* 2003). The regulation of *FLM* and *MAF2* involves temperature-associated alternative splicing events and *MAF3* activity is most pronounced at low temperatures (Gu *et al.,* 2013). In contrast, *maf4* and *maf5* mutants tend to flower slightly earlier and exhibit greater sensitivity to vernalisation exceeding 20 days. This suggests *MAF4* and *MAF5* play a role in the later stages of vernalisation (Kim *et al.,* 2013, 2015; Kang *et al.,* 2015).

During floral repression in winter, *FLC* can bind to genes alone or as part of protein complexes. As such, *FLC* contains several domains that can interact with other proteins. FLC's target activity varies depending on the other proteins it forms complexes with (Mateos *et al.,* 2015). For example, *FLC* forms complexes with several other other MADS-box transcription factors, including *SVP, MAF3, MAF4* and *FLM* (Gu *et al.,* 2013). Many of these complexes overlap in spatial and temporal expression. However, some complexes, such as those involving FLM, MAF3 and SVP alter in abundance as MAF3 and SVP are under circadian or temperature control (Fujiwara et al., 2008; Gu et al., 2013). Therefore, *FLC's* target specificity and efficacy is determined by the protein/s with which it forms heterodimers with (Li et al., 2008; Gu et al., 2013).

1.3.2.1 Other roles of FLC beyond vernalisation

Prior to vernalisation, *FLC* is actively transcribed to prevent precocious flowering, before being stably silenced by vernalisation. After vernalisation *FLC* is reactivated during late embryogenesis (Figure 1.3), this acts to reset the vernalisation requirement in subsequent generations (Sheldon *et al.,* 1999; Choi *et al.,* 2009; Crevillen *et al.,* 2014). *FLC* will interact with cis-regulatory elements of genes to either promote or inhibit their activity. Approximately 500 *FLC* binding sites in have been predicted in the *A. thaliana* genome (Deng *et al.,* 2011). Critically, *FLC* function is not limited to vernalisation but controls many processes throughout plant development (Soppe *et al.,* 2021). For example, *FLC* is involved in seed dormancy

(Chiang *et al.,* 2009; Chen *et al.,* 2014; Chen and Penfield, 2018); circadian clock function (Edwards *et al.,* 2005); the juvenile to adult transition (Deng *et al.,* 2011); floral organ identity (Deng *et al.,* 2011); outgrowth of axillary meristems (Wang *et al.,* 2009) and even leaf size in *Cardamine hirsuta* (Cartolano *et al.,* 2015).

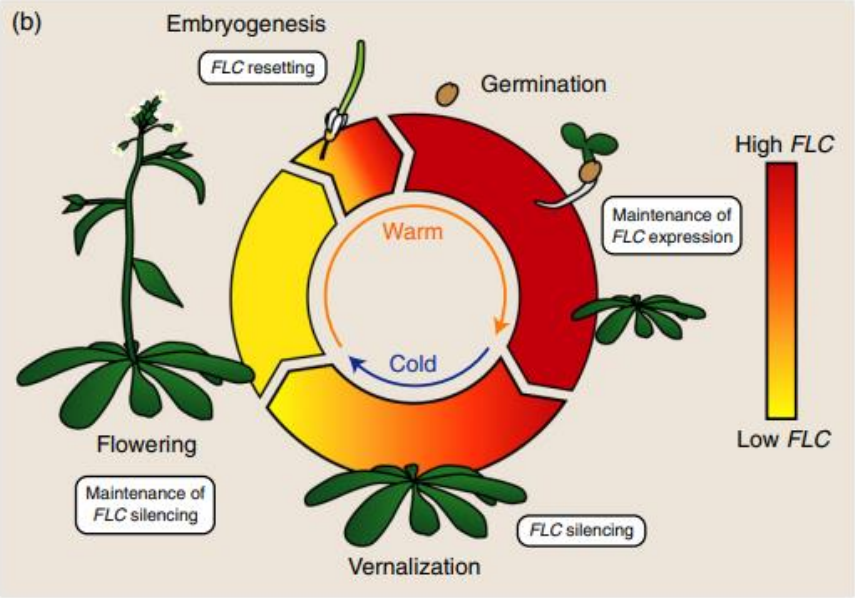


Figure 1.3 Expression of *FLC* throughout development in *A. thaliana*, reproduced from Berry and Dean (2015). Before and after vernalisation *FLC* is stably expressed, but vernalisation causes downregulation of *FLC* whilst embryogenesis causes upregulation of *FLC*

1.3.3 Control of *FLC* expression in *A. thaliana*

1.3.3.1 RNA processing factors maintain *FLC* repression in long and short days

General RNA processing factors are necessary for silencing and maintaining *FLC* repression in both long and short days, this transcriptional machinery was previously described as the 'autonomous pathway' ('AP') (Koorneef *et al.,* 1998; Levy and Dean, 1998). Twenty-three *FLC*

processing factors, outside of floral pathway genes, have been identified (reviewed in Cheng *et al.,* 2017). These *FLC* RNA processing proteins act in different ways to silence *FLC.* For instance, *FVE, LD* and *FLD* silence *FLC* through chromatin changes (Ki *et al.,* 2006; Liu *et al.,* 2007; Yu *et al.,* 2016), and *FVE* antagonises upregulation of *FLC* by the *FRI* complex (Lee and Amasino, 2013; Scho *et al.,* 2021). Meanwhile, *FPA, FCA* and *FY* mediate 3'-end antisense transcript silencing of *FLC* (Simpson *et al.,* 2003; Liu *et al.,* 2010), or post-translational modifications. *FCA* and *FPA* independently interact with *FY* interact to promote the use to the proximal *COOLAIR* antisense poly(A) site which acts to silence *FLC* (Hornyik *et al.,* 2010; Liu *et al.,* 2010).

1.3.3.2 *FLC* activation complexes lead to high *FLC* expression before vernalisation

Control of *FLC* expression is coordinated throughout the seasons. For instance, before winter *FLC* expression is high due to a combination of genes which includes *FRI.* In *A. thaliana,* functional *FRI* is partly responsible for the ecotype's vernalisation requirement (Johanson *et al.,* 2000, Gazzani *et al.,* 2003). *FRI* promotes the accumulation of *FLC* mRNA prior to winter (Simpson and Dean, 2002; Bezerra *et al.,* 2004; Geraldo *et al.,* 2009). To achieve this, *FRI* forms a scaffold protein with *FRIGIDA LIKE1 (FRL1), FRIGIDA ESSENTIAL1 (FES1), SUPPRESSOR OF FRIGIDA 4 (SUF4)* and *FLC EXPRESSOR X* (*FLX*), forming a transcription activator complex (FRI-C). This complex targets the *FLC* promotor and recruits chromatin modification factors, the SWR1 complex and additional transcription factors collectively leading to an upregulation of *FLC* (Choi *et al.,* 2011; Figure 1.4).

Mutant screens of early flowering *A. thaliana* lines identified several genes involved in various other *FLC* activation complexes. This led to identification of three complexes that activate *FLC,* including SWR1-C, FRI-C and PAF1-C (He *et al.,* 2004; Choi *et al.,* 2011; Bezerra *et al.,* 2004). Protein components of FRI-C physically interact with and recruit the SWR1-C protein complex to *FLC,* SWR1-C assists in the incorporation of the H2A.Z histone variant, which in turn increases *FLC* expression under warmer temperatures (Deal *et al.,* 2007; Kumar and Wigge, 2010). FRI-C also recruits other histone methyltransferases, namely ATX1, EFS and SDG25, which contribute to the increase in *FLC* transcription (Pien *et al.,* 2008; Berr *et al.,* 2009; Choi *et al.,* 2011).

1.3.3.3.1 The role of PAF1-C in *A. thaliana*

The Polymerase II Associated Factor Complex (PAF1-C) assists RNA Polymerase II in transcription elongation and promotes histone modifications (Tomson and Arndt, 2013). Forward genetic screens conducted on early-flowering mutants revealed that the protein components of the PAF1-C are involved in *FLC* activation. Notably, *paf1-c* mutants demonstrate decreased deposition of the active H3K4me3 and H3K36me2/3 histone marks but an increase of the repressive H3K27me3 mark (Zhang and van Nocker, 2002; Zhang *et al.,* 2003; He *et al.,* 2004; Oh *et al.,* 2004).

Among the PAF1-C components, the plant homolog of *CELL DIVISION CYCLE 3 (CDC73)*, known as *PLANT HOMOLOGOUS TO PARAFIBROMIN* (*PHP*), activity is limited to flowering time (Park *et al.,* 2010). *PHP* targets a select number of genes, including *FLC* and its clade members *FLM, MAF4* and *MAF5* (Yu and Michaels, 2010). However, other *paf1-c* subunit mutants induce more varied phenotypes, encompassing reduced plant size and floral organ abnormalities and lead to thousands of differentially expressed genes compared to wild-type (He *et al.,* 2004; Oh *et al.,* 2004).

1.3.3.3.2 The role of PHP from the PAF1C

The reason why the *cdc73/php* mutants' function is limited to *FLC* and its clade members is yet to be understood. In yeast, CDC73 chiefly prevents target silencing through effective termination of transcripts rather than their elongation (Kowalik *et al.,* 2015). Further, in humans CDC73 binds to a CPSF-CstF RNA processing complex to promote 3' mRNA processing (Rozenblatt-Rosen *et al.,* 2009). This suggests CDC73/PHP acts at the 3' end of target genes. This demonstrates similarity to the 3' activity on *FLC* observed in *FLC* RNA processing (previously known as the 'autonomous pathway'). FCA, FPA and FY, are associated with RNA binding and processing at the 3' end of *FLC.* They also interact with the same CPSF-CstF complex and enhance proximal polyadenylation of *COOLAIR* regulation (Swiezewski *et al.,* 2009; Hornyik *et al.,* 2010; Liu *et al.,* 2010). Interestingly, Yu and Michaels (2010) discovered that *cdc73/php* mutants suppressed late flowering and significantly lowered *FLC* expression in *fca, flk* and *fy* mutants, but in *fld, fve* or *ld* mutants *cdc73/php* only slightly accelerated flowering and showed no difference in *FLC* expression. These findings suggest CDC73/PHP function on *FLC* is related to RNA processing, but not histone modifications (Rataj and Simpson, 2013).

1.3.3.3 Downregulation of *FLC* during winter in *A. thaliana*

To facilitate spring flowering, *FLC* is downregulated by winter cold. The epigenetic silencing of *FLC* occurs in two stages. During the first two weeks of cold, the VAL1 protein associates with the nucleation region of *FLC* in intron 1 and then HDA19 is recruited, resulting in reduced *FLC* transcription (Questa *et al.,* 2016). During this phase antisense transcripts of *FLC,* originating from the 3' region of *FLC*, and together named *COOLAIR*, are upregulated and reduce *FLC* expression by altering transcription dynamics in a chromatin-dependent manner, such as ensuring the replacement of active H3K36me3 with repressive H3K27me3 (Csorba *et al.,* 2014; Rosa *et al.,* 2016).

Molecular genetic analyses and modelling have shown that long-term *FLC* shutdown primarily involves chromatin changes at the *FLC* locus. Long-term *FLC* shutdown involves recruitment of the Polycomb Repressive Complex 2 (PRC2) and the PLANT HOMEODOMAIN (PHD) proteins VERNALISATION INSENSITIVE 3 (VIN3) and its homolog VERNALISATION 5 (VRN5/VIL1) to the nucleation region of *FLC* (Wood *et al.,* 2006; Greb *et al.,* 2007). The PHD-PRC2 complex catalyses the deposition of H3K27me3, replacing the active histone marks H3K36me3 and H3K4me3 (Wood *et al.,* 2006; Young *et al.,* 2011; Muller-Xing *et al., 2014*).

The expression of VIN3 is influenced by cold temperatures across multiple timescales. *VIN3* is upregulated in response to several weeks of prolonged cold (Sung and Amasino, 2004). This is mediated through the cold-dependent accumulation of NTL8. NTL8 encodes a NAC domain transcription factor that directly binds *VIN3* and the *COOLAIR* promotor. NTL8 concurrently activates the transcription of COOLAIR *FLC* antisense (Zhao *et al.,* 2020; Zhao *et al.,* 2021). On a shorter timescale, *VIN3* transcripts are rapidly degraded following spikes of warm temperature (Hepworth *et al.,* 2018) and VIN3 expression varies on a diurnal cycle influenced by the circadian clock (Antoniou-Kourounioti *et al.,* 2018).

Two long noncoding RNAs (lncRNAs), COLDWRAP and COLDAIR, are also upregulated in response to cold. They are involved in the negative regulation of *FLC* potentially by recruiting PHD-PRC2 to *FLC.* (Heo and Sung, 2011; Kim *et al.,* 2017; Kim and Sung, 2017).

The active repression of *FLC* in response to cold happens alongside a reduction in *FLC* activation. For instance, during vernalisation, *FRI* undergoes proteasome-mediated degradation. In vernalising temperatures *FRI* forms nuclear condensates with *FRIGIDA like 1 (FRL1)* which sequester *FRI* away from the *FLC* promotor (Zhu *et al.,* 2021). The accumulation of these condensates is promoted by an alternatively spliced version of *COOLAIR,* which itself is induced by *FRI* response to cold. Importantly, this temperature-responsive condensate

formation is dynamic. During spikes of warm temperature, the association of FRI with FRL1 is reversible, leading to reactivation of *FLC* by *FRI* (Zhu *et al.,* 2021).

The winter cold response therefore involves numerous different inputs across multiple timescales. Different sensory inputs likely contribute to the formation of a distributed temperature-sensing network, enabling a precise response to complex and prolonged environmental stimuli (Antoniou-Kourounioti *et al.,* 2021*).*

1.3.3.4 Stabilisation of the *FLC* state after winter

In laboratory experiments when *A. thaliana* plants are returned to warmth after vernalisation treatment the repressive H3K27me3 mark on the nucleation region of *FLC* spreads across the entire *FLC* locus, causing stable repression of *FLC* (Jiang and Berger, 2017; Yang *et al.,* 2017). The chromatin modifying enzyme LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) plays a crucial role in facilitating the spreading of the H3K27me3 mark at the *FLC* locus (Berry *et al.,* 2017; Yang *et al.,* 2017). LHP1 physically associates with VAL1 and PRC2 subunits in what is believed to be a positive feedback mechanism for chromatin modification. In this mechanism, LHP1 binds with H3K27me3 and interacts with the PRC2 subunit MSI1 which deposits further H3K27me3 at the *FLC* locus (Derkacheva *et al.,* 2013; Yuan *et al.,* 2016). These changes are subsequently preserved through cell division (Mylne *et al.,* 2006). Whittaker and Dean (2017) propose that the process of cell division in warmer conditions could aid in the spreading of H3K27me3 across the locus. This is based on observations that mutants with altered DNA polymerase function cannot stably maintain histone modifications on *FLC* (Hyun *et al.,* 2013). This could explain why an immediate increase to high temperatures (30 °C) after vernalisation inhibits H3K27me3 deposition but not after sustained growth at 22 °C, which allows for cell division (Bouché *et al.,* 2015).

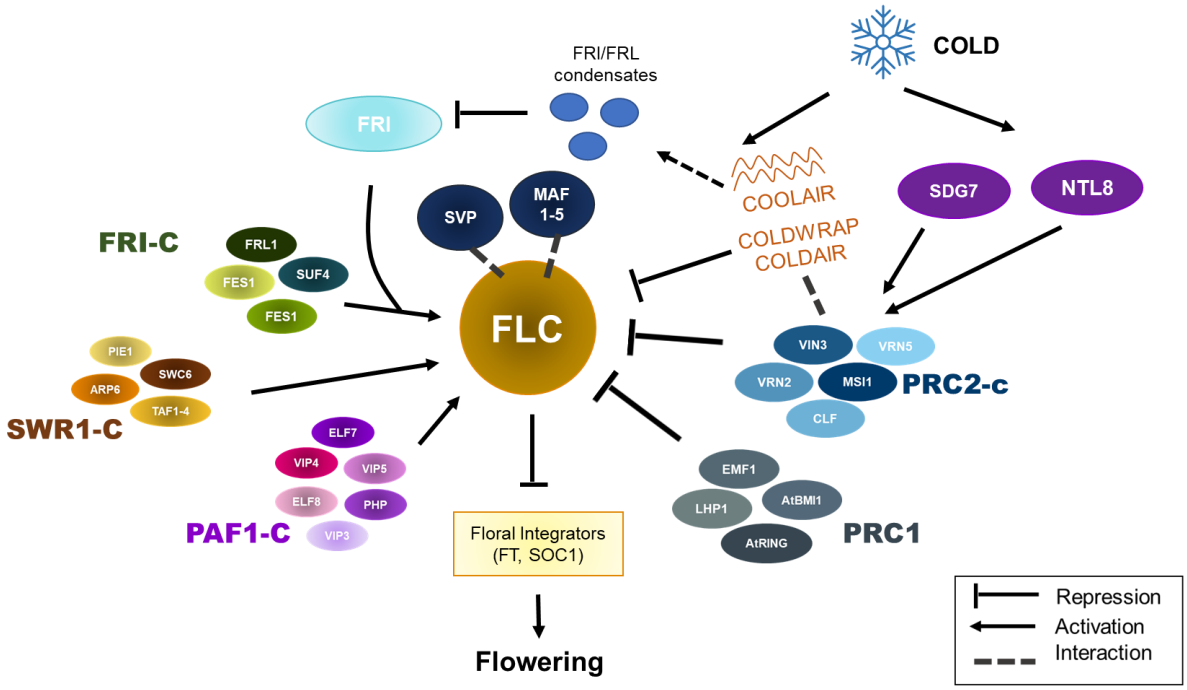


Figure 1.4 Scheme showing up and downregulation of *FLC*

1.3.4 Spring flowering

After vernalisation, spring conditions promote flowering through the action of multiple floral pathways. One of these is the photoperiodic pathway. Under long day conditions, FLAVIN BINDING, KELCH REPEAT F BOX 1 (FKF1) and GIGANTEA (GI) accumulate to high levels in the afternoon (Fornana *et al.,* 2009; Sawa *et al.,* 2007). FKF1 and GI form the FKF1-GI complex in a blue light dependent manner. The photo-induced FKF1-GI complex then degrades the *CONSTANS* transcriptional repressors CYCLING DOF FACTORs (CDFs), enabling *CO* transcription at the end of long days (Fornana *et al.,* 2009; Imaizumi *et al.,* 2005; Sawa *et al.,* 2007). In addition, in long days, the CO protein is stabilised by the far-red light photoreceptor PHYTOCHROME A (phyA) and cryptochrome 2 (cry2) which acts as a blue-light photoreceptor and FKF1 (Valverde *et al.,* 2004; Suárez-López *et al.,* 2001). Once stabilised, CO strongly induces *FLOWERING LOCUS T* (*FT*) around dusk through direct binding to the *FT* promotor and other *FT* regulators (Song *et al.,* 2012; Samach *et al.,* 2000; Valverde *et al.,* 2004). Following activation, *FT* subsequently promotes flowering (Figure 1.5)

Warmer spring temperatures can also promote flowering. *PHYTOCHROME INTERACTING FACTOR4 (PIF4)* expression is enhanced by warmth and then binds to the *FT* promotor in leaves activating it. In warmer temperatures *FT* also experiences lower H2A.Z occupancy

enabling its activity and flowering (Wigge, 2013; Kumar *et al.,* 2012). Warm temperatures also promote the alternative splicing of *FLM* in the shoot apical meristem (SAM) (Balasubramanian *et al.,* 2006), reducing the prevalence of *FLM-β* which under low temperatures forms a repressor complex with *SVP* to repress *SOC1* (Lee *et al.,* 2013; Pose *et al.,* 2013).

Once *FT* is transcribed and translated, FT is transported from the leaves to the shoot apical meristem (SAM). At the SAM, *FT* forms a complex with FLOWERING LOCUS D (FD). The FT-FD complex initiates the transcription of genes that promote flowering, including the floral integrator gene *SOC1*. Both *SOC1* and the *FT-FD* complex then activate genes involved in floral meristem identity including *APETELA 1* (AP1), *LEAFY* (*LFY)* and *FRUTITFUL* (FUL) (Abe *et al.,* 2005; Wigge *et al.,* 2005; Figure 1.5).

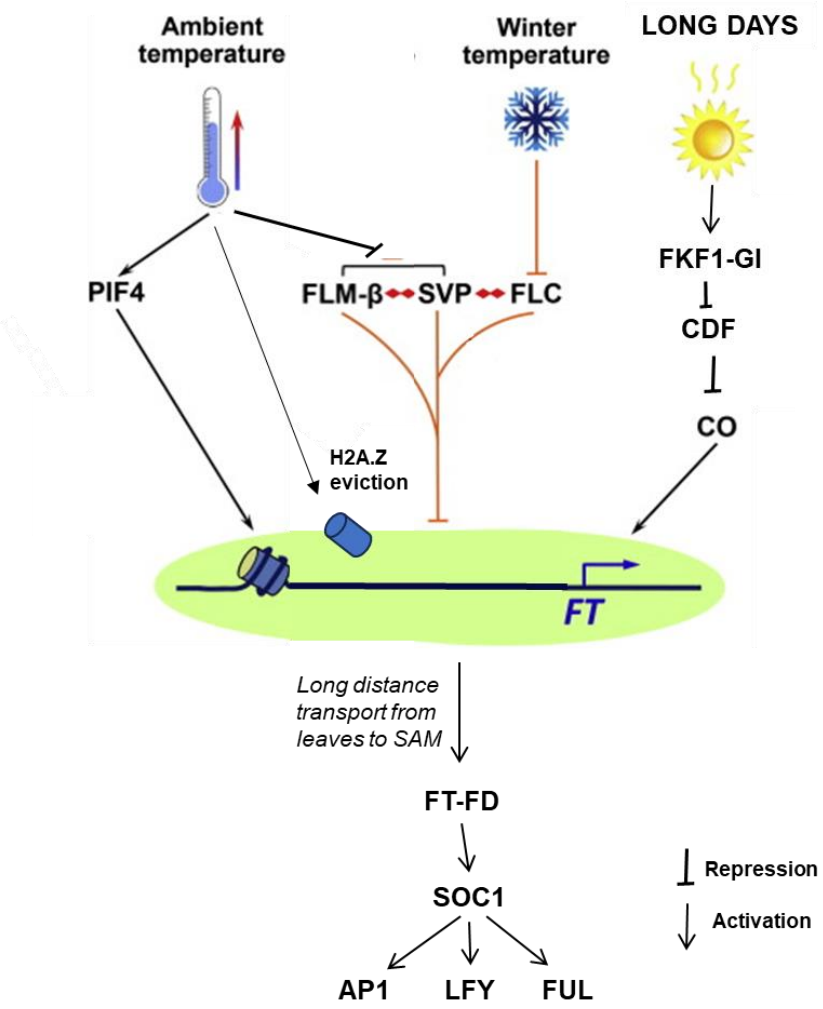


Figure 1.5 Spring flowering is promoted through the photoperiodic and ambient temperature pathways. Figure adapted from Yan *et al.,* 2014.

1.4 The control of vernalisation in the field

1.4.1 Laboratory conditions do not capture fluctuating field conditions

Unlike controlled laboratory experiments where most fundamental research has taken place, environmental conditions in the field are complex and varied. How vernalisation is integrated in fluctuating field conditions is complicated. For example, *A. thaliana* completed vernalisation sooner when the same average temperature was given in fluctuating compared to constant temperatures (Burghardt *et al.,* 2016; Zhao *et al.,* 2020a), suggesting that developmental phenomena in complex environments, like the field, may be different from those understood from controlled laboratory conditions. Indeed, the exact role of floral pathway genes discovered in laboratory studies is likely to be far more complicated. Wilczek *et al.* (2009) grew *A. thaliana* floral pathway mutants in the field and their laboratory phenotype was almost completely absent in some locations, suggesting they are not essential for flowering time in the field, likely as other processes are occurring that are not captured in control laboratory conditions.

1.4.2 The control of vernalisation and floral transition during chilling

In laboratory experiments plants are directly moved from chilling conditions into spring-like warm long days, this enables the transition to flowering through activation of the *FLC* targets *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*) by warmth and red light in the evening (Valverde *et al.,* 2004; Michaels *et al.,* 2005).

However, in the field chilling occurs over longer timescales. Practically this means that *A. thaliana* will saturate its vernalisation requirement during short days in autumn and make flowers ready to open in spring (Duncan *et al.,* 2015). Even in uncharacteristically warm years vernalisation saturation occurs before midwinter (Hepworth *et al.,* 2020), and *A. thaliana* accessions with strong vernalisation requirements will transition to floral meristems in winter not spring (Lu *et al.,* 2019). In *B. napus* too, inflorescence meristems are formed in mid-autumn before spring bolting and flowering (O'Neill *et al.,* 2019). *Arabis alpina* and *Arabidopsis lyrata* will also complete the vegetative to floral transition during vernalisation if chilling is long enough (Wang *et al.,* 2009; Lazaro *et al.,* 2018; Kemi *et al.,* 2019). Thus, it appears that autumn and winter floral initiation in short days is a common feature of the Brassicaceae. This is matched by autumnal silencing of all but two *FLC* genes in *B. napus,* silencing of the *FLC* orthologue *PEP1* immediately before winter floral transition in *A. alpina,* and low levels of *FLC*

expression in autumn seen in early flowering accessions of *A. thaliana* (O'Neill *et al.,* 2019; Hyun *et al.,* 2019; Hepworth *et al.,* 2020).

This is consistent with findings that *A. thaliana* can vernalise at temperatures up to 15 °C, *B. napus* up to 17 °C and even hexaploid bread wheat up to 18 °C (Tommey and Adams, 1991; O'Neill *et al.,* 2019; Dixon *et al.,* 2019). Theoretical studies have also predicted that *B. napus* can fulfil its vernalisation requirement in autumn (Habekotte, 1997). This suggests that in the field vernalisation is responsive to autumnal chilling, not just winter chilling, and this occurs alongside the floral transition in many plants.

In autumn and winter field conditions, *FT* expression is low due to the short daylength (Hyun *et al.,* 2019; O'Neill *et al.,* 2019). In *A. alpina* winter floral initiation therefore occurs in an *FT*-independent manner. This occurs through silencing of the *FLC* orthologue *PEP1* which acts on *SPL15* which can then promote floral initiation (Hyun *et al.,* 2019; Figure 1.6). *PEP1* also acts on genes involved in gibberellin (GA) metabolism. When *PEP1* is silenced during vernalisation higher GA levels further facilitate short day floral transition (Hyun *et al.,* 2016; Tilmes *et al.,* 2019). This is consistent with findings that both GA and *SPLs* are known regulators of *FT*-independent short-day flowering and both known targets of *FLC* (Hisamatsu and King, 2008; Wang *et al.,* 2009; Deng *et al.,* 2011; Mateos *et al.,* 2015; Tilmes *et al.,* 2019). This suggests that winter floral initiation occurs in an *FT*-independent manner and *FT* is instead active in later floral development in spring associated instead with bolting (Hyun *et al.,* 2019; O'Neill *et al.,* 2019; Figure 1.6). This contrasts with the established control of floral development from laboratory studies in *A. thaliana* which suggested only in long and warm spring days can floral initiation occur (Figure 1.6).

Also, unlike laboratory experiments, *FLC* is not always downregulated following vernalisation. For example, short periods of cold can induce *FLC* upregulation (Jung *et al.,* 2013) and high temperatures can promote *FLC* expression post-vernalisation to prevent precocious flowering (Gan *et al.,* 2014). In the perennial *A. alpina*, post-winter reactivation of the *FLC* orthologue *PEP1* is essential for the perennial habit (Hyun *et al.,* 2019) and acts to suppress outgrowth of axillary meristems, thus suggesting *PEP1* is responsible for controlling multiple stages of floral development.

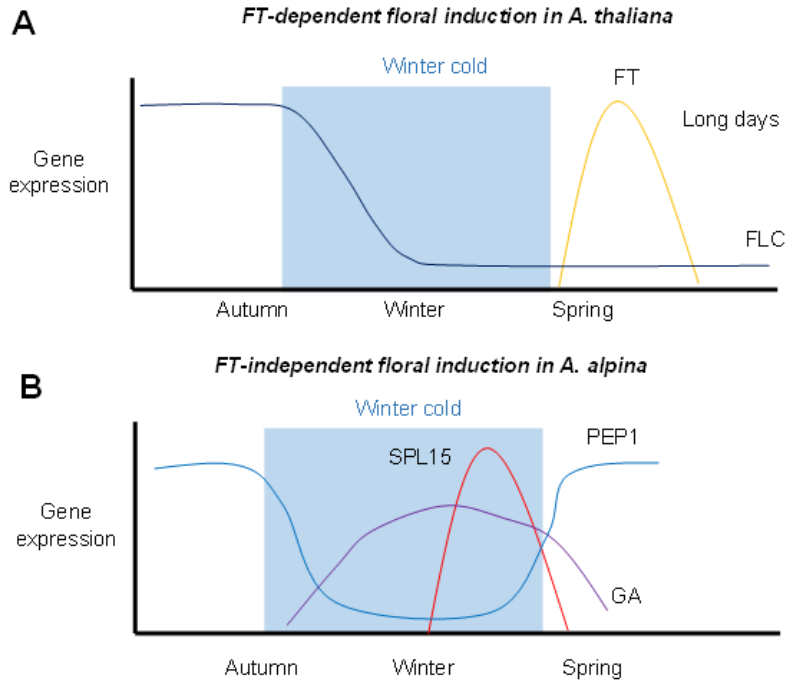


Figure 1.6. The control of *FT-dependent* floral initiation in *A. thaliana* and *FT-independent* floral initiation in chilling in *A. alpina*. A) The model of vernalisation popularised in *A. thaliana* laboratory experiments, whereby cold downregulation of *FLC* enables spring floral development only under long days when *FT* can be activated (Whittaker and Dean, 2017). B) The model of *A. alpina* floral induction during chilling occurs through mutual upregulation of GA and SPL15 following *PEP1* downregulation facilitating floral induction in short days (Tilmes *et al.,* 2019; Hyun *et al.,* 2019).

1.4.3 The role of *FLC* in oilseed rape

Being a close crop relative of *A. thaliana*, the role of multiple *FLC* genes in *B. napus* have been extensively studied. Due to a historic genome triplication, as well as a tandem or segmental duplication, the *B. napus* genome encodes up to nine *FLC* genes (Zou *et al.,* 2012; Cai *et al.,* 2014).

All nine *Bna.FLCs* act as floral repressors, albeit to differing extents, with differences in regulation (Tadege *et al.,* 2001; Jones *et al.,* 2018). Among these nine genes, the coding sequence is highly conserved (80-99%) yet differences in intron and promotor sequences, including transposon insertions, lead to diverse expression patterns across *Bna.FLC* genes

and crop types (Tadege *et al.,* 2001; Wang *et al.,* 2012; Hou *et al.,* 2012; Shah *et al.,* 2018; Schiessl *et al.,* 2019a; Raman *et al.,* 2019; Yin *et al.,* 2020). The retention of such a high number of paralogues implies *Bna.FLC* genes may be dosage sensitive or individual genes may have acquired new functions (Maere *et al.,* 2005). Indeed, there is evidence that individual paralogs have different vernalisation responses (Table 1.1). For instance, *Bna.FLC.A10* has the strongest effect on vernalisation (Tadege *et al.,* 2001; Long *et al.,* 2007; Hou *et al.,* 2012). While *Bna.FLC.A02, Bna.FLC.A03a, BnaFLC.A03b, Bna.FLC.C02, Bna.FLC.A10*are downregulated by cold (Raman *et al.,* 2016; Schiessl *et al.,* 2019a) but *Bna.FLC.C09a, Bna.FLC.C09b* and *Bna.FLC.C03a* have lost their cold responsiveness implying they may have sub functionalised (Schiessl *et al.,* 2019a), and *Bna.FLC.C03b* is a pseudogene (Zou *et al.,* 2012; Schiessl *et al.,* 2019a).

More recently, computational modelling has suggested that the cumulative expression of all *Bna.FLC* genes in *B. napus* determines vernalisation requirement rather than individual paralog expression (Calderwood *et al.,* 2021). Although, differential temporal expression of *Bna.FLCs* may still contribute to novel functionality. For instance, *Bna.FLC.C02* and *Bna.FLC.A03b* remain active following the floral transition in WOSR suggesting they may have a role beyond winter floral initiation (O'Neill *et al.,* 2019).

Table 1.1 Known functions of *Bna.FLC* genes

FLC gene	Known Function	Citation
BnaFLC.A02	Downregulated by cold	Raman et al., 2016; Schiessl et al., 2019a
	Associated with flowering time under vernalising conditions	Tudor *et al.,* 2020
	Associated with flowering time generally (not necessarily under vernalisation)	Scheissl *et al.,*2017; Raman *et al.,* 2016
	High expression required for winter crop types (MITE insertion attenuates cold induced repression in WOSR)	Hou *et al.,* 2012; Yin *et al.,* 2020

BnaFLC.A03a	Downregulated by cold	Raman et al., 2016; Schiessl et al., 2019a
BnaFLC.A03b	Downregulated by cold	Raman et al., 2016; Schiessl et al., 2019a
	Active after the floral transition in WOSR	O'Neill et al., 2019
	Differentially expressed between WOSR and SOSR	Schiessl *et al.,* 2019
BnaFLC.A10	Strongest effect on *B. napus* vernalisation	Tadege et al., 2001; Long et al., 2007; Hou et al., 2012
	Downregulated by cold	Raman et al., 2016; Schiessl et al., 2019a
	Duplicated in Swedes, selection against bolting	Schiessl *et al.,* 2017b
	Sequence variation at *COOLAIR* binding site accounts for crop type expression differences	Schiessl *et al.,* 2019
BnaFLC.C02	Downregulated by cold	Raman et al., 2016; Schiessl et al., 2019a
	Active after the floral transition in WOSR	O'Neill et al., 2019
	Associated with flowering time more generally (not necessarily under vernalisation)	Schiessl *et al.,* 2017; Raman *et al.,* 2016
	High expression required for winter crop types	Yin *et al.,* 2020
BnaFLC.C03a	Lost cold-responsiveness	Schiessl et al., 2019a

BnaFLC.C03b	Likely a pseudogene	Zou et al., 2012; Schiessl et al., 2019a
BnaFLC.C09a	Lost cold-responsiveness	Schiessl et al., 2019a
BnaFLC.C09b	Lost cold-responsiveness	Schiessl et al., 2019a

1.4.4 The role of *FLC* and other chilling responsive MADS-box genes in non-model plants

The role of *FLC* as a chilling responsive floral repressor is broadly conserved across the Brassicaceae and distantly related eudicot species such as soybean and citrus fruits, as well as monocot species like *Allium sativum* and to a lesser extent, cereals (Reeves *et al.,* 2007; Blümel *et al.,* 2015; Ben Michael *et al.,* 2020; Lyu *et al.,* 2020; Ruelens *et al.,* 2013; Agusti *et al.,* 2020).

However, the role of *FLC* as a chilling responsive floral repressor is not conserved in winter annuals and perennials even though vernalisation itself is a conserved process. For instance, in *Beta vulgaris, BtFLCL* plays a minor role in vernalisation (Vogt *et al.,* 2014), while in spinach and kiwifruit, *FLC* homologs act as floral promotors (Hirakawa et al., 2021; Voogd *et al.,* 2021). Certain plants such as Medicago and quinoa lack *FLC* homologs entirely and instead rely on other floral pathways to complete vernalisation (Hecht *et al.,* 2005; Golicz *et al.,* 2020). While in cereals, the *FLC* homologue *ODDSOC2* plays a minor role in vernalisation, with the mechanism instead being controlled by a core module consisting of *VRN1, VRN2* and *VRN3* (Yan *et al.,* 2003, 2004, 2006).

Despite this, a common theme across diverse plant lineages is that while the chilling response may not always be controlled by *FLC* itself, it is regularly controlled by other chilling responsive MADS-box genes. For instance, MADS-box genes control vernalisation in barley, bread wheat, oilseed rape, broccoli and Chinese cabbage (Greenup *et al.,* 2010; Yan *et al.,* 2003; Long *et al.,* 2007; Hou *et al.,* 2012; Irwin *et al.,* 2016; Wu *et al.,* 2012).

Many perennial tree species have also evolved adaptations to cold winters, but instead of vernalisation overwintering buds will undergo a dormant phase in autumn before spring bud break (Hovarth, 2009). In perennial fruit trees this process is also controlled by chilling

responsive MADS-box genes, the most prominent being the *DORMANCY ASSOCIATED MADS-box* (*DAM*) genes (Bielenberg *et al.,* 2008), which belong to the same subfamily of MADS-box genes as *SHORT VEGETATIVE PHASE (SVP)* from Arabidopsis (Srikanth and Schmid, 2011). Thus, it appears common that MADS-box transcription factors are involved in the chilling response between distantly related species with different life history strategies.

1.5 Control of perennial bud dormancy by chilling responsive MADS-box genes

Unlike annuals, perennials complete many growing cycles over the course of multiple years. In perennials shorter days or declining temperatures trigger endodormancy, which is where vegetative and reproductive growth is repressed even under favourable environmental conditions (Lang *et al.,* 1987). Endodormancy is 'broken' after a chilling requirement is met. Following sufficient chilling, buds transition to an ecodormant state, where they are still dormant but have recovered competency to grow under favourable environmental conditions. The molecular control of endodormancy is similar to the control of vernalisation, in that stable silencing of chilling responsive MADS-box genes is required in both processes.

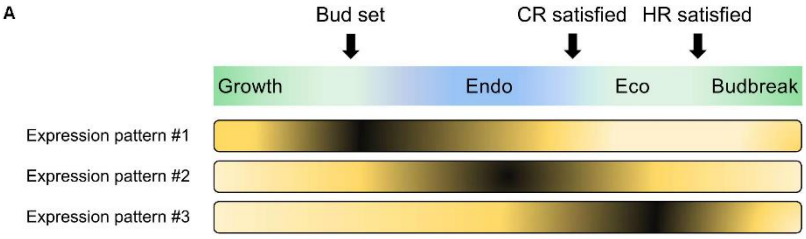
For example, during annual vernalisation, *FLC* forms protein complexes with *SHORT VEGETATIVE PHASE (SVP)* to prevent flowering and floral transition. *SVP* also forms complexes with floral repressors and promoters to control development of floral meristem with *AGL23* and *AP1* (Gregis *et al.,* 2006; Gregis *et al.,* 2008) and control gibberellin metabolism (Andres *et al.,* 2014).

Yet in perennial species, *SVP*-like genes control endodormancy induction and release. In Rosaceae species six *DAM* genes, closely related to *SVP,* are active throughout all dormancy stages, initially discovered from the peach *evergreen* (*evg*) mutant which had all six *DAMs* missing and could not enter dormancy (Bielenberg *et al.,* 2008; Jiminez *et al.,* 2009). Each *DAM* gene shows a distinct seasonal expression pattern (Falavinga *et al.,* 2019; Figure 1.7), but all are downregulated by endodormancy release (Falavinga *et al.,* 2019; Vimont *et al.,* 2019). For instance, in apples *(Malus domestica) MdDAM1* and *MdDAM4* are required to enter dormancy (Moser *et al.,* 2020; Wu *et al.,* 2021), but if *MdDAMb* and *MdSVPa* are overexpressed in apples bud break is delayed but growth cessation and endodormancy induction is unaffected (Wu *et al.,* 2017). This could therefore suggest their downregulation is required for exit from endodormancy. More recently, it has also been shown that *DAM* genes can form complexes with *SVP* and *FLC* genes in apple (Falavinga *et al.,* 2021). Analogous to *FLC* in annual species*, DAM* genes are silenced during prolonged cold by the repressive

chromatin mark H3K27me3 and antisense transcripts (Lloret *et al.,* 2017; De la Fuente *et al.,* 2015; Leida *et al.,* 2012; Zhu *et al.,* 2020).

However, *DAM* genes do not take the role of *FLC*. During Rosaceae evolution, *SVP*-like genes expanded and *FLC-like* genes were lost (Liu *et al.,* 2020), which may have favoured neo-functionalisation of *SVP-like* genes in dormancy control which is consistent with the multiple roles of *DAM* genes across dormancy (Figure 1.7). Instead in apple *MdoFLC* is suggested to inhibit growth after transition from endo- to ecodormancy as *MdoFLC* is seasonally expressed during ecodormant buds and has been identified from a QTL study of bud break in apple (Porto *et al.,* 2015; Kumar *et al.,* 2017; Miotto *et al.,* 2019).

A



B

Expression pattern #1	Expression pattern #2	Expression pattern #3
EesDAM1	*EesDAM2*	***AdeSVP1***
MdoDAM2	*MdoDAM1*	***AdeSVP2***
PmuDAM1	*MdoDAM3*	***AdeSVP4***
PmuDAM2	*MdoDAM4*	***MdoDAMb***
PmuDAM3	*PavMADS1*	
PmuSVP1	*PavMADS2*	
PmuSVP2	*PmuDAM4*	
PpeDAM1	*PmuDAM5*	
PpeDAM2	***PmuDAM6***	
PpeDAM3	*PseDAM4*	
PpeDAM4	*PseDAM5*	
	PpeDAM5	
	PpeDAM6	
	PpyMADS13-1	
	PpyMADS13-2	
	PpyMADS13-3	
	PpyDAM3	

Figure 1.7 Seasonal expression dynamics of different *DAM* and *SVP* genes and corresponding dormancy phases in perennial species reproduced from and summarised in Falavinga *et al.,* 2019. Black shading indicates expression peak of genes for each expression profile. CR is chilling requirement and HR is heating requirement needed before perennial development will proceed.

Work in hybrid aspen has further shown the key role of *SVP*, as well as the phytohormones abscisic acid (ABA) and gibberellin (GA) in control of bud dormancy (Singh *et al.,* 2018, 2019; Tylewicz *et al.,* 2018). In hybrid aspen lacking the *SVP-like (SVL)* gene vegetative meristems cannot enter dormancy, exactly like the *evg* peach mutant (Singh *et al.,* 2018). Dormancy occurs through *SVL* binding to *BRC1* which upregulates abscisic acid (ABA) biosynthesis and receptor genes as well GA2 oxidases which reduces gibberellin levels (Singh *et al.,* 2018). This mechanism mirrors the FT-BRC1 module that prevents lateral bud outgrowth of axillary meristems in *A. thaliana* (Niwa *et al.,* 2013). The dormant state is then maintained through a positive feedback loop between *SVL* and ABA in hybrid aspen, before long-term cold reduces ABA levels which in turn leads to bud break (Tylewicz *et al.,* 2019; Singh *et al.,* 2018). This is similar to perennial fruit trees, where ABA biosynthesis genes, some of which bind to *DAM* promotors, are activated at the beginning of dormancy while ABA catabolism genes towards the end of dormancy. Thus ABA levels are essential for dormancy control (Li *et al.,* 2003; Tuan *et al.,* 2017; Yang *et al.,* 2018; Li *et al.,*2018; Li *et al.,* 2019; Yang *et al.,* 2020).

1.6 Thesis Overview

This thesis will investigate the continued physiological requirement for winter cold after the floral transition in *B. napus* and investigate how this chilling requirement impacts yield. This thesis will also explore the genetic basis for this requirement. This will provide a foundation for future breeding efforts to adapt *B. napus* to a changing environment.

The first results chapter aims to understand the phenotypic variation to winter warming following floral initiation in a diversity set of *B. napus.* I test the hypothesis that the within the diversity panel there will be variation in the requirement for winter cold following the floral transition. To do this, I investigate the effect of winter warmth following the floral transition on *B. napus* floral development and seed characteristics in three independent experiments. I show that the physiological response to winter warming after floral initiation is genotype dependent. In WOSR winter warming causes a floral developmental delay and lower thousand grain weight (g) but in SWOSR and Swedes winter warming accelerates floral development, and in SOSR there is no effect on floral development. The floral delay in WOSR resembles perennial bud dormancy. This previously undefined bud dormancy stage is only present in certain WOSR and SOSR varieties implying it is under genetic control.

In the second results chapter, I aim to understand the molecular control of bud dormancy. To do this I used transcriptomics, comparing a variety with warming-induced bud dormancy to a second in which warming promotes early flowering. I show using gene ontology analysis and clustering analysis that the main differences between these varieties is in the behaviour of

genes known to have roles in dormancy processes. I also present evidence that varieties with no dormancy response to warming lack expression of *FLC* and MAF genes, providing evidence that *FLCs*, and their clade members, act as a potential regulators of winter bud dormancy in *B. napus.* A Genome Wide Association Study (GWAS) and Gene Expression Marker Analysis (GEM) further support this notion as they identify *Bna.FLC.C02* expression is correlated to flowering time responses to winter cold. I also show that SNP variation in the *FLC* regulator *Bna.PHP.A05* correlates with the effect of winter temperature on flowering time.

The final results chapter focusses on further analysis of the association identified between *Bna.PHP.A05* and flowering time. Here, I identify putative loss of function alleles of *Bna.PHP.A05* in early flowering varieties and show that mutation of PHP.A05 in *B. rapa* leads to early flowering. I show evidence that up to three haplotypes of *Bna.PHP.A05* have varying effects on flowering time in *B. napus.*

Chapter 2. Materials and Methods

2.1 Diversity Set Analysis

Ninety-six lines from the *B. napus* Diversity Fixed Foundation Set (Harper *et al.,* 2012) were sown in a concrete-floored polytunnel (Keder house; www.kedergreenhouse.co.uk) with maximum ventilation (sides down, doors open), no supplemental lighting or heating, automatic irrigation twice daily and overnight (22:00- 07:00) sulphur burning. WOSR, Swedes and Exotic lines were sown on 24/08/2020, while SWOSR and SOSR lines were sown twice on the 09/09/2020 and 21/09/2020 as past flowering time data indicated they flower earlier than WOSR lines. For each variety we used the sowing that most closely matched WOSR in the timing of floral initiation in late autumn or early winter. Indicator varieties from each crop type (Spring: Stellar DH; Semi-winter: Zhongshuang 11; Swede: Altasweet; Mid-winter: Vision; Late-winter: Dippes; Early-winter: Catana; Exotic: Slapska Slappy) were dissected throughout the growing season to determine the timing of floral transition, which was then confirmed by dissecting one plant of each line. Two weeks after the floral transition, three biological replicates of each variety were transferred to a warmer but unlit glasshouse maintained at 20 °C/16 °C Day/night temperatures for four weeks and compared to three biological replicates kept in a control control polytunnel. In practise the lines were transferred to the warming treatment in 5 cohorts depending on floral initiation date (Figure 2.1; Table 2.2). After treatment, all plants were returned to the polytunnel and potted into 5L pots with cereal mix compost and then sorted into a randomised incomplete block design using Gendex software (http://designcomputing.net/gendex/). In total there were 12 blocks with 48 plants in at plant density of 15 plants/m^2. Plants were scored for date to first flower opening and bud emergence using the BBCH (Biologische Bundesanstalt,Bundessortenamt und Chemische Industrie) scale (Weber and Bleiholder, 1990). Accumulated thermal time was calculated as degree days, calculated as hourly $\Sigma(T - T_b)/24$ where T was temperature in degrees Celsius and T_b is the base temperature of 3 °C (Habekotté, 1997). As plants reached the end of growing season (June – August, depending on variety) watering was stopped to allow matured plants to fully dry. Maturity was judged based on fully set pods, stems and pods starting to dry out and cessation of vegetative growth. When matured plants were dry, 20 representative pods were harvested from the primary raceme and secondary raceme material was collected. Primary and secondary raceme material was then threshed and analysed. Plant height (cm) was calculated on 31st March 2021 and at final harvest. Seeds from the twenty pods on the primary raceme were analysed for Thousand grain weight (TGW) (g), Weight (g), Number of seeds,

Seeds Per Pod (SPP), Area measurements (including Area, Width, and Length) using a MaRViN ProLine Seed Analyser (http://marvitech.de/en/) and a standard balance with 0.001g resolution.

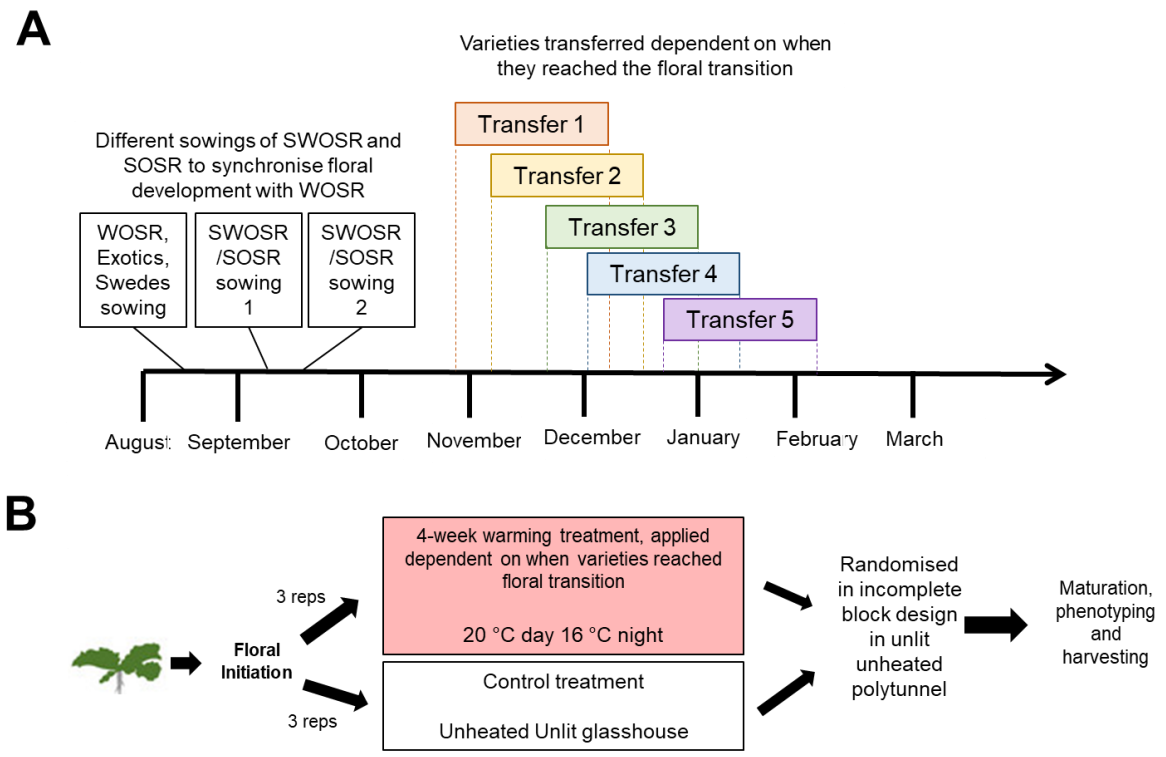


Figure 2.1. Schematic showing the experimental design for the winter warming experiment. A) timeline for sowing and treatment transfer. B) Schematic showing that after floral initiation 3 replicates of each variety were transferred to a warmer glasshouse and 3 remained in a control unlit control polytunnel.

Table 2.1 Lines Grown from DFFS Diversity set in 2020-2021 growing season, their corresponding crop type, sowing date, transfer number (T1: 20/11/2020 – 18/12/2020; T2: 28/11/2020 – 26/12/2020; T3: 17/12/2020 – 14/01/2021; T4: 11/01/2021 – 08/02/2021; T5: 04/02/2021 – 04/03/2021) and country of origin.

Line	Crop Type	Sowing Chosen	Transfer number	Country of Origin
ABUKUMANATANE	Exotics	26/08/2020	T4	JPN
BRAUNERSCHNITTKOHL	Exotics	26/08/2020	T5	FRA
CHEMBEREDZAGUMHANA	Exotics	26/08/2020	T5	GBR
COUVENABICA	Exotics	26/08/2020	T5	GBR
GROENEGRONINGERSNIJMOES	Exotics	26/08/2020	T5	FRA
Q100	Exotics	26/08/2020	T4	USA
RAGGEDJACK	Exotics	26/08/2020	T4	USA
RAPIDCYCLINGRAPE(CrGC5)	Exotics	26/08/2020	****	NOR
SIBERISCHEBOERENKOOL	Exotics	26/08/2020	T5	NZL
SLAPSKASLAPY	Exotics	26/08/2020	T4	NZL
Taisetsu	Exotics	26/08/2020	T4	NZL
Chuanyou2	Semiwinter OSR	09/09/2020	T1	GBR
Ningyou7	Semiwinter OSR	21/09/2020	T1	GBR
Shengliyoucai	Semiwinter OSR	09/09/2020	T1	NZL
SWUChinese1	Semiwinter OSR	21/09/2020	T1	NZL
SWUChinese2	Semiwinter OSR	21/09/2020	T1	NZL

Xiangyou15	Semiwinter OSR	09/09/2020	T1	FIN
ZhongshuangII	Semiwinter OSR	21/09/2020	T1	GBR
Zhouyou	Semiwinter OSR	21/09/2020	T1	GBR
Liho	Spring fodder	09/09/2020	T1	DEU
Bronowski	Spring OSR	09/09/2020	T1	FRA
CeskaKrajova	Spring OSR	09/09/2020	T1	GBR
CRESOR	Spring OSR	09/09/2020	T1	GBR
CUBSROOT	Spring OSR	09/09/2020	T1	GBR
Drakkar	Spring OSR	09/09/2020	T1	FRA
Duplo	Spring OSR	09/09/2020	T1	FRA
ERGLU	Spring OSR	09/09/2020	T1	FRA
HELIOS	Spring OSR	09/09/2020	T1	GBR
KARAT	Spring OSR	21/09/2020	T1	SWE
KAROO-057DH	Spring OSR	21/09/2020	T1	SWE
MAZOWIECKI	Spring OSR	21/09/2020	T1	GBR
MONTY-028DH	Spring OSR	21/09/2020	T1	GBR
01D-1330	Spring OSR	09/09/2020	T1	GBR
N02D-1952	Spring OSR	09/09/2020	T1	GBR

STELLARDH	Spring OSR	09/09/2020	T1	NZL
SURPASS400-024DH	Spring OSR	21/09/2020	T1	NZL
TANTAL	Spring OSR	09/09/2020	T1	NZL
Topas	Spring OSR	21/09/2020	T1	NZL
Tribune	Spring OSR	21/09/2020	T1	NZL
WEIHENSTEPHANER	Spring OSR	09/09/2020	T1	FIN
WESTAR	Spring OSR	21/09/2020	T1	FIN
Willi	Spring OSR	09/09/2020	T1	FIN
Altasweet	Swede	26/08/2020	T5	USA
HUGUENOT	Swede	26/08/2020	T5	GBR
JAUNEACOLLETVERT	Swede	26/08/2020	T4	POL
SENSATIONNZ	Swede	26/08/2020	T5	NZL
Tina	Swede	26/08/2020	T4	NZL
VIGEDH1	Swede	26/08/2020	T5	NOR
Wilhelmsburger	Swede	26/08/2020	T5	FIN
YORK	Swede	26/08/2020	T5	GBR
Aphidresistantrape	Winter fodder	26/08/2020	T5	GBR
CANARD	Winter fodder	26/08/2020	T4	GBR

DwarfEssex	Winter fodder	26/08/2020	T4	FRA
EnglishGiant	Winter fodder	26/08/2020	T5	FRA
MOANAMOANARAPE	Winter fodder	26/08/2020	T2	GBR
Apex	Winter OSR	26/08/2020	T3	DER
APEX-93_5XGINYOU_3	Winter OSR	26/08/2020	T2	GBR
Baltia	Winter OSR	26/08/2020	T3	GBR
BIENVENUDH4	Winter OSR	26/08/2020	T3	FRA
Cabernet	Winter OSR	26/08/2020	T3	GBR
Cabriolet	Winter OSR	26/08/2020	T2	GBR
CANBERRAXCOURAGE	Winter OSR	26/08/2020	T3	GBR
Capitol	Winter OSR	26/08/2020	T3	GBR
Castille	Winter OSR	26/08/2020	T2	GBR
Catana	Winter OSR	26/08/2020	T2	GBR
Coriander	Winter OSR	26/08/2020	T2	GBR
Dimension	Winter OSR	26/08/2020	T2	FRA
Dippes	Winter OSR	26/08/2020	T2	FRA
EUROL	Winter OSR	26/08/2020	T3	FRA
Excalibur	Winter OSR	26/08/2020	T2	FRA

Expert	Winter OSR	26/08/2020	T3	FRA
Flash	Winter OSR	26/08/2020	T2	FRA
HANSENXGASPARD	Winter OSR	26/08/2020	T2	GBR
HuronxNavajo	Winter OSR	26/08/2020	T3	JPN
IncaxContact	Winter OSR	26/08/2020	T3	JPN
JanetzkisSchlesischer	Winter OSR	26/08/2020	T2	AUT
Kromerska	Winter OSR	26/08/2020	T2	CSK
LEMBKESMALCHOWER(LENORA)	Winter OSR	26/08/2020	T3	DEU
Lesira	Winter OSR	26/08/2020	T2	DEU
LICROWNXEXPRESS	Winter OSR	26/08/2020	T2	DEU
MADRIGALXRECITAL	Winter OSR	26/08/2020	T2	YUG
Matador	Winter OSR	26/08/2020	T3	GBR
NORIN	Winter OSR	26/08/2020	T3	GBR
Palmedor	Winter OSR	26/08/2020	T2	GBR
POH285Bolko	Winter OSR	26/08/2020	T3	USA
Quinta	Winter OSR	26/08/2020	T3	USA
RAFALDH1	Winter OSR	26/08/2020	T2	USA
Ramses	Winter OSR	26/08/2020	T2	USA

Rocket	Winter OSR	26/08/2020	T2	NOR
Samourai	Winter OSR	26/08/2020	T3	NOR
SHANNONXWINNER	Winter OSR	26/08/2020	T2	NZL
SlovenskaKrajova	Winter OSR	26/08/2020	T2	NZL
TAPIDORDH	Winter OSR	26/08/2020	T3	NZL
Temple	Winter OSR	26/08/2020	T2	NZL
Verona	Winter OSR	26/08/2020	T3	NOR
Vision	Winter OSR	26/08/2020	T3	FIN

2.2 Winter warming repeat experiment

A subset of WOSR varieties was grown to confirm findings in an independent year. These lines were chosen as the represented the response of WOSR lines to winter warming. Conditions, treatment, phenotyping and data recording were identical as that for the diversity set analysis. As before, developmental progression was confirmed by apice dissection. Plant height (cm) was recorded on 30th March 2022.

Table 2.2 Lines grown from DFFS diversity set in 2021-2022 growing season. Corresponding crop type, sowing date, transfer date for each line given.

Cultivar	Crop Type	Flowering time (UH – H) in 2020-2021	Sowing date	Transfer date
Castille	Winter OSR	13.7 (**)	25/08/2021	24/11/2021
Excalibur	Winter OSR	17.3 (*)	25/08/2021	08/12/2021
HuronxNavajo	Winter OSR	12.7 (**)	25/08/2021	08/12/2021

IncaxContact	Winter OSR	11.2 (*)	25/08/2021	08/12/2021
LembkesMalchower (Lenora)	Winter OSR	18 (**)	25/08/2021	08/12/2021
Lesira	Winter OSR	12.3 (*)	25/08/2021	01/12/2021
LicrownxExpress	Winter OSR	22 (*)	25/08/2021	01/12/2021
POH285Bolko	Winter OSR	16.7 (**)	25/08/2021	08/12/2021
Rocket	Winter OSR	16 (**)	25/08/2021	01/12/2021
Temple	Winter OSR	16.7 (**)	25/08/2021	08/12/2021
Vision	Winter OSR	14 (***)	25/08/2021	01/12/2021
TapidorDH	Winter OSR	-2.6 (ns)	25/08/2021	01/12/2021
Palmedor	Winter OSR	0 (ns)	25/08/2021	01/12/2021

2.3 Controlled Environment Room Experiments

Two past growing seasons were simulated in two controlled environment room chambers. The two growing seasons chosen represented a cool winter high yielding year for oilseed rape (2010-11) and a warm winter low yielding year (2015-16). Temperature data used in the CERs was taken from the Met Office Integrated Data Archive System (MIDAS) CEDA Archive (www.catalogue.ceda.ac.uk). Coleshill station in the Midlands (Latitutde 52.48012, Longitutde -1.69072) was chosen as the local data point. The CERs were Conviron BDW80 growth room with an ARGUS controller (Conviron; Controlled Environments Limited, Canada), with 15-minute set points for temperature and lighting control. To accommodate plant growth in CER chambers light intensity was set at specific levels according to optimal light levels determined in past experiments (used for CER experiments detailed in Lu *et al.,* 2022), and to mimic photoperiod. This meant light levels started at 175 µmol/m²/s¹ on simulated 24th August before decreasing to the following levels and raising again on the following dates: October 1st: 150 µmol/m²/s¹; November 1st: 100 µmol/m²/s¹; November 21st: 75 µmol/m²/s¹; February 2nd: 100 µmol/m²/s¹; March 17th: 150 µmol/m²/s¹; May 1st: 175 µmol/m²/s¹; June 18th: 200 µmol/m²/s¹. Six *B. napus* DFFS lines were chosen for the experiment (Cabriolet, Castille, Catana, Dimension, Temple and Vision) as they represent commonly grown commercial WOSR varieties. Seeds were sown on simulated August 24th, 2010, and August 24th 2016 respectively. Plants were scored for date to first flower opening and bud emergence using the BBCH scale (Weber and Bleiholder, 1990). Mature plants were harvested on simulated August 15th, before

20 representative pods from the primary raceme were harvested and analysed for seed characteristic differences.

2.4 *Brassica rapa* mutant analysis

Brassica rapa M3 seed of the ji41194-b heterozygous premature stop-codon TILLING mutant was obtained from RevGENUK (Stephenson *et al.,* 2010). Seed was sown in a 9 cm pot before pricking out into a 2 L pot. Plants kept in lit glasshouse with 600 W LED lighting for a 16 hour day (00:00 – 16:00) from October 2022 – January 2023, 20 °C/16 °C day/night temperature, with twice daily automatic irrigation and overnight sulphur burning. Heterozygous mutant plants were crossed by hand in a glasshouse before seed was set, harvested and resown. Seedlings were confirmed as homozygous mutants or outcrossed wild-type by Sanger sequencing. 10 homozygous and 10 wild-type out-segregant seedlings where then transferred to a Hettich CER cabinet (Hettich Instruments, Tuttlingen, Germany). As *Arabidopsis thaliana* experiments had shown there was a stronger effect of cooler temperatures on the mutant phenotype (Nassim et al., 2022), plants were grown at 15°C Day and 12 °C night for 16-hour days, under ambient humidity, automatic irrigation twice a day and kept at 175 µmol/m²/s¹. Plants were then scored for time to BBCH51 and BBCH60 from sowing.

2.5 Vernalisation Experiment data analysis

Flowering time data from an independent experiment conducted by other members of the Penfield laboratory was used for analysis in this thesis. This experiment was a large-scale seed phenotyping experiment on 3456 plants using the 96 DFFS lines of *B. napus* described in 2.1. Plants were grown under twelve different conditions: vernalisation at 5 °C, 10 °C or 15 °C for either six or twelve weeks and then plants were matured in a glasshouse, kept at either 18 °C or 24 °C. Three plants of each line were sown for each condition as biological replicates. Sowing was staggered to ensure vernalisation completed at the same time across all varieties. Only days to BBCH51 and BBCH60 from the end of vernalisation was assessed (Weber and Bleiholder, 1990). Only WOSR and SOSR varieties with a C or T SNP call at the Cab041204.2:750 marker from Harper *et al.* (2012) were analysed, while all other SNP calls ignored for the analysis. Time to BBCH51 and BBCH60 from the end of vernalisation were then compared between C and T groupings using a linear mixed model. Then, SNPs in each gene were used to manually categorize haplotypes of either gene in IGViewer (Broad Institute, Robinson *et al.,*2011) with Darmor *bzh* version 4.0 reference (Chalhoub *et al.,* 2014).

Haplotypes were considered to be the same if there were less than 3 base changes across the entire gene. The time to BBCH51 and BBCH60 were then compared between each haplotype.

2.6 General Plant Growth Methods

All *Brassica napus* seeds used were from a subset of 96 fixed, as doubled haploids (DH) or at S4 and above, from the *Brassica napus* Diversity Fixed Foundation Set (DFFS) (Harper *et al.,* 2012). The subset was chosen to represent the phenotypic diversity, crop type and origins of the *Brassica napus* germplasm. Diversity set data available at: https://www.brassica.info/resource/plants/diversity_sets.php The subset included 45 winter oilseed rape (WOSR), 22 spring oilseed rape (SOSR), 8 semi-winters oilseed rape (SWOSR), 7 swedes and 11 exotic oilseed rape.

Seeds were sown into 9cm pots in peat-based soil (Table 2.3). One week after germination, seedlings were pricked out into individual 9cm pots with cereal mix compost (Table 2.3). After two weeks plants were potted into 1L pots with reduced fertiliser mix compost (Table 2.3). When plants reached the developmental stage of BBCH51 (Weber and Bleiholder, 1990) plants were potted into cereal mix in 5L pots.

Table 2.3. Compost ingredients for all experiments

Compost	Components	Purpose
Peat-based sowing soil (John Innes F2 Starter + GRIT)	10% Grit, 90% Levington F2, 4kg/m3 Dolomitic Limestone, 1.2kg/m3 Osmocote Start	Sowing *Brassica napus* and *Brassica rapa* seedlings
Cereal mix compost	65% peat, 25% loam, 10% grit, 3.0 kg/m3 dolomitic limestone, 1.3 kg/m3 haif multimix 14:16:18 +ME (0.2Mo), 3.0 kg/m3 Osmocote Exact 15:9:11 + 2MgO+TE 8-9 months	Initial 1L and final 5L soil for *Brassica napus* and final *Brassica rapa* 2L potting soil
Reduced fertiliser cereal mix compost	65% peat, 25% loam, 10% grit, 3.0 kg/m3 dolomitic limestone, 0.25 kg/m3 haif multimix 14:16:18 +ME (0.2Mo)	Intermediate low-fertiliser soil for 2L *Brassica napus* pots for overwintering
Levington F2 Starter + GRIT	10% GRIT, 90% PEAT	*Arabidopsis thaliana* seed sowing

2.7 Microscopy and dissections

To determine when the floral transition had occurred single shoot apices were manually dissected using a scalpel and photographed using a Leica M80 dissection microscope fitted with a Leica DFC295 digital camera. Floral transition was determined upon emergence of floral primordia (Figure 2.2).

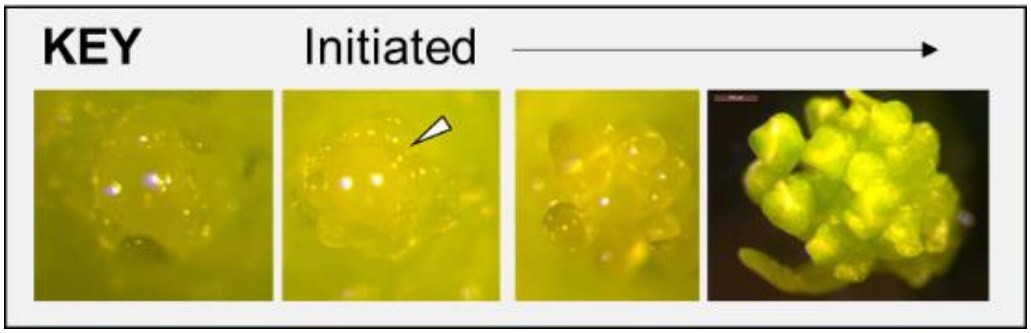


Figure 2.2 Schematic showing the emergence of floral primordia, indicated by white arrow

2.8 Photography

Unless otherwise detailed, plants were photographed side by side against a black background using a Canon PowerShot SX620 HS digital camera. Images were analysed using Image J software by Fiji (Schindelin *et al.,* 2012). Professional photography was carried out by Phil Robinson (Photographer, John Innes Centre).

2.9 Seed measurements

When each plant had reached maturity (pods had fully set, stems and pods dried out and growth had ceased) plants were harvested to analyse seed characteristics. Secondary racemes were collected for seed yield analysis. Twenty representative pods from the primary raceme were collected and seeds removed from the material by threshing. Seeds from those twenty pods were analysed for Thousand grain weight (TGW) (g), Weight (g), Number of seeds, Seeds Per Pod (SPP), Area measurements (including Area, Width, and Length) using

a MaRViN ProLine Seed Analyser (http://marvitech.de/en/) and a standard balance with 0.001g resolution.

2.10 Data Processing

Data for the Genome Wide Association Analysis was cleaned as follows. The entire dataset was checked for anomalous data using the following Z score formula:

$$Z = \frac{(x - \mu)}{\sigma}$$

where x is the data point, μ is the dataset mean, and σ is the standard deviation of the dataset. No anomalous results were identified using Z scores (Z > 2; or Z < -2) in any experiments.

Seed traits (TGW, SPP, Weight, Area, Pod Number, Number of seeds) were filtered by removing all data sets for individual lines where more than 10% of pods were missing out of the chosen twenty on the primary raceme (here < 18 pods). All data points for lines that were heavily diseased, damaged before or during material processing or had abnormal growth habits (fasciated, cojoined stems or missing primary raceme) were also removed. Entire lines were removed when there was wide variation between individual replicates in a treatment, measured by a standard deviation greater than 3. Where two out of three replicates for a line in a treatment had a similar measurement, the anomalous replicate was removed.

All exotic lines were removed from the data set due to inappropriate selection of the indicator variety meaning they received treatment well beyond moment they had florally initiated. Removing exotics, along with heavily disease lines left 86 of the 96 varieties, removing lines with wide variation left 78 lines for analysis.

2.11 Statistical software

All t-tests, ANOVA, Chi-squared and post-hoc tests was performed in OriginPro2022 (OriginLab Corporation, Northampton, MA, USA).

Linear modelling was performed in R version 4.3.0, using the glm feature available in base R, fit with a Gaussian family function. Pairwise comparison was then conducted using emmeans package. Plotting was performed using ggplot2 package in R.

2.12 DNA Extraction

Unless stated otherwise all genomic DNA from either *Brassica rapa* or *Brassica napus* was extracted using a modified protocol from Edwards *et al* (1991). Flash frozen (in liquid N_2) plant leaf or apex tissue (around 5mm2) was ground using a pestle and mortar then 400µl Edward's buffer was added (200mM Tris-HCl pH 8.0, 250mM NaCl, 25mM EDTA pH 8.0, 0.5% SDS). Each sample was then vortexed at room temperature until all material and buffer were mixed. Then samples were centrifuged for one minute at maximum speed in a benchtop centrifuge. 300 µl of supernatant and 300 µl of isopropanol were then mixed in a fresh 1.5ml microcentrifuge tube before centrifugation at maximum speed for 5 minutes. The supernatant was then discarded. The pellet was then dried with two 70% ethanol washes before air drying for 15 minutes. The pellet was then resuspended in 50 µl of distilled water before long term storage at -20°C.

2.13 PCR, Genotyping and Sequencing

All PCRs used the following reaction:

12.5 µl GoTaq G2 Green Master Mix (Promega Biotech) + 9.5 µl H_2O + 1 µl forward primer + 1 µl reverse primer + 1 µl template DNA (diluted with H_2O to 50 ng/ µl).

PCRs were run in a GStorm ThermoCycler. Initial denaturation was three minutes at 95 °C. This was followed by 35 cycles of: Denaturation (95 °C for 30s), Annealing (30s at calculated Tm of primer pair) and Extension (60s per kb at 72 °C). A final extension was carried out at 72 °C for 5 minutes.

Unless otherwise specified, 5 µl of amplified sample was run on a 1% agarose gel at 100V using a BioRad (www.biorad.com) gel electrophoresis tank powered by a Kikusui power pack (model PAB). For every 100ml of agarose gel, 5 µl of ethidium bromide was added. Unless otherwise specified the DNA ladder used was the 1kb Plus DNA Ladder (New England Biolabs).

List of all primers for each reaction used in this study is detailed in Table 2.4. All primers were designed using SnapGene Viewer (https://www.snapgene.com/snapgene-viewer).

All DNA for sequencing was purified using a Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit according to manufacturer instructions. 5 µl of purified DNA diluted to 50ng/ µl was sent with 5 µl of 5mM of the respective primer to Genewiz (Azenta Life Sciences, https://www.azenta.com/) for sanger sequencing.

Table 2.4 Primers used in this thesis

Name	Sequence	Description	Purpose

FP1BnPHPc	ATGGATCCGTTATCGGTGCTCAAGG	Forward Primer 25bp of CDS of Darmor (ensembl plants) of BnaA05g17020D (PHP)	Sequencing gDNA and cDNA
RP1BnPHPc	GAACAATTTAACTCACCAGTACTGG	Reverse Primer (reverse complemented) 25bp of CDS of C allele (from ensembl plants) of BnaA05g17020D	Sequencing gDNA and cDNA
FP1BnPromotorPHPc	TGCGAGAGAGAGAGATTCTGATTTC	Forward Primer of 2kb upstream region of BnaA05g17020D - potential promotor	Sequencing gDNA and cDNA
RP1BnPromotorPHPc	TCACCAACAACAGATCCTTAATTCA	Reverse of 2kb upstream region of BnaA05g17020D - potential promotor	Sequencing gDNA and cDNA
F1	GGAGGTACCGTGCTGTGATT	*B.napus* housekeeping gene forward	
R1-1	CGCTCCCATTGGTAACTTGT	AS ABOVE but reverse	

genotyping primer 1	GTAATCAACGTCCTCTCC	*B.rapa* genotyping for sequencing to check genotype	
Genotyping_FWD_2	GTTGGACCGAATCAGCATAATG	Rapa genotyping	

2.14 Diversity set gene analysis for *Bna.PHP.A05*

RNAseq data for each variety in the DFFS set was obtained from NCBI SRA (project number PRJNA309367; BioProject 309367). Individual fastq files were downloaded using the SRA Toolkit (version 3.0.5) before aligning to Darmor *bzh* version 4 reference sequence using TopHat (version 2.1.1). Individual bam files were then indexed using samtools (version 1.0.0) and visualised in IGViewer (Robinson *et al.,* 2011) using Darmor v4 as reference (Chalhoub et al., 2014). Consensus sequences were generated using samtools and bcftools (version 1.0.0) before seqtk (version 1.0.0) was used to convert files into fasta files which were viewed in Qiagen CLC Main Workbench (Qiagen, Hildenm Germany) where sequence data for each variety was compared.

SNPs in each gene were used to manually categorize haplotypes of either gene in IGViewer (Broad Institute, Robinson *et al.,*2011) with Darmor *bzh* version 4.0 reference (Chalhoub *et al.,* 2014). Haplotypes were considered to be the same if there were less than 3 base changes across the entire gene.

FLC haplotype analysis was determined from exome capture data (Woodhouse et al., 2021) of all *Bna.FLC* copies. Again, haplotypes were manually scored in IGViewer. Bait data used to generate dataset available in Steuernagel *et al.* (2021).

2.15 Protein sequence alignments

Protein sequences of Bna.PHP.A05, Bna.PHP.C05, *A. thaliana* PHP, *Sacchromyces Cerevisae* CDC73, *Homo sapiens* CDC73 were downloaded from uniprot (uniprot.org). Alignment was done in QIAGEN CLC Main Workbench (Qiagen, Hilden, Germany). The effect of changes in protein sequence were analysed by checking the alpafold prediction on Uniprot for each protein (Jumper *et al.,* 2021).

2.16 Associative Transcriptomics

2.16.1 Genome Wide Association Study (GWAS)

The GEM and GWAS Automation version 1.0 (GAGA pipeline for Gene Expression Marker (GEM) and Genome Wide Association Study (GWAS) Automation (available at https://github.com/bsnichols/GAGA; Nichols, Wells and Morris, unpublished) was used to analyse all traits of interest (Chapter 4; Table 4.3) to test for statistical associations between trait score and SNP identities. The GAGA pipeline was run in R version 4.3.1 for Windows. The pipeline uses GAPIT3 (Wang and Zhang, 2021) to run genetic association analysis. The SNP data used in the pipeline contained 355,536 SNPs from leaf transcriptome data of a subset of the DFFS set, as described in Harper et al. (2012). The Q matrix for kinship analysis in the pipeline was derived from STRUCTURE analysis performed by Guanyuang Lu at the John Innes Centre (Norwich, UK). The pipeline automatically selects the best fitting of FarmCPU, BLINK, GLM or MLM, incorporated in GAPIT3, for the data. FarmCPU, MLM and BLINK all account for population structure and kinship, whilst FarmCPU and BLINK better control Type I errors than GLM and MLM. BLINK can detect associations in the presence of linkage disequilibrium. BLINK and FarmCPU use a multi-locus model to test gene markers across a genome (Huang *et al.,* 2018; Liu *et al.,* 2016). BLINK runs two fixed effect models iteratively. The first tests each marker with associated markers fitted as covariates to control for population stratification. The second model selects those covariate markers associated with markers from the first test, this controls for spurious associations that arise in place of kinship (Zhang, 2014). As BLINK uses multiple markers as covariates, including markers with significant minor allele frequencies, pre-filtering of SNPs with an allele frequency > 0.05 was not performed. Therefore, I manually filtered genes from GWAS analysis that had fewer than 5 *B. napus* varieties with that SNP after results were generated.

When the GAGA pipeline runs GWAS analysis it determines significance of traits from false discovery rate (FDR). FDR is calculated individually for each trait and uses P-values for each trait to determine false discoveries. Results below the FDR are not considered to be significant.

Table 2.5 GAGA Pipeline models

Acronym	Model Type

FarmCPU	Fixed and random model Circulating Probability Unification
BLINK	Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway
GLM	General Linear Model
MLM	Mixed Linear Model

2.16.2 Gene Expression Marker Analysis

The GAGA pipeline also performs gene expression marker analysis. This uses gene expression data from leaf tissues of 21-day old seedlings generated by Harper *et al.* (2021) for the diversity set. GAGA removes all markers where gene expression was lower than 0.5 RPKM, leaving 117,784 markers and then performs a linear regression model with remaining markers to predict and compare gene expression with trait values of the trait being investigated. The GAGA pipeline automatically plots GEM outputs on Manhattan plots and produces significance tables. Manual searching of results identified any significant traits. GEM Analysis uses FDR to filter for significance in the same way as in GWAS. A more stringent Bonferroni correction was added manually to filter significance based on the number of markers in each set.

2.17 Transcriptomics

2.17.1 RNA Harvesting.

The WOSR Castille and SWOSR Ningyou7 from the DFFS set were grown for transcriptomic sampling to investigate the role of genetic control of bud dormancy. 80 plants for each line were grown. Each line was chosen for its flowering and temperature relationship. Castille was sown on 25/08/2021 while Ningyou7 was sown on 21/08/2021. Both lines were dissected until floral initiation. Two weeks after floral initiation half of remaining plants were exposed to a four-week warming treatment in a 20 °C/ 16 °C day/night glasshouse, as Ningyou7 and Castille developed at different times this meant treatment was provided at different times. Four single shoot apices were harvested for each timepoint and treatment. RNA harvesting occurred at four timepoints in both warmer and control conditions: one day before treatment began; 24 hours after treatment began; 7 days after treatment began and 14 days after treatment began (Figure 2.3). On each occasion, RNA was harvested two hours and 30 minutes after sunrise. Harvested tissue was immediately flash frozen in liquid nitrogen before extraction.

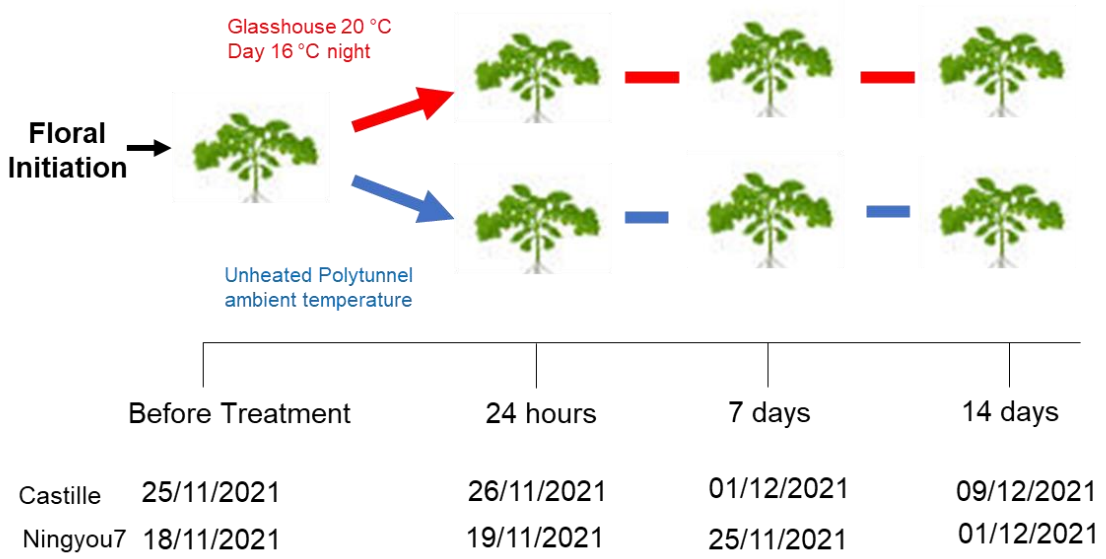


Figure 2.3 Dates and times for transcriptomic experiment sampling. All samples were harvested between 2 hours 30 minutes and 3 hours after sunrise. Samples taken from the John Innes Centre, Norwich, UK (Latitude 52.620, Longitude 1.222).

2.17.2 RNA Extraction

Frozen RNA apex tissue was ground to a fine powder in a 1.7ml Eppendorf tube using two sterilised 3.5mm stainless steel UFO Beads (http://thistlescientific.co.uk) and ground a Geno/Grinder® (http://spexsampleprep.com) for 1 minute at 30RPM speed. RNA extraction and DNase treatment were carried out using a EZNA® Plant RNA Kit according to their protocol (Omega Biotek Inc., http://omegabiotek.com/store/). Four biological replicates were used and Apex tissue from each replicate was harvested for each time point, the three with the highest 260/230 and 280/260 scores were then sent for sequencing. Using ddH_2O, all samples were brought to 50ng/µl before sending for transcriptomic sequencing. RNA samples were processed and sequenced by Novogene using a HiSeq 4000 system (Novogene, Cambridge, UK). Complementary DNA (cDNA) libraries were then constructed, 150-bp paired-end sequences with a minimum of 30 million reads were acquired per sample.

2.17.3 Transcriptomic data analysis

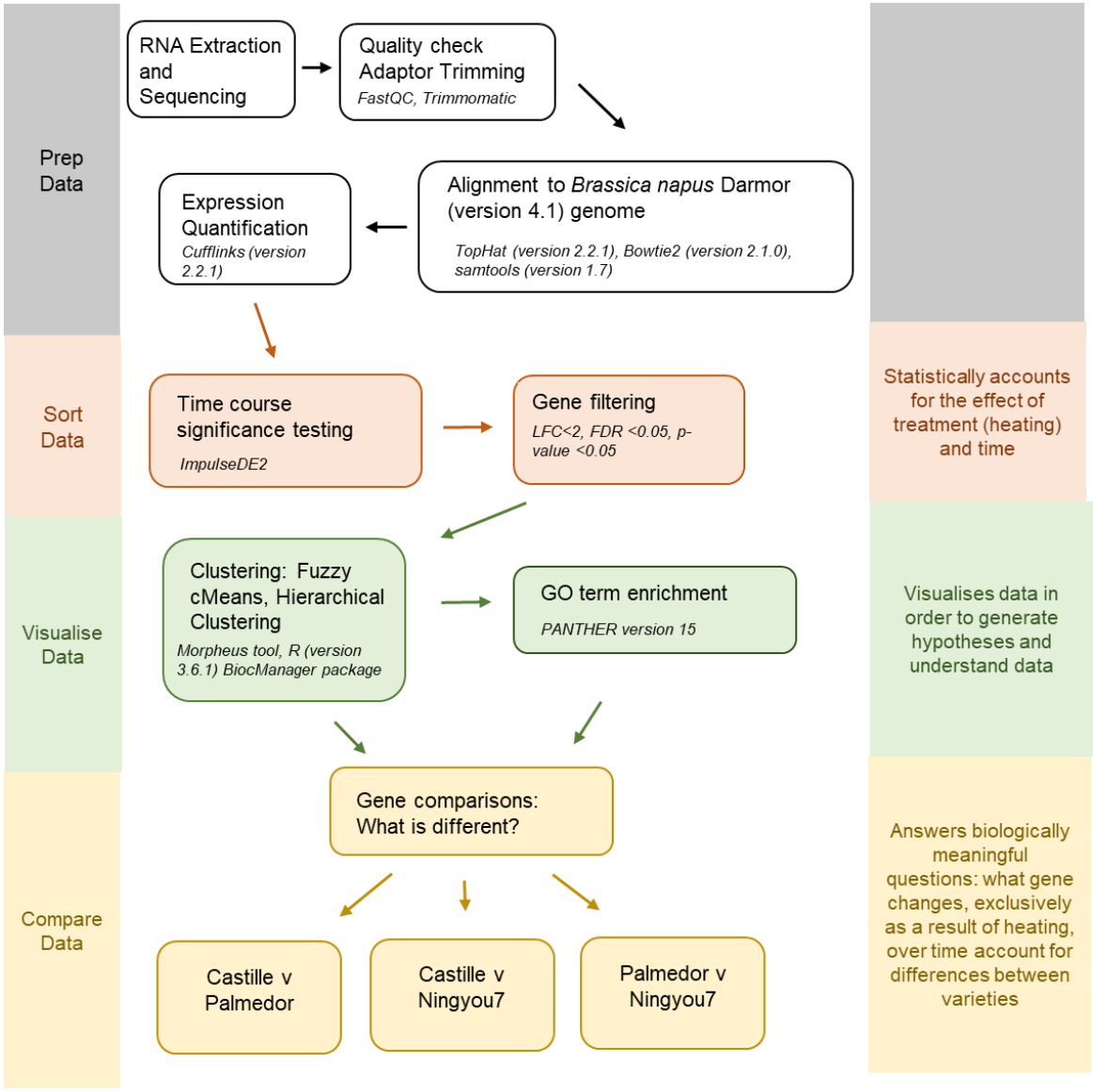
All transcriptomic analysis was performed the same way (Figure 2.4). Quality of reads was analysed running FastQC (Andrews, 2010) on raw sequencing reads. No further quality control

was necessary. Trimmomatic was used to trim adaptor sequences from reads (Bolger, Lohse, Usadel 2014). Reads were aligned to the Darmor-bzh reference genome (version 4.1.) (Chalhoub et al., 2014) downloaded from http://www.genoscope.cns.fr/brassicanapus/data using the alignment software TopHat (version 2.1.1), Bowtie2 (version 2.1.0) and samtools (version 1.7) with default parameters (Kim et al., 2013). Cufflinks (version 2.2.1) was used to align reads and output gene expression data as raw counts and Fragments Per Kilobase of transcript per Million mapped reads (FPKM) according to Trapnell *et al.* (2012). Pairwise comparison of individual varieties and timepoints was done using Cuffdiff (version 2.2.1) according to Trapnell et al (2012), while entire time course analysis was performed using count data and ImpulseDE2 (Fischer *et al.,* 2018). Genes where FPKM = 0 were removed from analysis. Significantly differentially expressed genes (DEGs) were chosen by filtering for $\log_2$ fold change > 2, false discovery rate < 0.05, p-value < 0.05 and FPKM equal to zero were all removed from analysis. To ensure expression levels were comparable between samples, raw counts were normalised using DESeq2 median of ratios method, using a publicly available R script and tutorial (Github; Jihe-Liu, tutorial and script available at: https://github.com/hbctraining/DGE_workshop).

Principal Component Analysis was performed on filtered gene expression data using an in-house script performed using prcomp from R stats (version 3.6.2) and visualised using ggplot2 from the tidyverse package in R (version 4.3.1 in Windows). Venn Diagrams describing the ifferentially expressed gene lists were generated using Bioinformatics and Evolutionary Genomics Venn Diagram Web Tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). Hierarchical clustering, k-means clustering and heatmap generation were generated using the Morpheus tool (https://software. broadinstitute.org/morpheus).

Before hierarchical clustering was performed on normalised count data, genes where there was no expression for each timepoint and data point were removed. Hierarchical clustering was carried out using one minus Pearson correlation, with an average linkage method. GO term enrichment analysis was performed using the PANTHER Overrepresentation Test (Panther 15.0, GO Ontology database DOI: 10.5281/zenodo.3727280, Release date 23-03-2020), the GO terms with p < 0.05 were taken as significantly enriched. GO terms were visualised using the enrichgo function in clusterProfiler (version 3.0.4) and visualised using the ggplot2 from the tidyverse package in R (version 4.3.1 for windows).

Figure 2.4 RNA-seq analysis pipeline. Summary of all RNA-seq analysis steps used within the thesis, referred to in section 2.17.3.



Chapter 3. Winter warmth post floral initiation delays floral development in winter type *Brassica napus.*

3.1 Introduction

The timing of flowering is crucial for successful plant reproduction. For instance, in the winter annual *Brassica napus* the timing of flowering influences final seed yield and oil quality (Raman *et al.*, 2019). In winter annuals vernalisation is genetically controlled through winter silencing of the floral repressor *FLOWERING LOCUS C* (*FLC*) (Henderson *et al.,* 2003) which prevents winter flowering. After winter cold, spring warmth and long days promote flowering through the photoperiod and ambient temperature pathways by acting on *FLOWERING LOCUS T* (*FT*) (Samach et al., 2000; Sawa et al., 2007; Blázquez et al., 2003; Kumar et al., 2012; Posé et al., 2013).

In contrast to the above model, it was discovered that Winter Oilseed Rape (WOSR) undergoes the floral transition in late autumn (O'Neill *et al.,* 2019). Winter floral initiation has also been seen in *A. alpina* and *A. lyrata* (Wang *et al.,* 2009; Kemi *et al.,* 2019) suggesting it may be common across Brassicaceae. Late autumn floral initiation in *B. napus* is matched by the silencing of six out of nine *FLC* copies (O'Neill *et al.,* 2019). In *A. alpina* the *FLC* homolog *PEP1* is also silenced to facilitate floral development during chilling, this occurs in an *FT*-independent manner during short winter days (Hyun *et al.,* 2019).

In most winter annuals, such as *A. thaliana,* after floral initiation warmer temperatures promote reproductive development (Wigge, 2013; Brightbill *et al.,* 2022). However, in WOSR *B. napus,* warmth in early winter is associated with lower yields (Brown *et al.,* 2019), after the point when WOSR is assumed to have undergone the floral transition (O'Neill *et al.,* 2019). A similar phenomenon, where late winter temperatures are associated with low yield in *B.napus*, has also been reported in China (He *et al.,* 2017).

It is unclear whether chilling of newly formed floral buds is important for *B. napus* development and yield formation. In preliminary work by Xiang Lu in the Penfield laboratory (unpublished data, Xiang Lu, Carmel O'Neill, Steve Penfield), we discovered that winter warming following floral initiation delayed reproductive development in one winter type oilseed rape Cabriolet, in a process independent of the control of floral initiation. However, it was unclear whether this was a general stress response or a widespread phenomenon in *B. napus,* this led to the experiments presented here.

Selective breeding for *B. napus* varieties adapted to different environments has produced different crop types that form distinct 'breeding pools', broadly separated by vernalisation requirement and flowering time (Schiessl *et al.,* 2020). These crop types are defined based on their growth type: winter oilseed rape (WOSR) is winter-hardy and depends on vernalisation for spring flowering, spring oilseed rape (SOSR) is grown as an annual and doesn't require prolonged cold to flower, semiwinter oilseed rape (SWOSR) are adapted to milder winters while Swedes have a strong vernalisation requirement but are grown for their tubers. Breeding to produce these crop types has resulted in variation at *FLC* genes (Wang *et al.*, 2011; Hou *et al.*, 2012; Schiessl *et al.*, 2017, 2019; Song *et al.*, 2020). As breeding has produced crop types with varying responses to winter temperatures, it is feasible there may also exist natural variation to post-floral initiation warmth.

3.1.1 Hypotheses and aims.

In this chapter I investigate phenotypic variation in the response to winter warming following floral initiation across a diversity set of *B. napus* varieties in three independent winter warming experiments. I use time to visible bud emergence (BBCH51), time to first flower emergence (BBCH60) and bolting as measures of flowering time (Weber and Bleiholder, 1991) and assess varieties for differences in key seed characteristics such as Thousand Grain Weight (TGW) and Seeds Per Pod (SPP).

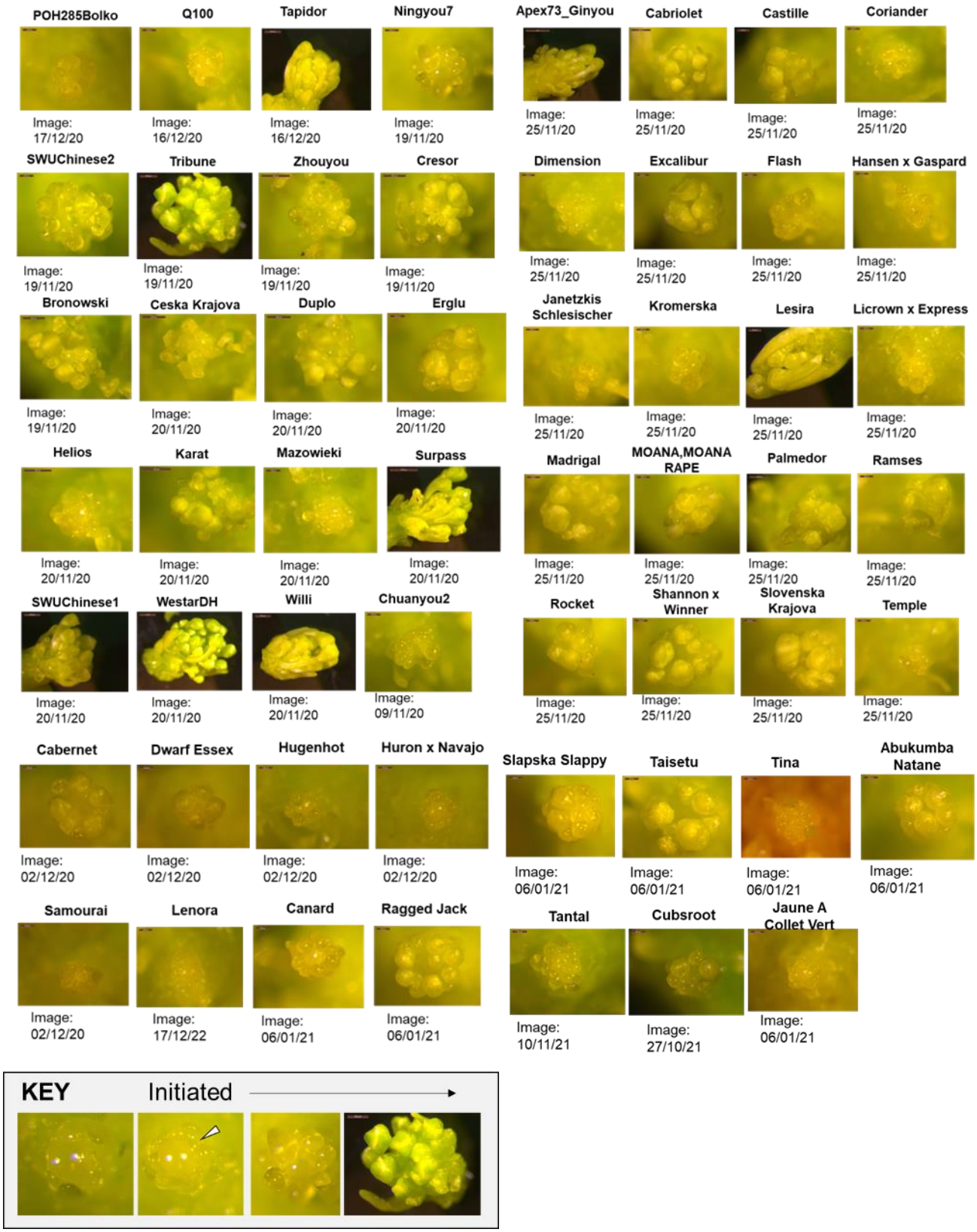
Due to historic breeding efforts to adapt *B. napus* to a wide range of environments, including conditions where there is reduced or no vernalisation requirements, I hypothesise there will also be different requirements for winter cold post floral initiation across *B. napus.*

As historically warmer temperatures after floral initiation have been associated with yield decline, I also hypothesise that WOSR plants grown in warm winters will produce fewer or lighter seeds, measured by TGW and SPP.

3.2 The effect of post floral initiation warmth on *B. napus* floral development

3.2.1 Post floral-initiation winter warning delays floral development in winter-type *B. napus*

To investigate the effect of winter warmth following floral initiation on the reproductive development of *B. napus* and the extent of variation to winter warmth within *B. napus,* I performed a large-scale winter warming experiment on a mixed-crop type diversity panel of *B. napus.* This diversity panel included 96 lines representing the phenotypic diversity of *Brassica napus* from the Diversity Fixed Foundation Set (DFFS) (Harper *et al.,* 2012). To take advantage of natural winter cold and ensure winter warming was applied at a uniform developmental stage across varieties, I aimed to synchronise floral initiation across the diversity set. I sowed all vernalisation-requiring later flowering WOSR, Exotic varieties and Swedes in late summer on 24th August 2020, and staggered later sowings for earlier flowering SWOSR and SOSR so that all lines passed through the floral transition in late autumn (Figure 3.1). Some crop types, like Exotics and some Swedes, did not undergo the transition to flowering until very late winter so were removed from subsequent analysis.


POH285Bolko
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Tapidor
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Cabriolet
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Castille
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Coriander
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SWUChinese2
Image: 19/11/20
Tribune
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Zhouyou
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Cresor
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Dimension
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Excalibur
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Hansen x Gaspard
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Bronowski
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Ceska Krajova
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SWUChinese1
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WestarDH
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Willi
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Chuanyou2
Image: 09/11/20
Rocket
Image: 25/11/20
Shannon x Winner
Image: 25/11/20
Slovenska Krajova
Image: 25/11/20
Temple
Image: 25/11/20
Cabernet
Image: 02/12/20
Dwarf Essex
Image: 02/12/20
Hugenhot
Image: 02/12/20
Huron x Navajo
Image: 02/12/20
Slapska Slappy
Image: 06/01/21
Taisetu
Image: 06/01/21
Tina
Image: 06/01/21
Abukumba Natane
Image: 06/01/21
Samourai
Image: 02/12/20
Lenora
Image: 17/12/22
Canard
Image: 06/01/21
Ragged Jack
Image: 06/01/21
Tantal
Image: 10/11/21
Cubsroot
Image: 27/10/21
Jaune A Collet Vert
Image: 06/01/21
KEY
Initiated

Figure 3.1. There is variation in floral development across a diversity set of *Brassica napus* in early autumn. A representative subset of the Diversity Fixed Foundation Set (DFFS) diversity set is represented here. Images represent dissections of *B. napus* apices in late autumn grown in a ventilated polytunnel in Norwich UK (Figure 2.1; Table 2.1). Varieties indicated by names and date of dissection indicated below images. The key indicates floral developmental progress, in the key the white arrow indicates floral initiation. The brown scale bar corresponds to 2 μm.

Following floral initiation, plants were grown in an unlit warmed glasshouse for four-weeks and floral development was compared to plants kept in control winter conditions in a control unlit polytunnel (Figure 3.2). The heating treatment approximately corresponds to the length and time when warm temperatures are associated with yield decline in WOSR in the UK (Brown *et al.,* 2019). As there was variation in when plants passed through floral initiation (Figure 3.1), the winter warming treatment was applied across five different transfers, based on when each variety florally initiated (Figure 3.2; Methods Section Figure 2.1).

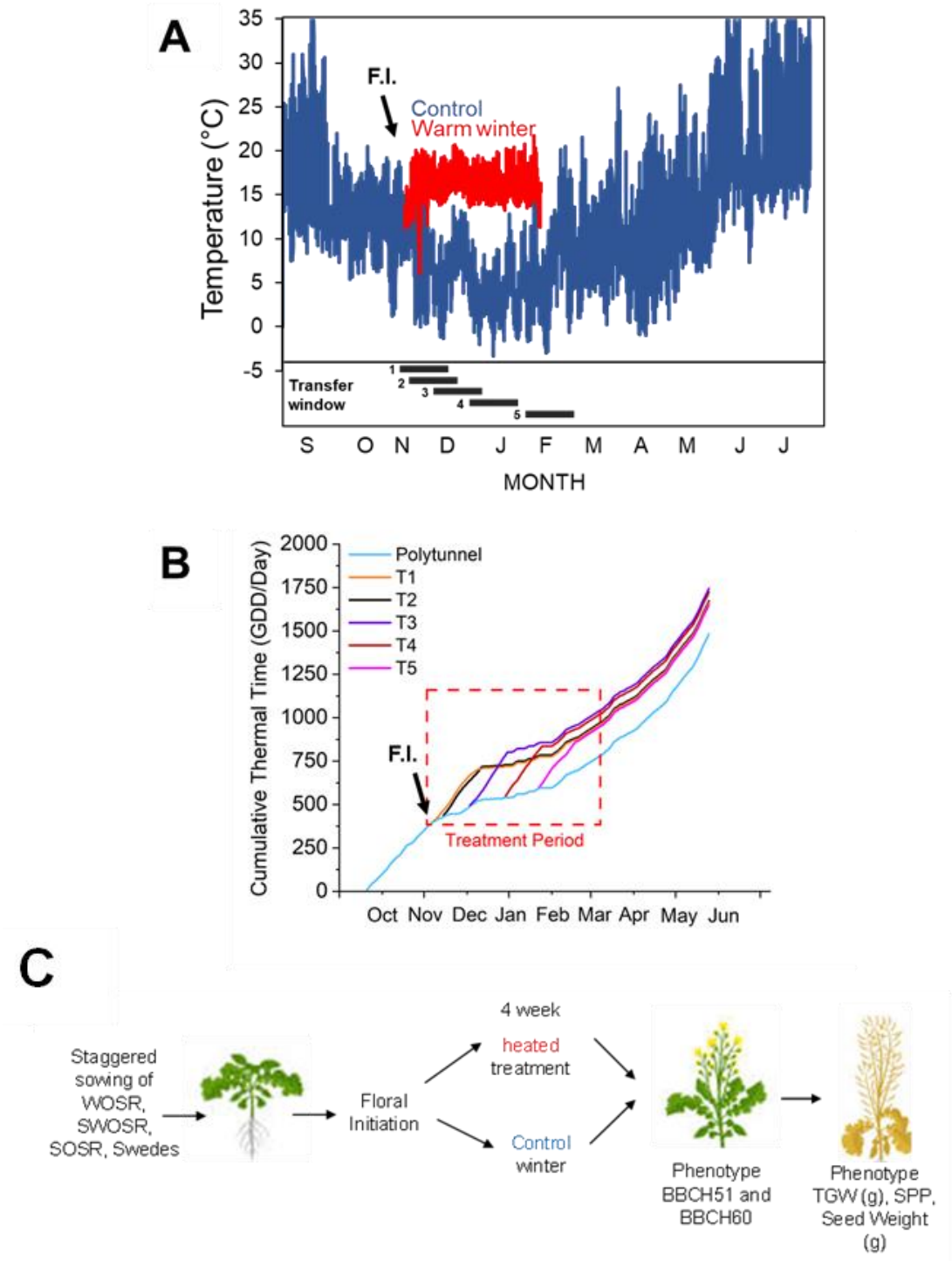


Figure 3.2 Comparison of ambient temperature and warming treatments in the 2020-2021 winter warming experiment. A) Temperature in control polytunnel (blue) and warmed glasshouse (red). FI refers to floral initiation (F.I.) of *B. napus* plants grown in control polytunnel. Transfer windows indicate the time at which different varieties were transferred to

the heating treatment depending on when they florally initiated (Methods Figure 2.1). B) Cumulative thermal time in the control polytunnel and each transfer (cumulative thermal time calculated according to Methods section 3.2.3). C) Experimental design of winter warming *B. napus* phenotyping experiment. BBCH51 refers to bud emergence and BBCH60 refers to first flower emergence. TGW is thousand grain weight (g), SPP is seed per pod.

To assess how floral development was affected by winter warming, I phenotyped all varieties according to the BBCH scale for time to BBCH51 (visible bud emergence) and BBCH60 (first flower emergence). Across varieties, preliminary analysis suggested plants were either advanced or delayed to BBCH51 (Figure 3.3A) or BBCH60 by winter warming (Figure 3.3B). In general, the effect of warming correlated with flowering time such that later flowering lines more frequently showed delayed floral development while earlier flowering lines were more likely to be advanced by warming (Figure 3.3 C). Whilst not completely uniform across crop types, winter warming of floral buds delayed floral development in WOSR, had no effect on SOSR, but advanced floral development in most SWOSR and Swedes (Figure 3.3). This indicates there is variation in responses to winter warming across *B. napus* varieties. This suggests that warming-induced growth delay may be genetically determined, as crop types have been selectively bred for different environments.

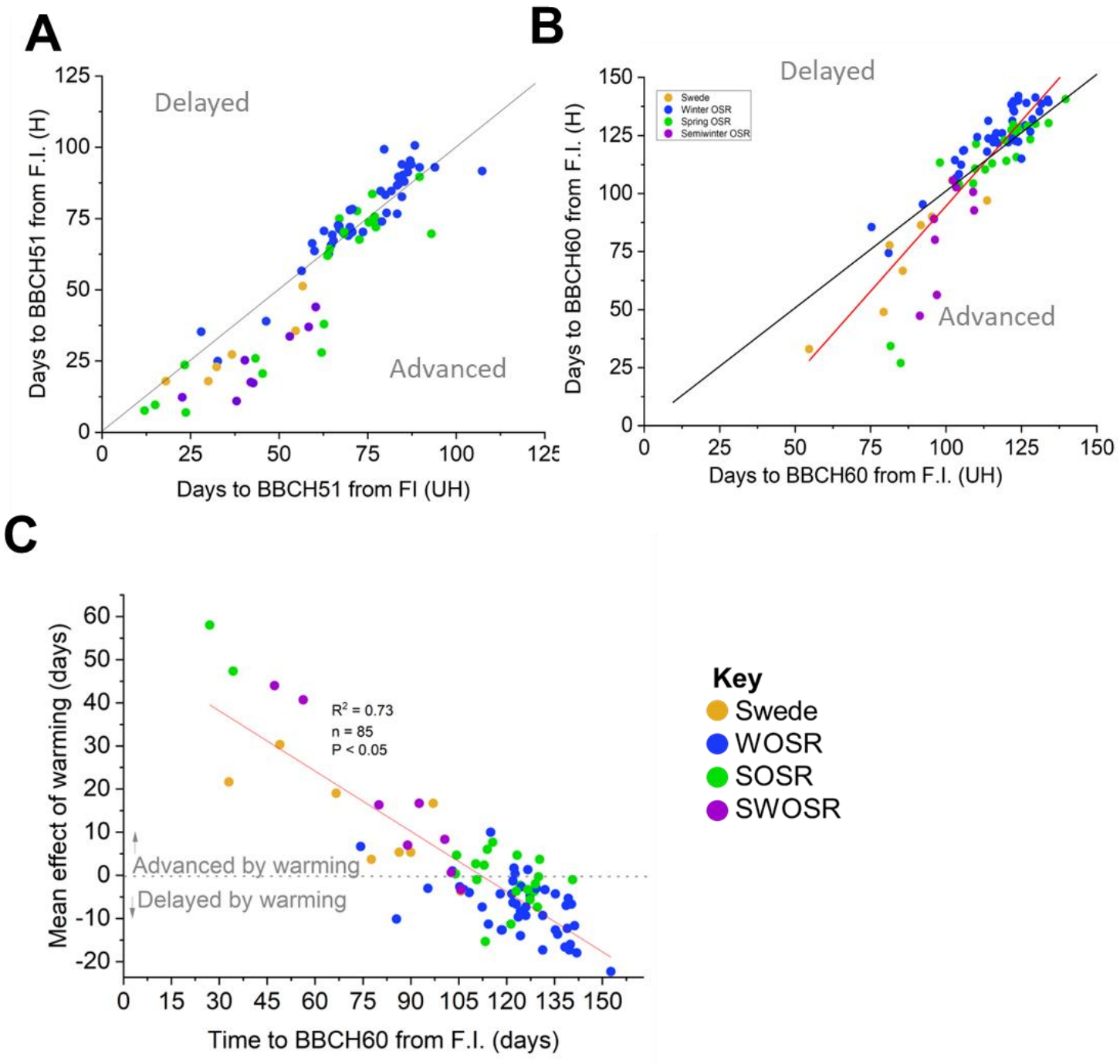


Figure 3.3 Late flowering varieties of *B.napus* are delayed to bud emergence (BBCH51) and first flower opening (BBCH60) in warmer winters compared to control winters, but early flowering varieties are accelerated. Individual dots represent the mean of three replicates grown for each *B. napus* (Methods Table 2.1) in a winter warming experiment (Methods Section 2.1). Dots on the black lines in A and B indicate flowering time was identical in both treatments, where a variety is above this line it indicates varieties that flowered later in the control than warmer treatment, points below indicate the opposite. Red lines indicate a linear regression model fitted to data with R^2 values presented. F.I. refers to floral initiation. H refers to heated treatment (warmer winter) and UH the unheated control

treatment A, B). Days to BBCH51 and BBCH60, respectively, from floral initiation (FI) in 2020-2021 experiment (Methods section 2.1). C) Time to BBCH60 from floral initiation (FI) against the mean effect of warming. Lines below the dotted grey line indicated varieties where flowering was delayed by warming and lines above this line indicate lines where flowering was advanced by warming.

3.2.2 Winter warming either advances or delays time to BBCH51 and BBCH60 depending on crop-type

To determine how time to BBCH51 and BBCH60 from floral transition was influenced by winter warmth across the diversity set, I ran several preliminary ANOVA analyses to determine which experimental factors significantly affected BBCH51 or BBCH60. Plants were randomised in an incomplete block design to account for any location effects (Methods section 2.1). To account for the effect of block I ran a one-way ANOVA model for both BBCH51 and BBCH60, where μ was the mean, L is block and ε was the residual term is represented here:

$$BBCH = \mu + L_i + \varepsilon_i$$

Table 3.1 One-way ANOVA for effect of location on BBCH51 and BBCH60 in winter warming experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	Location	1	18	18.0	0.036	0.849
	Residuals	400	198889	497.2		
BBCH51	Location	1	28	28.3	0.05	0.823
	Residuals	408	231946	568.5		

This indicated that the location of plants (blocking) had no effect on time to BBCH51 or BBCH60 (Table 3.1). Therefore, blocking effect was not considered in further analyses.

As preliminary analysis indicated there may be an effect of crop type on time to BBCH51 and BBCH60 in warm winters (Figure 3.3), I grouped varieties into their respective crop types (Harper *et al.,* 2012). Then I ran a one-way ANOVA for time to BBCH51 and BBCH60 accounting for crop type. CT is crop type and ε was the residual term:

$$BBCH = \mu + CT_i + \varepsilon_i$$

Table 3.2. One-way ANOVA for effect of crop type on BBCH51 and BBCH60 in 2020-2021 experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	Crop Type	5	80942	16188	54.34	<2e-16
	Residuals	396	117965	298		
BBCH51	Crop Type	5	117160	23432	82.45	<2e-16
	Residuals	404	114815	284		

This indicated crop types showed differences in time to BBCH51 and BBCH60 (Table 3.2).

Next, I determined whether plants being exposed to warming after floral initiation had a significant effect on time to BBCH51 or BBCH60 and whether this effect varied by crop type using two-way ANOVA where CT is crop type, T is treatment (warmed or control) and (T x CT) is the interaction term and ε was the residual term:

$$BBCH_{ij} = \mu + T_i + CT_j + (T_i \times CT_j) + \varepsilon_i$$

Table 3.3 Two-way ANOVA for effect of treatment and crop-type and treatment and crop type interaction on BBCH51 and BBCH60 in winter warming experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	Crop Type	5	80942	16188	54.34	<2e-16
	Treatment	1	38	38	0.136	0.713
	Treatment. Crop Type	5	9717	1943	7.004	2.78e-06
	Residuals	390	108210	277		
BBCH51	Crop Type	5	117160	23432	86.071	<2e-16
	Treatment	1	828	828	3.043	0.08187
	Treatment. Crop Type	5	5635	1127	4.140	0.00112
	Residuals	398	108351	272		

The interaction between crop type and treatment is significant (p = 2.78e-06) but treatment alone is not (p = 0.713). This indicates that the effect of treatment varies between crop types, and different crop types have a different response to reproductive warming (Table 3.3).

To understand how crop type influences bud development in warm winters, I analysed the time to BBCH51 and BBCH60 for each crop type (Figure 3.4). To visualise the effect of warming on time to BBCH51 and BBCH60 for each crop type I subtracted the time to BBCH51 and BBCH60 for the warming treatment from the time to BBCH51 and BBCH60 for the control treatment (Figure 3.4).

For BBCH51, Winter oilseed rape (WOSR) required 81 days after floral initiation on average in the warmed treatment (SEM = 1.155) and 77 days in the control (SEM =1.124), a difference of four days. For BBCH60, WOSR required 128 days after floral initiation in the warmed treatment (SEM = 1.112) but 119 in the control (0.877) (Figure 3.4), a difference of 9 days. This indicates that, in general, winter warming delayed flowering in WOSR, mainly by affecting time from BBCH51 to BBCH60.

Semi-winter oilseed rape (SWOSR), bred for a reduced vernalisation requirement, took 26 days to reach BBCH51 after floral initiation in the warmed treatment (SEM = 3.68) but 45 days in the control treatment (SEM = 3.63), a difference of 19 days. For BBCH60, SWOSR took 86 days after floral initiation in the warmer treatment (SEM = 5.012) but 102 days in the control treatment (SEM = 1.706), a difference of 16 days. This suggests that SWOSR are advanced to floral development by warming.

Swede varieties took 29.2 days to reach BBCH51 after floral initiation in the warmed treatment (SEM = 3.47) and 38.7 days in the control treatment (SEM = 4.32), a difference of 9.5 days. While Swedes took 67.4 days to reach BBCH60 in the warmed treatment (SEM = 7.48) and 83.57 days in the control treatment (SEM = 4.71), a difference of 16 days. This indicates that for most, but not all, Swedes floral development was accelerated by warming floral buds. Spring oilseed rape (SOSR) however did not show a uniform pattern, some varieties were advanced whilst some were delayed (Figure 3.4).

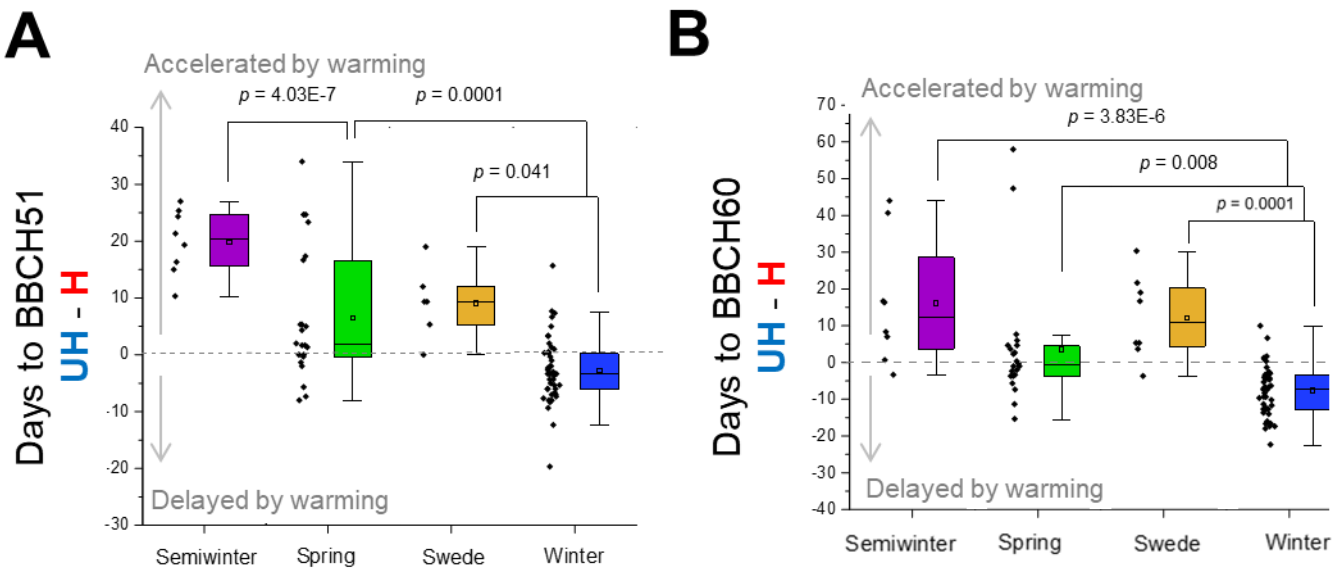


Figure 3.4. The crop type of *Brassica napus* (semiwinter, spring, swede, winter) predicts whether winter warming causes a delay or advancement to floral development . Each black dot indicates the mean of 3 replicates of each variety (Methods Table 2.1) A) Time to bud emergence (BBCH51) of unheated control (UH) subtracted by warmed (H) treatment. Values above zero indicate varieties that are advanced whilst values below zero indicate varieties that are delayed following warming, this is highlighted by grey arrows. B) Time to BBCH60 control subtracted by warmer. Values indicate the same as in A. Statistics performed using student's t-test with Welch's correction.

To understand individual genotype responses to winter warming I analysed individual varieties for time to BBCH51 and BBCH60. Not all varieties in every crop type grouping responded uniformly to warming (Figure 3.5; Figure 3.6).

For time to BBCH51, most WOSR were delayed to BBCH51. Although eight WOSR were significantly delayed to BBCH51 (Figure 3.5). Meanwhile, the only lines that were significantly advanced to BBCH51 were SOSR, Swedes and SWOSR varieties. However, there were individual varieties that showed delayed flowering from these crop types such as the SOSR Erglu, Cresor, Tantal, Helios and Willi and the SWOSR SWUChinese2. However, of these only Erglu was significantly delayed to BBCH51 following winter bud warming. This indicates that whilst crop type is a useful indicator of response to winter bud warming there is still a genotype specific effect.

For time to BBCH60 most WOSR were delayed to flowering following warming (Figure 3.6). However, one WOSR variety, Aphid resistant rape, was significantly advanced to flowering

(Figure 3.6). In SWOSR varieties, where bud warming almost universally advanced time to flowering, Xiangyou15 and Zhouyou were delayed to flowering because of bud warming however this effect was not significant (Figure 3.6). Meanwhile, the SOSR Helios was significantly delayed to BBCH60 (Figure 3.6). Overall, this indicates that while crop type is a useful predictor of time to BBCH60 following bud warming there is still specific genotype variation.

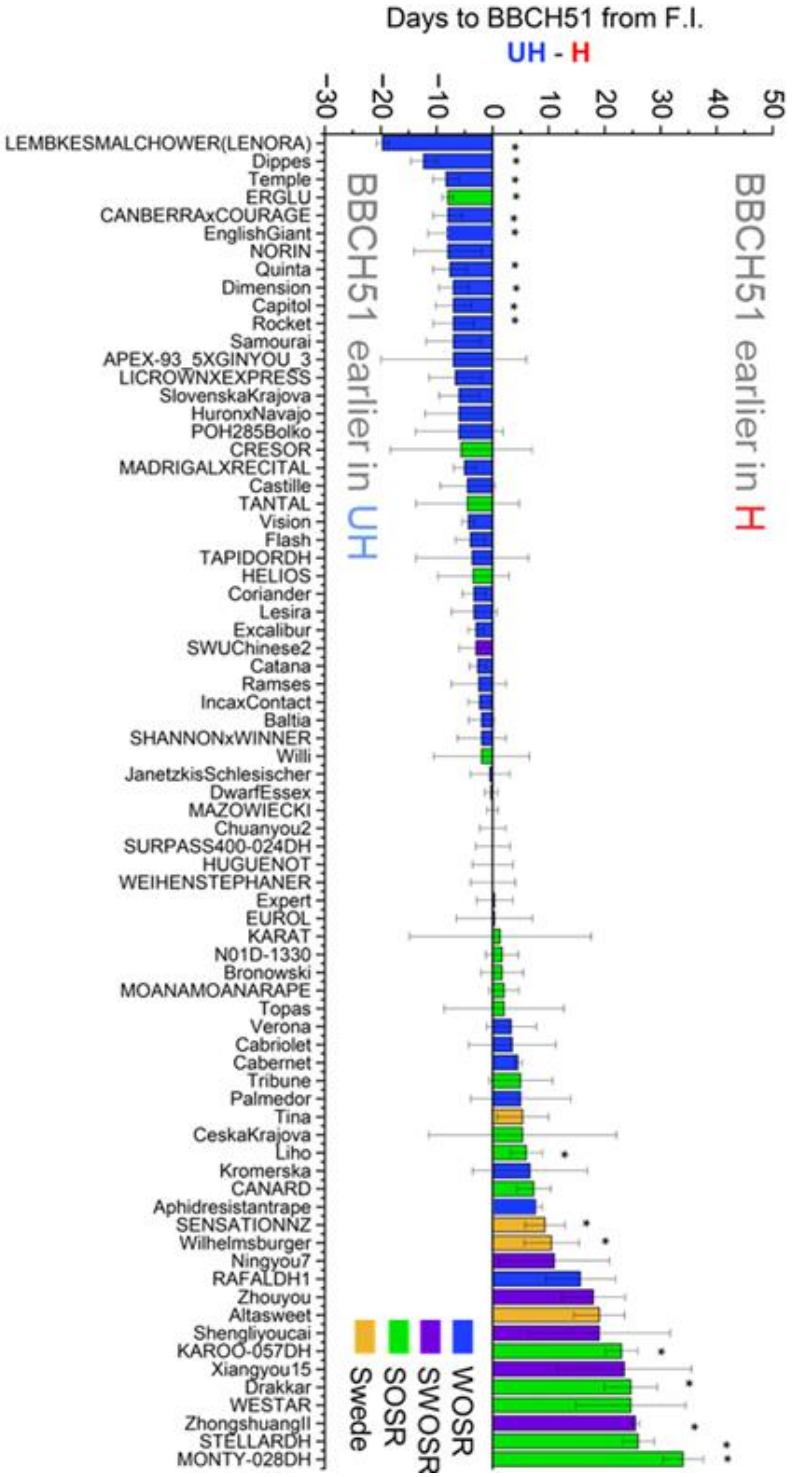


Figure 3.5. The time to BBCH51 between warmer and control treatments. Crop type is indicated by colours denoted in the key. Values represent the days to control minus the days to warmer to BBCH51, such that values above zero indicate varieties that were advanced to BBCH51 by warming plants post-floral initiation and those below zero indicate varieties that were delayed. Significance determined using students t-test where * where $p < 0.05$, $n = 6$ per genotype.

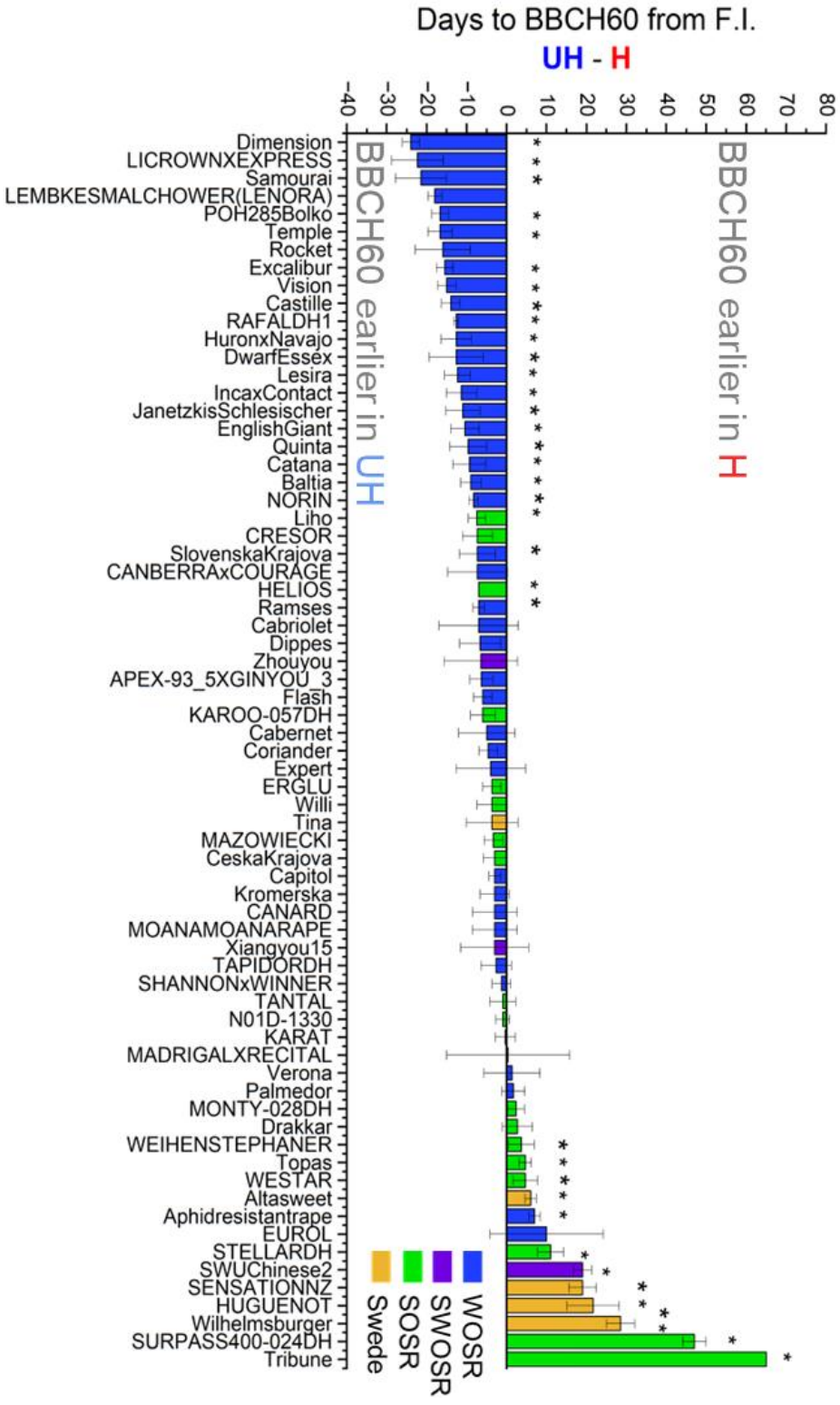


Figure 3.6. Time to BBCH60 between warmer and control treatments. Crop type is indicated by colours denoted in the key. Values represent the days to control minus the days to warmer to BBCH60, such that values above zero indicate varieties that were advanced to BBCH60 by warming plants post-floral initiation and those below zero indicate varieties that were delayed. Significance determined using students t-test * where $p < 0.05$, $n = 6$ per genotype.

3.2.3 When accounting for thermal time all varieties are delayed following winter warmth

Plant development is mediated through environmental and endogenous signals. To account for fluctuating temperatures across different transfers, I compared how floral development varied between varieties accounting for accumulated thermal time. I calculated the thermal time to BBCH51 and BBCH60 using the following formula, where BBCH represents either BBCH51 or BBCH60:

$$BBCH_{GDD} = \sum(T_{avg} - T_{base})$$

Where Growing Degree Days (GDD) is the cumulative growing degree days since the date of floral initiation until the date of flowering. T_{base} is the base temperature of *B. napus* under which no development occurs, assumed here as 3°C (Habekotte, 1997), T_{avg} is the daily mean temperature, calculated approximately as the mean of the maximum and minimum temperature where the returned value is never negative:

$$T_{avg} = \begin{cases} \frac{(T_{max} - T_{min})}{2} & for \ \frac{(T_{max} - T_{min})}{2} > 0 \end{cases}$$

This analysis showed that all varieties are delayed to BBCH51 and BBCH60 measured in GDD (Figure 3.7). It also indicated that in both treatments WOSR broadly took more thermal time than other crop types (Figure 3.7). This indicates that GDD was not a useful measure for distinguishing between crop types, so for future analysis I compared time to BBCH51 and BBCH60 for calendar time only.

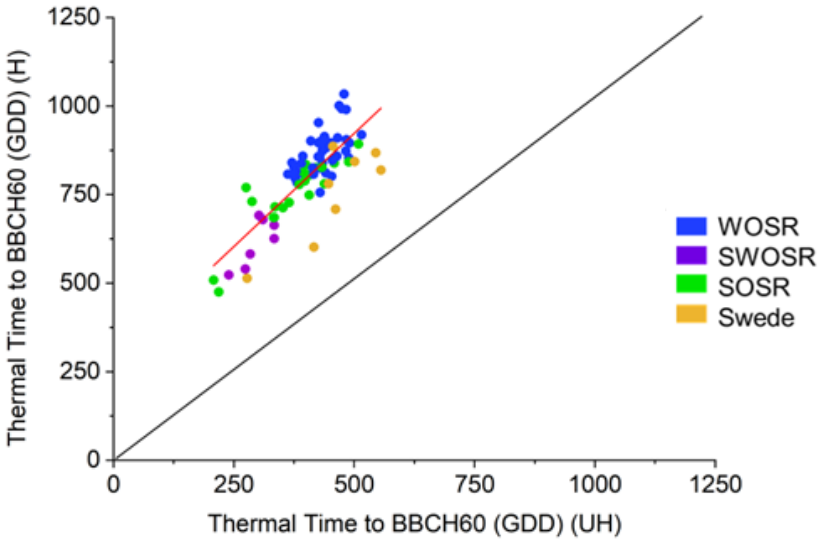


Figure 3.7 All *Brassica napus* varieties are delayed to flowering by winter warming when accounting for thermal time. Data points show each *B. napus* variety (Methods Table 2.1) mean flowering times in Growing Degree Days in warmer and control treatments. Varieties above the black line indicate varieties that required more thermal time to flower in the warmer treatment. Colours represent *B. napus* crop type: blue is winter, green is spring, orange is swede and purple is semiwinter. A) Thermal time to flower (BBCH60) in 2020-2021 experiment; B) Thermal time to BBCH60 in 2021-2022 experiment.

3.2.4 Warmed WOSR varieties were shorter at BBCH60 than control varieties

In *B. napus,* QTL studies on plant yield have implicated genes associated with plant height, indicating there is a relationship between plant height and yield (Raboanatahiry *et al.,* 2018). To understand whether the warming treatment affects plant height I measured plant height at BBCH60 (cm) and plant height at harvest. At BBCH60, only WOSR showed taller plants in the control treatment (Figure 3.8A) yet by harvest there was no difference in height except SWOSR which were taller in the warmed treatment (Figure 3.8B; Figure 3.9). Warmed SWOSR are taller at harvest likley due to the enhanced growth earlier in development when warming accelerates SWOSR development. For WOSR this indicates that winter warming affects plant height around floral development. By final harvest in WOSR the development of control plants was comparable to warmed plants. This suggests that WOSR varieties bolted later in warmer treatment, this is because height at BBCH60 is broadly considered to be a measure of when plants have bolted relative to flowering time. However, due to the scale of the experiment and time constraints, the exact time when control plants and warmed plants bolted or when they no longer show developmental differences in WOSR was not captured.

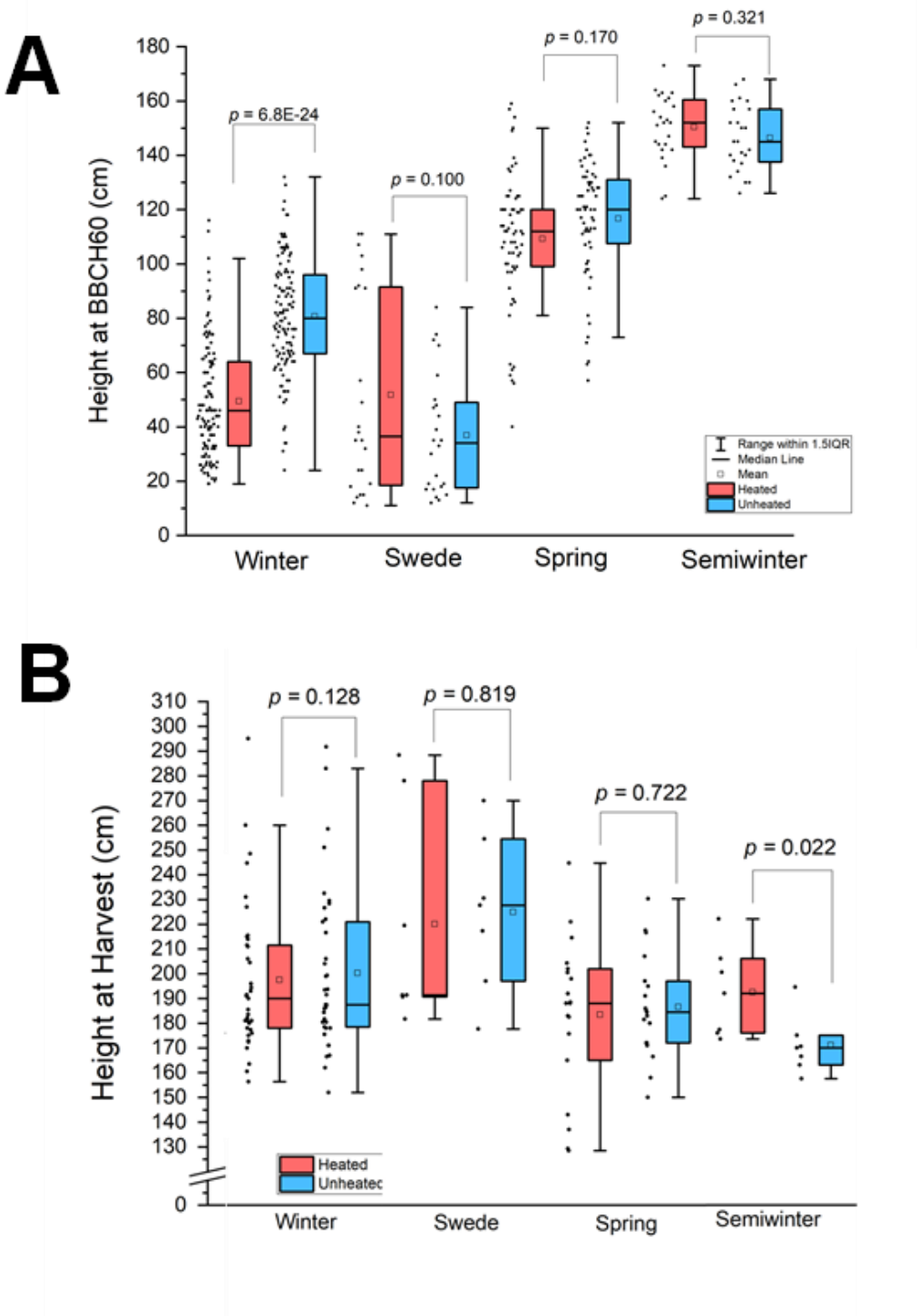


Figure 3.8. Different crop types of *Brassica napus* plant Height at first flower emergence (BBCH60) and harvest following either a warm winter (red) and control unheated winter (blue). A) Winter type *B. napus* is significantly shorter at BBCH60 following warming post floral initiation, all other crop types are unaffected. B) By harvest warmer plants catch up and there is no difference in plant height except warmer SWOSR are taller than control SWOSR. Two-way ANOVA with treatment and crop type as factors were used to test for significance. As in key, blue represents control and red represents warmer. Each data point is the mean of replicates of an individual variety, n = 6, per genotype.

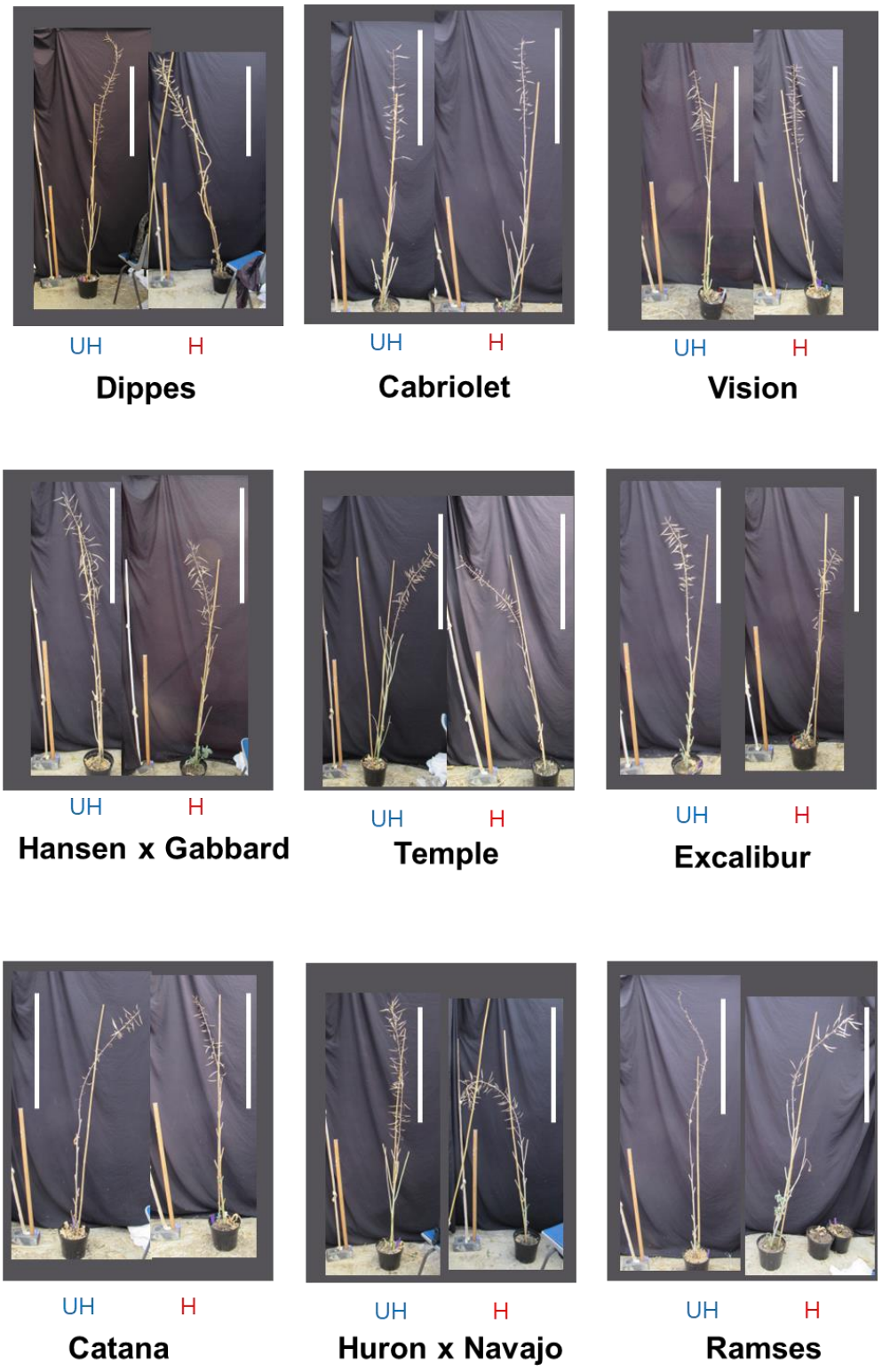


Figure 3.9. There is no difference in *Brassica napus* stature or height at harvest between winter warmed (H) or control winter (UH). Representative images taken from the 2020-2021 experiment showing differences between warmed and control plants at final harvest. White to scale metre rule on right hand side of every plant.

3.2.5 Post floral-initiation warmth reduces TGW (g) in WOSR

In *B. napus* final seed yield is determined mainly by seed weight, seeds per silique (or pod) and silique number. To determine the relationship between post floral initiation warming and seed traits, 20 pods were harvested from the primary raceme of *B. napus* plants and TGW, SPP and total seed weight (g) of seeds in all 20 pods was measured (Methods section 2.1).

I ran several preliminary ANOVA analyses, on the entire dataset, to determine which factors significantly affected seed characteristics across the dataset.

To account for the effect of block I ran a one-way ANOVA model for all the measured seed characteristics, where μ was the mean, L is block and ε was the residual term is represented here:

$$Seed\ Characteristic = \mu + L_i + \varepsilon_i$$

Table 3.4 One-way ANOVA for the effect of location on seed characteristics in winter warming experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
Total seed weight (g)	Location	1	0.87	0.8707	2.639	0.105
	Residuals	387	127.70	0.3300		
Seed per pod (SPP)	Location	1	20	19.55	0.551	0.458
	Residuals	402	14269	35.49		
Thousand Grain Weight (g)	Location	1	3.8	3.813	1.894	0.17
	Residuals	387	779.3	2.014		

This indicated that blocking had no effect on any seed characteristics in the dataset (Table 3.4). Therefore, blocking effect was not considered in further analyses.

Next, I ran a one-way ANOVA to test for the effect of crop type on seed characteristics in the dataset, where μ was the mean, CT is crop type and ε was the residual term is represented here:

$$Seed\ Characteristic = \mu + CT_i + \varepsilon_i$$

Table 3.5. One-way ANOVA for the effect of crop type on seed traits

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
Total seed weight (g)	Crop Type	5	26.1	5.220	19.51	<2e-16
	Residuals	383	102.5	0.268		
Seed per pod (SPP)	Crop Type	5	1056	211.15	6.351	1.09e-05
	Residuals	398	13233	33.25		
TGW (g)	Crop Type	5	277.3	55.46	41.99	<2e-16
	Residuals	383	505.8	1.32		

This indicated that crop type had a significant effect on all seed characteristics TGW, SPP and total seed weight in the entire dataset (Table 3.5).

Next, I determined whether the heating treatment after floral initiation had a significant effect on any seed characteristics across the dataset. As crop type had a significant effect on all seed characteristics, I ran a two-way ANOVA to test the effect of treatment and crop type where μ was the mean, CT is crop type, T is treatment and (T x CT) is the interaction term and ε was the residual term:

$$BBCH_{ij} = \mu + T_i + CT_j + (T_i \times CT_j) + \varepsilon_i$$

Table 3.6 Two-way ANOVA for effect of treatment and crop-type and treatment and crop type interaction on all seed characteristics in winter warming experiment

	Df	Sum Squares	Mean Squares	F Value	Pr (>F)
Crop Type	3	2.201	0.7338	5.819	8.76E-4

Total seed weight (g)	Treatment	1	26.353	26.353	208.97	0.0601
	Treatment. Crop Type	3	1.853	0.618	4.897	0.280
	Residuals	146	18.4123	0.1261		
Seed per pod (SPP)	Crop Type	3	575.93	191.976	6.50097	3.69E-5
	Treatment	1	0.325	0.325	0.011	0.91661
	Treatment. Crop Type	3	31.4647	10.488	0.355	0.785
	Residuals	146	4311.449	29.530		
TGW (g)	Crop Type	3	127.088	42.365	33.441	0.0001
	Treatment	1	0.086	0.086	0.0681	0.7945
	Treatment. Crop Type	3	2.899	0.966	0.763	0.001
	Residuals	145	183.682	1.2667		

This indicates temperature has no effect on total seed weight (g), SPP or TGW across the dataset (Table 3.6). However, the interaction between crop type and treatment was significant for TGW, this suggests that the effect of treatment depends on crop type for TGW (g).

To determine how TGW varied between treatments across the crop types, I performed a Tukey pairwise comparison for the treatment across every crop type for all seed traits analysed (Figure 3.10). This showed that for WOSR there was a significant difference in TGW (g) between warmed and control treatment ($p = 0.0041$, estimate size = 0.332158 g heavier in control, SE = 0.162). However, there was no significant difference in any other crop type (Figure 3.10). This suggests that in WOSR bud warming after floral initiation leads to lower TGW (g) (Figure 3.10).

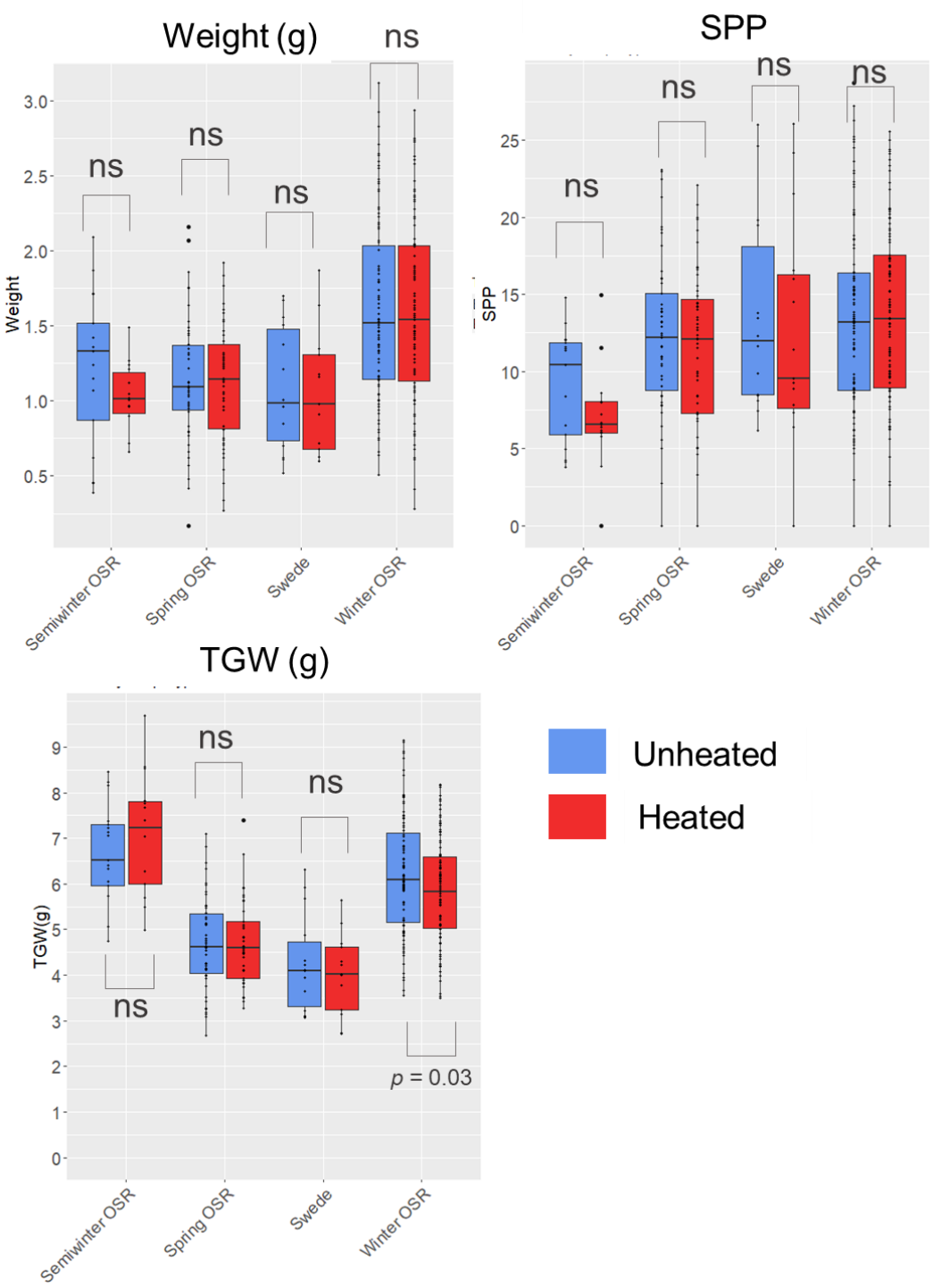


Figure 3.10 In the 2020-2021 experiment (Methods section 2.1), only winter type (WOSR) *Brassica napus* show significantly higher Thousand grain weught (TGW) in a control winter compared to a warmed winter (H). All other seed traits (Weight of seeds, seed size per pod, seeds per pod (SPP)) are not significantly different from each other. Weight is the weight of all seeds in representative 20 pods of the primary raceme. SPP was the number of seeds per pod on average. TGW was the weight of a thousand seeds in g. Significance determined by mixed linear effects model summarised in Table 3.11 and 3.12, ns = not significant

To understand the response of individual genotypes, I analysed individual crop types (Figure 3.11; 3.12; 3.13). This indicated that most varieties that showed significantly higher TGW (g) in the control treatment were WOSR, with one exception, Aphidresistant rape which showed significantly higher TGW (g) in the warmer treatment. Again, for SPP and Seed weight (g) most WOSR showed greater SPP and Seed Weight in the control treatment, with exceptions including Samourai, ShannonXWinner and Temple which showed higher SPP in the warmer treatment and Castille, Quinta and Palmedor which showed higher Seed weight in the warmer treatment. By contrast, the TGW (g), SPP and Weight of seeds for SOSR, SWOSR and Swedes for some varieties was higher in the warmed treatment and some in control (Figure 3.11; 3.12; 3.13), but there was no specific trend for each crop type. This indicates that winter warming post floral initiation negatively impacts TGW (g) in WOSR alone while other crop types showed a genotype specific response. This implies WOSR share a genetic basis for TGW (g) reduction in warm winters. It also suggests that seed characteristics are under genetic control that varies from genotype to genotype. However, as I did not collect final seed yield I cannot comment on how warming may influence final yield.

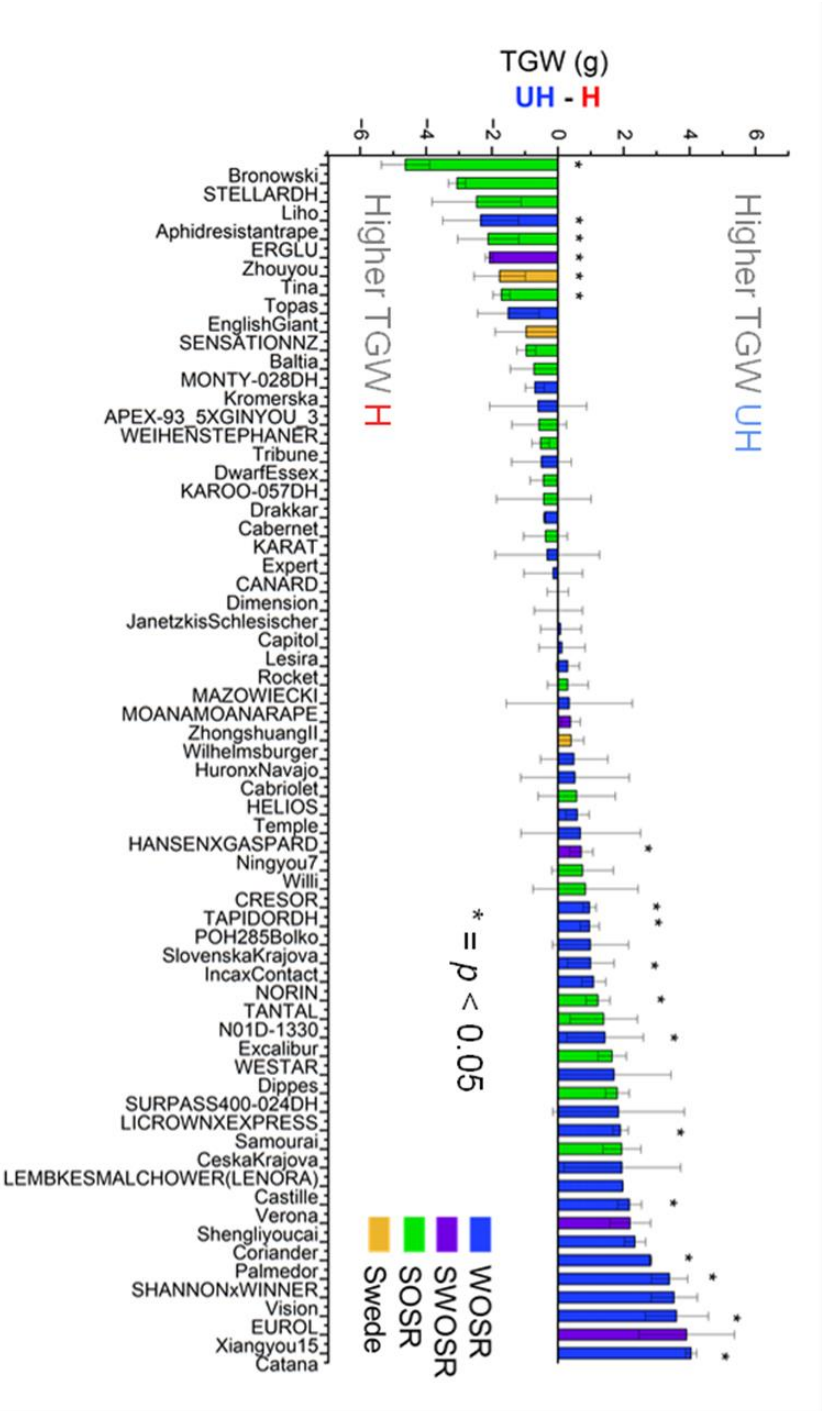


Figure 3.11. Individual varieties of *Brassica napus* show a range of thousand grain weight (g) (TGW) (g). Crop type is indicated by colours denoted in the key, where WOSR is winter type, SWOSR is semiwinter type, SOSR is spring type. Values represent TGW (g) in the control minus the TGW (g) in warmer, such that values above zero indicate varieties that had higher TGW (g) in control and those below zero higher TGW (g) in warmer. Significance determined using students t test and indicated with * to indicate $p < 0.05$, n = 6 per genotype.

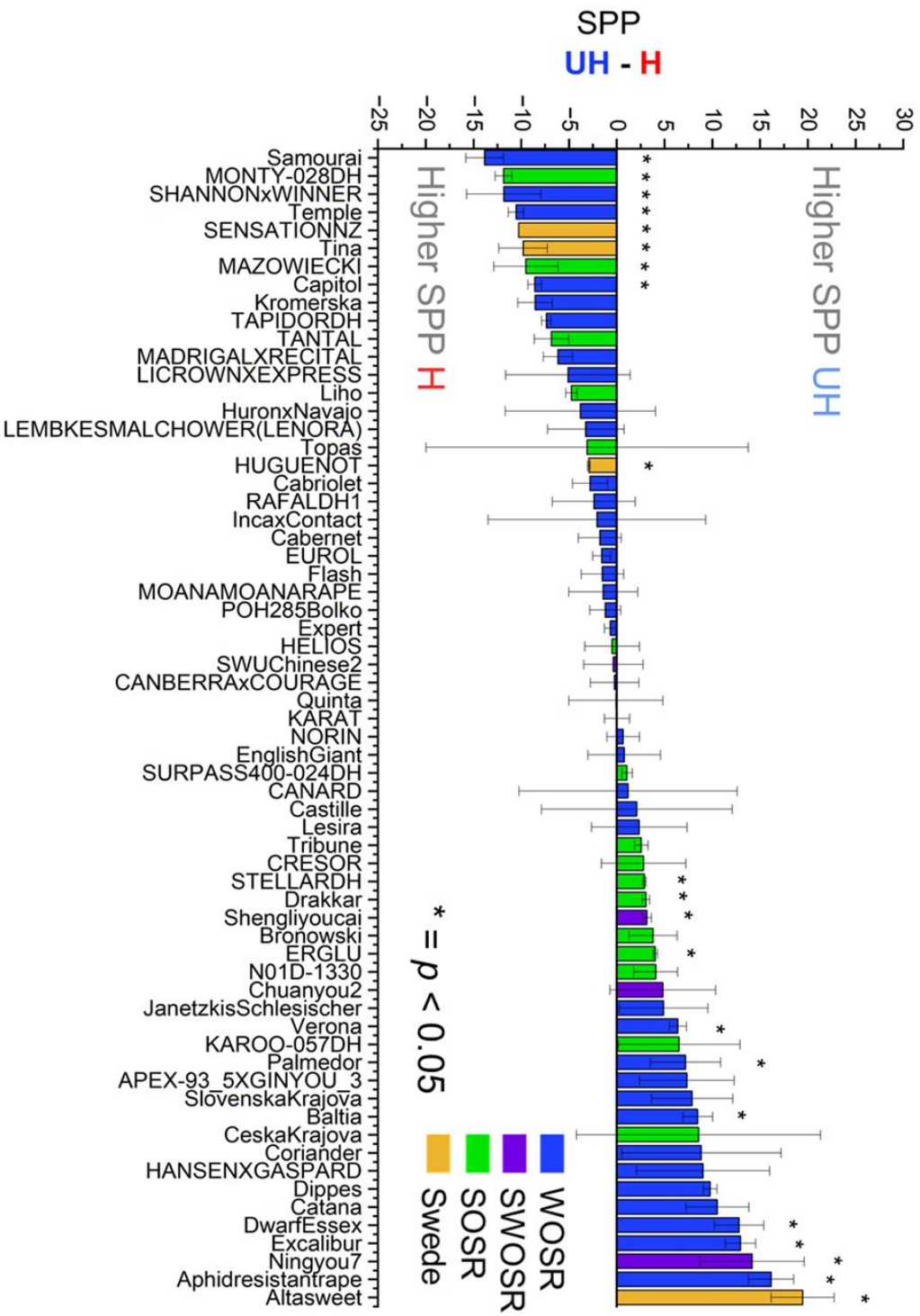


Figure 3.12. Individual varieties of *Brassica napus* show a range of values forSeeds Per Pod (SPP). Crop type is indicated by colours denoted in the key, where WOSR is winter type, SWOSR is semiwinter type, SOSR is spring type. Values represent SPP in the control minus the SPP in warmer, such that values above zero indicate varieties that had higher SPP in control and those below zero higher SPP in warmer. Significance determined using students t test and indicated with * to indicate $p < 0.05$, n = 6 per genotype.

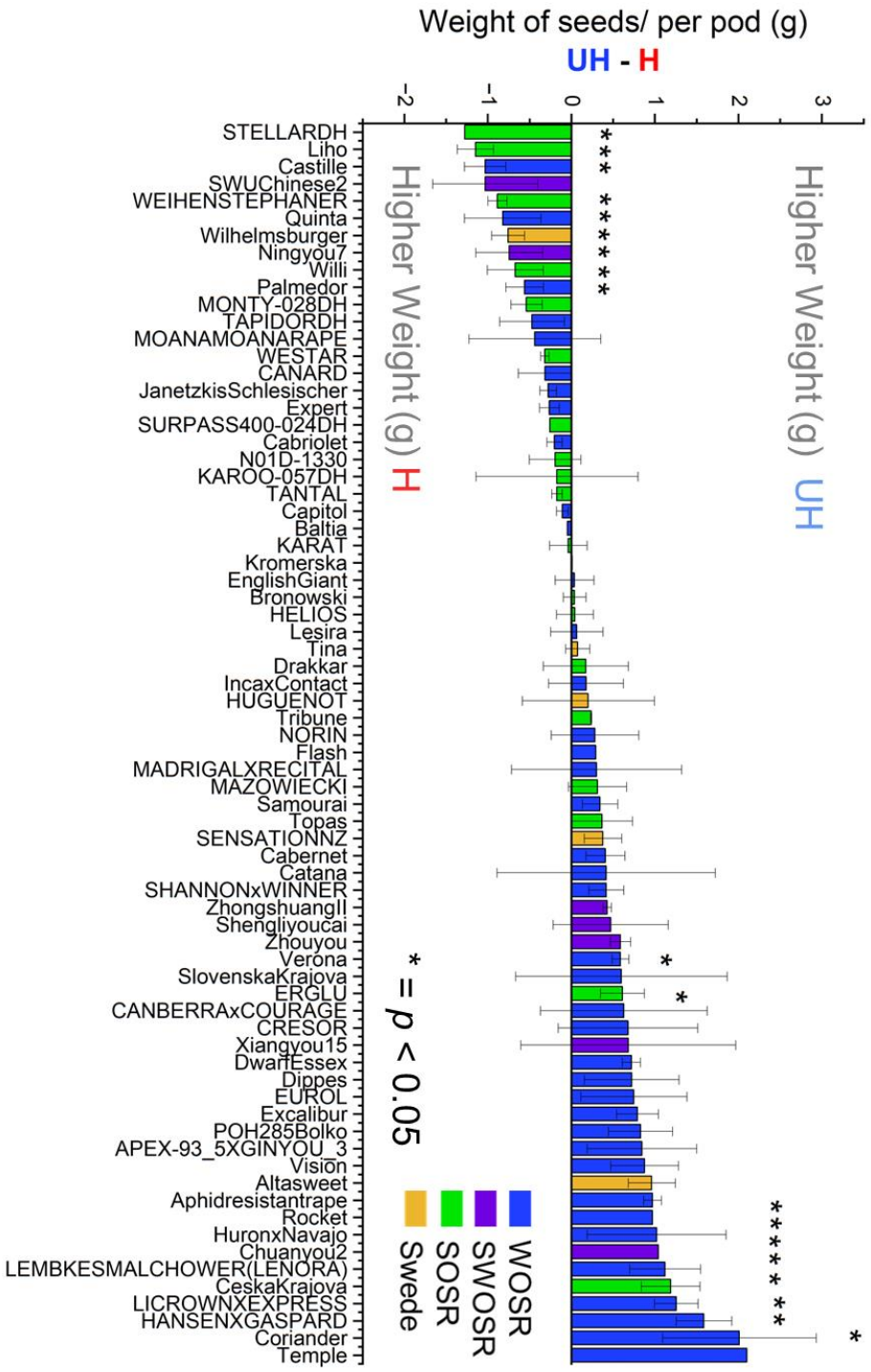


Figure 3.13. Individual varieties of *Brassica napus* show a range of average weight of all seeds in a pod. Crop type is indicated by colours denoted in the key, where WOSR is winter type, SWOSR is semiwinter type, SOSR is spring type.. Values represent Weight (g) in the control minus the Weight (g) in warmer, such that values above zero indicate varieties that had higher Weight (g) in control and those below zero higher Weight (g) in warmer. Significance determined using students t test and indicated with * to indicate p< 0.05, n = 6 per genotype.

3.3 Winter warming delays flowering in WOSR in a repeated experiment

To confirm findings from the previous experiment could be reproduced in an independent year with alternate weather conditions and that WOSR do show delayed reproductive development from bud warming I grew a subset of WOSR varieties from the winter warming experiment (Section 3.2). A subset was regrown as space limitations prevented growing the entire diversity set again. WOSR varieties grown in the subset were chosen to show the range of WOSR response to warmer winters. As before, plants were exposed to either control winter conditions in an control unlit polytunnel throughout winter or a four-week heating treatment following floral initiation (Methods section 2.2; Figure 3.14). Warmer winters almost universally delayed BBCH51 and BBCH60 for the WOSR subset (Figure 3.15), although there was wide variation in time to BBCH51 within individual varieties, this was not seen in BBCH60. This data confirmed that warmer winters delay time to BBCH51 and BBCH60 in WOSR, and that this is a robust response seen in independent experiments.

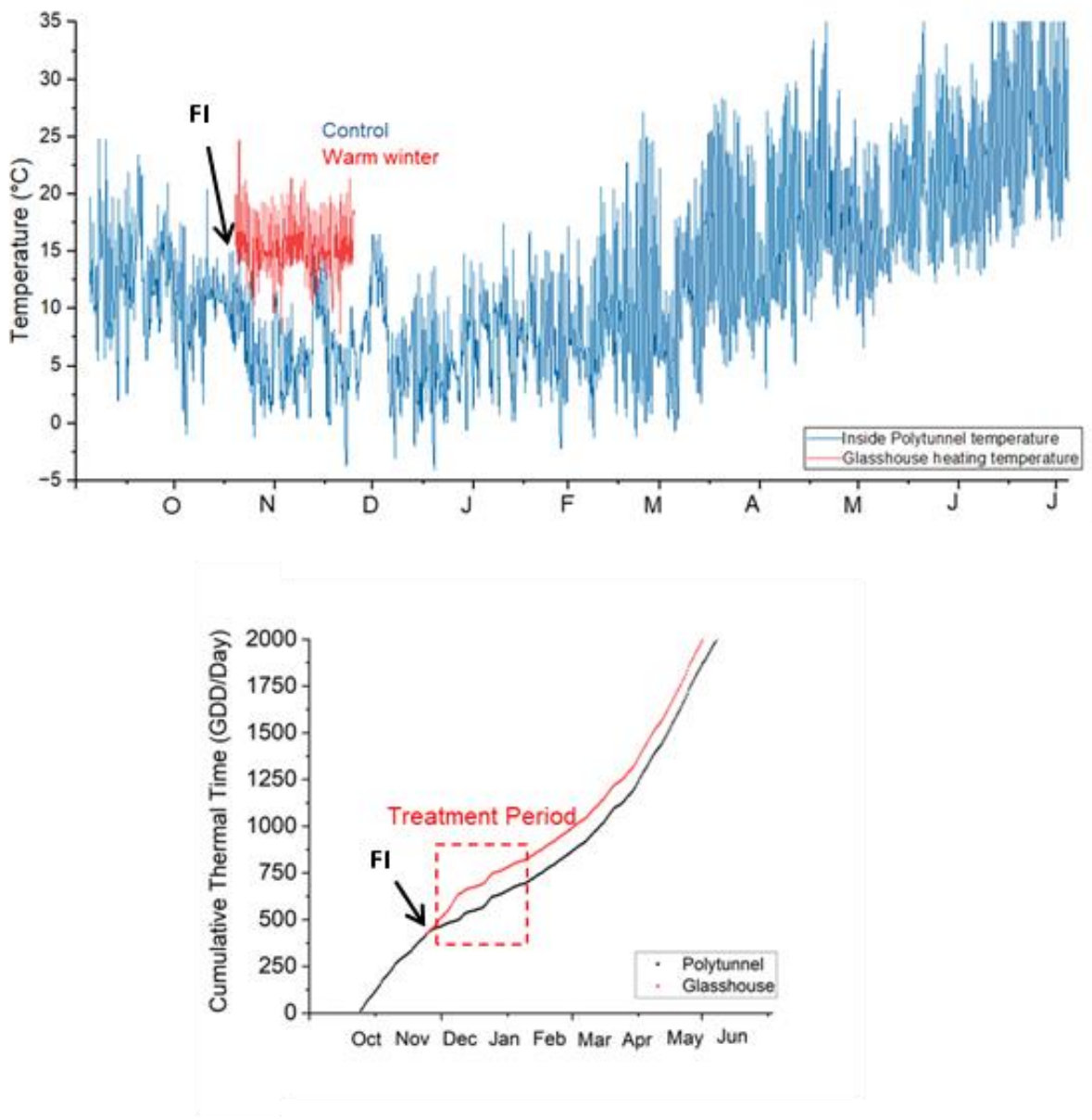


Figure 3.14 Comparison of ambient temperature and glasshouse heating treatment used in the 2021-2022 winter warming experiment (Methods section 2.2). A) Temperature recordings between control polytunnel (blue) and the warmer glasshouse (red), relative to floral initiation (F.I.) timing of *Brassica napus*. B) Cumulative thermal time between the control polytunnel and warmer glasshouse, red box indicates the time when plants were exposed to heating treatment (cumulative thermal time calculated according to Methods section 2.2.

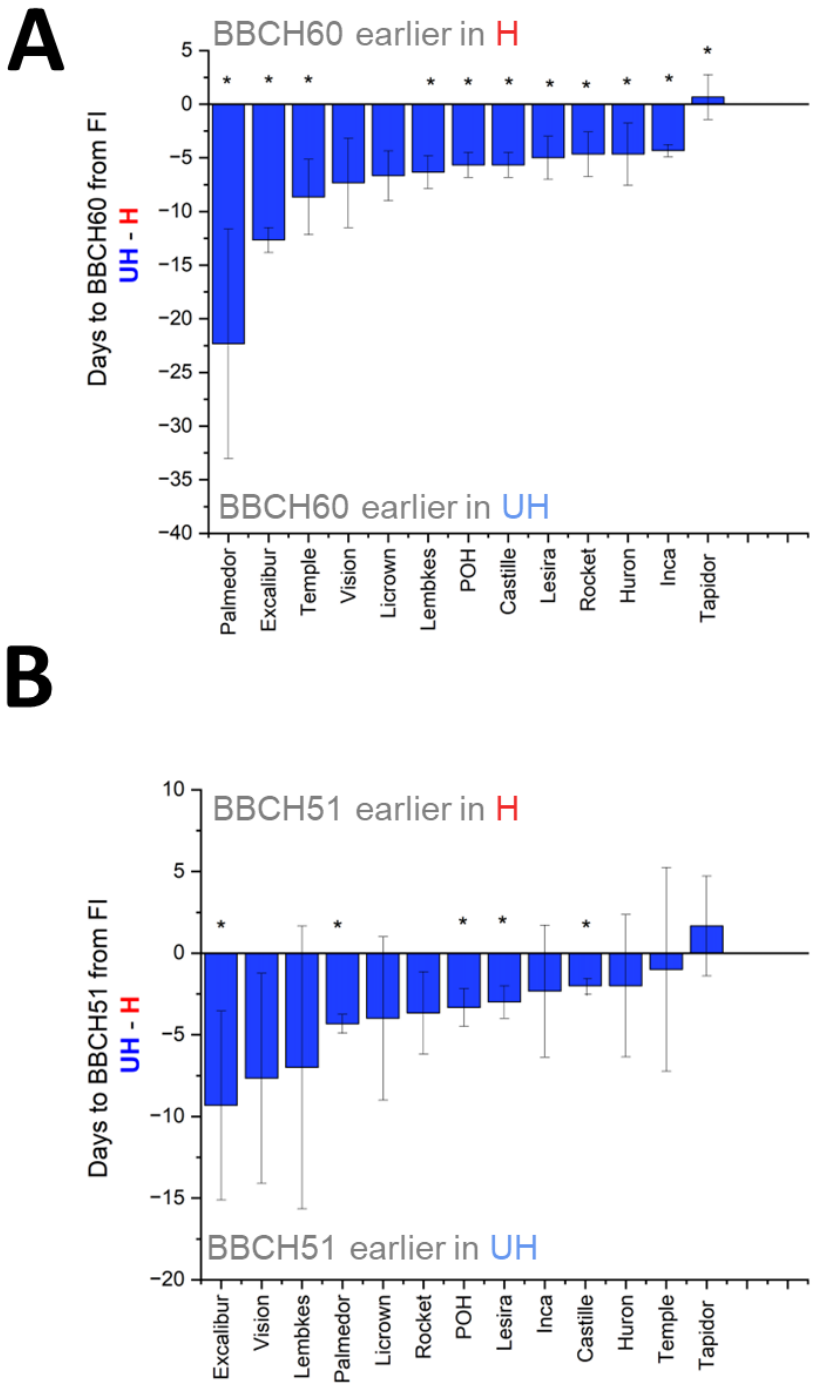
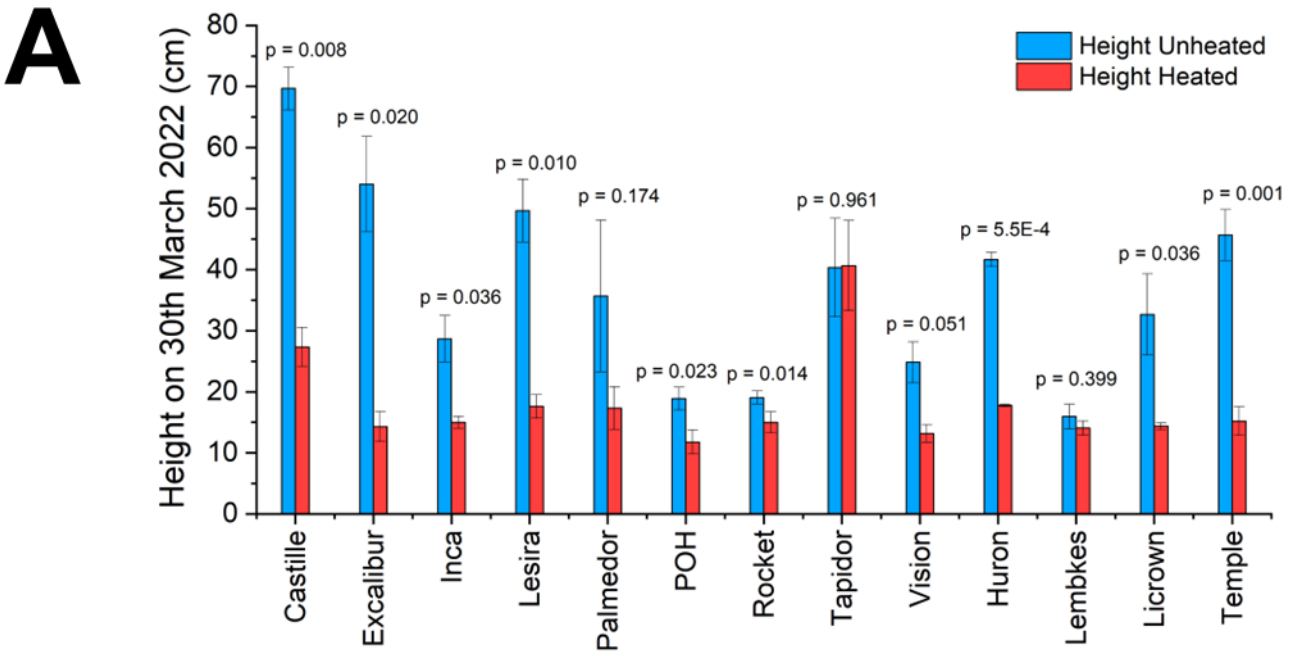


Figure 3.15. Most winter type *Brassica napus* reach first bud emergence (BBCH51) and first flower emergence (BBCH60) earlier in warmed winters compared to control treatments. Values represent the days to control minus the days to warmer to either BBCH51/60, such that values above zero indicate varieties that were advanced to BBCH51/60 by warming plants post-floral initiation and those below zero indicate varieties that were delayed. Significance determined using students t test and indicated with * where $p < 0.05$, $n = 6$ per genotype.

To understand how winter warming affects plant development, I measured the plant height as a proxy for bolting time between warmed and control varieties on 30th March 2022, to mark the beginning of spring. In nearly all WOSR varieties there was a significant difference in plant height. This indicates control WOSR plants bolted earlier than warmer WOSR plants (Figure 3.16).



B

Huron x Navajo

Castille

Vision

Rocket

Lembkes Malchower (Lenora)

Inca x Contact

Excalibur

Lesira

Temple

Tapidor DH

Licrown x Express

Palmedor

POH285Bolko

Figure 3.16 In early spring (30th March 2022) control winter type *Brassica napus* are taller than *B. napus* grown under a winter warming treatment (Methods Section 2.2) A) Plant height differences at spring bolting. Student's T test B) Images of plant height differences. Images with red bar indicate warmer treatment plants, images with blue bar indicate control plants. White bar is scale bar of 20cm, n = 6 per genotype.

To understand how winter warming affects *B. napus* bud development I scored bud development for three replicates of each variety for each treatment, according to the BBCH scale at the start of spring on 30th March 2022. In all lines except Tapidor and Rocket, warmer WOSR plants were at an earlier growth stage, between BBCH50 (flower buds formed but hidden by leaves), BBCH51 (flower buds visible from above) to BBCH52 (flower buds free and level with youngest leaves) (Figure 3.17) compared to control WOSR buds. Control WOSR plants were more developed (flower buds raised above youngest leaves). This confirms that in an independent year WOSR bud development is slowed following winter warming.

A

51 (Detail)

53 (Detail)

55

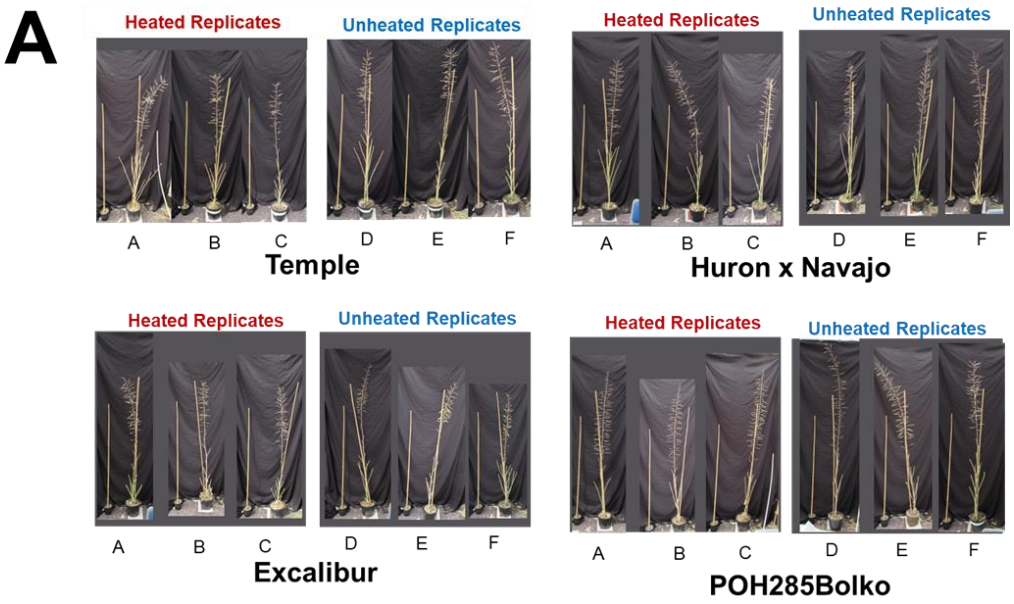
57

Principal growth stage 5: Inflorescence emergence

50	Flower buds present, still enclosed by leaves
51	Flower buds visible from above ("green bud")
52	Flower buds free, level with the youngest leaves
53	Flower buds raised above the youngest leaves
55	Individual flower buds (main inflorescence) visible but still closed
57	Individual flower buds (secondary inflorescences) visible but still closed
59	First petals visible, flower buds still closed ("yellow bud")

Figure 3.17 Floral bud development of winter type *Brassica napus* is slower following a warmed winter compared to a control winter Samples taken 30th March 2022. A) BBCH guide to *B. napus* bud development, according to Weiber and Bleiholder, 1990. B) Twelve winter oilseed rape lines representative of replicates for that line and treatment. Red indicates warmer treatment and blue indicates control treatment. Photos taken from 20cm above buds once spring growth resumed (30th March 2022). Warmer buds generally more compact and less open, indicating they are less developed,

To understand if delayed reproductive development impacted final plant stature, I measured the final plant height (cm) of each plant at maturity. There was no significant difference between plant height in the warmed or control varieties for all WOSR varieties (Figure 3.18). This indicates that winter warming delays WOSR bud development, but that the development of warmer varieties catches up with control varieties before maturation. However, I was unable to record when this happened.



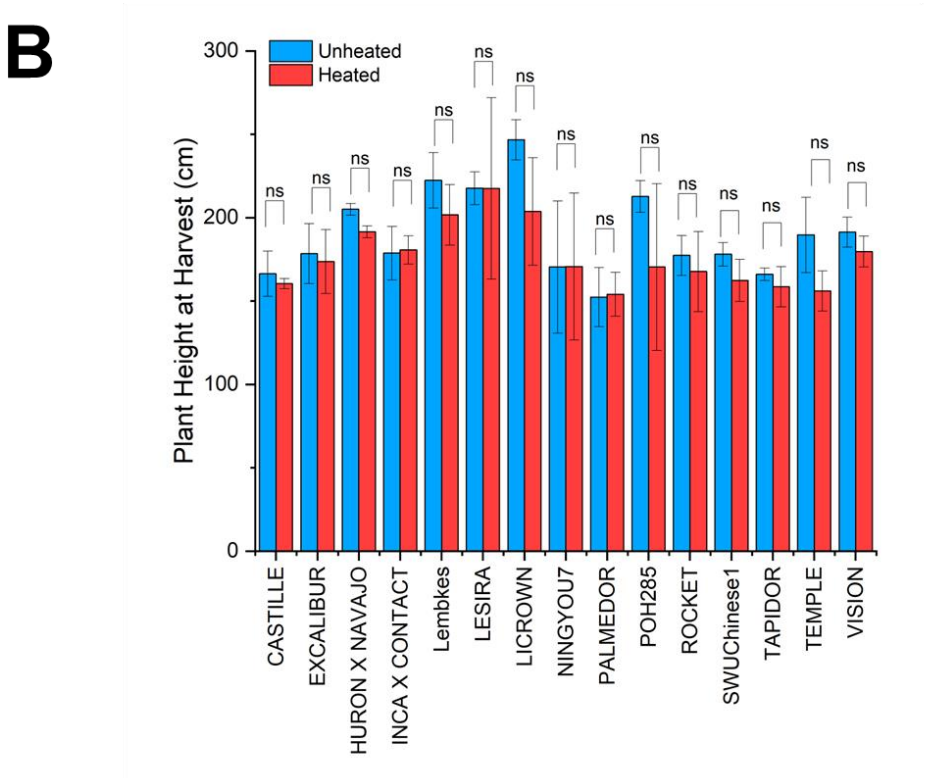


Figure 3.18. Winter type *Brassica napus* varieties show no difference in plant height at harvest when warmed after floral initiation in the 2021-2022 experiment (Methods Section 2.2). A) Four representative WOSR lines comparing warmer and control replicates. Metre rule in left hand side of every image (brown stick). B) Each WOSR variety shows no difference in plant harvest height (cm). ns indicates no significant difference determined by a two sample students t test, n = 6 per genotype.

To understand whether delayed reproductive development led to lower seed yield in WOSR varieties in the repeat experiment I harvested plants and scored for TGW (g), SPP, total seed area per pod and total weight of seeds per pod (g). All plants were grown in the same location under the same conditions, so a student's t-test was used to test for differences between treatments. No WOSR variety showed any significant difference between treatments for any seed traits (Figure 3.19). However, there was large variability within three replicates. This indicates the study size was likely insufficient to extrapolate findings about seed yield measures, so I cannot make conclusions about the effect of winter warming on WOSR seed yield.

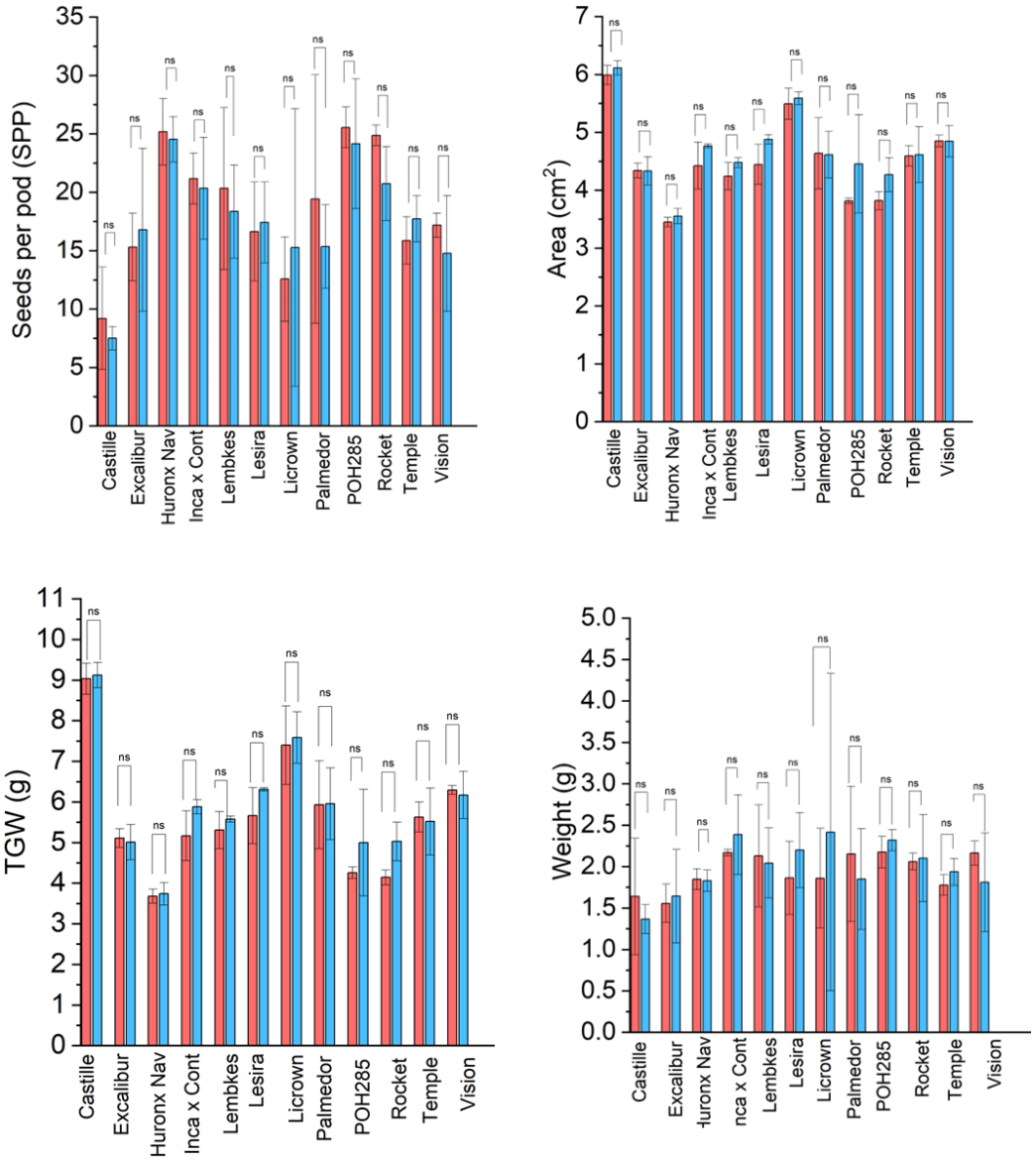


Figure 3.19 There is no significant difference in any seed traits between a control or winter warmed winter *Brassica napus* varieties in the 2021-2022 experiment (Methods section 2.2). Red indicates warmer treatment, blue indicates control treatment. Students t-test for significance comparison, ns = no significant difference, n = 6 per genotype.

3.4 Controlled Environment Room Experiments

3.4.1 Simulating growing seasons in Controlled Environment Rooms (CERs) shows that in warmer winters there is delayed reproductive development.

Historically, warmer winters have been associated with lower *B. napus* yields (Brown *et al.,* 2019). I wanted to understand whether variation in yields between growing seasons can be explained by differing temperatures between those growing seasons in isolation. This would confirm that temperature, instead of other aliased seasonal signals, is a strong determinant of final plant yield. I also wanted to confirm that the effects seen in 3.2 are representative of conditions that would be seen in the field.

To do this, I grew and scored six WOSR varieties representative of commercial UK *B. napus* cultivars in simulated conditions from two past growing seasons using Controlled Environment Rooms (CERs), adjusting temperature profiles between the simulations but keeping all other variables constant. I chose to simulate the 2010-11 growing season. The 2010-11 growing season had a comparatively cool winter (Figure 3.20) and had the highest yield for winter sown oilseed rape in England since 1999 (4.0 tonnes/hectare). By comparison, 2015-16 had a comparatively warm winter (Figure 3.20) and was one of the five lowest yielding years for winter sown oilseed rape in England since 1999 (3.1 tonnes/hectare) (Department for Environment, Food and Rural Affairs, Cereal and Oilseed Rape Production Survey for Winter sown Oilseed Rape in England, 2022, Accessible at: https://www.gov.uk/government/statistics/cereal-and-oilseed-rape-production).

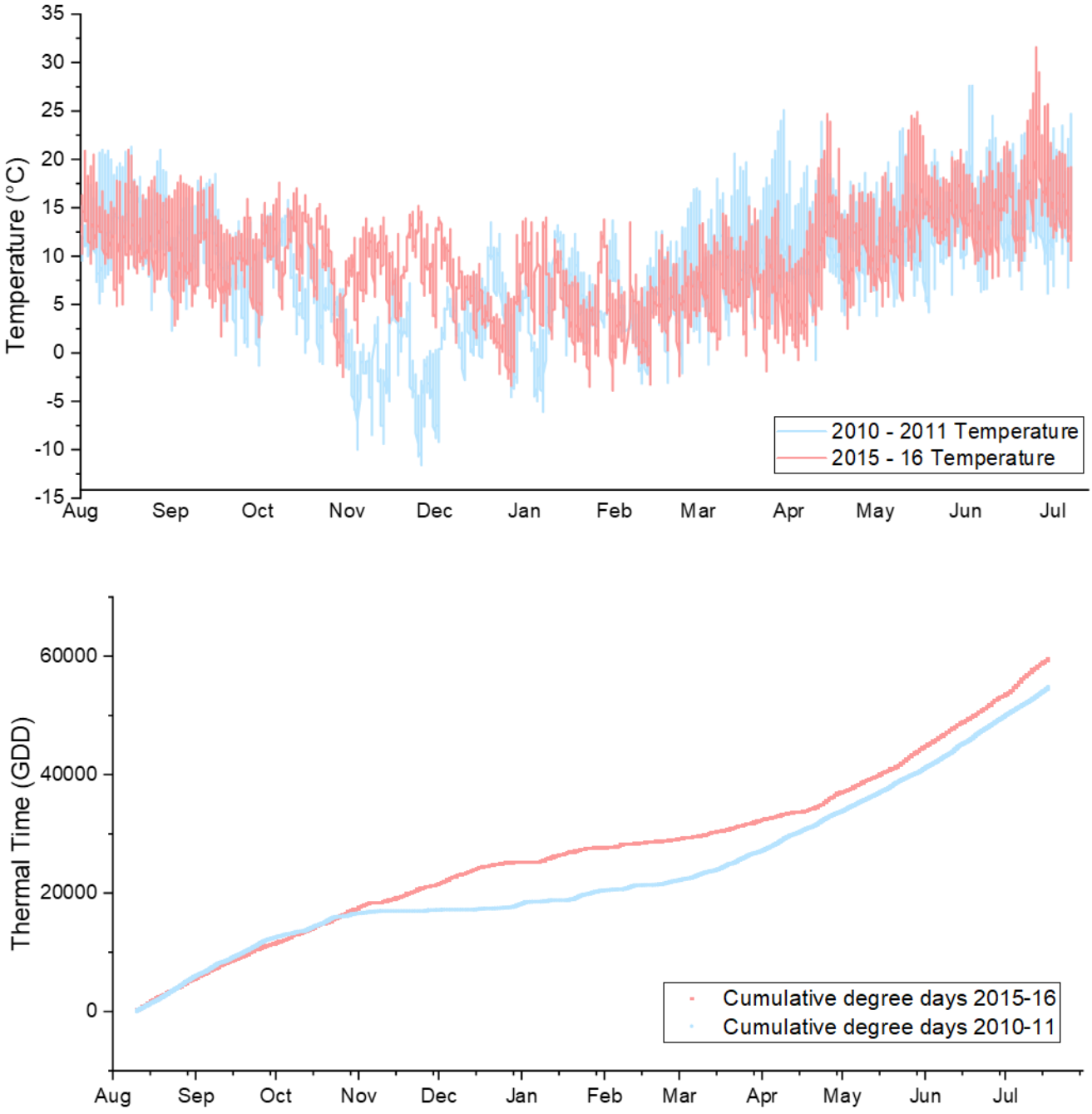


Figure 3.20. Temperature (°C) and Growing Degree Days (GDD) comparisons between the warm low yielding 2015-16 (red) growing season and the cool high yielding 2010-11 years (blue). Temperature data taken from weather station in Coleshill weather station (Methods section 2.1.2.3), GDD calculated according to Methods Section 2.2.3.

In the warmer 2015-16 growing season time to BBCH51 and BBCH60 was later than 2010-11 in almost all varieties (Figure 3.21). In 2015-16 varieties took from 3.1 to 13.1 days longer to BBCH51 after sowing than 2010-11, although there was no significant difference in days to BBCH51 in Vision. While in 2015-16 Temple took 8.9 days longer to reach BBCH51 (Figure 3.21). In 2015-16 all varieties took longer to reach BBCH60 from sowing than in 2010-11, from 11.9 to 26.6 days longer (Figure 3.21). The length of the difference in time to BBCH60 between 2015-16 and 2010-11 was large, for example Temple reached BBCH60 26.6 days later in 2015-16 than 2010-11. Differences this large in flowering time would alter whether flowers are exposed to spring frosts and change the environment for seed set occurs, either of these in isolation would affect final yield. The variation in response between varieties suggests that there is a genotype dependent response to winter warming. Furthermore, data here indicates that the warmer winter of 2015-16 delayed flowering in WOSR, mainly by affecting time from BBCH51 to BBCH60. This also indicates that real world temperature variation can drive similar delays to BBCH51 and BBCH60, albeit larger delays, seen in the winter warming experiments performed in polytunnels (Section 3.2, 3.3).

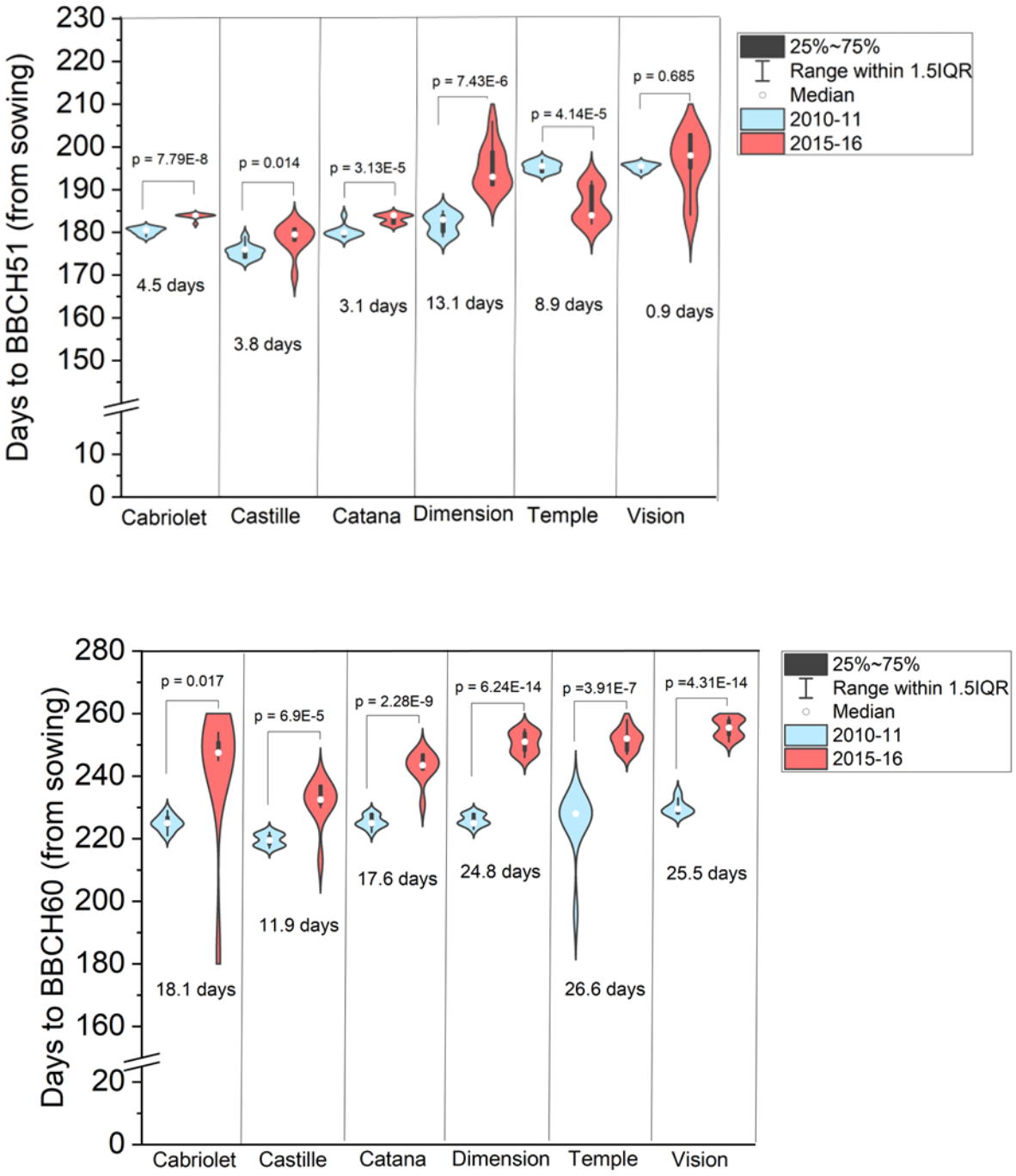


Figure 3.21 For all WOSR *Brassica napus* varieties, except Temple, time to BBCH51 and BBCH60 was later in 2015-16 (shown in red) than 2010-11 (shown in blue). Violin plots show data variability. Date from sowing as floral initiation date not checked for CER experiments. Significance tested by students t-test, n = 10 per genotype.

3.4.2 The 2015-16 warmer winter led to lower SPP compared to 2010-11 in varieties that set high seed in the CERs

To determine if the delayed reproductive development (Figure 3.21) in 2015-16, compared to 2010-11, also causes reduction of key measures of seed yield, I measured thousand grain weight (TGW) (g) and seed per pod (SPP) of 20 representative pods on the primary raceme on each plant in both simulated years. There was no significant difference (students t-test with Welch's correction) between TGW of either growing season for any variety (Figure 3.22). However, for SPP, Temple and Vision had significantly greater SPP in 2010-11 than 2015-16 (student's t-test, $p = 0.0219$, $p = 0.045$, respectively). For Cabriolet, Castille, Catana and Dimension however, SPP is far lower than would be expected in the field and both growing seasons show very low SPP. For these varieties there were fewer than 18 pods on the primary raceme (individual plants marked in pink in Figure 3.22). This highlights a key disadvantage of CER experiments, where some plant varieties, regardless of treatment, set low seed that is not representative of the field. Fertility issues in CER has been a common problem across experiments in the Penfield laboratory. This reduces our ability to extrapolate field relevant processes from these varieties. However, for varieties that still set high seed yield, such as Temple and Vision, this suggests that temperature differences between these years alone is sufficient to cause lower SPP, but not TGW.

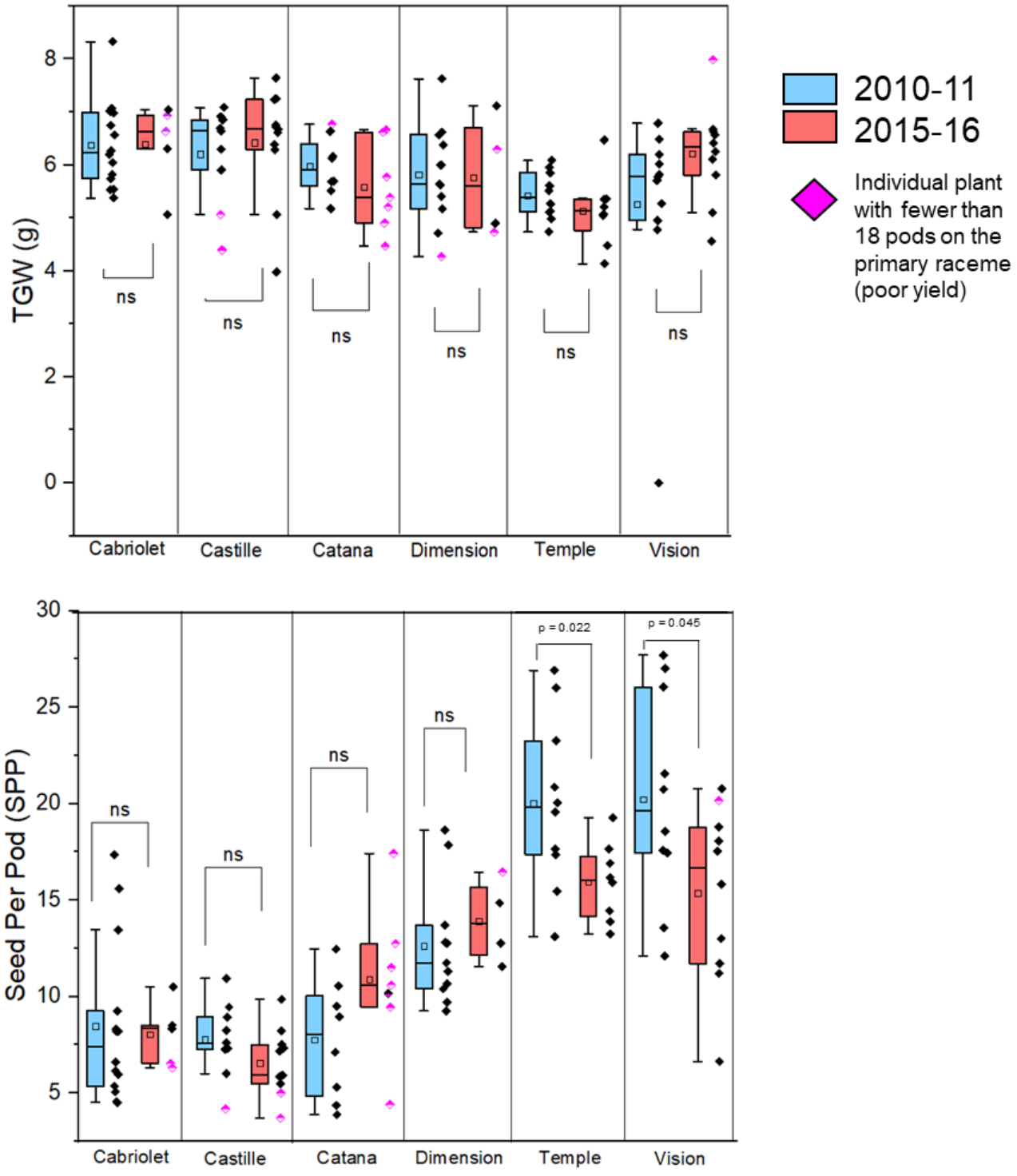


Figure 3.22 Varieties of *Brassica napus* resilient to yield defects seen in Controlled Environment Rooms (CER) have greater Seeds per Pod (SPP) in a CER simulation of 2010-11 than 2015-16. Seed trait differences between 2015-16 (red) and 2010-11 (blue). Student t-test with Welch's correction for significance testing, where ns = P>0.05. Pink half-filled points indicate individual lines that were unable to form the full 20 pods on the primary raceme needed for final development, n = 10 per genotype.

3.5 Discussion

Vernalisation has been described as the promotion of spring floral development following a period of prolonged winter cold, typically of one to three months at temperatures between 1 and 10 °C in *A. thaliana* (Simpson and Dean, 2002; Hepworth and Dean, 2015). Previous work in *A. thaliana* has shown that after vernalisation, warmer temperatures promote flowering through the photoperiod and the ambient temperature pathway (Samach *et al.,* 2000; Sawa *et al.,* 2007; Blázquez *et al.,* 2003; Kumar *et al.,* 2012; Posé *et al.,* 2013). However, recently it was shown that WOSR *B. napus* will proceed through the floral transition in late autumn (O'Neill *et al.,* 2019). Here, I show that contrary to expectation, warmth following the floral transition delays flowering in WOSR varieties. If there are warmer than average temperatures following the floral transition, there are also declines in seed characteristics that are associated with yield (Figure 3.10). This suggests temperatures post-floral initiation are important for winter *B. napus* development.

3.5.1 Winter warmth delays flowering in WOSR

Crucially, I show that simulating temperature variation alone, not other winter weather phenomenon, is enough to see floral delay and reduced SPP in WOSR varieties unaffected by CER fertility issues (Figure 3.22). These growing seasons differed in winter temperatures after the time when WOSR is assumed to have passed through the floral transition (Figure 3.20; O'Neill *et al.,* 2019) and resulted in significant delays to flowering. The two past growing seasons recreated here represented historic high and low yielding years so the large floral delay suggests the cause of these large yield differences may be due to temperature effects alone. For instance, in 2015-16 plants took from 11.9 to 26.6 days longer to BBCH60 than 2010-11 (Figure 3.21). This was on average 10-days longer than equivalent varieties grown in the polytunnel experiment in either treatment, excluding Castille and Dimension. Greater time to BBCH60 would shift the temperatures for seed maturation, potentially affecting final yield.

This delay is likely influenced by warmer spring temperatures further accelerating development in the 2010-11 CER (Figure 3.20). The greater floral delay in CER experiments than polytunnel and glasshouse experiments may be due to fluctuating winter temperature treatment in the CERs compared to the constant treatment in both glasshouse warming experiments. Whether winter temperature is constant or fluctuating influences the degree of vernalisation a plant will experience, so it is not unreasonable to assume that this difference

could also influence the chilling requirement following the floral transition (Burghardt *et al.,* 2016; Topham *et al.,* 2017; Zhao *et al.,* 2020a).

This highlights the need for representative experiments as laboratory heating experiments may underestimate the extent of floral delay from winter warming and CER experiments show fertility issues resulting in an inability to set final seeds (Figure 3.22). We also recently showed that in a field experiment on the WOSR Cabriolet there is a delay to flowering caused by winter warming, this supports the findings here, although in the field there was only a modest 5.3-day delay (Lu *et al.,* 2022), which is representative of the delays seen in some varieties in the diversity set experiment.

3.5.2 WOSR warmth induced floral delay resembles perennial dormancy

The necessity for winter cold to accelerate flowering in overwintering floral meristems of WOSR physiologically resembles perennial bud dormancy. It is unclear whether *B. napus* demonstrates bud dormancy or whether the delayed reproductive development seen in warmer years occurs by another mechanism. However, there remain key similarities between the *B. napus* winter bud physiology and perennial bud dormancy. Here in WOSR, buds form before winter and winter chilling of newly emerged buds promotes spring flowering independently of the control of the floral transition (Figure 3.6). Similarly, in perennials, buds form before winter and then remain dormant until bud burst in spring. In perennials prolonged cold after bud initiation is required to 'break' endodormancy (Lang, 1987; Lloret *et al.,* 2018). If there is insufficient chilling perennial species show delayed bud break (Atkinson *et al.,* 2013). This in turn alters the time of anthesis which is associated with poor formation of floral, fruit and seed structures that impact yield (Hovarth *et al.,* 2009; Atkinson *et al.,* 2013). Similarly, in *B. napus,* we showed that WOSR Cabriolet floral structures develop abnormal flowers in warm winters (Lu *et al.,* 2022). Crucially for perennial species, the extent of winter bud chilling determines the cultivation boundaries of perennial fruit trees in Southern America and Europe (Yamato *et al.,* 2010; Atkinson *et al.,* 2013). Limited southern cultivation of *B. napus* is a predicted consequence of warmer summers inducing drought stress (Jaime *et al.,* 2018), evidence here suggests reduced winter chill in Europe (Luedling, 2012) will further reduce the optimal cultivation limits of *B. napus.*

Winter floral transition appears common across the Brassicaceae. For example, biennial brussel sprouts (*B. oleraceae var. gemmifera*), cabbage (*B. oleraceae* var. capitata) and the perennial *A. alpina* and *A. lyrata* will undergo the floral transition during chilling conditions before spring (Stokes and Verkerk, 1951; Wang *et al.,* 2009; Kemi *et al.,* 2019). Indeed, the basis of the harvestable component of cauliflower, *B. oleracea var botrytis,* is dormant

inflorescence buds. This suggests that, at least for Brassicaceae winter floral initiation followed by a period of overwintering floral buds may be a common phenomenon. As such, the hitherto undiscovered secondary requirement for winter cold may also be important in other species. In wheat it has also been shown that lower winter temperatures increase spikelet number (Dixon *et al.,* 2018) and are associated with higher yields (Kettlewell *et al.,* 1999). Although, whether there is a potential dormancy stage, or yield penalty during warm winters, in winter annual species other than *B. napus* remains to be determined.

Crucially, breeding efforts over the last 40 years has led to the development of perennial fruit trees with 'low chill' requirements that can still be cultivated in warm winter conditions (Atkinson *et al.,* 2013; Campoy *et al.,* 2018; Fadón *et al.,* 2021 Fernandes *et al.,* 2023), indicating that bud dormancy is a genetically adaptable trait. Here, I have uncovered genotype variation both within crop types and between crop types in the response to winter bud warming (Figure 3.6). This suggests that there is also genetic variation in the presence or absence of bud dormancy in *B. napus.* However, more work is needed to establish the exact duration and intensity of this developmental delay induced by winter warming in *B. napus* buds.

3.5.3 Warmth induced bud dormancy is a potential mechanism for *B. napus* yield loss

In perennial trees, insufficient chill of overwintering buds is associated with yield decline through many effects including reduced bud break efficiency, which in turn delays anthesis to a less environmentally favourable time (Atkinson *et al.,* 2013). In *B. napus* the significance of this potential bud dormancy phenomena relates to its potential association with low yields. In agreement with a previous correlative study (Brown *et al.,* 2019), I show that the seed yield parameter of TGW is reduced in a winter warming experiment in WOSR when buds are exposed to warmer than average temperatures in early winter (Figure 3.10)

Intriguingly, in the diversity set experiment, individual varieties with the largest delay to BBCH60 from bud warming are also lines that have the largest TGW reduction following the warming treatment, e.g. Samourai, Catana, Vision, Lembkes. While Aphidresistant rape, the only WOSR that was statistically accelerated to flowering following bud warming was also one of the only WOSR varieties that showed reduced TGW in the control treatment (Figure 3.11). This suggests that the requirement for bud chilling varies between varieties and may be directly associated with flowering time and yield. Therefore, understanding the genetic control of the requirement for winter chill in *B. napus* buds and identifying varieties more resilient to winter warmth will be important to adapt WOSR to a warmer environment. Further work is also needed to establish the reasons why poor yield formation occurs in warmer years in WOSR.

3.5.4 The variability of response to winter warmth in *B. napus*

The variable response to post floral initiation chilling is highlighted by the strong crop type effect seen in bud warming. Whilst bud warming delays floral development and is associated with yield loss for most WOSR, bud warming accelerates floral development in SWOSR and Swedes but has varying effect in SOSR, both advancing some varieties but delaying others (Figure 3.4). The extent to which bud warming delays floral development correlates with floral timing, such that early flowering lines are accelerated to flowering by post floral initiation warmth while late flowering lines are delayed (Figure 3.3). This implicates flowering time genes which control flowering time in both winter and spring environments such as *Bna.FRI* and *Bna.FLC* genes (Wang *et al.,* 2011; Calderwood *et al.,* 2021a; Calderwood *et al.,* 2021b). This may explain why some late flowering SOSR lines show floral delay during bud warming, a short bud dormancy stage may have been kept in SOSR to delay flowering in late flowering lines (Figure 3.3). Although, it is also likely SOSR are unresponsive as they depend on photoperiod for development not temperature (Friedt and Snowdon, 2009).

3.5.5 Implications

It remains to be seen how widespread this phenology is in other overwintering winter annual crops, or whether the reproductive delay and yield penalty induced by warm winters is a bud dormancy stage in *B. napus*. However, our current understanding of how climate change will impact agricultural yields is informed through computational models of crop development. For oilseed rape, many of these models assume vernalisation is a single-step process and no crop model accounts for the need for winter cold in developing WOSR buds (Habekotte *et al.,* 1997; Deligiosa *et al.,* 2012; Weyman *et al.,* 2015). If our crop models are inaccurate, we risk underestimating real-world outcomes of climate change. This has been seen in maize, wheat, rice and soybean, where crop models underestimate yield loss seen in field experiments (Wang *et al.,* 2020). Therefore, discovering and characterising this novel bud dormancy response is important for climate adaptation efforts. Meanwhile, understanding the molecular mechanism controlling post floral initiation chilling requirement will be essential for producing WOSR varieties resilient to warmer winters. This is the focus of the remaining results chapters of this thesis.

Chapter 4. Genetic variation in warming induced flower bud dormancy in *Brassica napus.*

4.1 Introduction

B. napus has been bred for different environments, this has resulted in distinct breeding pools of unique crop types and genetic variation across different *B. napus* varieties. In Chapter 3 I uncovered phenotypic variation to the response of winter warming on developing floral buds. I showed that temperature after the floral transition influences flowering time in WOSR and I also present evidence that late winter temperature is important for optimal yield formation in *B. napus* consistent with past correlative studies (He *et al.,* 2017; Brown *et al.,* 2019). Therefore, understanding the genetic mechanism behind this warm winter floral delay phenomenon will be important for adapting *B. napus* to a changing environment.

A range of molecular genetic techniques have been used to understand the control of complex traits in *B. napus.* For instance, using comparative transcriptomics we can compare gene expression profiles of varieties with different phenotypes to understand the cause of phenotypic difference. In *B. napus* comparative transcriptomic studies have uncovered the genetic control of traits as diverse as root architecture, seed oil content and disease resistance (Dun *et al.,* 2017; Wang *et al.,* 2016; Wu *et al.,* 2016). Genome Wide Association Studies (GWAS) are also commonly used to find genes associated with a trait of interest. For instance, by associating phenotypic variation with the presence of single nucleotide polymorphisms (SNPs) across a population of plant varieties, GWAS' in *B. napus* have successfully identified genetic loci influencing traits such as plant height, yield, flowering time, and disease resistance (He *et al.,* 2017; Li *et al.,* 2014; Li *et al.,* 2016; Helal *et al.,* 2021; Wei *et al.,* 2016). More recently, gene expression marker (GEM) analysis has also been used to identify molecular markers associated with traits of interest in *Brassica* species (Harper *et al.,* 2012; Woodhouse *et al.,* 2021; Fell *et al.,* 2023). In GEM analysis, a transcriptomic sequence is aligned to a reference and the number of reads in RPKM (Reads per Kilobase of transcript per Million mapped reads) is recorded as the expression level for each gene. A regression model between trait value and gene expression across a set of varieties is then used to identify significant associations between gene expression markers and traits of interest. Together, comparative transcriptomics, GWAS and GEM analysis are all powerful tools for discovering the genetic control of key traits of interest in crop species.

4.1.1 Hypotheses and aims

In this chapter, I investigate the genetic control of delayed reproductive development in response to winter warming described in Chapter 3. I investigate transcriptomic differences between Castille, a winter oilseed rape that demonstrates delayed reproductive development under warm winters and Ningyou7, a semi-winter oilseed rape that shows accelerated reproductive development under warm winters, to understand more about the mechanism by which warmer winters delay flowering.

Castille and Ningyou7, the two varieties investigated here, are a winter and semi-winter variety, respectively. Selective breeding pressures led to the development of semi-winters from winters, this is controlled primarily by *FLC* genes and its partners and regulators. I hypothesise that the difference in response to bud warming between these two varieties is due to the differences in *FLC* gene function, their known interacting partners and regulators.

In Chapter 3 I identified variation in warm winter responses across a diversity set of *B. napus*. Here, in addition to comparative transcriptomics, I use a Genome Wide Association Study (GWAS) and Gene Expression Marker correlation analysis (GEM) to elucidate the control of flowering time in response to winter bud warming in *Brassica napus*.

4.2 Transcriptomic comparison of bud warming

4.2.1 Transcriptomic responses to warming in developing WOSR and SWOSR flower buds

Within *Brassica napus* there is extensive variation in the effect of winter warmth on flowering time (Chapter 3; Figure 3.6) and a strong crop-type effect (Chapter 3; Figure 3.4). This suggests the temperature response is under genetic control. To understand the genetic control of flowering time I generated an RNA sequence (RNA-seq) data series of developing flower buds for two *B. napus* cultivars which demonstrated opposing responses to post floral initiation warmth, selected using the data described in Chapter 3. Castille shows delayed reproductive development after winter warmth ($p < 0.001$) (Figure 4.1), but Ningyou7 shows accelerated development ($p < 0.001$) (Figure 4.1): thus the differences between the transcriptomic

responses of the two varieties are likely to be useful in understanding the underlying mechanism.

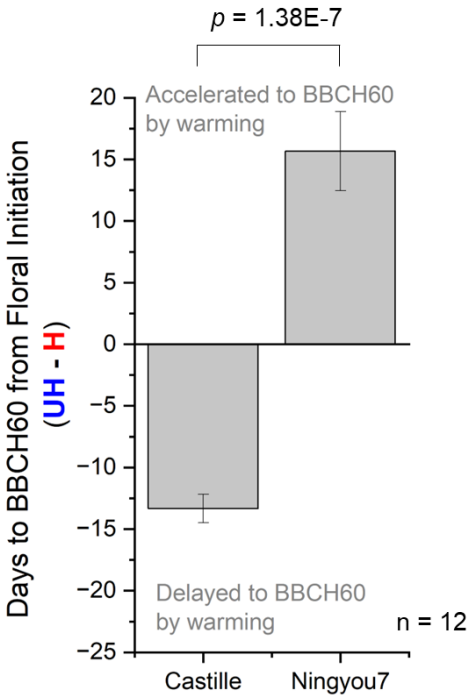


Figure 4.1. Winter warming after bud development delays flowering in WOSR Castille but accelerates flowering in Ningyou7. Timing of first flower emergence (BBCH60) between control and warm-treated WOSR, expressed as days after floral initiation. Here the effect of warming is accounted for by subtracting the mean time to BBCH60 in warmed treatment from the mean time to BBCH60 in the control treatment, such that values below zero indicate a delay to BBCH60 in warmed treatment and values above zero indicate an acceleration to BBCH60 in warmed treatment. Significance determined by students t-test.

As WOSR flowering is delayed by warmer temperatures (Chapter 3; Figure 3.4), I compared the transcriptomic responses of Castille and Ningyou7 between plants warmed in an unlit glasshouse and plants maintained in an unlit control polytunnel (Methods section 2.7) in time-series experiment over two weeks (Figure 4.2). Ningyou7 was sown after Castille to ensure it went through floral initiation at a similar time as Castille (Figure 4.2).

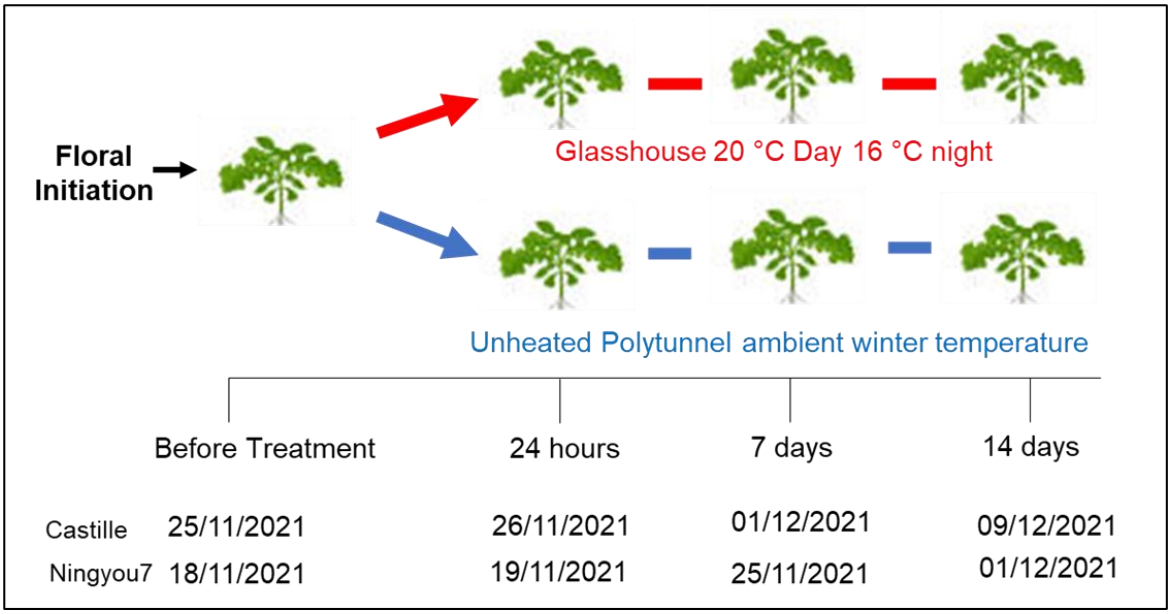


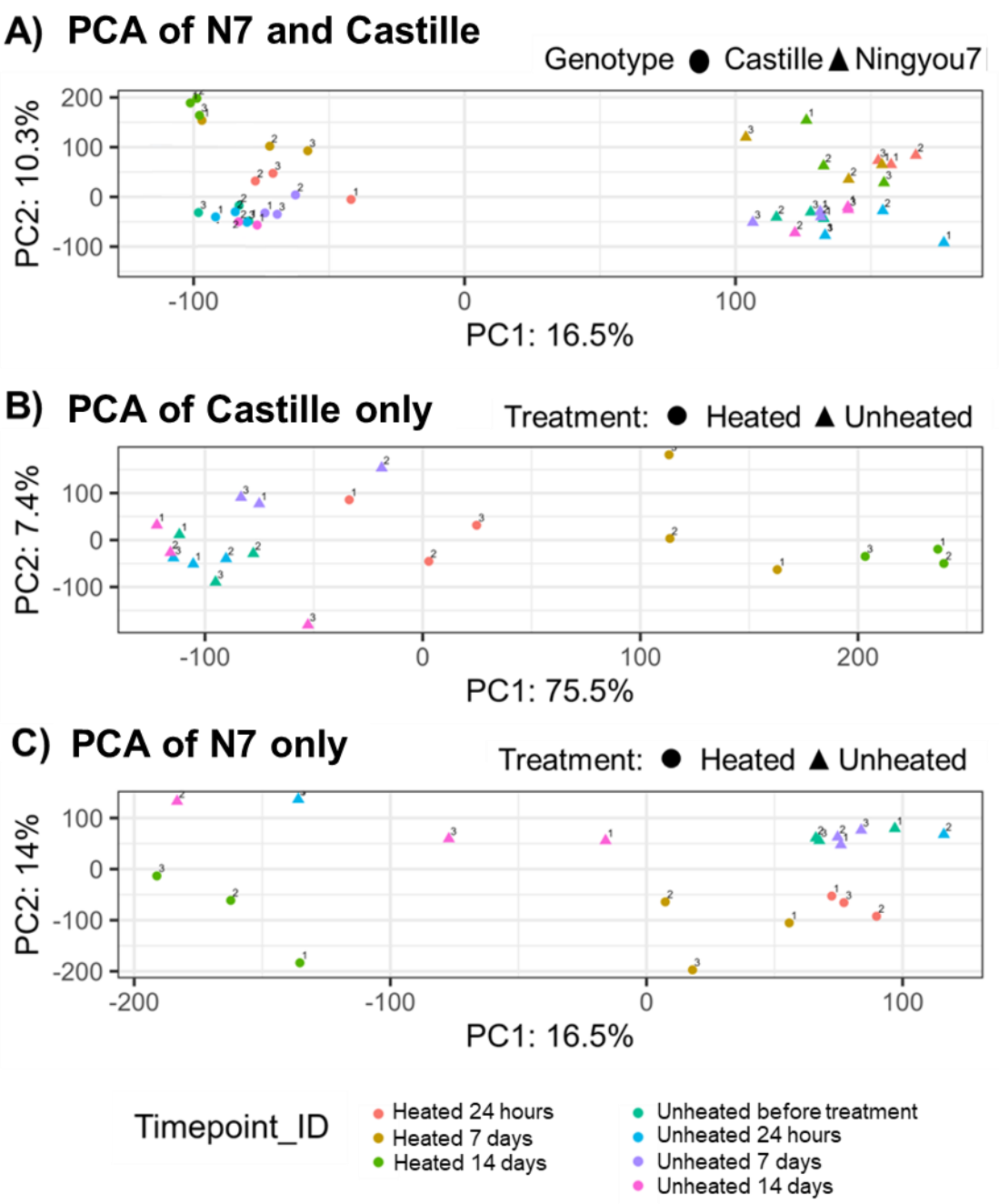
Figure 4.2. Schematic to represent the sampling schedule for collection of transcriptomic time series data. Each plant and timepoint indicates when the apex tissue was harvested. In total there were four timepoints (before treatment, 24 hours after treatment, 7 days after treatment, 14 days after treatment) and two comparative environments (control and warmer).

To identify any differences in the transcriptomes of two varieties I performed Principal Component Analysis (PCA) on all samples from the RNA time course (Figure 4.3A). PC1 explained 16.5% of the variation and separates samples based on genotype. PC2 explains 10.3% of the variation of samples although no clear pattern could be identified (Figure 4.3A). This suggested that Castille and Ningyou7 show divergent transcriptional responses.

To understand the transcriptomic response to winter warming in Castille I performed PCA on Castille timepoints only (Figure 4.3B). PC1 explained 75.5% of the variation in the Castille transcriptome, PC1 separated warmed and control samples, this suggests that the temperature treatment is the main cause of variation in the Castille transcriptome. PC2 explained less of the total variation (7.4%) of Castille timepoints, and it was unclear what PC2 explained (Figure 4.3B). To understand the transcriptomic response of Ningyou7 to winter warming I performed PCA for Ningyou7 samples only (Figure 4.3C). PC1 explained only 16.5% of variation in samples and appeared to be related to time as earlier samples were

separated from later samples (Figure 4.3C). PC2 explained 14% of transcriptomic variation and appeared to separate samples according to treatment (Figure 4.3C). This suggests that Castille has a strong response to post-floral initiation temperature but Ningyou7 does not.

Next, I performed differential gene expression analysis between Castille and Ningyou7 using ImpulseDE2. ImpulseDE2 was chosen to compare the effect of warming over the entire timecourse (Fischer *et al.,* 2018). Castille had 10,162 differentially expressed genes affected by heating over time and 5355 differentially expressed genes unique to Castille (Figure 4.3D). By comparison Ningyou7 had 5994 differentially expressed genes in response to warming and 1197 unique differentially expressed genes in response to warming (Figure 4.3D). This indicates that in Castille a larger number of genes show greater expression in response to warming than in Ningyou7, or a larger number of genes show a significant response to warming in Castille than Ningyou7


A) PCA of N7 and Castille
Genotype ● Castille ▲ Ningyou7
PC2: 10.3%
PC1: 16.5%
B) PCA of Castille only
Treatment: ● Heated ▲ Unheated
PC2: 7.4%
PC1: 75.5%
C) PCA of N7 only
Treatment: ● Heated ▲ Unheated
PC2: 14%
PC1: 16.5%
Timepoint_ID
Heated 24 hours
Heated 7 days
Heated 14 days
Unheated before treatment
Unheated 24 hours
Unheated 7 days
Unheated 14 days

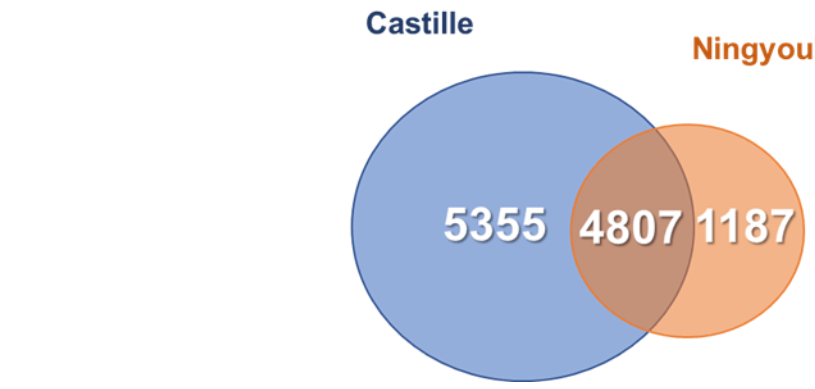

D) DEGs N7 v Castille
Castille
Ningyou7
5355
4807
1187

Figure 4.3. Heating causes significant transcriptional changes in Castille whilst time causes most transcriptional changes in Ningyou7. Principal component analysis to show the transcriptional response of Castille and Ningyou7. A) Principal component analysis shows genotype separates varieties. B) PCA analysis of Castille. PC1 represents treatment (warmer/control), PC2 represents time C) PCA analysis of Ningyou7 timepoints. PC1 represents time and PC2 represents treatment. D) Differentially expressed genes (DEGs) between Castille and Ningyou7 in response to warming over time, derived from Impulse DE2 analysis.

4.2.2. Bud dormancy-associated genes are differentially affected by warming between Castille and Ningyou7

To further understand the different transcriptome responses to temperature I performed hierarchical clustering on the gene expression across all Ningyou7 and Castille samples (Figure 4.4A). Hierarchical clustering confirmed the Castille transcriptome is predominantly affected by temperature, as warmer and control timepoints clustered separately (Figure 4.4B). In contrast for Ningyou7, samples clustered based on time regardless of whether samples were obtained from warmed or control plants (Figure 4.4B). This is further evidence that Castille shows a transcriptomic response to warming that is absent in Ningyou7.

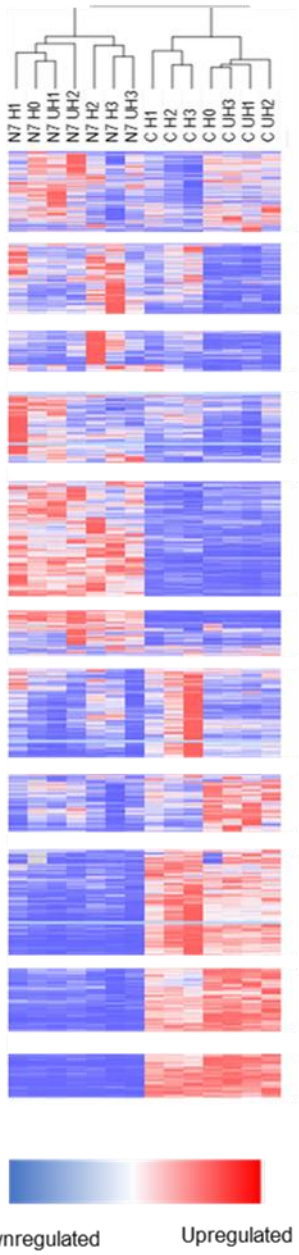
Eleven clusters were identified from this analysis (Figure 4.4A). Each cluster separated gene expression patterns according to genes that were upregulated in specific varieties at specific times and showed different responses to the heating treatment (Figure 4.4A). Each cluster was assigned a ‘cluster behaviour’ tag in Figure 4.4A, describing the association of these upregulated genes with the respective variety and timepoints when they were upregulated. Two of these eleven clusters showed expression patterns that explained the main transcriptional responses of either variety identified from previous PCA Analysis (Figure 4.3B; Figure 4.3C). Cluster 6 represented genes that were upregulated in late Ningyou7 timepoints while Cluster 7 represented genes that were upregulated in warmed Castille timepoints (Figure 4.4A).

To understand the function and class of the genes in each cluster, I performed gene ontology (GO) term analysis. Cluster 7 contains genes upregulated in response to warming in Castille but not in Ningyou7. GO term analysis of this cluster revealed that this cluster is enriched for genes involved in the response to abscisic acid (GO:0009737), abscisic acid-activated signalling pathway (GO:0009738), cell communication (GO:0007154) and signal transduction (GO:0007165) ($P<0.00005$) (Figure 4.4C). This suggests that warming induces a response to ABA and enhances cell communication in Castille but not Ningyou7.

Meanwhile, Cluster 6 contains genes upregulated over the course of time in Ningyou7 alone. GO term analysis of this cluster revealed that the cluster is enriched for genes involved in reproductive process (GO:0022414), regulation of flower development (GO:0009910), positive regulation of transcription (GO:0045944) ($P<0.00010$) (Figure 4.4C). This indicates that over the course of time floral development progresses in Ningyou7 but not Castille.

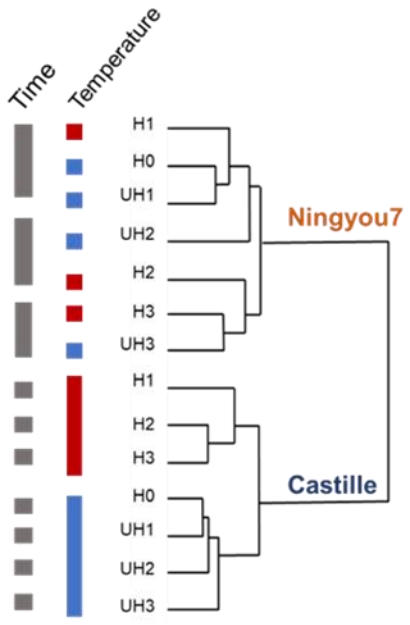
Taken together this indicates that warming induces ABA in Castille but promotes floral development in Ningyou7. As both Ningyou7 and Castille faced the same warming treatment this indicates that warming does not induce ABA as a stress response but induces ABA for another reason. Interestingly, ABA is a known dormancy regulator in perennial species. Furthermore, GO terms associated with ABA, cell communication and signal transduction have all been identified in transcriptomic experiments of perennial bud dormancy (Zhang *et al.,* 2018; Vimont *et al.,* 2019). Therefore, this provides evidence that Castille may be demonstrating bud dormancy, alongside evidence that Castille shows a flowering time delay in response to winter bud warming (Figure 4.1) which is another hallmark of dormancy.

A



Cluster Number	Cluster Behaviour (Gene Activity)
1	Upregulated in All Unheated samples
2	Upregulated in All Heated samples
3	Upregulated in Heated Ninyou7 samples
4	Upregulated in Early Ningyou7 samples
5	Upregulated across all Ningyou7 samples
6	Upregulated in Late Ningyou7 samples
7	Upregulated in Heated Castille samples
8	Upregulated in Unheated Castille samples
9	Upregulated across all Castille, more upregulation in Castille Heated samples
10	Upregulated across all Castille, more upregulation in Castille Unheated samples
11	Upregulated across all Castille samples

B



UH0: Unheated Before Treatment
UH1: Unheated 24 hours
UH2: Unheated 7 days
UH3: Unheated 14 days

H1: Warmed 24 hours
H2: Warmed 7 days
H3: Warmed 14 days

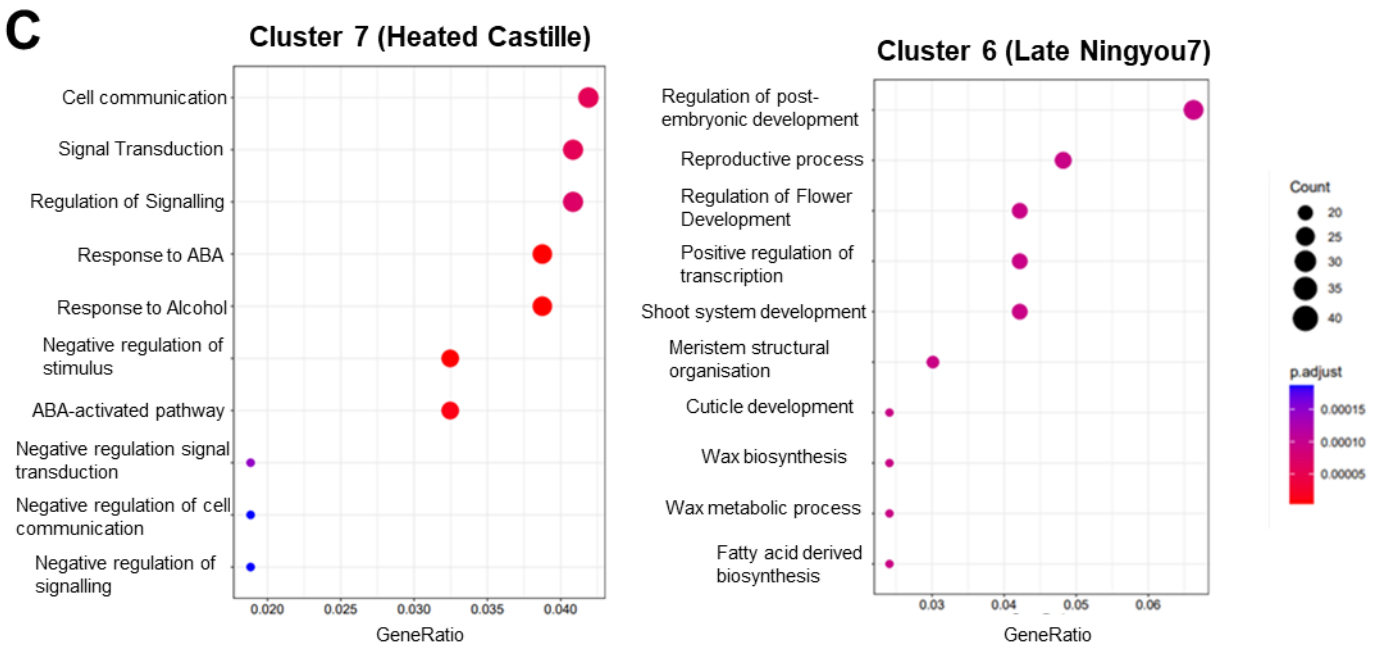


Figure 4.4 Hierarchical clustering of Castille and Ningyou7 warmer and control timepoints reveals floral development progresses in Ningyou7 but processes involved in floral delay, e.g. dormancy, may be active in Castille. A) Hierarchical clustering of timepoints separated into broad transcriptional categories of similar gene expression. Cluster behaviour indicates the behaviour of genes in each cluster. Mean of three replicates for each gene shown. B) Hierarchical clustering separates Ningyou7 timepoints according to time and Castille timepoints according to temperature. C) Gene ontology analysis of cluster 7 and cluster 6. Gene Ratio refers to the percentage of total Differentially Expressed genes (DEGs) in the given GO term.

To further understand how warming affects Castille and Ningyou7, I analysed gene expression patterns for individual genes in each Castille- and Ningyou7- associated cluster. To do this, I filtered cluster 6 and cluster 7 for specific genes that were upregulated over time in Castille in response to heating but were not in Ningyou7, showed a log fold change greater than 4 between varieties, and were associated with the GO terms identified from Figure 4.4C. The dormancy associated gene *DORMANCY-ASSOCIATED GENE 1* (*DRM1)*, ABA signalling genes *BRANCHED 1 (BRC1)* and *ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 3* (*ABF3)* and the ABA response gene *RD29* (Figure 4.5) increased in expression in response to warming in Castille but did not in Ningyou7. In hybrid aspen, *BRC1* and ABA biosynthesis genes are involved in bud dormancy control (Singh *et al.,* 2018). In addition, the callose synthase gene *GLUCAN-SYNTHASE-LIKE 11* (*GSL11)* was more highly expressed in Castille than Ningyou7 and expression was raised upon warmth in Castille (Figure 4.5). Callose has a well-established role in isolating dormant buds, however whether this callose

synthase is performing that function is not clear. The ABA signalling genes *HIGHLY-ABA-INDUCED 1* and *2* (*HAI1), (HAI2)* and *ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 2* (*ABF2)* were expressed in both Ningyou7 and Castille after warming. However, this upregulation was only maintained in Castille (Figure 4.5). This suggests these ABA signalling genes may either be upregulated following the initial temperature response in both varieties, or signal during a transient dormancy response in Ningyou7 which is then sustained in Castille. Taken together, the gene expression patterns here suggest that winter warming floral buds induces a well-known ABA-related bud dormancy module, which shares conserved function in woody perennials (Singh *et al.,* 2018).

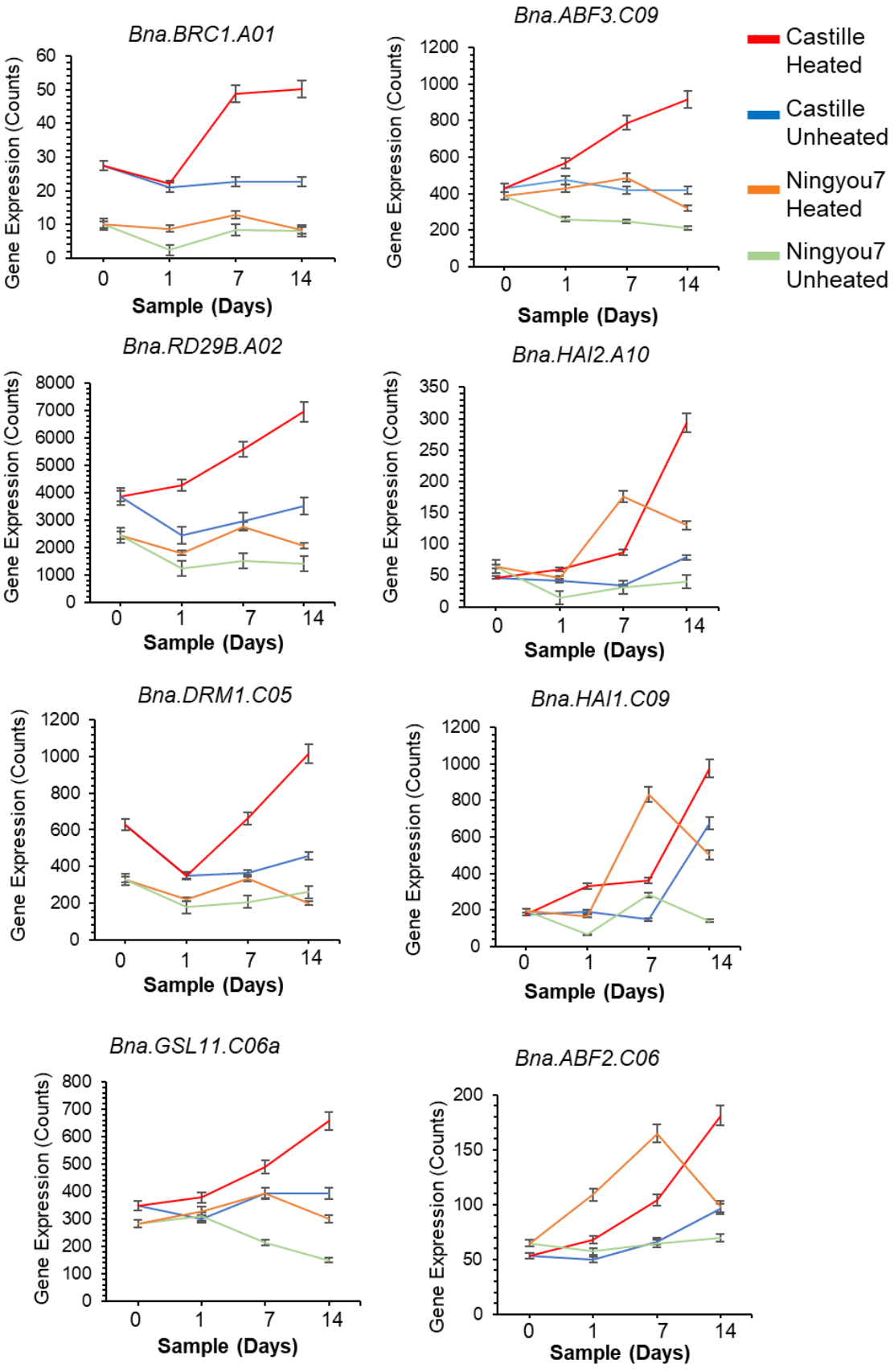

Bna.BRC1.A01
Bna.ABF3.C09
Bna.RD29B.A02
Bna.HAI2.A10
Bna.DRM1.C05
Bna.HAI1.C09
Bna.GSL11.C06a
Bna.ABF2.C06
Gene Expression (Counts)
Sample (Days)
Castille Heated
Castille Unheated
Ningyou7 Heated
Ningyou7 Unheated

Figure 4.5. Transcriptome analysis reveals that winter warming induces differential gene expression between Castille and Ningyou7 in key dormancy and abscisic acid genes. Expression responses of selected ABA-associated genes in Castille and Ningyou7 before treatment, after 1 day, 7 days and 14 days of treatment. Data show the mean ± SE of three biological replicates in normalised count data, normalised using DESeq2 (see Methods).

4.2.3 Ningyou7 is missing key *FLC* and *MAF* genes, which may drive the bud dormancy phenotype in Castille

Bud warming delays floral development in Castille but accelerates floral development in Ningyou7 (Figure 4.1). Alternatively, chilling promotes floral development in Castille but is not required in Ningyou7. To understand how warming prevents floral development in Castille, I compared the expression level of genes involved in the vernalisation pathway, a known controller of floral development during chilling, between Castille and Ningyou7, using normalised FPKM data. To analyse the response to warming, I calculated the log fold change between the mean gene expression of three control and three warmer biological replicates at each time point. I then compared the overall trends of gene expression in Castille and Ningyou7. The varieties were considered to have different expression patterns if the overall trends in gene expression in response to heating were inconsistent (e.g., one gene upregulated in one variety but downregulated in another in response to heating) or varied in magnitude (e.g., one gene upregulated in one variety at least >0.5 log fold change over time greater than the same gene in another variety).

Most flowering-associated genes show similar expression patterns over time in response to warming between Castille and Ningyou7 but a few floral genes showed different expression patterns between Castille and Ningyou7 (Figure 4.6): these include almost all *Bna.AP1* genes and one *Bna.SOC1* gene (Figure 4.6). *APETALA1 (AP1)* genes were on average 0.7x more highly expressed over time and *Bna.SOC1.A03* was 0.74x more highly expressed over time in Castille than Ningyou7. *AP1* and *SUPPRESOR OF CONSTANS 1 (SOC1)* are active in early floral development before AP1 is restricted to specific floral whorls and *SOC1* is downregulated later in floral development (Gregis *et al.,* 2006; Gregis *et al.,* 2009; Dorca-Forenll *et al.,* 2011). This suggests that Castille may be at an earlier stage of floral development than Ningyou7, this matches what is seen physiologically (Figure 4.1). However, the general trend for floral gene expression is that the up and downregulation of floral pathway genes is similar between Castille and Ningyou7. Therefore, floral pathway genes are not likely candidates for the bud dormancy phenotype.

However, key genes in the vernalisation pathway were either not expressed or missing in Ningyou7, including *FLOWERING LOCUS C (FLC)* and *MADS-AFFECTING FLOWERING (MAF*) genes. In Ningyou7, *Bna.FLC.C02, Bna.FLC.C03a, Bna.FLC.C03b, Bna.MAF4.A02a, Bna.MAF4.A02b* and *Bna.MAF3.A02* were not expressed in any sample and no reads were registered in RNA-seq analysis suggesting that these genes may be deleted in Ningyou7 rather than that they were downregulated prior to the floral transition (Figure 4.6A; Figure 4.6B; Figure 4.7). In Castille expression of most known *B. napus FLC* genes were detected, except for *Bna.FLC.C02,* suggesting it may be deleted in Castille (Figure 4.6B). This suggests that the absence of *FLC* and *MAF* genes in Ningyou7 may be candidates for the cause of the difference in bud dormancy response between the two varieties.

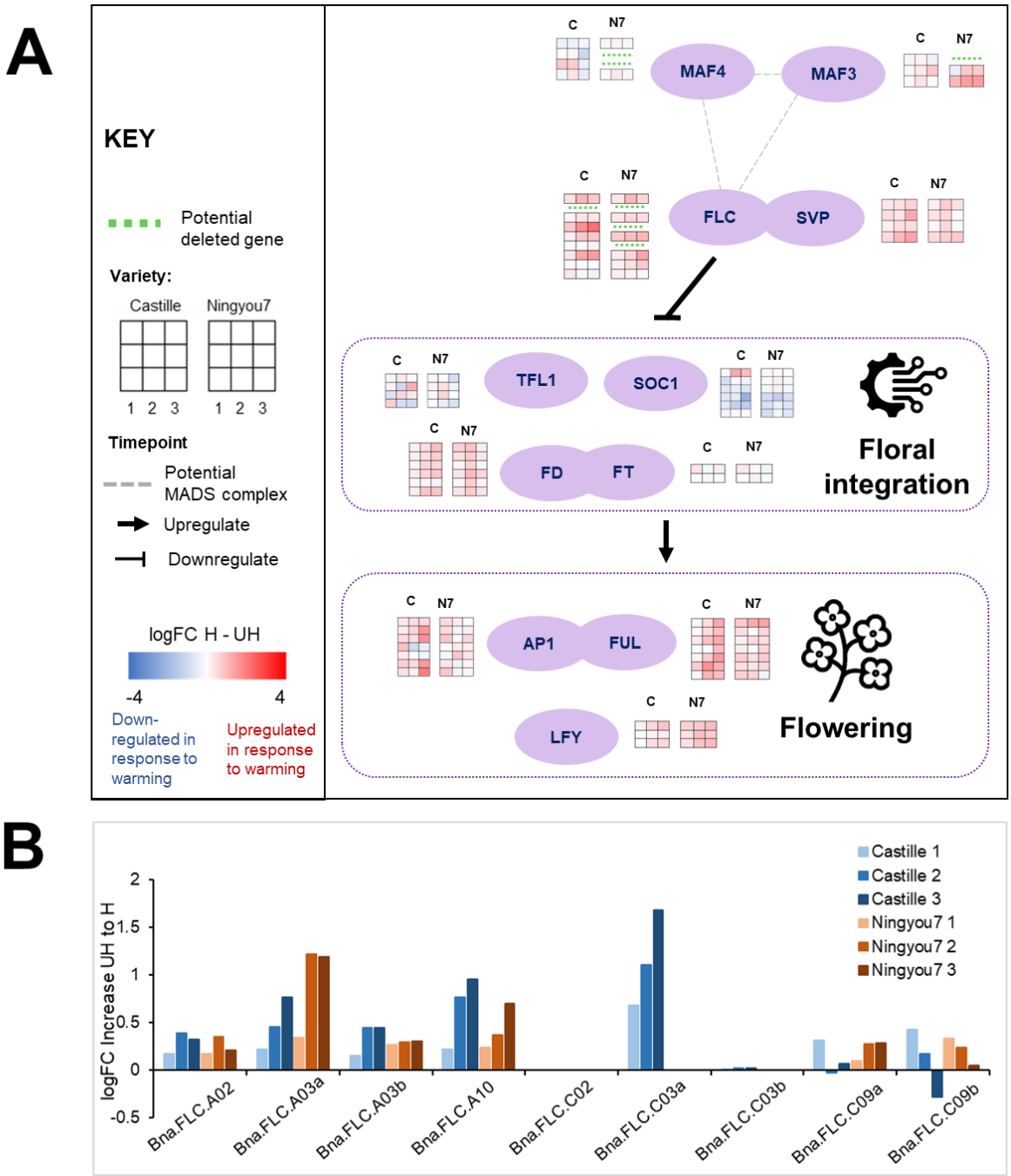


Figure 4.6 Vernalisation and key floral pathway transcription factor expression across time between Castille and Ningyou7. A) RNA sequencing (RNA-seq) results showing upregulation of vernalisation and floral pathway components after winter warming. Coloured boxes indicate log fold change between warmed and control plants, with each vertical box indicating a different gene copy of *Brassica napus*, such that red indicates upregulation and blue downregulation in response to warming. The total log fold change over time for warmer minus the control experiment, so genes in blue represent genes that were downregulated by warming over time and genes in red indicate genes that were upregulated by warming over time. B) Mean log fold changes between Castille and Ningyou7 for the nine *FLC* genes of count data normalised using DESeq2 (see Methods).

To determine whether other oilseed rape varieties have missing *FLC* and *MAF* genes, and how general this finding is, I analysed sequence data from five WOSR varieties known to demonstrate bud dormancy, and three SWOSR varieties that don't demonstrate bud dormancy. I used apex transcriptomic experiments that I or other members of the Penfield laboratory had generated for experiments that are not presented in this thesis (unpublished data, Samuel Warner, Becca Doherty). Transcriptomic data was aligned, quantified, and analysed as before (Methods section 2.7). In three SWOSR varieties transcripts for all three *MAF* genes on chromosome A02 are missing (*Bna.MAF3.A02, Bna.MAF.A02a, Bna.MAF4.A02b*) and *Bna.FLC.C03b, Bna.FLC.C03a* are also missing in all SWOSR lines. *Bna.FLC.C02* was present in one SWOSR, Zhongshuang11, but one WOSR, Castille, had transcripts for *Bna.FLC.C02* missing (Figure 4.7). This suggests that the absence of some *FLC* (*Bna.FLC.C03b* and *Bna.FLC.C03a*) and *MAF* (*Bna.MAF3.A02, Bna.MAF.A02a, Bna.MAF4.A02b)* transcripts across SWOSR could explain why the crop type lacks bud dormancy (Chapter 3; Figure 3.4) but shows that the lack of *Bna.FLC.C02* alone is not enough to cause bud dormancy loss. This is consistent with findings that suggested *Bna.FLC* genes on chromosome C3 in the SWOSR variety Zhongshuang11 would be pseudogenized due to a premature stop-codon (Chalhoub *et al.,* 2014; Sun *et al.,* 2017). Here I confirm that a common route to lower winter chilling requirement in SWOSR is through loss or mis-expression of *Bna.FLC* copies. Furthermore, in *A. thaliana MADS AFFECTING FLOWERING* (*MAF*) genes are clustered in a 22-kb tandem repeat region (Ratcliffe *et al.,* 2003). This suggests that the entire tandem repeat region of *MAF* genes on chromosome A02 is deleted in SWOSR varieties. However, more extensive analysis across other varieties is needed to determine how general this result is, especially for the importance of *Bna.FLC.C02* in other WOSR varieties.

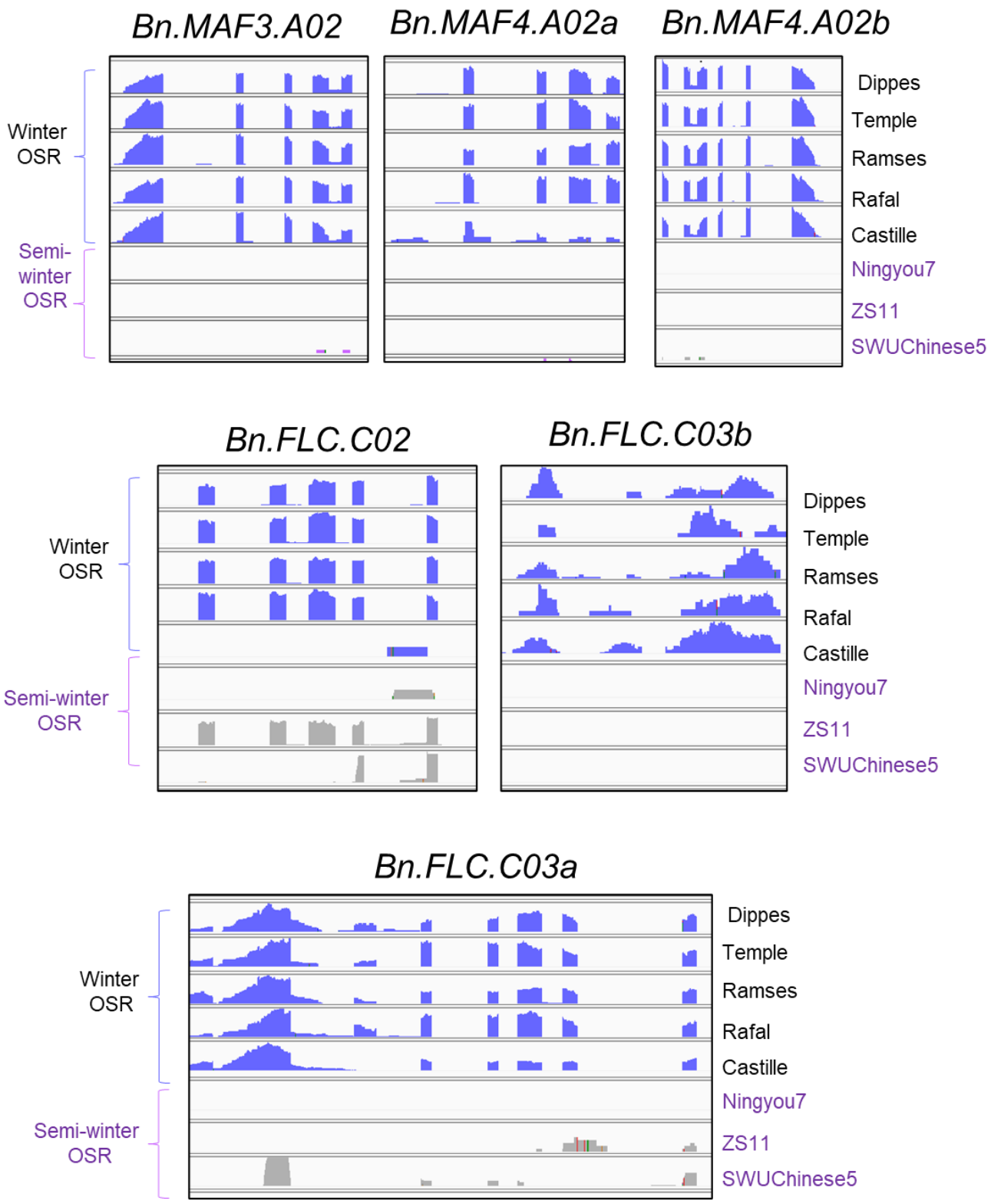


Figure 4.7. Presence or absence of *MAF* and *FLC* copies is common across crop types. Images from IGV Genome Browser using readily available transcriptomic sequence data from floral apices across past experiments. Short read sequences aligned against Darmor V4 Reference Genome. Blue

indicates WOSR where there are reads in the gene, grey indicates SWOSR. Where there are lines on the reads, red represents a single nucleotide polymorphism from Cytosine → Thymine and a green line represents a single nucleotide polymorphism from Guanine → Alanine.

4.2.4 The activity of FLC and its downstream targets may drive the difference in bud dormancy response.

To understand whether the missing *FLC* genes in Ningyou7 are responsible for the lack of a winter chilling requirement, I analysed whether the expression of known *FLC* targets in Arabidopsis differed between their homologs in Ningyou7 and Castille flower buds. I hypothesised that missing *FLC* genes in Ningyou7 may result in mis-expression downstream targets, depending on whether *FLC* functions as an activator or repressor of these genes.

I identified 2587 putative genes in *B. napus* that were homologous to the 615 known *FLC* gene targets identified in *A. thaliana* (Mateos *et al.,* 2015). I used a chi-squared test to compare between observed *FLC* targets and the expected number based on even distribution of 2587 targets across the whole *B. napus* genome in clusters from Figure 4.4A. I determined that *FLC* gene targets were overrepresented in clusters 2, 3, 5, 6, 7 and 8 (Table 4.1). These clusters broadly correspond to upregulation of genes involved in the warm temperature response in Castille, and the effect of time in Ningyou7 but not Castille. Over-representation of *FLC* targets in these clusters suggests that the biological processes corresponding to these clusters are regulated by *FLC.*

Table 4.1. Clusters of gene expression patterns in Ningyou7 and Castille and their corresponding overrepresentation analysis of putative *FLC* targets. Where clusters show overrepresention for more putative *FLC* targets then that cluster has more putative *FLC* targets than expected based a genome-wide distribution.

Cluster	Cluster Behaviour	Observed FLC targets in cluster	Expected FLC Targets	Over or under-represented	Chi-squared	P-value
1	Genes that are Upregulated in Control	20	14.54	Over (ns)	2.1134	0.146
2	Genes that are upregulated in Warmer	72	16.1	Over	200.3423	<0.00001
3	Genes that are upregulated in Warmer Ningyou7 timepoints	51	11.85	Over	133.27	<0.00001
4	Genes that are upregulated in Early Ningyou7 timepoints	8	8.04	Under (ns)	0.0002	0.989
5	Genes that are upregulated in all Ningyou7 timepoints	56	35.82	Over	11.832	0.00582
6	Genes that are upregulated in late Ningyou7 timepoints	14	5.1	Over	16.0036	0.000063
7	Genes that are upregulated in Warmer Castille timepoints	89	28.65	Over	131.921	<0.00001
8	Genes that are upregulated in Control Castille timepoints	41	13.77	Over	55.512	0.00001
9	Genes that are upregulated	33	27.58	Over (ns)	1.107	0.293

	across all Castille timepoints, more upregulated in warmer Castille timepoints					
10	Genes that are upregulated in all Castille timepoints, more upregulated in control Castille timepoints	22	22.74	Under (ns)	0.0247	0.875
11	Upregulated equally across all Castille timepoints	17	23.58	Under (ns)	1.9022	0.168

Next, I performed GO term analysis on the *FLC* target genes in each cluster. To understand how the absence of *FLC* genes in Ningyou7 may influence the presence or absence of bud dormancy I focussed on the clusters that represent the main transcriptional differences between Castille and Ningyou7, as identified previously. In cluster 7 (Genes that are upregulated in Warmed Castille timepoints), cellular response to abscisic acid (GO:0009737), abscisic acid-activated signalling pathway (GO:0006351) were overrepresented among putative *FLC* targets alongside several signalling associated GO terms (Figure 4.8). Whilst in cluster 6 (Genes that are upregulated in late Ningyou7 timepoints), *FLC* targets associated with floral development (GO:0048437), meristem structural organisation (GO:0009933), multicellular organismal reproductive process (GO:0032504) were overrepresented (Figure 4.8). This indicates there are clear differences in *FLC* gene target expression between Castille and Ningyou7. This implies the absence of *FLC* copies in Ningyou7 could prevent regulation of *FLC* gene targets responsible for initiating bud dormancy in response to warming, whilst causing up-regulation of *FLC*-activated gene targets involved in floral development that are otherwise repressed by warming in Castille.

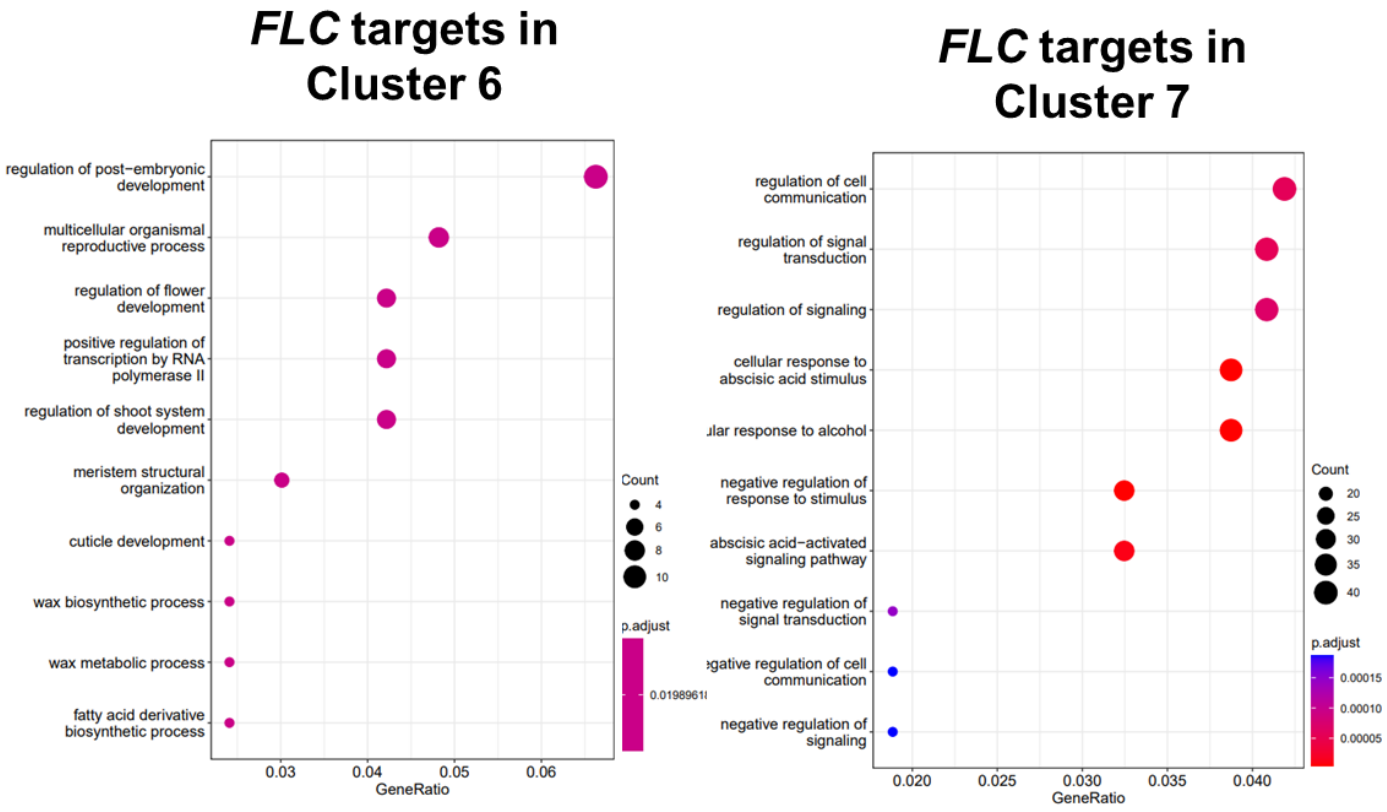


Figure 4.8. GO term analysis of putative *FLC* target genes shows *FLC* targets in Castille are enriched in ABA-associated dormancy module following bud warming while *FLC* targets in Ningyou7 are enriched in floral developmental processes. Figure represents the GeneRatio, adjusted p-value and count of every gene for each respective GO term, according to the *PANTHER* database of GO terms.

To confirm this, I determined which putative *FLC* gene targets are in cluster 7 (Upregulated in Warmer Castille) or 6 (Upregulated in Late Ningyou7). There were only a small number of *FLC* targets present exclusively in either of the clusters. *FLC* targets uniquely present in warmed Castille samples included genes associated with dormancy, abscisic acid, DNA-templated transcription, embryo development and flowering (Table 4.2). However, the predicted regulation by *FLC* based on Mateos *et al.* (2015) identified that some genes with similar functions were upregulated whilst some were downregulated, e.g. dormancy associated *DRM1* was upregulated whilst *BG2* was downregulated.

By contrast, in cluster 6 (Upregulated in Late Ningyou7) unique *FLC* target genes include genes involved in reproductive transitions, floral repression, transcriptional control, floral organ development and ABA catabolism. Every gene in each of these processes is predicted to be downregulated by *FLC* (Mateos *et al.,* 2015): e.g. *AGL15, SOC1, TPS1, HY5* and *CYP707A*. This suggests that the lower *FLC* activity in Ningyou7 permits expression of genes with diverse

roles in either floral development or growth promotion. While in Castille, higher *FLC* activity permits expression of genes associated with bud dormancy processes.

Table 4.2 Unique *FLC* targets that only appear in clusters 6 and 7 of hierarchical clustering indicate following heating in Castille genes associated with dormancy are upregulated but in Late Ningyou7 clusters genes to do with floral development are upregulated. Genes taken from analysis performed by Mateos *et al.* (2015)

Gene Clusters	**Associated Process**	**Unique *FLC* Targets (identified from Mateos *et al.*, 2015)**	**Up or downregulated by *FLC* (data from Mateos *et al.*, 2015)?**
Warmer and Castille	Dormancy associated	*DRM1* (AT1G28330), dormancy associated protein; *BG2* (AT3G57260)	*DRM1* Upregulated, *BG2* downregulated
	Abscisic acid associated	*RHA2A* (AT1G15100); *AN1-like* (AT2G36320); *DEAR2* (AT5G67190); *DIV2* (AT5G04760)	*DEAR2*, involved in negative regulation of cell division and abscisic acid, downregulated
	Regulation of DNA-templated transcription	*MYB52* (AT1G17950), AT1G28050, AT1G79060, AT3G15210, Galactose oxidase (AT3G59940), *TINY2* (AT5G11590)	*TINY2* upregulated
	Embryo development, fruit development, ovule development	*GRP2B* (AT2G21060), *ARL* (AT2G44080), *VDD* (AT5G18000), (AT5G18670)	
	Regulation of flowering	*VEL1* (AT4G30200)	
	Regulated by PIF4	*AT5G65920*	

Up in Ningyou7, focussing on Late Ningyou7	Involved in reproductive transitions: vegetative to reproductive and flowering	AT1G66330, *AGL15* (AT5G13790), *AT1G75390*,	*AGL15* downregulated, implicated in delaying transition to flowering;
	Floral repressors	*SOC1* (AT2G45660), AGL19 (AT4G22950)	*SOC1* downregulated
	Cell-growth, transcription control	*TPS1* (AT1G78580)	Downregulated
	Floral organ development, meiosis and fertility	*DTX35* (AT4G25640), *AP2-like* (AT5G57720), *ARF2* (AT5G62000)	
	ABA Catabolism and Signalling	*CYP707A* (AT5G45340), *HY5* (AT5G13790)	*HY5* upregulated and *CYP707A* downregulated

4.3 Identifying loci of interest involved in the bud dormancy response.

4.3.1 *Plant Homologous to PARAFIBROMIN (PHP)* is associated the effect of warming on floral development

Chapter 3 showed there is variation in bud dormancy activation in warm winters between *B. napus* varieties (Figure 3.4). I have also shown that there is clearly divergent transcriptomic responses to warming between a variety with bud dormancy, Castille, and one without, Ningyou7 (Figure 4.4). To further understand the genetic basis of bud dormancy, I used the results from the large-scale winter warming experiment from Chapter 3 as the basis of GWAS analysis here (Methods section 2.1).

I conducted Genome Wide Association Studies (GWAS) on traits associated with warmth induced floral delay, which is associated with the presence of bud dormancy (Table 4.3). The markers used in GWAS were generated by Harper *et al.* (2012) and represent variation in gene sequences such as Single Nucleotide Polymorphisms (SNPs). To perform the GWAS, I used the GEM and GWAS Automation Pipeline (GAGA), which uses GAPIT3 to perform GWAS (Github; Nichols, Wells, Morris et al unpublished data). In all GWAS here, the BLINK model was used. To ensure BLINK can perform optimally, the GAGA pipeline does not apply a minor allele frequency cut off, otherwise significant traits can be missed. Therefore, I manually applied a minor allele cut-off of 0.05 after analysis was run, so that a trait was only considered significant is the minor allele appeared in more than 5% of the population.

To assess the impact of winter warming on floral development, I conducted several GWAS on trait scores that incorporated data from both the warmed and control conditions, to assess the effect of winter warming on floral development (Table 4.3; Supplementary Figure 1). Across all traits considered, the '*Effect of warming on time to BBCH60 (Percentage Difference in Thermal Time to BBCH60 between Control and Warmer Conditions)'* and the '*Effect of warming on time to BBCH60 (Ratio of Thermal Time to BBCH60 between Control and Warmer Conditions)'* were the only traits where trait scores were significantly associated with any markers, both traits investigate the effect of temperature on time to BBCH60. This significance was determined by a p-value less than 0.05, FDR of 5% and each marker surpassing the minor allele frequency cut off 0.05 (Table 4.3; Supplementary Figure 1).

Table 4.3. Traits associated with bud dormancy that were analysed using GWAS. Markers were assessed as significant if they surpassed p value < 0.05, FDR, (P<0.05) and were higher than minor allele frequency (MAF ≤0.05). Each trait represents the effect of warming on floral development. The calculation for each trait is in brackets by the trait. E.g., the percentage difference trait was calculated by subtracting time in warmer conditions (H) from time in control conditions (UH) and then dividing it by time in control conditions, so a positive trait score indicates a longer time to reach BBCH51 under warmer compared to control conditions, whilst a negative score indicates the opposite.

Trait (All assess the effect of warming on floral development)	Significant markers surpassing minor allele frequency cut off 0.05?
Percentage difference in thermal time to BBCH60 between control and warmer conditions (UH – H/ UH)	YES – ONE
Ratio of thermal time to BBCH60 under control conditions compared to warmer conditions (UH ÷ H)	YES – ONE
Percentage difference in thermal time to BBCH51 between control and warmer conditions (UH – H/ UH)	None
Ratio of thermal time to BBCH51 under control conditions compared to warmer conditions (UH ÷ H)	None
Percentage difference in days to BBCH51 between control and warmer conditions (UH – H/ UH)	None
Ratio of days to BBCH51 under control conditions compared to warmer conditions (UH ÷ H)	None
Ratio of days to BBCH60 under control conditions compared to warmer conditions (UH ÷ H)	None
Percentage difference in days to BBCH60 between control and warmer conditions (UH – H/ UH)	None
Ratio of days between BBCH51 to BBCH60 under control conditions compared to warmer conditions (UH ÷ H)	None

Percentage difference in days between BBCH51 to BBCH60 between control and warmer conditions (UH – H/ UH)	None
Difference in thermal time to BBCH60 between control and warmer conditions (UH – H)	None
Difference in thermal time to BBCH51 between control and warmer conditions (UH – H)	None
Difference in time (days) to BBCH60 between control and warmer conditions (UH – H)	None
Difference in time (days) to BBCH51 between control and warmer conditions (UH – H)	None
Time (days) to BBCH51 for warmer only	None
Time (days) to BBCH60 for warmer only	None

Cab041204.2:750:T was the only marker that was significantly associated with both traits (Table 4.4; Figure 4.9). Cab041204.2:750:T is at position 750 in the first exon of *BnaA05g17020D* on chromosome A05. *BnaA05g17020D* shares 83.4% sequence similarity with *A.thaliana AT3G22590* encoding the Plant Homologous to Parafibromin (PHP) protein, a component of the Paf1 Complex (Paf1c). *PHP* upregulates *FLC* prior to vernalisation and delays the phase transition from vegetative to reproductive growth in Arabidopsis (He *et al.,* 2004; Yu and Michaels*,* 2010; Park *et al.,* 2010). This suggests that the effect of winter warming on flowering time is affected by a known regulator of *FLC*, *PHP* in *B. napus.* This relationship is investigated in depth in Chapter 5.

Table 4.4. Genome-wide association (GWA) markers for traits associated with the effect of warming on time to BBCH60 in *B. napus*. Significant *SNP* markers identified using where p-value < 0.05, Adjusted False Discovery rate greater than 5% and minor allele frequencies greater than 0.05.

Trait	Significant markers surpassing minor allele frequency cut off 0.05	P-value	FDR	Chromosome and Position	Arabidopsis homolog
Percentage difference in thermal time to BBCH60 between control and warmer conditions (UH – H/ UH)	Cab041204.2:750:T	1.55E-09	0.00027522	A05: 18672633	AT3G22590
Ratio of thermal time to BBCH60 under control conditions compared to warmer conditions (UH ÷ H)	Cab041204.2:750:T	1.55E-09	0.00027522	A05: 18672633	AT3G22590

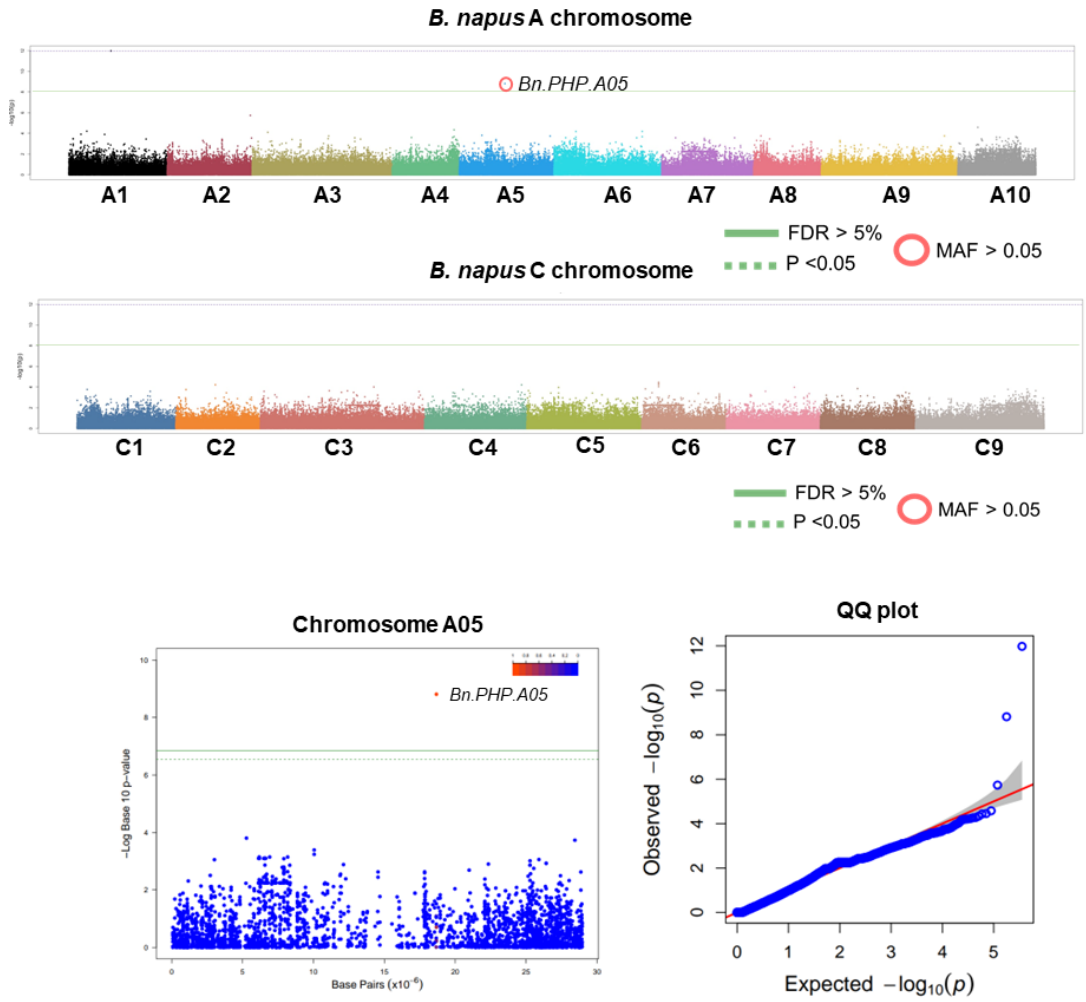


Figure 4.9. The Cab041204.2:750:T marker from *BnaA05g17020D* is significantly associated with the effect of warming on thermal time to BBCH60. Manhattan plot for A and C genomes of *Brassica napus* generated by GAPIT via GAGA pipeline using the best fitting GWAS model (BLINK here) for the combined trait *'Ratio of Thermal Time to BBCH60 between control conditions to warmer conditions'* for plants grown in the winter warming experiment from Chapter 3. GWAS model ran using GAGA pipeline (Methods section 2.16.1). Solid green line shows the Benjamini-Hochberg False Discovery Rate (FDR) of α =0.05, dotted green line shows p-value significance at α =0.05. QQ plot presented. Red circle indicates marker where the minor allele frequency (MAF) surpassed 0.05.

4.3.2 Expression of *Bna.FLC.C02* is associated with bud dormancy.

To further understand the genetic control of bud dormancy I investigated whether there was any correlation in gene expression and traits associated with warmth induced bud dormancy, using gene expression data for 21-day old seedlings generated by Harper *et al.* (2012). I used the same traits as analysed for GWAS (Table 4.3). I performed gene expression marker (GEM) analysis using the GAGA pipeline. However, when calculating the significance threshold, the GEM algorithm in the GAGA pipeline fails to account for the number of gene markers, this means on average there are 500-2000 significant GEMs per trait after accounting for FDR ($P<0.05$). Therefore, to isolate only the most significant GEMs I used the Bonferroni method to adjust the p-value:

$$\alpha_B = \alpha/m$$

Where α is the original p-value cut-off of 0.05, α_B is the adjusted p-value and *m* is the number of markers (117,784) used in the GEM analysis. The α_B is $4.24xE^{-7}$.

Using this cut-off, there were six traits that had significant GEMs (Table 4.5; Supplementary Figure 2). I identified two genes with known roles in flowering time control in GEM analysis: *Bna.FLC.C02* and the *LIKE HETEROCHROMATIN PROTEIN 1* (LHP1) on the C9 chromosome of *B. napus* (*BnLHP1.C09)* (Table 4.5; Figure 4.10). All other genes identified across traits were uncharacterised with no known *A. thaliana* homolog or had known *A. thaliana* homologs but unknown biological or molecular function. Due to time constraints, I focussed analysis on genes with known functions related to floral or bud development. GO term analysis identified there was no enrichment for specific classes of genes in all traits except enrichment for cellular processes in '*Ratio of Control Time to BBCH51 to Warmer Time to BBCH51'* (Table 4.5).

Table 4.5. Gene Expression Marker (GEM) analysis on traits associated with warmth induced bud dormancy identify *Bna.FLC.C02* and *Bna.LHP1.C09* as significantly associated with the effect of warming on flowering time. Total number of GEMs identified that surpassed the adjusted p-value (α_B, $4.24xE^{-7}$, Bonferroni) threshold, alongside GO term enrichment for those genes and any genes that were associated with developmental processes.

Trait	Number of genes after Bonferroni correction	GO term enrichment	Associated genes in top 30

Ratio of Control Time to BBCH51 to Warmer Time to BBCH51	286	Cellular process (GO:0009987)	
Ratio of Control Thermal Time to BBCH51 to Warmer Thermal Time to BBCH51	172	None	
Ratio of Control Time to BBCH60 to Warmer Time to BBCH60	47	None	*Bna.LHP1.C09*
Difference in time (days) to BBCH51 between control and warmer conditions (UH – H)	180	None	*Bna.FLC.C02*
Ratio of Control Thermal Time to BBCH60 to Warmer Thermal Time to BBCH60	6	None	
Ratio of Time between BBCH51 and BBCH60 in Control to Time between BBCH51 and BBCH60 in Warmer	5	None	
Difference in thermal time to BBCH60 between	0	None	

control and warmer conditions (UH – H)			
Difference in thermal time to BBCH51 between control and warmer conditions (UH – H)	0	None	
Difference in time (days) to BBCH60 between control and warmer conditions (UH – H)	0	None	

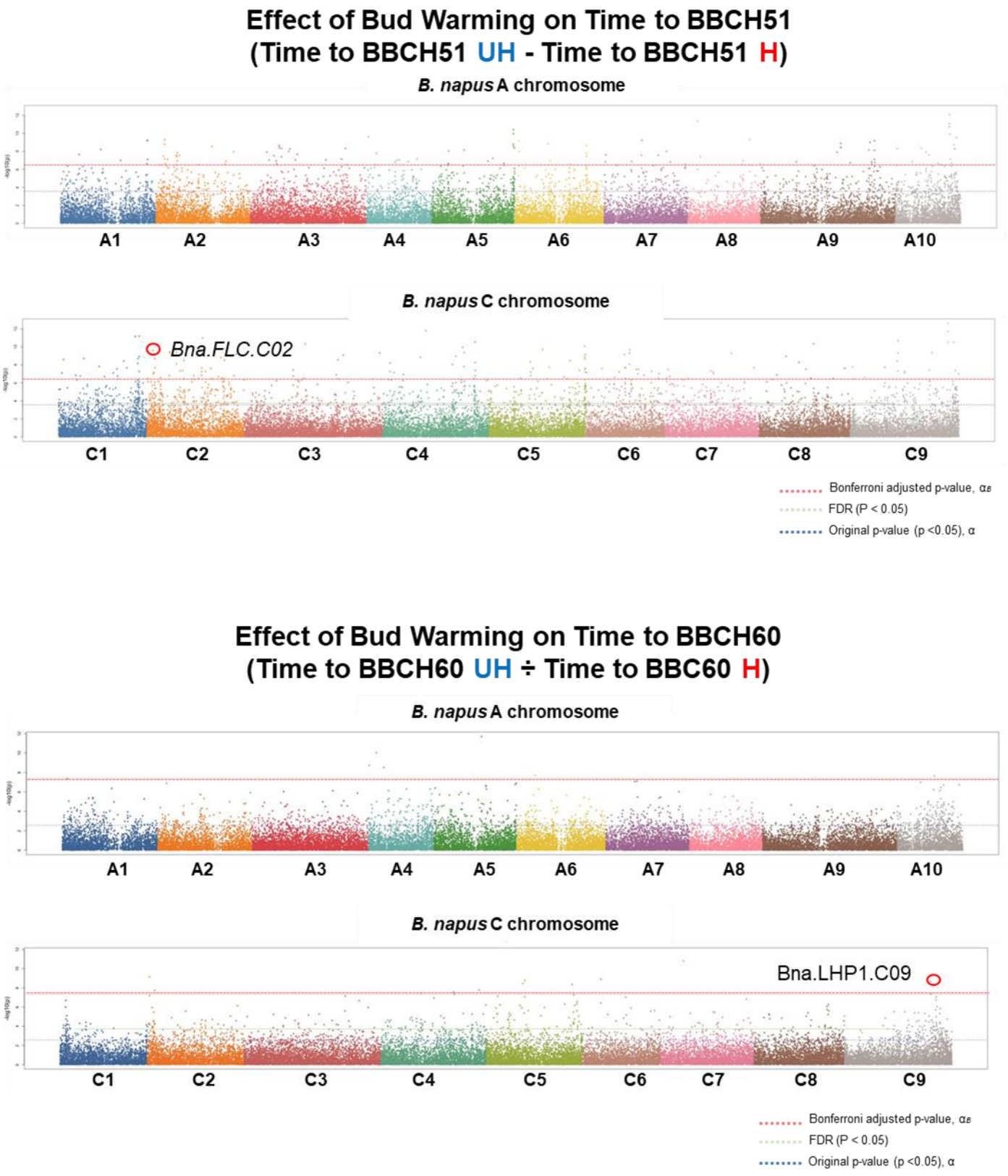


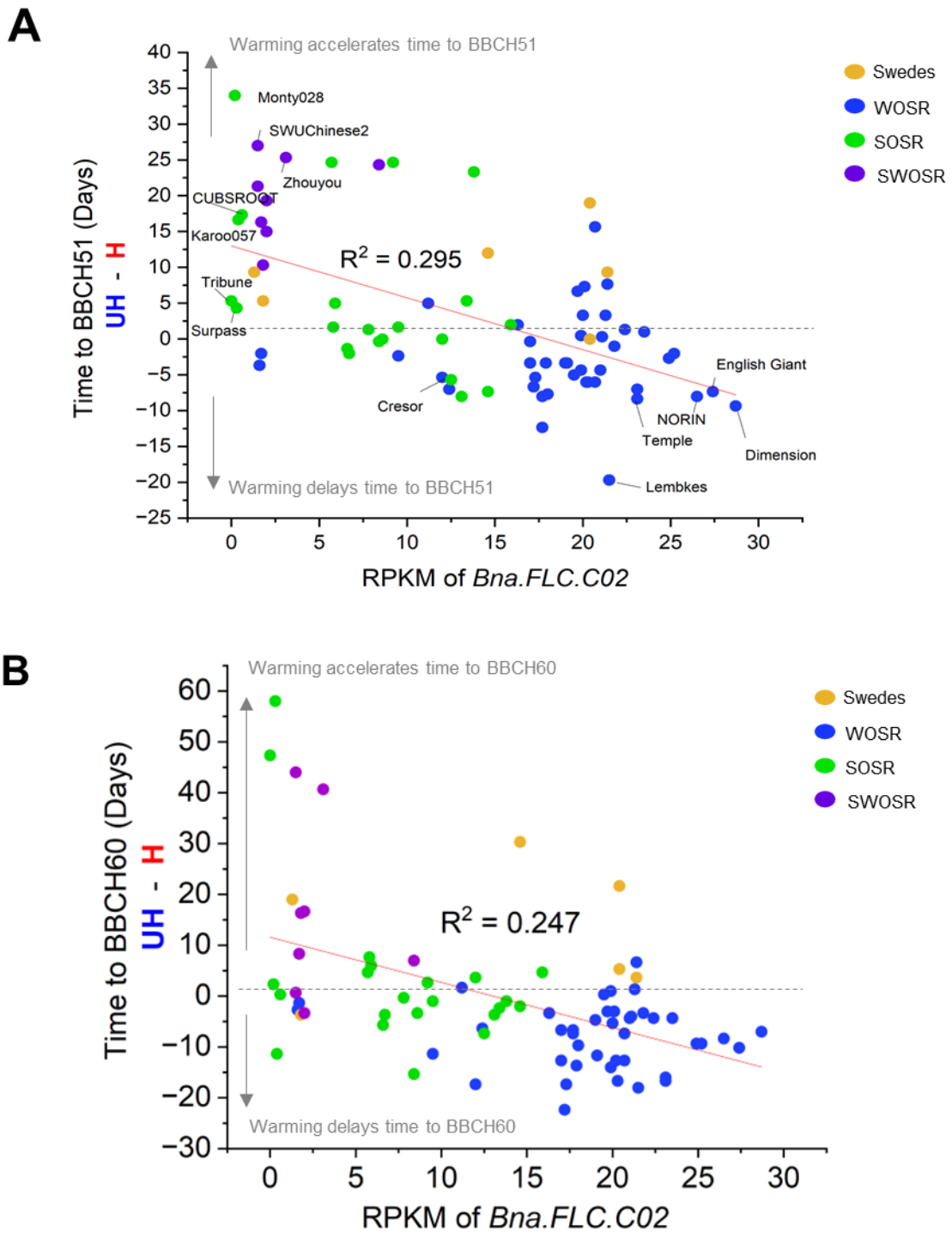
Figure 4.10. Gene expression markers (GEMs) associated with the effect of warming on floral development; expression of the genes highlighted is highly correlated (p < 4.24xE-7) with the effect of bud warming on floral development (BBCH51 and BBCH60). Genes of interest (associated with developmental processes) highlighted in red circles and labelled. Chromosomes indicated for *B. napus.* Cut off thresholds indicated in the key.

To understand the effect of the genes identified from GEM analysis I determined how their expression correlated with traits of interest (Figure 4.11; Figure 4.12). The expression of *Bna.FLC.C02* is significantly associated with the effect of bud warming on time to BBCH51, (Figure 4.11A). The expression of *Bna.FLC.C02* correlates with the effect of warming on time to BBCH51 (R^2 = 0.295), BBCH60 (R^2 = 0.247), as well as thermal time to BBCH51 (R^2 = 0.388) and BBCH60 (R^2 = 0.192). This correlation suggests that varieties where bud warming delayed time to BBCH51 and BBCH60 had higher *Bna.FLC.C02* expression and varieties where warming accelerated time to BBCH51 and BBCH60 had lower expression (Figure 4.11A-B). This also suggests that varieties with a greater thermal time requirement to BBCH51 and BBCH60 also had higher *BnaFLC.C02* expression (Figure 4.11C-D). Overall, this indicates that the 21-day leaf expression level of *BnaFLC.C02* is a good indicator of floral development following winter warming and that *BnaFLC.C02* is a candidate for the effect of post-floral initiation warmth on flowering time. Interestingly *Bna.FLC.C02* is silenced by chilling only after the floral transition (O'Neill et al., 2019). This suggests *Bna.FLC.C02* is a strong candidate for the genetic control of bud dormancy, as it is expressed when bud dormancy occurs and it's expression is correlated with many traits associated with bud dormancy.

I also identified that the expression of *Bna.LHP1.C09* was significantly associated with the effect of temperature on time to BBCH60 (ratio of time to BBCH60) (Figure 4.12). *Bna.LHP1.C09* expression positively correlated with the ratio of time (days) to BBCH60 (Figure 4.12), such that greater expression of *Bna.LHP1.C09* is weakly correlated with the extent that warming delays flowering (R^2 = 0.351). In *A. thaliana, LHP1* is necessary for the maintenance of the vernalised state (Mylne *et al.,* 2006) suggesting that higher *LHP1* expression could be associated with earlier vernalisation and flowering. In our diversity set there are four varieties with low *Bna.LHP1.C09* expression (Shengliyoucai, SWUChinese1, Tribune and Surpass): if these varieties are removed from the analysis the correlation between expression and the effect of warming on time to BBCH60 disappears (R^2 = 0.04; Figure 4.12B). This suggests that low expression of *BnLHP1.C09* may affect the time to BBCH60 in these varieties alone as *LHP1* is known to affect the silencing rate of *FLC* in *A. thaliana* (Mylne *et al.,* 2006).

Table 4.6. Gene expression markers (GEMs) associated with the effect of warming on floral development; expression of the genes listed is highly correlated ($p < 4.24xE-7$) with the effect of bud warming on floral development (BBCH51 and BBCH60). Chromosome location of each gene given. P value based on Manhattan plot analysis performed in GAGA pipeline; False Discovery Rate (FDR) = 0.001. A. thaliana homolog is the Arabidopsis Information Resource gene ID.

Trait	*B. napus* gene	*A. thaliana homolog*	Chromosome position	*P* value	Q value	FDR value
Ratio of Control Time to BBCH60 to Warmer Time to BBCH60	BnaC09g40240D (*Bna.LHP1.C09*)	AT5G17690.1 (LHP1/TFL2)	C09_ 050174208_ 050176135	3.41E-08	4.89E-05	1.07E-04
Ratio of Control Time to BBCH51 to Warmer Time to BBCH51	BnaC02g00490D (*Bna.FLC.C02*)	AT5G10140.4 (FLC)	C02_ 002415852_ 002415454.001	1.52E-08	6.08E-06	9.61E-06


A
Warming accelerates time to BBCH51
Warming delays time to BBCH51
Time to BBCH51 (Days)
UH - H
RPKM of Bna.FLC.C02
R2 = 0.295
Monty028
SWUChinese2
Zhouyou
CUBSROOT
Karoo057
Tribune
Surpass
Cresor
English Giant
NORIN
Temple
Dimension
Lembkes
Swedes
WOSR
SOSR
SWOSR
B
Warming accelerates time to BBCH60
Warming delays time to BBCH60
Time to BBCH60 (Days)
UH - H
RPKM of Bna.FLC.C02
R2 = 0.247
Swedes
WOSR
SOSR
SWOSR

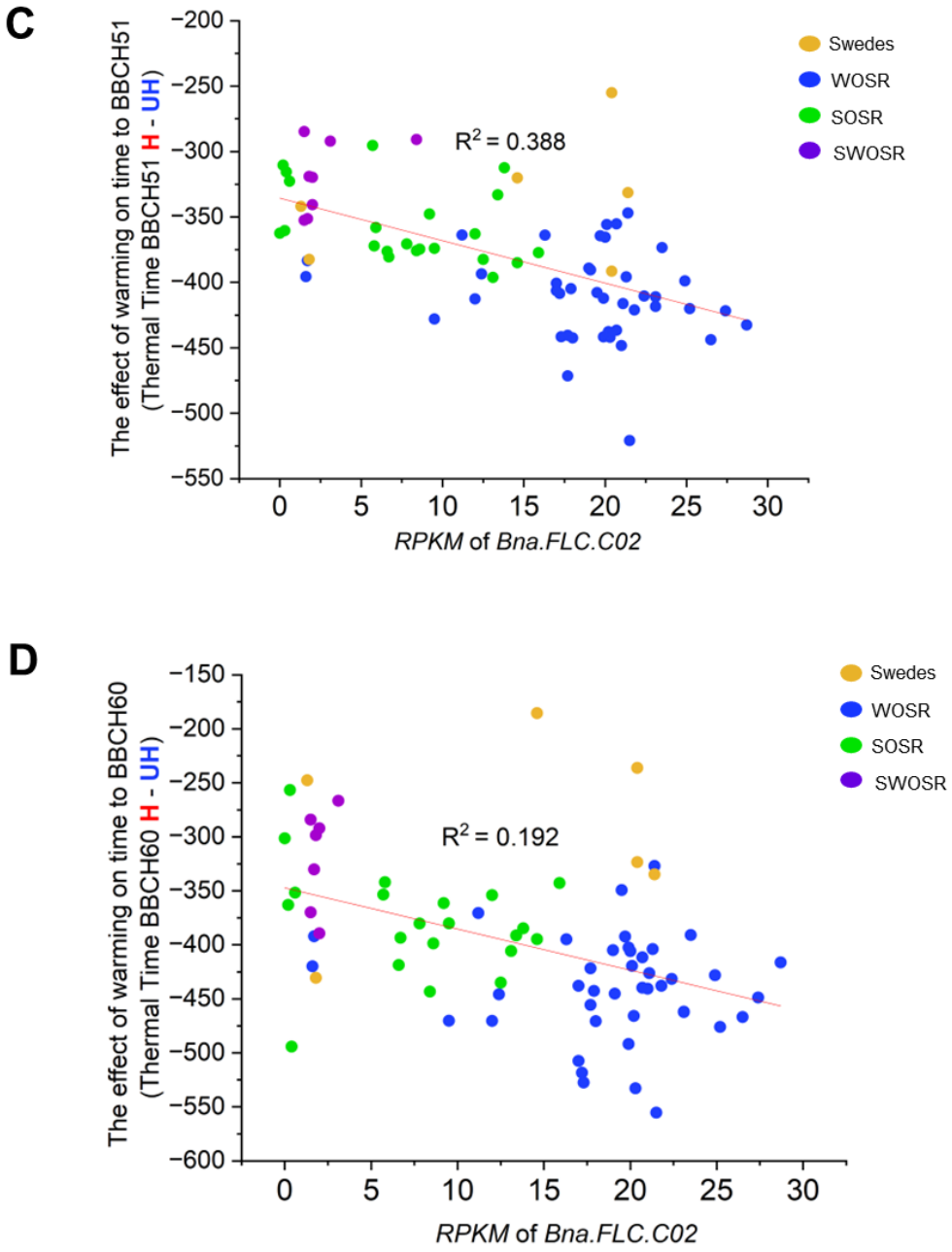


Figure 4.11. Expression of *Bna.FLC.C02* correlates with warmth induced floral delay A) *Bna.FLC.C02* negatively correlates with the ratio of time to BBCH51; values below zero (indicated by grey line) indicate varieties where warming delays time to BBCH51. Individual varieties referenced in the text are labelled here. B) *Bna.FLC.C02* negatively correlates with the ratio of time to BBCH60; values below zero (indicated by grey line) indicate varieties where warming delays time to BBCH60. Individual varieties referenced in the text are labelled here C) *Bna.FLC.C02* negatively correlates with the effect of warming on thermal time to BBCH51; lower thermal time values indicate varieties that required more thermal time to reach BBBCH51. D) *Bna.FLC.C02* negatively correlates with the effect of warming on thermal time to BBCH60; lower thermal time values indicate varieties that required more thermal time to reach BBBCH60.

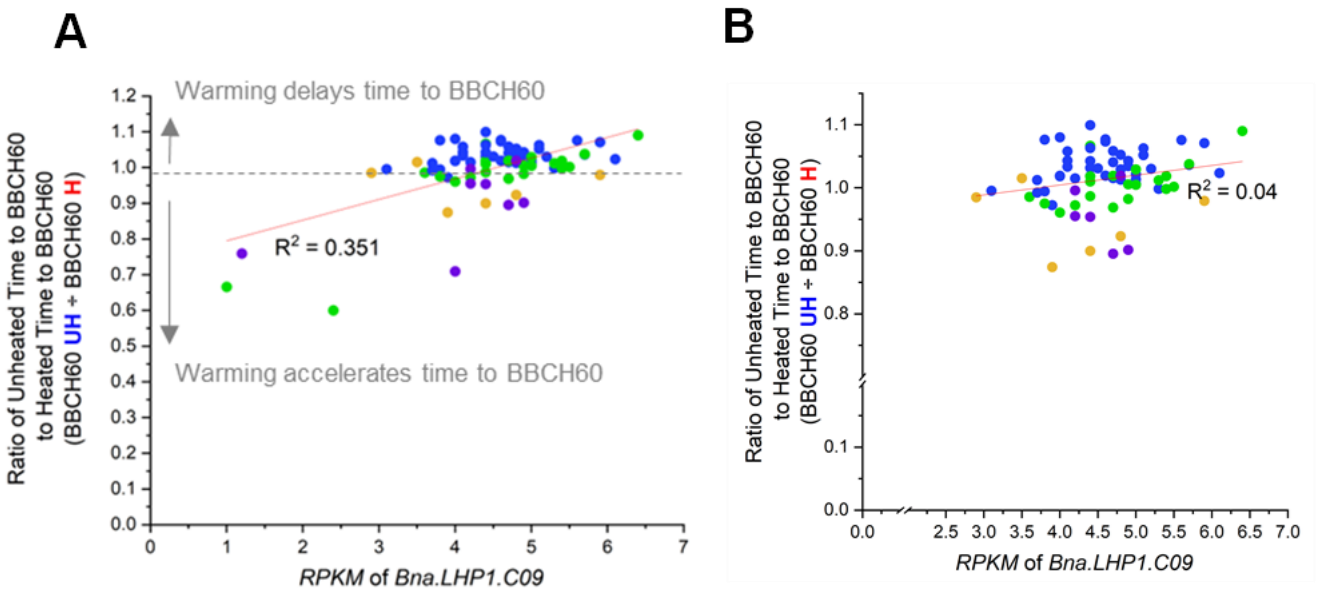


Figure 4.12. Expression of *Bna.LHP1.C09* correlates with warmth induced floral delay but does not when outliers are removed A) *Bna.LHP1.C09* positively correlates with the ratio of time to BBCH60; B) Once outliers are removed *Bna.LHP1.C09* does not correlate with ratio of time to BBCH60

4.4 Discussion

Unlike *A. thaliana*, *B. napus* does not remain vegetative before spring floral induction but undergoes the floral transition in late autumn (O'Neill *et al.,* 2019). In Chapter 3, I demonstrated that there is a requirement for continued cold following floral initiation in WOSR *B. napus* to promote floral development and optimal yield formation. Here, I have shown that the absence of chilling in developing WOSR flower buds induces the well-known ABA-dormancy module and upregulates dormancy genes (Figure 4.4C; Figure 4.5). This bears striking similarity to the genetic control of bud dormancy in perennial fruit trees (Hovarth *et al.,* 2009; Lloret *et al.,* 2018; Zhang *et al.,* 2018; Vimont *et al.,* 2019). I also present evidence that *FLC* plays a role in WOSR warm winter bud dormancy.

4.4.1 Bud dormancy is induced by warmth.

In Chapter 3 I showed that winter warmth, given after floral initiation, delays floral development for most WOSR varieties (Figure 3.4). In Castille, a WOSR variety exhibiting this delay, there is a clear transcriptional response to this warming that is absent in a variety that is not developmental retarded by warming (Figure 4.3).

In warmed Castille buds, I found enrichment for GO-terms associated with cell communication, DNA-templated transcription, dormancy associated genes and abscisic acid associated genes (Figure 4.4C; Figure 4.5). These GO terms were also identified in the control of dormancy in sweet cherry (Vimont *et al.,* 2019). Further analysis identified that in Castille, the transcriptional response to warming is associated with upregulation of genes associated with the ABA response (Figure 4.5) including *HAI1, HAI2, ABF2, ABF3,* and *RD29B* as well as the known dormancy associated gene *DRM1,* and callose synthase gene *GSL11* (Figure 4.5). Similarly, in the WOSR Cabriolet ABA-related genes, including *ABF3, AFP1, HAI1, NCED3* and *DRM1* orthologues, were also upregulated in response to bud warming in work we recently published (Lu *et al.,* 2022). This bears similarity to the upregulation of ABA biosynthesis and signalling genes that occurs during bud dormancy control in many perennial species including plum, peach, leafy splurge and sweet cherry (Zhang *et al.,* 2018; Hovarth *et al.,* 2009; Vimont *et al.,* 2019; Lloret *et al.,* 2018). The induction of ABA signalling and biosynthesis genes seen here is unlikely to do with heat stress or drought as watering was constant in all experiments presented here and there was no ABA induction in the SWOSR Ningyou7 which faced the same experimental conditions.

Work in hybrid aspen showed that *SVP-like* (*SVL*) induces bud dormancy through the *BRC1* dormancy module which in turn leads to upregulation of ABA biosynthesis gene *NCED3* (Singh *et al.,* 2019; Maurya *et al.,* 2020), in a similar mechanism to the *FT-BRC1* mediated control of axillary meristem outgrowth in *A. thaliana* (Niwa *et al.,* 2013). *BRC1* is also essential for bud dormancy establishment in apple (Falavinga *et al.,* 2021). Here, and in work we recently published, we show *BRC1* is upregulated in two WOSR varieties in response to bud warming (Figure 4.5; Lu *et al.,* 2022). Recently we also showed that in Cabriolet and Castille, raw ABA levels are higher in warm winters compared to controlled conditions (Lu *et al.,* 2022). Similarly, in deciduous trees, sweet cherry, apples and pear higher ABA levels are associated with establishing and maintaining the bud dormancy (Li *et al.,* 2018; Fadon *et al.,* 2020; Wang *et al.,* 2020; Chmielewski *et al.,* 2018). It is noteworthy that the only ABA-related gene expression in Ningyou7, which lacks bud dormancy, was associated with ABA catabolism (Table 4.2). ABA catabolism in bud dormancy is essential for the resumption of bud growth after dormancy and facilitating the expression of cell cycle genes in perennial fruit trees (Bai *et al.,* 2013, Tuan *et*

al., 2017, Yang *et al.,* 2020). This implies that Ningyou7 has a transient dormancy response that is maintained in Castille. This would explain the brief upregulation of ABA signalling genes *HAI1* and *ABF2* in Ningyou7 that are then downregulated (Figure 4.5). A time course experiment with greater resolution at earlier stages of bud warming would help elucidate this.

Taken together, this provides strong evidence that the developmental delay seen in WOSR during warm winters shares genetic and physiological similarity to perennial bud dormancy. Further work in *B. napus* using ABA synthesis or signalling inhibitors in warm winters or inducing ABA biosynthesis in control winters would help to confirm if the increase in ABA in warm winters itself drives this response.

4.4.2 Evidence that bud dormancy is likely mediated through specific *FLC* genes that respond to chilling in developing flower buds.

In chapter 3 I showed that SWOSR are universally advanced to flowering following post floral-initiation winter warmth (Chapter 3; Figure 3.4). SWOSR were bred for a milder cold requirement than WOSR (Werner *et al*., 2018). Calderwood *et al.* (2021) showed this has been achieved through comparatively low expression of most *Bna.FLC* copies before vernalisation. Here I have shown that, the SWOSR Ningyou7 lacks *BnaFLC.C02, Bna.FLC.C03a* and *Bna.FLC.C03b* expression during winter which is also seen in two other SWOSR varieties (Figure 4.6; Figure 4.7). The lack of reads in transcriptomic data suggests this is due to a lack of expression entirely, caused by a deletion, rather than through pre-floral initiation silencing. This is consistent with the *Bna.FLC* genes on chromosome C3 being pseudogenized in SWOSR (Sun *et al.,* 2017).

Crucially, this key difference in expression of these *FLC* copies is associated with alteration in expression of putative targets of *FLC* in *B. napus.* This implies that *FLC* may have a central role in bud dormancy control. The higher *FLC* activity in Castille warmed buds is associated with expression of putative *FLC* targets that are involved in processes related to dormancy, abscisic acid and DNA replication (Table 4.2; e.g. *DRM1, BG2, DEAR2, DIV2*). By contrast, the lower *FLC* activity in Ningyou7 is associated with expression of putative *FLC* target genes that are involved in floral development or ABA catabolism (e.g. *SOC1, ARF2, CYP707A*) (Figure 4.8; Table 4.2). However, it remains to be determined if these homologs of *FLC* targets from *A. thaliana* are also targets of *FLC* in *B. napus.*

Previously, *Bna.FLC.C03a* was shown to be silenced following the floral transition in WOSR Cabriolet (O'Neill *et al.,* 2019), while *BnaFLC.C03b* was not expressed in Cabriolet and is likely a pseudogene (O'Neill *et al.,* 2019; Schiessl *et al.,* 2019). Yet here in Castille

Bna.FLC.C03a is the most highly expressed *Bna.FLC* paralogue during winter (Figure 4.6). Presumably, while *Bna.FLC.C03a* may not have a role in vernalisation in early winter that leads to floral initiation (Schiessl *et al.,* 2019; O'Neill et al., 2019) it may have redundant role in the response to winter chilling in flower buds when *Bna.FLC.C02* is absent in Castille, although currently the only evidence for this is the reported expression dynamics of these *Bna.FLC* genes in Castille. Detailed analysis of the transcriptional levels of *Bna.FLC* paralogues during winter across a wider number of WOSR varieties would shed light on this.

4.4.3 The role of *Bna.FLC.C02* in flower bud responses to winter chilling

I have shown that *Bna.FLC.C02* expression level correlates with flowering time across a diversity set of *B. napus* (Figure 4.11). This is consistent with work we published recently that showed haplotype variation, including discovery of a potential insertion that may disrupt expression levels, at both *Bna.FLC.C02* and *Bna.FLC.A03b* is highly predictive of the presence of the bud dormancy phenotype (Lu *et al.,* 2022). *Bna.FLC.C02* is a good candidate for the response to winter cold alongside *Bna.FLC.A03b,* because both genes are not silenced until after the floral transition and are unaffected by temperatures before the floral transition (O'Neill *et al.,* 2019). Both *Bna.FLC.C02* and *Bna.FLC.A03b* are upregulated when winter buds are warmed (Lu *et al.,* 2022), which is when bud dormancy emerges (Chapter 3; Figure 3.4).

However, *Bna.FLC.C02* alone is not fully predictive of a bud dormancy response to winter warming. This is shown by the role of *Bna.FLC.C03a* and *Bna.FLC.C03b* in dormancy like processes in Castille, which lacks *Bna.FLC.C02* expression. As in perennials, *B. napus* bud dormancy is most likely controlled by combinatorial action of multiple genes. Indeed, the vernalisation requirement for flowering in *B. napus* was recently shown to be most closely related to the total expression of all *FLC* paralogues instead of individual paralogues as previously suggested (Calderwood *et al.,* 2021). In the Rosaceae, there are six *DORMANCY ASSOCIATED MADS BOX (DAM)* genes that collectively control dormancy (Bielenberg *et al.,* 2008). Emerging evidence suggests that each *DAM* gene controls different stages of dormancy through distinct seasonal expression patterns (Falavinga *et al.,* 2019; Vimont *et al.,* 2019; Falavinga *et al.,* 2021). Mutant studies comparing the bud dormancy phenotypes of multiple *Bna.FLC* knock-outs would help to identify the specific roles of each *Bna.FLC* gene in bud dormancy control.

4.4.4 The potential role of *MAF3* and *MAF4* in bud dormancy

I show that there is reduced expression, or deletions, of the *FLC* clade members *Bna.MAF4.A02a* and *Bna.MAF3.A02* in SWOSR varieties that lack bud dormancy (Figure 4.5). In *A. thaliana* both *MAF3* and *MAF4* are active late in winter. *MAF3* is responsible for maintaining the vernalised state while *MAF4* is responsible for preventing premature vernalisation (Gu *et al.,* 2013; Kim and Sung, 2013). Both *MAF3* and *MAF4* form multimeric complexes with *SVP* and *FLC* to control floral development (Gu *et al.,* 2013).

Data from the Penfield laboratory has shown *MAF3* and *MAF4* are active after the floral transition in the WOSR variety Cabriolet (Figure 4.13; O'Neill, Penfield, unpublished data). This is when warmth has been shown to induce bud dormancy. Here, I have shown that winter warmth upregulates *BnaMAF3,* but not *BnaMAF4* (Figure 4.6). As *MAF3* and *MAF4* are active after the floral transition and known to form complexes with *FLC* and *SVP,* it is possible they may have a role in controlling bud dormancy in *B. napus,* especially considering that *BnaMAF3* is upregulated in response to warmth after the floral transition. This would be analogous to how multimeric complexes in apple between different *DAM* and *SVP* genes has been suggested to control bud dormancy (Falavinga *et al.,* 2021). However, further work is needed to confirm that the presence or absence of *MAF3* and *MAF4* controls bud dormancy in *B. napus*. *B. napus* m*af3* and *maf4* mutants in WOSR varieties that demonstrate bud dormancy would prove this.

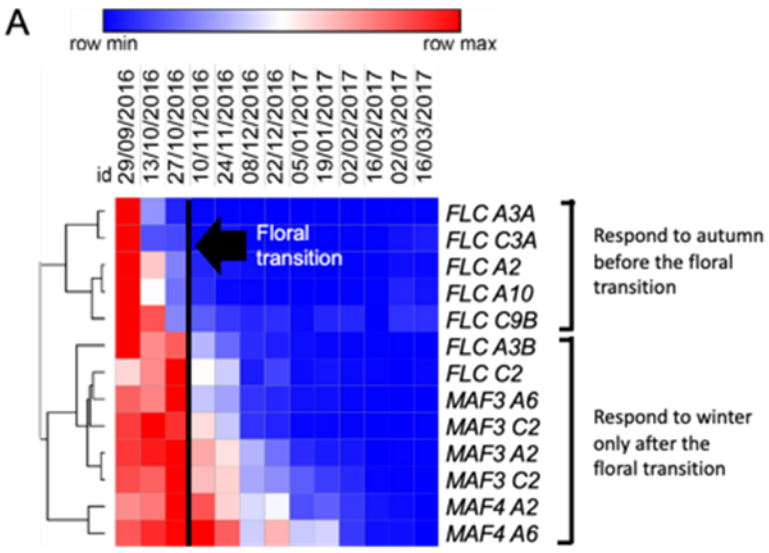


Figure 4.13 *MAF3* and *MAF4* genes in *B. napus* respond to winter temperatures after the floral transition. Data from O'Neill *et al.* (2019) field transcriptome analysis of winter type Cabriolet in Norwich UK. Data processing by Steve Penfield.

4.4.5 GWAS identified the role of an *FLC* regulator in bud dormancy control

The association of *Bna.PHP.A05* with the effect of warming on flowering time (measured by proxy of thermal time to BBCH60) further supports *FLC's* central role in bud dormancy. *Plant Homologous to Parafibromin* (*PHP*) is a member of the Polymerase II Associated Factor Complex (PAF1C) and its activity is limited to *FLC* and its clade members, including all *MADS AFFECTING FLOWERING (MAF2-5)* genes in Arabidopsis, where it deposits active H3K4me3 and H3K36me2/3 histone marks to increase expression of *FLC* (He *et al.,* 2004; Park *et al.,* 2010; Yu and Michaels, 2010; Nasim *et al.,* 2022). Interestingly, in warmth, WOSR Cabriolet *FLC.C02* and *FLC.A03b* have been shown to have enhanced H3K4me3 and be more highly expressed (Lu *et al.,* 2022). The role of *PHP* in bud dormancy will be investigated in depth in Chapter 5.

4.4.6 Conclusion

Taken together, the results here support the hypothesis that *FLC,* and possibly *FLC*-clade members, control bud dormancy in *B. napus*. In *A. thaliana, FLC* is known to bind to many genes involved in the ABA biosynthetic pathway (Deng *et al.,* 2011) and in a potential feedback-loop several abscisic acid signalling transcription factors *ABI3, ABI4* and *ABI5* are known to induce upregulation of *FLC* around floral initiation (Wang *et al.,* 2013; Shu *et al.,* 2016; Shu *et al.,* 2018). Therefore, bud dormancy in WOSR in warm winters, could be controlled by and through feedback regulation of *FLC* and ABA levels. This would be similar the control of ABA levels by DAM proteins in perennial species (Li *et al.,* 2019; Wang *et al.,* 2020). *FLC* genes in *B. napus* may therefore have evolved a function similar to *SVP-like* genes that act as dormancy regulating 'hubs' controlling ABA levels and cell division in perennial dormancy (Tylewicz *et al.,* 2018; Singh *et al.,* 2018, 2019; Azeez *et al.,* 2021).

Within perennial species, failure to break bud dormancy is associated with yield decline through inability to perform bud burst as well as floral and fruit abnormalities (Atkinson *et al.,* 2013). In warm winters, after floral initiation, WOSR experiences warmth-associated yield decline and floral abnormalities (He *et al.,* 2017; Brown *et al.,* 2019; O'Neill *et al.,* 2019; Lu *et al.,* 2022). Therefore, understanding the genetic control of WOSR *B. napus* dormancy allows for targeted breeding programmes to safeguard WOSR yield under climate change.

Chapter 5. The role of Plant Homologous to Parafibromin (*PHP*) in reproductive development in *Brassica*

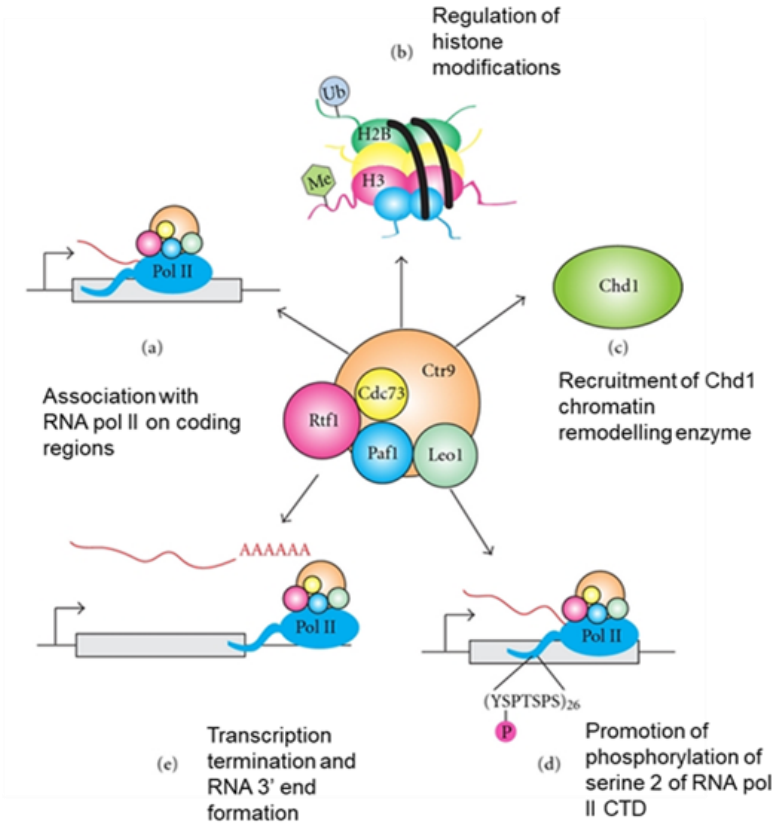
5.1 Introduction

Before vernalisation, *FLC* expression is high and this is associated with enrichment of active histone marks H3 Lys 4 (H3K4me3), H3 Lys 36 (H3K36me3) but reduced presence of repressive H3 Lys 27 (H3K27me3) (Kim, 2022). In *A. thaliana*, *FLC* activation complexes positively regulate *FLC* expression. These include FRI-C, SWR1-C and PAF1-C (Choi *et al.,* 2005; Lazaro *et al.,* 2008; Choi *et al.,* 2011; He *et al.,* 2004).

Of these *FLC*-activating complexes, PAF1-C is known to travel and physically interact with RNA Polymerase II (Pol II) at target genes to aid transcription elongation and influence chromatin structure (Francette *et al.,* 2021). PAF1-C has been extensively studied in budding yeast, *Saccharomyces cerevisiae,* because of its role in transcription elongation, and in humans because of its role in parathyroid carcinomas (Chen *et al.,* 2022; Cetani *et al.,* 2019). This means protein domains and function of individual protein components in the PAF1-C have been at least partially characterised, including description of interaction and histone modification domains (Figure 5.1; Sun *et al.,* 2017; Amrich *et al.,* 2012; Chen *et al.,* 2022).

Across the plantae, fungi and animalia kingdoms PAF1C is an evolutionarily conserved multi-unit protein complex consisting of Ctr9, Paf1, Leo1, Cdc73, and Rtf, while in humans there is an additional sub-unit Ski8 (Krogan *et al.,* 2002; Mueller and Jaehning, 2002; Squazzo *et al.,* 2002, Zhu *et al.,* 2005). Work in budding yeast has shown that each Paf1C subunit has a degree of functional specificity separate to other subunits, however overlapping functions of each subunit that can be hard to isolate (Figure 5.1; Francette *et al.,* 2021).

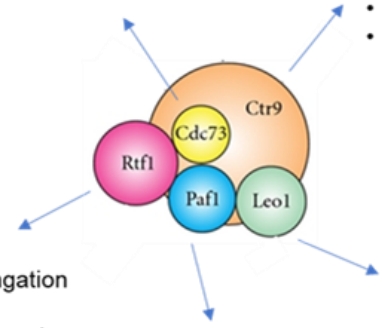
A



B

CDC73:
- Recruits Paf1 complex to target genes
- Maintains elongation efficiency of RNA Pol II
- Associates with target gene chromatin (anchors Paf1c to Pol II elongation complex through protein-protein interactions)
- Regulates transcription elongation

Ctr9:
- Performs histone modifications
- Recruits Paf1C to transcription machinery
- Essential for complex integrity
- Regulates transcription elongation



Rtf1:
- Transcription elongation factor
- Associates with target gene chromatin (anchors Paf1c to Pol II elongation complex through protein-protein interactions)
- Important for histone modification

Paf1:
- Essential for complex integrity
- Binds to RNA Pol II
- Regulates transcription elongation

Leo1:
- Binds Paf1c to RNA
- Performs histone modifications
- Maintains elongation efficiency of RNA Pol II
- Associates with target gene chromatin (anchors Paf1c to Pol II by RNA binding)

Figure 5.1 Multiple functions of the Paf1C and subunits, Figure reproduced and edited from Crisucci and Arndt (2011). A) During transcription elongation the Paf1c complex (a) associates with RNA pol II at coding regions, (b) regulates histone modifications; (c) recruits Chd1 an ATP-dependent chromatin remodelling enzyme; (d) later during transcription elongation Paf1C promotes phosphorylation of serine 2 on RNA pol II CTD; I Paf1C is important for proper transcription termination and RNA 3' end formation of both polyadenylated and non-polyadenylated transcripts. B) Function of Paf1C subunits in yeast. Figure adapted from Crisucci and Arndt (2011) with subunit information from Francette *et al.,* (2021)

In *A. thaliana*, PAF1-C forms a 670-kD protein complex (Park *et al.,* 2010) and, like yeast, associates with RNA Pol II (Oh *et al.,* 2004; He *et al.,* 2004). The Plant Paf1C subunits are named after the behaviour of their mutants: early flowering (*elf*) or vernalisation independent (*vip*). This includes yeast Paf1 (*AtELF7*), human Ski8 (*AtVIP3),* Leo1 (*AtVIP4*), Rtf1 (*AtVIP5*), Ctr9 (*AtELF8/AtVIP6*) and Cdc73 (*AtCDC73/AtPHP*) (Zhang and van Nocker, 2002; Zhang *et al.,* 2003, He *et al.,* 2004, Oh *et al.,* 2004).

A. thaliana mutants deficient in PAF1C subunits ELF7, VIP3, VIP4, VIP5, VIP6/ELF8 are associated with reduced *FLC* expression and show delayed flowering, reduced plant size compared to wild type and floral organ abnormalities (Oh *et al.,* 2004; He *et al.,* 2004; Park *et al.,* 2010). These mutants also have several hundred mis-expressed genes (He *et al.,* 2004; Oh *et al.,* 2008; Xu *et al.,* 2008; Yu and Michaels, 2010; Park *et al.,* 2010; Nasim *et al.,* 2022; Obermeyer *et al.,* 2022).

In plants, the homolog of *CDC73*, is often called *PLANT HOMOLOGOUS TO PARAFIBROMIN* (*PHP*), named after its homology to the human *CDC73* protein, also named PARAFIBROMIN (Park *et al.,* 2010). *A. thaliana* mutants defective in *CDC73* show reduced expression of a small subset of flowering associated genes such as *FLC* and its clade members *FLM, MAF4, MAF5* (Yu and Michaels, 2010; Nasim *et al.,* 2022). *A. thaliana php* mutants are early flowering but, unlike other Paf1C component mutants, are the same size as wild type plants and show no floral abnormalities (Park *et al.,* 2010; Yu and Michaels, 2010). The difference in flowering time between the *php* mutant and wild type is greater at lower temperatures (10°C vs 16°C vs 23°C), suggesting a direct involvement in the temperature response promoting the floral transition (Nasim *et al.,* 2022). The reduced *FLC* expression in *php* mutants is associated with reduced deposition of H3K4me3 in the proximal promotor and 5' section of intron 1 of *FLC,* increased deposition of the active H3K27me3 at 3' end of *FLC* and reduced H3K36me3 mark throughout *FLC* (Park *et al.,* 2010).

This suggests that *PHP* function in plants is limited to flowering time and may only be required for the association of the PAF1-C with specific flowering time genes.

5.1.1 Hypotheses and aims

In this chapter I investigate the role of *PHP.A05* from *B. napus* in temperature responsive flowering time control. This follows from the association between SNP variation at *BnaPHP.A05* and time to first flowering identified in Chapter 4.

The GWAS in Chapter 4 (Figure 4.9) identified that SNP variation at *BnaPHP.A05* was associated with the effect of temperature on time from floral initiation to first flower emergence. This implies *BnaPHP.A05* has a role in control of flowering in winter after floral initiation in *B. napus*, when two *Bna.FLC* genes are still active (O'Neill *et al.,* 2019). Due to the close evolutionarily relationship between *A. thaliana* and *B. napus*, I hypothesise the function of *PHP* is conserved in *B. napus* as in *A. thaliana*, and that *BnaPHP.A05* delays flowering via an effect on *FLC* expression in developing flower buds.

5.2 Genetic variation in *BnPHP.A05* controls flowering time in *B. napus*

5.2.1 A T SNP call in the Cab041204.2:750 marker in *BnPHP.A05* is associated with earlier flowering relative to a C SNP call.

In Chapter 4, I identified SNP variation at the Cab041204.2:750 marker on the *BnaA05g17020D* gene (hereafter referred to as *Bna.PHP.A05*), was associated with the effect of temperature on time from floral initiation to first flower emergence (Figure 4.9). This marker associated with variation at position 750 of the first exon of *BnaPHP.A05.* Across the diversity set, at the Cab041204.2:750 marker the Darmor *bzh* reference sequence had a CytosiI(C) as did 15 other varieties, while 49 varieties had a Thymine (T), 10 had an N (no data) and 2 had a Y ambiguous call , indicating the nucleotide was either C or T (Figure 5.2).

To understand how this SNP variation at position 750 in *BnaPHP.A05* correlates with flowering time I examined differences in floral development between varieties with T or C SNP calls

across multiple treatments and experiments. I compared the time to bud appearance (BBCH51) and first flower emergence (BBCH60) between using the diversity set floral development dataset outlined in chapter 3 using ANOVA analysis presented below. Calendar time, rather than thermal time, was chosen as it allowed for cross-dataset comparison where thermal data was not recorded. Calendar time is also important to establish whether genetic variation in *BnPHP.A05* is of commercial interest, where days to flowering is an important agronomic trait.

Within the diversity set, only WOSR and SOSR varieties had both C and T SNP calls: SOSR had 7 varieties with C and 12 with T. Meanwhile, WOSRs had 7 with C and 23 with T (Figure 5.2). Therefore, I focussed on the 49 varieties of WOSR and SOSR with either a C or T call for all analysis.

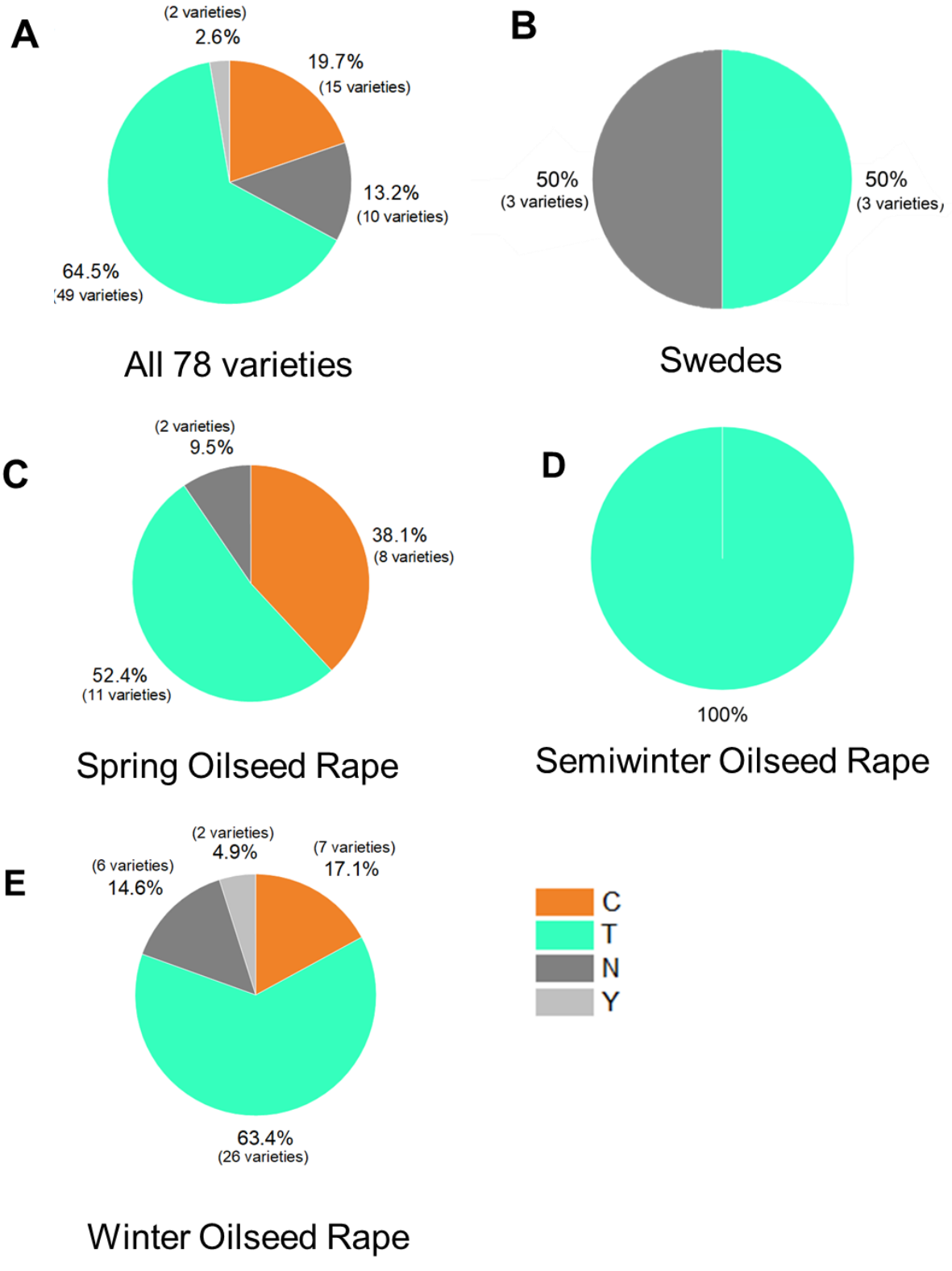

A
(2 varieties)
2.6%
19.7%
(15 varieties)
13.2%
(10 varieties)
64.5%
(49 varieties)
All 78 varieties
B
50%
(3 varieties)
50%
(3 varieties)
Swedes
C
(2 varieties)
9.5%
38.1%
(8 varieties)
52.4%
(11 varieties)
Spring Oilseed Rape
D
100%
Semiwinter Oilseed Rape
E
(2 varieties)
4.9%
(7 varieties)
17.1%
(6 varieties)
14.6%
63.4%
(26 varieties)
Winter Oilseed Rape
C
T
N
Y

Figure 5.2. Ratio of C, T, N and Y SNP calls at the Cab041204.2:750 marker of *BnaPHP.A05* for each crop type. A) All varieties, B) Swedes, c) Spring OSR, D) Semi winter OSR, E) Winter OSR. Key indicates haplotype colours in pie charts.

To determine if the C or T SNP call alone was associated with days to BBCH51 and BBCH60 from floral transition I ran two one-way ANOVA's as follows:

$$BBCH = \mu + PHP_i + \varepsilon_i$$

where BBCH is either BBCH51/60, *PHP* indicates the *PHP* SNP call of C or T and ε is the residuals term.

Table 5.1 One-way ANOVA for effect of PHP SNP call on time to BBCH51 and BBCH60 in the winter warming experiment.

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	PHP	1	7117.026	7117.026	24.138	0.0001
	Residuals	28	83463.276	294.923		
BBCH51	PHP	1	14171.747	14171.747	35.339	0.0001
	Residuals	289	115895.105	401.021		

Table 5.2 Tukey post hoc means comparison test for effect of PHP SNP call on days to BBCH60 following floral initiation.

		MeanDiff	SEM	Q Value	Prob	Alpha
BBCH60	T C	-11.20829	2.28163	6.9472	0.0001	0.05

Table 5.3 Tukey post hoc means comparison test for effect of PHP SNP call on days to BBCH51 following floral initiation.

		MeanDiff	SEM	Q Value	Prob	Alpha
BBCH51	T C	-15.400	2.59063	8.407	0.0001	0.05

One-way ANOVA indicated there is a significant effect of the SNP call on *BnaPHP.A05* on days to BBCH51 (p = 0.0001) and BBCH60 (p = 0.001) from floral transition (Table 5.1).

Varieties with a T SNP call reached BBCH51 on average 65.5 days after floral transition, 15.4 days earlier than C varieties (p = 0.0001), which took on average 80.9 days (Figure 5.3). Varieties with a T SNP call reached BBCH60 on average after 116.7 days after floral transition, 11.2 days earlier than varieties with a C SNP call (p = 0.0001), which took 127.9 days on average (Figure 5.3). Taken together, this shows that varieties with a T SNP call budded and flowered earlier than varieties with a C SNP call (Figure 5.3).

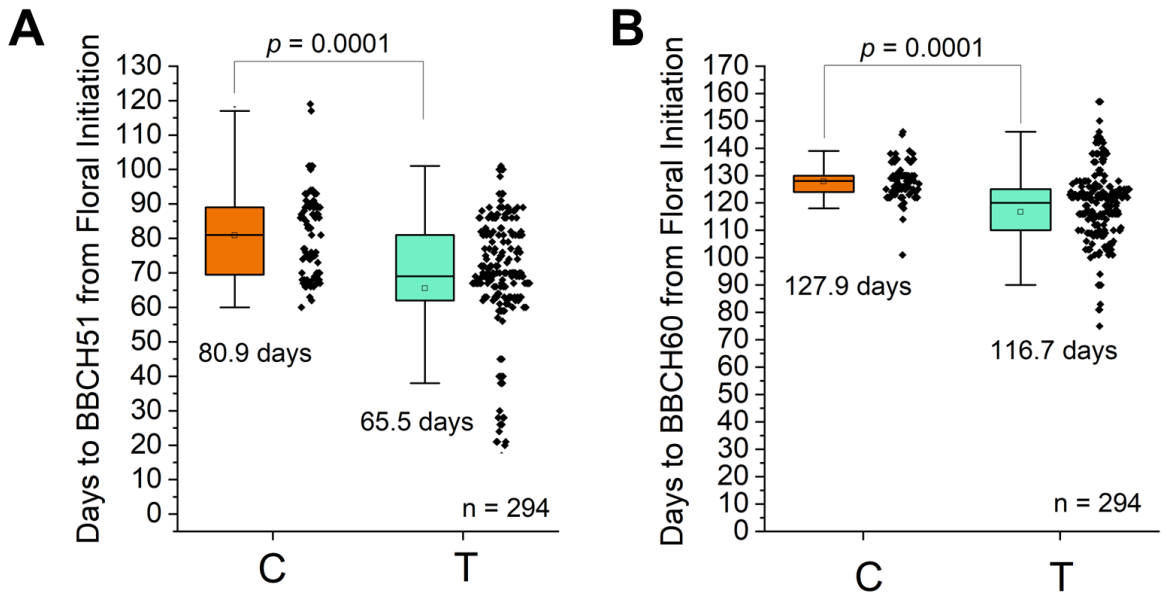


Figure 5.3 Varieties with T SNP call reach BBCH51 and BBCH60 earlier than the C SNP call. T SNP call in teal, C SNP call in orange. A) Days to BBCH51 from floral initiation. Mean time to BBCH51 for each variety given. B) Days to BBCH60 from floral initiation. Mean time to BBCH60 for each variety given. Significance determined using one-way ANOVA.

5.2.2 The early flowering of T SNP call is only associated with SOSR regardless of treatment

Analysis from Chapter 3 showed that that the effect of warming on time to BBCH51 and BBCH60 in *B. napus* is dependent on crop type (Chapter 3; Figure 3.4). This was also the case for the subset of C and T lines investigated here (Supplementary Tables 1-3). Therefore, to understand the effect of *PHP* SNP call, crop type and treatment together on time to BBCH51 and BBCH60, I ran two three-way ANOVA's to test the effect of the *PHP* SNP call, crop type and treatment on time to BBCH51 and BBCH60, as follows:

$$BBCH_{ijkt} = \mu + PHP_i + G_j + (PHP_i \times G_j) + W_k + (PHP_i \times W_k) + (G_j \times W_k) + (PHP_i \times G_j \times W_k) + \varepsilon_{ijkt}$$

where BBCH is either BBCH51/60, *PHP* indicates the *PHP* SNP call of C or T, *G* is crop type grouping (WOSR/SOSR) and *W* is the treatment (warming or control) and ε is the residuals term.

5.2.2.1 The effect of SNP call and crop type on time to BBCH51

I first ran the three-way ANOVA for time to BBCH51 (Table 5.4) on the diversity set data as before. This indicated that the effect of the *PHP* SNP call significantly affected time to BBCH51 ($p < 0.0001$; Table 5.4) as did crop type ($p < 0.0001$; Table 5.4), but treatment (warming/control) did not ($p = 0.31361$; Table 5.4).

The three-way ANOVA also indicated that there was a significant interaction between the effect of the *PHP* SNP call and crop types ($p < 0.0001$; Table 5.4) and there was a significant interaction between crop type, treatment, and the *PHP* SNP call ($p = 0.03139$; Table 5.4). This suggests that the effect of the *PHP* SNP call is dependent on the interaction between crop type and treatment together.

Table 5.4 Three-way ANOVA for effect of PHP SNP call, crop-type and treatment and the interactions between them on time to BBCH51 in the 2020-2021 diversity set experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH51	PHP	1	23013.067	23013.067	93.355	<0.0001
	Crop Type	1	20827.777	20827.777	84.490	<0.0001
	Treatment	1	251.203	251.203	1.019	0.31361
	PHP: Crop Type	1	6740.473	6740.473	27.344	<0.0001
	PHP: Treatment	1	162.963	162.963	0.661	0.41686
	Crop:Treatment	1	844.518	844.518	3.426	0.06522
	Crop:Treatment:PHP	1	1153.161	1153.161	4.678	0.03139
	Residuals	283	69762.554	246.511		

To understand this further, I performed a Tukey post-hoc analysis (Supplementary Table 4). This revealed that there was a significant difference in time to BBCH51 between C and T SNP calls in SOSR in the warmed treatment ($p < 0.0001$; Figure 5.4) and the control treatment (p

< 0.0001; Figure 5.4). However, there was no significant difference between time to BBCH51 in either treatment for WOSR (Figure 5.4; Supplementary Table 4).

Together, this suggests that the earlier BBCH51 seen in T SNP call (Figure 5.3) is due to early budding T SNP calls in SOSR varieties in both treatments (warmed/control) but not WOSR.

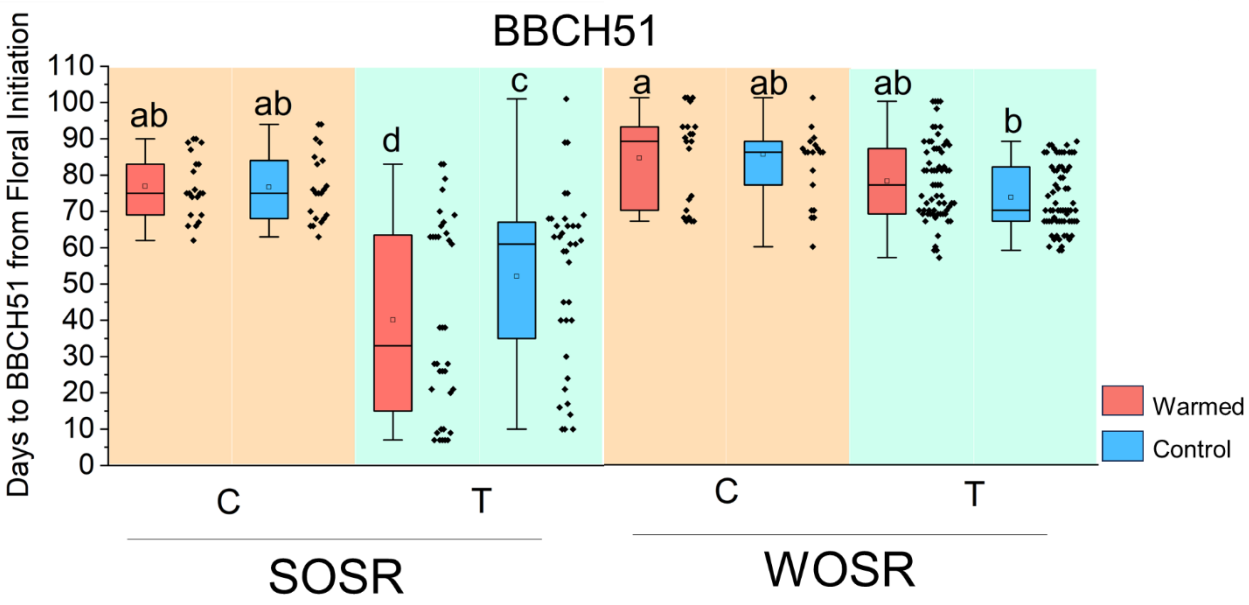


Figure 5.4. In SOSR there is a significant difference between time to BBCH51 in both the warmed and control treatment in varieties with a T SNP call. This is not the case for WOSR. Statistical significance determined by Tukey post hoc test on three way ANOVA with treatment, PHP SNP call and crop type as factors and all interaction terms, *n* = 294. Where varieties were significantly different they were marked with a letter interaction term, each C SNP call and each T SNP call were compared across both crop types and treatments.

5.2.2.2 The effect of SNP call and crop type on time to BBCH60

Next, I performed a three-way ANOVA for time to BBCH60 (Table 5.5) on the diversity set data as before. This indicated that the effect of the *PHP* SNP call significantly affected time to BBCH60 (p = 4.26E-4; Table 5.5) as did crop type (p < 0.0001; Table 5.5), but treatment (warming/control) did not (p = 0.51683; Table 5.5).

The three-way ANOVA also indicated that there was a significant interaction between the effect of the *PHP* SNP call and crop types (p <0.00213; Table 5.5). There was also a significant interaction between *PHP* SNP call and treatment (p = 0.01056; Table 5.5). This suggests that the effect of the *PHP* SNP call is dependent on crop type and treatment separately.

Table 5.5 Three-way ANOVA for effect of PHP SNP call, crop-type and treatment and the interactions between them on time to BBCH60 in the 2020-2021 diversity set experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	PHP	1	3189.013	3189.013	12.721	4.26E-4
	Crop Type	1	9736.105	9736.105	38.838	<0.0001
	Treatment	1	105.613	105.613	0.421	0.51683
	PHP: Crop Type	1	2409.065	2409.065	9.610	0.00213
	PHP: Treatment	1	1661.358	1661.358	6.627	0.01056
	Crop:Treatment	1	0.045	0.045	1.78E-4	0.98937
	Crop:Treatment:PHP	1	547.333	547.333	2.18333	0.14065
	Residuals	277	69440.252	250.687		

To understand this further, I performed a Tukey post-hoc analysis (Supplementary Table 4). This revealed there was a significant difference in time to BBCH60 between C and T SNP calls in SOSR in the warmed ($p < 0.0001$; Figure 5.5) and control treatment ($p < 0.0001$; Figure 5.5). However, there was no significant difference between time to BBCH60 in either treatment for WOSR (Figure 5.4; Supplementary Table 4). Together, this suggests that early flowering T SNP call only causes earlier flowering in SOSR varieties and that this occurs regardless of treatment.

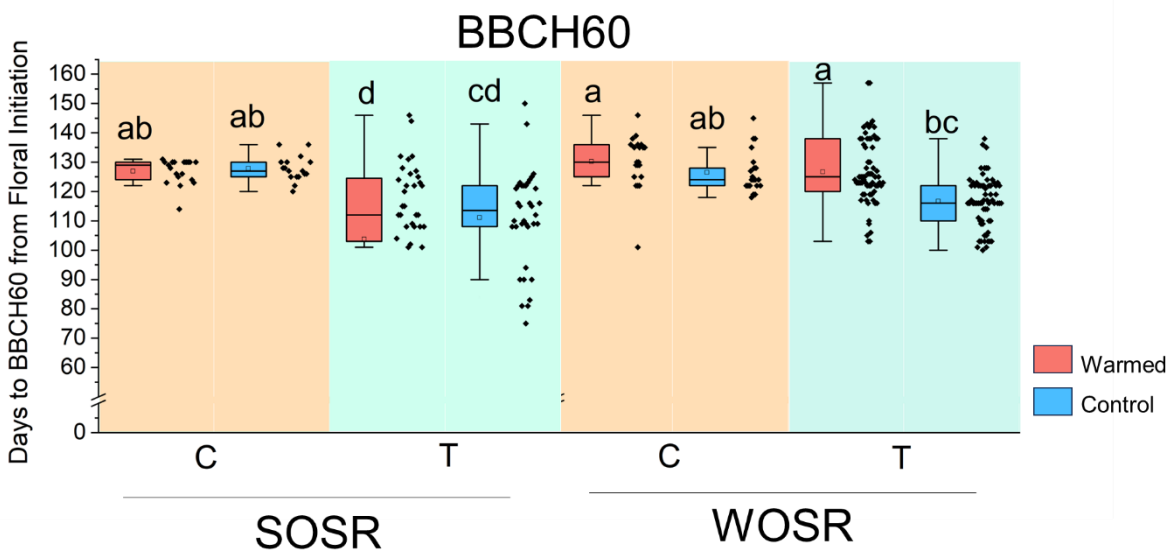


Figure 5.5. In SOSR there is a significant difference between time to BBCH60 in both the warmed and control treatment. This is not the case for WOSR. Statistical significance determined by Tukey post hoc test on three-way ANOVA with treatment, PHP SNP call and crop type as factors and all interaction terms, *n* = 294. Where varieties were significantly different they were marked with a letter interaction term, each C SNP call and each T SNP call were compared across both crop types and treatments.

Overall, this shows that the early BBCH51 and BBCH60 of the T SNP call is due to the effect of T SNP call in SOSR alone and occurs in both treatments. Loss-of-function *php* mutants in *A. thaliana* are earlier flowering compared to wild-type plants as functional *PHP* upregulates *FLC* expression which delays flowering (Yu and Michaels, 2010; Park *et al.,* 2010). This suggests that the T SNP call may show some loss-of-function compared to the C SNP call, but that this only occurs in SOSR varieties.

5.2.3 Varieties with a T SNP call reach BBCH51 and BBCH60 earlier than varieties with a C SNP call in another large-scale vernalisation experiment

To investigate whether *BnaPHP.A05* affects flowering time across diverse winter conditions, I examined the effect of the *PHP* SNP call at position 750, C or T, on days to bud emergence (BBCH51) and first flower opening (BBCH60) after floral initiation using data from an experiment run previously in the laboratory. This experiment was conducted to understand the effect of vernalisation temperature on floral development. This experiment used the same 96 *B. napus* varieties as in the diversity set experiment (section 5.2.1) (Harper *et al.,* 2012).

To simulate different winter conditions and their subsequent impact on flowering, plants were subjected to twelve different vernalisation treatments. *B. napus* varieties were vernalised at either 5°C, 10°C or 15°C, for six or twelve weeks. This was followed by flowering and seed maturation at either 18°C or 24°C. Sowing was staggered so that irrespective of whether plants underwent six or twelve weeks of vernalisation all plants finished vernalisation simultaneously. For consistency, I only analysed WOSR and SOSR with the C and T SNP calls and individual replicates that did not reach flowering were removed from the analysis. This left 1764 individual plants for analysis.

I ran several preliminary ANOVA analyses to determine which factors in this experiment were significant and affected time to either BBCH51 or BBCH60.

To determine if the crop type affected both BBCH51 and BBCH60 I ran a one-way ANOVA to test for a crop type effect on either BBCH51 or BBCH60, where μ was the mean, *G* is crop type grouping (WOSR or SOSR) and ε was the residual term is represented here:

$$BBCH = \mu + G_i + \varepsilon_i$$

Table 5.6 One-way ANOVA for effect of crop type on BBCH51 and BBCH60 in vernalisation experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	Crop Type	1	185651	185651	203.3	<2e-16
	Residuals	1580	1442500	913		
BBCH51	Crop Type	1	300045	300045	324.4	2e-16
	Residuals	1676	1550237			

This ANOVA (Table 5.10) indicated crop type significantly affected both time to BBCH60 (p = 2e-16) and BBCH51 (p = 2e-16).

Next, I ran an ANOVA for BBCH51 and BBCH60 accounting for vernalisation length, either six or twelve weeks. The formula was as follows:

$$BBCH = \mu + VL_i + \varepsilon_i$$

where BBCH represents BBCH51/60, VL stands for vernalisation length and e the residuals term.

Table 5.7 One-way ANOVA for effect of Vern Length crop type on BBCH51 and BBCH60 in vernalisation experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	VernLength	1	339033	339033	415.3	<2e-16
	Residuals	1579	1288995	816		
BBCH51	VernLength	1	265427	265427	280.4	2e-16
	Residuals	1674	1584649	947		

This ANOVA (Table 5.11) indicated vernalisation length had a significant impact on time to BBCH51 (p = 2e-16) and BBCH60 (p = 2e-16).

I then ran a one-way ANOVA to test the effect of the post-vernalisation flowering temperature. The formula was as follows:

$$BBCH = \mu + PVFT_i + \varepsilon_i$$

where BBCH represents BBCH51/60, *PVFT* stands for post-vernalisation flowering temperature and ε the residuals term.

Table 5.8 One-way ANOVA for effect of post-vernalisation flowering temperature on BBCH51 and BBCH60 in vernalisation experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	Transfer	1	1290	1290	1.252	0.263
	Residuals	1579	1626738	1030		
BBCH51	Transfer	1	12	11.7	0.011	0.918
	Residuals	1674	1850064	1105.2		

This ANOVA (Table 5.12) indicated post-vernalisation flowering temperature did not have a significant effect on either BBCH51 (p = 0.918) or BBCH60 (p = 0.263), so it was disregarded in future analyses.

I then ran an ANOVA to test the effect of vernalisation temperature on BBCH51 and BBCH60:

$$BBCH = \mu + VT_i + \varepsilon_i$$

where BBCH represents BBCH51/60, VT stands for vernalisation temperature and ε the residuals term.

Table 5.9. One-way ANOVA for effect of vernalisation temperature on BBCH51 and BBCH60 in vernalisation experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	VernTemp	1	258519	258519	298.2	<2e-16
	Residuals					
BBCH51	VernTemp	1	150950	250950	263	<2e-16
	Residuals	1676	1599333	954		

This ANOVA (Table 5.13) indicated vernalisation temperature did have a significant effect on time to BBCH51 (p = 2e-16) and BBCH60 (p = 2e-16).

I then ran an ANOVA to test the effect of either the C or T PHP SNP call on BBCH51 and BBCH60:

$$BBCH = \mu + PHP_i + \varepsilon_i$$

where BBCH represents BBCH51/60, PHP stands for PHP SNP call C or T and e the residuals term.

Table 5.10. One-way ANOVA for effect of PHP SNP call (C/T) on BBCH51 and BBCH60 in vernalisation experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	VernTemp	1	4425	4425	4/306	0.0381
	Residuals					
BBCH51	VernTemp	1	8683	8683	7.902	0.005
	Residuals	1676	1841600	1099		

This ANOVA (Table 5.14) indicated varieties with either a C or T SNP call are significantly different in the number of days to reach BBCH51 (p = 0.005) and BBCH60 (p = 0.0381) post-vernalisation.

Therefore, I performed two generalised linear models for time to BBCH51 and also time to BBCH60 accounting for significant effects (PHP SNP call, vernalisation length, vernalisation temperature, crop type) and testing for interaction between each term. The generalised linear models were performed as follows:

$$\begin{aligned} BBCH_{ijkl} = {} & \mu + G_i + VT_j \\ & + VL_k + PHP_l + (G_i \times PHP_l) + (G_i \times VL_k) + (G_i \times VT_j) + (PHP_l \times VL_k) \\ & + (PHP_l \times VT_j) + (VL_k \times VT_j) + e_{ijkl} \end{aligned}$$

where BBCH represents BBCH51/60, *G* is crop type grouping, *VT* stands for vernalisation temperature, *VL* is vernalisation length, *PHP* is the SNP call at *PHP* (C or T) and interaction terms in brackets and ε the residuals term.

5.2.3.1 The effect of SNP call, vernalisation temperature and length and crop type on time to BBCH51

The generalised linear effects model for time to BBCH51 (Table 5.11) indicated that the effect of *PHP* SNP call significantly affected time to BBCH51 (p = 8.09e-15; Figure 5.6), as did crop type (p < 2.2e-16), vernalisation length (p < 2.2e-16) and vernalisation temperature (p < 2.2e-16).

The generalised linear model also indicated that there was a significant interaction between crop type and *PHP* SNP call (p = 1.56e-16). There was also a significant interaction between PHP SNP call and vernalisation temperature (p = 5.81e-7).

This suggests that the effect of the *PHP* SNP call is dependent on vernalisation temperature and crop type. There were other significant interactions, but these did not implicate the effect of the *PHP* SNP call so were excluded from analysis.

Table 5.11 Analysis of Variance table from the generalised linear effects model for vernalisation experiment

		Df	Sum Sqaures	Mean Squares	F value	Pr (>F)	Sig
BBCH51	Crop Type	1	286972	286972	604.2736	<2.2e-16	***
	PHP_hap	1	29210	29210	61.5082	8.09e-15	***
	VernLength	1	254989	254989	536.9272	2.2e-16	***
	VernTemp	1	249881	249881	526.1707	2.2e1-6	***
	Crop_Type:PHP_allele	1	8920	8920	18.7838	1.56e-5	***
	Crop_Type:Vern_length	1	1449	1449	3.0506	0.0809	ns
	Crop_Type:Vern_temp	1	149774	149774	315.3781	<2.2e-16	***
	PHP_allele:Vern_length	1	343	343	0.7216	0.3958	ns
	PHP_allele:Vern_temp	1	11960	11960	25.1830	5.81e-7	***
	Vern_length:Vern_temp	1	24585	24585	51.7694	9.63e-13	***
	Residuals	1569	745125	475			

To understand this further, I performed Tukey post-hoc analysis on results from the generalised linear model (Figure 5.6). This revealed that in SOSR varieties with a T SNP call reached BBCH51 significantly earlier than varieties with a C SNP call at vernalisation temperatures of 10 °C and 15 °C (both $p < 0.0001$), but not at 5 °C ($p = 0.5874$) (Figure 5.6).

This also revealed that WOSR varieties with a T SNP call reached BBCH51 significantly earlier than varietis with a C SNP call at vernalisation temperature of 15 °C ($p < 0.0001$) but not at 5 °C or 10 °C ($p = 0.2749$; $p = 0.8923$ respectively).

Together this suggests that the T SNP call causes earlier BBCH51 in SOSR at vernalisation temperatures above 10 °C, but in WOSR the T SNP call only causes earlier BBCH51 at vernalisation temperature of 15 °C (Figure 5.6).

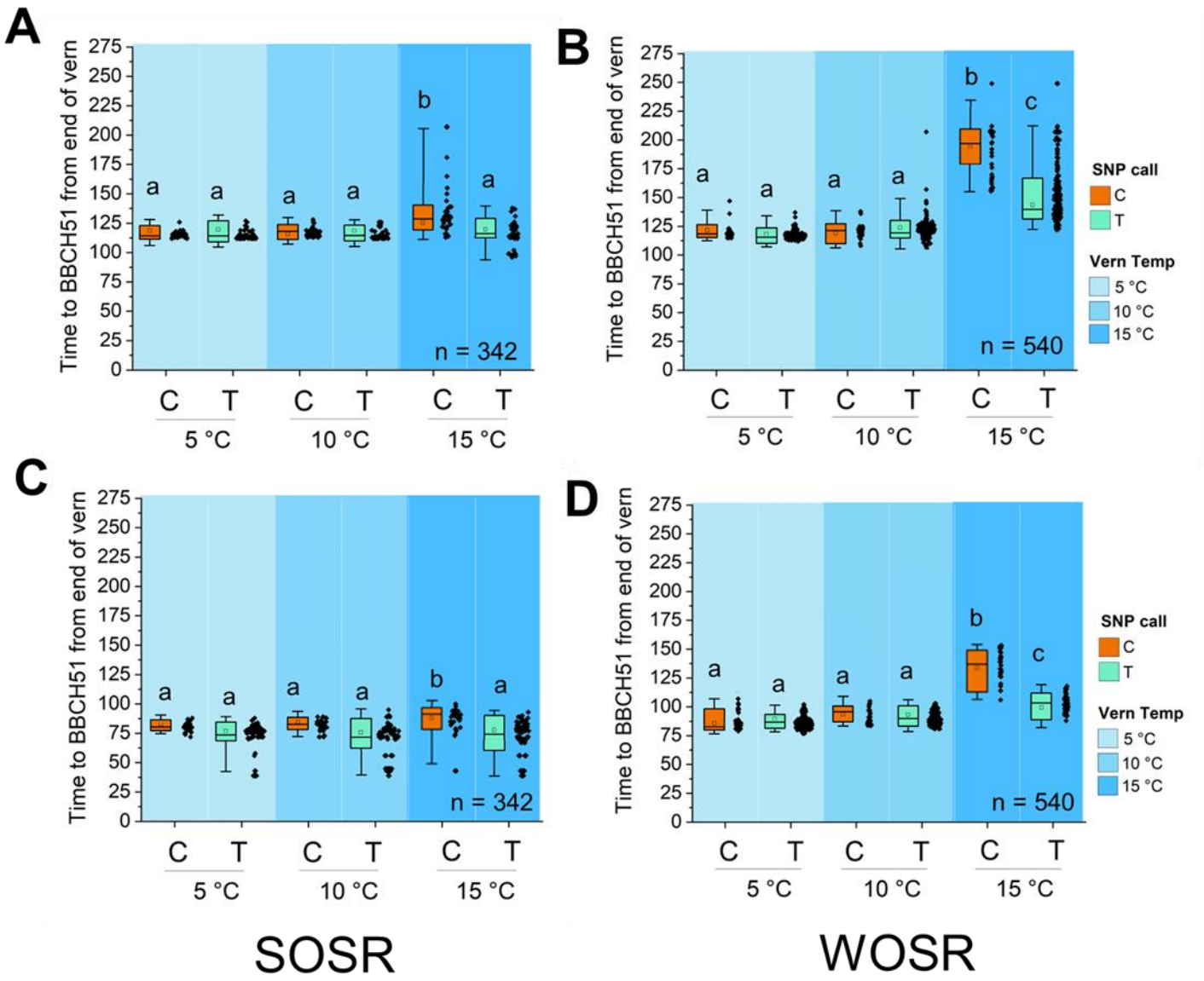


Figure 5.6. In SOSR varieties with the T SNP call reach BBCH51 significantly earlier than varieties with a C SNP call at vernalisation temperatures of 10 °C and 15 °C, while in WOSR varieties with a T SNP call reach BBCH51 significantly earlier at vernalisation temperatures of 15 °C only. Statistical significance determined by Tukey post hoc test on generalised linear model with vernalisation length, vernalisation temperature, PHP SNP call and crop type as factors and all interaction terms. Where varieties were significantly different they were marked with a letter interaction term, each C SNP call and each T SNP call were compared across all three vernalisation temperatures. Key on the right hand side indicates that teal box plots represent T SNP call varieties while orange box plots represent C SNP call varieties, while the different shades of blue represent the different vernalisation

temperatures. A and B represent the shorter 6 week vernalisation treatment whilst C and D represent the longer 12 week vernalisation treatment (Methods section 2.5).

5.2.3.2 The effect of SNP call, vernalisation temperature and length and crop type on time to BBCH60

Next, I ran a generalised linear effects model for time to BBCH60 (Table 5.12). This indicated that the effect of *PHP* SNP call significantly affected time to BBCH60 (p = 1.47e-112e-16; Figure 5.6), as did crop type (p < 2.2e-16), vernalisation length (p < 2.2e-16) and vernalisation temperature (p < 2.2e-16). The generalised linear model also indicated that there was a significant interaction between PHP SNP call and vernalisation temperature (p = 5.81e-7), but not crop type or vernalisation length (Table 5.12).

This suggests that the effect of the *PHP* SNP call is dependent on vernalisation temperature. There were other significant interactions, but these did not implicate the effect of the *PHP* SNP call so were excluded from analysis.

Table 5.12 Analysis of Variance table from the generalised linear effects model for vernalisation experiment

		Df	Sum Sqaures	Mean Squares	F value	Pr (>F)	Sig
BBCH60	Crop Type	1	185534	185534	425.2171	<2e-16	***
	PHP_hap	1	20183	20183	46.2569	1.47e-11	***
	VernLength	1	327603	327603	750.813	<2.2e-16	***
	VernTemp	1	271247	271247	621.6579	<2.2e1-6	***
	Crop_Type:PHP_allele	1	133	133	0.3049	0.58092	ns
	Crop_Type:Vern_length	1	13026	13026	29.8529	5.1e-8	***
	Crop_Type:Vern_temp	1	105186	105186	241.0706	2.2e-16	***
	PHP_allele:Vern_length	1	1481	1481	3.3949	0.0655	ns
	PHP_allele:Vern_temp	1	12215	12215	27.9948	1.38e-07	***

Vern_length:Vern_temp	1	6386	6386	14.6347	0.0001 36	***
Residuals	1570	685035	436			

To understand this further, I performed Tukey post-hoc analysis (Figure 5.7). This revealed that for both WOSR and SOSR, varieties with a T SNP call reached BBCH60 significantly earlier than varieties with a C SNP call at vernalisation temperatures of 15 °C only (both $p < 0.0001$), but not at 5 °C ($p = 0.523$) or 10 °C ($p = 0.23542$).

Together this suggests that the early flowering T SNP call causes earlier BBCH60 in both SOSR and WOSR but only at vernalisation temperatures of 15 °C (Figure 5.7).

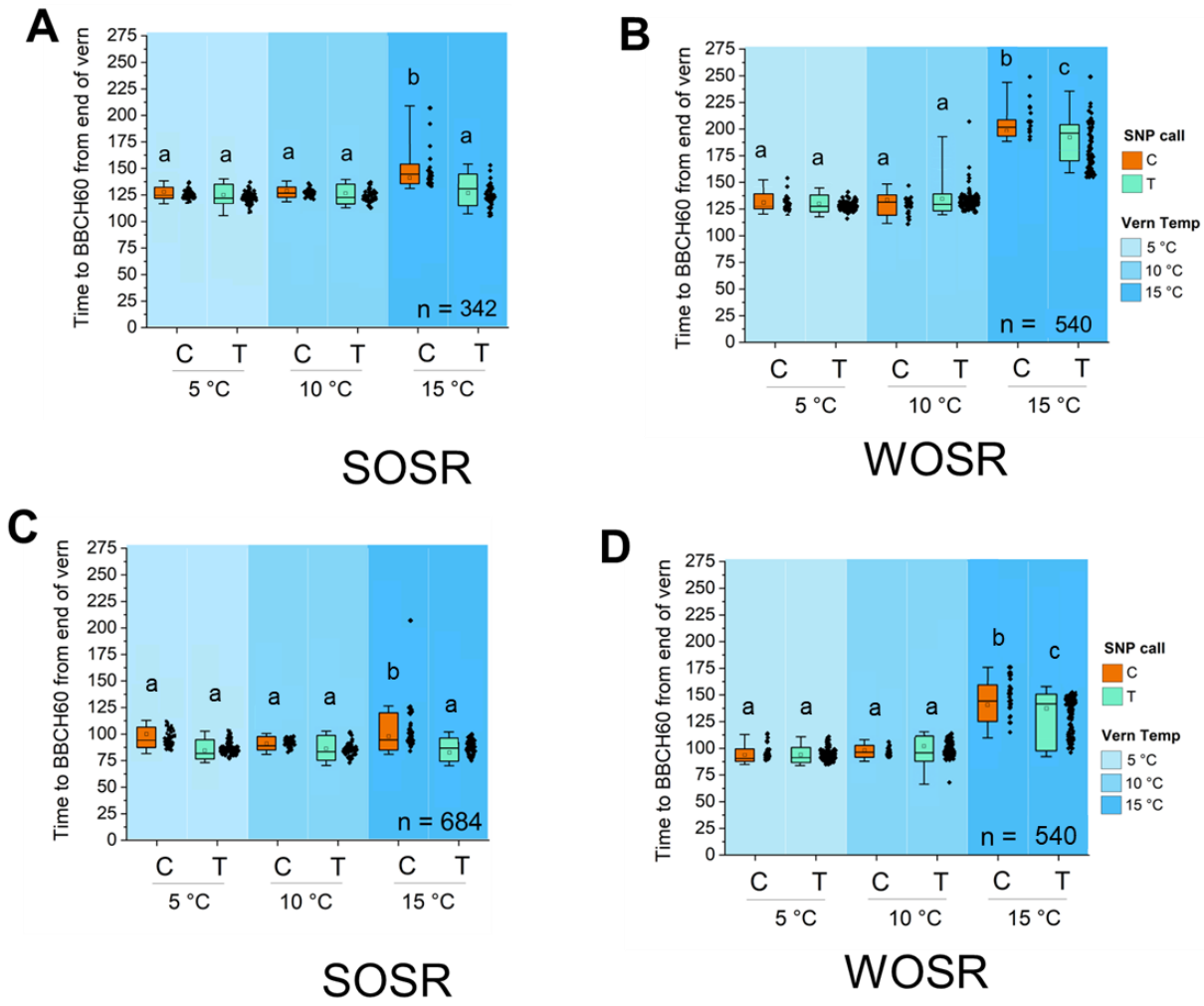


Figure 5.7. In WOSR and SOSR varieties with the T SNP call reach BBCH60 significantly earlier than varieties with a C SNP call at the vernalisation temperature of 15 °C only. Statistical

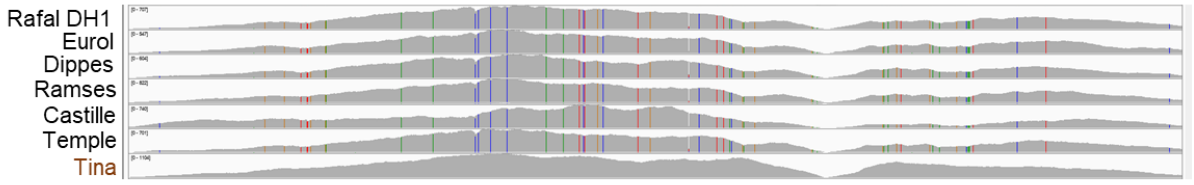
significance determined by Tukey post hoc test on generalised linear model with vernalisation length, vernalisation temperature, PHP SNP call and crop type as factors and all interaction terms. Where varieties were significantly different they were marked with a letter interaction term, each C SNP call and each T SNP call were compared across all three vernalisation temperatures. Key on the right hand side indicates that teal box plots represent T SNP call varieties while orange box plots represent C SNP call varieties, while the different shades of blue represent the different vernalisation temperatures. A and B represent the shorter 6 week vernalisation treatment whilst C and D represent the longer 12 week vernalisation treatment (Methods section 2.5).

Taken together, this suggests that the effect of the T SNP call in WOSR can only be observed at higher vernalisation temperatures around 15 °C (Figure 5.6; Figure 5.7). Meanwhile, in SOSR the T SNP call leads to earlier BBCH51, but not BBCH60, at vernalisation temperatures including 10 °C and 15 °C across two independent experiments (Figure 5.4; Figure 5.5; Figure 5.6; Figure 5.7). This also suggests the effect of *BnaPHP.A05* may be limited to warmer winter conditions.

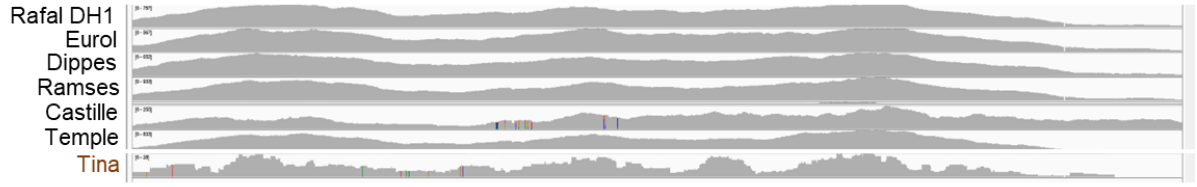
5.2.4 WOSR varieties with T SNP call of *BnaPHP.A05* in *Brassica napus* reach floral initiation earlier than those with C SNP call.

Varieties with C and T SNP calls show significant differences in time to bud emergence and flowering (Figure 5.4; Figure 5.5). Next, I wanted to determine whether SNP variation on *BnaPHP.A05* is associated with earlier or later floral initiation. To do this, I grew three *B. napus* varieties with a C SNP call and three with a T SNP call to compare time to floral transition. I chose WOSR varieties as SOSR are known to have genetic differences at *FLC* genes which can lead to earlier flowering and may mask the effect of *PHP* (Schiessl *et al.,* 2017, 2019). *BnaFLC* haplotype differences are known to affect flowering time in WOSR (Schiessl *et al.,* 2017; Schiessl *et al.,* 2019; Lu *et al.,* 2022), therefore I used exome capture data (Methods section 2.7; Tudor et al., 2020) to select WOSR varieties with the most similar *BnaFLC* haplotypes across varieties. Except for Castille which has a unique *BnaFLC.C02* haplotype (Lu *et al.,* 2022), all *FLC* haplotypes were identical between each variety (Figure 5.8).

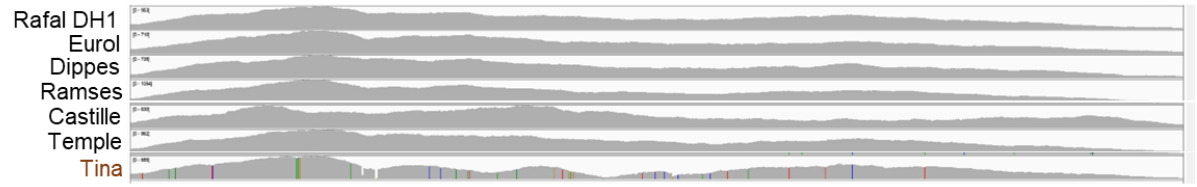
Bna.FLC.A02 (BnaA02g00370D)



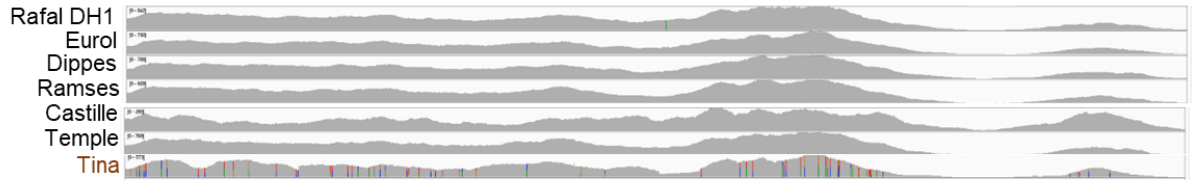
Bna.FLC.C02 (BnaC02g00490D)



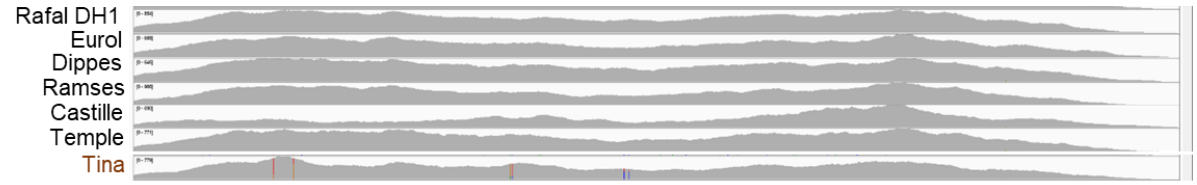
Bna.FLC.A03a (BnaA03g02820D)



Bna.FLC.C03a (BnaC03g16530D)



Bna.FLC.A03b (BnaA03g13630D)



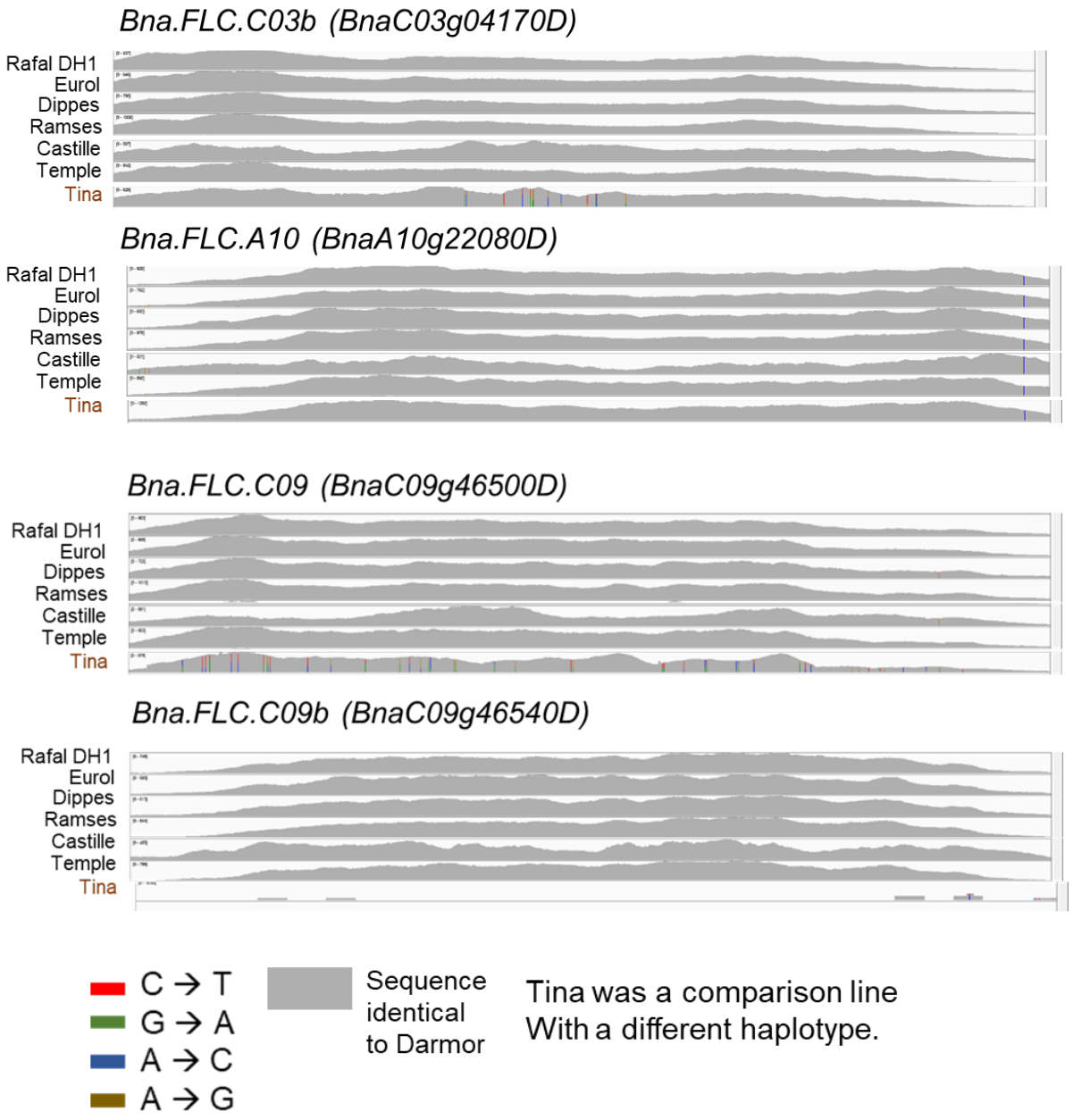
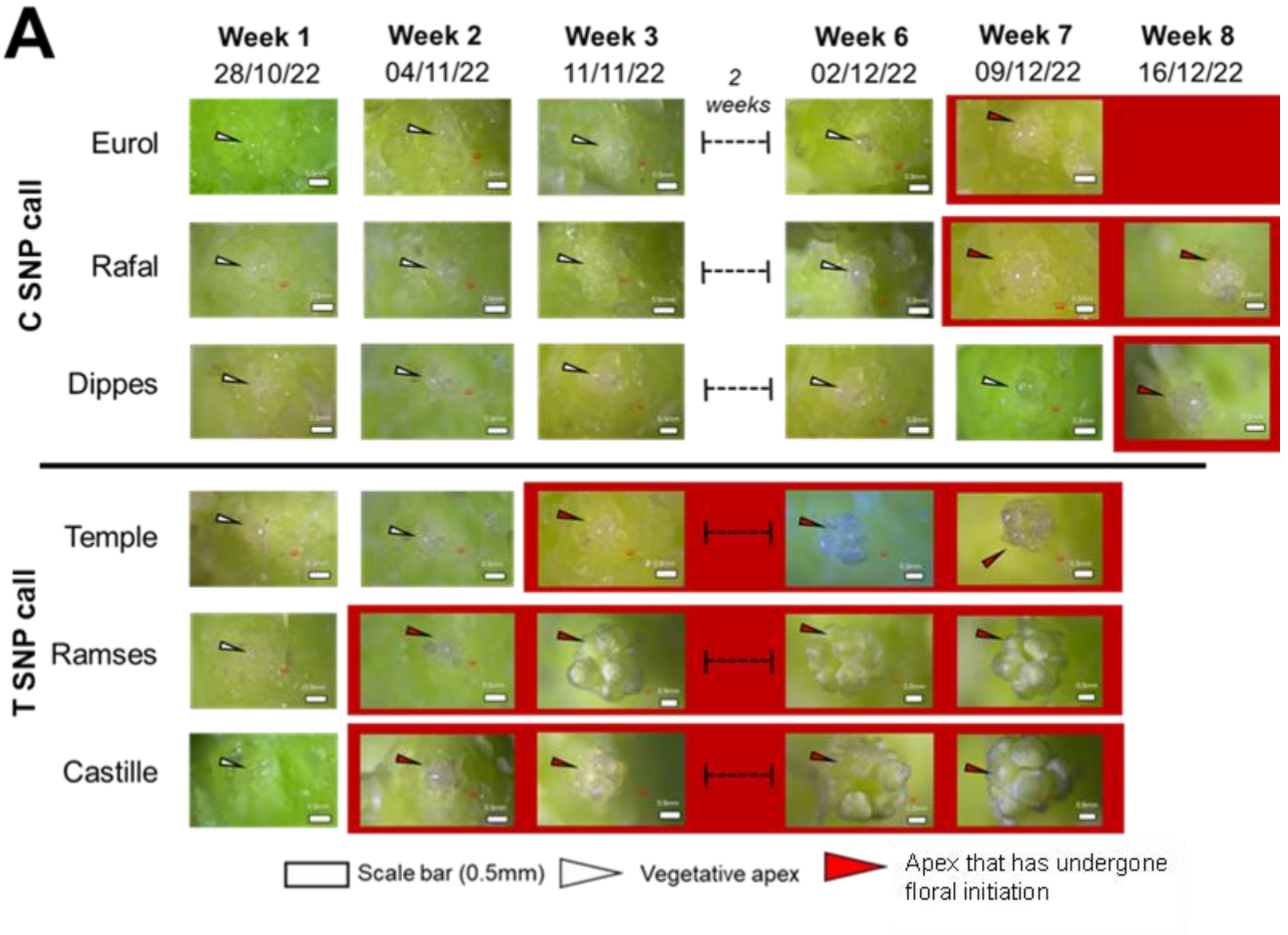


Figure 5.8 Six chosen *Brassica napus* lines share the same haplotype across all *FLC* copies, except Castille in *FLC.C02.* Tina is provided as a comparison line. Figure produced in IGViewer (methods section 2.5.6), Y axis represents read depth, grey indicates sequence is conserved in the Darmor *bzh* reference sequence (version 4.0) whilst coloured lines indicate single nucleotide polymorphisms with colours indicated by the key.

To understand how the C or T SNP call of *BnaPHP.A05* may affect early floral development, I grew Eurol, Rafal, Dippes, Temple, Ramses and Castille in a control unlit polytunnel. An uncharacteristically warm autumn meant lines transitioned to flowering later than expected, however varieties with the T SNP call on average transitioned to floral meristem 3-4 weeks before C lines, on average 34 days (Figure 5.9). This suggests that the T SNP call is associated with early floral initiation in WOSR, although this could be caused by other undetermined differences between varieties. Overall, this suggests that SNP variation in *PHP* may lead to early floral development, acting both before and after floral initiation.


A
Week 1
28/10/22
Week 2
04/11/22
Week 3
11/11/22
2 weeks
Week 6
02/12/22
Week 7
09/12/22
Week 8
16/12/22
C SNP call
Eurol
Rafal
Dippes
T SNP call
Temple
Ramses
Castille
Scale bar (0.5mm)
Vegetative apex
Apex that has undergone floral initiation

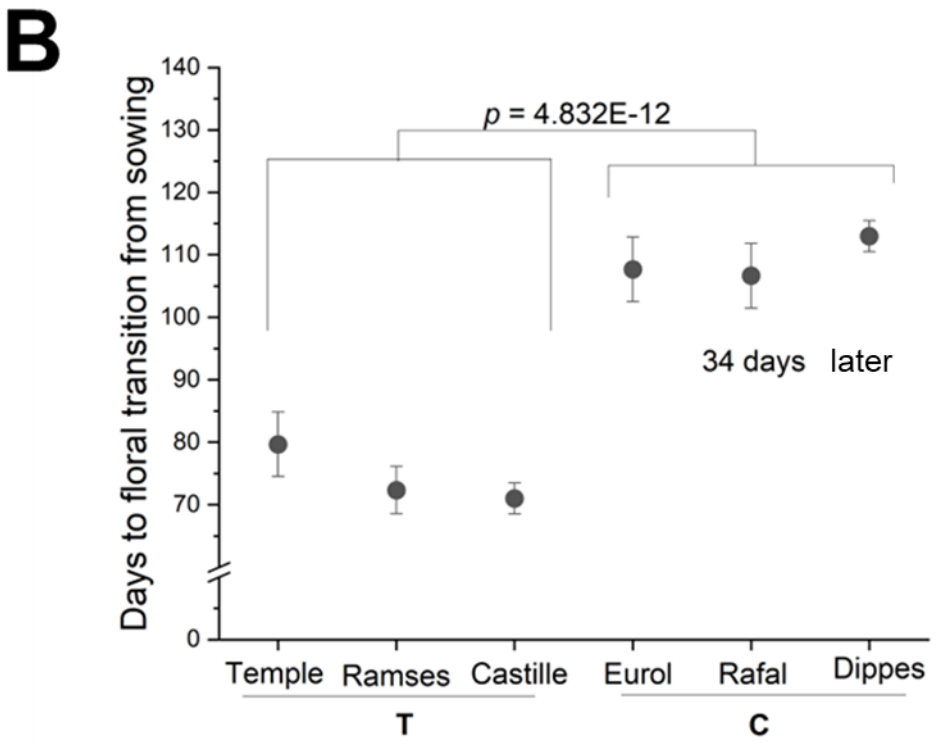


Figure 5.9 The C SNP call transitions to flowering 3-4 weeks earlier than the T SNP call. A) 8-week time course of three C SNP call lines and three T SNP call lines with the same *FLC* haplotypes (except Castille) grown in an unlit control polytunnel sown in late summer and grown until early winter. The key indicates floral transition marked by a red arrow whilst a white arrow marks a vegetative apex. Size of images are indicated by a 0.5mm scale bar. B) Days to floral transition from sowing comparing three T SNP call varieties to 3 C SNP call varieties. P value from students t-test

5.2.5 Variation in flowering time is only associated with variation at *BnaPHP.A05*, not *BnaPHP.C05*

Brassica napus has two homologues of *PHP* gene from Arabidopsis, one on chromosome A05 (BnaA05g17020D; *BnaPHP.A05*) and one on chromosome C05 (BnaC05g29870D;

BnaPHP.C05) (Supplementary Figure 4). The mRNA expression level of both *BnaPHP.A05* and *BnaPHP.C05* does not correlate with floral development (Supplementary Figure 5). This suggests the function of the gene is not related to transcription level. Therefore, to understand whether *SNP* variation at *BnaPHP.C05* influences floral development as it does in *BnaPHP.A05* (Figure 5.4; Figure 5.5), I analysed sequence data for *BnaPHP.C05*. Some lines show Single Nucleotide Polymorphisms (SNPs) relative to the Darmor *bzh* version 4 reference sequence, however presence of SNPs did not associate with changes in flowering time. Across all varieties, there are no non-synonymous changes, indels or any UTR differences across all plant varieties in *Bna.PHP.C05* (Figure 5.10). This suggests there is no meaningful variation in the *BnPHP.C05* gene that may affect its function. Therefore, I focussed analysis on *BnaPHP.A05.*

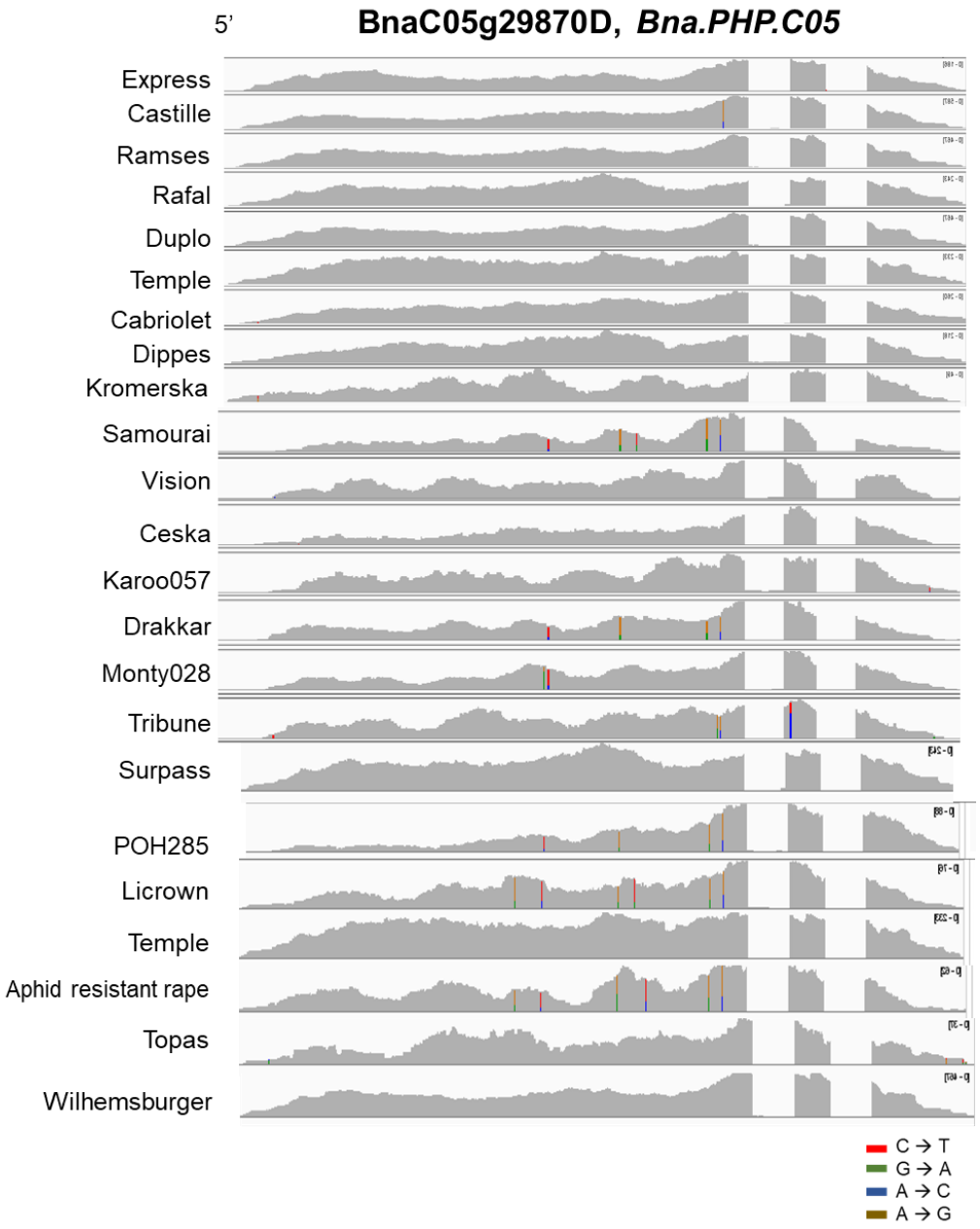


Figure 5.10 Presence of SNPs in *BnaPHP.C05* is not associated with alterations in flowering time. IGV viewer output of short read sequences aligned to *BnaPHP.C05.* SNPs coloured and key in bottom right. Most varieties have no SNPs relative to Darmor *bzh* version 4. Some varieties do show SNPs but the presence of these is shared in early and late flowering lines (e.g., Samourai, POH285 and Licrown are late flowering while Aphid resistant, Drakkar and Monty are early flowering yet all share SNPs in same position).

5.2.6 Variation in *BnaPHP.A05* at the haplotype scale

To understand why the T SNP call on *BnaPHP.A05* was associated with early flowering I analysed sequence data for the 48 SOSR and WOSR varieties with C and T SNP calls obtained from transcriptomic data of the DFFS diversity set. I assigned varieties into five major haplotype groups for *PHP.A05* (Figure 5.11).

HAP1 corresponded to the Darmor *bzh* reference sequence. HAP1 associates with the C SNP call in both SOSR and WOSR only.

HAP2 is characterised by many single-nucleotide polymorphisms (SNPs) and a shorter 5' UTR relative to the Darmor *bzh* reference (Figure 5.11) and is only ever associated with the T SNP call (appears in both SOSR and WOSR). Of the SNPs in HAP2, most were synonymous changes, however one SNP causes a non-synonymous base change from an Alanine to Valine in HAP2 compared to HAP1 at position 422 in exon 1 (Figure 5.11). This change was predicted to occur in a side chain with very low alphafold prediction quality pLDDT (predicted local distance difference test) score (Supplementary Figure 3). This, alongside the chemical similarity between these two non-polar amino acids, suggests it is unlikely to lead to the observed phenotypic difference compared to HAP1.

HAP3 shares many features with HAP2 (shorter 5'UTR, association with T SNP call, many SNPs relative to Darmor) but also contains two deletions in exon 1. HAP3 only occurs in SOSR varieties.

HAP4 is characterised by a retained 5'UTR and several SNPs compared to Darmor, HAP4 only ever associates with T SNP variation. HAP4 only occurs in SOSR lines.

HAP5 was associated T SNP call and contained several deletions in exon 1 and exon 2, however, HAP5 was only represented by the WOSR variety Temple so was not considered further.

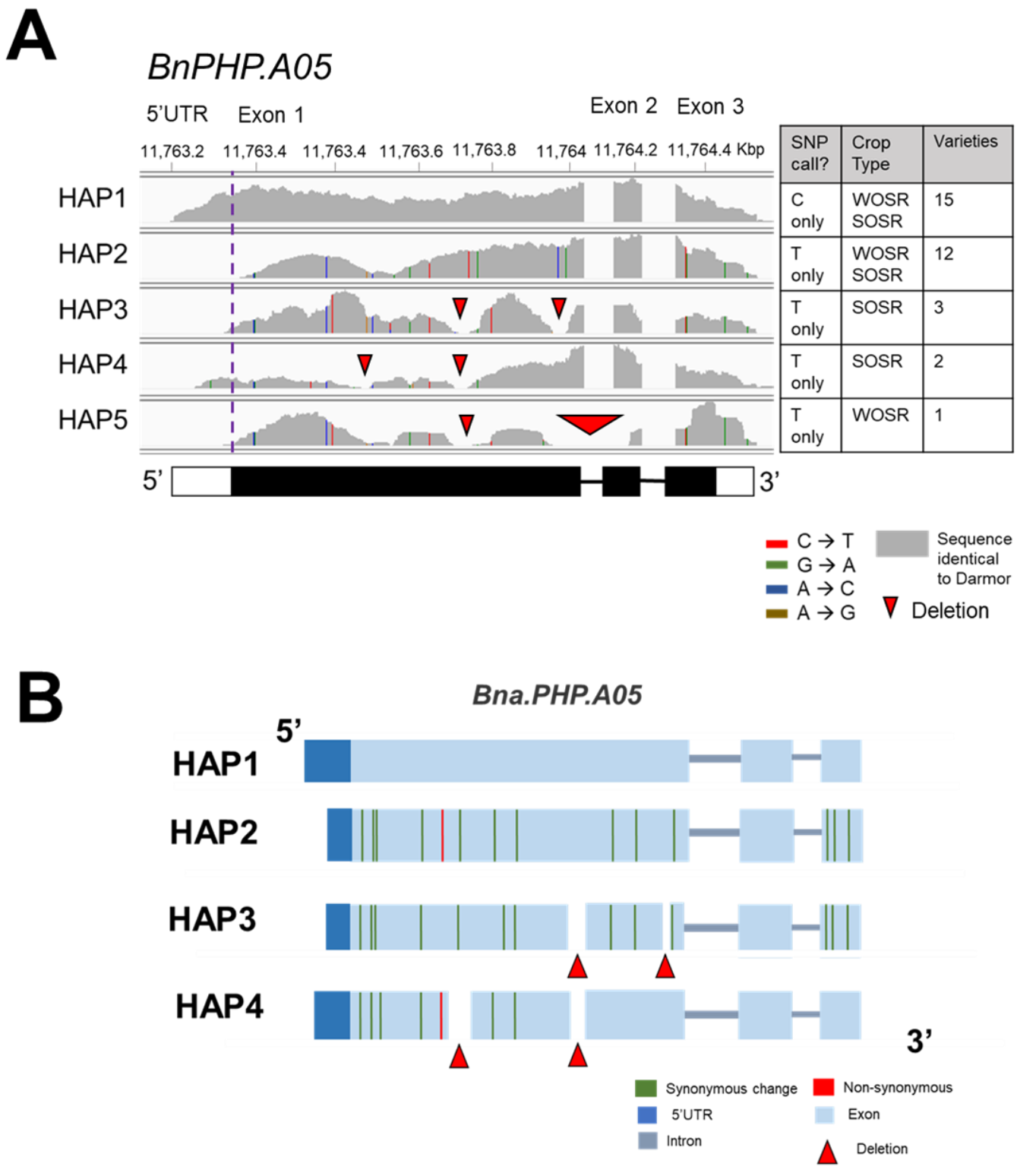


Figure 5.11 Haplotype analysis at *BnaPHP.A05* using transcriptomic data from the Renewable Industry products from Rapeseed (RIPR) project, showing polymorphisms relative to Damor *bzh* version 4 reference sequence. A) Gene view from IGViewer, Grey indicates read depth and

deletions appear where there is no read depth. The SNP call that each haplotype is associated with is indicated in the box on the right-hand side. Gene model below. Purple line indicates the end of 5'UTR and start of exon 1. Red arrow indicates presence of deletions. Coloured lines indicate base changes relative to Darmor, summarised in key. B) Gene model for *BnaPHP.A05* HAP1-4 (HAP5 excluded as only one variety), key indicates changes.

5.2.7 Shorter 5' UTRs, SNP variation and deletions in exons is associated with early BBCH51 and BBCH60

To understand how variation at *PHP* loci in these different haplotypes affected flowering time I examined the effect of the different haplotypes (HAP1-4) on days to bud emergence (BBCH51) and first flower opening (BBCH60) on the experiment described in section 5.2.3. This experiment was chosen for analysis as the study power was far larger than the diversity set analysis and the 10 °C vernalisation treatment in this experiment was comparable to the effects seen in the diversity set analysis, and significant effects were found regardless of treatment.

Previous analyses confirmed that vernalisation length, vernalisation temperature and crop type but not post-vernalisation flowering temperature had a significant effect on time to BBCH51 and BBCH60 (Section 5.2.2). Therefore, I performed a general linear model as in section 5.2.2, but instead of the C or T SNP calls, I used the new haplotypes.

$$BBCH_{ijkl} = \mu + G_i + VT_j + VL_k + HAP_l + (G_i \times HAP_l) + (G_i \times VL_k) + (G_i \times VT_j) + (HAP_l \times VL_k) + (HAP_l \times VT_j) + (VL_k \times VT_j) + e_{ijkl}$$

where BBCH represents BBCH51/60, *G* is crop type grouping, *VT* stands for vernalisation temperature, *VL* is vernalisation length, *HAP* is haplotype, interaction terms in brackets and ε the residuals term.

5.2.7.1 Shorter 5' UTRs, SNP variation and deletions in exons are associated with early BBCH51

I first performed generalised linear model for time to BBCH51 (Table 5.4). This indicated that there was a significant difference in time to BBCH51 between haplotypes ($p < 2.2e\text{-}16$). Interaction terms indicated that the effect of the haplotype on time to BBCH51 was dependent

on both crop type and vernalisation temperature (p = 0.031; p = 1.7E-07; respectively). Other significant interactions that were unrelated to the effect of *PHP* haplotype were ignored.

Table 5.13. Analysis of variance table from generalised linear effects model of the vernalisation experiment on time to BBCH51

		Df	Sum Sqaures	Mean Squares	F value	Pr (>F)	Sig
BBCH51	Crop Type	1	299841	299841	673.9418	< 2.2e-16	***
	PHP_hap	1	79813	79813	179.3924	< 2.2e-16	***
	VernLength	1	269116	269116	604.8836	< 2.2e-16	***
	VernTemp	1	267918	267918	602.1891	< 2.2e-16	***
	Crop_Type:PHP_hap	1	2082	2082	4.6806	0.03065	*
	Crop_Type:Vern_length	1	2113	2113	4.7483	0.02947	ns
	Crop_Type:Vern_temp	1	150639	150639	338.5855	< 2.2e-16	***
	PHP_hap:Vern_length	1	668	668	1.5009	0.2207	ns
	PHP_hap:Vern_temp	1	12272	12272	27.5829	1.70E-07	***
	Vern_length:Vern_temp	1	24846	24846	55.8445	1.26E-13	***
	Residuals	1665	740769	445			

To understand how the vernalisation temperature and crop type influenced the effect of the haplotypes on time to BBCH51, I performed post-hoc Tukey test on the output from the generalised linear model (Figure 5.12). This indicated that in SOSR, HAP4 was the earliest to BBCH51 of all haplotypes across all vernalisation temperatures (Figure 5.12 A). In SOSR, HAP3 was earlier than HAP1 at all vernalisation temperatures but only earlier than HAP2 after vernalisation at 10 °C and 15 °C (Figure 5.12 A). In SOSR, HAP2 was earlier than HAP1 after vernalisation at 10 °C and 15 °C but not 5 °C (Figure 5.12 A).

Meanwhile, for WOSR, HAP2 is only earlier to BBCH51 than HAP1 after vernalisation at 10 °C and 15 °C (Figure 5.12 B). This is similar to results from the diversity set analysis (Supplementary Figure 7).

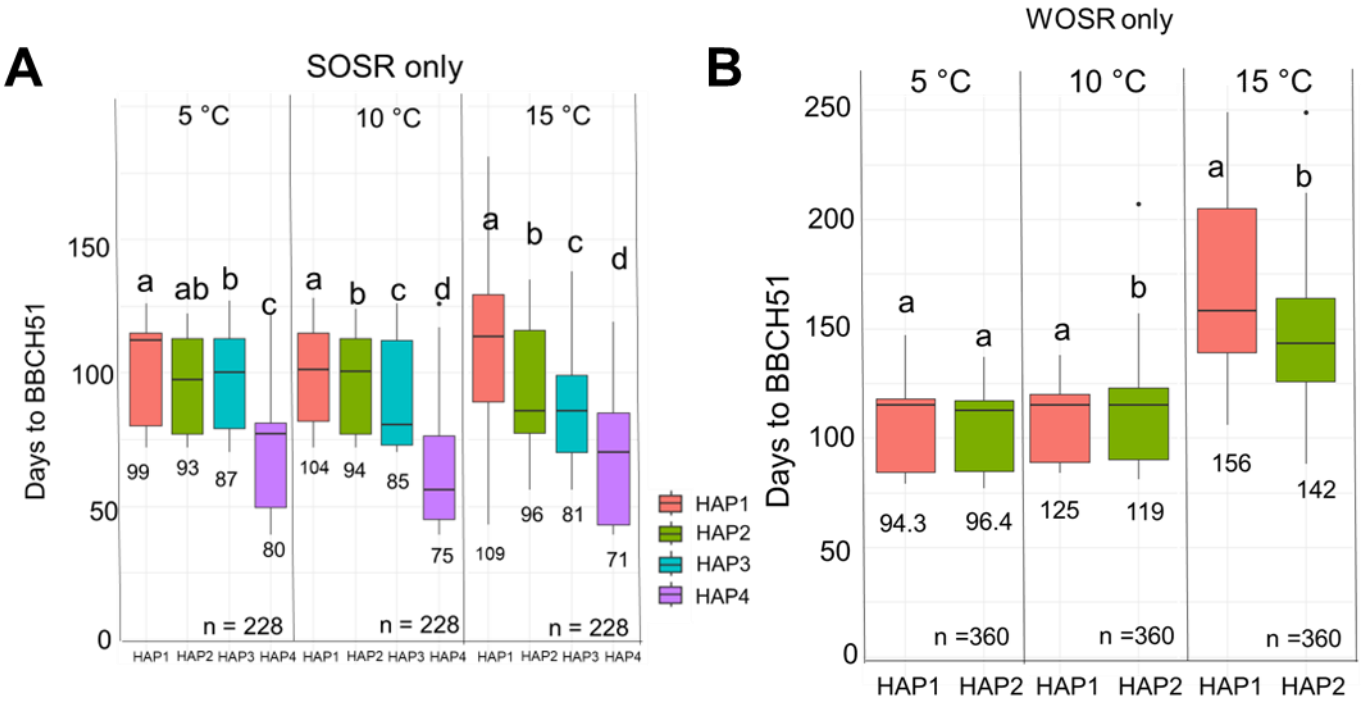


Figure 5.12 Association of *BnaPHP.A05* haplotypes with days to BBCH51 at 3 vernalisation temperatures in SOSR and WOSR. Significant differences at P < 0.05 are shown, calculated tukey post hoc test, comparisons within each vernalisation temperature not across all vernalisation temperatures. Mean days to BBCH51 for each haplotype at the respective temperature shown under each box plot. A) Days to BBCH51 from transfer date into vernalisation treatment for SOSR varieties; B) Days to BBCH51 from transfer date into vernalisation treatment for WOSR varieties

5.2.7.2 Shorter 5' UTRs, SNP variation and deletions in exons are associated with early BBCH60

Next, I performed a generalised linear model for time to BBCH60 (Table 5.5). This indicated that there was a significant difference in time to BBCH60 between haplotypes (p = 2.2e-11). Interaction terms indicated that the effect of the haplotype on time to BBCH60 was dependent on vernalisation temperature alone (p = 3.89E-5). Other significant interactions that were unrelated to the effect of *PHP* haplotype were ignored.

Table 5.14. Analysis of variance table from generalised linear effects model of the vernalisation experiment on time to BBCH60

		Df	Sum Sqaures	Mean Squares	F value	Pr (>F)	Sig
BBCH60	Crop Type	1	174983	174983	397.5089	< 2.2e-16	***
	PHP_hap	1	20001	20001	45.4358	2.22E-11	***
	VernLength	1	316608	316608	719.2359	< 2.2e-16	***
	VernTemp	1	262027	262027	595.2459	< 2.2e-16	***
	Crop_Type:PHP_allele	1	1358	1358	3.084	0.07927	ns
	Crop_Type:Vern_length	1	12911	12911	29.3305	7.07E-08	***
	Crop_Type:Vern_temp	1	102760	102760	233.4398	< 2.2e-16	***
	PHP_allele:Vern_length	1	1070	1070	2.4298	0.11925	ns
	PHP_allele:Vern_temp	1	7492	7492	17.0206	3.89E-05	***
	Vern_length:Vern_temp	1	7175	7175	16.3005	5.67E-05	***
	Residuals	1547	680989	440			

To understand how the vernalisation temperature and crop type influenced the effect of the haplotypes on time to BBCH60, I performed post-hoc Tukey test on the output from the generalised linear model (Figure 5.13). This indicated that in SOSR, HAP3 and HAP4 were earlier than HAP1 and HAP2 only after vernalisation at 15 °C, but not 10 °C or 5 °C (Figure 5.13 A). In SOSR, HAP2 was earlier to BBCH60 only after vernalisation at 15 °C, but not 10 °C or 5 °C (Figure 5.13 A).

Meanwhile in WOSR, HAP2 was only earlier to BBCH60 after vernalisation at 15 °C, but not 10 °C or 5 °C (Figure 5.13 B). As HAP2 corresponds to T SNP call and HAP1 to C SNP call the data for WOSR varieties is consistent with earlier results.

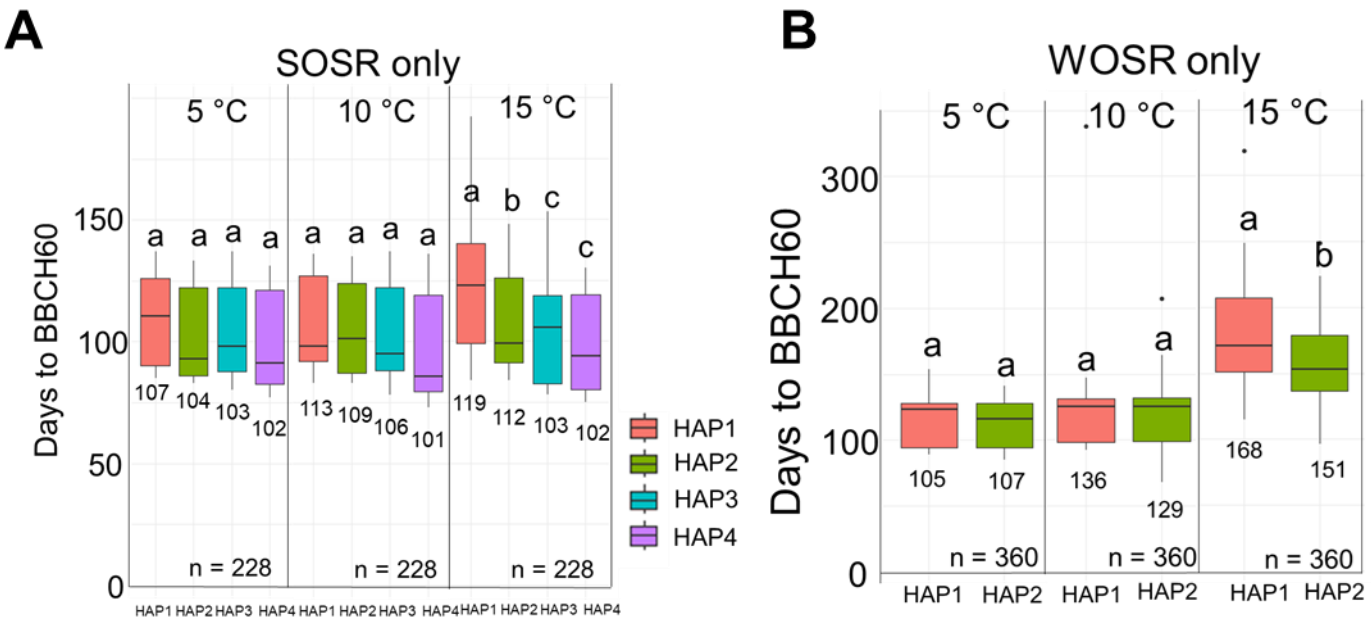


Figure 5.13 Association of *BnaPHP.A05* haplotypes with days to BBCH60 at 3 vernalisation temperatures in SOSR and WOSR. Significant differences at P < 0.05 are shown, calculated by students t-test, with Benjamin-Hochberg adjustment. Mean days to BBCH60 for each haplotype at the respective temperature shown under each box plot. A) Days to BBCH60 from transfer date into vernalisation treatment for SOSR varieties; B) Days to BBCH60 from transfer date into vernalisation treatment for WOSR varieties

Taken together, this suggests that *SNP* variation and the deleted 5'UTR in HAP2 leads to earlier BBCH51 in both SOSR and WOSR after vernalisation at 10 °C and 15 °C but not the lower vernalisation temperature of 5 °C, but only leads to earlier BBCH60 at 15 °C in both SOSR and WOSR (Figure 5.12; Figure 5.13). This is consistent with findings that WOSR, which only has HAP1 and HAP2 varieties, only shows floral time differences at higher vernalisation temperatures (Figure 5.6; Figure 5.7).

This also suggests that the deletions in exon 1 in SOSR HAP3 and HAP4 result in earlier BBCH51 at all vernalisation temperatures compared to the *Darmor* HAP1 (Figure 5.12), but that the deletions only lead to earlier BBCH60 after vernalisation at 15 °C (Figure 5.13). It also suggests that the deletions in HAP4 lead to earlier flowering than deletions in HAP3 (Figure 5.12).

5.2.8 HAP2 is associated with higher *BnPHP.A05* expression than other haplotypes

Next, I determined whether the expression level of *BnaPHP.A05* altered with each haplotype to determine if mRNA expression explained the flowering time variation between haplotypes. To do this, I compared expression level of *BnaPHP.A05* using RPKM values for 21-day old seedlings generated for the diversity set in Harper *et al.* (2012). Varieties with HAP2 showed significantly higher expression of *BnaPHP.A05* than other haplotypes (Figure 5.14). However, HAP3 and HAP4 did not differ in expression from HAP1 (Figure 5.14). This suggests that HAP2 causes increased expression of *BnaPHP.A05.* However, time constraints prevented investigation into protein abundance of each haplotype to determine if the increased mRNA expression of HAP2 alters translation as well as transcription.

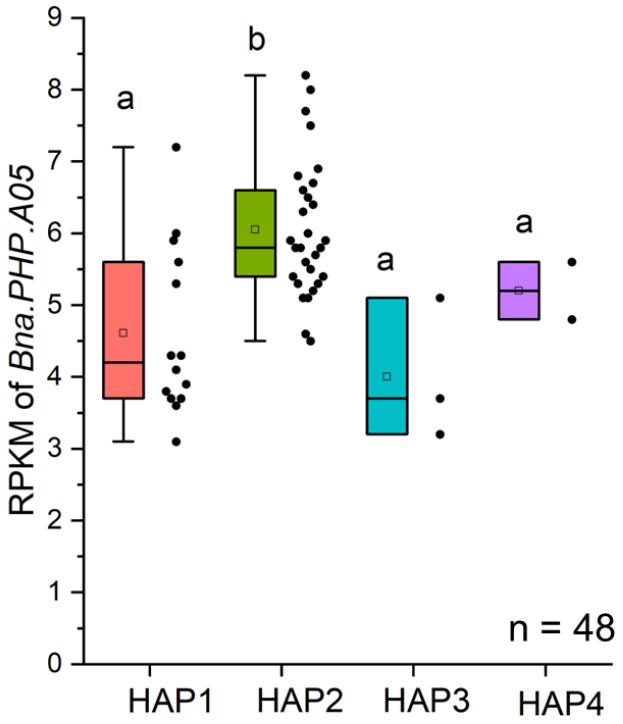


Figure 5.14 In HAP2 lines *BnaPHP.A05* is more highly expressed than all other haplotypes. Expression given in Reads Per Kilobase of transcript per Million mapped reads (RPKM), which accounts for transcript length. Significance determined by one way ANOVA with Tukey post hoc test between haplotypes, and marked by a and b groupings. Expression values correspond to 21-day old *B. napus* seedings from Harper *et al.* (2012).

5.2.9 In SOSR, HAP3 and HAP4 deletions in BnPHP.A05 occur in regions of potential importance for protein function

Extensive work in budding yeast and human CDC73 has characterised key protein domains of the CDC73 protein (Amrich *et al.,* 2010; Cetani *et al.,* 2019). Therefore, to understand how the deletions in HAP3 and HAP4 may affect BnPHP.A05 protein function I downloaded and aligned protein sequences from both PHP proteins in *B. napus,* the human CDC73 homolog, the budding yeast *Saccharomyces cerevisiae* CDC73 homolog and *A. thaliana* PHP protein in order to infer the potential impact of these deletions on key domains identified in other species.

The first deletion in HAP4 is 9-14bp long (starting at position 11763.7kbp) and occurs over amino acid positions 113 to 116 and 113 to 115, depending on the plant variety (Figure 5.15; Figure 5.16). The protein sequence in *B.napus* across this deletion shares no conservation with either human or yeast CDC73 (Figure 5.16). This suggests this protein region has diverged over evolutionary history and may not share similar functions with either human or yeast CDC73 function. Therefore, the role of this deletion cannot be easily inferred.

Next, HAP4 and HAP3 both share a 33-48bp deletion towards the end of exon 1 (starting at position 11763.7kbp) which occurs over amino acid position 155 to 161 (Figure 5.15). This deletion occurs in a region with limited conservation to yeast and human CDC73 (Figure 5.16). This deletion occurs across the interaction domain of the CDC73 yeast protein responsible for binding to other protein components of the Paf1c complex (Chen *et al.,* 2022; Sun *et al.,* 2017). This suggests the deletion could prevent association of PHP with other PAF1C components, thus preventing association of the PAF1C with genes in the *FLC* clade and preventing its activity on *FLC.* However, further protein studies would be needed to confirm if this was the case in *B. napus.*

The second deletion in HAP3 is 38-55bp long (starting at position 11763.9kbp) across amino acid positions 278-296 (Figure 5.15). This deletion causes the BnPHP.A05 protein to come out of frame. This deletion occurs over a region of the protein that is highly conserved across yeast, human, *B.napus* and *A. thaliana:* 11 out of 19 amino acids are conserved between *S. cerevisiae,* and *B. napus* and 14 out of 19 amino acids are conserved between *H. sapiens* and *B. napus* over this region (Figure 5.16). This suggests this region is critical for protein function. In *S. cerevisiae* and *H. sapiens* this region has been characterised as the Ras-like domain. In both *H. sapiens* and *S. cerevisiae*, the Ras-like domain is essential for CDC73 binding to other Paf1c proteins and histone ubiquitination and methylation of CDC73 target genes (Sun *et al.,*

2017; Amrich *et al.,* 2011; Ellison *et al.,*2023; Chen *et al.,* 2021). If the Ras-like domain is mutated the protein cannot function (Sun *et al.,* 2017). This suggests, the deletion in HAP3 may affect the ability of *PHP* to alter its target genes through chromatin modification or bind to other Paf1C subunits. This is consistent with findings that HAP3 is significantly earlier flowering than HAP1 and HAP2 and resembles the early flowering seen in loss of function *A. thaliana* mutants (Park *et al.,* 2010).

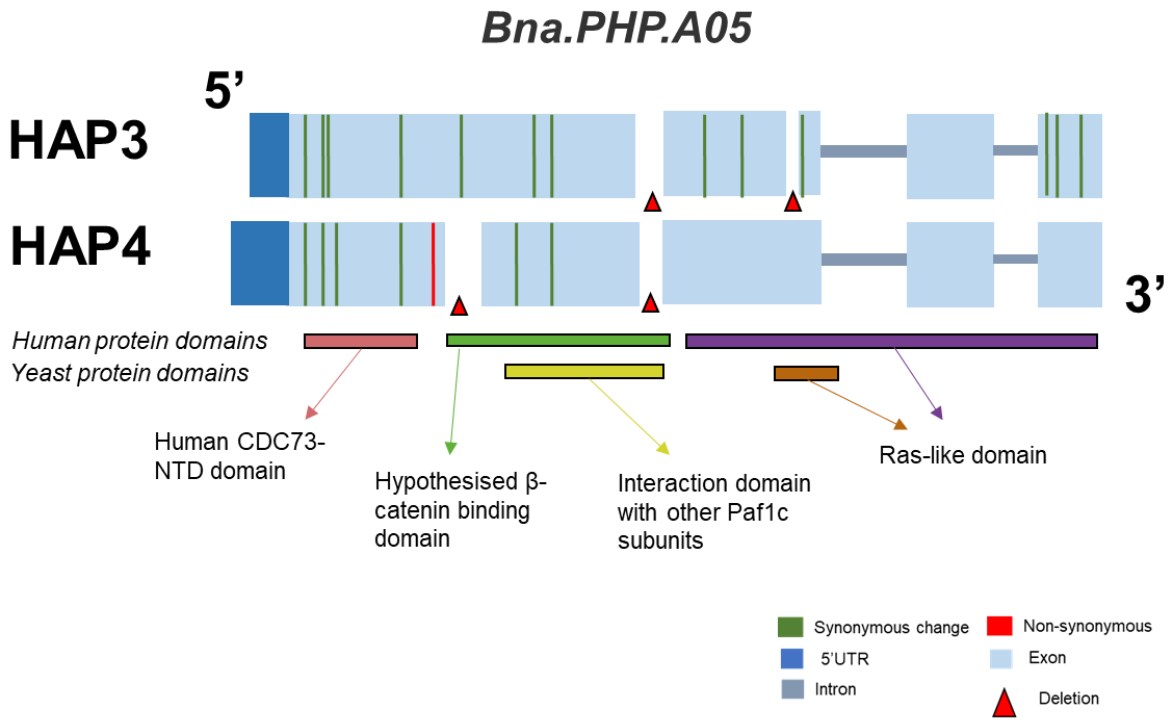
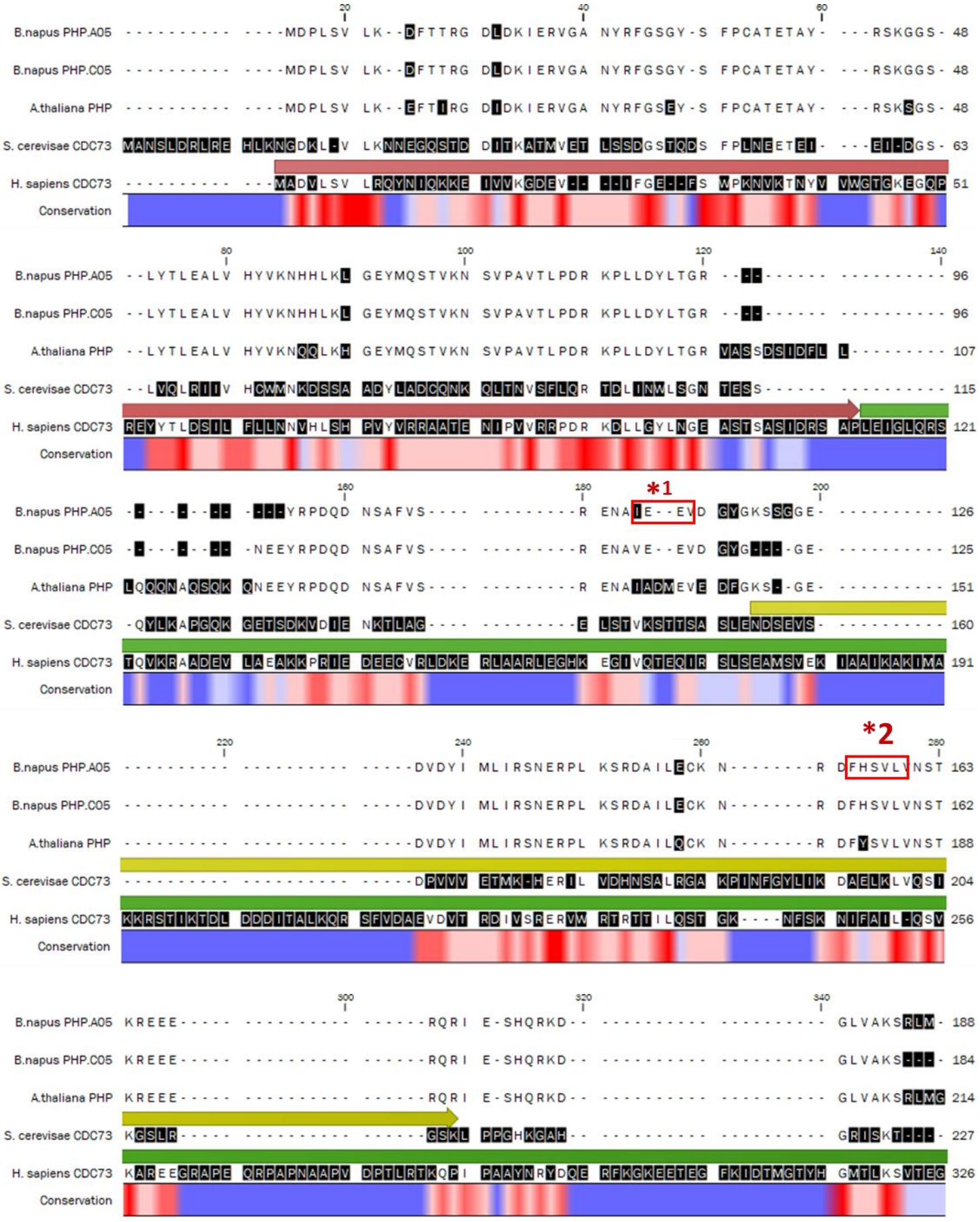
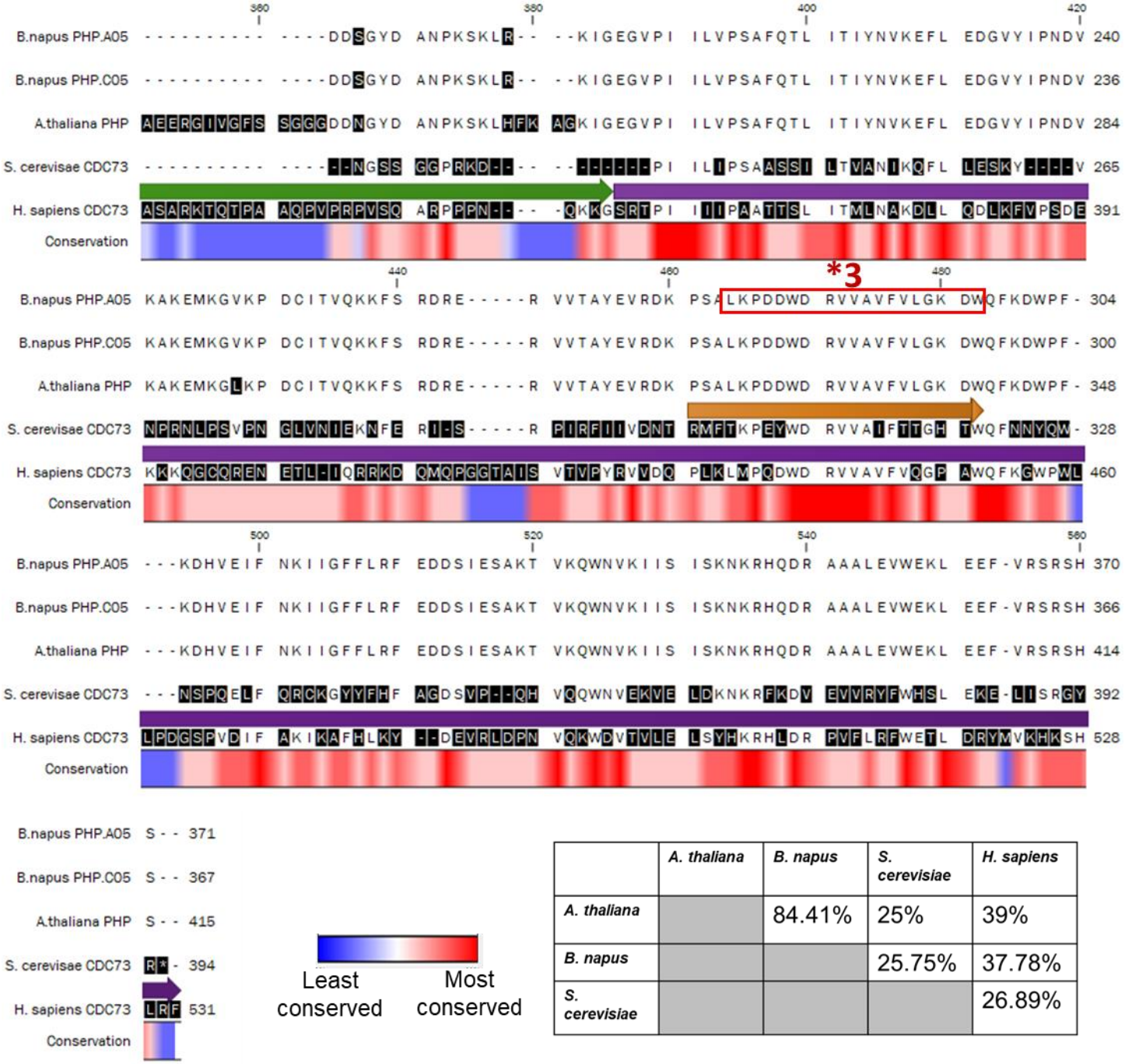


Figure 5.15 Gene model of HAP3 and HAP4 in *BnaPHP.A05* and corresponding interaction domains for both human and yeast CDC73. Coloured bands underneath the gene model represent the known protein domains of human and yeast CDC73 and where they would occur in the *B. napus* gene model based off alignment in Figure 5.16.


B.napus PHP.A05
B.napus PHP.C05
A.thaliana PHP
S. cerevisae CDC73
H. sapiens CDC73
Conservation
*1
*2



	A. thaliana	*B. napus*	*S. cerevisiae*	*H. sapiens*
A. thaliana		84.41%	25%	39%
B. napus			25.75%	37.78%
S. cerevisiae				26.89%

	Human CDC73-NTD	Sun *et al.*, 2017; Masi *et al.*, 2014	NoLS in this region, most pathogenic mutations associated with this region
	Potential human binding domain	Sun *et al.*, 2017	Hypothesised β-catenin interaction domain of CDC73
	Interaction domain with Pa1c proteins	Chen *et al.*, 2022;	*S. Cerevisae* interaction domain (amino acids 155-211) essential for binding to other components of the Pa1c
	Ras-like domain in human CDC73	Sun *et al.*, 2017	Involved in binding to other Pa1c proteins, essential for histone ubiquitination and methylation of CDC73 target genes
	Ras-like domain in *S. cerevisae*	Amrich *et al.*, 2011; Ellison *et al.*,2023; Chen *et al.*, 2021	Essential for binding of Paf1c to chromatin; essential for binding CDC73 with Spt6 and Rbp1, components which are involved in histone modification β-catenin interaction domain of CDC73. Involved in H2B monoubiquitination of target genes.

Figure 5.16 Protein alignment of *B. napus* PHP A05 and C05, *A. thaliana* PHP, *H.sapiens* CDC73, *S. cerevisae* CDC73. Each letter represents an amino acid in the protein sequence of the respective protein, black highlighted amino acids denote where amino acids do not align with at least two other protein sequences in the alignment. Domains of interest are coloured on the alignment as arrows immediately below the protein sequence they refer to and colours indicated in the key. Conservation of protein regions indicated in blue red colour bar and total conservation scores indicated in the table below the alignment. Alignment performed in CLC Main Workbench from protein sequences downloaded from Uniprot.

5.2.10 A *Brassica rapa* R-o-18 *php* mutant is earlier flowering than the outcrossed wild-type

In *A. thaliana, php* mutants result in early flowering through upregulation of the floral repressor *FLC*, with a stronger flowering time effect seen at 10 °C relative to 23 °C (Yu and Michaels, 2010; Park *et al.,* 2010; Nassim *et al.,* 2022).

To understand if *PHP* loss of function also causes early flowering in Brassicas, I phenotyped a *php* mutant *B. rapa* from the R-o-18 TILLING population (Stephenson *et al.,* 2010). I grew mutant line ji41194-b which has a premature stop codon replacing Glutamine at position 126 in the first exon (Figure 5.17) of *BraPHP*, the orthologous copy of *BnaPHP.A05*. This abolishes putative protein domains of importance for *PHP* function (Figure 5.17). I used a *B. rapa* mutant as the only mutants for *Brassica napus* is Cabriolet, a HAP2 variety, where a mutation may not show a strong effect as HAP2 is already earlier flowering (Figure 5.13).

B. rapa php mutants bolted before outcrossed wild-type plants, reaching BBCH51 6.89 days earlier ($p = 0.0031$) than the outcrossed wild type and BBCH60 4.55 days faster ($p = 0.0025$) (Figure 5.17). Therefore, it appears common across the Brassicaceae that loss-of-function mutations in *PHP* cause earlier flowering. This also suggests that HAP3 and HAP4 are loss-of-function mutations that lead to earlier flowering in *B. napus*, relative to HAP1.

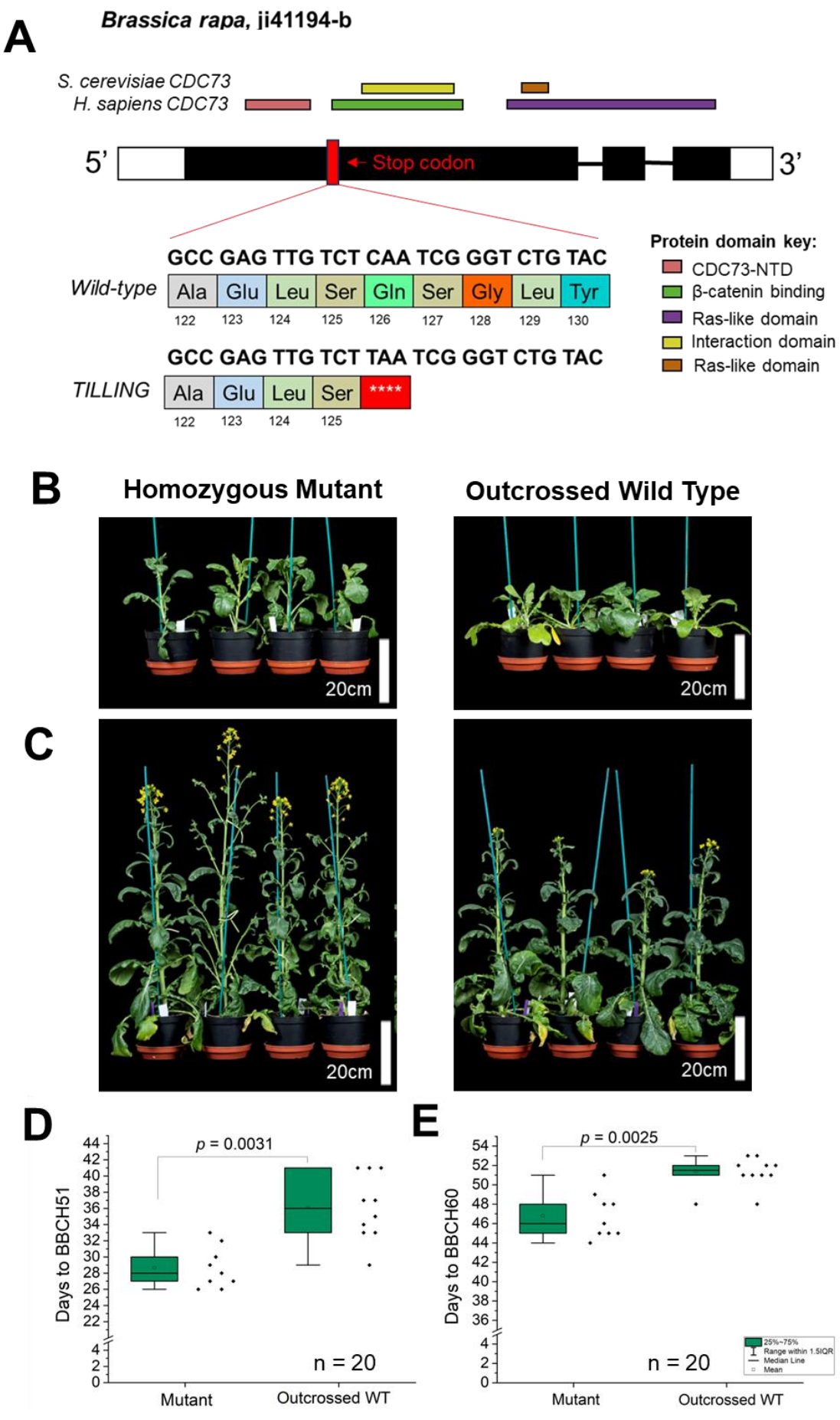

A
Brassica rapa, ji41194-b
S. cerevisiae CDC73
H. sapiens CDC73
5'
Stop codon
3'
GCC GAG TTG TCT CAA TCG GGT CTG TAC
Wild-type
Ala Glu Leu Ser Gln Ser Gly Leu Tyr
122 123 124 125 126 127 128 129 130
GCC GAG TTG TCT TAA TCG GGT CTG TAC
TILLING
Ala Glu Leu Ser ****
122 123 124 125
Protein domain key:
CDC73-NTD
β-catenin binding
Ras-like domain
Interaction domain
Ras-like domain
B
Homozygous Mutant
Outcrossed Wild Type
20cm
20cm
C
20cm
20cm
D
p = 0.0031
Days to BBCH51
n = 20
Mutant
Outcrossed WT
E
p = 0.0025
Days to BBCH60
n = 20
25%~75%
Range within 1.5IQR
Median Line
Mean
Mutant
Outcrossed WT

Figure 5.17 The *bra.php.a05* mutant bolts, reaches BBCH51 and BBCH60 earlier than outcrossed wild-type *Brassica rapa*. A) Premature stop codon in exon one of *B. rapa* mutant; amino acid positions indicated below amino acids. B and C) Mutant plants flower earlier than wild-type outcrossed lines, 20cm scale bar provided for comparison. D-E) Mutant lines reach BBCH51 and BBCH60 (after sowing) significantly earlier than outcrossed wild-type, significance determined by students t-test.

5.3 Discussion

The PAF1c is highly conserved across eukaryotes and contributes to many aspects of RNA polymerase II (PolII) transcriptional regulation, playing an essential role in transcription elongation, histone modifications and chromatin remodelling of target genes (Selth *et al.,* 2010). In plants, the PAF1c subunit *PHP* shows activity limited to control of flowering time, potentially through recruiting the Paf1c to flowering time specific genes and causing chromatin modification, although the exact reason for this remains unclear (Park *et al.,* 2010; Yu and Micahels, 2010). Here, I present evidence that *BnaPHP.A05,* but not *BnaPHP.C05,* shares a conserved role in controlling temperature responsive floral development, before and after floral development, in an important winter annual crop, *Brassica napus.*

5.3.1 Haplotype variation at *BnaPHP.A05* influences flowering time at higher vernalising temperatures.

GWAS analysis indicated that SNP variation at *BnaPHP.A05* was associated with the effect of temperature on thermal time to flowering. I identified that a T SNP, relative to a C SNP, was associated with early floral development, before and after the floral transition, in both SOSR and WOSR at vernalising temperatures above 10 °C (Figure 5.6; Figure 5.7). Later, I identified three early flowering haplotypes that were associated with the early flowering T SNP (HAP2, HAP3, HAP4) and one late flowering haplotype (HAP1) that was associated with a C SNP (Figure 5.11).

HAP1 was identical to the Darmor *bzh* reference sequence and did not show early flowering relative to the population average so was considered to be a functional copy of *BnPHP.A05.* HAP2 occurred in both WOSR and SOSR and was associated with early flowering in SOSR and WOSR at vernalising temperatures above 10 °C and earlier budding in WOSR at 10 °C and SOSR and WOSR at 15 °C (Figure 5.12). HAP2 differed from HAP1 in several SNPs and a shorter 5' UTR (Figure 5.11). Only one SNP resulted in an Alanine to Valine substitution.

However, considering the similarity and charge for both amino acids I concluded this was unlikely to result in a change to protein structure.

In *A. thaliana,* a deletion in the 5' UTR of the PAF1c *VIP3* genes causes the *ths1* touch insensitivity mutant (Jensen *et al.,* 2016). The *ths1* mutant is earlier flowering, has increased *VIP3* expression, similar to HAP2 (Figure 5.14), and shows reduced H3K36me3 deposition on *VIP3* target genes (Jensen *et al.,* 2016). Likewise, in *H. sapiens* a 5'UTR deletion in the *PHP* ortholog *CDC73* has been associated with HPT-JT syndrome, a syndrome that often occurs when the CDC73 protein is non-functional (Guarnieri *et al.,* 2017). This suggests that the 5'UTR deletion seen in *BnaPHP.A05* HAP2 could also affect flowering time and show reduced protein function. The 5'UTR is important for regulation of gene translation. Therefore, I hypothesise that a deletion in the 5' UTR may alter protein function in HAP2, through as-yet unknown post-translation modification. This in turn causes earlier flowering. It is unclear why the effect of HAP2 occurs at higher vernalising temperatures. A lack of time prevented me investigating protein abundance of HAP2 compared to the other haplotypes or determining the ability of the HAP2 BnaPHP.A05 protein to bind to target genes. This would be necessary to confirm the effect of the 5'UTR deletion in HAP2.

HAP3 and HAP4 were only present in SOSR and resulted in earlier budding at 10 °C and 15 °C, and earlier flowering at 15 °C only (Figure 5.11; Figure 5.12). Both HAP3 and HAP4 have a deletion in the BnaPHP.A05 protein (amino acid 115-161) that shares conservation with the interaction domain of yeast CDC73 (Figure 5.16; Chen *et al.,* 2022). In budding yeast, the *PHP* ortholog *CDC73* is essential for interacting with other PAF1c subunits and recruiting the PAF1c to target genes (Francette *et al.,* 2021). The target genes of *PHP* in *A. thaliana* are limited to flowering time genes including *FLC, FLM, MAF4* and *MAF5* (Park *et al.,* 2010; Yu and Michaels, 2010). This suggests that if the HAP3 and HAP4 deletion occurs over the interaction domain of *B. napus,* as in budding yeast, the BnPHP.A05 protein would have reduced ability to bind with other PAF1c components and show reduced recruitment to target genes, including *FLC* and its clade members. This in turn may explain the earlier flowering phenotype. However, it is not yet clear whether BnaPHP.A05 targets *FLC* in *B. napus.* Comparing expression of *Bna.FLC* or *Bna.MAF4-5* between functional BnPHP.A05 and non-functional BnPHP.A05 protein in HAP3 and HAP4 would confirm this.

In addition to the deletion at amino acid position 115-161 HAP3 lines also contain a mutation in a domain that shares very high conservation with Ras-like domains of human and budding yeast CDC73 (Figure 5.15). In humans and budding yeast, the Ras-like domain of CDC73 is important for binding to other Paf1c components and is essential for methylation and histone ubiquitination of target genes (Sun *et al.,* 2017; Amrich *et al.,* 2011; Ellison *et al.,* 2023; Chen

et al., 2021). A deletion in a potential domain involved in making histone modifications could prevent upregulation of *FLC* through deposition of H3K4me3, thereby leading to earlier flowering.

It is unclear why HAP4 buds significantly earlier than HAP3 but is not earlier flowering than HAP3 (Figure 5.11; Figure 5.12). HAP4 contains a deletion across the amino acid positions 113-116 (Figure 5.15). This region contains no conservation with other protein sequences from yeast or humans. In humans this region is associated with CDC73 binding to β-catenin (Sun et al., 2017). The closest homolog to β-catenin in *A. thaliana* is *ARABADILLO-1* and *2*, primarily active in lateral root development (Coates et al., 2006). *PHP* is involved in root angle development in tomato (Toal et al., 2018), although it is unclear how this may affect flowering time. Considering the lack of conservation of this region, it is likely this region has undergone protein changes associated with functions specific to plants. Further protein studies would be needed to confirm the significance of the unique deletion in HAP4.

However, all the deletions in HAP3 and HAP4 lead to earlier flowering. This is consistent with the early flowering *B. rapa* ji41194-b mutant where the interaction and Ras-like domains are knocked out and this causes earlier flowering (Figure 5.17).

5.3.1.1 Temperature specific haplotype effects

It is unclear why genetic differences at *Bna.PHP.A05* only affect time to BBCH60 at vernalising temperatures of 15 °C, but affect time to BBCH51 at vernalising temperatures of 5 °C, 10 °C and 15 °C. At higher vernalisation temperatures, *FLC* shutdown will be slower therefore *BnaPHP.A05* may still affect floral development at these higher temperatures. Overall, this phenomenon suggests that the activity of *BnaPHP.A05* primarily affects floral development by affecting time to budding, but not flowering, after floral initiation. This is consistent with findings that almost all *FLC* genes, targets of *PHP,* in *B. napus* are silenced before flowering including late acting *BnaFLC* genes (O'Neill *et al.,* 2019). Flowering itself may instead be conferred through floral promotive signals of long warm days of spring. This is similar to control of winter short-day floral transition seen in *A. alpina. A. alpina* can undergo floral transition if it is maintained in prolonged chilling conditions, this occurs through silencing of the *FLC* homolog *PEP1* and action of gibberlin and *SPL15* (Hyun *et al.,* 2019; Tilmes *et al.,* 2019). If *A. alpina* is returned to warm long days it will flower instead through action of the photoperiod pathway (Hyun *et al.,* 2019).

5.3.2 Potential role of *BnaPHP.A05* in bud dormancy control

I had previously hypothesised that *MAF4* and *MAF5* are involved in bud dormancy control in *B. napus* (Chapter 4; Figure 4.13). *BnaFLC.C02* and *BnaFLC.A03b* have also been implicated in bud dormancy control in my work and work published alongside this PhD (Chapter 4; Figure 4.11: Lu *et al.,* 2022). The basis for enhanced *BnaFLC.C02* and *BnaFLC.A03b* expression during bud dormancy is through deposition of H3K4me3 mark, a mark associated with *FLC* upregulation by *PHP* (Park *et al.,* 2010). As *BnaPHP.A05* controls floral development after floral transition and is known to target *FLC, MAF4* and *MAF5* in *A. thaliana* (Yu and Michaels, 2010), it is possible that *PHP* could also be implicated in dormancy control. This is further supported by the fact that all Swedes and SWOSR, crop types that don't show bud dormancy, all have the T SNP associated with earlier floral development (Figure 5.2). Indeed, the original GWAS trait where *BnPHP.A05* was discovered is associated with the effect of temperature on floral development after the floral transition (Chapter 4; Figure 4.9), a trait strongly associated with the presence of bud dormancy.

BnPHP.A05 affects time to flowering both before and after floral initiation (Figure 5.8). This suggests that *BnPHP.A05* may upregulate the five *Bna.FLC* copies that control floral timing but are downregulated before floral initiation (O'Neill *et al.,* 2019) as well as the two *Bna.FLC* copies, *Bna.FLC.C02* and *Bna.FLC.A03b,* that we recently showed are only downregulated after floral initiation and control bud dormancy (Lu *et al.,* 2022). We recently showed that in warmer temperatures when bud dormancy is activated (Figure 3.4; Figure 3.16), the two *Bna.FLC* copies active after floral initiation show enhanced H3K4me3 deposition, a mark deposited by *PHP* (Park *et al.,* 2010). However, as yet there is no direct evidence that *BnaPHP.A05* leads to upregulation of these dormancy associated genes. A transcriptional time series, or *BnPHP.A05* mutants in a HAP1 background would confirm this. If this was the case, *BnaPHP.A05* would be a key target for breeders interested in reducing the warm winter yield penalty associated with bud dormancy or as a general target for earlier flowering.

5.4.3 Implications

The timing of flowering in *B. napus* is of considerable agronomic importance. Here I have identified genetic variation in *PHP*, an *FLC* regulator, is associated with flowering time control in both vernalisation requiring WOSR varieties and SOSR varieties and *B. rapa* R-o-18 which do not require vernalisation (Figure 5.17; Figure 5.4: Figure 5.5). In R-o-18 *B. rapa, BraFLC.A03a* influences flowering time independently of *SOC1* and vernalisation (Calderwood *et al.,* 2021b). It is still unclear whether all or only some *FLC* paralogs in *B. napus* are targeted by *BnaPHP.A05,* however this suggests that *BnaPHP.A05* could lead to upregulation of *FLC* copies regardless of their role in vernalisation. This would provide a key target gene for breeders interested in altering flowering time across *B. napus* crop types.

Chapter 6: General Discussion

6.1 Chapter Summaries

6.1.1 Chapter 3: Winter warmth post floral initiation delays floral development in winter type *Brassica napus*

The UK is experiencing warmer and more variable winter temperatures (Luedling, 2012). Recently, it was shown that winter oilseed rape (WOSR) *Brassica napus* will pass through the floral transition in late November (O'Neill et al., 2019) when temperatures are close to 10 °C. This is consistent with theoretical predictions that suggest the vernalisation requirement in *B. napus* is fulfilled during autumn (Habekotte, 1997) and that temperatures as high as 17 °C are sufficient for vernalisation in *B. napus* (Tommey and Evans, 1991). However, this is contrary to the expectation that winter annuals only undergo floral development under inductive long days and warm temperatures of spring, suggested from laboratory studies in *A. thaliana* (Whittaker and Dean, 2017). Interestingly, warmer than average conditions in late November and early December, after which WOSR is assumed to have undergone the floral transition, are associated with reduced yield (Brown *et al.,* 2019). A similar effect has also been observed in China (He *et al.,* 2017). This suggested that chilling of developing buds in winter is important for optimal yield production in WOSR *B. napus,* in a process that is independent of the control of floral initiation.

To understand the importance of temperature after floral initiation in *B. napus*, I phenotyped a diversity set of *B. napus* from the Diversity Fixed Foundation Set (Harper et al., 2012) exposed to transient warm or control winter conditions following floral initiation. I investigated floral development and seed characteristics to understand the effects of post floral initiation warmth on *B. napus* development. Phenotyping a large diversity set allowed me to determine the extent of variation to warmth after floral initiation in *B. napus.*

In the diversity set analysis, I determined that the response to winter warmth is influenced by crop type (Figure 3.4). Most WOSR varieties showed significantly delayed floral development following transient winter warming of developing buds (Figure 3.4), this was also true in a repeat experiment (Figure 3.15). I concluded that this shows physiological similarities to how dormant perennial buds respond to warming during perennial bud dormancy. For the first time, this demonstrates that temperature immediately after floral transition can delay flowering time

in a major winter annual crop. Meanwhile, SWOSR and Swedes showed significantly advanced floral development while some SOSR varieties are advanced and some delayed following post floral initiation warming. This indicated that there is there is variation to winter warming across *B. napus.* This also suggested that breeding between crop types led to differences in response to post floral initiation warmth.

To ensure that it was temperature variation alone that was contributing to the effect of temperature on development, and this reflected real world conditions, I recreated conditions from a high yielding (2010-11) growing season with a cool winter and a low yielding (2015-16) growing season with a warm winter in Controlled Environment Rooms (CERs). I kept all other conditions consistent between treatments. I showed that recreating temperature conditions from past growing seasons alone is sufficient to see delays to floral development. This led to floral delays between 11.9 to 26.6 days (Figure 3.21). In the field these delays would shift reproductive development into harsh summer conditions.

Interestingly, this effect of winter warming on floral development correlated with flowering time: warming delays the development of late flowering lines but advances the development of early flowering lines (Figure 3.3). This suggested that there was a genetic control of this floral delay and may explain why there is such a strong crop type effect as flowering time genes are known targets differentiating crop types (Schiessl *et al.,* 2019). This discovery formed the basis for further investigation in this thesis.

Next, I confirmed results from a correlative study (Brown *et al.,* 2019) to show that warming after floral initiation leads to reductions in seed characteristics implicated in yield formation in WOSR varieties only. In WOSR varieties, TGW (g) was significantly lower in warmed compared to control conditions (Figure 3.10). Equally, WOSR plants unaffected by CER fertility issues produced fewer seeds per pod in the warmer winter CER experiment compared to the cooler winter (Figure 3.22). In the diversity set experiment, the extent of TGW reduction in WOSR lines related to the extent to which floral delay was impacted by warming. This suggested that warming induced floral delay may directly influence yield formation, through as-yet undefined mechanisms.

Overall, the results from this chapter underscore the importance of detailed phenotypic experiments in crop species. Many studies predicting the effects of climate change on oilseed rape have assumed that vernalisation is a winter-long phenomena. Yet here I uncovered a previously unknown requirement for winter chilling after floral initiation that has implications on final yield formation. Critically for breeding efforts in a changing climate, I uncovered phenotypic variation both within and across crop types in the presence of post floral initiation floral delay and TGW reduction in WOSR lines.

6.1.2 Chapter 4: Genetic variation in warming induced flower bud dormancy in *Brassica napus*.

B. napus is the third most important oil crop globally and of huge economic importance across the world (Wang et al., 2018). Recently it was discovered it undergoes autumnal, not spring, floral initiation (O'Neill et al., 2019), after which warm temperatures are associated with yield loss (Brown et al., 2019; Lu et al., 2022). In Chapter 3, I discovered that if temperatures are warmer than average following floral initiation there is delayed reproductive development and reduction in key yield components. In Chapter 4 I investigated the genetic control of delayed reproductive development. To do this, I compared transcriptomic responses to warming between a variety that was developmentally retarded by winter warmth (Castille, a WOSR) and one that was not (Ningyou7, a SWOSR).

To identify genes that regulate warmth induced bud dormancy, I compared warmed and control transcriptomic profiles over a time course for the contrasting varieties. Hierarchical clustering and PCA analysis of all samples demonstrated that Castille has a strong transcriptional response to warmth that is not present in Ningyou7. Under warming conditions, Castille showed enrichment for processes associated with bud dormancy including response to abscisic acid (GO:009737) (Figure 4.4). Key ABA and dormancy associated genes *BRC1, ABF3, RD29B* and *DRM1* were all upregulated in warmer Castille samples (Figure 4.5). By contrast, Ningyou7 appeared to not show genetic markers of bud dormancy and instead progressed with reproductive development, with reproductive processes (GO:0022414) and regulation of floral development (GO:0009910) occurring (Figure 4.4). This potential bud dormancy response in Castille resembles what has been reported in WOSR Cabriolet (Lu et al., 2022) and BRC1-mediated dormancy control in hybrid aspen (Singh et al., 2018). This suggests that in WOSR Castille, warmth induces dormancy through a well-known process that occurs in perennials.

As chilling promotes floral development but warming promotes reproductive delay in Castille, I next compared the expression of floral and vernalisation related genes between the two varieties. I identified that three copies of the key floral repressor, *Bna.FLC.C02, Bna.FLC.C03a* and *Bna.FLC.C03b* were missing in Ningyou7 as was *Bna.MAF4.A02a*, *Bna.MAF4.A02b*, and *Bna.MAF3.A02,* but only *Bna.FLC.C02* was missing in Castille (Figure 4.7). I then identified that, except for *Bna.FLC.C02,* two other SWOSR varieties were also missing these genes (Figure 4.6B; 4.7). Critically, I then determined that putative *FLC* targets expressed in warmed Castille samples related to ABA and dormancy associated genes but the putative *FLC* targets expressed in Ningyou7 samples were associated with floral organ development and

reproductive transitions (Figure 4.8). This suggested that higher *FLC* activity in Castille permits expression of genes associated with bud dormancy while lower *FLC* activity in Ningyou7 permits expression of genes associated with floral development and growth potential.

Next, I used a GWAS study and Gene Expression Marker (GEM) Analysis to identify genomic markers associated with the effect of warmth on time to flowering. A marker associated with *BnPHP.A05* was associated with the effect of temperature on time to flowering. Plant Homologous to PARAFIBROMIN (PHP) is a component of the PAF1-c and upregulates *FLC* and its clade members through deposition of the active H3K4me3 mark and, unlike other PAF1c components, has a role limited to flowering time (Yu and Michaels, 2010; Park et al., 2010). This again implicated *BnaFLC* control in the response to warmth after the floral transition.

GEM analysis identified that *Bna.FLC.C02* expression is higher in plant varieties that took longer to reach BBCH60 in response to warmth (Figure 4.11). This is intriguing as *Bna.FLC.C02* is one of few *FLC* copies active after floral initiation (O'Neill et al., 2019) and haplotype variation at *Bna.FLC.C02* was associated with the presence of the bud dormancy phenotype (Lu et al., 2022). This suggested that *Bna.FLC.C02* has a prominent role in time to flowering in response to temperature after floral initiation.

6.1.3 Chapter 5: The role of Plant Homologous to Parafibromin (PHP) in reproductive development in Brassica

Plant Homologous to PARAFIBROMIN (PHP) is a component of the PAF1C complex. *php* mutants in *A. thaliana* are known to be early flowering (Yu and Michaels, 2010; Park *et al.,* 2010). Here, I present the first study in a crop species showing the conserved role of *PHP* in flowering time control. I explored Single Nucleotide Polymorphism (SNP) and haplotype variation at the *B. napus PHP* copy on chromosome A05 and C05. I determined that *BnPHP.C05* did not affect flowering time (Figure 5.10). However, I identified specific haplotypes of *BnPHP.A05* that led to earlier flowering in both winter and spring type *B. napus.* This data is directly relevant to *B. napus* breeders interested in altering the key agronomic trait of flowering time (Figure 5.11; Figure 5.12).

GWAS Analysis in Chapter 4 was used to identify that SNP variation at Cab041204.2:750 marker on the BnaA05g17020D gene (*BnPHP.A05*), which corresponded to position 750 in exon 1, was associated with the effect of winter warming on time from floral initiation to first flower emergence. Across the DFFS diversity set *B. napus* either had a Cytosine (C), Thymine (T), No data (N) or an ambiguous call (Y) at Cab041204.2:750. To assess the importance of SNP variation at Cab041204.2:750 I analysed the time to bud emergence and first flower

emergence between C and T SNP calls. In both winter and spring type *B. napus* varieties with a T SNP call reach BBCH51 earlier but only in spring type *B. napus* do varieties with a T SNP call reach BBCH60 earlier across two independent large-scale experiments (Figures 5.4 – 5.7). Varieties with a T SNP call also reached floral initiation earlier than varieties with a C SNP call, this suggests that the T SNP call was associated with early floral development before and after floral initiation (Figures 5.9).

Next, I determined that the early flowering T SNP call was associated with three different early flowering haplotypes of *BnPHP.A05* (HAP2, HAP3, HAP4) assumed to be loss of function. The C SNP call was associated with the only later flowering haplotype (HAP1). Of the early flowering haplotypes, HAP2 occurred in both winter and spring type *B. napus* and was associated with early flowering when plants had been vernalised at temperatures above 10 °C (Figure 5.12; Figure 5.13). The earlier flowering HAP2 had minor SNP variation and a shorter 5'UTR (Figure 5.11). I hypothesised that post-translational modifications arising from the shorter 5'UTR caused the flowering time differences in HAP2 compared to HAP1. Meanwhile, HAP3 and HAP4 were even earlier flowering than HAP2 but only present in spring type *B. napus* (Figure 5.12; Figure 5.13). HAP3 and HAP4 had deletions in regions that correspond to the interaction domain in *S. cerevisaeae* CDC73 which in budding yeast is known to affect the ability of CDC73 to interact with other PAF1C subunits. HAP3 also had a deletion in a region that corresponds to the Ras-like domain of CDC73 in both *S. cerevisaeae* and humans, which affects the ability of CDC73 to interact with other PAF1C subunits and for PAF1C to perform chromatin modifications (Figure 5.15; Figure 5.16). HAP4 had a unique deletion in a region of the protein that shares no conservation with other PHP like proteins. I later confirmed the effect of the HAP3 and HAP4 deletions in an investigation on the *B. rapa* mutant of *PHP,* which reached BBCH51 and BBCH60 significantly earlier than the outcrossed wild-type (Figure 5.17). This suggested HAP3 and HAP4 were loss of function mutants.

6.2 Implications of Research

Here, I investigated the role of winter warmth following autumnal floral initiation in *B. napus* and discovered that there is a requirement for chilling in developing floral buds in winter-type *B. napus.* This chilling requirement affects flowering time, the length of the vegetative phase and has implications on final yield for *B. napus*.

I conclude that WOSR shows bud dormancy in response to winter warmth. This is because winter warming delays reproductive development, while expression of ABA signalling and dormancy associated genes increases and growth-related genes decreases. These are all

hallmarks of bud dormancy (Vimont *et al.,* 2018; Falavinga *et al.,* 2019). I provide evidence that this is controlled through *FLC.*

6.2.1 The potential role of *FLC* in controlling bud dormancy

In perennial species, *SVP-like* or *DORMANCY ASSOCIATED MADS BOX* (*DAM*) genes control bud dormancy (Singh *et al.,* 2018; Falavinga *et al.,* 2019; Vimont *et al.,* 2019; Zhang *et al.,* 2018). *DAM* genes are epigenetically silenced over winter like *FLC* is during vernalisation (Lloret *et al.,* 2018). *DAM* genes have been shown to bind to *BRC1* and ABA biosynthesis genes to control differing stages of dormancy in apple (Falavinga *et al.,* 2021). In other perennial species *DAM* expression corresponds with the expression of ABA signalling and biosynthesis genes (Hovarth *et al.,* 2009; Vimont *et al.,*2019; Lloret *et al.,* 2018).

However, *B. napus* does not have *DAM* genes. Here, I suggest that *Bna.FLC* genes and potentially *Bna.MAF* genes control the response to bud dormancy instead. This is evidenced by the lack of *Bna.FLC.C03b, Bna.FLC.C03a, Bna.MAF3.A02, Bna.MAF4.A02a, Bna.MAF4.A02b* expression in SWOSR varieties that lack bud dormancy compared to WOSR varieties that show bud dormancy (Figure 4.7). It is also evidenced by the fact that higher *FLC* activity in the WOSR Castille variety is associated with expression of dormancy and ABA biosynthesis and signalling genes, including genes that *DAM* genes are known to bind to such as *BRC1* (Figure 4.8; Table 4.2). Further evidence is provided by Gene Expression Marker Analysis (GEM), which directly implicated *Bna.FLC.C02* in the effect of heating on time to BBCH60, a trait associated with bud dormancy (Figure 4.10). The expression level of *Bna.FLC.C02* correlates with the presence of bud dormancy (Figure 4.10). Both *Bna.FLC.C02* and *Bna.FLC.A03b* are unaffected by temperature before floral initiation (O'Neill *et al.,* 2019) and are only silenced after the floral transition (Lu *et al.,* 2022). Under warm winter conditions, when bud dormancy occurs in WOSR (Figure 3.4), both *Bna.FLC.C02* and *Bna.FLC.A03b* are more highly expressed and show greater deposition of the active H3K4me3 mark (Lu *et al.,* 2022), suggesting the expression of these two *Bna.FLC* genes are strong candidates for the control of bud dormancy.

Taken together, this suggests that expansion of *Bna.FLC* gene copy number in *B. napus* may have facilitated the evolution of a secondary requirement for winter cold following floral initiation, this would be consistent with previously reported individual roles of *Bna.FLC* genes (Table 1.1). There is likely some redundancy to the control of bud dormancy, as is seen in Castille, a WOSR with bud dormancy but without *Bna.FLC.C02* expression (Figure 4.7). This is consistent with suggestions that expansion of *DAM* genes during Rosaceae evolution facilitated control of multiple stages of bud dormancy (Liu *et al.,* 2020) as well as the findings

that temporal expression of different *MdDAM* genes control different stages of bud dormancy in apple (Falavinga et al., 2019). Inducing the expression of individual *FLC* genes in transgenic *B. napus* in control winters and determining the extent to which they phenocopy warm winter plants at different stages of bud dormancy would confirm whether the expression of *FLC* itself can induce bud dormancy in *B. napus.* Comparative transcriptomics between *FLC* induced and non-induced lines could then determine the putative targets of *FLC* genes involved in bud dormancy. This would help identify how warmer winters are also associated with yield decline.

I also showed that the known *FLC* regulator *BnaPHP.A05* is associated with the time to flowering both before and after the floral transition (Figure 5.3; Figure 5.9). This suggests *BnaPHP.A05* upregulates all *FLC* genes in *B. napus* regardless of their role in vernalisation or bud dormancy. This is supported by findings in both SOSR and *B. rapa* plants with loss of function *PHP* copies that are early flowering but do not require vernalisation (Figure 5.5; figure 5.6; Figure 5.17). Therefore, future study on the effect of bud dormancy should focus on *BnaFLC.C02* and *BnaFLC.A03b* which appear to have a more specific effect.

However, it is not yet clear whether *FLC* by itself, or the *Bna.MAF* genes, can directly induce bud dormancy, although *FLC* is known to induce seed dormancy in *A. thaliana* (Chiang et al., 2009; Hughes et al., 2019). A potential role of *FLC* in bud dormancy has been shown in *A. alpina*. In *A. alpina,* the *FLC* orthologue *PEP1* represses gibberellin during winter to prevent floral development (Tilmes et al., 2019). After vernalisation, when *PEP1* is silenced, increases in gibberellins and *SPL15* promote the floral transition during chilling and short days in an *FT*-independent manner (Tilmes et al., 2019; Hyun *et al.,* 2019), after this the floral bud overwinters while *PEP1* is reactivated to prevent axillary bud outgrowth before spring flowering (Lazaro et al., 2018; Hyun et al., 2019; Figure 1.6). It is feasible that the five *Bna.FLC* genes silenced immediately before floral initiation in *B. napus* are involved in the initial vernalisation response whilst *Bna.FLC.C02* and *Bna.FLC.A03b* may control bud dormancy.

Emerging evidence also suggests *FLC* can have a direct role in bud dormancy in some perennial species. In apple, it was shown that a *MdFLC-MdSVPa* complex can bind to the ABA biosynthesis gene *NCED4* which may be important for dormancy induction (Falavinga et al., 2021). *FLC* is also highly expressed during dormancy in plum alongside *DAM* and *SVP* genes (Zhang *et al.,* 2018), suggesting a direct role of *FLC* in dormancy control in perennials. By contrast in *A. thaliana flc* mutants are known to have reduced expression of many genes implicated in bud dormancy control including ABA biosynthetic genes (Edwards et al., 2006; Deng et al., 2011; Mateos et al., 2015). Transgenic studies in *A. thaliana* have also shown that the ABA responsive gene *ABA-INSENSITIVE 5 (ABI5),* which plays a key role in ABA-arrested seed germination, can promote *FLC* expression through binding to the *FLC* promotor to delay

the floral transition in long and short days (Wang et al., 2013; Xiong et al., 2019). *ABA-INSENSITIVE 4* (*ABI4)* will also upregulate *FLC* and repress GA biosynthesis (Shu et al., 2016, 2018), an important mediator of flowering under short days. As ABA and GA are central regulators of bud dormancy, this suggests that *FLC*-like genes could control chilling responses involved in bud dormancy.

The link between *FLC* expression and flowering time is well established in *A. thaliana*, as is the necessity for winter cold to lower the expression of *FLC* (Blümel *et al.,* 2015; Henderson *et al.,* 2003). If *FLC* can also control bud dormancy in *B. napus,* as evidence here suggests, this implicates a well-known floral repressor in final yield formation, as the presence of bud dormancy associates with lower final yield in WOSR (Figure 3.10). In overwintering perennial buds, disruption to bud dormancy can cause yield abnormalities through delayed anthesis and disrupted floral bud formation (Atkinson *et al.,* 2013). We recently showed similar floral defects occur in warm winters in *B. napus* (Lu *et al.,* 2022). Further work is needed to understand the effect of *BnaFLC* copies on final yield formation, such as identifying *Bna.FLC* targets active in floral organs that lead to these floral defects that may cause low yields. This information will be invaluable to breeding climate resilient rapeseed.

6.3 Concluding Remarks

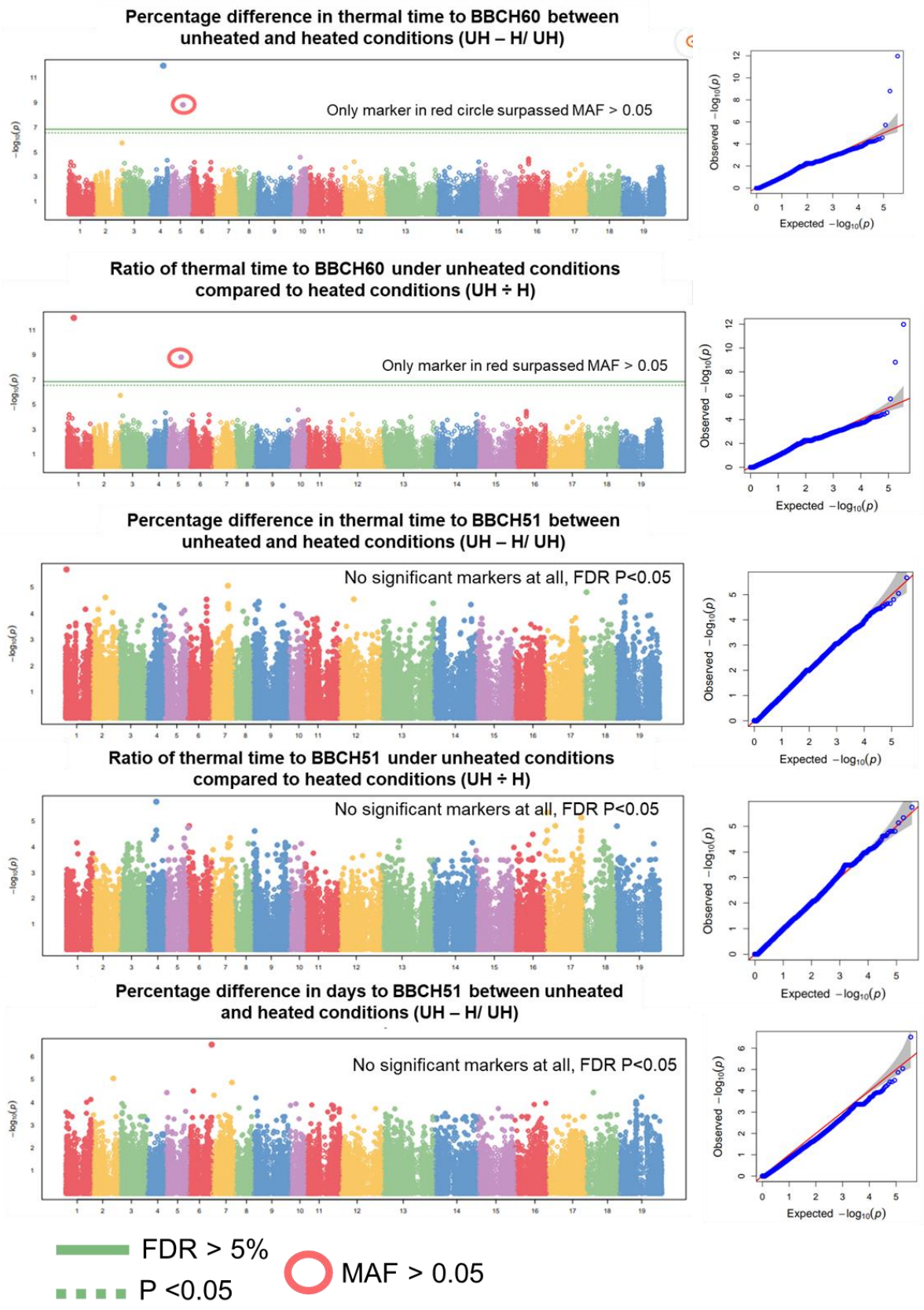
The aim of this project was to characterise the requirement for winter cold after the floral transition in winter-type *B. napus.* This has been achieved through extensive phenotyping of a diversity set of *B. napus* and subsequent genetic and transcriptomic analysis.

As discussed, I present evidence that following autumnal floral initiation, winter-type *B. napus* requires a continued period of cold to promote floral development and optimal yield formation. In warmer winters floral buds enter dormancy marked by delayed reproductive development and induction of ABA signalling and dormancy associated genes. Most climate models of rapeseed development do not yet account for this secondary requirement for winter chilling so may lead to underestimates of the effect of warmer winters on *B. napus* (Habekotte *et al.,* 1997; Deligiosa *et al.,* 2012; Weyman *et al.,* 2015). Integrating this effect into future models and understanding the prevalence of this phenomenon across other species is important in understanding and adapting to changing temperatures.

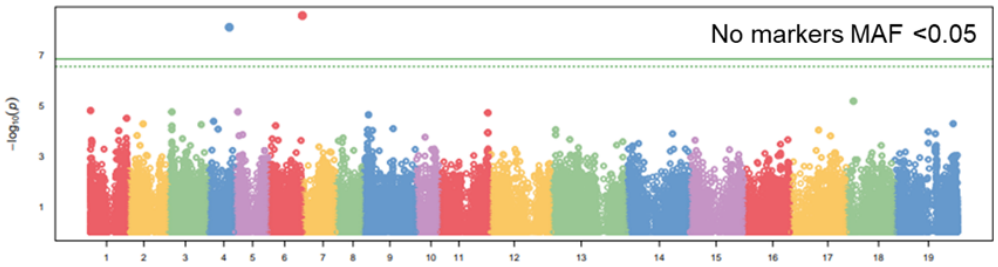
Here I provide evidence that late acting *Bna.FLC* genes likely regulate bud dormancy, although further evidence is needed to prove this. I also uncover genetic variability in this novel bud dormancy phenotype. I also uncover variation at the *FLC* regulator *BnPHP.A05* is associated

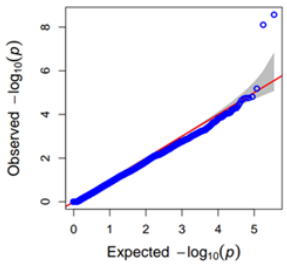
with early flowering before and after the floral transition and as such provides a direct avenue for breeding earlier flowering lines.

Appendix A: Supplementary Materials for Chapter 4

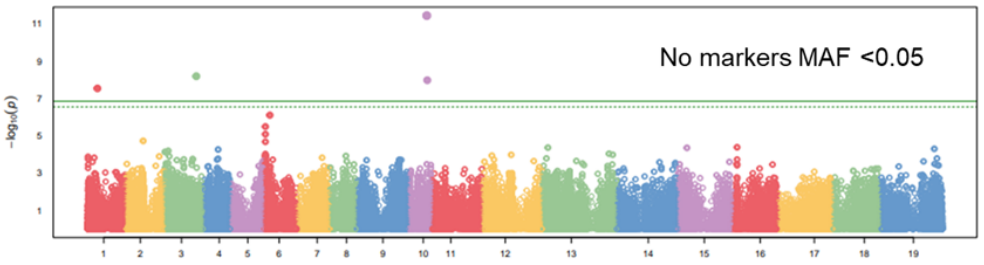


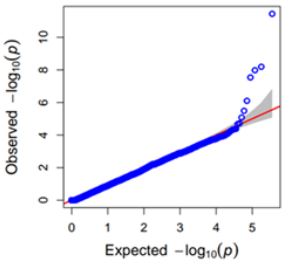
Ratio of days to BBCH51 under unheated conditions compared to heated conditions (UH ÷ H)



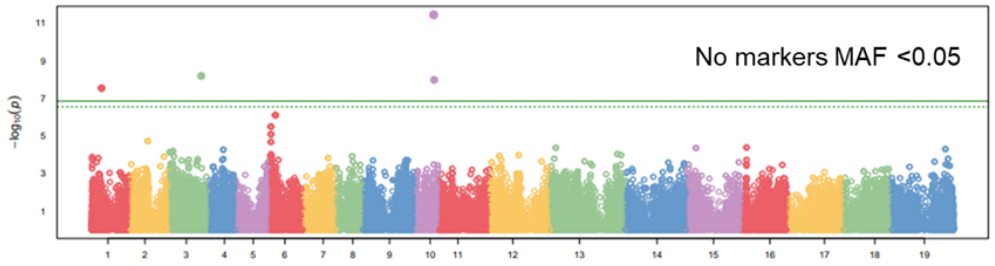


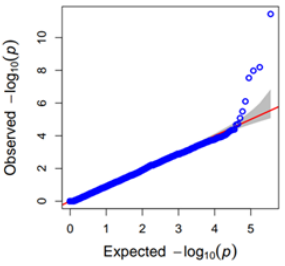
Ratio of days to BBCH60 under unheated conditions compared to heated conditions (UH ÷ H)



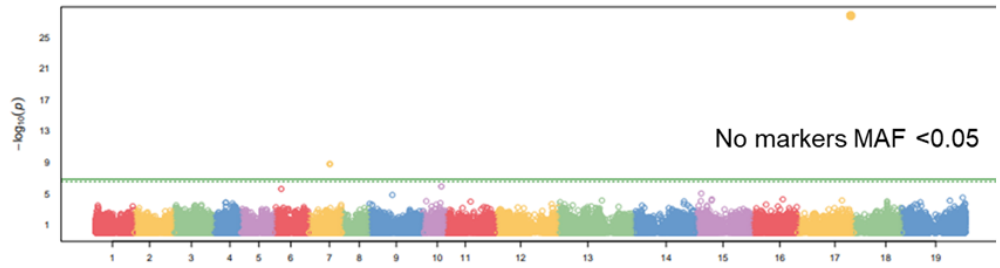


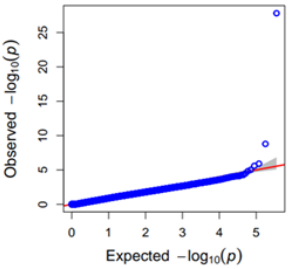
Percentage difference in days to BBCH60 between unheated and heated conditions (UH – H/ UH)



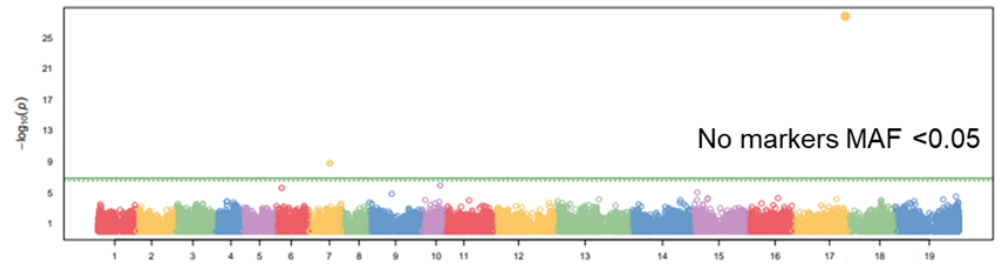


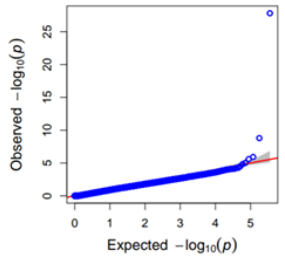
Ratio of days between BBCH51 to BBCH60 under unheated conditions compared to heated conditions (UH ÷ H)



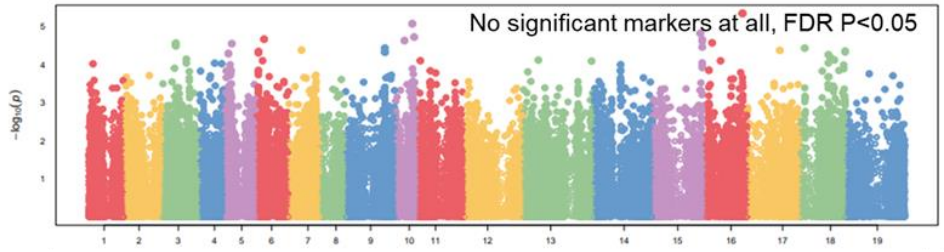


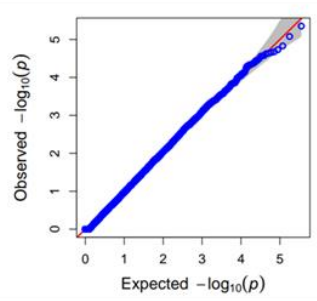
Percentage difference in days between BBCH51 to BBCH60 between unheated and heated conditions (UH – H/ UH)



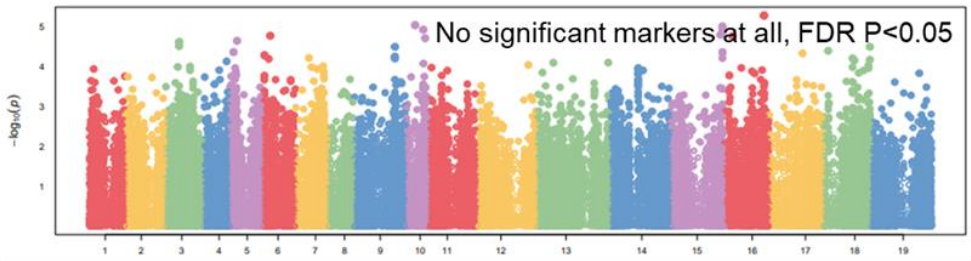


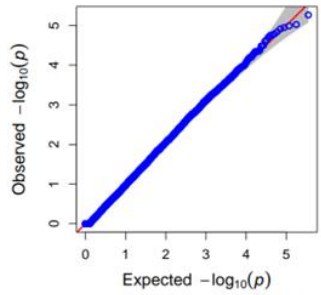
Ratio of SPP in unheated conditions compared to heated conditions (UH ÷ H)



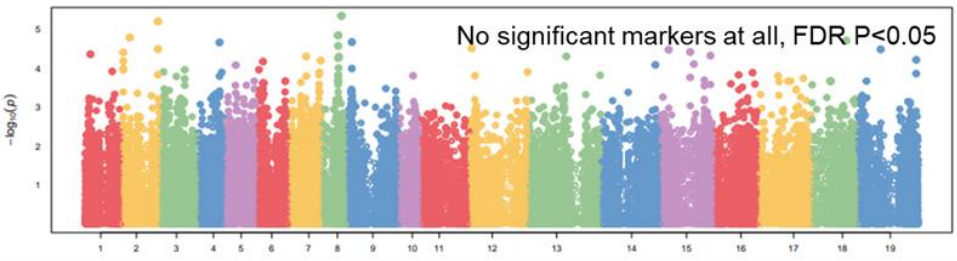


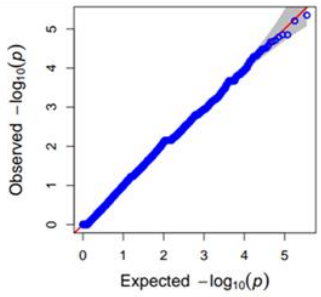
Percentage difference between SPP between unheated and heated conditions (UH – H/ UH)



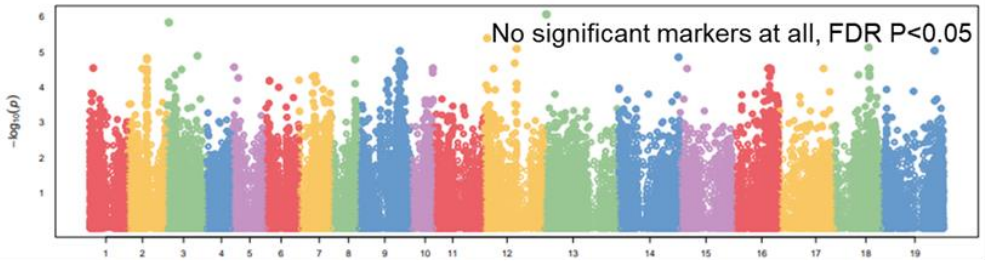


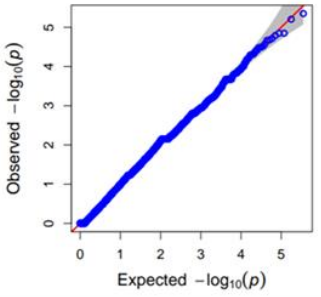
Percentage difference between TGW between unheated and heated conditions (UH – H/ UH)



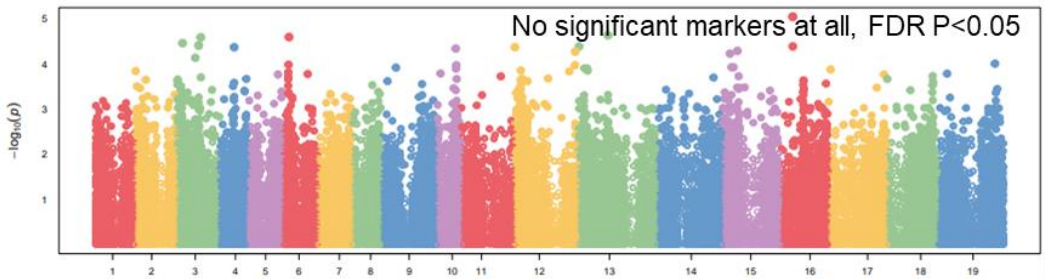


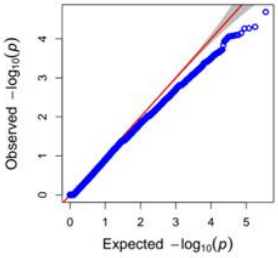
Ratio of TGW in unheated conditions compared to heated conditions (UH ÷ H)

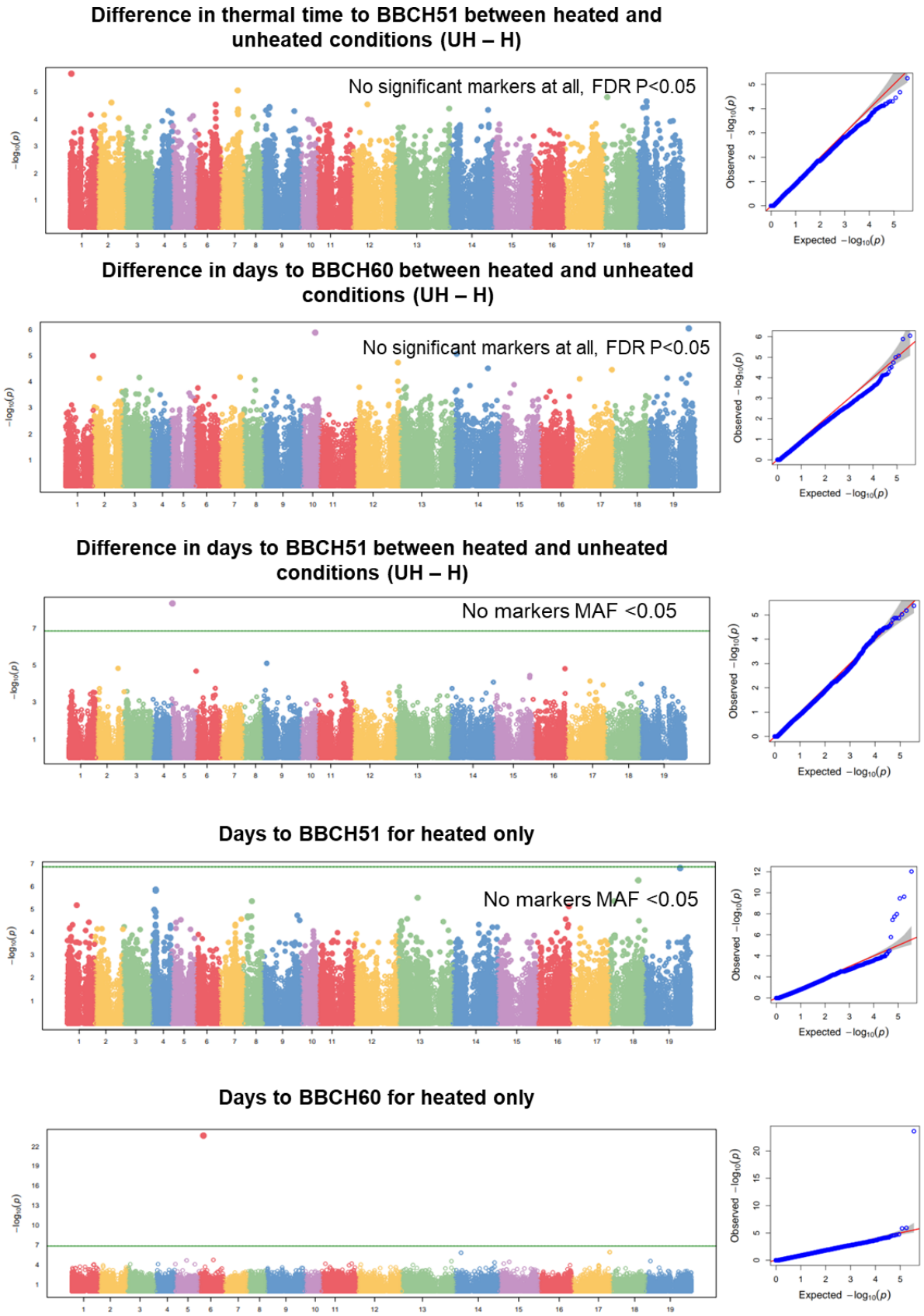




Difference in thermal time to BBCH60 between heated and unheated conditions (UH – H)

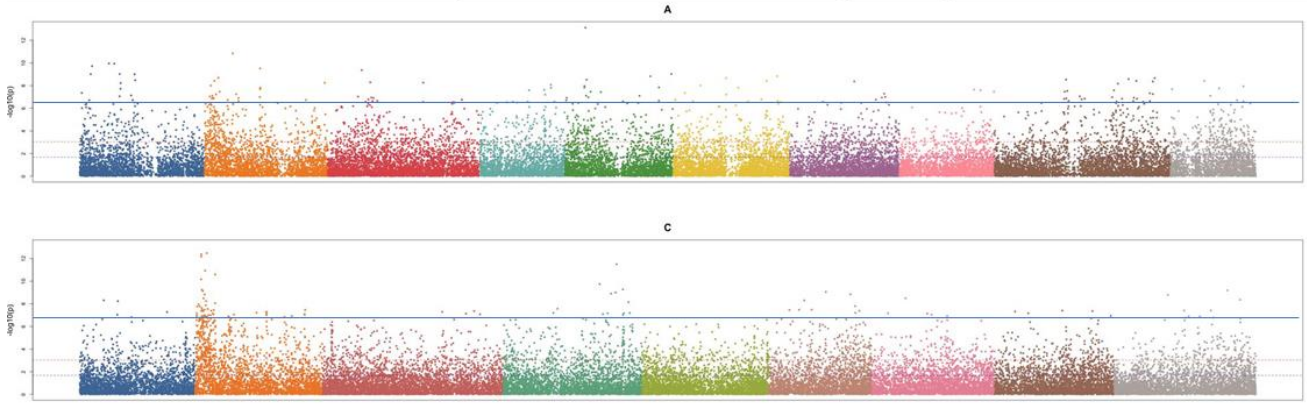




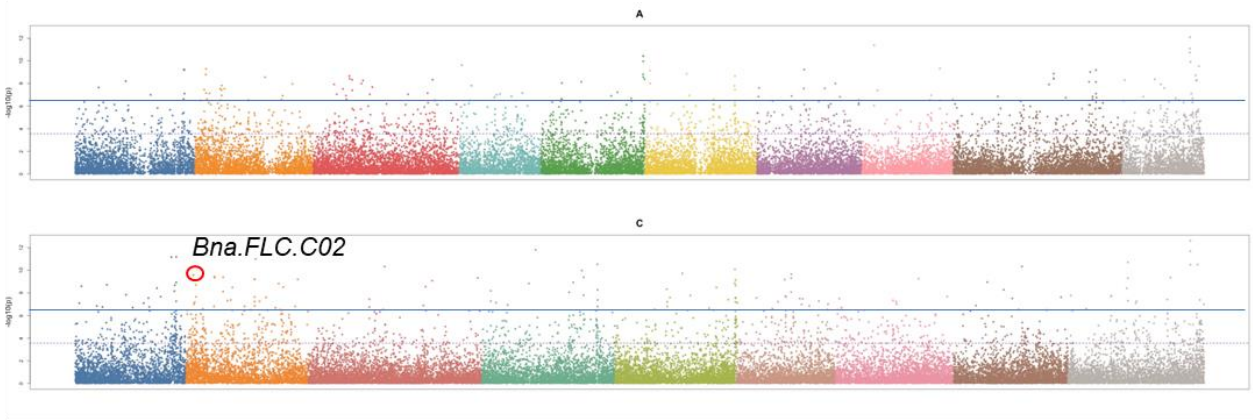


Supplementary Figure 1. Manhattan plots and QQ plots for each trait on the effect of heating on floral development run in GWAS analysis in Chapter 4.

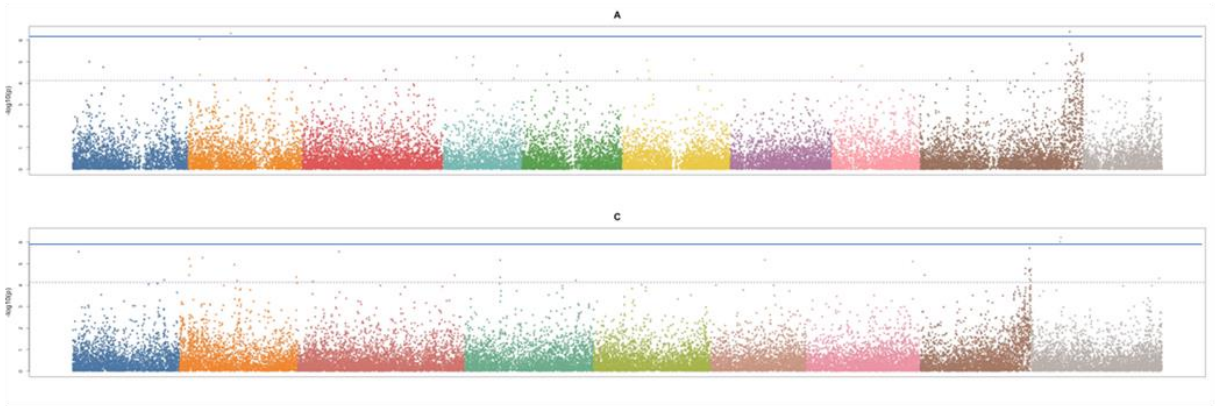
Ratio of time to BBCH51 from floral transition under unheated conditions compared to heated conditions (UH ÷ H)



Ratio of thermal time to BBCH51 from floral transition under unheated conditions compared to heated conditions (UH ÷ H)



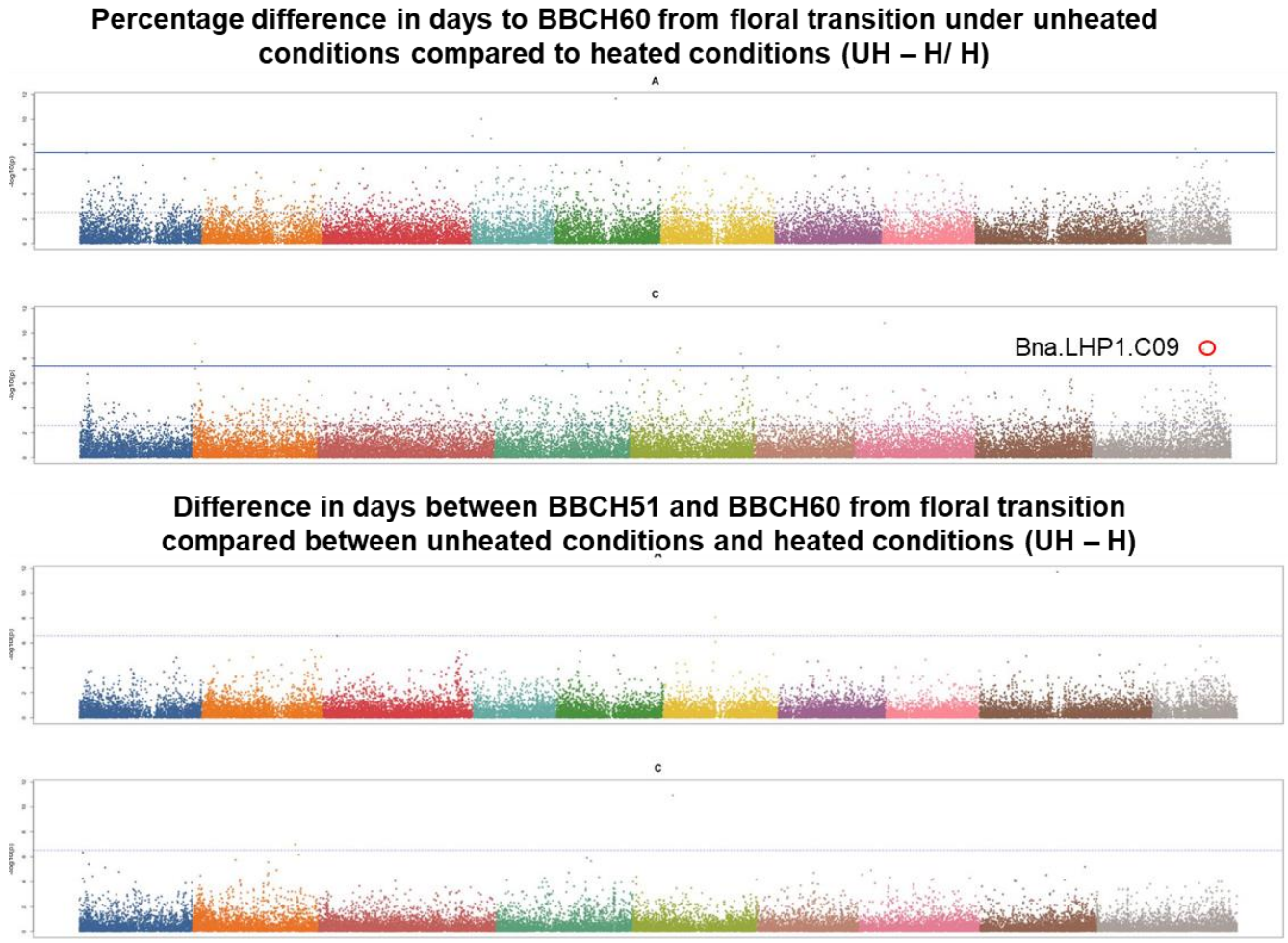
Ratio of thermal time to BBCH60 from floral transition under unheated conditions compared to heated conditions (UH ÷ H)



Bonferonni cut off

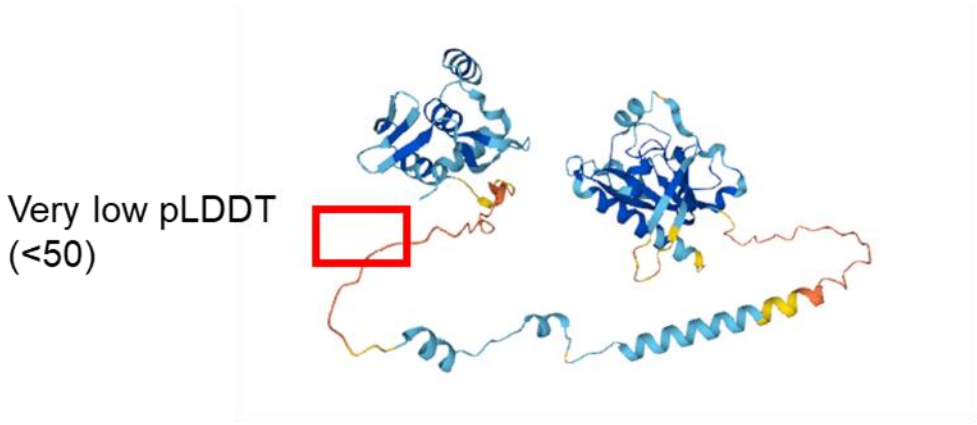
P <0.05

Significant trait

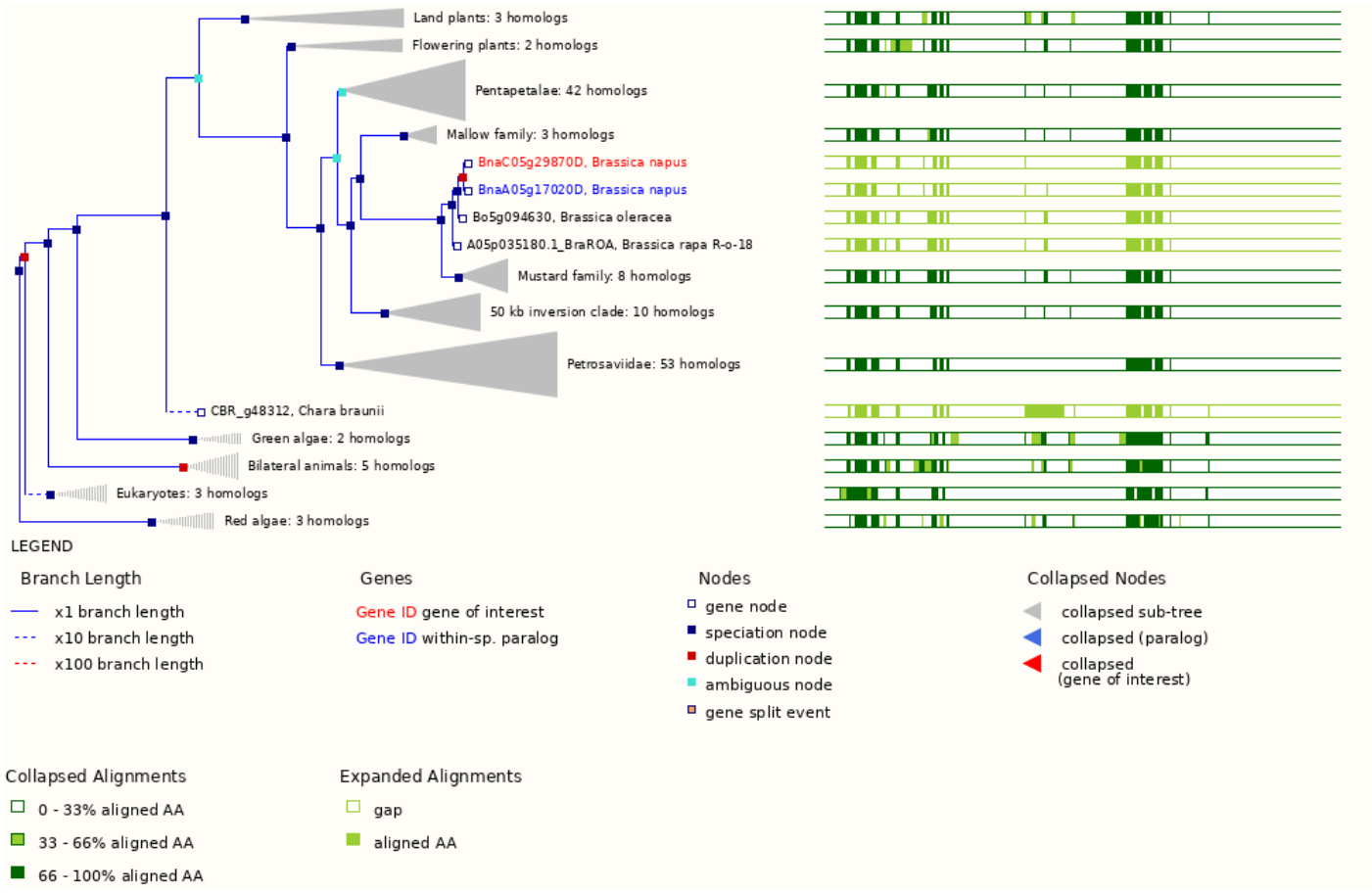


Supplemental Figure 2. GEM Manhattan plots for traits associated with the effect of warming on floral development.

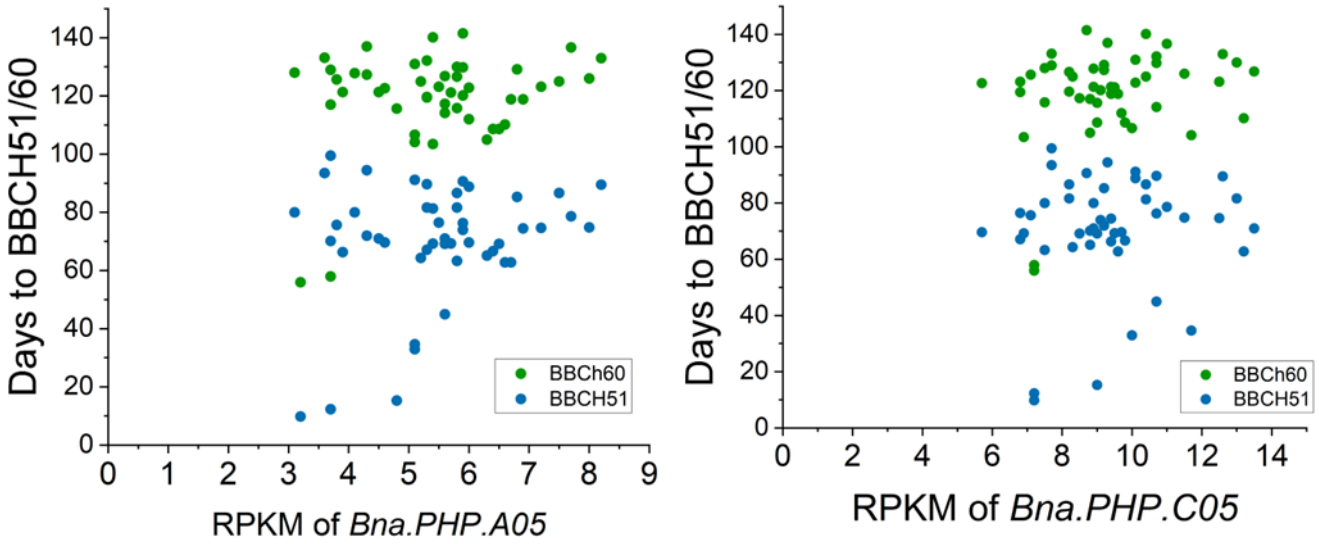
Appendix B: Supplementary Materials for Chapter 5



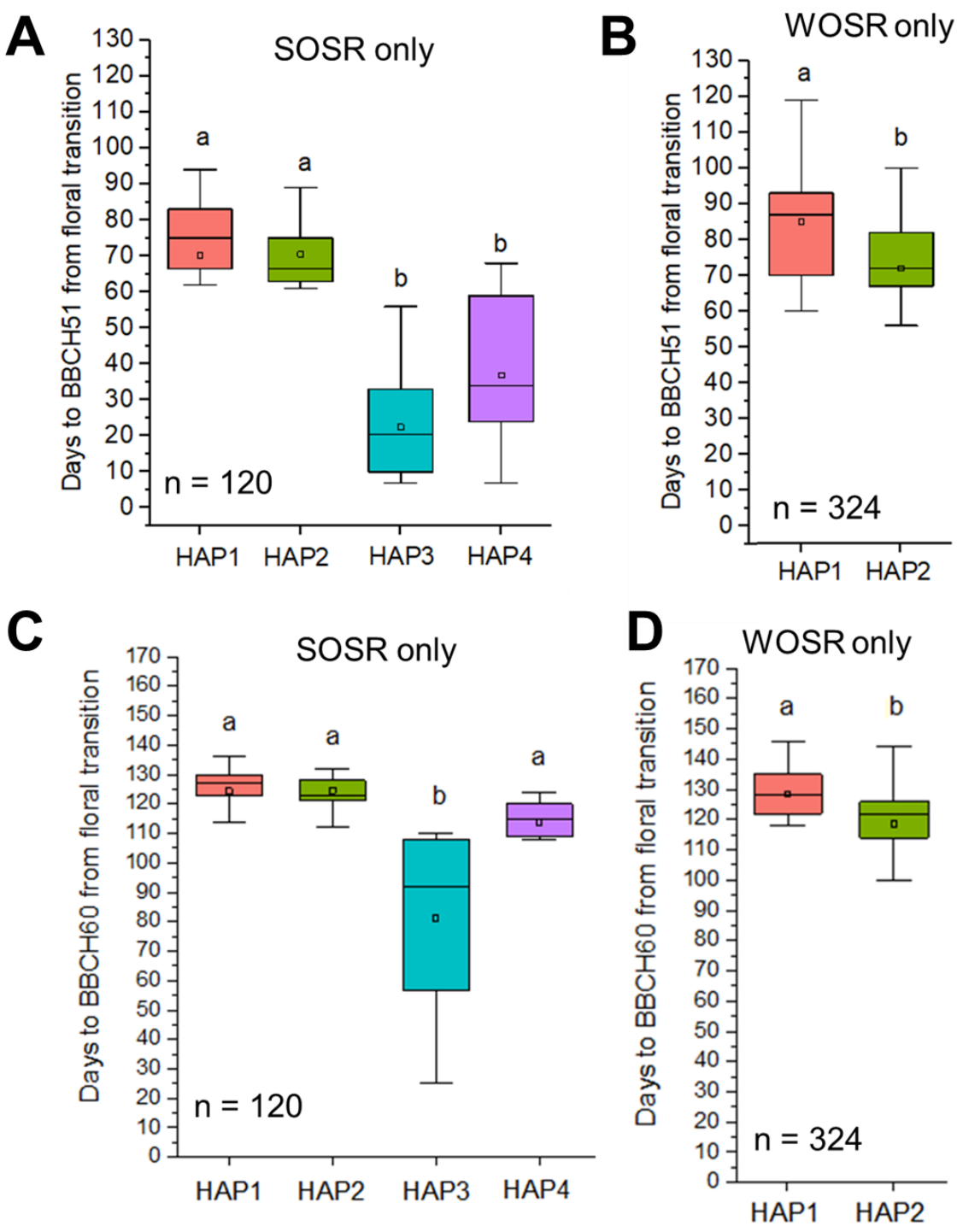
Supplementary Figure 3. A) Non-synonymous substitution in T lines occurs in a side branch of the protein with little predicted function.



Supplementary Figure 4. Gene copies and Gene tree of *BnaPHP.A05* taken from Ensembl Plants



Supplementary Figure 5. mRNA expression of *BnaPHPA05* and *BnaPHPC05* do not correlate with BBCH51 or BBCh60



Supplementary Figure 6. Association of BnaPHP.A05 haplotypes with days to BBCH51 and BBCH60 in the diversity set analysis in SOSR and WOSR. Significant differences at $P < 0.05$ are shown, calculated by students t-test, with Benjamin-Hochberg adjustment. Mean days to BBCH51 or BBCH60 for each haplotype at the respective temperature shown under each box plot. A) Days to BBCH51 from transfer date into vernalisation treatment for SOSR varieties; B) Days to BBCH51 from

transfer date into vernalisation treatment for WOSR varieties; C) Days to BBCH60 from transfer date into vernalisation treatment for SOSR varieties; D) Days to BBCH60 from transfer date into vernalisation treatment for WOSR varieties

Supplementary Table 1. Two-way ANOVA for effect of treatment and crop-type and treatment and crop type interaction on days to BBCH51 and BBCH60 from floral transition in winter warming experiment

		Df	Sum Squares	Mean Squares	F Value	Pr (>F)
BBCH60	**Crop Type**	1	5620.206	5620.206	19.47	0.0001
	Treatment	1	250.81357	250.81357	0.86901	0.35203
	Treatment. Crop Type	1	2940.99698	2940.99698	10.18985	0.00157
	Residuals	281	81102.30865	288.62032		
BBCH51	**Crop Type**	1	29360.14013	293601.14013	85.40236	0.0001
	Treatment	1	318.83118	318.83118	0.92741	0.33635
	Treatment: Crop Type	1	1995.39857	1995.39857	5.80419	0.01662
	Residuals	287	98666.59818	343.78606		

Supplementary Table 2 Tukey post hoc means comparison test for crop type, treatment and crop type x treatment interaction, on days to BBCH60 following floral initiation.

		MeanDiff	SEM	Q Value	Prob	Alpha
BBCH60	**Winter Spring**	9.17404	2.0438	6.25444	0.0001	0.05
	SOSR UH v SOSR H	4.6885	3.270	2.02765	0.47935	0.05
	WOSR UH v WOSR H	-8.56	2.555	4.7379	0.00506	0.05

Supplementary Table 3 Tukey post hoc means comparison test for crop type, treatment and crop type x treatment interaction, on days to BBCH51 following floral initiation.

		MeanDiff	SEM	Q Value	Prob	Alpha
BBCH51	**Winter Spring**	20.56824	2.22665	13.06353	0.0001	0.05

	SOSR UH v SOSR H	7.50877	3.473	3.0575	0.13648	0.05
	WOSR UH v WOSR H	-3.22	2.787	1.63378	0.65554	0.05

Supplementary Table 4. Tukey post-hoc test means comparison between C and T SNP calls

Cab041 204.2: 750:T	CROP	Treatment	Cab0412 04.2: 750:T	CROP	Treatment	MeanDiff	SEM	q Value	Prob	LCL	UCL
C	Spring OSR	--	C	Winter OSR	--	-8.16667	3.37874	-3.41826	0.07617	-16.89844	0.5651
C	Spring OSR	--	T	Spring OSR	--	30.68849	3.00626	14.43658	<0.0001	22.91934	38.45764
C	Spring OSR	--	T	Winter OSR	--	0.96899	2.73566	0.50092	0.98474	-6.10086	8.03883
C	Winter OSR	--	T	Spring OSR	--	38.85516	3.00626	18.27837	<0.0001	31.08601	46.62431
C	Winter OSR	--	T	Winter OSR	--	9.13566	2.73566	4.72272	0.00524	2.06581	16.2055
T	Spring OSR	--	T	Winter OSR	--	-29.7195	2.25954	-18.60097	<0.0001	-35.5589	-23.8801
C	--	Warmer	C	--	Control	-0.40476	3.37874	-0.16942	0.99938	-9.13653	8.32701
C	--	Warmer	T	--	Warmer	21.58769	2.87529	10.61792	<0.0001	14.157	29.01837
C	--	Warmer	T	--	Control	17.8317	2.873	8.77752	<0.0001	10.40693	25.25647
C	--	Control	T	--	Warmer	21.99245	2.87529	10.817	<0.0001	14.56176	29.42314
C	--	Control	T	--	Control	18.23646	2.873	8.97676	<0.0001	10.81169	25.66123
T	--	Warmer	T	--	Control	-3.75599	2.25954	-2.35081	0.3457	-9.59539	2.08341

--	Spring OSR	Warmer	--	Spring OSR	Control	-5.89484	3.00626	-2.77307	0.20543	-13.66399	1.87431
--	Spring OSR	Warmer	--	Winter OSR	Warmer	-22.75755	2.87529	-11.19332	<0.0001	-30.18824	-15.32686
--	Spring OSR	Warmer	--	Winter OSR	Control	-21.02346	2.873	-10.34864	<0.0001	-28.44823	-13.59869
--	Spring OSR	Control	--	Winter OSR	Warmer	-16.86271	2.87529	-8.29393	<0.0001	-24.2934	-9.43202
--	Spring OSR	Control	--	Winter OSR	Control	-15.12862	2.873	-7.44695	<0.0001	-22.55339	-7.70384
--	Winter OSR	Warmer	--	Winter OSR	Control	1.73409	2.73566	0.89645	0.92103	-5.33575	8.80394
C	Spring OSR	Warmer	C	Spring OSR	Control	0.2381	4.77826	0.07047	1	-14.35367	14.82986
C	Spring OSR	Warmer	C	Winter OSR	Warmer	-7.52381	4.77826	-2.22681	0.76516	-22.11557	7.06795
C	Spring OSR	Warmer	C	Winter OSR	Control	-8.57143	4.77826	-2.53687	0.6248	-23.16319	6.02033
C	Spring OSR	Warmer	T	Spring OSR	Warmer	36.82143	4.25149	12.24826	<0.0001	23.83831	49.80454
C	Spring OSR	Warmer	T	Spring OSR	Control	24.79365	4.25149	8.24735	<0.0001	11.81053	37.77677
C	Spring OSR	Warmer	T	Winter OSR	Warmer	-1.16986	3.87221	-0.42726	0.99999	-12.99474	10.65501
C	Spring OSR	Warmer	T	Winter OSR	Control	3.34594	3.86541	1.22416	0.98879	-8.45817	15.15005
C	Spring OSR	Control	C	Winter OSR	Warmer	-7.7619	4.77826	-2.29728	0.73521	-22.35367	6.82986
C	Spring OSR	Control	C	Winter OSR	Control	-8.80952	4.77826	-2.60734	0.59079	-23.40129	5.78224
C	Spring OSR	Control	T	Spring OSR	Warmer	36.58333	4.25149	12.16906	<0.0001	23.60022	49.56645

C	Spring OSR	Control	T	Spring OSR	Control	24.55556	4.25149	8.16815	<0.0001	11.57244	37.53867
C	Spring OSR	Control	T	Winter OSR	Warmer	-1.40796	3.87221	-0.51422	0.99996	-13.23284	10.41692
C	Spring OSR	Control	T	Winter OSR	Control	3.10784	3.86541	1.13705	0.99281	-8.69627	14.91195
C	Winter OSR	Warmer	C	Winter OSR	Control	-1.04762	4.77826	-0.31006	1	-15.63938	13.54414
C	Winter OSR	Warmer	T	Spring OSR	Warmer	44.34524	4.25149	14.75098	<0.0001	31.36212	57.32835
C	Winter OSR	Warmer	T	Spring OSR	Control	32.31746	4.25149	10.75006	<0.0001	19.33434	45.30058
C	Winter OSR	Warmer	T	Winter OSR	Warmer	6.35394	3.87221	2.3206	0.72499	-5.47093	18.17882
C	Winter OSR	Warmer	T	Winter OSR	Control	10.86975	3.86541	3.97685	0.09609	-0.93436	22.67386
C	Winter OSR	Control	T	Spring OSR	Warmer	45.39286	4.25149	15.09946	<0.0001	32.40974	58.37597
C	Winter OSR	Control	T	Spring OSR	Control	33.36508	4.25149	11.09854	<0.0001	20.38196	46.3482
C	Winter OSR	Control	T	Winter OSR	Warmer	7.40156	3.87221	2.70321	0.54418	-4.42332	19.22644
C	Winter OSR	Control	T	Winter OSR	Control	11.91737	3.86541	4.36013	0.04597	0.11326	23.72148
T	Spring OSR	Warmer	T	Spring OSR	Control	-12.02778	3.64946	-4.66092	0.02422	-23.17242	-0.88313
T	Spring OSR	Warmer	T	Winter OSR	Warmer	-37.99129	3.19959	-16.79208	<0.0001	-47.76214	-28.22044
T	Spring OSR	Warmer	T	Winter OSR	Control	-33.47549	3.19136	-14.83427	<0.0001	-43.22212	-23.72978
T	Spring OSR	Control	T	Winter OSR	Warmer	-25.96352	3.19959	-11.47582	<0.0001	-35.73437	-16.19267

T	Spring OSR	Control	T	Winter OSR	Control	-21.44771	3.19136	-9.5043	<0.0001	-31.19342	-11.702
T	Winter OSR	Warmer	T	Winter OSR	Control	4.5158	2.66526	2.39613	0.69099	-3.62332	12.65492

Glossary of commonly used terms

Abbreviation	Definition
ABA	Abscisic acid
DTF	Days to flowering
BBCH51	Bud emergence, according to BBCH scale (Weber and Bleiholder, 1990)
BBCH60	First flower emergence, according to BBCH scale (Weber and Bleiholder, 1998)
GA	Gibberellic acid
WOSR	Winter Oilseed Rape
SWOSR	Semi-winter Oilseed Rape
SOSR	Spring Oilseed Rape
SPP	Seeds Per Pod
TGW	Thousand Grain Weight
SNP	Single Nucleotide Polymorphism
ENSO	El-Nino Southern Oscillation
NAO	North Atlantic Oscillation
CR	Chilling Requirement
APSIM	The Agricultural Production Systems sIMulator
QTL	Quantitative Trait Loci
GWAS	Genome Wide Association Study
GEM	Gene Expression Marker Analysis
DFFS	Diversity Fixed Foundation Set
T1-5	Transfer 1 – 5
CER	Controlled Environment Room
GAGA	GWAS and GEM Automation Pipeline
SEM	Standard Error of the Mean
GDD	Growing Degree Days
ANOVA	Analysis of Variance
FI	Floral Initiation

RPKM	Reads Per Kilobase Of Transcript Per Million Reads Mapped
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
PCA	Principal Component Analysis
GO	Gene Ontology
ZS11	Zhongshuang 11
FDR	False Discovery Rate
UTR	Untranslated Region
HAP	Haplotype
PRC2	Polycomb Repressive Complex 2

Gene name abbreviations	**Complete Gene Name**
FLC	*FLOWERING LOCUS C*
PHP	*PLANT HOMOLOGOUS TO PARAFIBROMIN*
SOC1	*SUPPRESSOR OF CONSTANS 1*
LHP1	*LIKE HETEROCHROMATIN PROTEIN 1*
BRC1	*BRANCHED 1*
FRI	*FRIGIDA*
LFY	*LEAFY*
SPL	*SQUAMOSA PROMOTOR LIKE*
AP2	*APETALA 2*
FD	*FLOWERING LOCUS D*
MAF1-5	*MADS AFFECTING FLOWERING 1-5*
FLM	*FLOWERING LOCUS M*
SVP	*SHORT VEGETATIVE PHASE*
PAF1-C	*POLYMERASE ASSOCIATED FACTOR COMPLEX 1*
CDC73	*CELL DIVISION CYCLE 73*
VIN3	*VERNALISATION INSENSITIVE 3*
CO	*CONSTANS*
PEP1	*PERPETUAL FLOWERING 1*
DAM	*DORMANCY ASSOCIATED MADS BOX*
SVL	*SVP-LIKE*

ABF3	*ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 3*
NCED3	*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3*

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