MOLECULAR TOOLS FOR UNDERSTANDING AND PREDICTING FLOWERING IN CAULIFLOWER

by

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Stephen Ridge
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ABSTRACT

Synchronous flowering between parent lines is essential for pollination and seed set in commercial hybrid cauliflower seed production. However, weather-related variation in reproductive development and attempts to produce novel hybrids by crossing parents with markedly different flowering phenology make synchronous flowering difficult to achieve. Molecular technology presents an opportunity to understand and manage this variability.

Prospects for understanding the molecular control of flowering time in cauliflower have benefited greatly from recent work with the closely-related model species *Arabidopsis thaliana*. Over the last decade, genetic analysis in Arabidopsis has identified a large number of flowering genes, enabling the development of a model describing the molecular pathways that regulate floral induction through environmental cues such as photoperiod and vernalisation. Although elements of this model are conserved in *B. oleracea*, the scenario has been complicated by ancient genome triplication and rearrangements within the genome. The degree of genetic diversity generated by such events has given rise to a wide range of flowering habits within the species. In this study, we investigated the contribution of the *BoFLC* gene family to flowering time variation and examined the vernalisation-regulated expression of key flowering genes.

The flowering behaviour of 54 homozygous cauliflower lines ranging from tropical to winter varieties was characterised and plants were screened for polymorphisms in flowering time candidate genes including members of the vernalisation-mediated *BoFLC* family. A functional allele of the *BoFLC2* gene was identified for the first time in an annual brassica variety, along with an allele disrupted by a frameshift mutation in exon 4. A strong correlation between flowering time and *BoFLC2* genotype was observed in field and glasshouse trials. In a segregating F_2 population derived from a cross between late-flowering (functional *BoFLC2*) and early-flowering (mutated *BoFLC2*) cauliflower lines, the *BoFLC2* gene behaved in a dosage-dependent manner and *BoFLC2* genotype was found to account for up to 65% of flowering time variation. The identification of this gene as an important

determinant of flowering time within brassicas grown as annual crops is potentially valuable to plant breeders, with possible applications as a genetic input for QTL-based models of flowering time, as a marker for screening and classifying flowering time in cauliflower germplasm, or as a target for genetic modification.

RT-qPCR was used to study the effects of vernalisation on the expression of several key flowering time candidate genes in a wide range of flowering types. For the first time in cauliflower, it was shown that vernalisation reduced levels of *BoFLC2* and *BoFLC3* transcript and upregulated expression of the flowering signal integrator *BoFT*, with colder temperatures and increased vernalisation duration amplifying these effects and hastening curd initiation. *BoFT* was not significantly upregulated in vernalised plants less than four weeks old, whilst the final level of *BoFLC2* and *BoFLC3* downregulation was independent of plant age. *BoFLC2* gradually increased with plant age in unvernalised plants, possibly reflecting the transition from reproductive juvenility to maturity. In early-flowering lines with mutated *BoFLC2*, overall expression of *BoFLC2* was lower and *BoFT* expression significantly higher supporting the idea that *BoFLC2* plays a key role in maintaining the vegetative state. A homologue of Arabidopsis *VIN3* was also isolated for the first time in a brassica crop species, and was found to be upregulated in ten-week-old plants by as little as two days of vernalisation at 5°C, in contrast to findings in Arabidopsis, where prolonged exposure to cold was required to elicit upregulation. Minor differences in the transcriptional dynamics of these genes were observed between apex and leaf tissue.

Devernalisation and floral reversion have been reported to contribute to asynchronous or uneven flowering in cauliflower seed production, forcing growers to avoid warm and dry areas that would otherwise be conducive to high-yielding crops. The effects of devernalising conditions on curd development and gene expression were investigated. In contrast to findings in Arabidopsis, the vernalisation-induced downregulation of *BoFLC2* was unstable, with expression returning to pre-vernalisation levels after return to warm conditions. By contrast, *BoFT* was stably upregulated, possibly suggesting the involvement of a *BoFLC*-independent process.

The strong correlations observed between gene expression and flowering time in controlled-environment experiments were validated with gene expression analysis of field-grown cauliflower parent lines and cauliflowers grown outdoors in pots. ‘Natural’ outdoor

vernalising temperatures were capable of downregulating *BoFLC2* and upregulating *BoFT*, and a strong correlation was found between both *BoFLC2* and *BoFT* expression and curding and flowering time. These findings indicate a need for further investigation of the potential for this molecular tool to be used as a predictive assay for curd initiation and flowering time, or as a management tool for assessing the effect of crop treatments such as growth regulator applications.

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INTRODUCTION & LITERATURE REVIEW

A. Outline

There are approximately 40 species in the *Brassica* genus (Curtis & Morris 1993), including several important crop species that contribute significantly to world food supplies. This chapter provides background information on the genus and introduces topics that will receive more detailed attention in the research chapters of this thesis. In part B of this chapter, the origins of six major *Brassica* species are briefly outlined. The genetic diversity within this genus has been exploited to generate a host of specialised varieties from which all manner of products are derived. In particular, the species *Brassica oleracea* exhibits a remarkable variety of cultivated forms, including cauliflower (var. *botrytis* L.) – the focus of this research project – in which the immature inflorescence (curd) constitutes the edible part of the plant. In part C, an overview of the physiology of cauliflower curd development and flowering is provided, and in part D, consideration is given to management of the reproductive process from the perspective of commercial hybrid seed producers, using examples from the Tasmanian seed industry. Part E considers the prospects for understanding the molecular control of flowering time in cauliflower, which have benefited greatly from recent work with the closely-related model species *Arabidopsis thaliana*. The current model of the Arabidopsis floral induction pathway is reviewed, and the present knowledge of brassica flowering genetics is considered. Finally, the aims of this thesis are presented in Part F.

B. Origins, Diversity & Economic Importance of *Brassica*

The cultivated species of the *Brassica* genus (see Table 1) represent the group of crops most closely related to the model species *Arabidopsis thaliana* (Lan & Paterson 2000; Town et al. 2006). Comparative genome mapping in brassica suggests that following the divergence of these two genera, the *Brassica* genus underwent whole-genome triplication (Lagercrantz & Lydiate 1996; Lagercrantz 1998). Subsequent frequent and extensive chromosomal fusions and rearrangements are believed to have led to the formation of the three *Brassica* species typically regarded as diploid (*B. nigra*, n=8; *B. oleracea*, n = 9; and *B. rapa*, n = 10) but which may actually be descended from a common hexaploid ancestor (Lagercrantz & Lydiate 1996; Lukens et al. 2003).

Table 1. Common and taxonomic names of significant members of the *Brassica* genus.

COMMON NAME	TAXONOMIC NAME
Brassica (Cole) Crops	***B. oleracea* L.**
Cauliflower	var. *botrytis* L.
Broccoli	var. *italica* Plenck
Kale	var. *acephala* DC
Cabbage	var. *capitata* L.
Brussels Sprout	var. *gemmifera* Zencker
Kohlrabi	var. *gongylodes* L.
Turnips	***B. rapa* (syn. *B. campestris* L.)**
True Turnip	subsp. *rapifera* Metz.
Chinese Mustard, Pak-Choi	subsp. *chinensis* Jusl.
Chinese Cabbage, Pe-Tsai	subsp. *pekinensis* (Lour.) Rupr.
Swedes	***B. napus* L.**
Swede/Rutabaga	var. *napobrassica* (L.) Mill.
Winter Oilseed Rape	*var. oleifera* DC.
Black Mustard	***B. nigra* Koch**
Indian or Brown Mustard	***B. juncea* (L.) Coss.**
Ethiopian Mustard	***B. carinata* Braun**

B. rapa is thought to be the first of these wild pseudodiploid species to be cultivated, probably for its high oil content (Thompson 1979). This species is believed to have originated in the highland regions of the fertile crescent (Rakow 2004) where it exhibits rapid vegetative growth and produces copious seed (Dixon 2007). As it spread northward into Scandinavia and westward to Eastern Europe, turnip forms emerged (Hancock 2004), and a wide range of leafy, heading vegetable forms were domesticated as the species was

introduced to China and Japan. Wild *B. oleracea* species naturally inhabit cool and damp habitats on the rocky sea cliffs of the Mediterranean coast. They have a slow, even growth rate which enables them to conserve water and nutrients (Dixon 2007). Cultivated forms of this species are generally believed to have arisen by selection on the polymorphisms already available within wild *B. oleracea*, but some authors have suggested that these groups may have developed through hybridisation with other closely-related *Brassica* species (e.g. Snogerup 1980). Broccoli, cauliflower and Brussels sprout are the most recently-formed varieties, appearing as recently as 500 years ago (Hancock 2004). *B. nigra* exists naturally as a small, weedy annual found growing in shallow soils of the rocky Southern Mediterranean coast (Dixon 2007). Its seeds have significant fatty oil content, and are also ground for use as a spice.

Hybridisation among these diploid lines is believed to have led to the creation of three allopolyploid members of the *Brassica* genus (U 1935), where the chromosome compositions of these species are similar to the sum of their presumed diploid progenitors (Axelsson, Shavorskaya & Lagercrantz 2001; Osborn 2004). The amphidiploid *B. carinata* (n=17) is believed to have arisen as a result of hybridisation between *B. nigra* and *B. oleracea* (Kimber & McGregor 1995). No wild forms of this species have been reported, and its cultivation is restricted to the Ethiopian plateau. It exhibits the high oil content of *B. nigra* and the slow, steady growth of *B. oleracea* (Rakow 2004). *B. juncea* (n=18) is a large-leafed species derived from natural hybridisation between *B. nigra* and *B. rapa,* probably in the south of modern Iran (Rakow 2004). It has the rapid growth of *B. rapa* and the oil content of *B. nigra,* for which it is cultivated in India. Cultivated vegetable varieties are consumed throughout Asia. Wild forms of the third hybrid, *B. napus* (n=19), are generally found in coastal regions of Northern Europe (Rakow 2004). It is a very important source of vegetable oil worldwide. The genetic relationship of these tetraploid species to their diploid progenitors is shown in Figure 1.

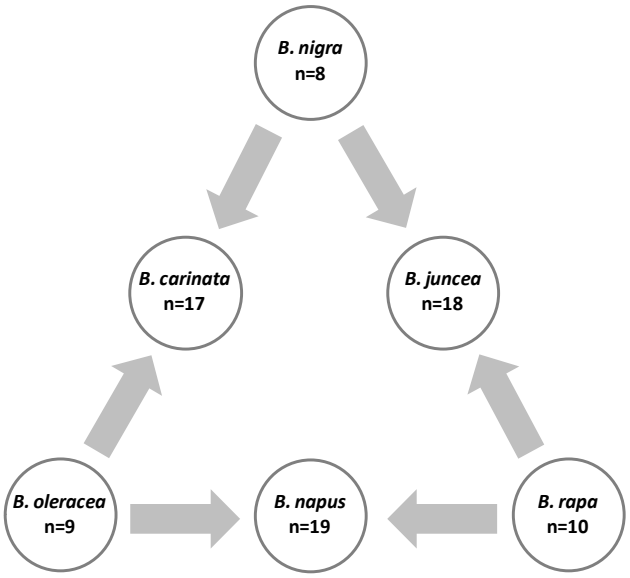


Fig. 1. Gene flow 'triangle of U' diagram showing the genetic relationship of three amphidiploid brassicas to their diploid progenitors (U 1935). This model has been supported by several different molecular lines of evidence (for example; Song, Osborn & Williams 1990; Snowdon et al. 2002). n = haploid chromosome number.

The complex, multiple-polyploid origins of the *Brassica* genus have resulted in a group of plants with unique genetic diversity and metabolic flexibility (Osborn 2004). This, combined with both natural and active selection for specific characteristics and further combination with closely-related species, has resulted in such an astonishing range of phenotypes that the *Brassica* genus provides the greatest diversity of products utilised by man from a single genus (Nieuwhof 1969; Murphy 2007).

These products may be divided into two groups: oilseeds and vegetables. In terms of monetary value, rapeseed oil is the most important brassica product (Suwabe, Morgan & Bancroft 2008). Historically, rapeseed oil has been used for industrial applications such as illumination and lubrication. In the late 1970s, Canadian breeders developed 'Canola' cultivars with low (<2%) erucic acid content in the oil and less than 30 µmol/g meal of aliphatic glucosinolates (Canola Council of Canada 2011). The oil from these cultivars is safe and palatable for human and animal consumption, and also well-suited to biodiesel production. These qualities have seen worldwide production increase rapidly (Mendham & Salisbury 1995), and in 2009/2010, approximately 60.62 million tonnes were produced, making rapeseed the second largest source of vegetable oil in the world behind soybean (United States Department of Agriculture 2011). The *Brassica* species with the highest oil yield potential is *B. napus* (Mendham & Salisbury 1995), but Canola-quality varieties of *B.*

rapa, *B. juncea* and *B. carinata* have been, or are being developed, and are also used in certain agro-ecological niches. For example, *B. rapa* is preferred in areas where winters are too cold for *B. napus*, and *B. juncea* is the major oilseed on the Indian subcontinent due to its heat and drought tolerance. Several of these species also contain varieties that are commonly used in soil conditioning as a green manure and biofumigant due to their high glucosinolate content (Brown & Caligari 2008).

Although there are several important vegetable or forage forms of the oil-producing brassicas (e.g. turnip, rutabaga, Chinese cabbage and mustard), the vast majority of all brassica vegetables belong to the *Brassica oleracea* species. This species represents a large fraction of the total *Brassica* diversity, containing an extremely wide range of vegetable forms, with edible components ranging from stems (kohlrabi) and leaves (cabbage and kale) to axillary buds (Brussels sprouts) and inflorescence structures (cauliflower and broccoli). Brassica vegetable products consumed by humans are increasingly thought of as 'functional foods', with research suggesting that they are protective against cancer (Van Poppel et al. 1999; Finley 2003), coronary heart disease (Willcox, Catignani & Lazarus 2003), and several other major illnesses, due to compounds including organosulphates (most notably glucosinolates), antioxidants, flavonoids and polyphenols (for a review, see Vasanthi, Mukherjee & Das 2009).

Matching this morphological diversity, the *Brassica* genus exhibits a wide range of flowering behaviour, with biennial and annual types found among closely-related cultivars of the same species. In cauliflower and broccoli, the flowering habit is characterised by an arrested stage of inflorescence development, which is itself highly variable and subject to environmental influences. The following section focuses on *B. oleracea* flowering, especially on cauliflower curd development and flowering physiology, and on the environmental inputs that regulate this process.

C. Physiology of Reproductive Development in *B. oleracea*

The transition from vegetative to reproductive growth is a critical phase in the life cycle of flowering plants, especially in monocarpic species, whose life cycle only allows a single opportunity to sexually reproduce (Bernier & Perilleux 2005). The timing of transition to flowering depends on integration of endogenous factors such as size, or number of leaves (Bernier & Perilleux 2005), GA biosynthesis (Wilson, Heckman & Somerville 1992) and environmental cues such as photoperiod, light quality, temperature and stress (Simpson, Gendall & Dean 1999). The biological function of such endogenous requirements is to prevent the plant from flowering before it has accumulated the necessary reserves required for energy-intensive reproductive development (Boss et al. 2004). Photoperiod and/or vernalisation requirements serve to synchronise the flowering of conspecific, cross-pollinating plants, and also ensure that flowering occurs when conditions are conducive to fruit set and seed production (Putterill, Laurie & Macknight 2004). For example, plant species that live in climates with a cold winter have developed a requirement for an extended period of vernalisation to ensure that flowering does not occur immediately prior to the onset of winter, or during winter, when reproductive tissue may be damaged by frost (Sung & Amasino 2004b).

Temperature is the main environmental factor influencing *B. oleracea* flowering time. Most cultivars exhibit at least some flowering time response to vernalisation, but the degree to which vernalisation is required for flowering shows substantial genetic variation within the genus: cultivars vary from a near-obligate requirement (e.g. cool-temperate spring cultivars) to virtually none (e.g. tropical cultivars) (Friend 1985). The other characteristic feature of flowering phenology in *B. oleracea* is the existence of a juvenile phase, during which the plant is insensitive to vernalisation. This trait also shows substantial genetic variation and may range in length from ten to nearly 100 leaves (Wien & Wurr 1997). This means that, unlike *A. thaliana* and other *Brassica* species (e.g. *B. campestris*, *B. juncea*, *B. napus*), seed vernalisation in *B. oleracea* is generally not effective (Friend 1985).

Ancestral forms of *B. oleracea* may have also shown a response to long photoperiods (Long Day, or LD) but any substantial LD requirement has been largely bred out of commercial cultivars, and small residual responses in some cultivars are only seen in vernalised plants (Heide 1970). There is some evidence that members of the gibberellin group of

phytohormones – particularly endogenous gibberellin A_1 (GA_1) and GA_3 (gibberellic acid, or simply GA) – are involved in the overall bolting process, although it is not clear whether the effect occurs at the level of floral initiation or is restricted merely to elongation of the inflorescence stem (Guo et al. 2004). Nevertheless, GA_3 application has been reported to precipitate flowering in some cultivars that have received marginal levels of vernalisation (Friend 1985). Devernalisation has also been reported, in which exposure to sufficiently high temperatures following a period of vernalisation can revert its effects (e.g. Erwin 2007).

Due to the unique nature of inflorescence development in cauliflower, the flowering process is somewhat more complex. Cauliflower is an annual plant that is characterised by a dome-shaped mass of tissue known as a curd, which is composed of proliferated inflorescence meristems (Sadik 1962). It is distinguished from its close relative broccoli by the ontological stage of arrest at marketable maturity; cauliflower curds consist of arrested inflorescence meristems, whereas broccoli heads are not arrested until flower buds are fully differentiated (Gray 1982).

The existence of this intermediate curd stage defines two separate phases in the flowering of cauliflower; induction of curding, and subsequent 'bolting' of the curd to produce normal brassica flowers. The diversity of curd morphology and developmental physiology within the cauliflower cultivar alone is massive (Crisp 1982), with vernalisation requirements ranging from obligate in winter/spring varieties to facultative or non-existent in tropical and early-maturing types, and a juvenile phase of highly variable length (Sadik 1962; Atherton, Hand & Williams 1987). Control of the bolting phase is poorly understood, but reports of a post-curd induction cold requirement for bolting in lines that do not require vernalisation for curd initiation suggest that these processes may be regulated by distinct physiological mechanisms (Guo et al. 2004). A limited amount of evidence suggests that GA may play a role in the flowering process in vernalised plants (e.g. Leshem & Steiner 1968; Booij 1990; Guo et al. 2004).

Flowering time is clearly important for the continuity of plant species in nature, and plants have accordingly adapted to their environments in order to maximise their reproductive success. In agricultural species that are sexually propagated, or whose marketable part is a reproductive structure, such as cauliflower, the flowering process often requires careful

management. Indeed, the very survival of the curding trait in *Brassica* species depends heavily on human selection, cultivation and management of flowering: the reproductive fitness of cauliflower is relatively low, partly due to the fact that over 90% of the apical meristems in the cauliflower curd abort with the onset of flowering and the excess undifferentiated tissue is especially susceptible to attack by saprophytic pathogens (Crisp & Walkey 1974). The use of inbred parent lines in hybrid seed production makes this an even more 'unnatural' process, requiring extremely intensive management. In the following section, the Tasmanian hybrid seed industry is briefly reviewed, and the commonly used methods of managing flowering time in hybrid cauliflower seed crops are considered.

D. The Tasmanian Hybrid Vegetable Seed Industry & Management of Flowering Time

Much of the background information on the Tasmanian vegetable seed industry contained in this section was provided by the manager of Tasmanian seed research company seedPurity, C. Spurr (2011, pers. comm., 8 Aug). All climatic data is sourced from the Australian Bureau of Meteorology web site <http://www.bom.gov.au>.

Vegetable seed production is a small, but important sector of the agricultural industry in Tasmania, with approximately 100 growers producing around 350 ha of seed with a total value to seed companies of approximately A$7 million. Hybrid brassica crops constitute the largest proportion of this figure, with a production area of around 200 ha generating a product worth approximately A$5 million in total. Hybrid cauliflower and cabbage crops represent approximately 90% of brassica seed production, with broccoli and other brassicas making up the remaining 10%. In 2001, hybrid cauliflower seed was the second most widely-planted vegetable seed crop in the state, behind cabbages. Hybrid cauliflower seed constituted the highest value vegetable seed crop in Tasmania, with an estimated total value of A$2 million. Other important hybrid seed crops include onion, carrot and lettuce. The industry is largely export-oriented, with the vast majority of non-specialist growers working for contract multipliers who supply overseas (mostly Dutch) vegetable breeding companies such as Rijk Zwaan, Bejo Zaden and Enza Zaden. Small-scale domestic markets include producers of home-gardening supplies, such as Mr Fothergills, Yates and Hortico.

Despite being a relatively small player in the global seed market, Tasmania plays an important role in the production regimes for these vegetable breeders. Being in the southern hemisphere, it is strategically located for off-season production, allowing breeders to multiply seeds faster and ensuring continuity of supply. Continuity of supply is essential for the maintenance of market share. Furthermore, production divisions in Australia enable vegetable breeding companies to spread their production risk over multiple growing locations.

Most of Tasmanian brassica seed production occurs in the south east and on the central east coast. The Coal River and Derwent Valleys are particularly important production areas. There are also several important sites in the northwest and central north of the state. Like

some inland regions of South East Australia such as Mt Gambier, SA and Daylesford, VIC, these locations have an excellent climate for temperate vegetable seed production. Cool winters (3.2°C -13.1°C in July at Campania in the Coal River Valley) are generally sufficient for adequate vernalisation. Mild springs (8.6°C-20.7°C in November in the Coal River Valley) are conducive to insect pollination, although late frost is occasionally problematic. Summer in Tasmania is typically mild and dry, with an average of rainfall of 37.4 mm occurring in January in the Coal River Valley. These dry conditions are essential to enable good seed maturation.

Seed crops are low-volume commodities, meaning that Tasmania's isolation does not radically disadvantage producers through expensive sea freight costs. Furthermore, since Tasmanian brassica hybrid seed crops are typically grown in very different areas to other brassica vegetable crops, there are fewer problems associated with achieving the isolation zones required for the production of hybrid seed than there are in the more intensive agricultural environments of, for example, northern Europe.

In addition to these natural virtues, Tasmania has a skilled grower base capable of reliably producing high-quality, high-value seeds in a small area. Production of these crops is increasingly technically demanding, and high quality agricultural infrastructure, such as irrigation, spraying, harvesting and processing equipment allows intensive and cost-effective management. However, despite these favourable circumstances, there are a number of inherent difficulties associated with hybrid seed production, and it remains a high-risk enterprise requiring a level of attention to detail and agronomic crop knowledge well beyond that needed to grow the same crop as a vegetable.

The parent lines used to produce commercial hybrid brassica seed crops are typically inbred for a minimum of six generations to ensure homozygosity. This is essential for phenotypic uniformity of the F_1 generation. The effects of inbreeding depression mean that these parent lines are often weak, susceptible to disease, and liable to produce less viable pollen. As a consequence, special agronomic management is required to produce healthy plants.

A second major issue facing growers is management of the flowering process. Synchronicity in flowering time between brassica parent lines is essential to ensure pollen transfer from pollen donor genetic lines to male sterile genetic lines in order to produce hybrid seed.

However, achieving synchronous flowering has been complicated through the use of varieties with difficult seed production characteristics. For example, seed producers face the challenge of ensuring flowering in F_1 hybrid varieties specifically bred for the desirable vegetable quality of bolting resistance. Attempts to improve heterosis and produce novel hybrids through crossing widely divergent parents with markedly different flowering phenology have also helped to make synchronous flowering difficult to attain. Furthermore, seasonal variation contributes to unpredictable flowering time. Low yields or entire crop failures are common due to poor synchronicity of flowering between parent lines (Verdial et al. 2001). These inherent difficulties require careful management, regardless of where the crop is grown.

As elsewhere, flowering time in Tasmanian cauliflower seed production is usually managed by site selection and time of planting, which is determined through trial and error (c.f. George 1999). Whilst this is generally successful, seasonal temperature variation and lack of experience with new cultivars often results in poor or asynchronous flowering. Tasmanian seed producers often apply plastic coverings in order to modify the crop microclimate and accelerate plant development. The application of plant growth regulating chemicals (such as GA) has also been employed, with varying degrees of success (Spurr & Geard 2010). In cauliflower crops, early flower buds are frequently trimmed to promote development of later flower buds and delay flowering. These practices provide a limited degree of management of flowering time, but are sub-optimal and sometimes result in significant yield penalties.

Seed producers around the world would benefit greatly from improved methods of predicting flowering time, such as assays based on expression of key genes in the flowering process. The development of reliable models to forecast time of flowering across a wide range of genotypes would also be of great benefit. Several physiological models have been developed to enable prediction of cauliflower flowering under field conditions (e.g. Wurr et al. 1993; Pearson, Hadley & Wheldon 1994). Although such phenology models offer reasonable predictive power, their primary input has thus far been restricted to temperature. Consequently, there is considerable scope for the incorporation of additional levels of complexity to these models in order to account for genetic variability and underlying biological pathways. In particular, the integration of genetic and molecular information would potentially enable the development of robust, computational approaches to the modelling of flowering time in cauliflower, as has already been achieved in Arabidopsis

(Chew et al. 2012) and several crop species including bean (*Phaseolus vulgaris* L.), soybean (*Glycine max* L. Merr) and pea (*Pisum sativum* L.) (White & Hoogenboom 1996; Messina et al. 2006; Wenden et al. 2009). Although such models are unlikely to replace the need for field phenotyping, especially where large genotype x environment (GxE) effects are expected, recent improvements in genome sequencing technology and tools for analysing genome-scale gene expression and gene regulatory networks (such as chromatin immunoprecipitation sequencing, or ChIP-seq) have the potential to form the basis for genetically-informed models with greatly enhanced capabilities for predicting the behaviour of complex traits, such as flowering time (c.f. Yant 2012).

Alternative management strategies that offer a greater degree of manipulation of flowering time are also required. A clearer understanding of expression of key genes during induction of curding and flowering may also help in this regard by providing clues as to the potential value of growth regulators and other management strategies in promotion of flowering and identifying appropriate application regimes.

Furthermore, a detailed understanding of the molecular mechanisms that regulate cauliflower flowering time would be valuable to breeders of *B. oleracea* seeking to extend the geographical range of cultivation. For instance, cultivation of *B. napus* in Canada has typically been restricted to milder southern latitudes with longer growing seasons where seed may fully ripen before the first frosts of autumn (Lagercrantz et al. 1996). In recent years, the development of rapid-flowering, early-maturing varieties has enabled cultivation of *B. napus* in colder regions of northern Canada, but early frosts remain problematic and there are ongoing efforts to develop earlier-flowering *B. napus*. Rahman et al. (2011) recently reported an attempt to introduce early flowering time alleles from *B. oleracea* var. *alboglabra* into *B. napus*. Improved knowledge of the key genes involved in regulating flowering time of *B. oleracea* cultivars would not only assist the development of cultivars better-suited to existing agro-environments, but also the development of cultivars suited for new conditions predicted by climate change. Over the last decade, genetic analysis of Arabidopsis flowering time variants has identified a large number of genes that regulate the switch from vegetative to reproductive development. This has enabled the construction of a model describing an integrated network of molecular pathways that control flowering in response to environmental cues such as photoperiod and vernalisation. In the following section, we

briefly review the current molecular model of the Arabidopsis flowering pathway, and consider the potential to extend this model to cauliflower.

E. Genetic Control of Flowering Time in Arabidopsis & Potential for Extension to *B. oleracea*

Arabidopsis thaliana (L.) is a small, weedy annual, native to Europe, Asia and Northern Africa. The earliest non-taxonomic mention of the organism in the literature of scientific botany concerned the description of a flowering mutant discovered in Germany in 1873 (Meyerowitz 2001). Following several decades of sporadic scientific investigation, the 1940s saw the publication of research demonstrating the suitability of Arabidopsis as a model genetic organism based on its high fecundity, short generation time, small size, natural self-fertilisation combined with an amenability to cross-fertilisation, and ease of mutagenesis (Laibach 1943). Interest in the organism gradually increased throughout the 1950s, 60s and 70s and large collections of Arabidopsis mutants and ecotypes were assembled. In the 1980s, the release of a detailed genetic map (Koornneef et al. 1983) along with a series of rapid technical advances in molecular genetics saw the widespread adoption of Arabidopsis as a model genetic organism. This was supported by research demonstrating the small size of the Arabidopsis nuclear genome and the lack of dispersed repetitive DNA. In 1998, it was designated as a Model Genetic Organism (Fink 1998) and by 2000, all five chromosomes had been fully sequenced (Lin et al. 1999; Mayer et al. 1999; Salanoubat et al. 2000; Tabata et al. 2000; Theologis et al. 2000). Since its adoption as a model genetic organism, Arabidopsis has been the subject of intense investigation as a model for reproductive development. In particular, the rapid growth and development of modern biotechnology has led to considerable effort expended in the isolation and analysis of flowering-time genes. This work has resulted in a detailed model of the molecular processes which regulate genes in the floral induction pathway (see Figure 2 for a schematic overview of flowering time control in Arabidopsis).

As noted previously, the timing of floral induction is under the control of both environmental inputs (such as vernalisation and photoperiod) and endogenous cues (such as the juvenile-to-adult transition). The genetic and biochemical routes by which these signals initiate flowering are commonly referred to as ‘flowering pathways’ (Amasino 2010). Genetic and molecular analyses of Arabidopsis have identified four interdependent flowering pathways: the photoperiod and vernalisation pathways, which mediate the response to environmental signals, and the autonomous and GA pathways, which arise from endogenous changes, apparently independently of environmental sensing (Moon et al. 2005). These pathways form

a complex network that ultimately converges on three important regulatory genes including *FLOWERING TIME* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1* or *AGL20*), and *LEAFY* (*LFY*). These genes are known as 'floral integrators' because they integrate the balance of signals from the different flowering pathways and convey the resulting outcome to floral meristem identity genes (MIG) in the shoot apical meristem (SAM) (Simpson & Dean 2002). *LFY* may be regarded as both an integrator gene and a MIG; together with *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*) and *UNUSUAL FLORAL ORGANS* (*UFO*) MIGs, it is involved in the specification of floral fate to lateral meristems (Schultz & Haughn 1991; Mandel et al. 1992; Bowman et al. 1993; Wilkinson & Haughn 1995).

Boss et al. (2004) divide these four flowering pathways into two groups: those that 'enable' floral induction (which include the vernalisation and autonomous pathways), and those that 'promote' floral induction (including photoperiod and GA pathways). A number of floral repressors have been identified that inhibit the expression of floral integrator genes. 'Enabling' pathways function by inhibiting these repressors, thereby rendering the SAM 'competent to flower' (Bernier 1988). Once the SAM has acquired competence to flower, pathways that promote floral induction are effective in stimulating the transition to flowering. The pathways which enable flowering are considered first, beginning with the important floral repressor *FLOWERING LOCUS C* (*FLC*).

FLC encodes a MADS-box protein that binds directly to a region of DNA in the first intron and promoter of *FT* and *SOC1*, respectively (Michaels & Amasino 1999; Sheldon et al. 1999; Helliwell et al. 2006). This prevents transcription of the *FT* and *SOC1* integrator genes, thereby inhibiting flowering. In naturally occurring winter-annual Arabidopsis ecotypes, *FLC* expression is positively regulated by *FRIGIDA* (*FRI*) and other related genes including *FRIGIDA LIKE 1* (*FRL1*) and *FRL2* (Michaels, Bezerra & Amasino 2004; Schlappi 2006). Null alleles of *FRI*, or *FLC* alleles that are not upregulated by *FRI* confer a rapid-flowering habit, and allelic variation in these genes is thought to be a significant source of flowering time variation (Gazzani et al. 2003).

The vernalisation pathway enables flowering to occur by downregulating *FLC* expression. The first known step in this pathway involves the expression of *VERNALIZATION INSENSITIVE 3* (*VIN3*) in response to a prolonged cold signal perceived by the SAM. VIN3

is a PLANT HOMEODOMAIN (PHD)-finger-containing protein (Sung & Amasino 2004b) which is involved in de-acetylation of histone H3 in the *FLC* chromatin. This has the effect of condensing the DNA structure and preventing *FLC* mRNA transcription and translation, thereby allowing expression of *FT* and *SOC1* (Sung & Amasino 2004a; He & Amasino 2005). This initial establishment of a hypoacetylated environment also creates favourable conditions required for the constitutively expressed *VERNALIZATION 1* (*VRN1*) and *VRN2* genes (Gendall et al. 2001; Levy et al. 2002) to methylate H3. Although *VIN3* expression is transient, with mRNA decreasing to undetectable levels within three days of return to warmer temperatures, the methylation regulated by *VRN1* and *VRN2* is a structural change which is propagated through mitotic cell divisions in the absence of the inducing cold signal, accounting, at least partially, for epigenetic 'memory' of the vernalised state (Gendall et al. 2001; Levy et al. 2002; Amasino 2004; Bastow et al. 2004; Sung & Amasino 2004a).

The autonomous pathway also influences flowering time through regulation of *FLC*, although this pathway functions independently of the environment. A suite of genes including *LUMINIDEPENDENS* (*LD*), *FLOWERING LOCUS D* (*FLD*), *FPA*, *FCA*, *FY* and *FVE* are involved in repressing *FLC* expression (Simpson & Dean 2002) and maintaining it at a base level through a variety of mechanisms including chromatin remodelling (e.g. He & Amasino 2005), and RNA metabolism (e.g. Simpson et al. 2003). Although no input signals have been conclusively identified as regulators of autonomous gene expression (Simpson 2004), it has been noted that expression of *FCA* is very low during early seedling development, gradually increasing in the days after germination (Macknight et al. 1997). This may suggest a mechanism by which *FLC* levels are gradually reduced as the plant develops, thereby preventing precocious flowering (Boss et al. 2004). In accessions that are early-flowering due to null *FRI* alleles, mutations in any of these autonomous pathway genes restore the late-flowering phenotype in both long and short days (LDs and SDs) (Koornneef, Hanhart & Van der Veen 1991); in winter-annual accessions, *FRI* functions epistatically to the autonomous pathway to upregulate *FLC* and prevent flowering (Michaels, Bezerra & Amasino 2004). Regardless of whether increased *FLC* expression is due to upregulation by *FRI* or mutations in the autonomous pathway, vernalisation induces flowering through the epigenetic repression of *FLC* (Michaels, Bezerra & Amasino 2004). Interestingly, Hepworth et al. (2002) suggest that while FLC is bound to the *SOC1* promoter, the ability of flowering pathways to upregulate *SOC1* and promote flowering is compromised. Downregulation of

FLC removes this 'block' and enables other flowering pathways to affect expression of the floral integrators.

Once these 'enabling' factors have rendered the SAM competent to flower, pathways that promote the transition to flowering are effective in stimulating that change. LDs induce flowering in Arabidopsis, and this process begins with photoreceptors: light-sensitive proteins that undergo conformational changes upon absorption of a specific wavelength (Putterill, Laurie & Macknight 2004). Two of the most important photoreceptor families are the phytochromes (*PHYA-E*; red and far-red light absorbing) and cryptochromes (*CRY1* and *CRY2*; blue and UV-A light absorbing). When the chromophore (light-sensitive component) of these photoreceptors absorbs its specific wavelength of light, the photoreceptor changes conformation and interacts with downstream genes. For example, when *PHYB* is exposed to red light, it is activated and relocated to the cell nucleus where it forms a complex with the PIF3 transcription factor (Millar 2003). This complex binds to the promoters of genes encoding MYB transcription factors COLD CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), causing them to be upregulated. Both of these genes are important components of the circadian clock.

Circadian clocks are molecular oscillators that operate on a rhythmical, c.a. 24-hour basis. Their rhythms persist in the absence of external cues, but may be precisely entrained to a 24-hour cycle by diurnal variations in light or temperature (McWatters et al. 2000; Spalding & Folta 2005; Liew et al. 2009). This not only serves to synchronise regular physiological events with local day- and night-time, but also plays a role in initiating once-off developmental processes such as flowering. Indeed, altered flowering time is often the most obvious effect of mutations in circadian clock genes (e.g. Hicks et al. 1996; Doyle et al. 2002; Mizoguchi et al. 2002; Hazen et al. 2005), and variation in these genes is likely to be an important source of flowering time variation in many plant species. Until the mid-2000s, the best model of the circadian core oscillator comprised a single feedback loop involving the reciprocal regulation of the aforementioned *CCA1*, *LHY* and *TIMING OF CHLOROPHYLL A/B BINDING PROTEIN EXPRESSION 1* (*TOC1*) (e.g. Alabadí et al. 2001). In recent years however, a very large number of clock genes have been identified, and the vast complexity of the molecular processes controlling circadian clocks is being realised. Computational modelling studies have seen the extension of the 'single-loop model' into a 'three-loop' model comprising (at least) three networked, interlocking feedback loops with transcriptional

and post-transcriptional regulation and multiple points for light input and entrainment (Locke et al. 2006; Zeilinger et al. 2006).

The circadian clock is responsible for the photoperiod-regulated, rhythmic modulation of several flowering-specific genes, with the notable inclusion of *CONSTANS* (*CO*), an important transcription factor involved in the upregulation of floral integrator genes (Putterill et al. 1995; Suárez-López et al. 2001). In the morning, there is an abundance of proteins known as CYCLING DOF FACTORS (CDFs) which bind to, and repress the *CO* promoter (Imaizumi et al. 2005). Throughout the day, exposure to blue light increases activity of a photoreceptor named *FLAVIN-BINDING, KELCH REPEAT, F BOX PROTEIN 1* (*FKF1*). In the afternoon of LDs, FKF1 and the circadian protein GIGANTEA (GI) form a complex that is involved in CDF degradation, thereby enabling *CO* expression (Sawa et al. 2007). Coincident light perception by *CRY1*, *CRY2* and *PHYA* is thought to contribute to CO stabilisation, leading to upregulation of the floral integrator genes. In SD, peak transcription of *CO* mRNA does not occur until darkness has fallen, preventing protein accumulation and flowering.

Although the biochemical function of CO is not fully understood, its primary target has been identified as floral integrator *FT* (Wigge et al. 2005). Photoperiod is perceived by plant leaves, and it is here that *CO* upregulates *FT*. FT is then transported through the phloem to the SAM where it interacts with a transcription factor called FD to upregulate MIGs (Wigge et al. 2005). *TWIN SISTER OF FT* (*TSF*) is a close homologue of *FT* and appears to be another target of CO. Its transcriptional responses to environmental conditions closely match those of *FT*, and it is suggested that it plays an independent, yet largely redundant role to *FT* in floral integration (Yamaguchi et al. 2005). *TERMINAL FLOWER 1* (*TFL1*) is another close homologue of *FT*, but in contrast to *TSF*, it delays flowering by competing with FT for FD binding (Ahn et al. 2005). SOC1 was also thought to be a direct target of CO (Samach et al. 2000), but more recent studies (e.g. Wigge et al. 2005) suggest that *FT* is an intermediary in the *CO-SOC1* interaction (see also Turck, Fornara & Coupland 2008; Lee & Lee 2010). The influence of CO on *LFY* expression is more indirect still, and is thought to be mediated through positive feedback loops involving *SOC1* and *AGAMOUS-LIKE 24* (*AGL24*) (e.g. Liu et al. 2008).

Gibberellins have been shown to be essential for bolting in Arabidopsis (Koornneef & Van der Veen 1980; Griffiths et al. 2006). Experiments with GA-deficient *ga1-3* biosynthetic mutants have also shown GA to be necessary for flower induction in the absence of photoperiod-inductive conditions (Wilson, Heckman & Somerville 1992). Blázquez et al. (1998) demonstrated that GAs directly promote *LFY* expression through interaction with its promoter, stimulating floral induction. Evidence that *SOC1* is regulated by GA has been provided (Moon et al. 2003), and *FT* has also been proposed as a target of GA activity (Mutasa-Göttgens & Hedden 2009); Boss et al. (2004) note that *LFY* over-expression is unable to fully complement the late flowering behaviour of *ga1-3*, and suggest that this may be due to altered expression of *SOC1/FT* in *ga1-3*.

Many of the genes and genetic pathways involved in the Arabidopsis floral induction process have been conserved to some degree in other plant species. Consequently, there is a dramatic expansion of interest in the application of this knowledge to a wide range of crop species using comparative and functional genomics approaches. For example, the complete sequence of the rice (*Oryza sativa* L.) genome has allowed comparisons to be made between rice and Arabidopsis flowering pathways (Izawa, Takahashi & Yano 2003). The recent releases of the complete genome sequence of model species such as poplar (*Populus trichocarpa* Torr. & Gray) and soybean have enabled similar analysis of the flowering genetics of perennial trees and crop legumes, respectively (Brunner & Nilsson 2004; Hecht et al. 2005). Understanding the flowering process in other important cereal crops such as wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) has been greatly aided by comparative genetic approaches within temperate cereals using the framework established in Arabidopsis (Cockram et al. 2007). Other crop species in which Arabidopsis flowering time homologues have been cloned and investigated include tomato (*Solanum lycopersicum* L.), sunflower (*Helianthus annuus* L.) and onion (*Allium cepa* L.) (e.g. Molinero-Rosales et al. 1999; Lifschitz et al. 2006; Taylor, Massiah & Thomas 2010; Blackman et al. 2011).

The close relationship of *Brassica* and *Arabidopsis* has naturally led to comparative genetic studies of the molecular pathways controlling floral induction in brassica crops. The recent release of a *B. rapa* reference-quality complete genome sequence (Wang et al. 2011b), combined with ongoing sequencing of the *B. oleracea* and *B. napus* genomes and subsequent plans to sequence *B. nigra*, *B. juncea* and *B. carinata* genomes is likely to stimulate further investigation into this area. At the time of writing, partial sequences have been reported for

B. oleracea homologues of *LFY* (*BoFH*), *AP1*, *APETALA3 (AP3)*, *CAL*, *FRUITFULL (FUL)*, *FLC*, *FT*, *TFL1* and *FRI* genes (Anthony, James & Jordan 1993; Carr & Irish 1997; Mimida et al. 1999; Kop 2002; Lin et al. 2005; Vorobiev, Pankin & Khavkin 2006). Several flowering time candidate genes that appear to be specific to cauliflower have also been identified, including the putative MIG *BoREM1* (Franco-Zorrilla et al. 1999) and *CCE1* which is thought to be involved in the control of meristem development or arrest (Palmer et al. 2001). Partial sequences of several important flowering time homologues have been reported in other *Brassica* species, including *SOC1* in *B. rapa* (Kim et al. 2003) and *VRN1* in *B. rapa* (Kwon et al. 2004). Figure 2 is a simplified diagram of the Arabidopsis flowering pathway presented alongside a 'working model' of the *B. oleracea* flowering pathway, which includes the *B. oleracea* flowering genes mentioned above positioned according to their location in the Arabidopsis flowering pathway; it is clear that the identities of many *B. oleracea* flowering genes are yet to be discovered.

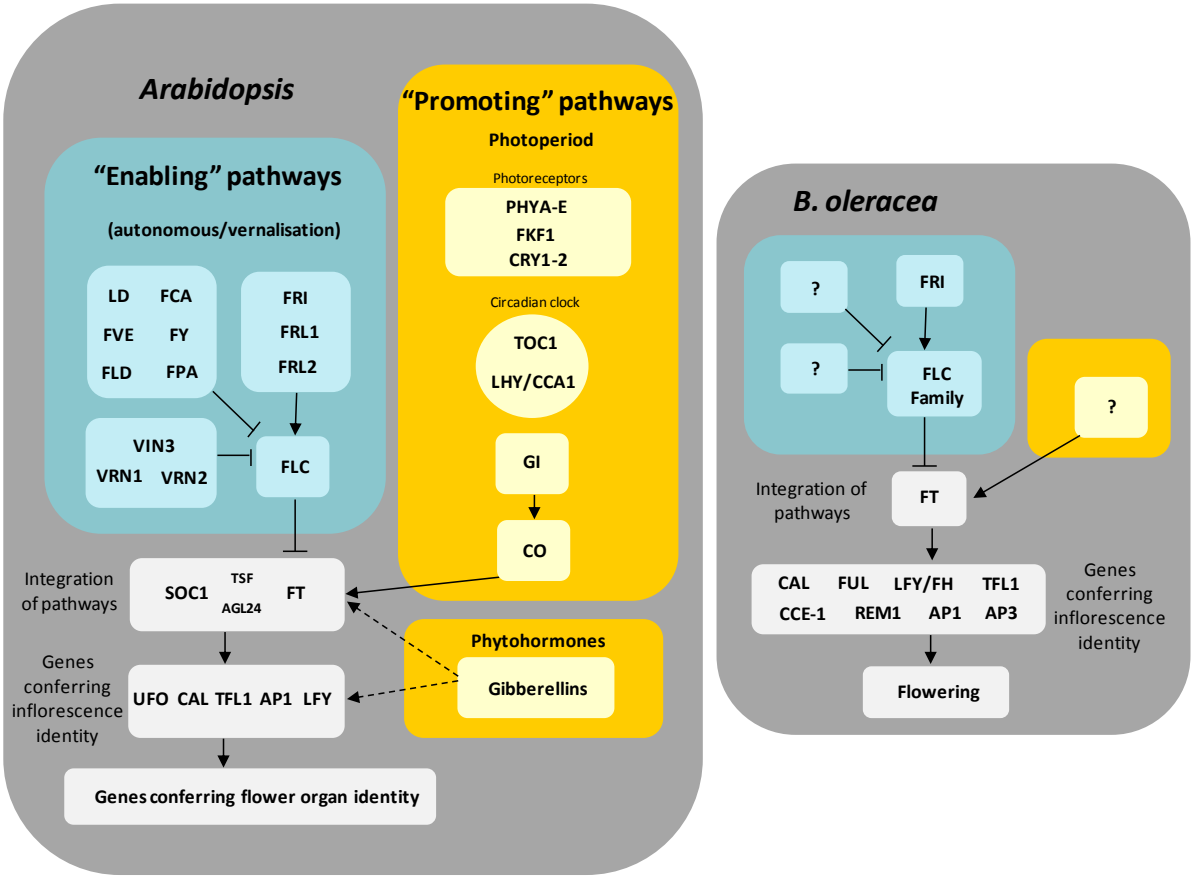


Fig. 2. Comparison of Arabidopsis and *B. oleracea* flowering pathways. Positive and negative regulatory actions are denoted by arrows, and lines with bars, respectively. Dashed lines indicate more speculative interactions. The location of the genes pictured within the *B. oleracea* diagram is based on

(*continued overleaf*)

Fig. 2. (*continued*) their location in the Arabidopsis pathway. Question marks indicate processes or pathways for which no homologous *B. oleracea* genes have been positively identified.

Efforts to characterise these flowering candidate genes have been made, revealing both parallels and differences to their Arabidopsis homologues. For example, in Arabidopsis, *CAL*, *AP1* and *LFY* are involved in the specification of floral meristem identity. In *ap1/cal* Arabidopsis mutants, the floral meristem behaves as an inflorescence meristem reminiscent of the cauliflower curd (Bowman et al. 1993). The identification of a non-functional *CAL* homologue in cauliflower (Kempin, Savidge & Yanofsky 1995), the isolation of a wild-type *CAL* allele in broccoli (Carr & Irish 1997) and the finding that *BoAP1-a* and *Bo-CAL* had additive roles in head arrest stage (Smith & King 2000) supported the idea that these genes were the primary regulators of developmental arrest in cauliflower (see also Lowman & Purugganan 1999). However, other studies have shown that although these genes are important to cauliflower curd development, and although there is considerable homology between the Arabidopsis and *Brassica* homologues, their patterns of expression in *B. oleracea* are inconsistent with existing models of Arabidopsis floral initiation and development (Anthony, James & Jordan 1993; Duclos & Björkman 2008). In addition, expression patterns of several other *Brassica* flowering homologues (including *TFL1* and *FUL*) are inconsistent with their roles in Arabidopsis, and it is likely that additional genes are involved in the complex process of curd development and bolting.

In contrast to the research on inflorescence development, relatively little work has been done on genes acting earlier in the floral initiation process in *B. oleracea*. Some attention has recently been given to the *FLC* gene family, due to the central role of Arabidopsis *FLC* in the vernalisation response. A number of genetic and QTL analyses have identified strong flowering time QTLs in the *B. napus*, *B. oleracea* and *B. rapa* chromosomal regions syntenic to the region near the top of Arabidopsis chromosome 5, where *FLC* and several other flowering time genes including *CO* and *FY* are located (e.g. Lagercrantz & Lydiate 1996; Osborn et al. 1997; Bohuon et al. 1998; Rae, Howell & Kearsey 1999; Schranz et al. 2002; Okazaki et al. 2007) and some of these authors have specifically identified these brassica loci as being homologous to *AtFLC*.

Several members of this gene family have been isolated and characterised in various brassica crop species (Tadege et al. 2001; Schranz et al. 2002; Martynov & Khavkin 2004; Li et al. 2005; Lin et al. 2005; Kim et al. 2007; Okazaki et al. 2007; Razi et al. 2008). Studies have shown that *FLC* is strongly down-regulated in response to cold treatment in *B. napus* (Tadege et al. 2001), *B. rapa* (Li et al. 2005; Kim et al. 2007) and *B. oleracea* var. *capitata* L. (Lin et al. 2005). These authors also reported instances where pre-vernalisation *FLC* transcript levels and *FLC* repression responsiveness appear to be correlated with flowering time and vernalisation requirement. Lin et al. (2005) isolated a cabbage homologue of the important flowering time integrator gene *FT* and found that expression of this transcript in cabbage apices was inversely related to *BoFLC* expression. Regulation of *FLC* and *FT* expression may also explain differences in the stage at which vernalisation is effective, which may in turn affect juvenility duration, and it is also possible that the stability of *FLC* repression may be linked with devernalisation phenomena reported in many *Brassica* species (Friend 1985). Furthermore, *FT* is known to play an ongoing role in Arabidopsis meristem maintenance during inflorescence development (Smith et al. 2010), raising the possibility that it may also play an analogous role in cauliflower curd development.

F. Research Motivation & Objectives

Research and development efforts in vegetable seed production currently lag behind those in vegetable breeding and vegetable crop production. There are major opportunities for productivity gains through application of fundamental research findings to seed production practice. *B. oleracea* seed constitutes the largest sector of the Australian vegetable seed industry, but industry sources estimate that up to 50% of *B. oleracea* seed crops grown in Australia produce sub-optimal yields due to problems with completeness or timing of flowering, with producers around the world facing the same problem. Current industry solutions to the problem of asynchronous flowering are sub-optimal and often incur a significant yield penalty. A better understanding of the physiological and molecular processes that regulate reproductive development in *B. oleracea* would provide new ways of monitoring and understanding progress towards flowering and would enable the development of more effective strategies for the management and manipulation of flowering in a production environment.

This project will attempt to address this issue by investigating the effects of vernalisation on the molecular and physiological aspects of floral induction, curd formation and flowering in a diverse range of cauliflower genotypes. In order to do this, recent discoveries relating to the genetic mechanisms involved in the Arabidopsis floral induction pathway will be extended to cauliflower. Rapid advances in knowledge of the molecular control of flowering have not been matched by development of new crop management strategies, and it is hoped that this study will enable the development of an integrated physiological and molecular approach to a practical problem. Five related areas of investigation, corresponding to the five research chapters in this thesis, may be identified:

1. Genetically evaluating the contribution of *Arabidopsis thaliana* flowering time candidate genes to variation in reproductive development, especially focusing on the contribution of allelic variation in brassica *FLC* to curd initiation and flowering time variation.
2. Characterising the reproductive behaviour of a range of cauliflower genotypes in multiple growth environments and investigating associations with flowering gene functionality.
3. Understanding sources of variation in vernalisation response, flowering time and

juvenility across a range of genotypes within the cauliflower group, and characterisation of these differences at the molecular level by monitoring the transcriptional dynamics of key genes known to regulate these processes.

4. Investigating the molecular basis of devernalisation and reversion by examining the expression of genes in the floral induction pathway.
5. Considering the feasibility of molecular assays to predict flowering time in production environments.

Predicting timing of key reproductive events and implementation of effective strategies to alter flowering times are critical to commercial hybrid brassica seed production. It is hoped that these five areas of investigation will, collectively, lead to an improved understanding of regulation of flowering at the molecular level in *B. oleracea*. This would facilitate development of (a) predictive tools – for instance, QTL-based crop growth models or gene expression assays that can be used to predict flowering time (b) management tools based on the use of molecular methods to assess effects of crop treatments and (c) breeding tools, such as markers that can be used to screen *B. oleracea* germplasm for molecular variation likely to be associated with traits of interest, or identification of genes that may be useful in the production of transgenic crops. Furthermore, it is hoped that this project will provide insights into the ways that basic genetic mechanisms for the environmental control of flowering have been adapted in different species and in different cultivars within the same species. In particular, analysis of key flowering genes would provide significant new information about the genetic and physiological control of important features in cauliflower development, such as juvenility, devernalisation, and curd development, that are either not represented or not well understood in Arabidopsis or in any other species.

Chapter II

GENERAL MATERIALS & METHODS

A. Introduction

The research presented in this thesis was initiated as a collaborative research project between the University of Tasmania, Rijk Zwaan Nederland B. V. (De Lier, Netherlands) and seedPurity Pty Ltd. (Margate, Tasmania). The project was funded through an Australian Research Council Linkage Grant and commenced in April 2006. This thesis formed a major part of the project and was aligned with a second component, focussed on crop agronomy to manipulate flowering time in brassica seed crop parent lines, undertaken by commercial partner seedPurity. The PhD research was conducted in two stages between April 2006 and June 2009 and between July 2010 and October 2011 (part-time).

The project made use of proprietary brassica inbred parent lines from the Rijk Zwaan breeding programme. Research focused on evaluation of the contribution of flowering time candidate genes to flowering variability between these parent lines and describing transcriptional dynamics of key genes both during 'normal' flowering and also under conditions promoting devernalisation. Preliminary investigations of the feasibility of using molecular assays to predict flowering time under commercial seed crop production conditions were also made; this was a minor component of the PhD project but formed an important linkage to the crop agronomy research undertaken in the larger project. All research was undertaken in Tasmania, Australia, apart from that presented in Chapter III, which was undertaken during a four-month visit to Rijk Zwaan facilities at Fijnaart, Netherlands, and the soil-based glasshouse trial in Daylesford, VIC described in Chapter IV.

In this chapter, general materials and methods common to two or more of the five research chapters are described. Modifications and methodology specific to the research covered in individual chapters is presented in the relevant chapter.

B. Plant Materials

Fifty-four homozygous inbred cauliflower proprietary parent lines, 41 homozygous inbred cabbage proprietary lines and two homozygous inbred swede (*B. napus* var. *napobrassica*) proprietary lines were provided by Rijk Zwaan Nederland B. V. (De Lier, 2678 ZG, Netherlands). Table 2 summarises the breeders' information available for these lines.

Table 2. Brassica variety/flowering classifications. The 54 cauliflower lines had been grouped into four flowering classes by Rijk Zwaan breeders, based on time from planting to full flower under field conditions in Fijnaart, Noord Brabant, Netherlands: Class 1: Early (50-70 days); Class 2: Medium Early (70-80 days); Class 3: Medium Late (80-120 days); Class 4: Late (120-180 days); Class 5: Very Late (180-250 days). Other Brassica lines were categorised only as 'white', 'savoy', 'red' or 'swede', with some described as 'easy bolting'. Specific flowering time data for the cabbage or swede varieties was not possible to obtain.

CAULIFLOWER LINE	CAULIFLOWER FLOWERING CLASS	CABBAGE/SWEDE LINE	CABBAGE/SWEDE VARIETY
1	Late	55	White
2	Very Late	56	White
3	Very Late	57	White (Easy Bolting)
4	Very Late	58	White
5	Very Late	59	White
6	Med Early	60	White
7	Very Late	61	White
8	Med Early	62	White
9	Late	63	White
10	Early	64	White
11	Very Late	65	White
12	Very Late	66	White
13	Late	67	White
14	Late	68	White (Easy Bolting)
15	Very Late	69	White (Easy Bolting)
16	Late	70	White
17	Late	71	White
18	Med Late	72	White (Easy Bolting)
19	Late	73	White
20	Med Early	74	White
21	Med Late	75	White
22	Very Late	76	White (Easy Bolting)
23	Med Early	77	White
24	Late	78	White
25	Late	79	White
26	Very Late	80	White (Easy Bolting)
27	Med Late	81	Swede
28	Very Late	82	Swede
29	Med Late	83	Savoy
30	Early	84	Savoy
31	Early	85	Savoy
32	Early	86	Savoy
33	Med Late	87	Savoy

(continued overleaf)

Table 2. *(continued)*

CAULIFLOWER LINE	CAULIFLOWER FLOWERING CLASS	CABBAGE/SWEDE LINE	CABBAGE/SWEDE VARIETY
34	Med Early	88	Savoy
35	Early	89	Savoy
36	Med Early	90	Red
37	Early	91	Red
38	Early	92	Red
39	Early	93	Red
40	Early	94	Red
41	Med Late	95	Red
42	Med Early	96	Red
43	Med Early	97	Red
44	Med Late		
45	Med Early		
46	Late		
47	Med Early		
48	Med Early		
49	Med Late		
50	Med Late		
51	Late		
52	Med Late		
53	Med Late		
54	Early		

C. Scoring of Curd Development

Many of the experiments undertaken in this project required assessment of the flowering sequence in cauliflower plants. A semi-quantitative scale to describe the developmental stage of curds was developed to allow consistent assessment throughout the project. This Curd Development Key is shown in Figure 3.

D. Plant Growth Medium

Pot-grown plants were grown in general purpose potting mix (pH approx. 6.3, EC approx. 1.31), which was a blend of composted pine bark (80%) and coarse sand (20%). Where seeds were germinated in seed raising mix, a 50/50 blend of peat/coarse sand was used.

Stage 1: Visible curd is compact

Stage 2: Early stages of curd 'breaking'

Stage 3: Early-order inflorescence branches elongating; flower buds not formed or rudimentary

Stage 4: Late-order branches elongating and flower buds fully formed

Stage 5: Less than 12 flowers fully open

Stage 6: More than 12 flowers fully open

Fig. 3. Curd Development Key. Stage 1 corresponds to a curd that is visible without damaging excavation amongst apical leaves, and that is compact, with no discernable elongation of peduncles. Stage 2 corresponds to a 'loose' curd that is beginning to 'break'. Stage 3 corresponds to a curd whose early-order inflorescence branches are elongating, but where individual flower buds are not yet formed, or are rudimentary. Stage 4 curds have fully-formed flower buds (similar to the individual buds of a broccoli head). Stage 5 corresponds to a curd that has one or more flowers fully open (circled), but less than twelve flowers fully open. Stage 6 is the highest developmental category, and curds have more than 12 flowers open, and may have started to produce fruit.

E. Gene Isolation

Genes were isolated using an eight-step approach: (1) DNA extraction; (2) DNA quantification; (3) primer design; (4) PCR; (5) bacterial cloning; (6) colony PCR; (7) plasmid DNA extraction; and (8) sequencing.

1. DNA Extraction

Genomic DNA was isolated from individual plants using a modified CTAB protocol. Harvested plant tissue was frozen in liquid nitrogen and ground to a fine powder in a mill mixer by adding a tungsten carbide bead to each tube containing the frozen plant tissue. 500 µL of extraction buffer (100 mM Tris-HCl pH 8, 1.4 M NaCl, 20 mM EDTA, 2% w/v CTAB, 20 mM 2-β-mercaptoethanol) were added to each tube prior to incubation at 60°C for 15 min. 500 µL of Chloroform:Isoamylic alcohol (24:1) was then added to the tubes and the contents were mixed by gentle inversion. The samples were centrifuged at 14000 rpm for 1 min and the upper aqueous phase was transferred to new tubes, and extracted a second time with 500 µL of Chloroform:Isoamylic alcohol (24:1). 1 mL of precipitation buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% w/v CTAB) was added, and tubes were mixed gently and allowed to rest at room temperature for 10-15 minutes until a thread-like precipitate formed. The precipitate was collected by centrifugation (10 min, 14000 rpm) and dissolved in 300 µL 1.5M NaCl containing 1 µL RNase A (25 mg/mL). The solution was incubated at 50°C for 15 minutes or until the pellet was fully dissolved. DNA was precipitated by adding 600 µL 100% ethanol and was collected by centrifugation (10 minutes at 14000 rpm). After centrifugation, the pellet was washed in 70% ethanol, air-dried, and re-suspended in 50 µL sterilised distilled water (SDW).

2. DNA Quantification

Two alternative methods of quantifying DNA were employed throughout this research. In the first, DNA concentration was measured in a 1X TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) using a fluorescent nucleic acid stain (Quant-iT™ PicoGreen® dsDNA quantification reagent; Invitrogen, Carlsbad, CA, USA) with a *Pico*fluor™ handheld fluorometer (Turner Designs, Sunnyvale, CA, USA). The Blue LED fluorescence channel was selected (excitation/emission of 474/515 nm), and a Lambda DNA standard supplied with the nucleic acid stain was used for calibration. Simpler and more accurate quantification

became possible following the acquisition of a NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MS, USA) which was utilised according to manufacturer's instructions <http://www.nanodrop.com/Library/nd-8000-v2.0-users-manual-8.5x11.pdf>.

3. Primer Design

All primers were designed with the aid of Primer3 <http://www.frodo.wi.mit.edu/primer3>. Primer sequences are provided in the relevant chapters.

4. Polymerase Chain Reaction (PCR)

All PCRs were performed in 50 µL reactions comprising 5 µL of (approx.) 50 ng/µL DNA and 45 µL master mix. Master mix reagents and proportions are detailed in Table 3. Generally, one of two Taq systems were used: either Mango Taq (Bioline, London, UK) with the included 5x Bioline buffer and $MgCl_2$ solution; or TasPol (an in-house Taq enzyme produced at the University of Tasmania) with a 10x PCR buffer comprising 500 mM KCl, 100 mM Tris-HCl pH 8.8, 1% Triton X-100 and 15 mM $MgCl_2$. For a small number of difficult PCRs, Titanium Taq Polymerase (Clontech Laboratories Inc., Mountain View, CA, USA) and its associated reagents were utilised according to manufacturer's instructions. PCRs were carried out in thermal cyclers with heated lids, and as a general rule, comprised an initial 5 min template denaturation step at 94°C, followed by 35 cycles of denaturing (94°C for 1 min), 1 min of annealing at the appropriate temperature, and extension at 72°C for 1 min per 1 Kb, with a final elongation step of 72°C for 10 min. Annealing temperatures were optimised according to the length and purine/pyrimidine composition of the primers. PCR products were visualised by agarose gel electrophoresis, with gel concentration dependant on the expected fragment size. Products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) following the manufacturer's microcentrifugation protocol.

Table 3. Standard PCR set-up. Substantial departures from the master mix composition shown below are noted in the relevant section.

REAGENT	AMOUNT/REACTION (TASPOL)	AMOUNT/REACTION (MANGO)
PCR Buffer (Taspol: 10x; MangoTaq: 5x)	5 µL	10 µL
$MgCl_2$	0 µL	2 µL
dNTPs (10 mM)	1 µL	1 µL
Forward Primer (10 µM)	1 µL	1 µL
Reverse Primer (10 µM)	1 µL	1 µL
DNA polymerase	0.5 µL	0.1 µL
DNA template (added separately)	5 µL	5 µL
Sterile Distilled Water (SDW)	36.5 µL	29.9 µL
Total	**50 µL**	**50 µL**

5. Cloning

Purified PCR products were ligated into a pGEM-T plasmid vector using the Promega pGEM-T Easy Vector System (Promega, Madison, WI, USA) using the 10 µL protocol. Electrocompetent *E. coli* cells (50 µL) were transformed by adding 5 µL of the ligation mix and electroporating at 1.2 kV. 400 µL Luria Broth (LB; 10 gL^{-1} NaCl, 5 gL^{-1} Bacto Yeast extract, 10 gL^{-1} Bacto Tryptone, pH 7.5) was added to each tube of transformed bacteria, and bacteria were incubated at 37°C with shaking for 60 min. After this period of recovery time, each transformation was spread across two LB agar plates (225 µL of transformation mix per plate) containing 100 µg/mL ampicillin and 1 µL/mL 4% X-gal (5-bromo-4-indolyl-beta-D-galactopyranoside) which were incubated at 37°C overnight. Successful cloning of an insert into the pGEM-T plasmid interrupted the coding sequence of the beta-galactosidase gene, allowing recombinant clones to be identified by their white colour (in contradistinction to the blue colour indicative of colonies that were still capable of metabolising X-gal due to the absence of an insert in their plasmid).

6. Colony PCR

Seven to ten transformed white colonies were picked up with a sterile toothpick, placed in 5 µL SDW, and denatured at 95°C for 5 min. A 20 µL aliquot of PCR master mix (Table 4) containing primers specific for the inserted sequence was added to each tube, a PCR was performed, and the product was visualised by gel electrophoresis according to the protocol mentioned above.

Table 4. Colony PCR set-up.

REAGENT	AMOUNT/REACTION
PCR Buffer (5x)	5 µL
$MgCl_2$	1 µL
dNTPs (10 mM)	0.5 µL
Forward Primer (10 µM)	0.5 µL
Reverse Primer (10 µM)	0.5 µL
DNA polymerase	0.2 µL
Transformed bacteria (added separately)	5 µL
Sterile Distilled Water (SDW)	12.3 µL
Total	**25 µL**

7. Plasmid DNA Extraction

Individual colonies of bacteria whose DNA produced PCR fragments of an appropriate size were subcultured in 5 µL Terrific Broth (TB; 12 g/L Bacto Tryptone, 24 g/L Bacto yeast extract, 4 mL/L glycerol, 25 mL/L KHPO) with 100 µg/mL ampicilin, and incubated at 37°C overnight in a shaker-incubator. Plasmids were isolated from the culture using the centrifugation method of the Wizard® *Plus* SV Minipreps DNA Purification System (Promega, Madison, WI, USA) according to manufacturer's instructions.

8. Sequencing

Initially, purified PCR products and plasmids were sequenced in-house at the University of Tasmania. Over the course of the project, products and plasmids were also sequenced at the Australian Genome Research Facility (AGRF; Gehrmann Laboratories, University of Queensland, St Lucia, QLD, Australia) and Macrogen (Seoul, Korea). Sequences were aligned and analysed using Sequencher® v4.0 sequence analysis software (Gene Codes Corp., Ann Arbor, MI, USA).

F. Gene Expression

In this section, the five-step approach employed in this project for the measurement of mRNA transcript levels in plant tissues is described: (1) tissue sampling; (2) RNA extraction; (3) RNA quantification; (4) reverse transcription; and (5) RT-qPCR. Specific details relating to the individual genes analysed (e.g. primer details and annealing temperatures) are provided in Chapter V.

1. Tissue Sampling

Young leaf and apex tissue was harvested for RNA extraction and gene expression analysis. Clean scalpel blades were used to remove tissue, which was immediately frozen in an aluminium foil package in liquid nitrogen. Young leaves were defined as the youngest non-fully expanded leaf of sufficient size to extract adequate RNA (usually 25 mm – 30 mm from leaf apex to the point where the petiole becomes distinct from the mid rib). In vegetative apex samples, care was taken to exclude as many young leaves and leaf primordia as possible, but small amounts of stem and leaves were invariably present in samples, which may be more accurately described as 'apically-enriched tissue'. Sufficient tissue was generally unable to be obtained from plants younger than three weeks of age, and in these cases, it was necessary to combine tissue from three to four replicate plants. Where plants had commenced reproductive development, a small amount of tissue was removed from the centre of the curd.

2. RNA Extraction

RNA extraction and purification was performed in a nuclease-free environment with the SV Total RNA Isolation System (Promega, Madison, WI, USA), according to manufacturer's instructions. Prior our laboratory's adoption of this Promega system, a small number of RNA extractions were performed with an alternative RNeasy® Mini system (Qiagen, Hilden, Germany) following manufacturer's instructions.

3. RNA Quantification

As with the DNA quantification described above, two alternative methods of quantifying RNA were employed. The first method involved the measurement of RNA concentration in a 20X TE solution (200 mM Tris-HCl, 20 mM EDTA, pH 7.5) using a fluorescent nucleic acid stain (Quant-iT™ RiboGreen® quantification reagent; Invitrogen, Carlsbad, CA, USA) with the same configurations on the same handheld fluorometer as described previously. A ribosomal RNA standard (16S and 23S rRNA from *E. coli*) supplied with the nucleic acid stain was used for calibration. Simpler and more accurate quantification became possible with the acquisition of a NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MS, USA) which was utilised according to manufacturer's instructions <http://www.nanodrop.com/Library/nd-8000-v2.0-users-manual-8.5x11.pdf>.

4. Reverse Transcription

Three methods of synthesising first strand cDNA from total RNA were employed in this research according to changing availability, price and laboratory practice. Outcomes were found to be similar between all three methods. The most common method used the MMLV High Performance Reverse Transcriptase System (Epicentre Biotechnologies, Madison, WI, USA). 1 µg RNA in 13 µL nuclease-free water was incubated at 65°C for 5 min in order to denature any secondary structures, and was then immediately placed on ice. A master mix was prepared according to Table 5a and a 7 µL aliquot was added to the RNA. For each sample, a negative control without reverse transcriptase (RT-) was prepared in order to check for genomic DNA contamination. The RT-PCR was performed at 37°C for 60 min, followed by a final 85°C, 5 min incubation step to denature the RT enzyme. For use in RT-qPCR, the synthesised cDNA was diluted 1:4 with SDW.

The second method used to synthesise cDNA was the Omniscript® Reverse Transcriptase system (Qiagen, Hilden, Germany). All reagents are listed in Table 5b and the remaining procedure was identical to the MMLV system, except for the initial use of 2 µg RNA in 14 µL H_2O with 6 µL master mix, and the omission of the final 85°C denaturation step. The third method used was the ImprompII Reverse Transcriptase system (Promega, Madison, WI, USA). 1 µg RNA in 10.5 µL nuclease-free was incubated for 5 min at 70°C and iced for 2 min prior to the addition of a 9.5 µL aliquot of master mix (Table 5c). Once again, RT-

reactions were prepared, and the RT-PCR consisted of a 5 min annealing step at 25°C, a 60 min extension step at 42°C, and a final 15 min enzyme deactivation step at 70°C. For use in RT-qPCR, the synthesised cDNA was diluted 1:4 with SDW.

Table 5. RT-PCR set-up. Master mix reagents and proportions are shown for three different systems; (a) MMLV High Performance Reverse Transcriptase System; (b) Omniscript Reverse Transcriptase system and (c) ImprompII Reverse Transcriptase system.

a.

REAGENT	RT+	RT-
Epicentre RT buffer (10x)	2 µL	2 µL
DTT (100 mM)	2 µL	2 µL
dNTPs (10 mM)	1 µL	1 µL
Oligo-dT primer (10 µM)	1 µL	1 µL
ScriptGuard RNase inhibitor	1 µL	1 µL
MMLV Reverse Transcriptase (200 U/µL)	1 µL	0 µL
RNA/nuclease-free water (added separately)	13 µL	13 µL
SDW	0 µL	1 µL
Total	**20 µL**	**20 µL**

b.

REAGENT	RT+	RT-
10x Buffer RT	2 µL	2 µL
dNTPs (10 mM)	1 µL	1 µL
Oligo-dT primer (10 µM)	1 µL	1 µL
RNase inhibitor (10 U/µL)	1 µL	1 µL
Omniscript Reverse Transcriptase	1 µL	0 µL
RNA/nuclease-free water (added separately)	14 µL	14 µL
SDW	0 µL	1 µL
Total	**20 µL**	**20 µL**

c.

REAGENT	RT+	RT-
RT buffer (5x)	4 µL	4 µL
$MgCl_2$ (25 mM)	2.5 µL	2.5 µL
dNTPs (10 mM)	1 µL	1 µL
Oligo-dT primer (10 µM)	0.5 µL	0.5 µL
RNase inhibitor	0.5 µL	0.5 µL
ImprompII Reverse Transcriptase	1 µL	0 µL
RNA/nuclease-free water (added separately)	10.5 µL	10.5 µL
SDW	0 µL	1 µL
Total	**20 µL**	**20 µL**

5. Real-Time Quantitative PCR (RT-qPCR)

RT-qPCRs were set up with a CAS-1200N Robotic Liquid Handling System (Qiagen, Hilden, Germany; formerly Corbett Life Science) and run in a Rotor-Gene RG3000 (Qiagen, Hilden, Germany; formerly Corbett Life Science). Each 10 µL reaction contained 2 µL of first-strand cDNA, 5.4 µL Quantace Sensimix *Plus* SYBR (Bioline, London, UK), 0.3 µL forward primer and 0.3 µL reverse primer. All RT-qPCRs commenced with a 10 min, 95°C

denaturation step, followed by 50 cycles of denaturation (95°C for 5 sec), and extension (T_m for 40 sec; T_m for individual genes is recorded in Chapter V). Runs concluded with a single melt step, ramping from 65°C to 95°C. Two technical replicates were performed for each sample. Relative transcript levels for each Gene of Interest (GOI) were normalised against an *ACTIN* (*ACT*) reference gene (e.g. Hecht et al. 2011) using non-equal amplification efficiencies (E) and deviation of cycle threshold (ΔCT: the cycle threshold of the reference gene minus the cycle threshold of the GOI in an individual sample) according to the following equation by Pfaffl (2001):

$$\text{Relative transcript level} = (E_{GOI})^{\Delta CT} \times 100\%$$

Unless otherwise stated, all data shown represents the mean ± SE of three biological replicates, with each replicate comprising material from one individual plant (except where plants were younger than three weeks, in which case tissue was pooled from three to four replicates).

G. Statistical Analyses

All statistical analyses were performed using SPSS 17.0.1 (IBM Corp., Somers, NY, USA) unless specifically stated otherwise. One-way ANOVAs were used to compare mean values. Where data met the assumptions of normality and equality of variance, a univariate general linear model was employed. Where these assumptions were not met, a non-parametric Kruskal-Wallis test was used. One-tailed tests were used for all bivariate Pearson correlations.

Chapter III

THE CONTRIBUTION OF *BoFLC2* TO FLOWERING TIME IN CAULIFLOWER

A. Introduction

The genomes of modern *Brassica* species are thought to be the product of successive rounds of hybridisation, polyploidisation, and chromosomal fusions and rearrangements following their divergence from ancestors of present-day *Arabidopsis thaliana* (Town et al. 2006; Murphy 2007). These complex events have resulted in multiple copies of many Arabidopsis genes (Kowalski et al. 1994), the structure and function of which are often conserved despite extensive chromosomal reorganisation (Osborn 2004). The repressor of Arabidopsis flowering *FLOWERING LOCUS C* (*FLC*) is one such gene that has been the subject of particular interest because of its central role in the vernalisation/autonomous flowering pathways. It has been suggested that the wide range of flowering time behaviour observed within the *Brassica* genus may be, at least partially, due to increased potential variation in the dosage-regulated expression of this gene (Schranz et al. 2002). However, where genes are duplicated, the possibility for altered or reduced functionality exists (Wang et al. 2006) and it is likely that there is at least some variability in the functionality and contribution of the various copies of *FLC* to *Brassica* flowering time. The following brief review of the recent history of research on *Brassica FLC* genes indicates that this is the case.

Early references to *FLC* in the *Brassica* crop species were made when flowering time QTLs were identified in the *B. napus* and *B. rapa* chromosomal regions collinear to the chromosomal position of *AtFLC* (the distal end of the short arm of chromosome 5; Osborn et al. 1997; Kole et al. 2001). Tadege et al. (2001) reported the isolation of five *FLC* genes in the allopolyploid *B. napus* designated *BnFLC1-5* (where *BnFLC1* denotes the sequence with the highest amino acid identity to *AtFLC* and *BnFLC5* the lowest). Early-flowering Arabidopsis ecotypes transformed with cDNA constructs of each of these genes exhibited

delayed flowering time, with *BnFLC1-3* causing the most pronounced effect. Flowering was similarly delayed by expression of Arabidopsis *FLC* in transformed *B. napus.*

Given that the diploid *Brassica* species are presumed to have arisen as a result of whole-genome triplication (Lagercrantz & Lydiate 1996; Lagercrantz 1998), they are expected to contain three *FLC* copies. Schranz et al. (2002) isolated these three expected copies in *B. rapa* (*BrFLC1*, *BrFLC2* and *BrFLC3*) along with a fourth unexpected homologue designated *BrFLC5*, the origins of which do not appear to be due to polyploidisation. Schranz et al. (2002) also isolated *BoFLC1*, *BoFLC3* and *BoFLC5*, but the *BoFLC2* sequence expected from findings in *B. rapa* could not be identified in the rapid-cycling *B. oleracea* variety used. Genes were named according to their similarity with the *BnFLC* genes described by Tadege et al. (2001). Alleles for at least two of the *BrFLC* genes (*BrFLC1* and *BrFLC2*) were reported to function in an additive manner to delay flowering time (data from Schranz et al. (2002) reported by Osborn 2004). Martynov & Khavkin (2004) isolated two *FLC* homologues in *B. juncea,* one being an orthologue of *BrFLC3,* the other being a possible *BrFLC5* orthologue.

Pires et al. (2004) resynthesised a number of *B. napus* allopolyploids from a single chromosome doubled amphihaploid derived from a cross between *B. rapa* and *B. oleracea* parent lines. The same primers as Schranz et al. (2002) were used to amplify *FLC* genes in both parent lines and the resynthesised *B. napus,* but again, the putative *BoFLC2* gene was not detected in the rapid-cycling *B. oleracea* parent (nor was the *BoFLC2* orthologue found in the resynthesised *B. napus*, although the *BrFLC2* orthologue was). Subsequent selfed generations of *B. napus* resulted in distinctly early or late lineages, and analysis of these two groups revealed chromosomal rearrangements leading to different complements of parental *FLC* genes and changes in *FLC* transcript level. Due to a suspected non-reciprocal transposition, duplicated *FLC2* copies from the *B. rapa* locus were found in early flowering *B. napus* lines, whereas the late flowering lines had no *FLC2*, leading the authors to postulate that this particular *BrFLC2* allele is non-functional, or has diverged function, consistent with observation that the *B. rapa* parent was even earlier-flowering than the rapid-cycling *B. oleracea* parent line. Lin et al. (2005) isolated two *AtFLC* homologues in cabbage (*B. oleracea* var. *capitata* L.) and designated them *BoFLC3-2* and *BoFLC4-1* according to their amino acid similarity to the *BnFLC* genes described by Tadege et al. (2001). Of these two genes, the authors identified *BoFLC4-1* as the more significant contributor to flowering.

In 2007, Okazaki et al. cloned *BoFLC1*, *BoFLC3* and *BoFLC5* (previously described by Schranz et al. 2002) in cabbage and broccoli (*B. oleracea* var. *italica* Plenck). They also isolated a novel sequence with 98% homology to *BoFLC4* and 91% homology to *BrFLC2*, designating it *BoFLC2*. Coding sequences within a 1.8 kb amplicon of this gene showed 100% similarity with the *BoFLC4-1* gene cloned by Lin et al. (2005) with the exception of a single-base deletion in exon 4 of the broccoli gene. Predicted amino acid sequences indicated that this deletion creates a frame-shift mutation which results in an in-frame stop codon (TAA) in exon 4, presumably resulting in a non-functional gene. All six cultivars and two strains of annual *B. oleracea* (broccoli and cauliflower) used in this study were identified as possessing this mutated form of *BoFLC2*, whereas all cultivars of cabbage, kale (*B. oleracea* var. *acephala* DC) and Brussels sprout (*B. oleracea* var. *gemmifera* Zenker) carried wild-type (functional) *BoFLC2* alleles. *BoFLC2* appeared to be absent or highly diverged in a rapid-cycling *B. oleracea* variety, consistent with the conclusions of Schranz et al. (2002) and Pires et al. (2004). A cross between broccoli and cabbage produced an F_2 generation that segregated for the *BoFLC2* allele, where flowering time data showed a clear relationship between late-flowering phenotypes and the non-mutant *BoFLC2* genotype. Analysis suggested that flowering time was controlled in a quantitative manner, with *BoFLC2* the only *FLC* gene that mapped to a known flowering time QTL.

Even more recently, Razi et al. (2008) assessed the possibility of *BoFLC1* and *BoFLC3-5* being candidate genes for known flowering time QTLs in a *B. oleracea* F_2 population. A cross was made between a doubled haploid (DH) rapid-cycling line in which *BoFLC4* was not detected and an annual DH broccoli line described as 'late-flowering' which carried the mutant *BoFLC4* allele described by Okazaki et al. (2007) as *BoFLC2*, however, none of the four *BoFLC* genes were found to be associated with differences in flowering time in the population studied. This is not surprising, given that both of the parents used to generate the mapping population were early-flowering lines in which the *BoFLC2* gene was shown to be defective or absent.

Several observations can be made from the above studies: (a) genes in the *FLC2*/*FLC4* clade are the members of the *FLC* family most commonly identified as being important in terms of flowering time regulation in members of the *Brassica* genus (this was emphasised recently by Zhao et al. (2010b) who identified *BrFLC2* as a candidate gene for vernalisation response QTL in *B. rapa*); (b) *BoFLC2* and *BoFLC4* are very similar genes with only minor

differences contained in non-coding regions. The fact that they have never been identified together in the same material supports the view that they are actually minor sequence variants of the same gene (Razi et al. 2008); (c) The presence of *BoFLC2* has never been detected in rapid-cycling lines; (d) the non-mutant *BoFLC2* allele has never been identified in early-flowering or 'annual' type brassicas (e.g. cauliflower, broccoli), with all cultivars surveyed to date containing the mutated allele; (e) the mutated *BoFLC2* allele has never been identified in late-flowering or 'biennial' brassicas (e.g. cabbage, Brussels sprouts), with all cultivars surveyed to date possessing the functional allele.

When taken together, these observations suggest that *BoFLC2* functionality may underpin important differences between annual and biennial brassica cultivars, particularly with respect to flowering time. However, no research has been conducted to demonstrate the contribution of this gene to flowering time *within* either annual or biennial brassica types, both of which have broad flowering time ranges across cultivars. In this study, populations of inbred annual and biennial brassica parent lines were screened to test for the presence of both alleles within these individual groups, and correlations between flowering time behaviour and *BoFLC2* genotype were assessed in order to test the hypothesis that *BoFLC2* functionality is a key determinant of flowering time, not only between annual and biennial groups, but also within these groups.

In this chapter, the contribution of *BoFLC2* functionality to flowering time within annual and biennial groups was further investigated by assessing flowering time and curd development in populations segregating for functional and mutant *BoFLC2* alleles derived from crosses between two members of the same group (cauliflower x cauliflower). This population also provided an opportunity to map additional flowering time candidate genes and identify flowering time QTLs. In order to do this, a number of key flowering time candidate genes besides *BoFLC2* were isolated in the parent lines that were used to generate these crosses, and unconserved regions of these genes were screened for polymorphisms. Markers for these polymorphisms were designed in order to identify associations with flowering time variation in the segregating population. Identification of additional flowering time QTLs could potentially be used in subsequent Marker Assisted Selection (MAS) programmes or serve as inputs for plant growth models.

B. Materials & Methods

1. Cauliflower & Cabbage Parent Lines

Seeds of the 54 homozygous inbred cauliflower parent lines, 41 homozygous inbred cabbage parent lines and two homozygous inbred swede parent lines described in Chapter II were germinated in Petri dishes at 22°C. After one week, cotyledons and shoot tips were harvested and DNA was extracted using the CTAB method.

2. *BoFLC2* CAPS Marker Design

To detect the single base deletion in exon 4 of *BoFLC2* described by Okazaki et al. (2007), DNA fragments were amplified using primers designed from published sequence DQ222849 derived from *B. oleracea* var. *capitata* cv. 'Reiho' (Ishi seed company, Japan) intron 3 (BoFLC2-PvuII-F: 5'-AGGGCCTAGAGGGCATACAT-3') and intron 6 (BoFLC2-PvuII-R: 5'-TTTTGAGGCTCTCGACACAA-3'). Denaturation was for 5 min at 94°C, followed by 35 cycles of denaturing, annealing and extension at 94°C for 1 min, 59°C for 1 min and 72°C for 1 min, respectively, with a final elongation step of 72°C for 10 min. The product was digested with PvuII for 8 hours at 37°C and the polymorphism was identified by 2% agarose gel electrophoresis. Representatives of each genotype were cleaned using the Promega Wizard® SV Gel and PCR Clean-Up System, and sequenced by the Australian Genome Research Facility Ltd. (Gehrmann Laboratories, University of Queensland, St Lucia, QLD).

3. Establishment of F_2 Populations

The following work was conducted in the south of the Netherlands at the Rijk Zwaan Research and Development facility, Fijnaart. In spring 2003, an individual plant from a late-flowering cauliflower proprietary parent line designated A was crossed with an individual plant from an early-flowering cauliflower proprietary parent line designated B. A second, similar cross was made between a late-flowering cauliflower proprietary parent line designated C and an early-flowering cauliflower proprietary parent line designated D. Both late-flowering lines had a flowering time of approximately 120 days, and both early-flowering lines flowered after approximately 70 days. Crosses were performed in a glasshouse at ambient temperatures in natural light by manually transferring pollen at bud stage to prevent self-fertilisation. Line A had been inbred for two generations (S_2), line B had

been inbred for four generations (S_4) and lines C and D had been inbred for six generations (S_6). Seeds were harvested, and F_1 plants from AB and CD crosses were raised in the glasshouse. In spring 2005, the F_1 plants were manually self-pollinated and the resulting AB and CD F_2 seed was sown in the glasshouse on 15th February 2008, along with A, B, C and D parent lines, S_3 seed of the parent line A, and the CD F_1 seed.

On 16th April 2008, all seedlings were planted into the field at a spacing of 50 cm between plants and 75 cm between rows. There were approximately 30 seedlings of each parent line, the CD F_1 population and the S_3 A population, and approximately 380 F_2 seedlings from each cross. These details are summarised in Figure 4. Conditions and management of both glasshouse and field environments were similar to those used in commercial Rijk Zwaan breeding programmes, and plant growth was consistent with normal crop development patterns. In this population, DNA was isolated from two 8 mm young leaf discs per plant using a mag Plant DNA Isolation Kit (AGOWA, Berlin, Germany) in conjunction with a KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA) according to manufacturer's specifications.

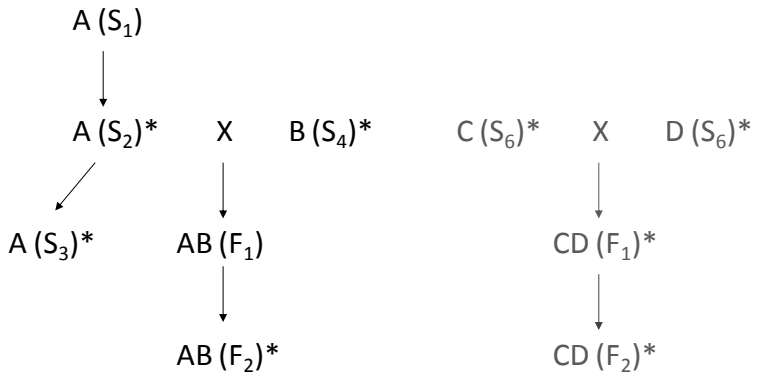


Fig. 4. Production of AB and CD segregating populations. Late-flowering line A (S_2) was crossed with early-flowering line B (S_4) and late-flowering line C (S_6) was crossed with early-flowering line D (S_6). The resulting F_1 plants were self-pollinated and the subsequent F_2 generations were produced. In addition, the S_2 generation of the A line was self-pollinated to produce an S_3 line. Those generations marked with an asterisk (*) were included in the April 2008 field planting. There were approximately 380 plants in each of the segregating F_2 populations and approximately 30 plants in the A (S_2), B (S_4), A (S_3), C (S_6), D (S_6) and CD (F_1) populations.

4. Phenotyping F_2 Populations

The first plants began to initiate curds in late June 2008. Weekly observations of developmental stage and curd diameter in all plants began on 3rd July 2008, shortly after the earliest curds were detectable. Observations were made every 6-12 days until the 11th August 2008, 117 days after transplanting into the field. Curd appearance was recorded as the number of days after transplanting when the curd became visible without the need for damaging excavation amongst the apical leaves. Plants that had not produced visible curds after 117 days were given a maximum score of 127 days in order to differentiate them from those plants for which curds were first recorded on the 117th day. Developmental stage was determined according to the Curd Development Key shown in Figure 3. Plants that had been obviously affected by animal browsing were excluded from both curd appearance and developmental stage analyses. Fungal infection was widespread throughout the brassica trial (perhaps due to an unusually wet and warm summer), and diseased curds displaying advanced symptoms of fungal infection were also excluded from the developmental stage analysis. The AB F_2 population and the B parent line were particularly affected by fungal infection, necessitating their exclusion from all developmental stage analyses and measurements of curd diameter (curd initiation appeared to be unaffected by disease).

5. Additional Marker Design

An attempt was made to clone and sequence a large number of flowering time candidate genes in order to identify polymorphisms between the crossed parents, with the intentions of using CAPS markers to construct a linkage map, and to investigate whether these genes contributed to flowering time variation. Primers were designed to span introns, promoters or other regions of greater variability. Primer details are listed in Table 6, primer locations within the gene relative to introns are summarised in Figure 5 and full details of PCR conditions, product sizes and results are provided in Table 7.

Only two of these genes were found to have polymorphisms suitable for use as CAPS markers. *BoFLC4-1* primers RZ-FLC41-F1 (5'-GAAATATGGAAAGCGGGTGA-3') and RZ-FLC41-R1 (5'-AGAGGTGATGCGCCTAGAAA-3') were designed from published sequence AY306124 (Lin et al. 2005), within the promoter/5'UTR. The same PCR

conditions as those used in the *BoFLC2* PCR were used, and products were digested with AseI for 2.5 hours at 37°C.

BoREM1 primers RZ-REM1-INT2-F (5'-TAATTTCAGCGAGGGAAACG-3') and RZ-REM1-INT2-R (5'-TGACACGAGCTCTGAGGAAA-3') were designed from published sequence AF051772 (Franco-Zorrilla et al. 1999), spanning the 71 bp intron 2. The same PCR conditions as those used in *BoFLC2* and *BoFLC4-1* were used, and the product was digested with MboII for one hour at 37°C. Sequencing of each genotype was performed by the Australian Genome Research Facility Ltd. (Gehrmann Laboratories, University of Queensland, St Lucia, QLD).

6. Statistical Analyses

Chi square values were calculated using the formula:

$$\chi^2 = \Sigma\left(\frac{(O_i - E_i)^2}{E_i}\right)$$

Where: χ^2= Pearson's cumulative test statistic
O_i = the observed frequency for class *i*
E_i = the expected frequency for class *i*

In order to calculate the contribution of a given gene of interest (GOI) to a given trait, the following formula was used:

$\sigma^2_{phenotype} = \sigma^2_{genotype} + \sigma^2_{environment}$

The $\sigma^2_{genotype}$ component was further partitioned into σ^2_{GOI} and $\sigma^2_{genetic\ background}$. One-way ANOVA was used to determine total ($\sigma^2_{genotype} + \sigma^2_{environment}$) and residual ($\sigma^2_{genetic\ background} + \sigma^2_{environment}$) variance for the F_2 population, and residual variance for parent lines ($\sigma^2_{environment}$). Using these values, the contribution of the GOI to the phenotypic and genetic variance of a given trait was calculated by dividing σ^2_{GOI} by $\sigma^2_{phenotype}$ and $\sigma^2_{genotype}$, respectively. All statistical analyses in this chapter were performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

Table 6. Forward and reverse primers used to amplify flowering time candidate genes in cauliflower parents A, B, C and D. Reference sequence refers to the sequence used to design the primers (GenBank accession number and reference).

PRIMER	SEQUENCE	REFERENCE SEQUENCE
***AGL20* Primers**		
RZ-AGL20-F1	5'-GGGCAAAACTCAGATGAAGC-3'	AY345237 (Kim et al. 2003)
RZ-AGL20-F2	5'-TGCAACAAGCAGACAAGTGA-3'	AY345237 (Kim et al. 2003)
RZ-AGL20-R1	5'-TCGACCAGACTTCGATTTCA-3'	AY345237 (Kim et al. 2003)
RZ-AGL20-R2	5'-ATCCCCACTTTTCAGTGAGC-3'	AY345237 (Kim et al. 2003)
RZ-AGL20-R3	5'-TTGGGCTACTCTCTTCGTCA-3'	AY345237 (Kim et al. 2003)
***AP1* Primers**		
RZ-AP1a-F1	5'-CTTACGCCGAGAGACAGCTT-3'	AJ505845 (Kop 2002)
RZ-AP1a-F2	5'-AACGTTCTTAGGGCGCAAC-3'	AJ505845 (Kop 2002)
RZ-AP1a-R1	5'-CAGCCAAGGTTGCAGTTGTA-3'	AJ505845 (Kop 2002)
RZ-AP1a-R2	5'-TTGCTGACTTTGGACACTGA-3'	AJ505845 (Kop 2002)
RZ-AP15PF	5'-TTGGTTCATACCAAAGTCT-3'	Primer from (Smith & King 2000)
RZ-AP15PR	5'-ATCCCTTTTAAAGGAACTA-3'	Primer from (Smith & King 2000)
***CAL* Primers**		
RZ-CAL4F	5'-CATACAAACGCAAACATCTC-3'	Primer from (Smith & King 2000)
RZ-CALI6R	5'-GGACAAAGCCCACCTACATG-3'	Primer from (Smith & King 2000)
RZ-CALnestF	5'-TCTTATCTTTGAGTTTGTGAAGA-3'	Primer from (Labate et al. 2006)
RZ-CALnestR	5'-TGTGCACATATGCTCATGATT-3'	Primer from (Labate et al. 2006)
***CCE1* Primers**		
RZ-CCE1-F1	5'-GGAGCAAAAATCCGTAGCTG-3'	AF227978 (Palmer et al. 2001)
RZ-CCE1-F2	5'-CCCATTCTCCATTCGTGTTT-3'	AF227978 (Palmer et al. 2001)
RZ-CCE1-F4	5'-CTCAACTGACACATGCTTTGG-3'	AF227978 (Palmer et al. 2001)
RZ-CCE1-R1	5'-AAAAACACCACCCAGAAGGA-3'	AF227978 (Palmer et al. 2001)
RZ-CCE1-R3	5'-TCGGAAAGGATTGGAGTGAC-3'	AF227978 (Palmer et al. 2001)
RZ-CCE1-R4	5'-GCGATTTACATTCGAAGCTAGG-3'	AF227978 (Palmer et al. 2001)
***BoFH* Primers**		
RZ-FH-INT1-F1	5'-GTGATTCCGGCACTCATCTT-3'	Z18362 (Anthony, James & Jordan 1993)
RZ-FH-INT1-F2	5'-ACGCCGTCATTTGCTACTCT-3'	Z18362 (Anthony, James & Jordan 1993)
RZ-FH-INT2-F1	5'-ACGAAGGTGAGGATGACGAC-3'	Z18362 (Anthony, James & Jordan 1993)
RZ-FH-INT2-F2	5'-AAAGATCGTGGCGAAAAATG-3'	Z18362 (Anthony, James & Jordan 1993)
RZ-FH-INT1-R1	5'-CCCTGGCTCCGTTACAATAA-3'	Z18362 (Anthony, James & Jordan 1993)
RZ-FH-INT1-R2	5'-GTCGTCATCCTCACCTTCGT-3'	Z18362 (Anthony, James & Jordan 1993)
RZ-FH-INT2-R1	5'-GCGTACCTGAACACCTGGTT-3'	Z18362 (Anthony, James & Jordan 1993)
RZ-FH-INT2-R2	5'-AGCGTTAAAAACGGCGTCTA-3'	Z18362 (Anthony, James & Jordan 1993)
***BoFLC1* Primers**		
RZ-FLC1-F1	5'-CGACCATGGATGCGTAAGTA-3'	AM231517 (Razi et al. 2008)
RZ-FLC1-F2	5'-AAAAGCTCGACCCTCAAAGC-3'	AM231517 (Razi et al. 2008)
RZ-FLC1-F3	5'-GGCTTTTGATTATGGACAAACC-3'	AM231517 (Razi et al. 2008)
RZ-FLC1-F4	5'-GGAGTCCATCTTTCCACGTT-3'	AM231517 (Razi et al. 2008)
RZ-FLC1-F5	5'-AACGGATAACCCGAAAAACC-3'	AM231517 (Razi et al. 2008)
RZ-FLC1-R1	5'-CCAATCTGCCCTAAAAACCA-3'	AM231517 (Razi et al. 2008)
RZ-FLC1-R2	5'-TCGCTTTATTTGAACGTGGA-3'	AM231517 (Razi et al. 2008)
RZ-FLC1-R3	5'-AACCCAACTTGGAATCAAACC-3'	AM231517 (Razi et al. 2008)
RZ-FLC1-R4	5'-TGATCTTGAGGTCCGGTTTC-3'	AM231517 (Razi et al. 2008)
RZ-FLC1-R5	5'-TCAATGCCCACAACAAGAAA-3'	AM231517 (Razi et al. 2008)
***BoFLC2* Primers**		
BoFLC2-PvuII-F	5'-AGGGCCTAGAGGGCATACAT-3'	DQ222849 (Okazaki et al. 2007)
BoFLC2-PvuII-R	5'-TTTTGAGGCTCTCGACACAA-3'	DQ222849 (Okazaki et al. 2007)

(continued overleaf)

Table 6. (*continued*)

PRIMER	SEQUENCE	REFERENCE SEQUENCE
***BoFLC3* Primers**		
RZ-FLC3-F1	5′-CGTGGTCCATGCAGTAAATG-3′	AM231518 (Razi et al. 2008)
RZ-FLC3-F2	5′-CCATGCAGTAAATGGCAATG-3′	AM231518 (Razi et al. 2008)
RZ-FLC3-R1	5′-TTGTCCAGCCCTAATTCCAC-3′	AM231518 (Razi et al. 2008)
RZ-FLC3-R2	5′-GTTTTTCGGATCCGGATTTT-3′	AM231518 (Razi et al. 2008)
***BoFLC3-2* Primers**		
RZ-FLC32-F1	5′-TGTCGGATAAGCCTCGTACC-3′	AY306125 (Lin et al. 2005)
RZ-FLC32-F2	5′-AGCAGAAACGGGAACCTACA-3′	AY306125 (Lin et al. 2005)
RZ-FLC32-R1	5′-CATTGCCATTTACTGCATCG-3′	AY306125 (Lin et al. 2005)
***BoFLC4-1* Primers**		
RZ-FLC41-F1	5′-GAAATATGGAAAGCGGGTGA-3′	AY306124 (Lin et al. 2005)
RZ-FLC41-F2	5′-GGGATTGCGCAAAATTCTAA-3′	AY306124 (Lin et al. 2005)
RZ-FLC41-R1	5′-AGAGGTGATGCGCCTAGAAA-3′	AY306124 (Lin et al. 2005)
RZ-FLC41-R2	5′-GCGTGGACGGCTAGTGTATT-3′	AY306124 (Lin et al. 2005)
***BoFLC5* Primers**		
RZ-FLC5-F1	5′-CCATTCGAGTTCGGATATTG-3′	AM231519 (Razi et al. 2008)
RZ-FLC5-F2	5′-TGATGAAGGAAGACCCTTGG-3′	AM231519 (Razi et al. 2008)
RZ-FLC5-F3	5′-ATCGGCAATCGTTGTGTGTA-3′	AM231519 (Razi et al. 2008)
RZ-FLC5-R1	5′-CGATGCGTTTTAACGACAAG-3′	AM231519 (Razi et al. 2008)
RZ-FLC5-R2	5′-TTCTCTATCATGGCGGTTCC-3′	AM231519 (Razi et al. 2008)
RZ-FLC5-R3	5′-CCCAAGACAAAAGACCCAAG-3′	AM231519 (Razi et al. 2008)
***BoFRI* Primers**		
RZ-BoFRI-F1	5'-GCAGTGGAAACATTCAAACG-3'	DQ503574 (Unpublished)
RZ-BoFRI-F2	5'-AAACGCCACTACGACGACTT-3'	DQ503574 (Unpublished)
RZ-BoFRI-R1	5'-TCCAGGATCAGAAGCGAAAC-3'	DQ503574 (Unpublished)
RZ-BoFRI-R2	5'-ATAAGCTTTGCGCCCTTGTA-3'	DQ503574 (Unpublished)
***BoFT* Primers**		
RZ-FT-F1	5′-GGGTTACATACGGCCAAAGA-3′	Appendix I
RZ-FT-F2	5′-ATGGTGGATCCAGATGTTCC-3′	Appendix I
At-FT-F1a-seq	5′-TCAACACAGAGAAACCACCTG-3′	AF152096 (Kardailsky et al. 1999)
RZ-FT-R1	5′-CGAGATTGTATAGCGCAGCA-3′	Appendix I
RZ-FT-R2	5′-GGTTGCTAGGACTTGGAACATC-3′	Appendix I
At-FT-R3a-seq	5′-CATCACCGTTCGTTACTCG-3′	AF152096 (Kardailsky et al. 1999)
***BoREM1* Primers**		
RZ-REM1-INT1-F	5′-CCACAACCCTCTCTGTTTCA-3′	AF051772 (Franco-Zorrilla et al. 1999)
RZ-REM1-INT2-F	5′-TAATTTCAGCGAGGGAAACG-3′	AF051772 (Franco-Zorrilla et al. 1999)
RZ-REM1-INT3-F	5′-TTTCCTCAGAGCTCGTGTCA-3′	AF051772 (Franco-Zorrilla et al. 1999)
RZ-REM1-INT1-R	5′-GTTGCCGTCCAGTTTCACTT-3′	AF051772 (Franco-Zorrilla et al. 1999)
RZ-REM1-INT2-R	5′-TGACACGAGCTCTGAGGAAA-3′	AF051772 (Franco-Zorrilla et al. 1999)
RZ-REM1-INT3-R	5′-TTAGCCCAGCCTCTTGTGAT-3′	AF051772 (Franco-Zorrilla et al. 1999)
***BoTFL1* Primers**		
RZ-TFL1-MIM-1F	5′-GGAGAATATGGGAACTAGAG-3′	AB017530 (Mimida et al. 1999)
RZ-TFL1-MIM-6R	5′-GATTAACTCGATGTGACCTATC-3′	AB017530 (Mimida et al. 1999)
***VIN3* Primers**		
RZ-VIN3-F3	5'-TGTTGTGCTTTTCCCTTTGC-3'	ES5935031 (The Brassica Database)
RZ-VIN3-R3	5'-CCCATACCCATCTTCCTTGA-3'	ES5935031 (The Brassica Database)

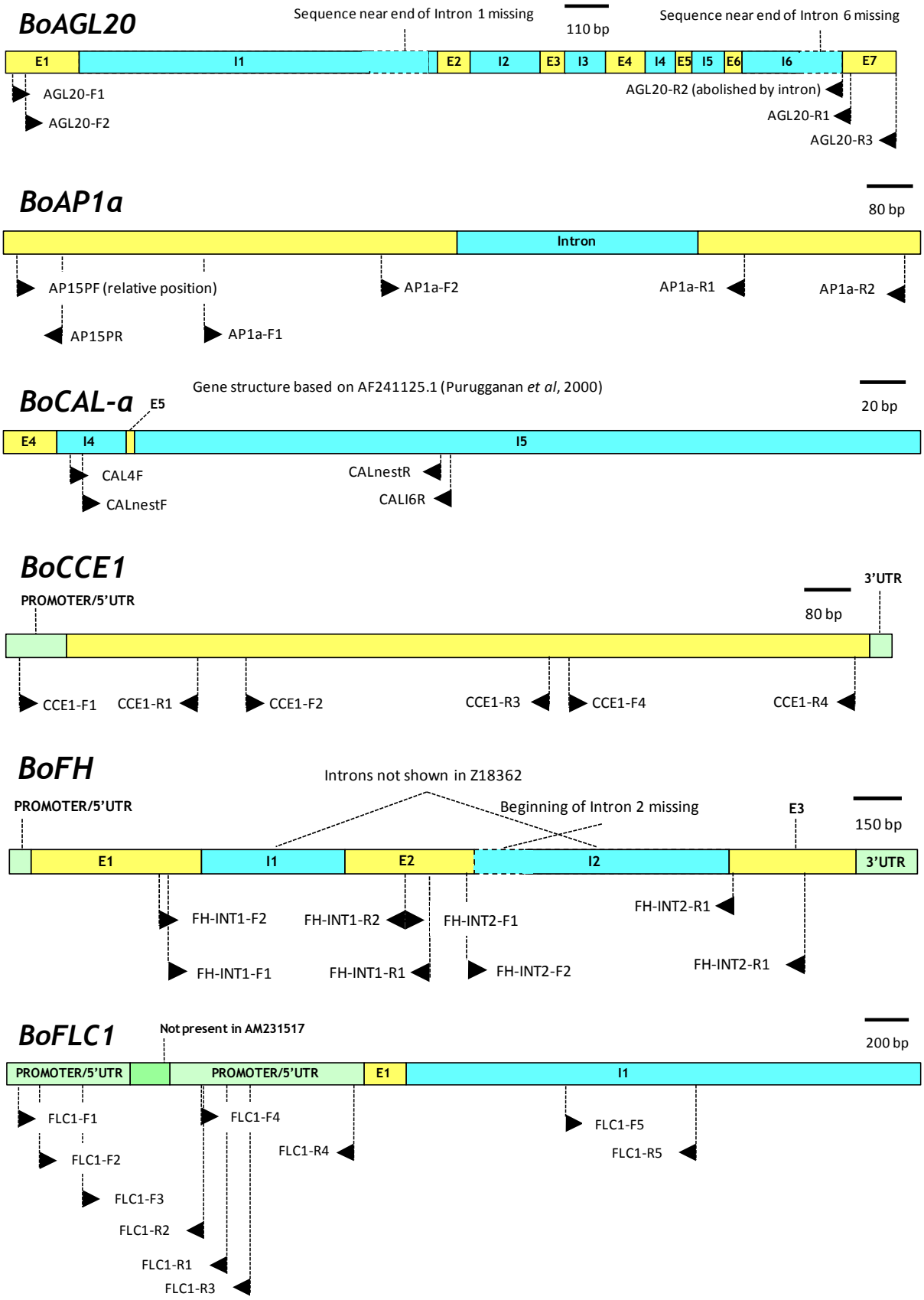


Fig. 5. **Primer locations within genes**. Diagrams are based on the sequence denoted by reference sequence listed in Table 6. (*continued overleaf*)

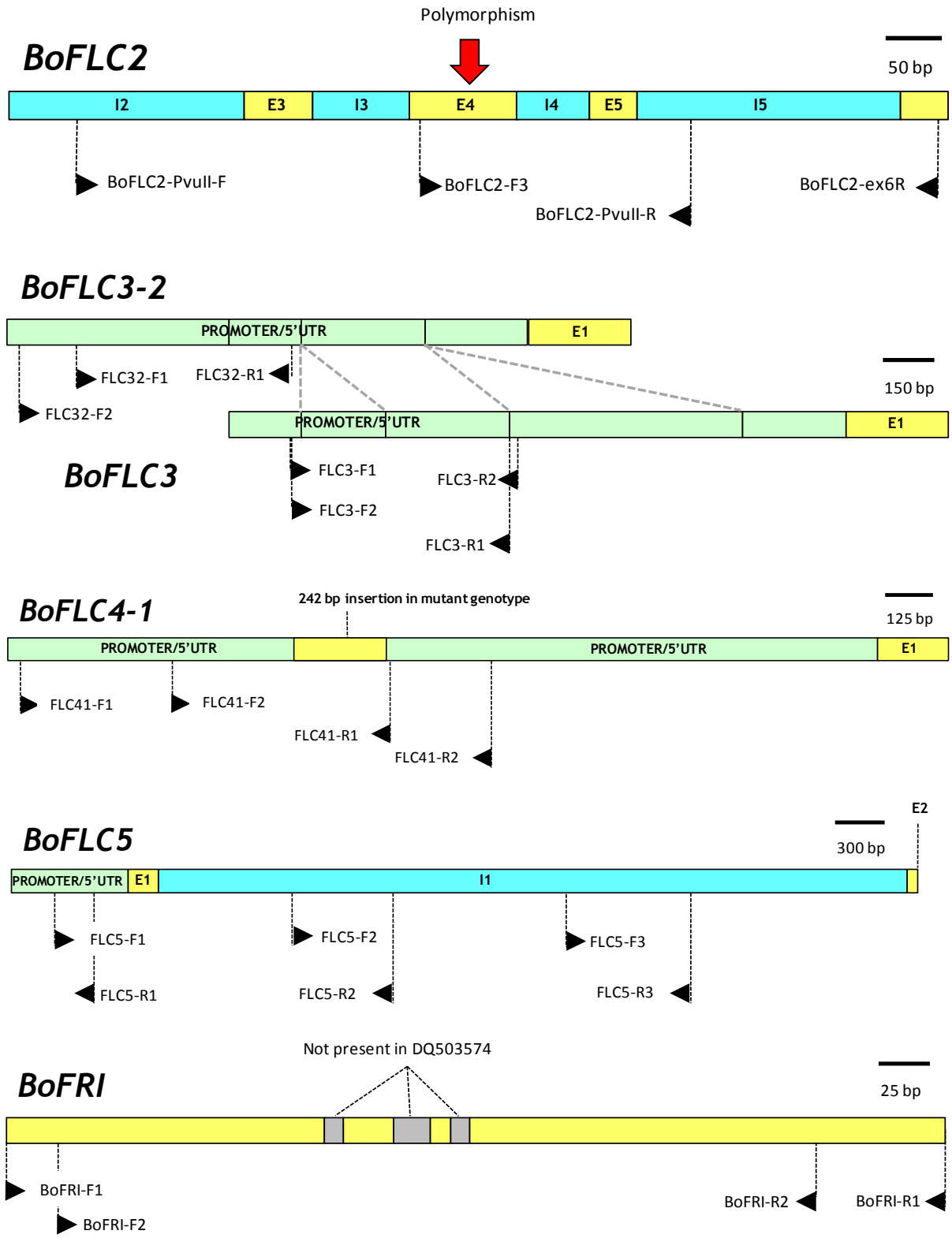


Fig. 5. (*continued overleaf*)

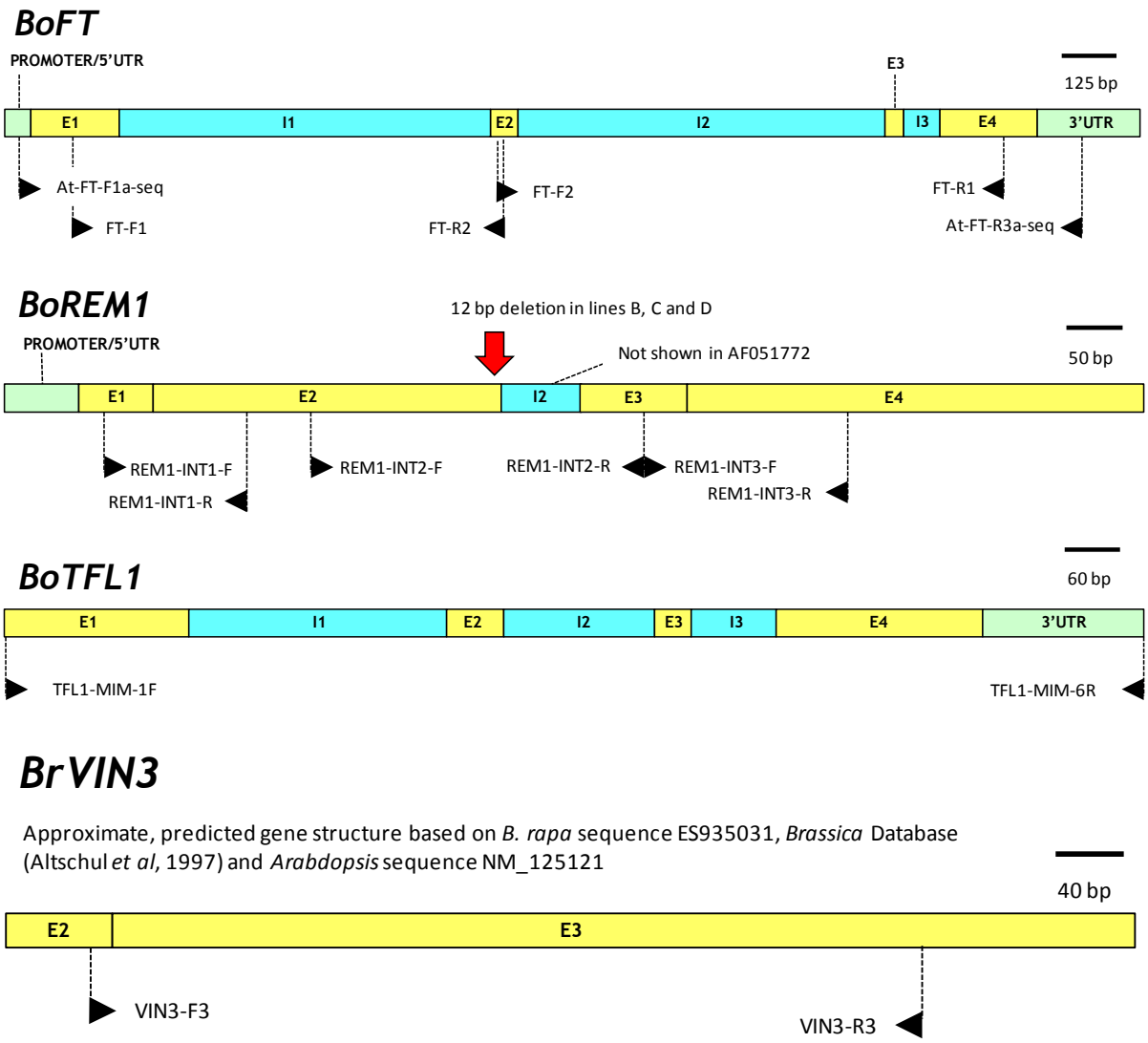


Fig. 5. (*continued*)

Table 7. PCR details for all primers tested on parent lines A, B, C and D. T_m refers to PCR annealing temperature; figures in the cDNA column refer to cDNA size (bp) based on reference sequence data, figures in the gDNA column refer to expected gDNA product size; Band A-D columns indicate the observed size of the product; Sequenced column values indicate whether or not the products were sequenced. All PCRs were denatured for 5 min at 94°C, followed by 35 cycles of denaturing (94°C for 1 min), annealing (at the T_m listed in table, for 1 min) and extension at 72°C for 1 min, with a final elongation step of 72°C for 10 min. Cycled denaturation, annealing and extension durations in PCRs marked with an asterisk (*) were only 45 seconds. Where PCRs failed, numerous attempts at optimisation were made with various combinations of T_m, extension time, DNA concentration, $MgCl_2$ quantities – these are not included in this table.

PCR	GENE	FORWARD	REVERSE	T_m	cDNA	gDNA	BAND A	BAND B	BAND C	BAND D	SEQUENCED
1*	*AGL20*	AGL20-F1	AGL20-R1	59	642	?	~2250	~2250	~2250	~2250	Y (A & C unclear)
2*	*AGL20*	AGL20-F2	AGL20-R2	59	479	?	Failed	Failed	Failed	Failed	N
3*	*AGL20*	AGL20-F1	AGL20-R2	59	509	?	Failed	Failed	Failed	Failed	N
4*	*AGL20*	AGL20-F2	AGL20-R1	59	503	?	~2100	~2100	~2100	~2100	Y (unclear)
5*	*AGL20*	AGL20-F1	AGL20-R3	59	578	?	~2170	~2170	~2170	~2170	Y (unclear)
6*	*AGL20*	AGL20-F2	AGL20-R3	59	548	?	Failed	Failed	Failed	Failed	N
7	*AP1*	AP15PF	AP15PR	52	110	?	Failed	Failed	Failed	Failed	N
8*	*AP1*	AP1a-F1	AP1a-R1	59	538	?	Failed	Failed	Failed	Failed	N
9*	*AP1*	AP1a-F2	AP1a-R2	59	505	?	924	924	924	924	Y
10	*CAL*	CAL4F	CALI6R	57	?	?	Multiple	Multiple	Multiple	Multiple	N
11	*CCE1*	CCE1-F1	CCE1-R1	59	323	?	~320	~320	~320	~320	N
12	*CCE1*	CCE1-F1	CCE1-R2	59	354	?	Failed	Failed	Failed	Failed	N (Error in R2 primer seq)
13	*CCE1*	CCE1-F2	CCE1-R3	59	541	?	~540	~540	~540	~540	N
14*	*CCE1*	CCE1-F4	CCE1-R4	59	511	?	511	511	511	511	Y
15	*BoFH*	FH-INT1-F1	FH-INT1-R1	59	320	?	~750	~750	~750	~750	N
16	*BoFH*	FH-INT1-F2	FH-INT1-R2	59	271	?	718	718	718	718	Y
17	*BoFH*	FH-INT2-F1	FH-INT2-R1	59	247	?	Multiple	Multiple	Multiple	Multiple	N
18	*BoFH*	FH-INT2-F2	FH-INT2-R2	59	273	?	~1100	~1100	~1100	~1100	Y
19	*BoFLC1*	FLC1-F1	FLC1-R1	59	.	791	Failed	Multiple	Multiple	Multiple	N
20	*BoFLC1*	FLC1-F1	FLC1-R2	59	.	683	Multiple	Multiple	Multiple	Multiple	N
21	*BoFLC1*	FLC1-F2	FLC1-R1	59	.	694	Failed	Failed	Failed	Failed	N
22*	*BoFLC1*	FLC1-F3	FLC1-R3	59	.	603	~850	~850	~850	~850	Y
23*	*BoFLC1*	FLC1-F4	FLC1-R4	59	.	717	~720	~720	~720	~720	Y
24*	*BoFLC1*	FLC1-F5	FLC1-R5	59	.	613	613	613	613	613	Y
25	*BoFLC2*	BoFLC2-PvuII-F	BoFLC2-PvuII-R	59	.	572	572	571	572	571	Y

(continued overleaf)

Table 7. *(continued)*

PCR	GENE	FORWARD	REVERSE	T_m	cDNA	gDNA	BAND A	BAND B	BAND C	BAND D	SEQUENCED
26	*BoFLC3*	FLC3-F1	FLC3-R1	59	.	663	Failed	Failed	Failed	Failed	N
27	*BoFLC3*	FLC3-F2	FLC3-R2	59	.	677	Failed	Failed	Failed	Failed	N
28	*BoFLC3*	FLC3-F1	FLC3-R2	59	.	683	Failed	Failed	Failed	Failed	N
29	*BoFLC3*	FLC3-F2	FLC3-R1	59	.	657	Failed	Failed	Failed	Failed	N
30	*BoFLC3-2*	FLC32-F1	FLC32-R1	59	.	649	~650	~650	~650	.	N
31	*BoFLC3-2*	FLC32-F2	FLC32-R1	59	.	812	812	812	812	812	Y
32	*BoFLC4-1*	FLC41-F1	FLC41-R1	59	.	744	744	986	744	986	Y
33	*BoFLC4-1*	FLC41-F2	FLC41-R2	59	.	610	610	852	610	852	Y
34	*BoFLC5*	FLC5-F1	FLC5-R1	59	.	252	252	252	252	252	Y
35	*BoFLC5*	FLC5-F2	FLC5-R2	59	.	634	634	634	634	634	Y
36	*BoFLC5*	FLC5-F3	FLC5-R3	59	.	773	773	773	773	773	Y
37*	*BoFRI*	BoFRI-F1	BoFRI-R1	59	425	?	Failed	Failed	Failed	Failed	N
38*	*BoFRI*	BoFRI-F2	BoFRI-R2	59	357	?	390	390	390	390	Y
39*	*BoFRI*	BoFRI-F2	BoFRI-R1	59	419	?	~450	~450	~450	~450	Y (unclear)
40*	*BoFRI*	BoFRI-F1	BoFRI-R2	59	372	?	Failed	Failed	Failed	Failed	N
41	*BoFT*	FT-F1	FT-R1	59	375	?	~1750	Failed	~1750	~1750	N
42	*BoFT*	FT-F1	FT-R2	59	153	?	1001	1001	1001	1001	Y
43	*BoFT*	FT-F2	FT-R1	59	256	?	1175	1175	1175	1175	Y
44	*BoFT*	At-FT-F1a-seq	At-FT-R3a-seq	56	675	?	Failed	Failed	Failed	Failed	N
45	*BoREM1*	REM1-INT1-F	REM1-INT1-R	59	147	?	~250	~250	~250	~250	Y (unclear)
46	*BoREM1*	REM1-INT2-F	REM1-INT2-R	59	245	?	316	304	304	304	Y
47	*BoREM1*	REM1-INT3-F	REM1-INT3-R	59	201	?	~300	~300	~300	~300	Y (unclear)
48	*BoTFL1*	TFL1-MIM-1F	TFL1-MIM-6R	59	.	1241	~1240	~1240	~1240	~1240	Y
49*	*VIN3*	VIN3-F3	VIN3-R3	59	507	?	.	.	.	.	N

C. Results

1. *BoFLC2* Marker Validation

PCR products from *BoFLC2* functional alleles yielded restriction fragments of 356 bp and 216 bp while the PvuII restriction site was abolished in the 571 bp fragments of the mutant allele (hereafter denoted by lower case letters, i.e. *boflc2*), which remained uncut (Figure 6).

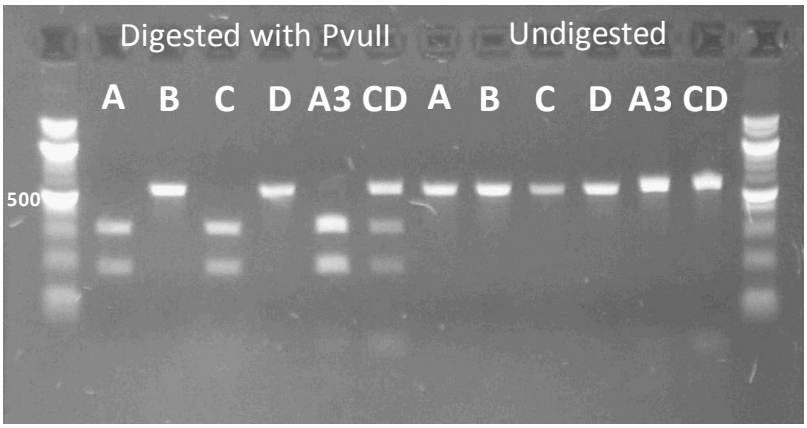


Fig. 6. ***BoFLC2* CAPS marker restriction pattern.** The undigested 571 – 572 bp PCR products of four parent lines (A-D), the inbred S_3 generation of parent line A (A3), and the F_1 progeny of a CxD cross are shown on the right. Digestion with PvuII resulted in the restriction pattern seen on the left, with A, C and A3 cut into 356 and 216 bp fragments. Gel is 2% agarose.

2. Screening Cauliflower & Cabbage Parent Lines for *BoFLC2* Mutation

Cauliflower and cabbage homozygous parent lines were screened for the presence of the single base deletion in exon 4 of *BoFLC2* using the CAPS marker described above. All 43 cabbage lines carried functional *BoFLC2* with the exception of one white cabbage line which was heterozygous. Of the 54 cauliflower parent lines, 33 carried *boflc2* and 21 carried *BoFLC2* (Figure 7).

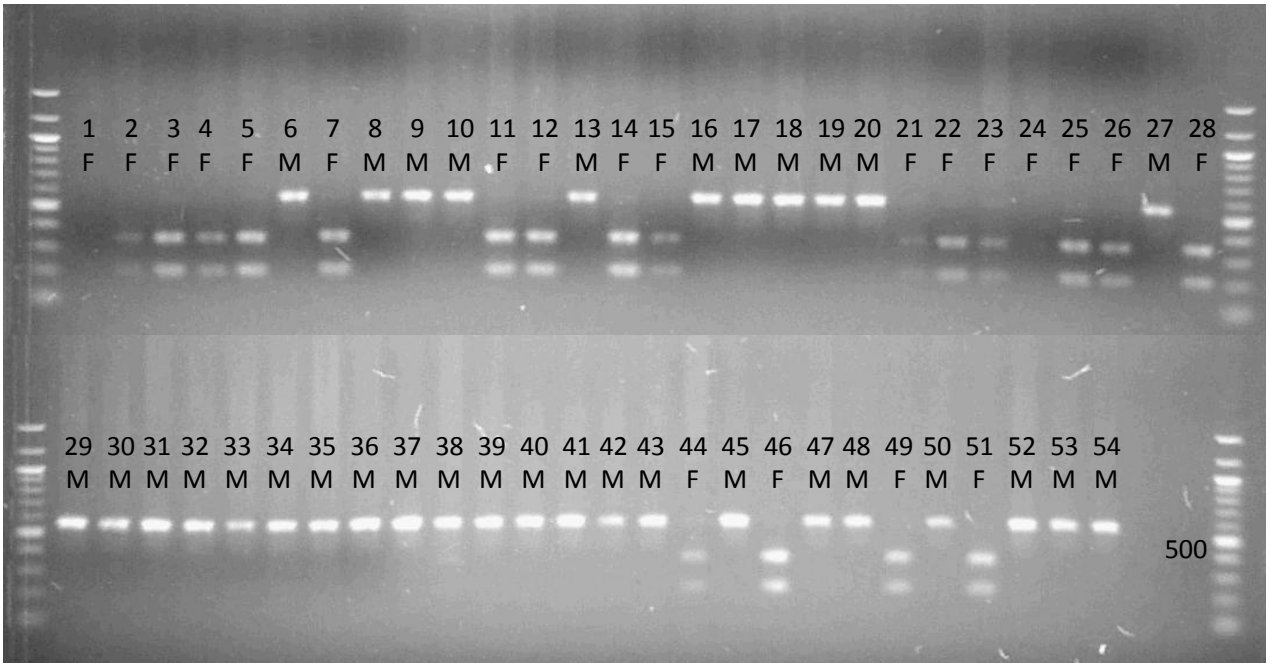


Fig 7. Digested *BoFLC2* fragments. Cauliflower line numbers (corresponding to those listed in Table 2) and genotype (mutant = M and functional = F) are shown. Gel is 1.5% agarose.

Figure 8 shows the distribution of *BoFLC2* genotype across the five flowering time classes. All lines in the Early class possessed the mutated form of the gene (*boflc2*). All but one of the lines in the Medium Early class carried *boflc2*. In the Medium Late class, eight lines carried the mutated allele, and three lines carried functional alleles. Five lines in the Late class carried *boflc2*, and six lines carried functional alleles. All Very Late lines possessed *BoFLC2*.

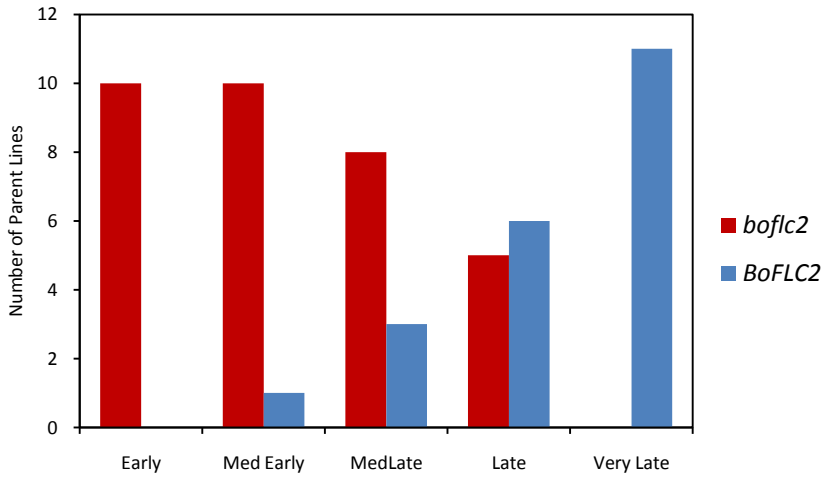


Fig. 8. Distribution of *BoFLC2* genotypes across the five cauliflower flowering time classes. *BoFLC2* parent lines are denoted by blue bars, and *boflc2* lines are denoted by red bars.

3. Characterisation of F_2 Population & Parent Lines

Following on from the positive identification of both *BoFLC2* and *boflc2* alleles in cauliflower lines, four different parent lines with suitable physiological and commercial qualities were selected for further examination. A subset of plants from the late-flowering parents A and C were identified as possessing homozygous functional *BoFLC2* (alternatively referred to as +/+ for the remainder of this chapter) and a subset of plants from early-flowering parents B and D were found to possess homozygous *boflc2* (alternatively referred to as -/- hereafter). These results are shown in Figure 6. Subsequent genotyping of the entire population of field-grown parental lines identified one and three heterozygous (+/-) plants in the S_2 and S_3 generations of the A parent line, respectively. One heterozygous plant was observed out of the total 30 plants in the S_6 parent line C. Heterozygous parent lines were excluded from all subsequent statistical analyses. All plants in the B and D parent lines were homozygous for the mutant *boflc2*, and all CD F_1 plants were heterozygous for this allele.

Genotyping of the AB F_2 populations revealed a segregation of 102:175:97 (homozygous functional:het:homozygous mutated) for the *BoFLC2* gene. This conforms to the expected theoretical 1:2:1 distribution ratio of 93.5:187:93.5 (df = 2; χ^2_{crit} with α at 0.01 = 9.21; χ^2_{obs} = 1.67). In the CD F_2 population, a segregation of 120:186:71 (homozygous functional:het:homozygous mutated) was observed, representing a significant departure from the theoretical expected distribution ratio of 94.25:188.5:94.25 (df = 2; χ^2_{crit} with α at 0.01 = 9.21; χ^2_{obs} = 12.80).

Figure 9 shows that the average length of time until curds became visible was significantly ($P<0.0001$) different between the three *BoFLC2* genotypes in the segregating AB F_2 population (Kruskal-Wallis one-way ANOVA). The average length of time from transplanting until curd visibility in +/+ F_2 plants was 88.2 days, compared to 84.6 days in +/- and 80.0 days in -/- plants. This effect was more extreme in the parent lines, with the S_2 parent line A curding after 122.3 days and the S_6 parent line B curding after just 78.0 days. These figures are significantly different from those for the corresponding genotypes in the segregating F_2 population ($P<0.001$). Also, in the S_3 A parent line, curds appeared significantly faster (116.5 days) than in the A parent line that was only inbred for two generations. Approximately 84% of -/- plants in the F_2 population had already formed curds by the first week of observation (78 days after transplanting). Approximately 58% of +/-

plants had formed curds by the first week, followed by approximately 48% of +/+ plants. Several +/+ and +/- plants in the F_2 population and a number of plants in both A parent lines had not produced visible curds at the time of the final recording.

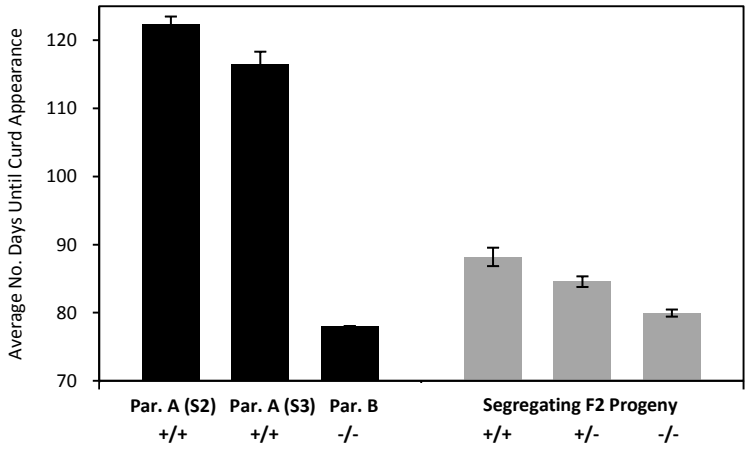


Fig. 9. Average number of days from transplanting until curds were visible in three *BoFLC2* genotypes in late-flowering parent A, early-flowering parent B, an S_3 population of parent A, and a segregating F_2 population. +/+ denotes plants with homozygous *BoFLC2*, -/- denotes plants with homozygous *boflc2*, and +/- denotes heterozygotes. Bars represent the standard error of the mean.

Figure 10 shows that in the second (CD) F_2 population, the average length of time until curds became visible was also significantly different between the three *BoFLC2* genotypes. The average length of time from transplanting until curd visibility in +/+ F_2 plants and parent line C was 94.5 and 90.7 days, respectively; in -/- plants, the duration was an average of 82.1 days and 81.2 days for parents. F_2 +/- curds became visible after an intermediate period of 88.0 days; however, in the heterozygote F_1 generation, curds became visible after just 80.1 days – a similar period of time to the D parent. Significant ($P<0.0001$) differences in time until curd visibility were observed between all three genotypes in the F_2 population (Kruskal-Wallis one-way ANOVA). Significant ($P<0.0001$) differences were also observed between the C/C parent plant and the other two genotypes, but no significant difference was found between the C/D genotype and the D/D genotype. Around 64% of the -/- plants had already developed a visible curd 78 days after planting, in comparison to 29% of the +/- plants and only 12% of the plants with the +/+ genotype. Only one plant (a heterozygote) had not initiated a visible curd by the final week of measurement.

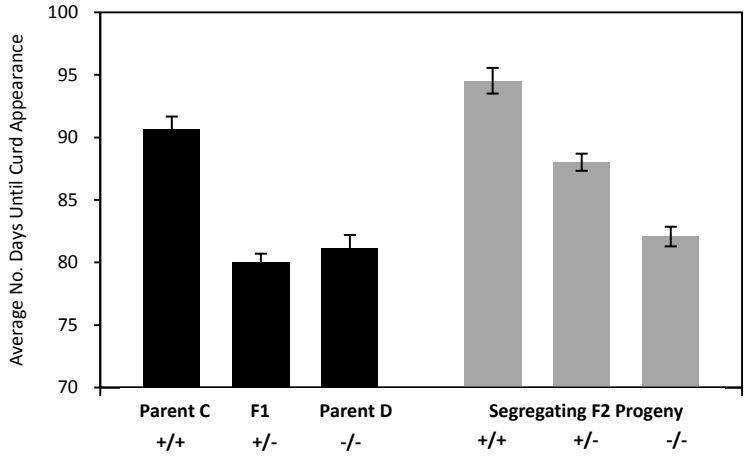


Fig. 10. Average number of days from transplanting until curds were visible in three *BoFLC2* genotypes in late-flowering parent C, early-flowering parent D, F_1 plants and a segregating F_2 population. +/+ denotes plants with homozygous *BoFLC2*, -/- denotes plants with homozygous *boflc2*, and +/- denotes heterozygotes. Bars represent the standard error of the mean.

Comparison of developmental stage between genotypes in the CD population was conducted at the fixed point of 117 days after transplanting. Figure 11 shows that at this point, F_2 plants with homozygous *BoFLC2* had an average developmental stage of 2.6, corresponding to a 'loose' curd just prior to peduncle elongation (see Figure 3 for curd development key). Heterozygous plants had an average stage of 3.6, which corresponds to a curd with elongating peduncles which has not quite formed distinct flower buds. An average developmental stage of 5.0 was observed in plants with homozygous *boflc2* corresponding to a flowering plant with less than 12 flowers fully open. The developmental stages of parent lines and plants from the F_1 population were similar to their corresponding genotypes in the F_2 population (average stages of 2.4, 4.3 and 4.8 in parent C, F_1 and parent D, respectively). Significant ($P<0.0001$) differences in development between the three genotypes were observed in both the F_2 population and the parent lines/F_1 plants (Kruskal-Wallis one-way ANOVA). Although variance within genotypes was found to be homogenous (Bartlett's test), a Shapiro-Wilk test failed to support the null hypothesis that the data were normally distributed.

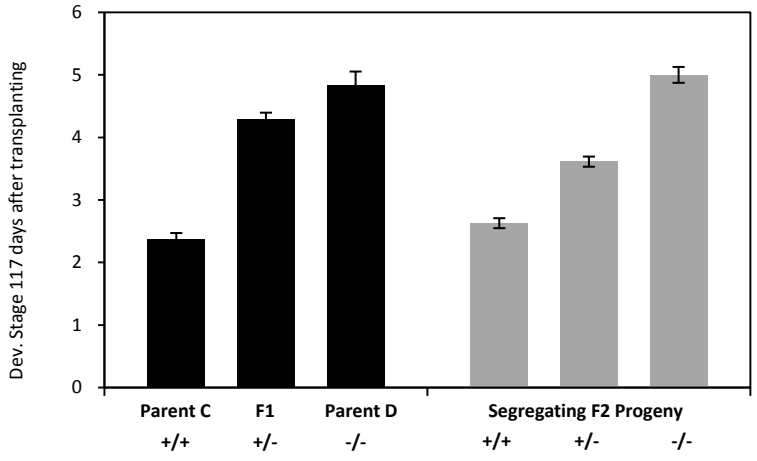


Fig. 11. Average developmental stage of curds in three *BoFLC2* genotypes in late-flowering parent C, early-flowering parent D, F_1 plants and a F_2 segregating population 117 days after transplanting. +/+ denotes plants with homozygous *BoFLC2*, -/- denotes plants with homozygous *boflc2*, and +/- denotes heterozygotes. Bars represent the standard error of the mean. Developmental stage figures are based on Figure 3.

Curd size was recorded throughout the observation period in CD F_2 plants. In the first week of measurement (78 days after planting), the average curd size of -/- plants was 11.1 cm, greater than that of the +/- plants (5.2 cm), which in turn was greater than the average curd size of +/+ plants (2.0 cm; see Figure 12). In the -/- genotype, curd size increased steadily before reaching a plateau. The other two genotypes appear as though they would also follow this general trend, but recording ceased before a clear plateau was reached. Despite significant differences ($P<0.0001$) in average maximum observed curd size (-/- = 26.7 cm; +/- = 25.0 cm; +/+ = 22.1cm) it appears that all three genotypes were tracking to reach a roughly similar maximum size, and by fitting quadratic trend lines to the data, similar maximum average curd diameters are predicted for each genotype (24.9 cm, 23.1 cm and 25.9 cm for -/- , +/-, +/+, respectively). However, while the homozygous mutant *boflc2* lines would be predicted to reach this maximum size after 108 days, the heterozygotes would require a further 6 days to reach this predicted size, and the homozygous functional lines would require yet another 26 days. Using a separate slopes model and comparing the slope between average Y values at the first two time points in the -/- line and the average Y values at the first three points in the +/- and +/+ lines, the slopes (indicating rate of curd growth) in the three genotypes were significantly different ($P=0.0427$), with the rate of curd growth slower in lines with more functional copies of *BoFLC2*.

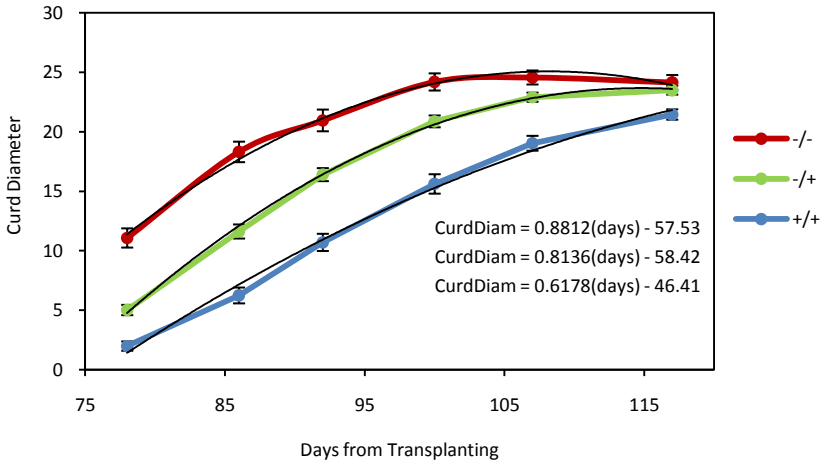


Fig. 12. Average curd size in the CD segregating F_2 population. +/+ denotes plants homozygous for *BoFLC2*, -/- denotes plants homozygous for *boflc2*, and +/- denotes heterozygotes. Polynomial trend lines are displayed, along with slopes of linear regression lines for early time points (first two for -/- and first three for +/- and +/+). Bars represent the standard error of the mean.

4. Identification of Polymorphisms in Parent Lines

From the 49+ PCRs, 25 DNA fragments from each of the four parent lines A, B, C and D were selected for sequencing and marker identification. Sequence data and alignments with corresponding reference sequences may be viewed in Appendix II. Five of these fragments yielded messy sequences, probably due to heterogeneity. Of the legible sequences, most were found to differ to some extent from the reference sequence from which the primers were designed. In the majority of cases, these differences consisted mainly of infrequent SNPs, but several products contained substantial insertions (most notably PCRs 22-24 and 38, with PCR number corresponding to figures listed in Table 7). Some reference sequences were based on cDNA, and in these cases, intron sequences are also provided in Appendix II.

In most cases (PCRs 9, 14, 16, 18, 23, 24, 31, 34-36, 38), the sequenced region was found to be identical in all four parent lines. In the case of PCR 1, parent lines A and C yielded illegible sequences, but the incomplete sequences of parents B and D were found to be identical. In PCRs 42, 43 and 48, parents A and B were practically identical to one another, and parents C and D were practically identical to one another, but, unusually, there were multiple polymorphisms between the A/B parents and the C/D parents. In PCR 22, a SNP was noted between A/C and B/D parent lines, but no suitable CAPS marker could be designed to distinguish it. PCRs 25 (*BoFLC2*), 32 (*BoFLC4-1*) and 46 (*BoREM1*) yielded

sequences that contained polymorphisms for which the CAPS markers described in the Materials and Methods section were designed. As well as the previously-described deletion of an exonic guanine nucleotide and introduction of a premature stop codon (TAA) in the *boflc2* sequence, two SNPs were noted in intron 4 of the functional allele (Figure 13).

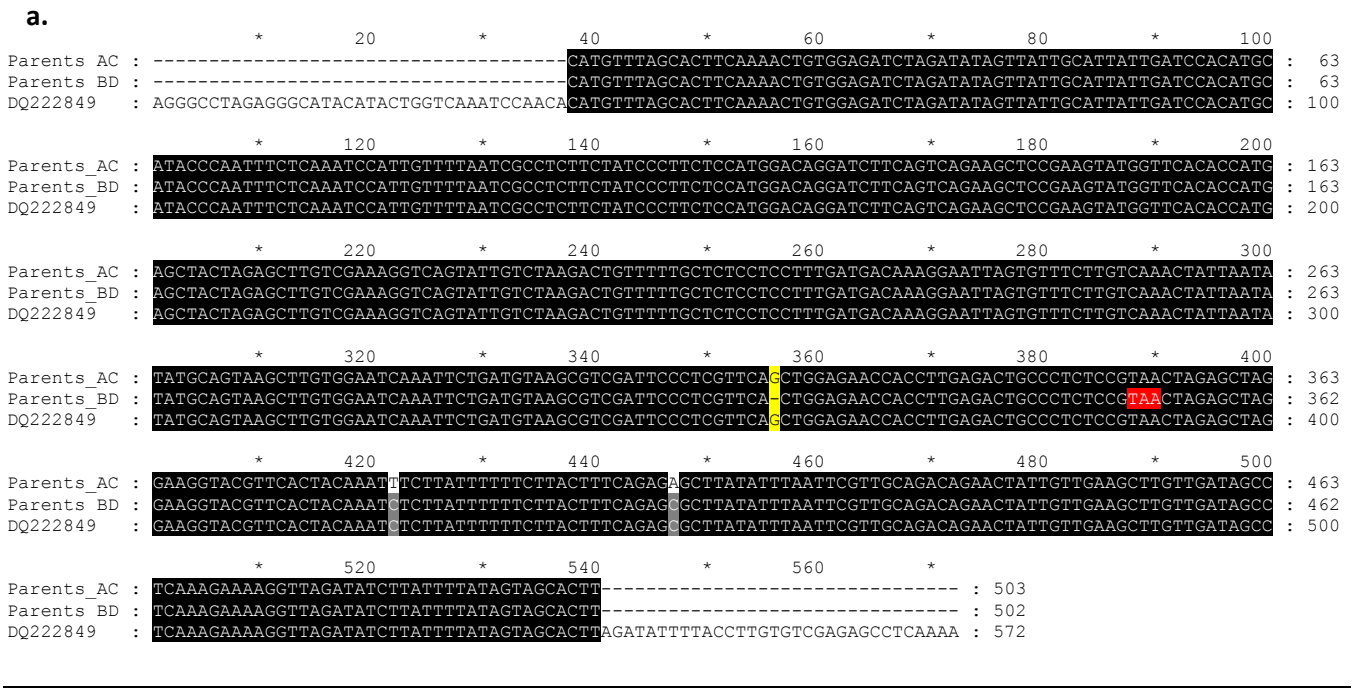


Fig. 13. ***BoFLC2* gDNA, cDNA and predicted amino acid sequence for PCR 25.** Part (a) shows gDNA sequences; reference sequence DQ222849 (Okazaki et al. 2007) begins and ends at forward and reverse primer locations. The DQ222849 (late-flowering cabbage variety 'Reiho') sequence was identical to the available sequence of late-flowering cauliflower parents A and C except for two SNPs in intron 4. Early-flowering parents B and D were identical to the reference sequence except for the notable deletion of a guanine nucleotide in exon 4 (highlighted in yellow), resulting in a premature termination codon (PTC), highlighted in red. Part (b) shows both cDNA and translated cDNA sequences for parents A/C (blue) and B/D (red). cDNA sequences commence at the beginning of exon 3 and conclude at the end of exon 5. Once again, the deletion of a guanine nucleotide in exon 4 is highlighted in yellow, and the resultant PTC in parents B/D is highlighted in red.

The primers used in *BoFLC4-1* PCRs 32 and 33 overlapped one another, enabling a contiguous sequence of the promoter/5'UTR to be assembled (Figure 14). The primers of both PCRs 32 and 33 spanned a 242 bp insertion, which was itself sufficiently large to have enabled agarose gel-based differentiation. However, the primary means of distinction between genotypes used in this work was through the use of the CAPS marker described previously, which utilised the primer combination of PCR 32.

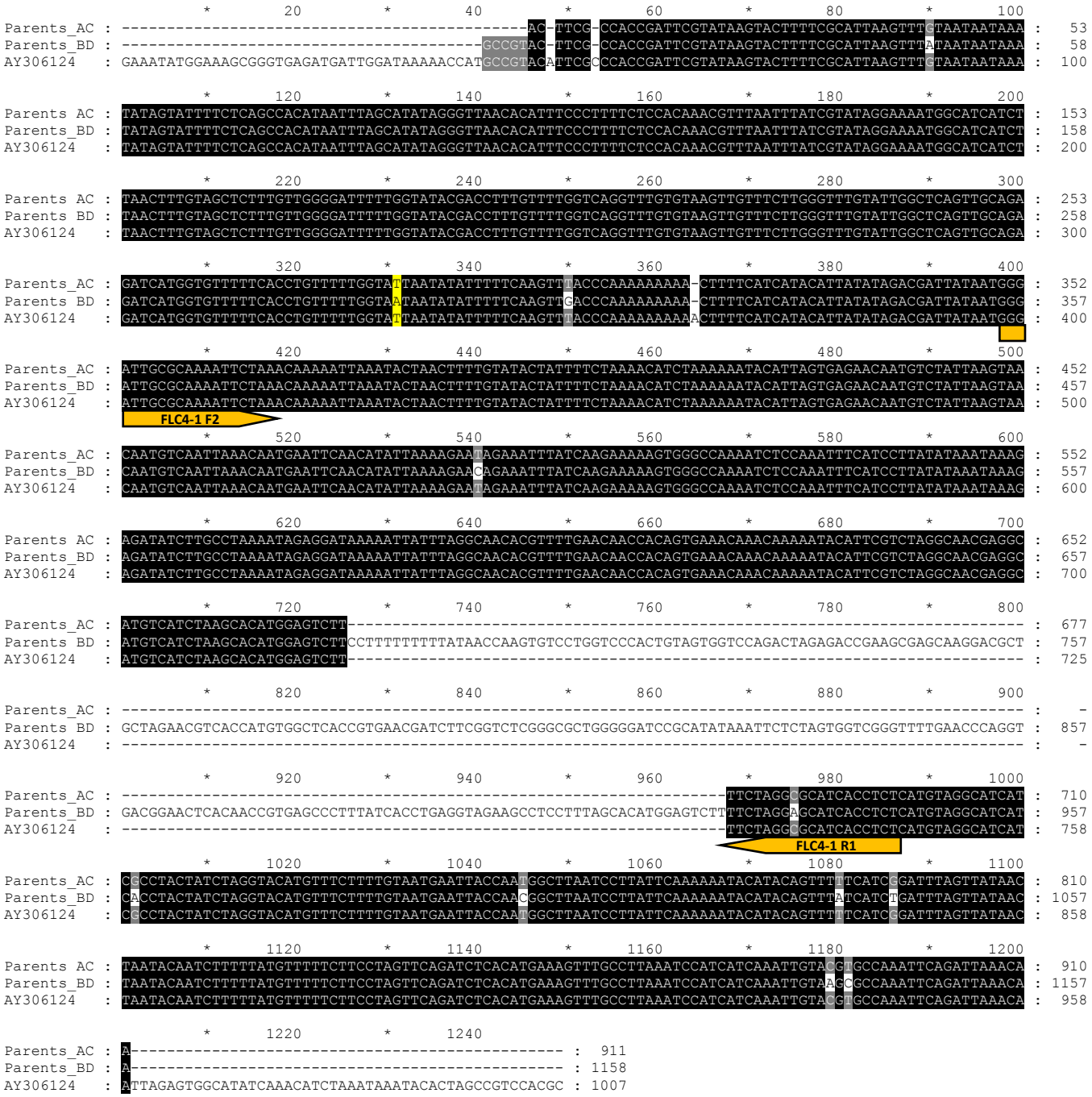


Fig. 14. Contiguous *BoFLC4-1* Sequences for PCRs 32 and 33. Reference sequence AY306124 (Lin et al. 2005) begins at the start of primer FLC41-F1 and ends at the finish of FLC41-R2. The positions of 'internal' primers FLC41-F2 and FLC41-R1 are indicated by orange arrows. (*continued overleaf*)

Fig. 14. (*continued*) Primer combinations 32 (FLC41-F1 and FLC41-R1) and 33 (FLC41-F2 and FLC41-R2) both spanned the 242 bp insertion that was present in parents B/D. This alone was sufficient to identify the different alleles, but for greater clarity, the primary means of distinguishing *BoFLC41* genotype was by digesting the PCR 32 product with AseI; the presence of an A instead of a T abolished the restriction site (highlighted in yellow) in parents B and D.

Sequences of PCR 46 (*BoREM1*) were identical in parents B, C and D. In each of these lines, a 12 bp region 1 bp upstream of intron 2 was absent, as compared to reference sequence AF051772 and Parent A (Figure 15) There is one bp difference between the reference sequence and parent A (a guanine nucleotide in the reference sequence becomes an adenosine nucleotide in parent A) in this 12 bp region.

```
                *        20         *        40         *        60         *        80         *       100
Parent_A : TAATTTCAGCGAGGGAAACGTCTTGGTTTTTAGACACAATGGAGAAGAGATCTTTCATGTGGCTGTTTCCAGTGAATCTGATGATGATGAGTCCGATGAT : 100
Parent_B : TAATTTCAGCGAGGGAAACGTCTTGGTTTTTAGACACAATGGAGAAGAGATCTTTCATGTGGCTGTTTCCAGTGAATCTGATGATGATGAGTCCGATGAT : 100
Parent_C : TAATTTCAGCGAGGGAAACGTCTTGGTTTTTAGACACAATGGAGAAGAGATCTTTCATGTGGCTGTTTCCAGTGAATCTGATGATGATGAGTCCGATGAT : 100
Parent_D : TAATTTCAGCGAGGGAAACGTCTTGGTTTTTAGACACAATGGAGAAGAGATCTTTCATGTGGCTGTTTCCAGTGAATCTGATGATGATGAGTCCGATGAT : 100
AF051772 : TAATTTCAGCGAGGGAAACGTCTTGGTTTTTAGACACAATGGAGAAGAGATCTTTCATGTGGCTGTTTCCAGTGAATCTGATGATGATGAGTCCGATGAT : 100

                *       120         *       140         *       160         *       180         *       200
Parent_A : ACTGACGACAGTGAATCTGATGATGAGTCCAATGACACTGACGACAGTGAATCTGATGATTCCGAAGATAATGATGAAGGTAAGTTGTTTGATATGTTTT : 200
Parent_B : ACTGACGACAGTGAATCTGATGATGAGTCCAATGACACTGACGACAGTGAATCTGATGATTCCGA----------AGGTAAGTTGTTTGATATGTTTT : 188
Parent_C : ACTGACGACAGTGAATCTGATGATGAGTCCAATGACACTGACGACAGTGAATCTGATGATTCCGA----------AGGTAAGTTGTTTGATATGTTTT : 188
Parent_D : ACTGACGACAGTGAATCTGATGATGAGTCCAATGACACTGACGACAGTGAATCTGATGATTCCGA----------AGGTAAGTTGTTTGATATGTTTT : 188
AF051772 : ACTGACGACAGTGAATCTGATGATGAGTCCAATGACACTGACGACAGTGAATCTGATGATTCCGAAGATAATGGTGAA---------------------- : 178

                *       220         *       240         *       260         *       280         *       300
Parent_A : ATTTTTTGGTTTAGTGGAAATGTAAGTGAAGATTGTTGTTATGATTGCAGGGGACAGTAGTTTGGTGAACAAGGAAGCAGATTCTTCATCAGACTGTTTC : 300
Parent_B : ATTTTTTGGTTTAGTGGAAATGTAAGTGAAGATTGTTGTTATGATTGCAGGGGACAGTAGTTTGGTGAACAAGGAAGCAGATTCTTCATCAGACTGTTTC : 288
Parent_C : ATTTTTTGGTTTAGTGGAAATGTAAGTGAAGATTGTTGTTATGATTGCAGGGGACAGTAGTTTGGTGAACAAGGAAGCAGATTCTTCATCAGACTGTTTC : 288
Parent_D : ATTTTTTGGTTTAGTGGAAATGTAAGTGAAGATTGTTGTTATGATTGCAGGGGACAGTAGTTTGGTGAACAAGGAAGCAGATTCTTCATCAGACTGTTTC : 288
AF051772 : ----------------------------------------------GGGGACAGTAGTTTGGTGAACAAGGAAGCAGATTCTTCATCAGACTGTTTC : 229

                *
Parent A : CTCAGAGCTCGTGTCA : 316
Parent B : CTCAGAGCTCGTGTCA : 304
Parent_C : CTCAGAGCTCGTGTCA : 304
Parent_D : CTCAGAGCTCGTGTCA : 304
AF051772 : CTCAGAGCTCGTGTCA : 245
```

Fig. 15. ***BoREM1* Sequence for PCR 46.** Sequences from parent lines A-D are aligned against reference sequence AF051772 (Franco-Zorrilla et al. 1999). Forward and reverse primers are located respectively at the beginning and end of the sequence. The intron can be clearly seen between positions 179 and 249. The absence of the 12 bp region in parent line B abolished the MboII restriction site, enabling distinction to be made between parents A and B.

5. *BoFLC4-1* Genotyping of F_2 Populations & Parent Lines

Figure 16 shows the *BoFLC4-1* CAPS marker restriction patterns of parents A-D. Parents A and C produced 784 bp fragments and a 986 bp fragment was amplified in parents B and D. Although the 242 bp insertion was sufficient to enable discrimination based on size alone, it was occasionally necessary to digest products, cutting the functional lines into 331 bp and 413 bp fragments, while leaving the mutant product intact. The promoter region in which

this marker was designed (based on AY306124; Lin et al. 2005) was not part of the sequence that was used to design the *BoFLC2* marker (DQ222849; Okazaki et al. 2007), meaning that it was initially unclear whether this marker was specific for *BoFLC4-1*, or whether it would also mark the *BoFLC2* gene.

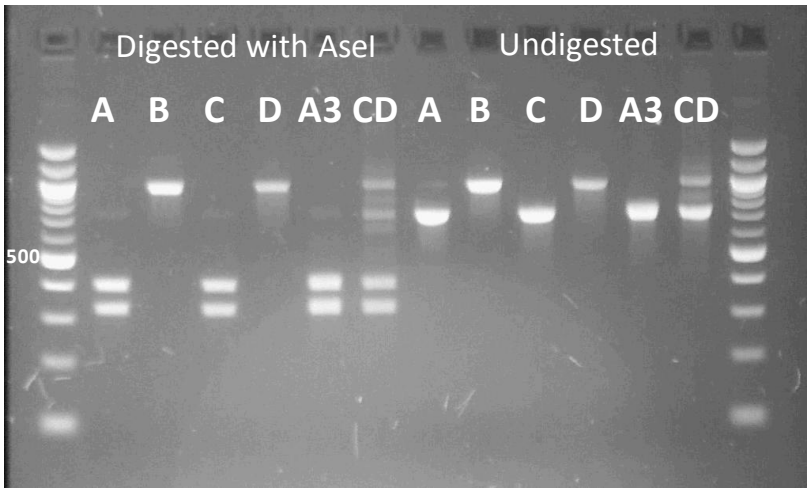


Fig. 16. ***BoFLC4-1*** **CAPS marker restriction pattern.** The undigested 784 and 986 bp PCR products of four parent lines (A-D), the inbred S_3 generation of parent line A (A3), and the F_1 progeny of a CxD cross are shown on the right. Digestion with AseI resulted in the restriction pattern seen on the left, with A, C and A3 cut into 331 and 413 bp fragments. Gel is 2% agarose.

Strong linkage was detected between the *BoFLC4-1* and *BoFLC2* markers in the AB F_2 population, with functional *BoFLC2* alleles mostly co-segregating with the *BoFLC4-1* alleles in which the 242 bp insertion within the promoter was absent (the allele present in parent A). Only six recombinant lines were identified. In five of these lines, the *BoFLC2* genotype was +/-, while the (somewhat ambiguous) *BoFLC4-1* genotype was homozygous for the 'parent A' *BoFLC4-1* allele. In the sixth recombinant line, the *BoFLC2* genotype was -/-, and the *BoFLC4-1* genotype was heterozygous. In the 30 S_3 A parent plants, four plants were observed to possess uncommon *BoFLC2*/*BoFLC4-1* allele pairings (two *BoFLC2* +/+ plants were heterozygous for the *BoFLC4-1* marker, and two *BoFLC2* +/- plants were homozygous for the 'parent A' *BoFLC4-1* allele). Of the 32 S_2 A parent plants, one +/- *BoFLC2* plant was heterozygous for the *BoFLC4-1* marker. All plants in the *boflc2* parent B line contained the *BoFLC4-1* allele with the insertion.

Genotyping of the CD F_2 population suggested perfect co-segregation of the *BoFLC4-1* and *BoFLC2* markers. However, in approximately six lines that contained heterozygous *BoFLC2*, the *BoFLC4-1* genotype was difficult to determine, with the ambiguity arising from the relative faintness of the band of the 'early-flowering' allele (that is, D parent allele that contained the 242 bp insertion. As a consequence, it is possible that a small number of +/+ F_2 plants in the CD population that were also designated as *BoFLC4-1* heterozygotes may in fact be recombinants. Only one of the 30 S_6 parent C plants carried the uncommon allele pairing (a +/- *BoFLC2* plant was homozygous for the 'parent C' *BoFLC4-1* allele). All *boflc2* parent D plants carried the more common allele pairing.

6. *BoREM1* Genotyping

Figure 17 shows the 316 bp *BoREM1* product amplified in parent A and the 304 bp product in amplified in parent B. Parent A produced restriction fragments of 56, 120, 64, 35 and 41 bp while the product from parent B contained one less restriction site, producing fragments of 56, 172, 35 and 41 bp

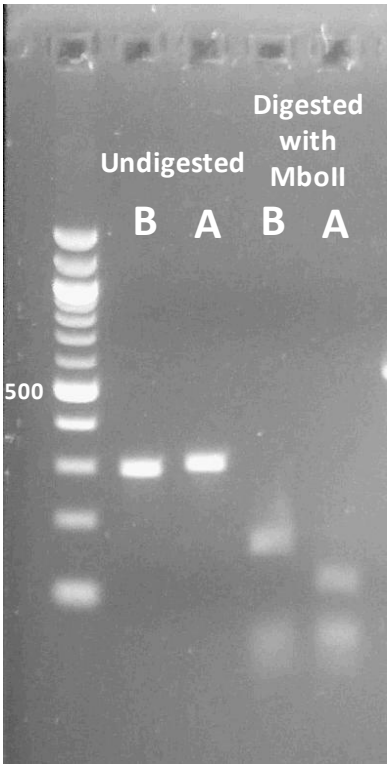


Fig. 17. *BoREM1* CAPS marker restriction pattern. The undigested 304 and 316 bp PCR products of two parent lines (B and A) are shown on the left. Digestion with MboII resulted in the restriction pattern seen on the right, with B cut into 56, 172, 35 and 41 bp fragments, and A cut into 56, 120, 64, 35 and 41 bp fragments. Gel is 2% agarose.

Genotyping of parent A (S_3) showed that two out of 30 plants had the polymorphism normally associated with the B parent line. The marker only worked in 15 of the 29 B

parents, but all were found to have the expected genotype. Genotyping the AB F_2 population was not possible due to an inability to obtain clear restriction fragment patterns.

D. Discussion

1. Screening Cauliflower & Cabbage Parent Lines for *BoFLC2* Mutation

This study provides the first record of non-mutant *BoFLC2* alleles in cauliflower varieties, and supports the idea that the previously described *boflc2* mutation (which is predicted to result in a loss of function due to a PTC) is a critical determinant of flowering time within the annual brassica sub-group. It is clear that the proportion of cauliflower parent lines with functional alleles becomes higher as the flowering class gets later, reflecting the role of *BoFLC2* as a flowering inhibitor. There are no functional alleles in the Early class, one in the Medium Early class and three in the Medium Late class, which represents just four out of the 32 lines in the three earliest flowering groups. The Very Late category consists solely of functional *BoFLC2* homozygotes. However, the presence of *BoFLC2* alleles in plants classed as Medium Early and Medium Late, combined with the relatively large number of *boflc2* genotypes in the Late flowering class is somewhat incongruous. In order to investigate the basis for these anomalies, the flowering behaviour of these specific cauliflower lines is investigated further in Chapter IV.

The detection of a single *BoFLC2* putative heterozygote amongst the 43 cabbage lines also potentially represents the first record of a *boflc2* allele in a biennial brassica cultivar. The suspected heterozygosity of this line is itself unusual, considering that it is an inbred parent line. If any mutant alleles were to be found within the cabbage lines, it would have been reasonable to expect them to be within those lines designated as 'easy bolting'. However, the white cabbage line in question was not classified as such, and Rijk Zwaan line descriptions do not contain any information to suggest that this line is especially early-flowering. No specific flowering time trials were carried out for this line to assess its flowering behaviour relative to other cabbage lines, but it would be informative to observe whether the presence of one non-functional allele in this line is associated with an earlier transition to reproductive development, relative to the other 42 cabbage lines. Moreover, it would be interesting to genotype this parent's seed lot to ascertain whether +/+ and -/- genotypes are also present, and to determine whether inbreeding this parent line produces progeny that segregate for the two different forms of *BoFLC2*, and whether *BoFLC2* genotype is associated with flowering time in cabbage, as it is in cauliflower.

More broadly speaking, the relative infrequency of the mutant allele in these cabbage lines supports the idea that functional *BoFLC2* is an important (if not essential) feature of biennial brassica cultivars. However, the known diversity of flowering time within these cabbage parent lines and lack of correlation with the presence of *boflc2* alleles indicates that flowering time variation within biennial brassica cultivars is largely independent of *BoFLC2* functionality. It could therefore be suggested that the functionality of the *BoFLC2* gene contributes in a large measure to the basic identity of the brassica as biennial variety, but that variation in the expression or functionality of other flowering time genes (such as other *BoFLC* genes) is responsible for 'fine-tuning' flowering time within the limitations set by the functional *BoFLC2* genotype.

The development of cultivated varieties more suited to local environments is an important objective of plant breeding efforts. Identification of a strong association between *BoFLC2* allelic variation and floral induction in 'natural' populations of cauliflower inbred parent lines is potentially of great practical and commercial value to this effort. For example, the functional marker described above could be used to screen germplasm and select favourable cauliflower and cabbage genotypes in breeding programmes without the need for lengthy growing periods in multiple climatic conditions to accurately determine flowering behaviour from phenotypic analysis. The number of crosses necessary in doubled haploid (DH) breeding programmes could be reduced by using this marker to help select parent lines with suitable combinations of alleles. In some cases, *BoFLC2* could be modified or transformed to alter timing of floral induction without significant negative pleiotropic effects on yield or general fitness. For example, (Kim et al. 2007) reported a substantial delay in Chinese cabbage (*B. rapa* ssp. *pekinensis*) floral induction by overexpression of *FLC*. In *B. oleracea* cultivars where delayed curd induction or flowering is sought, such an approach may find a similar application. Furthermore, this marker could be used for quality control of seed lots (as potentially illustrated by the identification of heterozygotes in the A and C cauliflower parent lines, and in the cabbage parent line described above) (see Jung & Müller 2009).

2. Characterisation of Parent Lines & F_2 Populations

As noted, parent line A was less inbred than the other parent lines. This may explain why four out of the five *BoFLC2* heterozygotes found in parent lines were parent A (one in the S_2 generation and three in the S_3 generation). The remaining (ambiguous) heterozygote was

found in the other late-flowering line, parent C (S_6). While finding heterozygous lines after six generations of inbreeding is unusual, a low frequency of +/- may be expected. As noted previously, all heterozygous parent lines were excluded from statistical analysis, but the fact that a potentially heterozygous line was used to make the crosses would be expected to affect the segregation of the *BoFLC2* alleles (and potentially other genes) in subsequent generations – especially in the AB F_2 population, where the relative frequency of heterozygous lines was higher in the parent lines. This may partially explain the greater phenotypic variation that was observed in the AB F_2 plants when compared to CD F_2 plants.

The genetic and phenotypic analysis of segregating *BoFLC2* F_2 populations derived from crosses between the late-flowering (+/+) and early-flowering (-/-) cauliflowers (as opposed to a group of unrelated cauliflower lines) enabled a more 'controlled' method of assessing *BoFLC2* influence on flowering time. Another benefit of this experiment was that it enabled the incomplete dominance and quantitative nature of the *BoFLC2* gene to be studied through analysis of heterozygotes; Okazaki et al. (2007) state that the annual habit in *B. oleracea* is dominant over the biennial habit, but the conditions used in this study exposed dosage effects and incomplete dominance of the *BoFLC2* gene. Furthermore, the use of these F_2 populations enabled the collection of flowering time data at a level of detail not possible for the parent lines used in this study, which were classified into different flowering classes based on in-house trials and inaccessible data.

The correlation of the *BoFLC2* genotype with curd appearance date in the AB F_2 population is plain; plants with more functional copies of *BoFLC2* produced curds later (Figure 9). The fact that this correlation remains clear despite parent lines not being 'thoroughly' inbred (which would potentially result in greater heterozygosity of other genes and more phenotypic 'noise'), emphasises the strong influence of *BoFLC2* on the transition to reproductive development. Compared to the differences in curd initiation between +/+ and -/- genotypes in the F_2 population, the difference in curd initiation between +/+ and -/- in parent lines is even more extreme, primarily due to the very late appearance of A (S_2) curds. The differences between genotypes in the F_2 population may be smaller because none of the parent lines used was isogenic. This means that in addition to *BoFLC2*, many other genes that influence flowering time would be segregating and influencing the phenotype of the F_2 population, resulting in less extreme phenotypes than in parent lines. It was also noted that the average curd appearance date for parent line A plants inbred for an additional generation

(S_3) was slightly earlier than those inbred for just two generations (S_2). Without specific knowledge of which characteristics formed the basis for selection in the inbreeding programme, it is difficult to account for this finding.

The CD cross produced an F_2 population that was greatly preferred for analysis of *BoFLC2* influence. Both C/D parents and F_2 population were less affected by disease than the AB plants, meaning that, as well as curd initiation, subsequent curd development and flowering time could also be investigated. Furthermore, the C/D parents were more highly inbred (six generations as opposed to two and four for the A and B parents, respectively). A significant departure from the expected theoretical *BoFLC2* distribution ratio was observed in the CD F_2 population; specifically, the +/+ fraction of the offspring was unusually large, and the -/- fraction was unusually small. This may possibly be due to preferential transmission of the functional allele by gametic selection.

Despite the somewhat distorted segregation ratio, the importance of the *BoFLC2* gene to the flowering phenotype in the CD F_2 population is also clear. In the segregating population, the *BoFLC2* gene appears to function in a dosage-dependent manner with curd formation delayed in an additive manner by additional copies of the functional allele (Figure 10). Similarly, as the number of functional alleles increased, the developmental stage at a fixed time point was observed to decline (Figure 11). The non-normality of the data in this method of classifying flowering time was expected due to the non-uniform development of curds, combined with the fact that the index used to classify developmental stage is not a perfect reflection of actual development; for example, the length of time required for a curd to pass through stages 2-5 may be brief relative to the amount of time spent as a curd (stage 1) or in full flower (stage 6). This means that observations of developmental stage at a fixed point in time are expected to result in positively or negatively skewed distribution. *BoFLC2* also appeared to delay developmental stage in an additive manner in F_1, C and D parent lines, but this was not the case for measurements of curd appearance, with F_1 curds appearing at a similar stage to curds in parent D (Figure 10). It is possible that compared to the CD F_2 population, the effect of *BoFLC2* heterozygosity on this phenotypic trait is modified or masked in the CD F_1 population by its presumed near-complete background heterozygosity predicted by the higher degree of inbreeding of C and D parent lines. This could only be confirmed by performing additional crosses.

Applying the formula $\sigma^2_{phenotype} = \sigma^2_{genotype} + \sigma^2_{environment}$ to the observed variance under a model of normal distribution would roughly equate to the *BoFLC2* gene explaining 40% of total phenotypic variance in flowering time, and 65% of the genetic variance in flowering time, where flowering time is based on the developmental stage of development 117 days after transplanting, and assuming environmental variance in the F_2 population is the same as the average of the variance in the homozygous parent lines (parental lines were used as environmental controls). As mentioned earlier, it appears likely that the remaining variation may be explained by other flowering time genes. For example, as this thesis was going to press, data was published suggesting that allelic variation at the *BoFRIa* locus may be responsible for variation in *B. oleracea* flowering time (Irwin et al. 2012).

A very recent QTL study by Uptmoor et al. (2011) using a *B. oleracea* population derived from a cross between late/early flowering types indicated that flowering time variability was primarily due to differences in vernalisation response. However, neither parent used to generate that population (the same as that used by Razi et al. (2008)) possessed functional alleles of *BoFLC2* and no other *BoFLC* genes were found to co-segregate with flowering time. The authors raise the possibility that *FLC*-independent pathways are responsible for the vernalisation response seen in their population, and this may also be the case in the populations generated in this study. However, it is also possible that other members of the *BoFLC* family may be contributing to the flowering response. In the previously mentioned study by Pires et al (2004), segregation of the *BnFLC3* explained 29% of the phenotypic variation in flowering time, with plants containing *B. oleracea FLC3* alleles flowering significantly earlier than those with *B. rapa FLC3* alleles. In the (likely) absence of fully-functional *FLC2* in that segregating *B. napus* population, it seems as though *FLC3* is a significant determinant of flowering time. In the same way, it is possible that variation in genes such as *BoFLC3* could contribute to the remaining variability in the AB and CD populations. However, this cannot be established due to the fact that no polymorphisms were detected in *BoFLC3* using the primers employed here, meaning that it is unknown whether this gene was segregating. Further examination of the promoter and introns of *BoFLC3* would enable the contribution of the *BoFLC3* gene to flowering time in these populations to be evaluated.

In both F_2 populations, it would have been preferable to commence observations earlier in order to capture the precise week of curd initiation in those plants that had already formed

curds by the first week of observation. This is particularly so in the AB population, where 84% of -/- plants had already formed curds by the first week of observation (followed by 58% of +/- and 48% of +/+ plants). In the CD population, the overall curd initiation was later (64% of the -/-, 29% of the +/- and 12% of the +/+); nonetheless, in both populations, the percentage of initiated plants decreased as the number of functional *BoFLC2* alleles increased, meaning that it is likely that the findings reported here would be equally, if not more pronounced had observations begun earlier.

Differences in average curd size at the beginning of the observation period are likely to be directly related to the differences in initiation date. It was not unexpected to observe that in the later-initiating genotypes, the average curd sizes were smaller in the initial observation stages (Figure 12). Even though significant differences were observed in average maximum observed curd size, the differences in curd initiation date make it somewhat misleading to compare these values: a more accurate measure of maximum curd size was taken to be the predicted maximum size, based on the quadratic trend line. This predicted maximum size was similar for all three genotypes, indicating that *BoFLC2* functionality does not have any bearing on the final curd size. Indeed, it was observed that within each of the three genotypes, some curds begin to bolt and flower when the curd size is very small, and others do not bolt and flower until the curd is quite large. As well as being influenced by other genes, it is expected that this characteristic is very much determined by environmental factors.

It is worth noting that there is a significant delay in reaching the maximum predicted curd size in +/- and +/+ genotypes. Again, this may be partially explained by the delayed initiation of +/- and +/+ curds compared to -/- lines. For example, Figure 12 shows that +/- lines were, on average, six days slower to reach this maximum predicted size than the -/- lines, which is likely to be because they also appeared an average of six days later than the -/- lines. However, the +/+ curds, which appeared an average of twelve days later than the -/- lines did not reach this maximum predicted size until 32 days later than the -/- lines. This is consistent with the idea that the rate of curd growth, as well as curd development, is slower in genotypes with more functional copies of *BoFLC2*. Although the statistical calculations of curd growth rate are based on comparisons between older curds in the -/- genotype and younger curds in the +/- and +/+ genotypes, the quadratic nature of the curves used to generate these predicted maximum averages means that even if the curds in all three

genotypes were able to be compared at the same age, the differences in slope (and therefore rate of growth) would actually be even more pronounced.

3. Additional Markers

The degree of divergence between crossed parent lines was uncertain (Rijk Zwaan breeders were only at liberty to indicate that they were 'quite' different). Therefore, it is not possible to say whether it is surprising that very few polymorphisms were found between parent lines. Observed differences between the sequenced products in Table 7 and their corresponding reference sequences were generally SNPs; however, in some situations, significant differences exist, and in many cases, intron locations and sequences have been identified. Therefore, even though few polymorphisms were identified, this information, combined with the trial of different primer pairs and optimisation of PCR conditions, will potentially be useful for future work where mapping of brassica flowering genes is needed. A 'wider' cross between early-flowering broccoli and late-flowering cabbage has already been made, and it is hoped that this information can be used in the future to map these candidate genes on the resulting F_2 population.

Some ambiguity still remains as to whether *BoFLC2* and *BoFLC4* are one and the same gene; alignments of *BoFLC2* sequence DQ222849 and *BoFLC4-1* sequence AY306124 reveal a number of SNPs and several insertions/deletions, but all are confined to introns, and may simply be natural variation within the gene between the cultivars. On the one hand, the six apparent recombinants seen in the AB population would normally suggest two separate identities for each of these genes. On the other hand, there are several reasons not to discount the idea that the two genes are the same. For example, the perfect co-segregation of *BoFLC4-1* with *BoFLC2* in the large CD F_2 population (although slightly ambiguous in several instances) points towards a common identity. Five out of the six ambiguous recombinants detected in the AB F_2 population could possibly be explained by the fact that parents B and D differed by a SNP in the FLC4-1 R1 primer sequence. This could mean that that plants which were heterozygous for the *BoFCL2* alleles were incorrectly being recorded as homozygous for the 'A' parent allele due to poor amplification of the 'B' parent allele in heterozygotes. However, this theory does not explain why only five of the plants should be subject to this ambiguity, nor does it account for the clear recombination observed in one plant that was -/- for *BoFLC2*, but heterozygous for the *BoFLC4-1* allele.

Another factor in favour of the 'same gene, but incorrect *BoFLC4-1* identification' theory is that populations of both A and C parents contained plants recorded as +/- for *BoFLC2*, but homozygous 'A' for *BoFLC4-1*, whereas none of the plants in the B or D parent lines (i.e. the two lines with a SNP in the primer region) contained heterozygous *BoFLC4-1* genotypes. However, even allowing for these possible genotyping errors, a simple lack of recombination does not enable us to distinguish between the two possibilities: (a) that the genes are the same, or (b) that they are different, but closely linked. The only way to establish this conclusively would be to demonstrate how the two pairs of sequence variants are physically linked by isolating a single DNA molecule containing them both.

Given that *BoREM1* is expected to have a role the initiation of floral meristems ((Franco-Zorrilla et al. 1999), there was thought to be some potential that a marker based on polymorphisms in its sequence would be associated with curd initiation. Although a suitable marker for the AB cross was designed, our inability to obtain clear restriction fragments in the AB F_2 population prevented a proper assessment of its relationship to the timing of reproductive transition. This is another area that would benefit from further investigation.

E. Conclusions

This research provided the first record of functional *BoFLC2* alleles in an annual *B. oleracea* cultivar, and the first record of a *boflc2* allele in a biennial *B. oleracea* cultivar. Genotyping of unrelated cauliflower parent lines showed that the mutant allele was associated with an early transition to reproductive development with a small number of exceptions: additional assessment of flowering behaviour of all these lines will be reported in Chapter IV. This finding is consistent with other research suggesting that flowering time variation in Arabidopsis has arisen, at least partly, through the generation of non-functional or weak *FLC* alleles (Gazzani et al. 2003).

This research also represents the first time that the contribution of *BoFLC2* functionality to flowering time has been assessed in a segregating F_2 population derived from a cross between 'annual' brassicas. The findings support the idea that not only is *BoFLC2* functionality an important contributing factor to annual/biennial flowering habits in brassica, but that it is also a key determinant of curd growth rate and flowering time within the annual cultivars, functioning in a dosage-dependant manner and contributing up to 65% of the genetic variance within the CD F_2 population. Within the biennial group of brassicas, flowering time diversity must be explained by other unknown genetic factors, and it is likely that these other factors also contribute to flowering time in annual cultivars to some extent. Although a linkage map was unable to be constructed in the cauliflower x cauliflower cross, the primers and candidate gene information presented here should assist future attempts to do so. The data support the view that *BoFLC4-1* is the same gene as *BoFLC2*, but conclusive evidence was not obtained in this experiment.

The development of novel cultivars that are better-suited to local environments and changing climatic conditions is a key goal of plant breeding efforts. The identification of *BoFLC2* as an important contributor to flowering time within brassica species grown as annual crops is potentially of great practical and commercial value to plant breeders, with possible applications as a marker for screening and classifying flowering time in cauliflower germplasm, or as a target for genetic modification. From this perspective, it is also important to understand how this gene and others in the cauliflower flowering pathway are regulated by vernalisation at the molecular level in order to be aware of possible pleiotropic effects. In

Chapter V, the expression dynamics of several important flowering genes, including *BoFLC2*, are investigated.

Chapter IV

CHARACTERISING FLOWERING TIME IN CAULIFLOWER PARENT LINES

A. Introduction

In the previous chapter, functionality of the *BoFLC2* gene was shown to make an important contribution to flowering behaviour in two segregating cauliflower populations, with functional alleles associated with later curd initiation and flowering. Furthermore, screening for *BoFLC2* genotype in Rijk Zwaan cauliflower parent lines revealed both *BoFLC2* and *boflc2* alleles in a range of unrelated flowering time accessions, with the *BoFLC2* genotype strongly associated with delayed flowering in data provided by Rijk Zwaan breeders. However, several instances were observed where flowering classifications appeared incongruous in light of the *BoFLC2* genotype. For example, several *BoFLC2* parent lines had been classed as Medium Early or Medium Late, and a relatively large number of *boflc2* genotypes were designated as Late flowering. In order to establish whether these inconsistencies were authentic, or whether they were simply artefacts of a particular unspecified method of designating flowering class, further trials were necessary to characterise the reproductive behaviour of the cauliflower parent lines in a range of growing conditions. First-hand observation of curding and flowering behaviour would potentially enable identification of the physiological basis of these incongruities, and provide a more complete understanding of the association between *BoFLC2* genotype and differences in reproductive development. In this short chapter, the curding and flowering behaviour of cauliflower parent lines grown in three different environments is reported.

B. Materials & Methods

For each of the three flowering time trials, seeds of each of the cauliflower parent lines described in Table 2 (except for line 1, of which there was insufficient seed, and lines 22, 26 and 28, which germinated poorly) were sown into a vermiculite-based seed raising mix on the 18th of February 2009 ('early' sowing) and the 11th of March 2009 ('late' sowing) and raised by Hills Transplants (Devonport, Tasmania).

1. Pot-Based Glasshouse Trial

Seven replicates of each parent line from the early sowing were transplanted into general purpose potting mix in 4.5 L pots on the 2nd of April (43 days after sowing) when seedlings had four to five fully expanded leaves. Plants were grown in a glasshouse under natural light and photoperiod conditions. Between the 2nd of April and the 19th of October, temperatures ranged between 4°C and 23.4°C, with an average temperature of 14°C, and between the 20th of October and the 8th of February (when recording ended), temperatures ranged between 8.8°C and 32.6°C, with an average temperature of 19.7°C. Plants were fertilised with Hoagland's solution (Hoagland & Broyer 1936) twice weekly. Plants were irrigated using drip tape located at the potting mix surface to ensure no water settled on curds. Following sowing, developmental stage was recorded on a weekly basis for 51 weeks. The trial setup is shown in Figure 19a-d.

2. Soil-Based Glasshouse Trial

The second glasshouse-based trial was conducted in Daylesford, Victoria at Rijk Zwaan Australia. All cultural practices used in the preparation and management of this trial were based on Rijk Zwaan's commercial seed crop management practices. Seedlings were transplanted into the red ferrosol soil at a spacing of approximately 350 mm. The trial was set out as a randomised complete block design. Three replicates (ten plants per replicate) of eight parent lines (2, 12, 17, 24, 40, 48, 50 and 51) from the early sowing were transplanted on the 8th of April 2009 (49 days after sowing), and three replicates (ten plants per replicate) of each of the parent lines from the late sowing were transplanted on the 28th of April (49 days after sowing). This trial site is shown in Figure 19f.

3. Field Trial

The open field trial was located at Oyster Cove in Southern Tasmania (43°06'09"S, 147°16'00"E, WGS84; climatic data shown in Figure 18). The site was on sandy soil which had previously been under pasture for cattle production. The site was sprayed with Roundup CT (450 g/L Glyphosate; 3 L/ha), and Mycrobor (21% boron; 10 kg/ha), sodium molybdite (400 g/ha) and dolomite lime (2 T/ha) were applied. In March 2009, the site was ploughed and fertilised with 1 ton/ha of Bejo proprietary fertiliser (3:5:14 + trace elements) and 1.6 m wide raised beds were formed with two rows per bed, each 800 mm apart. Prior to planting, the site was sprayed with Goal (240 g/L oxyfluorfen; 3 L/ha), Stomp440 (440 g/L pendimethalin; 2 L/ha) and Roundup CT (450 g/L Glyphosate; 2.5 L/ha). Seedlings were hand-transplanted at a spacing of approximately 500 mm, and were covered with frost cloth until curds were visible to prevent browsing by birds. The trial was set out as a randomised complete block design. Three replicates of each parent line from the early sowing were transplanted on the 7th and 8th of April (48-49 days after sowing), with each replicate comprising ten plants. Three replicates of each parent line (except 3, 4, 12 and 33) from the late sowing were planted into the field on the 5th of May 2009 (56 days after sowing), with each replicate again comprising ten plants. Plant development was scored regularly until late November 2009. The field site is shown in Figure 19e.

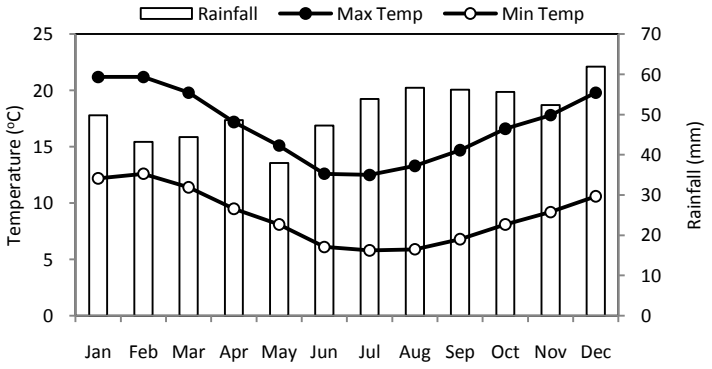


Fig. 18. Long-term mean monthly minimum and maximum temperature and rainfall data for Bull Bay (Lauriston) weather station, approximately 10 km from field site at Oyster Cove (Australian Bureau of Meteorology 2011).

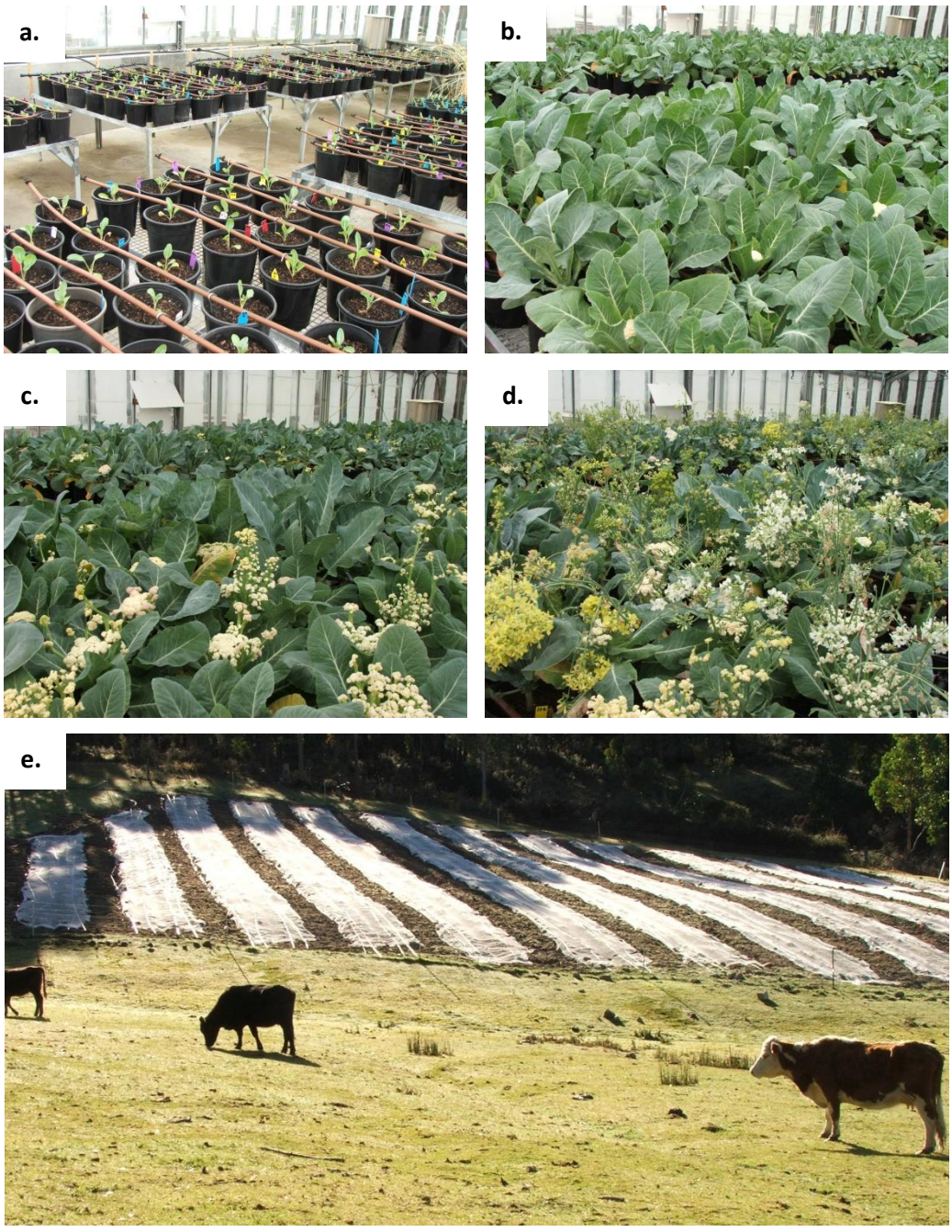


Fig. 19. Flowering time trials. a – d: Pot-based glasshouse trial at UTAS showing plant growth and development. e: Field trial site at Oyster Cove with frost cloth covering plants. f: Soil-based glasshouse trial at Rijk Zwaan, Daylesford.

C. Results

These flowering trials revealed that five of the cauliflower parent lines (21, 23, 27, 44 and 49) were of the 'Roman', or 'romanesco' variety (Kieffer, Fuller & Jellings 1998), with fractally-arranged pyramidal curds (Figure 20). Although not grown in these trials, parent line 1 had previously been identified as a romanesco variety. In addition, parent lines 9, 13 and 17 formed small, tightly bunched 'heads' of leaves prior to detection of a visible curd. Dissection of these 'bunchy' apices when first observed typically revealed an apparently vegetative, but very wide, or expanded apex. Dissection of more advanced apical 'heads' often revealed curds that were up to 12 mm in diameter. This tight packing of leaves complicated efforts to routinely distinguish the point at which curds were formed; curd induction was defined as the point at which curds became visible, and so the obscuration of sizeable curds tended to result in later-than-expected curd induction times for these lines.

Fig. 20. Romanesco parent line 27 (left), tightly-packed leaves prior to curding in 'bunchy' parent line 17 (centre) and 'standard' white-curded parent line 48 (right).

Figure 21 reprises Figure 8 (Chapter III), with the inclusion of data showing the proportion of romanesco and 'bunchy' parent lines in each *BoFLC2* genotype and each flowering class. As noted in the previous chapter, the vast majority of *boflc2* parent lines had been assigned to the three earliest flowering classes (Early, Medium Early, and Medium Late), with only four *BoFLC2* lines represented in these three classes. Figure 21 shows that all four of these *BoFLC2* parent lines were romanesco varieties. One *boflc2* line classed as Medium Late, and one *BoFLC2* line classed as Late were also found to be romanesco varieties. The three lines which formed bunched heads prior to visible curd initiation were all *boflc2* that had been assigned to the Late flowering group.

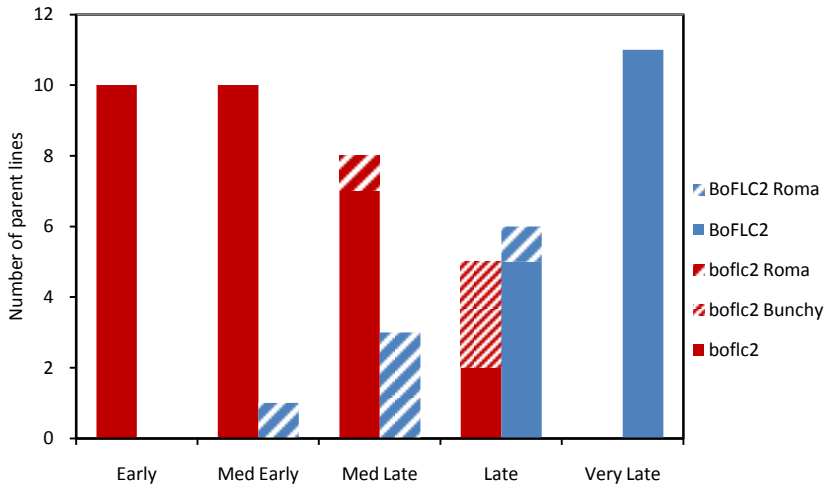


Fig. 21. Representation of romanesco and 'bunchy' flowering types between *BoFLC2* genotypes and across the five flowering time classes. Mutant lines are denoted by red bars, and functional lines are denoted by blue bars. The thick-striped component of each category indicates the proportion of lines that are Romanesco (denoted 'Roma' in the legend), and the thin-striped components represent the proportion of lines that formed 'heads' with tightly-packed leaves (denoted 'Bunchy' in the legend).

The pot-based glasshouse trial yielded curd initiation data broadly consistent with the classifications provided by Rijk Zwaan pre-breeders (Figure 22a); in general, parent lines classed as Early required the least time to form curds and initiate flowers, while the Late and Very Late lines required the most time, and in three cases, did not form curds at all. Medium Early and Medium Late lines were distributed between these groups. Despite considerable differences in genetic background between these parent lines, a clearly significant ($P<0.0001$) differentiation in curd initiation time was observed between *boflc2* lines, which initiated curds after an average of 15.5 weeks, and *BoFLC2* lines, which initiated curds after an average of 30.6 weeks. The four 'out-of-place' *BoFLC2* romanesco lines were the earliest-curding of the parent lines with the functional allele, and the three 'bunchy' plants were the latest-curding *boflc2* plants. Furthermore, data from this trial suggests that the designation of the two non-bunchy *boflc2* lines as Late may be dubious, with these parent lines both forming curds after a relatively short period of time. Therefore, the distinction in curd initiation between *BoFLC2* and *boflc2* parent lines is sharp, allowing for the deceptive 'transition' which is primarily due to parent lines with unusual curd initiation behaviour that complicates the *BoFLC2* gene effect.

As with curd initiation, there is a clear separation of *BoFLC2* and *boflc2* groups based on flowering time with the exception of romanesco and bunchy parent lines (Figure 22b).

BoFLC2 lines took an average of 40.9 weeks to reach full anthesis (flowering stage 6), which was significantly different (P<0.0001) from *boflc2* lines, which reached this stage after an average of just 26.9 weeks. Curd initiation time was usually strongly indicative of flowering time, but some lines that initiated curds at a young age remained in the arrested phase and did not proceed to flowering for a considerable length of time (e.g. line 47). Other parent lines which initiated curds very early were slow to flower (parent line 30) or failed to flower altogether (parent line 37) because of severe fungal infection.

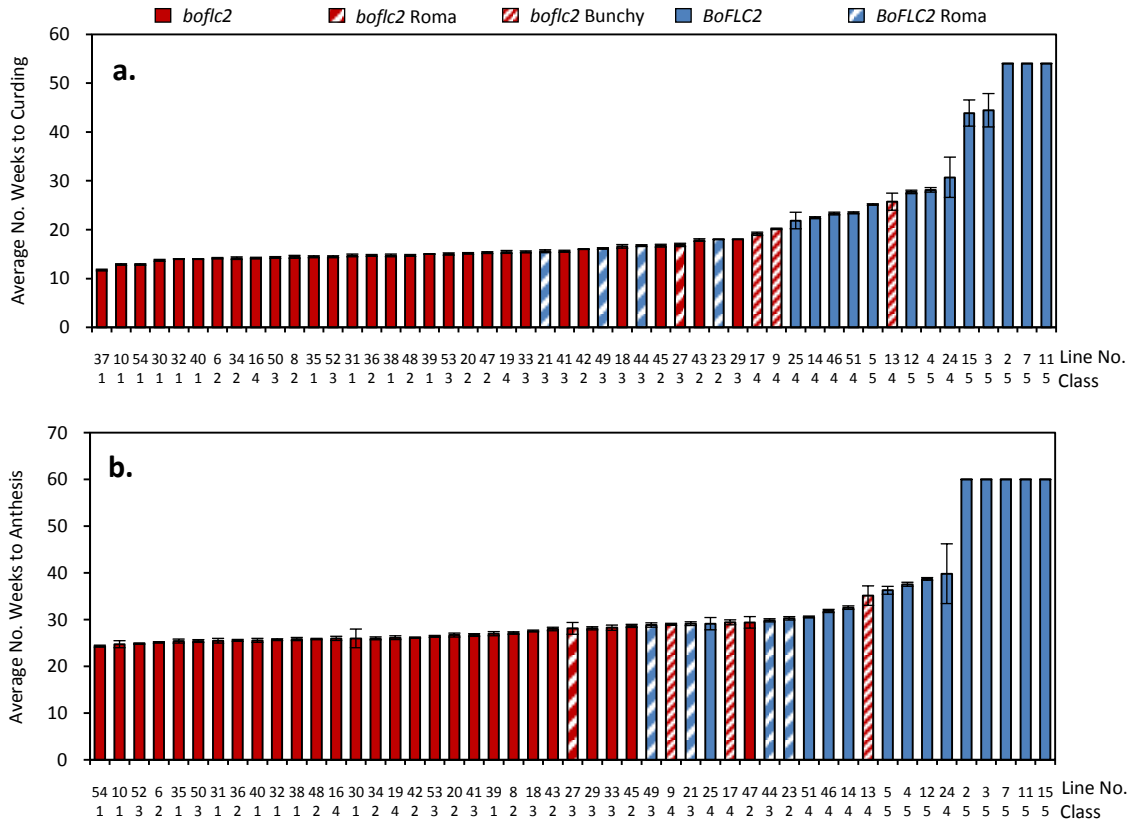


Fig. 22. Average number of weeks from sowing to (a) curding and (b) anthesis in cauliflowers grown in pots under glasshouse conditions. All values are the average of seven replicates. Red and blue columns denote mutant (*boflc2*) and functional (*BoFLC2*) parent lines, respectively. Where plants did not initiate visible curds, they were assigned a maximum value of 54 weeks, and where plants did not flower, they were assigned a maximum value of 60 weeks. Columns with thick stripes represent romanesco parent lines, and columns with thin stripes denote *boflc2* lines that formed a tightly-bunched 'head' of leaves prior to curd formation. The flowering class of each parent line (as described in Table 2) is shown beneath the line number, where class 1 = Early; 2 = Med Early; 3 = Late Early; 4 = Late; 5 = Very Late. Bars denote standard error of the mean.

In 2009, many regions in South East Tasmania experienced their wettest winter and spring on record. During the winter months, 664.4 mm fell at Snug Plains (approximately 3 km from

the Oyster Cove field site) and during the spring months, 491.4 mm was recorded (Australian Bureau of Meteorology). This extremely wet weather adversely impacted plant growth in the field trial. Seedling establishment was very slow, and many plants 'buttoned' shortly after transplanting (Figure 23a). Furthermore, this period of unusually high precipitation was attended by duck flocks of unprecedented size, which inflicted severe and extensive browsing damage before plants could be covered (Figure 23b). In many cases where seedlings were small, plants were entirely consumed, or otherwise never fully recovered, remaining grossly undersized throughout the entire observation period. In particular, the late-planted cauliflowers, which were smaller during the period of peak duck activity, were less able to recover from browsing damage and very few lines formed visible curds, meaning that results from this planting could not be used.

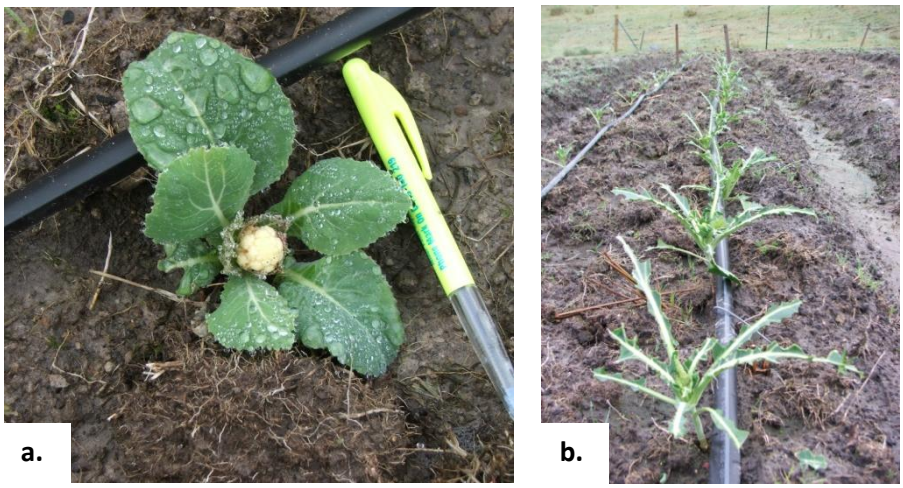


Fig. 23. Cauliflower line 37 exhibiting a 'buttoned' curd (a) and a typical example of damage done to a relatively advanced plant due to duck browsing (b).

Figure 24 shows the average number of days from sowing until visible curds were detected in field-grown cauliflowers. Once again, *BoFLC2* parent lines were, in general, slower to form curds than *boflc2* lines; 12 of the *BoFLC2* lines are ranked in the 'later' half of parent lines, compared to just five in the 'earlier' half. The average number of days to curd formation is significantly different (P=0.035) between the two genotypes (271.6 for *BoFLC2* and 257.2 for *boflc2*), but the difference between the two, and the level of significance is much less than in the previous trial, even when allowing for parent lines with unusual (romanesco or 'bunchy') flowering behaviour.

This may be partially explained by the fact that many of the *boflc2* lines were much less suited to the wet and cool field conditions and consequently, grew less vigorously and were slower to induce curds. A large majority of *boflc2* parent lines that formed curds later than expected (most notably 8, 29, 30, 34, 36, 43, 45, 46 and 48) comprised many dead or missing plants, and were typically very stunted and diseased due to both duck browsing and adverse weather, preventing them from developing curds as they normally would. Furthermore, several of the *BoFLC2* parent lines that formed curds unexpectedly early 'buttoned' (c.f. Figure 23) soon after transplanting. These two environmental/cultural effects obscured the effect of the *BoFLC2* gene.

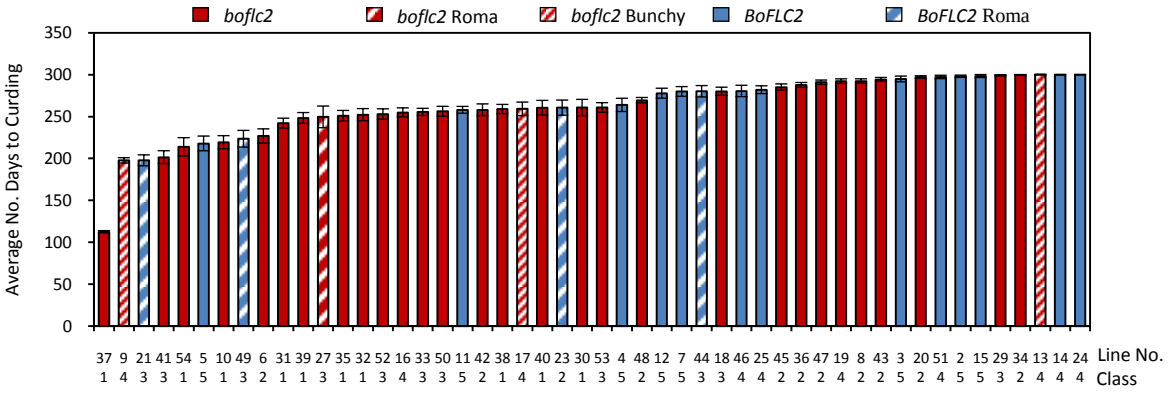


Fig. 24. Average number of days from sowing to visible curd formation in cauliflowers grown at the Oyster Cove field site. Values are the average of up to 30 early-sown plants (three replicates with 10 duplicates in each replicate), with dead or missing plants excluded from the analysis. Red and blue columns denote mutant (*boflc2*) and functional (*BoFLC2*) parent lines, respectively. Where plants did not initiate visible curds, they were assigned a maximum value of 300 days. Columns with thick stripes represent romanesco parent lines, and columns with thin stripes denote *boflc2* lines that formed a tightly-bunched 'head' of leaves prior to curd formation. The flowering class of each parent line (as described in Table 2) is shown beneath the line number, where class 1 = Early; 2 = Med Early; 3 = Late Early; 4 = Late; 5 = Very Late. Bars represent the standard error of the mean.

No significant difference (P=0.829) was observed in plant age at curd induction between *BoFLC2* and *boflc2* parent lines in late-sown cauliflowers grown at Daylesford, with both genotypes forming curds at an average of approximately 218 days (Figure 25a). Removal of curding data for romanesco and 'bunchy' parent lines separated the two groups somewhat (giving *BoFLC2* lines an average age at curd induction of 223.7 days, compared to 218.8 days for *boflc2* lines) and improved the P value (P=0.144); however, differences remained statistically insignificant. The cluster of *BoFLC2* parent lines, including lines 3, 25, 4, 12, 51,

15 and 7, is a noticeable feature of Figure 25a. Although differences in health and development between parent lines, and particularly between those with differing *BoFLC2* alleles, were far less pronounced than in the Oyster Cove trial, it was observed that once again, many of the *boflc2* lines that flowered later than the lines in this cluster had multiple dead or missing plants. This may indicate health problems due to incompatibility between parent line and environment, and may partially account for the later-than-expected (according to breeders' flowering classifications) curd induction in many *boflc2* lines. Similar results were found when ages at anthesis were compared between the *BoFLC2* and *boflc2* lines (data not shown).

No significant difference in anthesis age between *BoFLC2* and *boflc2* genotypes was identified in the small subset of early-sown parent lines (*BoFLC2* = 265.2 days; *boflc2* = 259.1 days; P = 0.100). However, removal of the 'bunchy' line 17 reduced the average anthesis age of *boflc2* lines to 256.4, representing a weakly significant (P=0.030) difference in flowering time. Apart from the incongruous positioning of parent line 24, plant age at anthesis increased with flowering class (Figure 25b). Plant age at curd induction could not be compared between genotypes because of insufficient data resolution during the period where curds were forming.

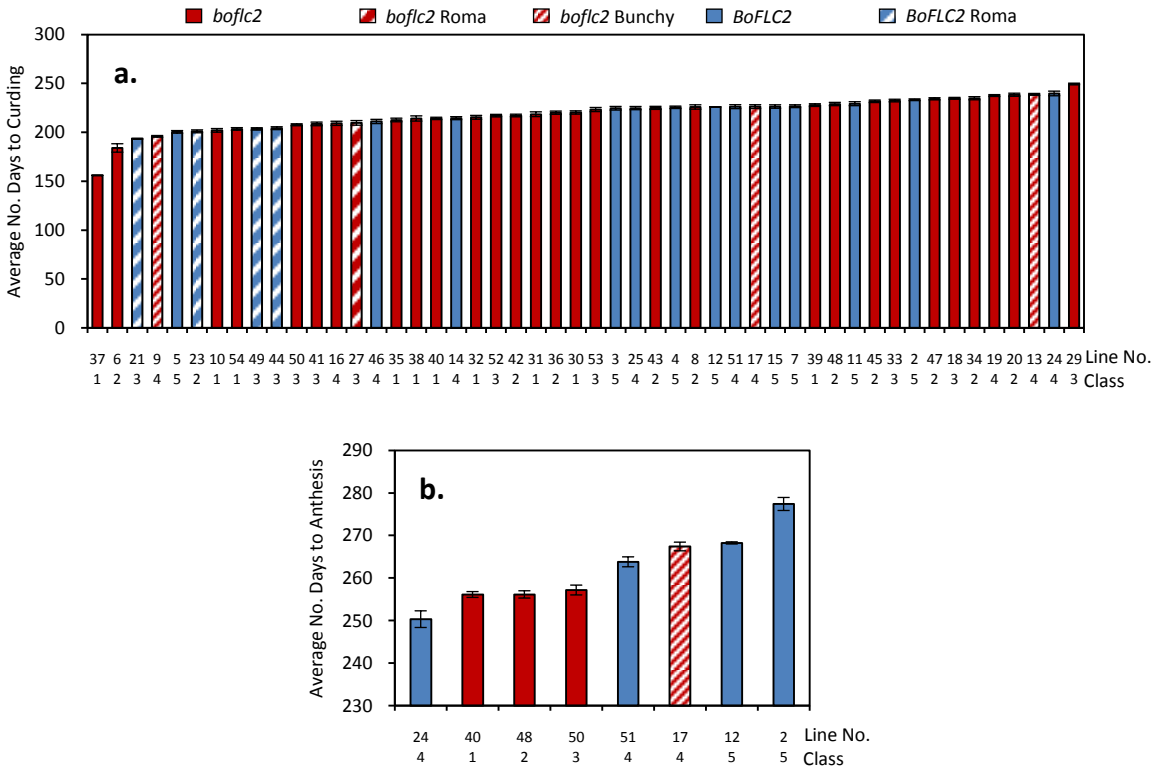


Fig. 25. Average number of days from sowing to visible curd formation in late-sown cauliflowers (a) and average number of days from sowing to anthesis (stage 6) in early-sown cauliflowers (b) grown at the Daylesford field site. Values are the average of up to 30 plants (three replicates with 10 duplicates in each replicate), with dead or missing plants excluded from the analysis. Red and blue columns denote mutant (*boflc2*) and functional (*BoFLC2*) parent lines, respectively. Columns with thick stripes represent romanesco parent lines, and columns with thin stripes denote *boflc2* lines that formed a tightly-bunched 'head' of leaves prior to curd formation. The flowering class of each parent line (as described in Table 2) is shown beneath the line number, where class 1 = Early; 2 = Med Early; 3 = Late Early; 4 = Late; 5 = Very Late.

D. Discussion

In the previous chapter, it was demonstrated for the first time that functionality of *BoFLC2* has an important influence on reproductive behaviour in segregating cauliflower populations, and that this allele was also associated with the flowering time categorisations of unrelated parent lines made by Rijk Zwaan pre-breeders. In this chapter, curd induction and flowering time data were collected from parent lines grown in a range of environments. Allowing for the vagaries associated with the production of cauliflowers in environments to which they were unsuited, these trials not only validated the Rijk Zwaan categories, but also enabled identification of the physiological basis for inconsistencies in flowering behaviour between the *BoFLC2* and *boflc2* genotypes and strengthened the case for *BoFLC2* as a fundamental determinant of flowering time. This information is foundational to subsequent experiments involving analysis of flowering time gene expression in a range of different parent lines.

'Romanesco' and 'bunchy' phenotypes were the two main curding variants observed among the 50 parent lines. The incongruous classifications of several parent lines by Rijk Zwaan pre-breeders (in view of *BoFLC2* genotype) may be explained by such unusual flowering behaviour which 'overrides', complicates, or disrupts the relatively simple *BoFLC2* effect. As noted, romanesco types were consistently amongst the earliest-curding *BoFLC2* lines. This may possibly be because the romanesco character is associated with some other unknown genetic factor that contributes to earlier flowering in spite of the *BoFLC2* functionality. This is the first study to show that romanesco varieties may possess both functional and non-functional *BoFLC2* alleles, and that within the romanesco group of cauliflowers, *BoFLC2* functionality does not significantly delay flowering. This apparent 'exception to the rule' is an important consideration for breeders seeking to employ the *BoFLC2* gene as a marker for late flowering in romanesco varieties.

Some 'bunchy' lines formed curds relatively early (e.g. Figures 24 and 25a), while in other situations, they tended to be ranked among the latest-curding and flowering *boflc2* lines (Figure 22a and 22b). It is possible this variation in ranking was simply due to limitations in the technique used to score curds; that is, timing of curd initiation may not have differed between trials, but a greater degree of 'bunching' (due to sub-optimal growth environments, for example) may have obscured curds for longer and resulted in a misleading record of curd

induction. Alternatively, this 'bunching' character may be associated with other genetic factors that cause a genuine delay in curd induction, regardless of *BoFLC2* genotype.

Despite the genetic variability responsible for such differences in reproductive physiology across these parent lines, a clear division in the timing of curd induction and flowering was observed between *BoFLC2* and *boflc2* genotypes in the glasshouse trial (Figures 22a and 22b). This emphasises the robustness of the *BoFLC2* effect on flowering time and gives further support to the idea that *BoFLC2* functionality is a major determinant of flowering time. The results from this glasshouse trial are largely supported by Rijk Zwaan breeders' classifications.

However, in less-controlled growth conditions, the effect of *BoFLC2* was obscured, and significant departures from expected reproductive behaviour were observed. The primary reason for this ambiguity appears to be the late curding of several *boflc2* parent lines. This unusually late curding is explained by the fact that many of these *boflc2* lines are adapted for growth in tropical environments, and the cold environments in which they were grown were unsuitable for their normal growth and development. Browsing damage placed additional pressure on the growth of lines in the Oyster Cove trial. Both *BoFLC2* and *boflc2* parent lines grew vigorously in the warm, stable environment of the pot-based glasshouse trial, but *BoFLC2* lines were slow to flower, reflecting their requirement for vernalisation and highlighting differences between the two genotypes. In the Daylesford and Oyster Cove trials, the colder, less-controlled conditions obscured the differences between genotypes not only by retarding growth of many typically early-flowering lines, and increasing variability between replicates, but also by satisfying the vernalisation requirements of both genotypes, and 'condensing' breadth of curd induction ages. Consequently, the results from the pot-based glasshouse trial appear to reflect the differences between *BoFLC2* and *boflc2* genotypes most reliably.

E. Conclusions

Having previously established the contribution of *BoFLC2* functionality to reproductive behaviour in segregating populations, this chapter reinforces the role of *BoFLC2* as a key determinant of flowering time by demonstrating a significant relationship between genotype and curding/flowering time in unrelated parent lines. A clear distinction in curding and flowering was seen between *BoFLC2* and *boflc2* lines in controlled, glasshouse conditions, but this was obscured by poor plant growth in less controlled trials. Romanesco forms and 'bunchy' leaf arrangements were identified as the physiological basis of anomalous flowering time classifications by pre-breeders. Despite considerable genetic variation between parent lines and diverse flowering behaviour, the *BoFLC2* allele stands out as a critical determinant of flowering time. Understanding the contribution of this gene to flowering time in our inbred parent lines is necessary for proper interpretation of gene expression results in the following chapter.

Chapter V

EXPRESSION DYNAMICS OF FLOWERING TIME CANDIDATE GENES

A. Introduction

In the preceding chapters, it was proposed that functionality of the *BoFLC2* gene was a significant determinant of both annual/biennial flowering behaviour in *Brassica oleracea*, and also of flowering time variation within the annual brassica cultivars. The identification of this strong genetic association with flowering time could potentially be helpful in accelerating commercial breeding programmes, whether as the target of genetic modification or as a marker for flowering time. However, while knowledge of the *BoFLC2* genotype allows broad predictions to be made concerning the timing of curd initiation and flowering, temperature fluctuations and seasonal variation during brassica seed crop production often affect the transition to reproductive development in unpredictable ways. A better understanding of the molecular basis of reproductive variability could potentially alleviate this problem by enabling the development of new ways of modelling, monitoring and managing progress towards flowering in a production environment.

The molecular basis of reproductive variability has been intensively studied in Arabidopsis, and a detailed model of flowering pathways has emerged (see Figure 2 for an overview). *FLC* plays a critical role in Arabidopsis autonomous and vernalisation pathways which 'enable' flowering to occur. It represses flowering by downregulating floral integrator genes, the most notable of which is *FT* (Michaels et al. 2005). Prolonged exposure to cold temperatures induces the expression of genes such as *VIN3,* which is involved in structural changes to *FLC* chromatin that inhibit its expression, enabling flowering to occur (Sung & Amasino 2004b). The transcriptional dynamics of such flowering genes in response to both vernalisation and photoperiod treatments have been investigated thoroughly in both rapid-cycling and winter-annual Arabidopsis varieties under laboratory conditions and recently,

Aikawa et al. (2010) examined the expression of flowering genes (including *FLC*) over a two-year period in a population of perennial *Arabidopsis halleri* plants grown outdoors under fluctuating natural environmental conditions, showing that that seasonal trends of temperature were detected at the level of *FLC* regulation.

However, there has been comparatively little research into the influence of variable environmental conditions on gene expression in brassica crop species. Only a small number of flowering genes have been identified, and their transcriptional responses to environmental signals have been tested under a very limited range of conditions. Consequently, there remains much to be understood about the molecular regulation of flowering in brassica, in terms of both the identity of the genes involved, and in terms of defining transcriptional responses to environmental variability that lead to altered curd initiation and flowering time. The following brief review of the recent history of research on brassica flowering gene regulation summarises the present state of affairs.

Most research relating to the molecular regulation of brassica curd initiation research has focussed on members of the *FLC* family. Tadege et al. (2001) were the first to report a conserved role for *FLC* genes in brassica with the finding that 35S-driven expression of each of the five *B. napus FLC* sequences in transformed Arabidopsis delayed flowering. *BnFLC* mRNA transcript levels in three-week-old *B. napus* shoot tips were observed to be correlated with late flowering behaviour and responsiveness to vernalisation, and the *BnFLC* transcript levels of all five genes were substantially reduced following ten weeks of vernalisation at 5°C when two weeks old. Each of the five *B. napus* genes were expressed differentially in Arabidopsis leaf, apex and stem tissue, but were not expressed at all in root tissue.

Subsequent research by Li et al. (2005) and Kim et al. (2007) demonstrated a correlation between vernalisation, *FLC* expression and flowering in *B. rapa* ssp. *pekinensis*. Seeds vernalised at 1-2°C reduced *BrpFLC* expression and went on to flower faster; this effect was reduced in less-responsive anti-bolting varieties (Li et al. 2005) and overall *BrpFLC* expression was lower in early-flowering cultivars (Kim et al. 2007). Zhao et al. (2010b) recently identified *BrFLC2* as a candidate gene for a vernalisation response QTL in pak choi (*B. rapa*), showing that it was downregulated by vernalisation of seedlings of both late- and early-flowering cultivars, with this effect diminished when vernalisation was applied to older plants.

Lin et al. (2005) isolated two *FLC* homologues designated *BoFLC3-2* and *BoFLC4-1* (equivalent to the *BoFLC2* gene which formed the subject of the Chapter III) in three cabbage (*B. oleracea* var. c*apitata*) varieties with a range of flowering habits. *BoFLC3* transcript was not detected in any unvernalised tissues at any stage of growth. *BoFLC4* transcript levels were low in unvernalised seedling apices, increasing with plant age to similar levels for all three flowering types. Vernalisation at 4°C for 2-6 weeks of eight-week-old plants downregulated *BoFLC4* in apex and leaf tissue, with the effect more rapid and responsive in the earlier-flowering variety; sequence data indicates that the later-flowering cabbage carried functional *BoFLC2*, but no data were provided as to which allele the relatively early-flowering variety possessed. In contrast to both Arabidopsis and *B. rapa*, vernalisation of cabbage seed had no effect on flowering time or *BoFLC4* expression. Okazaki et al. (2007) found no difference in expression levels of *BoFLC2* between unvernalised *boflc2* broccoli and unvernalised *BoFLC2* cabbage leaves and apices, and also reported strong expression of *BoFLC3* in both varieties; however, no investigation of *BoFLC* response to vernalisation treatments was made.

Apart from members of the *BoFLC* family, the brassica flowering genes to have received the most attention in terms of molecular characterisation are those relating to curd proliferation, developmental arrest and flower initiation (e.g. Anthony, James & Jordan 1996; Carr & Irish 1997; Duclos & Björkman 2008). Upstream of these MIGs, the only other gene whose expression has been studied is *BoFT* which was isolated from a late-flowering cabbage (Lin et al. 2005). In contrast to *AtFT*, which is principally expressed in cotyledons and leaves (Abe et al. 2005), expression of *BoFT* was high in apices of young seedlings before decreasing with plant age. Although it was upregulated by vernalisation at eight weeks of age in shoot apices, it was unaffected by seed vernalisation, in contrast to *AtFT*. Expression in leaves was unaffected by vernalisation. Homologues of several other important genes further upstream in the Arabidopsis vernalisation pathway, such as *VIN3*, remain unidentified in brassica crops.

These findings indicate that although the basic regulation and apparent function of *FLC* and *FT* are broadly conserved in members of the Brassicaceae, there is considerable variability in the details of expression responses to vernalisation, even between closely-related members. As such, it is reasonable to suggest that there are significant differences in flowering gene expression behaviour between cabbage and cauliflower, given the wide differences in

flowering behaviour between these members of the *B. oleracea* complex. The two crops are grown for completely different organs – one vegetative, and the other reproductive. As a result, breeding of the two sub-species has involved selection for late flowering and bolting-resistance in 'biennial' plants such as cabbage and more modest vernalisation requirements in members of 'annual' *B. oleracea* such as cauliflower. Furthermore, the wide differences in flowering time *within* the cauliflower group (for example, between *BoFLC2* and *boflc2* strains), also suggest differences in gene regulation within the *B. oleracea* crops grown as annuals.

Although *BoFLC2*, *BoFLC3* and *BoFT* have been isolated in cabbage and the expression response of cabbage *BoFLC2* and *BoFT* to vernalisation have been studied with a limited degree of resolution (Lin et al. 2005), a detailed investigation of *BoFLC* and *BoFT* expression dynamics has never been undertaken in cauliflower. Such an investigation carried out in a range of cauliflower flowering types would help to establish the role of these genes in flowering time variability and would lay the foundations for practical methods of predicting flowering time based on gene expression data. It would also offer the opportunity to study the molecular basis of features such as juvenility that are not well-represented in Arabidopsis or other members of the Brassicaceae such as *B. rapa.* In *B. oleracea,* strains with both long and short juvenile phases exist, with most commercial cauliflower and broccoli cultivars selected for a longer juvenile phase to help reduce losses from premature inflorescence initiation (buttoning) in response to low field temperatures encountered after transplanting in the field (Friend 1985).

Of particular interest are questions relating to the timing, location and overall level of gene expression between different flowering types, especially between *BoFLC2* and *boflc2* lines. The responsiveness of gene expression changes to different vernalisation treatments is also worthy of investigation. Furthermore, previous studies of gene expression dynamics in *B. oleracea* have been 'low-resolution', with semi-quantitative RNA gel-blotting or gel-based RT-PCR methods used to measure mRNA transcript levels at infrequent time points. A RT-qPCR approach would allow high-resolution profiling of expression dynamics in response to different vernalisation treatments to be generated, thereby enabling changes that occur over a short space of time to be determined.

In this chapter, four gene expression studies are reported, and a picture of the molecular regulation of curd induction by vernalisation is developed. Key flowering genes *BoFLC2*, *BoFLC3* and *BoFT* are isolated, and their transcriptional response to vernalisation is investigated. Furthermore, a homologue of *VIN3*, the important repressor of Arabidopsis *FLC*, is isolated and characterised for the first time in a brassica crop species. In the first experiment, the effects of vernalisation length on gene expression in shoot apex and leaf tissue are investigated in a late-flowering variety. In the second experiment, the effect of plant age when vernalised is studied, and in the third, vernalisation temperature effects are examined in late- and early-flowering varieties. Finally, these different vernalisation treatments are examined in a range of flowering types with differing *BoFLC2* functionality. By evaluating the transcriptional responses of these key flowering genes to multiple vernalisation treatments in highly-controlled conditions, the groundwork is laid for analysis of gene expression under 'noisy', natural environments in Chapter VII.

B. Materials & Methods

1. Gene Isolation & Expression Analysis

BoFLC2 and *BoFLC3* primers were designed directly from existing National Center for Biotechnology Information (NCBI) reference sequences <http://www.ncbi.nlm.nih.gov>. *FT* primers were designed from the Arabidopsis *FT* sequence, and subsequently refined with reference to our *BoFT* sequence. The BLAST programme was used to identify similar sequences to Arabidopsis *VIN3* cDNA on the Biological Sciences Research Council (BBSRC) Brassica Database <http://www.brassica.bbsrc.ac.uk>. A 581 bp *B. rapa* sequence with 80% homology to the *A. thaliana* sequence was identified. The Arabidopsis and brassica sequences were aligned, and primers were designed in conserved regions, with subsequent refinements based on our *BoVIN3* sequence. Several genes known to be expressed constitutively were also selected for use as 'housekeeping genes' in RT-qPCR. The BLAST programme was used to identify NCBI *B. oleracea* sequences with homology to Arabidopsis genes *ACTIN*, *UBIQUITIN* and *HISTONE1* and primers were designed in conserved regions. Gene fragments from cauliflower shoot apices and young leaf cDNA were amplified, ligated into plasmids, cloned and sequenced. Plasmids containing fragments of the gene of interest were serially diluted for use as a standard curve in the RT-qPCR, except in the case of *BoVIN3*, where standard curves were made by serially diluting cDNA fragments. Primer details are listed in Table 8. The final primer combinations used for all subsequent RT-qPCRs are shown in Table 9.

Table 8. Forward and reverse primers used for gene isolation and RT-qPCR. Reference sequence refers to the sequence used to design the primers (GenBank accession number and reference).

PRIMER	SEQUENCE	REFERENCE SEQUENCE
***BoACT* Primers**		
BoACT-1F	5'- ATGGCTGAGGCTGATGACATTC -3'	AF044573 (Yu, Parthasarathy & Nasrallah 1998)
BoACT-2F	5'- GAGATTCAGGTGTCCAGAGG -3'	AF044573 (Yu, Parthasarathy & Nasrallah 1998)
BoACT-1R	5'- CCCTGAGACAGCTTAGAAGC -3'	AF044573 (Yu, Parthasarathy & Nasrallah 1998)
BoACT-2R	5'- GGCTGGAACAAGACCTCTGG -3'	AF044573 (Yu, Parthasarathy & Nasrallah 1998)
BoACT-LLF	5'- CCGAGAGAGGTTACATGTTCACCAC -3'	Dr. Li Li (Pers. Comm; 2005)
BoACT-LLR	5'- GCTGTGATCTCTTTGCTCATACGGTC -3'	Dr. Li Li (Pers. Comm; 2005)
***BoHIS* Primers**		
BoHIS-1F	5'- GCCCTTACGCTATAGCAAAGC -3'	BH008415 (Katari et al. 2001)
BoHIS-1R	5'- AGGCTCTGATCTTCACGAGC -3'	BH008415 (Katari et al. 2001)
***BoFLC2* Primers**		
FLC41-5'F	5'- CAAATTAGGGCACAAAGGGT -3'	AY306124 (Lin et al. 2005)
FLC41-3'R	5'- CACAAGTTTTGGACTTTAAGG -3'	AY306124 (Lin et al. 2005)
FLC41-E3F	5'- CGAAGTATGGTTCACACCATGAGC -3'	AY306124 (Lin et al. 2005)

(continued overleaf)

Table 8. *(continued)*

PRIMER	SEQUENCE	REFERENCE SEQUENCE
FLC41-E4R	5'- CGGAGAGGGCAGTCTCAAGGTGGTT -3'	AY306124 (Lin et al. 2005)
***BoFLC3* Primers**		
FLC32-5'F	5'- CAAATTAGGGCACAGGGACC -3'	AY306125 (Lin et al. 2005)
FLC32-3'R	5'- TCAGCCCCGTCTAAAGGTGG -3'	AY306125 (Lin et al. 2005)
FLC32-E1F	5'- CCTCCTCCGGAAAGCTCTACAGC -3'	AY306125 (Lin et al. 2005)
FLC32-E4R	5'- TGTCCACGCTTACACCAACGAC -3'	AY306125 (Lin et al. 2005)
***BoFT* Primers**		
AtFT-F1a-seq	5'- TCAACACAGAGAAACCACCTG -3'	AF152096 (Kardailsky et al. 1999)
AtFT-F2a-seq	5'- GAGAAACCACCTGTTTGTTCAAG -3'	AF152096 (Kardailsky et al. 1999)
AtFT-R3a-seq	5'- CATCACCGTTCGTTACTCG -3'	AF152096 (Kardailsky et al. 1999)
AtFT-F3b-qPCR	5'- GCCAAAGAGAGGTGACTAATGG -3'	AF152096 (Kardailsky et al. 1999)
AtFT-F4b-qPCR	5'- TTGGATCTAAGGCCTTCTCAGG -3'	AF152096 (Kardailsky et al. 1999)
AtFT-R1b-qPCR	5'- CCAACCAATGGAGATATTCTCGG -3'	AF152096 (Kardailsky et al. 1999)
AtFT-F5c-qPCR	5'- GATATCCCTGCTACAACTGG -3'	Primer from (Tseng et al. 2004)
AtFT-R2c-qPCR	5'- AAACTCGCGAGTGTTGAAGTTC -3'	Primer from (Yamaguchi et al. 2005)
BoFT-F3b-qPCR	5'- GCCAAAGAGAGGTGACAAATGG -3'	Appendix I
BoFT-R1b-qPCR	5'- CCAACCAATGGAGATATTCTCGT -3'	Appendix I
***BoUBQ* Primers**		
BoUBQ-1F	5'- CACCATCGACAATGTCAAGG -3'	BH677406 (Ayele et al. 2005)
BoUBQ-2F	5'- CCCTTCATCTTGTGTTGAGG -3'	BH677406 (Ayele et al. 2005)
BoUBQ-2R	5'- GCCAACGCCATCAAAGAAGG -3'	BH677406 (Ayele et al. 2005)
BoUBQ-3R	5'- CCTGCAGTTCACAGCCCTCG -3'	BH677406 (Ayele et al. 2005)
***BoVIN3* Primers**		
VR-BoVIN3-F1	5'- CTTTTCCCTTTGCAGGTGTC -3'	ES5935031 (The Brassica Database)
VR-BoVIN3-F2	5'- GGTGTCGTGTGCACTGAGTT -3'	ES5935031 (The Brassica Database)
VR-BoVIN3-R1	5'- AAGCTTCTTCTGGCTGCTTG -3'	ES5935031 (The Brassica Database)
VR-BoVIN3-R2	5'- CGGCTCCACGAGTTTAAAAG -3'	ES5935031 (The Brassica Database)
AtVIN3-F2	5'- AGATAATGAATGCATTGAG -3'	NM_125121 (Unpublished)
AtVIN3-F3	5'- AAGCTGCTTCGCTCTCAAAG -3'	NM_125121 (Unpublished)
AtVIN3-R3	5'- CAATCCCATACCTATCTTGC -3'	NM_125121 (Unpublished)
AtVIN3-R4	5'- CTTGCAGGGTGTTACAAGCA -3'	NM_125121 (Unpublished)
AtVIN3-R5	5'- CCCAGCTTCATCTCCATCAT -3'	NM_125121 (Unpublished)
AtVIN3-R6	5'- AGAGCTTCCATTGCCTGAGA -3'	NM_125121 (Unpublished)
AtVIN3-R7	5'- TGCGGCATTATTGATCTCAG -3'	NM_125121 (Unpublished)
BoVIN3-F4	5'- TAGTGATCTTCTCGGATGCTG -3'	Appendix III
BoVIN3-F5	5'- GCGCTCGGAAGTGAAGATAC -3'	Appendix III
BoVIN3-R8	5'- GCCTCATCCATGAGTTCCAA -3'	Appendix III

Table 9. Primer combinations and annealing temperatures used in RT-qPCRs.

GENE	PRIMERS	T_m (°C)
BoACT	BoACT-2F/BoACT-LLR	60
BoFLC2	FLC41-E3F/FLC41-E4R	58
BoFLC3	FLC32-E1F/FLC32-E4R	58
BoFT	AtFT-F3b-qPCR/ BoFT-R1b-qPCR	58
BoHIS	BoHIS-1F/BoHIS-1R	58
BoUBQ	BoUBQ-1F/ BoUBQ-2R	60
BoVIN3	BoVIN3-F4/ BoVIN3-R8	56

BoVIN3 was initially isolated as part of a series of devernalisation studies, of which the experiment in Chapter VI was a part (see also Rajandran 2008). These studies were

conducted prior to the experiments in this chapter. The isolation of *BoVIN3* was undertaken with the assistance of an honours student; see Rajandran (2008) for supplementary details.

2. Vernalisation Experiments

In the first three vernalisation experiments described here (a-c), seeds were sown directly into 2 L pots containing general purpose potting mix topped with a thin layer of seed raising mix. All plants were grown in controlled environment growth cabinets under a mixture of 36 W cool white fluorescent lamps and 60 W incandescent lamps with an output of 100 µM m^{-2} s^{-1} as part of a 16 hour photoperiod regime.

a. Experiment 1: vernalisation duration

Seeds of cauliflower parent line 1 (a romanesco *BoFLC2* variety classed as 'late' flowering) were sown every 14 days, over the course of 16 weeks and grown in 22°C growth cabinets. As plants reached ten weeks of age, 5-6 replicates were transferred to 5°C growth cabinets for two, four and six week vernalisation treatments, while 5-6 replicates remained in non-vernalising conditions. At the conclusion of the six week vernalisation treatment, leaf and shoot apex samples were harvested for RNA extraction from three replicates of unvernalised plants aged 2, 4, 6, 10, 12, 14 and 16 weeks, and from the 12-, 14- and 16-week-old plants that had received two, four and six weeks of vernalisation, respectively. Three replicates of both vernalised and unvernalised 16-week-old plants and two replicates of each of the 12- and 14-week-old vernalised and unvernalised plants were transferred to a glasshouse environment for weekly observations of curd development.

b. Experiment 2: plant age when vernalised

Parent line 1 cauliflower seeds were sown every week and grown at in 22°C growth cabinets. In the 11th week, three replicates of plants aged one to ten weeks were transferred to 5°C growth cabinets, while three replicates of plants aged one to ten weeks remained at 22°C. In the 13th week, three replicates of plants aged one to 12 weeks were transferred to 5°C growth cabinets, while three replicates of plants aged one to 12 weeks remained at 22°C. In the 14th week, leaf and apex tissue of three replicates of unvernalised plants (UV; aged 1-13 weeks), three replicates of plants that had been vernalised for one week (1V; aged 2-13 weeks) and three replicates of plants that had been vernalised for three weeks (3V; aged 4-13 weeks)

were harvested for RNA extraction. Because plant growth largely ceased at 5°C, expression data was plotted against 'plant physiological age', where time spent at 5°C was disregarded (for example, a five-week-old plant that had been vernalised for three weeks when two weeks of age was considered to have a physiological age of two weeks). Plants harvested at a physiological age of one week did not possess any true leaves, so samples comprising cotyledon/apex tissue were harvested instead, as with Lin et al. (2005).

c. Experiment 3: vernalisation temperature

Supplies of line 1 seeds became unexpectedly unavailable, preventing its physiological and molecular responses to vernalisation from being fully characterised. Line 51 (a 'late'-flowering *BoFLC2* variety) was duly selected as a replacement. Line 40 (an 'early'-flowering *boflc2* variety) plants were also grown to allow comparison of gene expression behaviour between early- and late-flowering varieties. Every 14 days, over the course of 16 weeks, seeds from these two varieties were sown and raised in 22°C growth cabinets. As plants reached ten weeks of age, three replicates from each line were transferred to 5°C, 10°C and 15°C growth cabinets for two, four and six week vernalisation treatments, while three replicates remained in non-vernalising conditions. At the conclusion of the six week vernalisation treatment, leaf and shoot apex samples were harvested for RNA extraction from three replicates of unvernalised plants aged 2, 4, 6, 10, 12, 14 and 16 weeks, and from the 12-, 14- and 16-week-old plants that had received two, four and six weeks of vernalisation, respectively.

d. Experiment 4: effects of vernalisation on visible curd formation & gene expression in late- & early-flowering genotypes

Plants from eight different cauliflower parent lines were sown into trays with 25 mm cells containing general purpose potting mix (line details provided in Table 10). Seedlings were raised in 22°C controlled environment growth cabinets, and after three weeks, were transferred to 2 L pots containing general purpose potting mix, and randomly allocated between two identical controlled environment cabinets maintained at a constant 22°C. Plants ranging from three to ten weeks of age were transferred to 5°C, 10°C and 15°C growth cabinets for periods ranging from 2-35 days. These treatments, and the notation used to describe each treatment, are summarised in Table 11.

At the conclusion of each treatment, leaf and shoot apex tissue was harvested from three replicates (excluding the control) and four replicates from every vernalisation treatment were returned to UV conditions for observation of flowering behaviour. Due to space constraints, sowings were staggered so that treatments 3-UV, 3-35V, 6-UV and 6-35V were planted 10 weeks after the main planting of treatments 10-UV, 10-2V, 10-7V, 10-21V, 10-35V10, 10-35V15 and 15-UV. UV controls were planted 16 weeks after the main planting. Scoring of leaf number and curd formation lasted 33 weeks for the treatments in the main planting, 32 weeks for the controls, and 38 weeks for the 3-UV, 3-35V, 6-UV and 6-35V treatments. Growing degree-days (GDD) were calculated using a heuristic, standard formula (e.g. McMaster & Wilhelm 1997), modified to allow for the fact that both UV and V temperatures were constant (i.e. with no day/night or max/min temperature):

$$T_{DAILY} - T_{BASE}$$

Where T_{DAILY} = growth cabinet temperature for a 24 hr period and T_{BASE} = base temperature at which cauliflower growth had been observed to cease; in this case, 4°C. In some situations, the 'plant physiological age' concept, as described previously, was employed.

Table 10. Cauliflower inbred, homozygous parent lines used in Experiment 4. Flowering class refers to the categories designated by Rijk Zwaan breeders. '*BoFLC2* alleles' indicates whether the line was homozygous for the functional *BoFLC2* allele or the mutant *boflc2* allele, as determined by genotyping in Chapter III.

CAULIFLOWER LINE	FLOWERING CLASS	*BoFLC2* ALLELES
2	Very Late	*BoFLC2*
12	Very Late	*BoFLC2*
17	Late	*boflc2*
24	Late	*BoFLC2*
40	Early	*boflc2*
48	Med Early	*boflc2*
50	Med Late	*boflc2*
51	Late	*BoFLC2*

Table 11. Summary of Experiment 4 vernalisation treatments. Four plants were maintained in non-vernalising conditions (22°C) for the duration of the experiment, serving as unvernalised controls. For all other treatments, the initial digit in the treatment name indicates the age (weeks) at which plants were vernalised and the following digits indicate the duration (days) of the vernalisation treatment (or 'UV' if plants were unvernalised). Vernalisation temperature was 5°C in all treatments except two; for these 10°C and 15°C treatments, the vernalisation temperature is also included in the treatment name.

TREATMENT	AGE WHEN VERN.	VERN. TEMP	AGE WHEN SAMPLED
UV (control)	N/A	N/A	N/A
3-UV	N/A	N/A	3 weeks
3-35V	3 weeks	5°C	8 weeks
6-UV	N/A	N/A	6 weeks
6-35V	6 weeks	5°C	11 weeks
10-UV	N/A	N/A	10 weeks
10-2V	10 weeks	5°C	10 weeks and 2 days
10-7V	10 weeks	5°C	11 weeks
10-21V	10 weeks	5°C	13 weeks
10-35V	10 weeks	5°C	15 weeks
10-35V10	10 weeks	10°C	15 weeks
10-35V15	10 weeks	15°C	15 weeks
15-UV	N/A	N/A	15 weeks

C. Results

1. Flowering Gene Isolation

At the time of gene isolation, no sequence information for *BoFT* was available on NCBI. Subsequent to isolation and sequencing, several *B. oleracea FT* sequences with 98% homology to the *BoFT* described here were added (e.g. HM030997, HM030998). An alignment of the translated sequences of these genes, along with Arabidopsis *FT* and *TSF* is presented in Figure 26, and the annotated gDNA sequence of *BoFT* is included in Appendix I. An 831 bp contig of *BoVIN3* cDNA was constructed from *BoVIN3* PCR sequences. The 277 amino acids encoded by this cDNA showed the highest sequence similarity to the uncharacterised translated *B. rapa* sequence that was used when designing primers. The putative *B. oleracea* VIN3 polypeptide also showed a high degree of similarity to the VIN3 protein NP_200548, with both containing the containing the DUF1423 conserved domain. A 67 bp intron corresponding to intron 3 in the Arabidopsis sequence was also sequenced (not shown). Figure 27 shows an alignment of *BoVIN3, AtVIN3* and *BrVIN3* translated sequences, and the partial cDNA sequence of *BoVIN3* is included in Appendix III. Figure 28 is a schematic diagram of Arabidopsis *VIN3* gene structure, including the location of primers that were used for the isolation of the *B. oleracea* homologue. Sequences of *BoFLC* and reference genes had already been published (see Table 8 for references) and are not presented here.

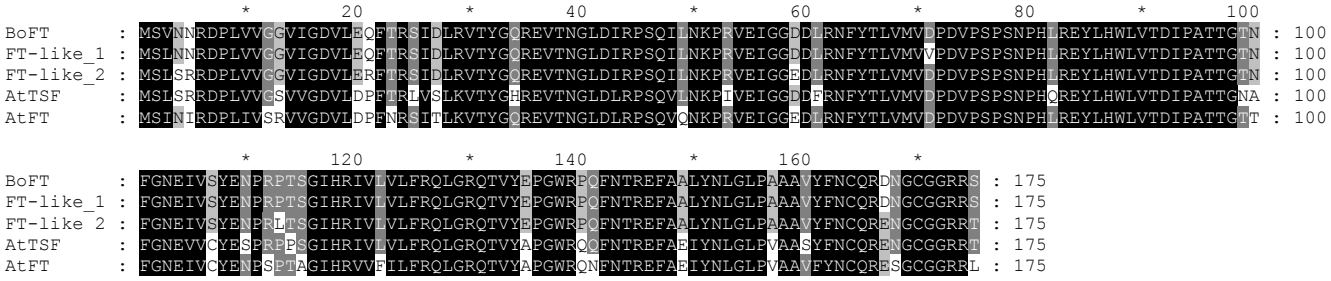


Fig. 26. Alignment of *FT* translated sequences. *BoFT* denotes the sequence generated in this study. *FT-like 1* and *FT-like 2* (HM030997 and HM030998) are two similar sequences isolated in *B. oleracea*. *AtTSF* and *AtFT* (NM_118156 and AF152096) are provided for comparison.

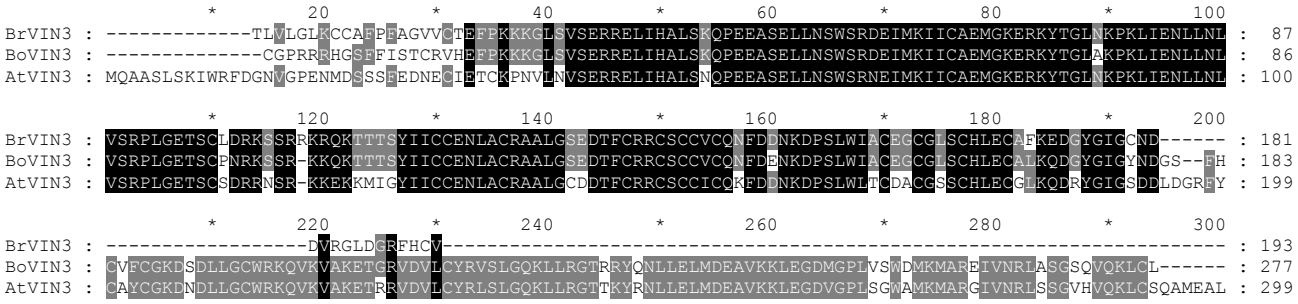


Fig. 27. **Alignment of *VIN3* translated sequences.** *BrVIN3* denotes the uncharacterised *B. rapa* sequence ES5935031, *BoVIN3* is the sequence isolated in this study, and *AtVIN3* is partial Arabidopsis sequence NP_200548.

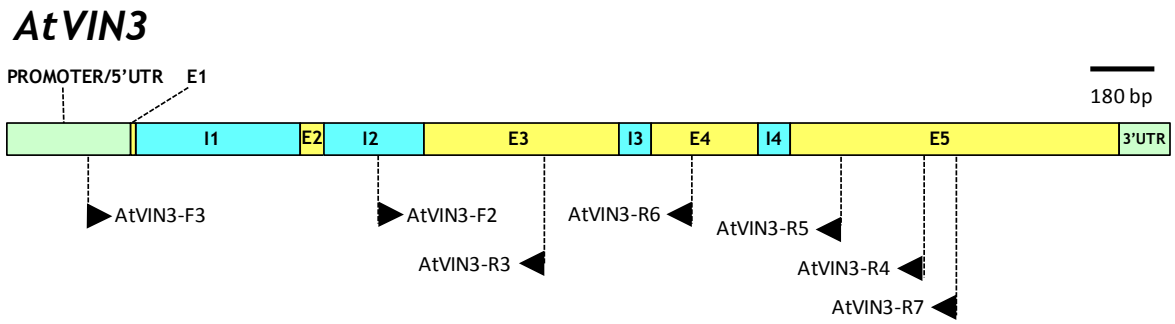


Fig. 28. **Gene structure of Arabidopsis *VIN3*.** The location of primers that were designed from Arabidopsis sequence NM_125121 are displayed. Gene structure is based on TAIR sequence AT5G57380 <http://www.arabidopsis.org>.

2. Vernalisation Experiments

a. Experiment 1: vernalisation duration

A direct relationship between vernalisation duration and curd initiation/development was observed, with longer periods of vernalisation associated with faster curding and curd development upon return to glasshouse conditions (Figure 29). Cauliflowers that had been exposed to two weeks of vernalisation at 5°C when ten weeks of age did not initiate curds for six weeks after being moved into the glasshouse (i.e. an average physiological age of 16 weeks). By contrast, plants that received four weeks of vernalisation initiated curds at an average physiological age of 14 weeks, and those that received six weeks of vernalisation formed curds at an average physiological age of just 11 weeks (one week after being returned to glasshouse conditions). Unvernalised plants did not form curds during the 19-week post-vernalisation period. Differences in age at visible curd formation between all three

vernalisation treatments were statistically significant ($P<0.05$). Corresponding differences in curd development were also noted; at a physiological age of 16 weeks, curds that had been exposed to six weeks of vernalisation were in full flower; those that had received four weeks of vernalisation were only just elongating; plants that had been vernalised for two weeks had very small curds (Figure 30).

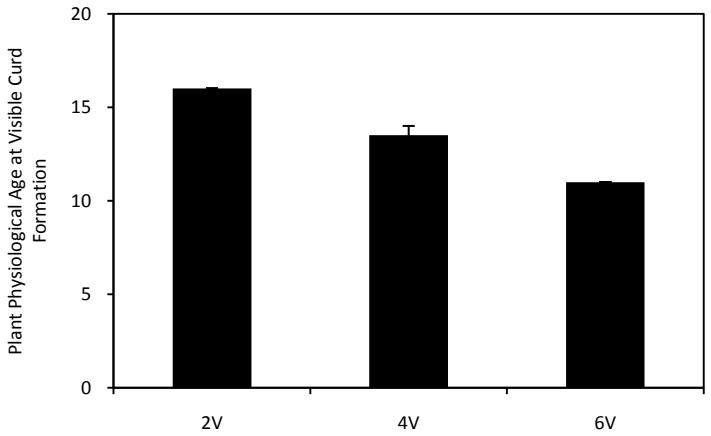


Fig. 29. Average physiological age of cauliflower plants at visible curd formation. 2V, 4V and 6V denote two, four and six week vernalisation treatments. 2V and 4V figures represent the average of two replicates, while 6V figures represent the average of three replicates. Unvernalised plants did not flower.

Fig. 30. Effect of vernalisation duration on curd development six weeks after transfer from vernalising conditions to the glasshouse (a physiological age of 16 weeks). Part (a) shows the very small curd visible in plants that had been vernalised for two weeks. Part (b) shows a plant vernalised for four weeks with a curd in the early stages of elongation. Part (c) shows an unvernalised plant (UV) compared to two plants that had been vernalised for six weeks.

Transcript levels of both *BoFLC2* and *BoFLC3* were dramatically reduced by two weeks of vernalisation (Figure 31). Continued vernalisation reduced expression further still, but subsequent reductions in transcript were small compared to the initial decrease. *BoFLC2* was expressed at a much lower overall level than *BoFLC3.* In apices, *BoFLC2* and *BoFLC3* expression increased slowly, but steadily throughout early plant growth, up until ten weeks of age. This gradual increase was less evident in leaf tissue. A sharp reduction in *BoFLC3* transcript of 16-week-old unvernalised plants was noted in leaves, but not apices. Apart from these two minor differences, expression of the two *BoFLC* genes was similar in shoot apex and leaf tissue. *BoFT* was upregulated by vernalisation. In leaf tissue, the initial upregulation after two weeks of vernalisation was small, with the response increasing

substantially in proportion to the length of vernalisation treatment. In apex tissue, the upregulation response to vernalisation length was more uniform. Leaf *BoVIN3* was upregulated by two and four weeks of vernalisation in some replicates, but there was considerable variation in this response and increases were not statistically significant ($P>0.05$). Apex *BoVIN3* was similarly upregulated by vernalisation with the effect decreasing with longer periods of vernalisation but once again, this was not statistically significant ($P>0.05$).

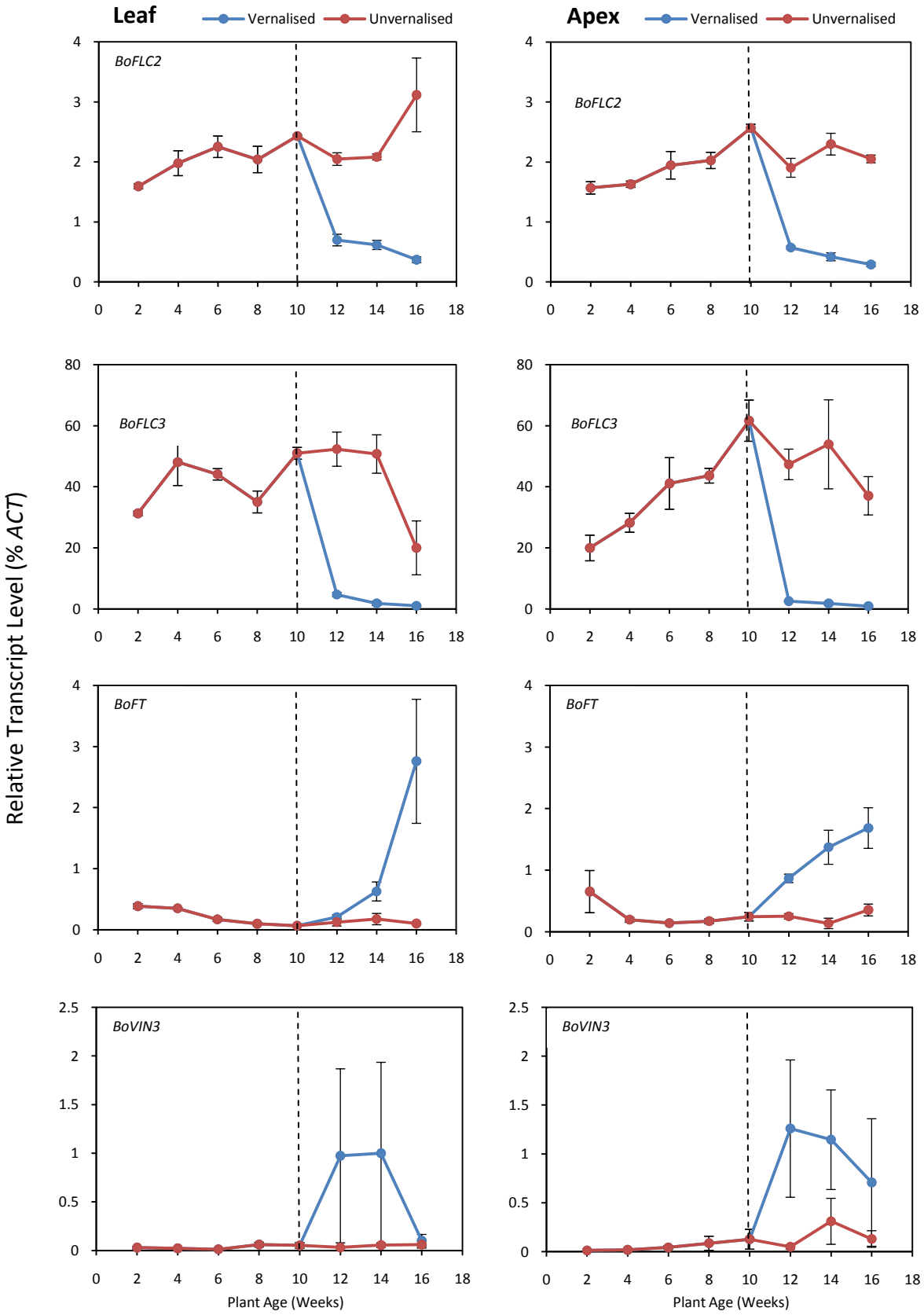


Fig. 31. Average *BoFLC2*, *BoFLC3*, *BoFT* and *BoVIN3* expression in shoot apex and leaf tissue of *BoFLC2* parent line 1 following vernalisation at ten weeks of age. The timing of vernalisation is indicated by a dashed line. Bars represent the standard error of the mean (n=3).

b. Experiment 2: plant age when vernalised

Transcript levels of both *BoFLC2* and *BoFLC3* were reduced by vernalisation independently of plant age (Figure 32). As in the previous experiment, the degree of downregulation was proportional to the length of vernalisation. *BoFLC2* expression rose steadily throughout plant growth, whereas *BoFLC3* expression was largely static throughout the course of this experiment. During the first two weeks of growth, *BoFT* expression was relatively high in unvernalised plants, and *BoFT* transcript levels were lower in vernalised one- and two-week-old seedlings than in unvernalised one- and two-week-old seedlings. By the third week of growth, *BoFT* expression in unvernalised plants was very low, with expression upregulated in vernalised plants proportionally to the length of vernalisation. A substantial increase in the level of *BoFT* upregulation by three weeks of vernalisation was seen in plants vernalised at four weeks of age, and after this point, the degree of *BoFT* upregulation was generally independent of plant age at vernalisation, apart from a sharp decrease in the level of *BoFT* upregulation by three weeks of vernalisation in plants vernalised at eight weeks of age. *BoVIN3* expression gradually increased with plant age in unvernalised plants. Vernalisation upregulated *BoVIN3* mRNA transcript relative to levels in unvernalised plants, regardless of plant age at vernalisation. There was little difference in uprgulated *BoVIN3* expression levels between plants vernalised for one and three weeks.

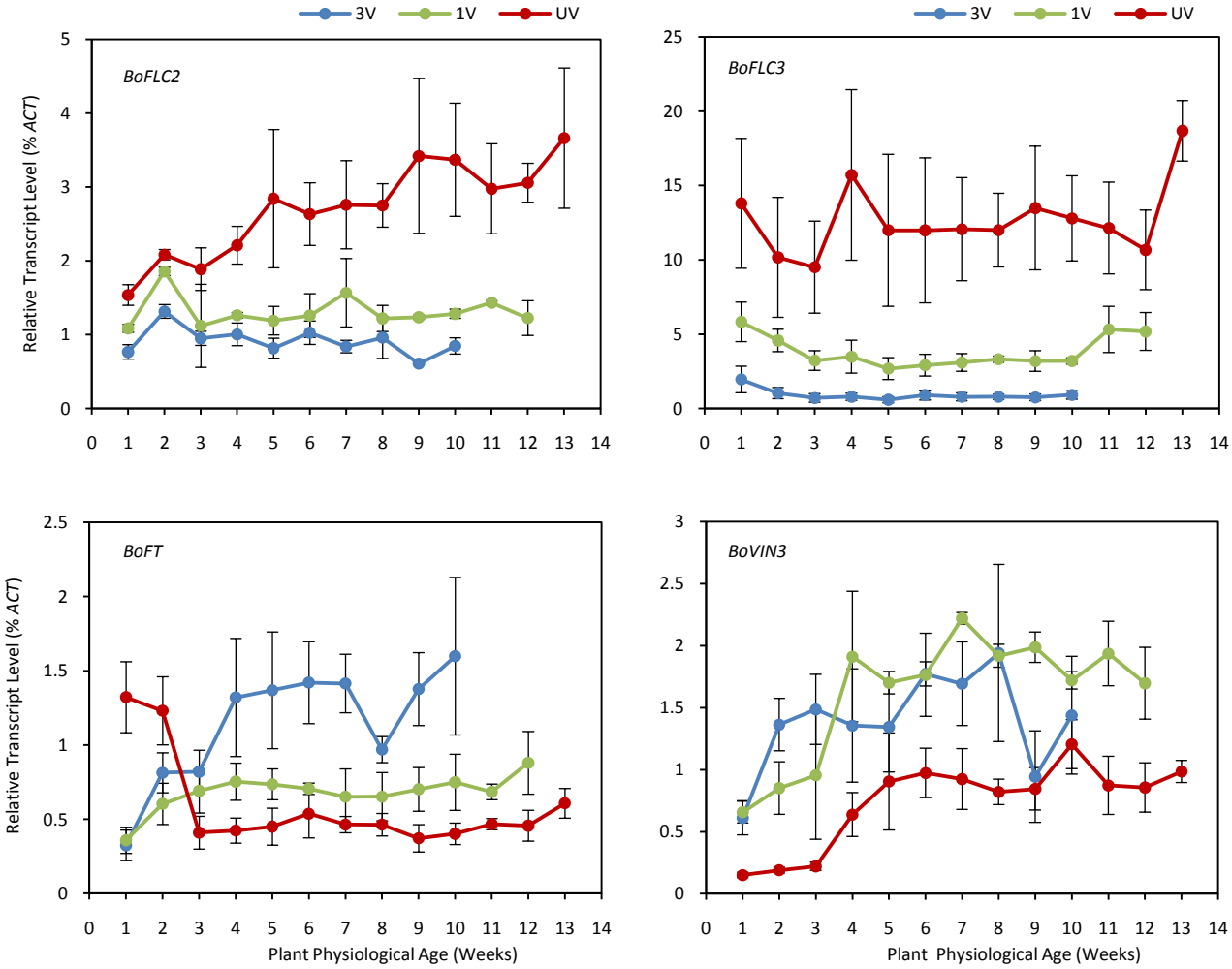


Fig. 32. Average *BoFLC2*, *BoFLC3*, *BoFT* and *BoVIN3* expression in apical tissue of cauliflower line 1 plants vernalised at different ages for different durations. UV (red) denotes unvernalised plants, 1V (green) denotes one week of vernalisation and 2-3V (blue) indicates two to three weeks of vernalisation. Bars represent the standard error of the mean (n=3).

c. Experiment 3: vernalisation temperature

BoFLC3 was downregulated by vernalisation in both apex and leaf tissue of late-flowering line 51 (Figure 33). Longer, colder chilling temperatures induced greater downregulation. Vernalisation temperatures of 15°C and 10°C had little effect on average *BoFT* expression in apex tissue. Vernalisation at 5°C was associated with a larger increase in *BoFT* expression, but this was not statistically significant (P>0.05). Similarly, vernalisation at 5°C was the only treatment to result in upregulation of *BoFT* in leaf tissue. In line 40 apex tissue, *BoFLC3* was downregulated by vernalisation, with colder vernalisation temperatures amplifying this effect. *BoFT* expression in unvernalised line 40 apices was higher in plants exposed to 5°C and 15°C

vernalisation treatments than in those that were not vernalised. In this case, the milder of the two vernalisation treatments was associated with greater *BoFT* upregulation.

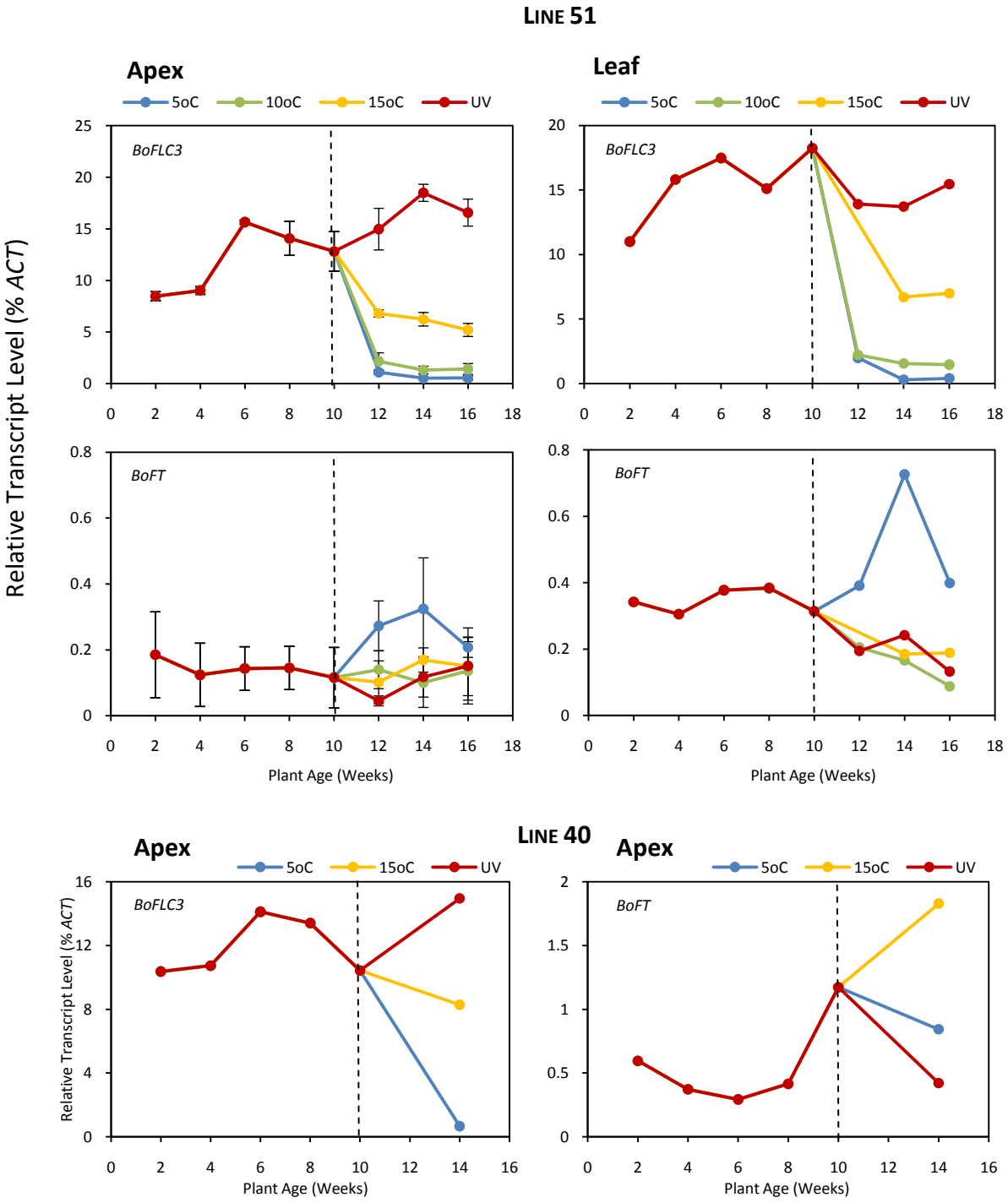


Fig. 33. *BoFLC3* and *BoFT* expression in line 51 leaf and shoot apex tissue and line 40 shoot apex tissue following vernalisation at 5°C, 10°C and 15°C at ten weeks of age. Leaf expression profiles for line 51 are averages of three replicates; all other profiles are based on one replicate only. The timing of vernalisation treatment is indicated by a dashed line. Bars represent the standard error of the mean.

d. Experiment 4: effects of vernalisation on flowering and gene expression in late- & early-flowering genotypes

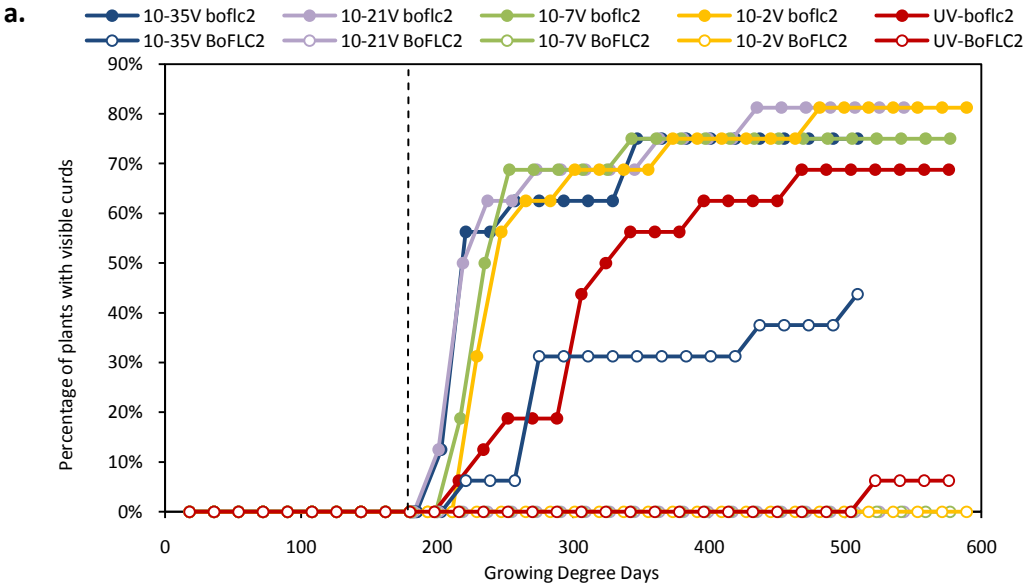
In vernalised plants that had subsequently been returned to 22°C cabinets, numerous instances of floral reversion were noted, where inflorescence organs had reverted to vegetative growth. The severity of these symptoms ranged from mild cases where bracts subtending the floral stalks of the curds were visible throughout the curd structure (Figure 34a) to more extreme cases where curd development ceased completely, and normal vegetative growth resumed (Figure 34b). Given the capacity of these post-vernalisation 22°C conditions to induce floral reversion, it is also possible that devernalisation may have significantly delayed curd induction in some cases. In particular, line 17, which normally develops a tightly-bunched 'head' of leaves prior to curd formation, was observed to persist in this phase of growth for a much longer period of time than usual prior to curd induction (e.g. Figures 34c and d), and in many cases, the precise point at which curds 'became visible' was difficult to distinguish (Figure 34e). The common occurrence of such a gradual transition from leaf, to bract, to curd production made leaf number an inappropriate measure of plant growth stage, necessitating the use of GDDs as a means of comparing between treatments. Furthermore, a large proportion of cauliflower curds became diseased, showing symptoms of fungal infection (Figure 34f). Affected curds usually failed to proceed to anthesis, or if bolting and flowering did occur, it was significantly delayed. Consequently, data relating to flowering time was disregarded, and observations of reproductive development were restricted to visible curd formation.

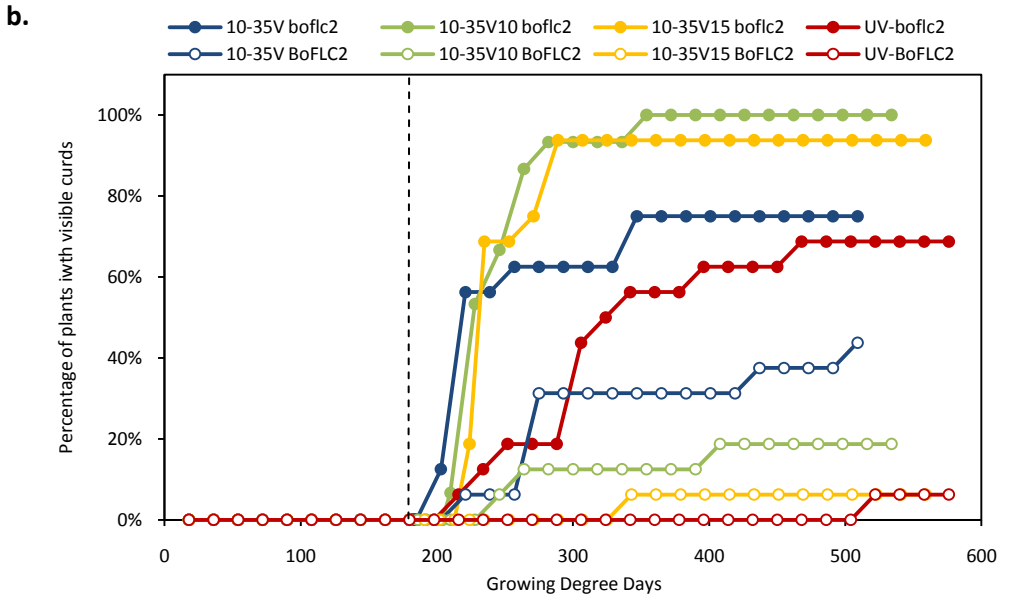
Fig. 34. Curd irregularities observed after transfer of plants from vernalising conditions to 22°C. Part (a) shows bracts protruding through the curd of a line 12 plant, 12 weeks after return to 22°C following 35 days of vernalisation at 5°C. Part (b) shows an example of inflorescence reversion in a line 50 plant, 14 weeks after return to 22°C following 21 days weeks of vernalisation at 5°C. Part (c) depicts the excessive 'bracty' growth observed prior to curd induction in many line 17 plants (17 weeks after return to 22°C following two days of vernalisation at 5°C). Part (d) shows a second example of the prolonged 'bunchy head' phase of a line 17 plant, 18 weeks after return to 22°C following 35 days of vernalisation at 15°C. Part (e) shows the difficulties of precisely determining the date of visible curd formation in line 17 plants – a 'head' has been formed, but no obvious curd tissue is present (eight weeks after return to 22°C following 21 days at 5°C), and part (f) shows the fate of most curds after return to UV conditions.

Figure 35a shows that none of the unvernalised *BoFLC2* cauliflowers had initiated curds by the end of the 32 week (576 GDD) observation period except for one of the four line 24 replicates, which eventually initiated a curd after 29 weeks (522 GDD). None of the *BoFLC2* plants exposed to 5°C for two, 7 or 21 days when ten weeks of age formed curds during the observation period. A number of *BoFLC2* plants formed curds shortly after the most 'extreme' vernalisation treatment (35 days at 5°C when ten weeks old), including all four line 12 replicates and several plants from lines 2 and 24. By contrast, 69% of the unvernalised *boflc2* plants eventually formed curds, with plants in the earliest line (40) forming curds as young as 12 weeks of age (216 GDD). None of the UV *boflc2* plants from line 17 produced curds. Vernalisation of *boflc2* lines at 5°C substantially increased the percentage of plants to initiate curds (81% in 10-2V and 10-21V and 75% in 10-7V and 10-35V). The uniformity of early curd induction was also increased, with longer vernalisation lengths amplifying this effect. Thirty-five and 21 day vernalisation treatments decreased the number of GDDs required for earliest visible curd formation in the *boflc2* lines. All of these vernalisation treatments except for the 7 day treatment were associated with curd initiation in at least one replicate from line 17; however, plants from this line were always slower and less likely to curd than plants from the other three *boflc2* lines.

Figure 35b shows that decreasing the temperature of 35 day vernalisation treatments of ten-week-old plants corresponded with decreases in the number of GDDs required for earliest curd formation in the *BoFLC2* parent lines (522, 343, 246 and 221 GDDs for UV, 10-35V15, 10-35V10, and 10-35V, respectively). Furthermore, the total percentage of initiated curds was higher in plants vernalised at a lower temperature (44%, 19% and 6% in the 5°C, 10°C and 15°C/UV treatments, respectively). Interestingly, 35 days of vernalisation at these milder temperatures was associated with a higher proportion of curding plants, than observed in plants exposed to colder, but shorter vernalisation treatments. In *boflc2* lines, plants that received milder vernalisation treatments were slightly slower to initiate their earliest curds, but the chief difference between the vernalisation temperature treatments was the total percentage of plants to initiate curds; 100% of the 10°C-treated plants and 94% of the 15°C-treated plants initiated curds, compared to 75% following the 5°C vernalisation treatment. This may be largely explained by the fact that in the 10°C and 15°C treatments, 4/4 and 3/4 line 17 replicates formed curds, respectively, compared to only 1/4 line 17 plants forming curds in the 5°C treatment.

No *BoFLC2* plants exposed to 5°C at three weeks of age went on to form curds (Figure 35c), and only one plant (line 24) initiated a curd after vernalisation at six weeks of age (i.e. no difference to the unvernalised control). As noted, the same vernalisation treatment applied to older plants (ten weeks old) resulted in a substantial number of curding plants. In *boflc2* lines, three-week-old plants exposed to 35 days of vernalisation at 5°C produced curds more uniformly and rapidly than the UV plants, but the total number of plants with curds was not substantially larger. A lengthy period between vernalisation treatment and the beginning of visible curd formation was noted. No line 17 plants produced curds. Plants vernalised at six weeks of age responded to their vernalisation treatment more rapidly than those vernalised when three weeks old, flowering earlier, but with less uniformity and with fewer plants eventually forming curds. Plants vernalised when ten weeks old immediately began to initiate curds.


a.
10-35V boflc2
10-21V boflc2
10-7V boflc2
10-2V boflc2
UV-boflc2
10-35V BoFLC2
10-21V BoFLC2
10-7V BoFLC2
10-2V BoFLC2
UV-BoFLC2
Percentage of plants with visible curds
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
100
200
300
400
500
600
Growing Degree Days


b.
10-35V boflc2
10-35V10 boflc2
10-35V15 boflc2
UV-boflc2
10-35V BoFLC2
10-35V10 BoFLC2
10-35V15 BoFLC2
UV-BoFLC2
Percentage of plants iwth visible curds
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80%
60%
40%
20%
0%
0
100
200
300
400
500
600
Growing Degree Days

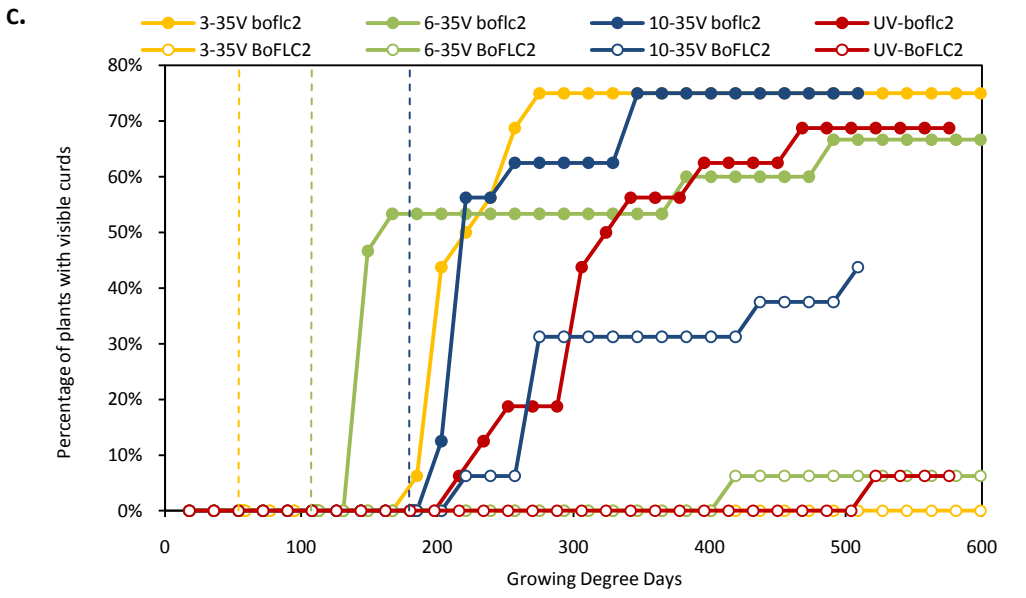

c.
3-35V boflc2
6-35V boflc2
10-35V boflc2
UV-boflc2
3-35V BoFLC2
6-35V BoFLC2
10-35V BoFLC2
UV-BoFLC2
Percentage of plants with visible curds
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
100
200
300
400
500
600
Growing Degree Days

Fig. 35. (Previous page). Percentage of curds initiated following vernalisation treatments. Part (a) and (b) show the effect of vernalisation length and temperature, respectively, on visible curd induction, and (c) shows the effect of plant age at vernalisation on curd induction. Eight different flowering types were evaluated; four *BoFLC2* lines, and four *boflc2* lines. Percentage values shown here represent the % of plants with curds in each of these combined two groups (data for individual lines not shown). Each of the four lines in the *BoFLC2* and *boflc2* groups had four replicates, meaning that these percentage values relate to the % of a group of 16 samples. Data is plotted against GDD to account for differences in plant development arising from different vernalisation treatments. The dotted lines represent the point at which vernalisation treatments were applied. In the case of (c), the yellow line represents the point at which three-week-old plants were vernalised, the green line indicates where the six-week-old plants were vernalised, and the blue line shows where the ten-week-old plants were vernalised.

Many of the gene expression patterns observed in these eight cauliflower parent lines were similar to those observed in the previous experiments described in this chapter. Furthermore, transcript profiles of all flowering candidate genes were highly uniform within the group of four functional *BoFLC2* parent lines, and also within the group of four mutant *boflc2* parent lines. In both *BoFLC2* and *boflc2* lines, leaf mRNA transcript levels of *BoFLC2* and *BoFLC3* were reduced by 35 days of vernalisation when ten weeks of age, with colder vernalisation associated with greater downregulation (Figure 36). The degree of downregulation was, once again, proportional to the length of vernalisation. Two days of vernalisation was sufficient to induce downregulation of both *BoFLC2* and *BoFLC3* in both genotypes, but this was not statistically significant ($P>0.05$). Although the general patterns of expression in response to these vernalisation treatments were similar between *BoFLC2* and *boflc2* genotypes, the overall transcript levels of *boflc2* in the four mutant parent lines were approximately five times lower than those of *BoFLC2* in the functional parent lines; for example, mean *boflc2* expression levels in unvernalised mutant plants were similar to mean *BoFLC2* expression levels in functional plants that had been exposed to 35 days of vernalisation at 5°C. The overall mean transcript levels of *BoFLC3* in the *BoFLC2* genotypes were slightly lower than those in the *boflc2* genotypes.

Initial *BoFT* mRNA levels in leaves of most *boflc2* parent lines were relatively high during early plant development (i.e. at three weeks of age), before gradually decreasing as the plant matured. Vernalisation at ten weeks of age elicited a characteristic upregulation of *BoFT*, with the effect amplified by longer vernalisation treatments (Figure 36). In all *boflc2* lines, the 5°C treatment induced the most extreme upregulation, followed by the 10°C treatment.

Vernalisation at 15°C had little effect on mean *BoFT* expression in *boflc2* lines. The *BoFT* response to vernalisation was much weaker in *BoFLC2* parent lines, with vernalisation at 5°C failing to even raise transcript levels above the naturally higher levels seen during early plant growth. Milder vernalisation temperatures did not stimulate any detectable increase in mean *BoFT* expression in *BoFLC2* plants. Overall mean *BoFT* transcript levels were approximately ten times lower in *BoFLC2* parent lines than in *boflc2* parent lines.

There were relatively minor differences in *BoVIN3* expression level and profile between *BoFLC2* and *boflc2* parent lines (Figure 36). Expression in leaves was initially high during early growth of both *BoFLC2* and *boflc2* plants. Unexpectedly, vernalisation at 5°C for as little as two days was sufficient to elicit upregulation in both genotypes (significant only in *BoFLC2* lines; P=0.014). Vernalisation durations of 7 and 21 days at 5°C produced a slight increase in *BoVIN3* expression, and a chilling for 35 days at 5°C resulted in a substantial upregulation in both *BoFLC2* and *boflc2* genotypes. This effect was slightly diminished by milder vernalisation temperatures, but differences between the 5°C, 10°C and 15°C treatments were not statistically significant (P>0.05).

Figure 37 shows that the age at which plants were vernalised had little effect on *BoFLC2* and *BoFLC3* expression in either *BoFLC2* or *boflc2* genotypes. Once again, overall *boflc2* transcript levels were several orders of magnitude lower than *BoFLC2* levels. In all *BoFLC2* genotypes except line 12, 5°C vernalisation treatments elicited a greater upregulation of *BoFT* when applied to older plants. However, these changes in transcript level are very small compared to the upregulation observed in *boflc2* varieties. Vernalisation of three-week- and ten-week-old plants resulted in upregulation of *BoFT* to roughly similar levels; however, unusually, vernalisation of six-week-old plants consistently failed to upregulate *BoFT* in any of the *boflc2* parent lines. In both *BoFLC2* and *boflc2* parent lines, *BoVIN3* responsiveness to vernalisation increased with age.

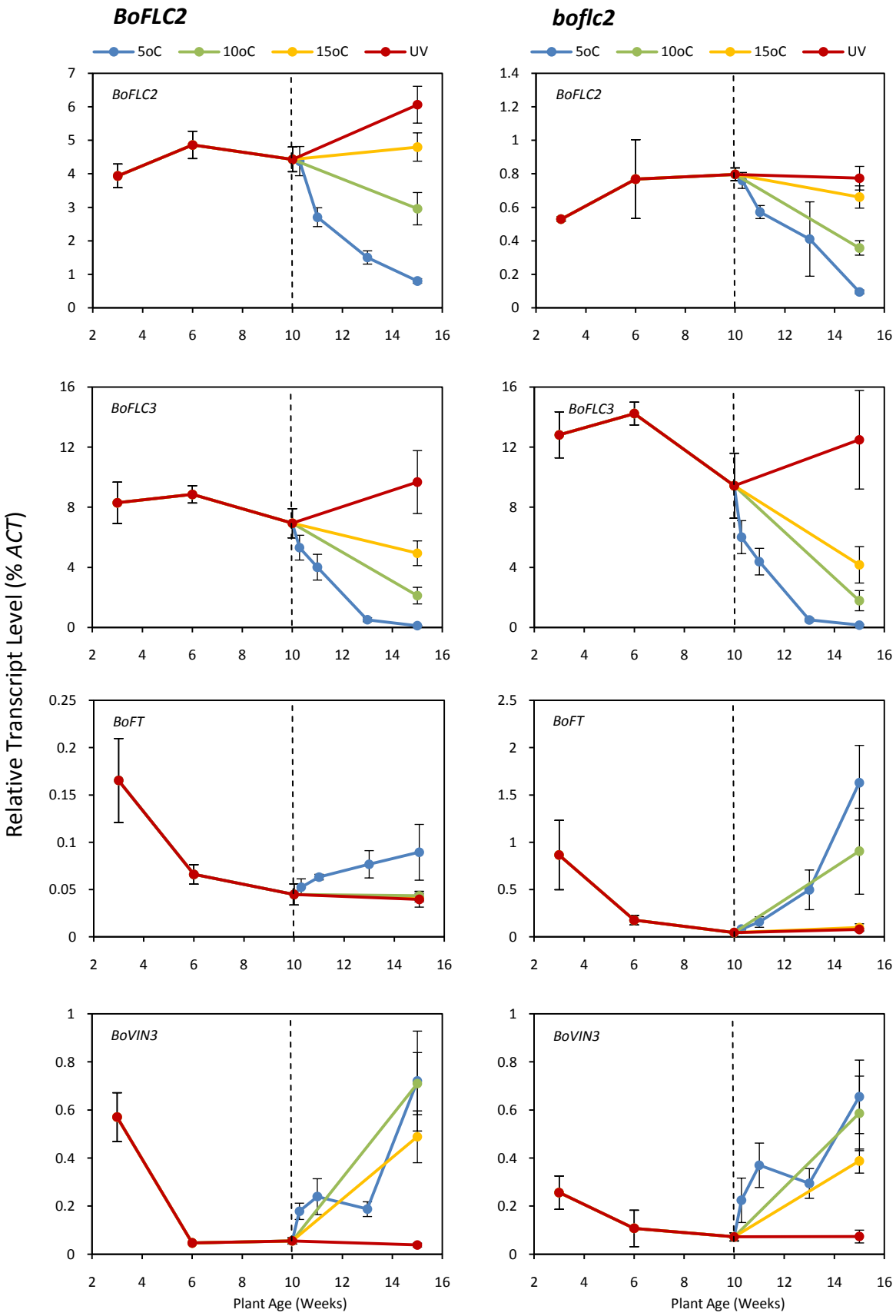


Fig. 36. Average *BoFLC2*, *BoFLC3*, *BoFT* and *BoVIN3* expression in *BoFLC2* and *boflc2* parent line leaves following vernalisation at ten weeks of age. Each series represents the average expression of the four different parent lines (one replicate per line) within the *BoFLC2* or *boflc2* genotype. The timing of vernalisation treatment is indicated by a dashed line. Bars represent the standard error of the mean.

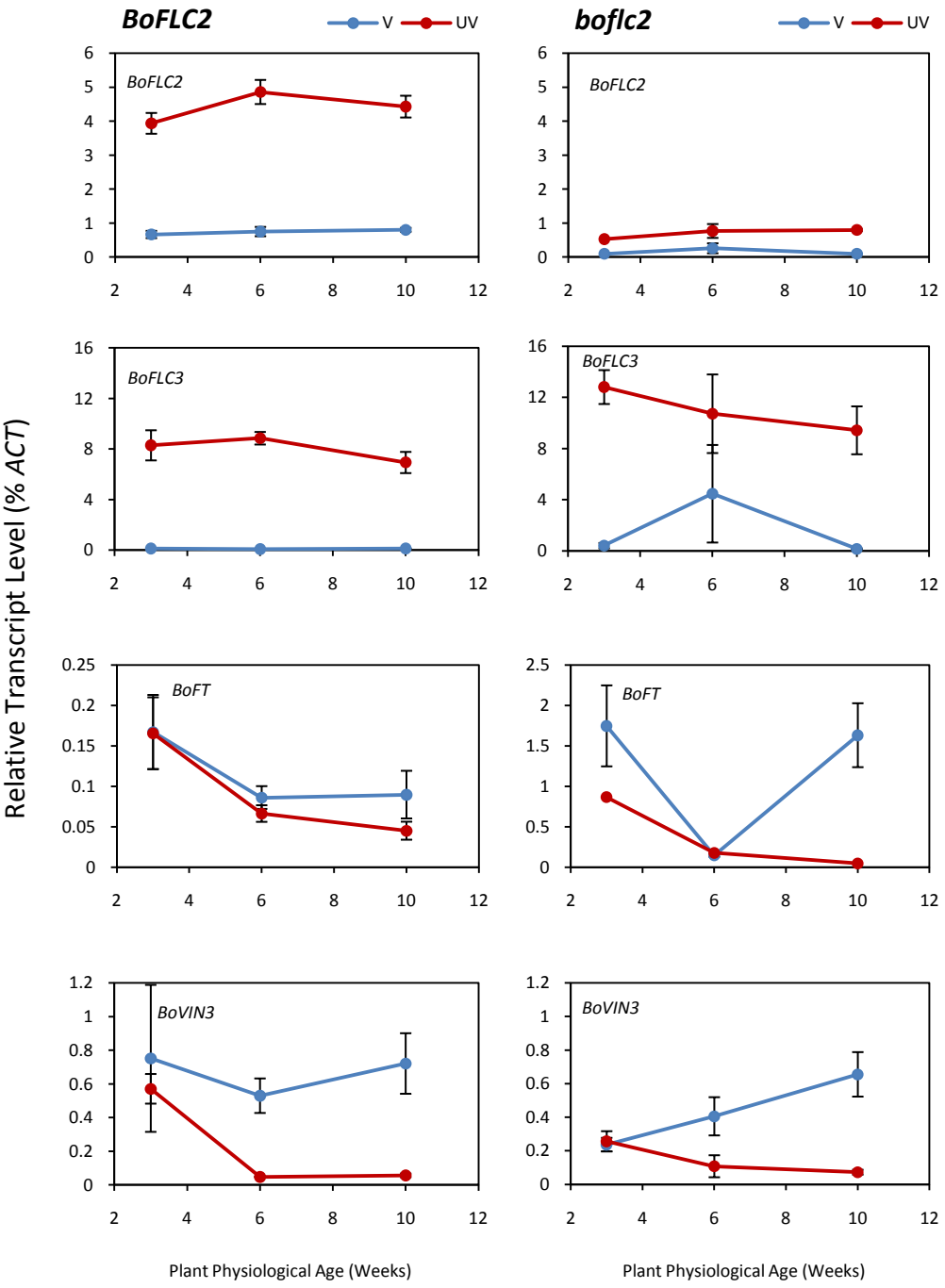


Fig. 37. Average candidate gene expression in *BoFLC2* and *boflc2* parent line leaves following vernalisation at three, six and ten weeks of age. Each series represents the average expression of the four different parent lines (one replicate per line) within the *BoFLC2* or *boflc2* genotype. Expression data is plotted against Plant Physiological Age, in order to account for the lack of growth at vernalising temperatures. Bars represent the standard error of the mean.

D. Discussion

In this study, several important flowering time genes were isolated and characterised in cauliflower. It was shown for the first time that the basic downregulation of *BoFLC2* and *BoFLC3* is conserved in cauliflower. *BoFT*, a downstream integrator of flowering, was characterised in cauliflower for the first time, and was found to be upregulated in response to vernalisation, and a homologue of the epigenetic repressor of *FLC*, *VIN3* was isolated for the first time in a brassica crop species, and shown to be upregulated by vernalisation.

In contrast to studies in cabbage, only minor differences in gene expression behaviour were noted between apex and leaf tissue, showing that *BoFT* is expressed in apices as well as in remote leaf tissues, and that *BoFLC* and *BoVIN3* expression is not restricted to dividing apical meristem cells. In both of these tissues, vernalisation effects on *BoFLC2*, *BoFLC3* and *BoFT* expression were amplified with increased vernalisation length and decreased vernalisation temperature. Vernalisation for 2-21 days weeks elicited a small upregulation of leaf *BoVIN3*, but vernalisation of 35 days was required for a more substantial upregulation. The level to which *BoFLC2* and *BoFLC3* mRNA apex transcript levels were reduced by vernalisation was independent of plant age, but *BoFT* was less responsive to vernalisation at very young ages suggesting the involvement of upstream genes other than *BoFLC* in regulating juvenility length.

As discussed in the introduction of this chapter, previous authors had reported that brassica varieties with different flowering behaviour also carried *FLC* alleles with different overall expression levels prior to vernalisation (Tadege et al. 2001; Kim et al. 2007). Others had reported differential *FLC* responsiveness to vernalisation in different flowering types (Li et al. 2005; Lin et al. 2005). In this study, no fundamental differences in vernalisation-mediated expression dynamics were observed between late-flowering *BoFLC2* and *boflc2* cauliflowers. Rather, the primary differences seen between the two flowering classes were related to the overall transcript level of *BoFLC2*, which was approximately five times higher in functional lines than in *boflc2* lines, and *BoFT*, which was approximately ten times lower in *BoFLC2* than in *boflc2* plants. The lower levels of *BoFT* expression and the associated reduction in vernalisation responsiveness are probably due to repression by the functional *BoFLC2*. These findings have potential practical applications, with the possibility that relative

expression levels of *BoFLC2* and/or *BoFT* could be used to predict curd induction in seed production environments.

Curd formation and flowering time data collected for experiment 1 indicated that increasing vernalisation length had the expected effect of hastening visible curd formation and flowering in parent line 1. In experiment 4, *boflc2* plants were capable of producing curds without any vernalisation; and yet, a vernalisation response was evident, with all vernalisation length treatments increasing the percentage of plants with reproductive structures. Curd induction in *BoFLC2* lines was unaffected by 5°C vernalisation treatments shorter than 35 days. Okazaki (2007) described brassicas grown as annuals, such as cauliflower and broccoli, as 'non-vernalising' types, however these results show that even the earliest-flowering cauliflower varieties exhibit a response to low temperatures by increasing apex growth and forming flower buds more rapidly (c.f. Wurr et al. 1995). Fellows, Reader & Wurr (1997) showed that milder vernalisation temperatures ranging from 5°C up to 15°C were more effective at inducing curd formation in calabrese broccoli, and this is broadly consistent with the curd induction response to temperature observed in these *boflc2* lines. By contrast, colder vernalisation temperatures resulted in a larger percentage of curded plants in *BoFLC2* lines. A vernalisation response was also noted in *boflc2* lines when vernalised at a young age, in contrast to *BoFLC2* lines which were largely unaffected by vernalisation treatments applied at an age of less than ten weeks (as is the case in other functional *BoFLC2* brassicas, such as cabbage). The high incidence of floral reversion observed in plants returned to 22°C raises the possibility that devernalisation may also have influenced flowering time; further studies where post-vernalisation behaviour is observed would be better conducted in cooler glasshouse conditions. Many of the phenotypic differences between vernalisation treatments and between flowering types were strongly linked to gene expression behaviour, as discussed below.

Initial experiments showed that expression profiles were mostly similar between young leaf and shoot apex tissue, both in terms of overall level of expression, and in terms of expression changes associated with developmental stage (Figures 31 and 33). Because of this, expression data was only generated for one tissue type for some experiments. Lin et al. (2005) observed strong *BoFLC2* expression increases and *BoFT* decreases with plant age, and these trends were also observed in most of these experiments; Figures 31, 32 and 36 show, to varying degrees, a general trend of upregulation of *BoFLC2* throughout early development,

and decreasing *BoFT* transcript levels throughout the earliest weeks of seedling development in both leaf and apex tissue. These early changes in gene expression may be associated with the transition from juvenility to maturity; it is possible that *BoFLC2* must be expressed at a certain threshold level before it is capable of being downregulated by vernalisation, although the mechanism for such behaviour is unknown. Average *BoVIN3* expression in plant apices increased gradually with plant age during early growth (Figures 31 and 32), but this was not the case in young leaves (Figure 36). Given the gradual upregulation of *BoFLC2* in unvernalised plant tissue, and the expected antagonistic role of *BoVIN3*, the natural increase of *BoVIN3* in unvernalised apices is unexpected, but it is possible that the level at which *BoVIN3* is expressed in unvernalised tissue is insufficient to upregulate *BoFLC*.

This study supports the idea that the function of *BoVIN3* is conserved in cauliflower. The *VIN3* homologue isolated here was consistently upregulated in response to vernalisation in all plant tissues, with no significant differences observed between early- and late-flowering parent lines. This suggests that expression response of this *BoVIN3* allele to vernalisation is not a major contributing factor to variability in vernalisation responsiveness or flowering time in the cauliflower lines investigated. In contrast to the Arabidopsis model, in which prolonged cold temperatures (approx. 20 days at 2-4°C) are required to induce expression (or more accurately, relieve the repression; Kim, Zografos & Sung 2010) of *VIN3*, results from this study show that *BoVIN3* is significantly upregulated by as little as two days of vernalisation (Figure 36) and that there is little difference between expression levels in plants vernalised for two, 7 and 21 days. In experiment 4, *BoVIN3* expression in leaves increased substantially after 35 days of vernalisation. It is possible that the upregulation of *BoVIN3* observed following shorter vernalisation treatments (i.e. those less than 35 days) is insufficient to elicit stable downregulation of *BoFLC*, accounting for the lack of curd initiation in *BoFLC2* plants.

Lin et al. (2005) raised the possibility that *BoVIN3* may be involved in the differential response of seed as opposed to plant vernalisation in cabbage, suggesting that *BoVIN3* may only be upregulated during vernalisation of mature cabbages, but not in vernalised seedlings. Figure 32 shows that *BoVIN3* transcription was upregulated by vernalisation, regardless of plant age. This may indicate that *BoVIN3* responsiveness is not a key determinant of juvenility length in cauliflower, but because no curd induction data was able to be obtained for this experiment, it is not possible to be conclusive about this. Furthermore, subsequent

results shown in Figure 37 clearly indicate that the degree of *BoVIN3* upregulation in response to vernalisation increases with plant age in both *BoFLC2* and *boflc2* parent lines. This is consistent with curd induction data, where curds were initiated most rapidly in plants vernalised at an older age. Consequently, *BoVIN3* responsiveness to vernalisation at different plant ages remains a possible contributor to juvenility length, but does not appear to be responsible for variability in juvenility between the *BoFLC2* and *boflc2* lines investigated here.

The downregulation of cauliflower *BoFLC* genes in response to vernalisation is consistent with the behaviour of this gene family in other members of the Brassicaceae. The response of both *BoFLC2* and *BoFLC3* to vernalisation occurred very rapidly, with two days at 5°C sufficient to induce downregulation. Colder temperatures and longer vernalisation treatments amplified this effect. Although this expression response was very similar in both *BoFLC2* and *boflc2* varieties, the overall level of *BoFLC2* transcript was much higher than that of the premature termination codon (PTC)-carrying *boflc2*, probably due to nonsense-mediated decay (NMD) of the *boflc2* mRNA. While NMD commonly acts to degrade mRNA harbouring PTCs leading to reduced levels of transcript (Chang, Imam & Wilkinson 2007), transcription of genes carrying PTCs is still possible, with other pre-translational suppression or post-translational mechanisms capable of regulating the expression of the gene. Okazaki et al. (2007) suggest that this is the case in *BoFLC2-GC*.

Other authors have reported similar strong correlations between overall *FLC* expression levels and flowering time in both *B. rapa* (Kim et al. 2007) and *B. napus* (Tadege et al. 2001) and it is possible that such differences in expression have a similar basis to those observed here. Despite the lower overall expression level, the pattern of *boflc2* expression response to vernalisation was similar to that seen in *BoFLC2* plants. This is in direct contrast to findings in cabbage, where *BoFLC2* transcript levels were similar between a range of flowering types prior to vernalisation, with the point of difference between varieties lying in the degree of reduction of *BoFLC2* expression following vernalisation (Lin et al. 2005). Although *boflc2* responsiveness to vernalisation is retained, this allele possesses a mutation which affects the post-transcriptional behaviour of the gene, meaning that downregulation and vernalisation responses are likely to be without direct effect. Nonetheless, residual responses are useful in that they are likely to be indicative of the behaviour of genes in upstream regulatory pathways.

Whereas Lin et al. (2005) found that *BoFLC3* transcript levels were too low to detect using a probe of 3'-UTR of *BoFLC3*, this study showed that *BoFLC3* is strongly expressed in cauliflower, at levels far greater than that of *BoFLC2*, and is also regulated by vernalisation in a similar manner to *BoFLC2*. Therefore, it seems probable that *BoFLC3* could play at least a minor role in contributing to the differences in age at visible curd formation observed between the different vernalisation treatments in *boflc2* parent lines. Furthermore, recent studies have shown that in species with expanded flowering gene families, transcriptional cross-regulation between different members of the same family may occur (e.g. Hecht et al. 2011); these results suggest that there is no such cross-regulation between *BoFLC3* and *BoFLC2*, with *BoFLC3* regulation entirely independent of *BoFLC2* expression and functionality.

BoFLC2 and *BoFLC3* were downregulated by vernalisation in both *BoFLC2* and *boflc2* parent lines, regardless of plant age. In this regard, *BoFLC* vernalisation responsiveness is more closely aligned to that of seed-vernalisation-responsive members of the Brassicaceae, such as *A. thaliana*, *B. rapa* and *B. napus*, than the more closely-related cabbage, whose *BoFLC* expression is unaffected by vernalisation at young ages (Lin et al. 2005). This suggests that the differences in curd initiation recorded for the vernalisation treatments at different plant ages are not primarily due to differences in *BoFLC2* or *BoFLC3* transcription.

The upregulation of *BoFT* in response to vernalisation is consistent with the behaviour of the upstream genes investigated in this study; that is, *BoFT* increases proportionally and in response to downregulation of *BoFLC*, which in turn follows upregulation of *BoVIN3*. Cauliflower *BoFT* was upregulated in both leaves and apices, unlike cabbage *BoFT*, which was unaffected by vernalisation in leaf tissue (Lin et al. 2005). The overall level of *BoFT* expression was approximately tenfold lower in *BoFLC2* parent lines, reflecting strong suppression by *BoFLC2*. In experiment 4, average *BoFT* transcript levels were low in unvernalised 15-week-old plants and in plants vernalised at 15°C; nonetheless, many of these plants went on to produce curds, suggesting that increases in *BoFT* expression occurred subsequent to this time point. Average *BoFT* expression in late-flowering parent lines was not upregulated by vernalisation of three-week-old plants; when vernalisation was applied to older plants, mild upregulation was observed. This suggests that some factor, other than *BoFLC*, is contributing to cauliflower juvenility. The *BoFT* expression profile is broadly similar to the *BoVIN3* profile, but since *BoVIN3* is likely to act directly upon the *BoFLC* gene

(which is itself upregulated independently of plant age), some other molecular factor must be at work in preventing *BoFT* upregulation in young plants. It is also possible that delayed *BoFT* responsiveness is mediated by a transcriptional activator, as opposed to putative negative regulators such as *BoFLC* and *BoVIN3*. Recent studies in Arabidopsis have indicated that proteins such as CO and GI are both directly involved in the activation of *FT* (Tiwari et al. 2010; Sawa & Kays 2011). Therefore, it is possible that a homologue of one of these genes, or activity of any of the DNA-binding proteins and protein complexes associated with their recruitment could potentially control the responsiveness of *BoFT* to vernalisation in the *Brassica* species.

Information that is potentially useful from a practical perspective may be derived from observations of the absolute values involved in the *BoFLC2* and *BoFT* expression profiles of late- and early-flowering plants in experiment 4. For example, vernalisation at 5°C of ten-week-old *BoFLC2* plants for 35 days reduced average *BoFLC2* expression in leaves to 0.80% *ACT* and upregulated average leaf *BoFT* expression to 0.089% *ACT*. This treatment was the only vernalisation treatment sufficient to induce curd formation in a substantial number of *BoFLC2* plants (44%). Many *boflc2* plants produced curds even without any vernalisation (69%), and in unvernalised 15-week-old plants, *BoFT* transcript levels were 0.078% *ACT*; similar to that seen in vernalised *BoFLC2* lines. These findings suggest the possibility that expression levels of around 0.80% *ACT* for *BoFLC2* and around 0.078-0.089% *ACT* for *BoFT* represent approximate thresholds that must be reached before curd initiation can occur.

In order to account for differences in pre-vernalisation expression levels, the downregulation of *BoFLC2* and upregulation of *BoFT* may also be expressed as a percentage; for example, transcript levels of leaf *BoFLC2* at ten weeks of age in lines 2, 12, 24 and 51 decreased in response to 35 days of vernalisation at 5°C by 79%, 78%, 87% and 81%, respectively. This treatment resulted in upregulation of leaf *BoFT* by 48%, 35%, 57% and 42% for lines 2, 12, 24 and 51, respectively (compared to the 90-99% *BoFT* transcript increases observed in leaves of *boflc2* lines). This is consistent with results from experiment 1 which showed plants vernalised for two weeks exhibited a 73% downregulation of leaf *BoFLC2* and a 60% upregulation of leaf *BoFT*, and eventually formed curds, but that induction was slow compared to plants that had been vernalised for longer. Naturally, detailed modelling of expression and flowering behaviour in multiple flowering types would be required in order to construct a robust model that could be used to predict flowering time in production

environments. Although such an effort is beyond the scope of this study, some initial investigations into the potential for *BoFLC2* and *BoFT* to be used as flowering time predictors in cauliflower are made in the Chapter VII.

E. Conclusion

By studying genes at multiple locations throughout the flowering pathway, this study represents the first attempt to gain a broader view on the molecular regulation of the cauliflower flowering pathway. As a first attempt, it should therefore be recognised that a considerable amount of work remains to fully elucidate the cauliflower flowering pathway. In particular, the polyploid nature of this species means that there are likely to be multiple additional undiscovered genes even within the families of those genes investigated here which also make interact with one another and make significant contributions to the floral transition.

While the functionality of the *BoFLC2* gene appears to be the chief genetic factor influencing curd induction and flowering time in the cauliflower inbred parent lines studied here, it is clear that there is much of practical value to be learnt from studies of flowering gene expression. With the use of RT-qPCR techniques to generate high-resolution expression profiles, this research has shown for the first time that the basic downregulation response of the genes *BoFLC2* and *BoFLC3* to vernalisation is conserved in cauliflower, and the upstream regulator *BoFT* is upregulated by vernalisation. Furthermore, the isolation and characterisation of *BoVIN3* represents the first record of this key gene in a brassica crop species, with transcription data indicating a conserved function, and a role in the vernalisation pathway. Curd formation behaviour was consistent with the expected function of these genes. These findings have potential applications, with the possibility that relative expression levels of *BoFLC2* and/or *BoFT* could be used to predict curd induction in seed production environments.

Chapter VI

DEVERNALISATION, GENE EXPRESSION & FLOWERING TIME

A. Introduction

Asynchronous flowering between parent lines is one of the most serious problems facing cauliflower breeders and producers of hybrid seed crops. One factor that may contribute to asynchronous or uneven flowering in these crops is annulment of the vernalisation effect by a phenomenon commonly referred to as 'devernalisation' (Purvis & Gregory 1952). This concept is well-known, with anecdotal evidence from members of the seed industry suggesting that it may be responsible for significant lost production in some brassica genotypes, especially Kohlrabi. In order to prevent crop failure as a consequence of devernalisation, the seed industry has been forced to avoid growing crops in warmer and drier areas – including glasshouse-based systems – that are normally conducive to high-yielding and high-quality seed crops. As a result, the available land and planting window is more limited for brassica seed production than almost any other vegetable seed crop (A. Baelde 2011, pers. comm. 18 April). Despite being a recognised, serious problem, the physiological and molecular mechanisms of devernalisation remain poorly understood, as do practical methods for its prevention and correction.

Devernalisation is characterised by the failure of plants to proceed to the reproductive phase after an extended period of 'relative cold' that is normally sufficient for floral initiation (Wiebe, Habegger & Liebig 1992). As noted earlier, numerous autonomous and environmental factors besides vernalisation combine to influence the transition from vegetative to reproductive development. For example, a plant which has received vernalisation of a duration and intensity normally sufficient to induce reproductive development may not flower if it is physiologically immature when vernalised, or if it has not

experienced the necessary photoperiodic stimuli (e.g. Booij & Struik 1990; Ramin & Atherton 1994).

However, the term 'devernalisation' is typically reserved for situations where a delay or failure to progress to the generative phase is specifically attributed to a loss or reduction of the vernalisation impetus in plants that are otherwise competent to flower (Wiebe, Habegger & Liebig 1992). Furthermore, this term is most commonly applied when the reversal of the vernalised condition is suspected to be principally due to high post-vernalisation temperatures; although inhibited flowering due to inadequate irradiance and light quality during vernalisation, or post-vernalisation exposure to environmental conditions opposite to those that induce flowering (such as short day conditions) is also sometimes described as 'devernalisation' (Engelen-Eigles 1996; Thomas & Vince-Prue 1997).

Previous reviewers have suggested that the minimum temperature capable of inducing partial devernalisation of cauliflower plants is within the 20 to 25°C range, depending on the cultivar (Friend 1985; Wurr et al. 1993; Erwin 2007). Researchers have employed temperatures of 30°C (Dahanayake & Galwey 1998) to 37°C (Yu et al. 2010) in order to specifically assess devernalising effects on brassica. Other studies have shown that the devernalisation effect, in terms of delayed flowering, is more pronounced when hot temperatures occur during, (Gultormsen 1981; Saito & Saito 2003) or immediately following (Michaels & Amasino 2000; Erwin 2007) an extended period of vernalisation, suggesting that a 'stabilisation' period is needed following vernalisation in order to maintain the vernalised state (Erwin 2007).

Antivernalisation and floral reversion are two phenomena closely related to devernalisation. Antivernalisation describes the delay or prevention of flower induction and floral development in plants exposed to high temperatures prior to vernalisation. In some *Brassica* cultivars, antivernalisation (such as would occur during unseasonably warm autumn days) may be a significant cause of delayed flowering; for example, Dahanayake & Galwey (1998) found that primed *B. napus* var. *annua* seeds, exposed to 30°C for just two days prior to eight weeks of vernalisation at 4°C, exhibited significantly delayed flowering time compared to plants that were not antivernalised. The precise nature of the connection between antivernalisation and devernalisation remains unclear.

Whereas devernalisation refers to the prevention of floral initiation by a reversal of the vernalised condition, floral reversion describes a return to vegetative growth following a period of reproductive development (Okamuro et al. 1996). Tooke et al. (2005) identify two distinct types of floral reversion; inflorescence reversion, where vegetative growth occurs after, or during and within inflorescence development; and flower reversion in which the flower organs are themselves altered or replaced with vegetative structures. Reversion of 'committed' inflorescence meristems has been observed in cauliflower following exposure to high temperatures following the transition from the vegetative to reproductive phase (Anthony, James & Jordan 1996).

Growing understanding of the molecular pathways controlling vernalisation-responsive flowering in Arabidopsis provides the potential to understand the above phenomena more fully. In Arabidopsis, prolonged cold temperatures (approx. 20 days at 2-4°C) induce the expression (or more accurately, relieves the repression; Kim, Zografos & Sung 2010) of *VIN3*, which encodes a PLANT HOMEODOMAIN (PHD)-finger-containing protein (Sung & Amasino 2004b). This protein is thought to be involved in the de-acetylation of Histone H3 in the chromatin of flowering inhibitor *FLC*, increasing its positive charge and encouraging high-affinity binding to the DNA backbone, thereby condensing DNA structure, preventing *FLC* transcription and allowing expression of the floral promoter and integrator genes such as *FT* and *SOC1*, and meristem identity genes such as *AP1* which participates in, and whose expression seems to indicate, floral determination (Carr & Irish 1997; Hempel et al. 1997; Sung & Amasino 2004a). The initial establishment of a hypoacetylated environment by *VIN3* also creates favourable conditions necessary for the constitutively expressed *VRN1* and *VRN2* genes (Gendall et al. 2001; Levy et al. 2002) to methylate *FLC* Histone H3. Although *VIN3* expression is transient, with mRNA decreasing to undetectable levels within three days of return to warmer temperatures, the methylation regulated by *VRN1* and *VRN2* is a structural change which is propagated through mitotic cell divisions in the absence of the inducing cold signal, accounting, at least partially, for epigenetic 'memory' of the vernalised state (Gendall et al. 2001; Levy et al. 2002; Amasino 2004; Bastow et al. 2004; Sung & Amasino 2004a). This mechanism provides a framework for understanding why devernalisation is not usually observed in Arabidopsis, and suggests that aspects of the process have altered functionality in species such as *Brassica* where devernalisation is common.

Given its role in the epigenetic suppression of *FLC*, *VIN3* is a likely candidate for involvement in the devernalisation and floral reversion phenomena observed in cauliflower. In the previous chapter, the isolation of a *VIN3* orthologue in cauliflower was described, with expression data supporting the idea that its vernalisation-regulated role in *BoFLC* repression is conserved. Questions remain as to whether partial induction of *BoVIN3* results in partial repression of *BoFLC* that is mitotically stable, or whether *VIN3* must be fully expressed before orthologous *VRN* genes can suppress *BoFLC* in an epigenetic fashion. Furthermore, the question of how long or cold a vernalisation treatment must be before *VIN3* is fully upregulated and vernalisation is saturated requires attention. Analysis of *VIN3* expression in *B. oleracea* plants exposed to devernalising conditions would allow such questions to be addressed. As the most 'upstream' gene currently known in the Arabidopsis vernalisation pathway, *VIN3* seems likely to be central to the mechanism of devernalisation.

Because *VIN3* is expressed exclusively at relatively low temperatures, expression analysis of *BoFLC*, the next gene downstream, would provide further valuable information about the molecular effects of a post-vernalisation return to hot conditions. We have shown that *BoFLC2* and *BoFLC3* are downregulated by cold temperatures in cauliflower, and according to the Arabidopsis model, expression of genes in the *FLC* family would be expected to remain low upon return to warm conditions. It would be interesting to observe whether this is so in *B. oleracea*, as stable repression of *BoFLC* upon return to high temperatures would seem to preclude the possibility of any literal 'devernalisation' involving a reversal of the key genes in the vernalisation floral inductive pathway (although it is possible that the devernalisation process affects elements of homologous *FLC*-independent pathways (c.f. Michaels & Amasino 2001; Schönrock et al. 2006). Increases in *FLC* transcript abundance in response to warm conditions post-vernalisation have never been reported in either Arabidopsis or *Brassica*. *BoFLC* upregulation after a short period of vernalisation would not be at variance with current opinion; however, increases in expression following long periods of continuous vernalisation would indicate that repression of *BoFLC* is unstable, and may underpin the process of devernalisation. We would expect expression profiles of integrator genes such as *BoFT* to be inversely related to *BoFLC* expression.

Two cauliflower homologues of the Arabidopsis meristem identity gene (MIG) *AP1* have already been isolated: *BoAP1-a* (Anthony, James & Jordan 1996) and *BoAP1-c* (Smith 1999). A third non-functional gene designated *BoAP1-b* (Lowman & Purugganan 1999) has also

been reported. *BoAP1-a* is thought to play the most important role in specifying the conversion of reiterating curd meristems to floral primordial and subsequent flowering (Anthony, James & Jordan 1996). The expression of *BoAP1-a* was examined by Anthony, James & Jordan (1996), who found that *BoAP1-a* expression was greatly reduced in the vegetative shoots of reverted cauliflowers that had been exposed to high temperatures. Similarly, Kop et al. (2003) identified a temperature-*BoAP1-a* interaction that affected bract development. Although the authors note that most cauliflower plants are characterised by recessive *Boap1-a* alleles that do not suppress bracting, expression analysis of this gene could be helpful in validating the aforementioned upstream regulatory processes.

Exposure of cauliflower plants to devernalising conditions, examination of the physiological response, and analysis of the expression of these various genes throughout the vernalisation pathway would clarify which aspects of the process are primarily impacted by devernalisation. The ability to determine the point in the pathway at which the vernalised state is reversed would inform practical methods for predicting, preventing or correcting the disorder.

B. Materials & Methods

1. Experimental Design, Plant Growth Conditions & Scoring

Seeds from parent line 50 (homozygous for the *boflc2* mutant allele) were sown into 56 2 L pots containing general purpose potting mix topped with a thin layer of seed raising mix. Once established, plants were thinned to one plant/pot. Plants were grown in 22°C controlled environment growth cabinets under a mixture of 36 W cool white fluorescent lamps and 60 W incandescent lamps with an output of 100 µM m^{-2} s^{-1} as part of a 16 hour photoperiod regime.

When the plants were seven weeks old, they were given one of six different treatments (six replicates per treatment); (a) two days of vernalisation at 5°C followed by three weeks at a 'devernalising' temperature of 32°C (treatment 2D); (b) two days of vernalisation at 5°C followed by three weeks at an 'optimal' temperature of 22°C (treatment 2O); (c) seven days at 5°C followed by three weeks at 32°C (treatment 7D); (d) seven days at 5°C followed by three weeks at 22°C (treatment 7O); (e) 28 days at 5°C followed by three weeks at 32°C (treatment 28D) and (f) 28 days at 5°C followed by three weeks at 22°C (treatment 28O).

At the end of treatments 2D and 2O, plants were 10 weeks and 2 days old. At the end of treatments 7D and 7O, plants were 11 weeks old. At the end of treatments 28D and 28O, plants were 14 weeks old. However, during the period spent at 5°C, plant growth was slowed or stopped. This made it unrealistic to compare treatments as though they were different ages. Instead, the time spent at 5°C was disregarded, and the 'physiological age' of the plants in each treatment was assumed to be 10 weeks. Accordingly, six plants that were aged seven weeks were maintained at 22°C (unvernalised or UV) for three further weeks (that is, until they were 10 weeks old) to act as controls. All treatments are summarised in Table 12.

Table 12. Summary of vernalisation/post-vernalisation treatments. The collective name for treatments with a common vernalisation duration are listed in the 'treatment' column. Specific treatments are denoted by an abbreviation comprising the vernalisation duration and a D for devernalising post-vernalisation temperatures of 32°C, or an O for 'optimal' post-vernalising temperatures of 22°C. Treatments were applied to seven week old plants. In addition, unvernalised control plants (UV) were maintained at 22°C at seven weeks of age for a further three weeks.

TREATMENT	ABBREVIATION	5°C VERN DURATION	POST-VERN TREAT (3 WEEKS)
2V	2D	2 days	32°C
	2O	2 days	22°C
7V	7D	7 days	32°C
	7O	7 days	22°C
28V	28D	28 days	32°C
	28O	28 days	22°C
Unvernalised	UV	N/A	N/A

At the conclusion of these treatments, leaf and apex tissue from two out of the six replicates from each treatment, and also from the 10 week old controls, was harvested for RNA extraction and analysis of gene expression as detailed below. The remaining four plants from each treatment (and 10 week old controls) were removed from growth cabinets and placed in glasshouse conditions where temperatures averaged between 14.5°C and 19.3°C (absolute minimum = 9°C and absolute maximum = 26°C) with photoperiods extended to 18 hours by 400 W high-pressure sodium lamps). Curd developmental stage was recorded each week according to the Curd Development Key (Figure 3) and throughout the experiment leaf/cicatrix numbers were noted. Because treatments differed in duration, timing of sowing was staggered so that the different treatments began on different dates. This allowed all treatments to conclude on the same date, thereby ensuring that all plants experienced the same post-treatment growth conditions.

In addition to harvesting plant tissues at the conclusion of each of the treatment and control periods, two additional replicates of leaf and apex material were harvested from UV plants aged 3, 5, 7 and 14 weeks. Furthermore, two additional replicates that had received two, seven or 28 days of vernalisation were immediately harvested with no subsequent period of growth in warmer conditions, devernalising or otherwise.

Tissue Harvest, RNA Extraction, RT-PCR and qPCR were performed according to the method described in Chapter II. RT-qPCR primer details and annealing temperatures for *BoVIN3, BoFLC2, BoFLC3, BoFT* and constitutive gene *BoACT* are listed in Chapter V.

2. *BoAP1* Isolation

Several primers were designed in order to amplify *BoAP1-a* and *BoAP1-c*. Primer details are shown in Table 13.

Table 13. Primers used in *BoAP1* isolation.

PRIMER	SEQUENCE	REFERENCE SEQUENCE
1. BoAP1-F1	5'-CTTTCCAATTGGTTCATACCAA-3'	Z37968 (Anthony, James & Jordan 1995)
2. BoAP1-F2	5'-CTCTTCTTGTAGTTTCTTAATGG-3'	Z37968 (Anthony, James & Jordan 1995)
3. BoAP1-R	5'-CATTTGCCGAACTATATAATGG-3'	Z37968 (Anthony, James & Jordan 1995)
4. BoAP1-a-F	5'-CTTGTTTTGTTTGGTTCTCTTAG-3'	AJ505845 (Kop 2002)
5. BoAP1-a-R	5'-ACTTGCTAATTATAAGAACATGG-3'	AJ505845 (Kop 2002)
6. BoAP1-c-F	5'-GGCTCTTTTAGAAGAAATAATAG-3'	AJ505846 (Kop 2002)
7. BoAP1-c-R1	5'-TAGCCAAAATATACTATGCGTC-3'	AJ505846 (Kop 2002)
8. BoAP1-c-R2	5'-CCTTGCCAAAATATATTAATTGG-3'	AJ505846 (Kop 2002)

Five primer combinations were used to amplify *BoAP1* fragments; (a) primers 1 and 3; (b) primers 2 and 3; (c) primers 4 and 5; (d) primers 6 and 7 and (e) primers 6 and 8. These combinations were tested on cDNA from several different sources including small cauliflower curds, mature cauliflower curds and cauliflower flowers. PCR denaturation was for 3 min at 94°C, followed by 35 cycles of denaturing, annealing and extension at 94°C for 30 sec, 50°C, 56°C and 58°C for 1 min and 72°C for 1 min, respectively, with a final elongation step of 72°C for 10 min. PCR products were cleaned and were then sequenced by the Australian Genome Research Facility Ltd. (AGRF).

Subsequently, primer combination (d) was used in a PCR on gDNA extracted from parent lines 36, 40 and 51 (denaturation at 94°C followed by 35 cycles of denaturing, annealing and extension at 94°C for 45 sec, 50°C for 45 min and 72°C for 3 min, respectively, with a final elongation step of 72°C for 10 min). The products from these PCRs were cloned with plasmid vectors, and sequenced by AGRF.

C. Results

1. Vernalisation & Post-Vernalisation Temperature Effects on Reproductive Development

As observed in previous vernalisation experiments (Chapter V), the average physiological age at which curds became evident decreased as vernalisation length increased (Figure 38); however, none of the differences in physiological age at curd appearance were statistically significant except in those plants vernalised for 28 days ($P<0.0001$). These 28D and 28O plants produced visible curds just one week after vernalisation (a physiological age of eight weeks, compared to plants in other treatments which did not form curds for up to 12 weeks after vernalisation (up to 19.5 weeks, phys. age). On average, 2D plants were slightly slower to form curds than 2O plants, but again, the difference was not statistically significant. There was no difference in physiological age of curd appearance between 7D and 7O treatments, nor was there any difference between 28D and 28O treatments.

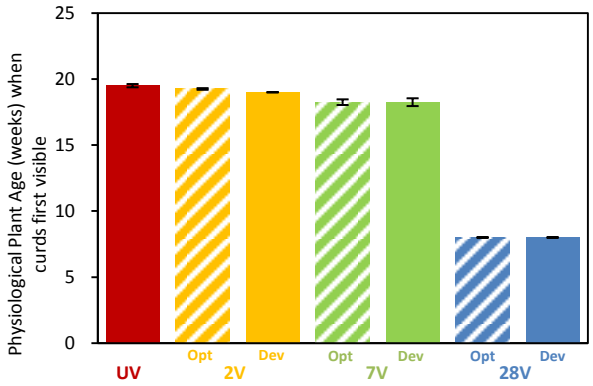


Fig. 38. Physiological age of cauliflowers when curds became visible. Physiological age refers to plant age in weeks, not counting weeks spent at 5°C. UV denotes unvernalised plants; 2V, 7V and 28V denote vernalisation duration in days; and Opt and Dev denotes optimal (22°C) or devernalising (32°C) post-vernalisation conditions, respectively. Bars represent the standard error of the mean (n=4).

Leaf number was used as an alternative method of quantifying the physiological age of the plants at visible curd formation – plants with fewer leaves were taken to be of a younger physiological age. The same general trend as above was observed, the only notable variation being that the 2D plants had an average of 20.25 leaves at curd formation, which was significantly different ($P=0.041$) from the 2O plants, which had an average of 24 leaves. If

taken at face value, this would suggest that the 2D plants formed curds at a younger physiological age than the 2O plants.

Although the post-devernalisation temperature conditions did not significantly affect the time until curd appearance (except for the plants vernalised for two days, when leaf number was used to quantify plant age), there were notable differences in curd morphology between the optimal and devernalising treatments, particularly between 28D and 28O treatments. Plants vernalised for 28 days and then grown in devernalising conditions produced curds with protrusions of numerous small bracts, and in some cases, the normal curd inflorescence meristems appeared to have reverted to vegetative apical buds. Upon removal from devernalising conditions, these bracty 'vegetative' buds within the curd structure recapitulated the earlier developmental process, giving rise to small, but otherwise normal curds, which in turn, elongated and flowered. By contrast, 28O plants produced curds that appeared 'normal', except for the fact that, shortly after curd formation, they began to produce distinct flower buds without any noticeable prior elongation of peduncles. These differences are shown in Figure 39. These morphological differences were not evident to the same degree in 2D/2O and 7D/7O treatments.

28D curd at conclusion of vern/post-vern treatment: small curd with bract-like growth

28O curd at conclusion of vern/post-vern treatment: large curd with distinct flower buds

28D curd one week after treatment: apical buds appear vegetative

28O curd one week after treatment: elongation of early-order branches bearing distinct flower buds

28D curd two weeks after treatment: apical buds appear vegetative, minimal elongation of branches

28O curd two weeks after treatment: elongation of later-order branches with fully-formed flower buds

28D curd three weeks after treatment: 'vegetative' buds open to reveal miniature curds

28O curd three weeks after treatment: several flower buds fully opened (corresponds to stage 5)

Fig. 39 (Previous page) Effect of post-vernalisation temperature on curd morphology. Both 28D (left column) and 28O (right column) plants had formed curds during the post-vernalisation stage of their treatment. By the end of this treatment, 28O curds had formed distinct flower buds, and within three weeks of treatment, curds were in the initial stages of flowering. In contrast, 28D plants had started to form 'vegetative' structures within the curd by the end of the treatment. Over the ensuing two weeks, curds expanded and the 'vegetative' apical buds developed. Three weeks after the treatment, the 'vegetative' structures opened to reveal compact masses of apical buds which appeared as a typical, but very small individual cauliflower curds. These eventually elongated and flowered in the normal manner of cauliflower curds.

In addition to the morphological differences between treatments 28O and 28D described above, the rate of growth was notably different between these two treatments, with the 28O curds growing much more rapidly (Figure 40). The growth rate of 2D and 7D plants was also reduced compared to 2O and 7O treatments, however the differences were not so great as observed between 28D and 28O.

Fig. 40. Effect of post-vernalisation temperature on curd growth rate. Both plants were exposed to 28 days of vernalisation at 5°C when seven weeks old. The plant on the left (28O) was then grown for three weeks at an 'optimal' temperature (22°C), while the plant on the right was subjected to three weeks of 'devernalisation' at 32°C. In both treatments, curds were first observed one week after being returned to warm conditions (two weeks before this photograph was taken). The plants grown in optimal conditions produced much larger curds than those grown at devernalising conditions. Also note the reduced epicuticular wax and curled leaf margins in the 28D plant.

This slower curd growth and development is reflected in flowering time data (Figure 41), where significant (P=0.042) differences were recorded in the average physiological age at anthesis between 28D and 28O plants; despite forming curds at the same age, the average physiological age of flowering in 28D plants was 18 weeks, compared to 15.5 weeks in 28O. In 2D/2O and 7D/7O treatments, flowering time was also consistently slower in devernalised plants, but these differences were not statistically significant. Generally speaking, treatments with longer vernalisation durations flowered faster, although 2D plants were slower to flower than plants that did not receive any vernalisation (not statistically significant).

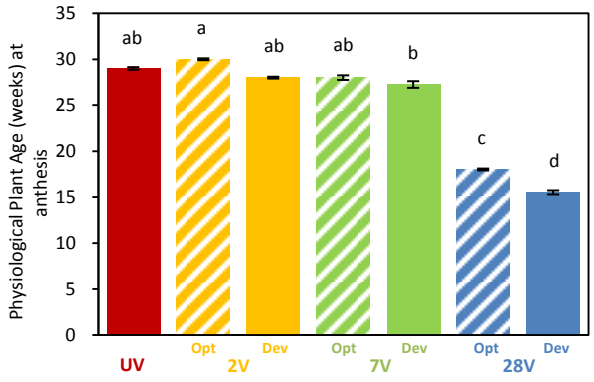


Fig. 41. Physiological age of cauliflowers at anthesis (stage 6). Physiological age refers to the plant's age (in weeks), not counting weeks spent at 5°C. UV denotes unvernalised plants; 2, 7 and 28 figures indicate the number of days of vernalisation; and D and O suffixes denote devernalising (32°C) or optimal (22°C) post-vernalisation conditions, respectively. Different letters above bars indicate significant (P<0.05) differences between treatments (LSD = 2.40). Bars represent the standard error of the mean (n=4).

2. Vernalisation & Post-Vernalisation Temperature Effects on Flowering Gene Expression

BoFLC2 expression in young leaves of parent line 50 was downregulated by seven and 28 days of vernalisation when plants were seven weeks old, but this was only significant in the 28V treatment (P=0.003; Figure 42). Two days of vernalisation were unexpectedly associated with a slight, but statistically significant (P=0.049) increase in *BoFLC2* transcript. Subsequent return of 2V plants to optimal conditions resulted in little *BoFLC2* expression change, and expression in plants returned to devernalising conditions was slightly lowered. In 7V and 28V treatments, the vernalisation-induced downregulation of *BoFLC2* was not stable, and gene expression returned to pre-vernalisation levels or higher once plants were returned to optimal conditions or exposed to devernalising temperatures. The average expression level of *BoFLC2* in 7V plants increased more when returned to optimal

temperatures than when exposed to devernalising conditions. By contrast, average expression of leaf *BoFLC2* in 28D plants was higher than that of 28O plants, although when the two replicates which comprised this point were examined individually, it was noted that this was only the case in one replicate. Post-vernalisation upregulation was highest, relative to expression levels immediately following vernalisation, in those plants vernalised for 28 days.

Figure 42 shows that *BoFLC3* expression profiles in young leaves were very similar to *BoFLC2* expression profiles. All three vernalisation treatments were associated with reductions in mRNA transcript levels, although the downregulation induced by the 2V treatment was not significant (P=0.166). Once again, the degree of downregulation was dependent on the length of vernalisation, and once again, the vernalisation-induced downregulation of *BoFLC3* was not stable; regardless of vernalisation duration, gene expression generally returned to pre-vernalisation levels or higher once plants were returned to optimal conditions or exposed to devernalising conditions. The increase in expression upon return to warm conditions was relatively larger in plants with a lower level of expression immediately subsequent to vernalisation. The average expression of *BoFLC3* was consistently higher in plants exposed to optimal conditions than those exposed to devernalising conditions, and the difference between these two treatments diminished with increasing vernalisation length; however, none of the differences in expression between plants exposed to 22°C and 32°C were statistically significant. As reported in the previous chapter, the overall level of expression was much higher in *BoFLC3* than in the mutant *BoFLC2*.

BoFT expression levels were not notably different to expression levels in unvernalised plants following 2V or 7V vernalisation treatments (Figure 42). In contrast, *BoFT* was significantly (P=0.005) upregulated after 28 days of vernalisation. In 2V and 7V vernalisation treatments, exposure to warm post-vernalisation temperatures was associated with moderate increases in *BoFT* expression, but there was no significant difference in expression between 32°C and 22°C treatments. The most striking feature of the *BoFT* expression profile was the upregulation of *BoFT* in both 28D and 28O plants post-vernalisation, relative to expression levels immediately following vernalisation, which was particularly dramatic in the 28D plants.

BoVIN3 expression data was more variable than that of other genes examined in this study (Figure 42). As in Figure 36, initial expression levels were high in one replicate, but this was not the case in the second replicate. No significant increases in transcript levels were measured immediately following any of the vernalisation treatments. In both 7V and 28V treatments, plants exposed to devernalising conditions had higher levels of *BoVIN3* transcript than those returned to optimal conditions, but again, there was considerable variability and this difference was not statistically significant.

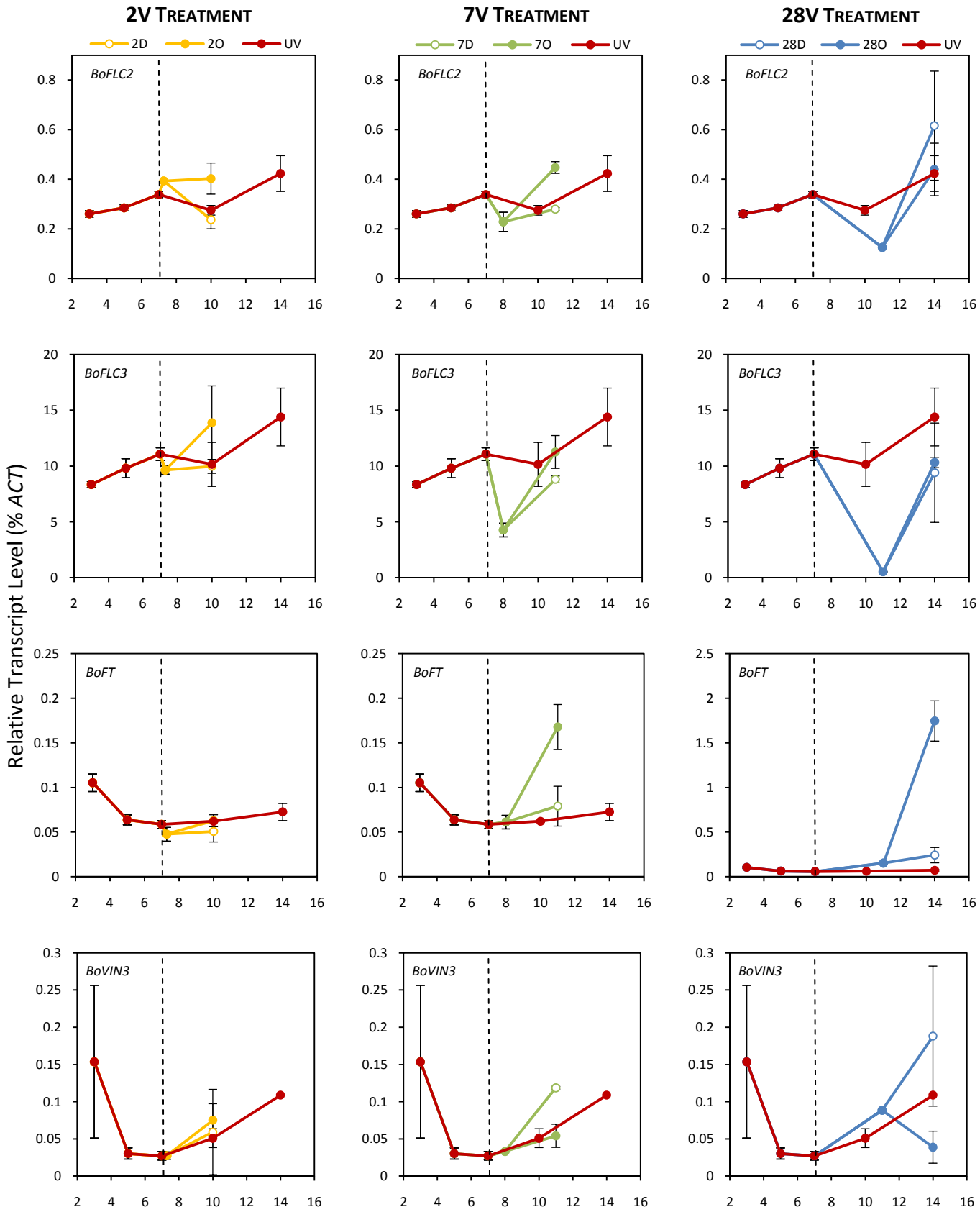


Fig. 42. *BoFLC2, BoFLC3, BoFT* and *BoVIN3* expression profiles for different vernalisation and post-vernalisation temperature treatments. Timing of vernalisation is indicated by a dashed line. Data points represent the average of two samples. Note the tenfold difference in scale between the 28V treatment and other vernalisation treatments in *BoFT* profiles. Bars represent the standard error of the mean (n=2).

3. *BoAP1* Isolation & Sequencing

Isolation of *BoAP1* genes was complicated by the presence of multiple very similar genes, and in the limited timeframe available for this work, suitable qPCR primers were not able to be successfully designed.

Primer combination (d) was the only combination that amplified a product fit for sequencing. The fragment sequence was identical to AJ505846 (Kop 2002). A PCR on genomic DNA was performed in order to identify the intron location in this sequence so that appropriate qPCR primers could be designed. Sequences were messy, indicating multiple products. In an attempt to isolate the PCR products, they were cloned in a plasmid vector and sequenced. The sequenced insert bore no significant similarity to any *AP1* genes; using the BLAST programme, the cloned products were found to show up to 93% homology with *Brassica rapa* subsp. *pekinensis* chloroplast sequence DQ231548.

D. Discussion

1. Vernalisation & Post-Vernalisation Temperature Effects on Reproductive Development

It is well-established that the timing of visible curd formation is directly related to vernalisation length in many cauliflower cultivars and the findings in both this chapter and the previous chapter are consistent with this principle. Unvernalised plants (grown at 22°C before transfer to 14.5°C – 19.3°C glasshouse conditions) eventually flowered, indicating that the vernalisation response seen in *boflc2* parent line 50 is non-obligate. This facultative response indicates that other genes (possibly including *BoFLC3*) are responding to, and being regulated by vernalisation, or alternatively, that ambient glasshouse temperatures are sufficient to induce vernalisation. Two days of vernalisation at 5°C were insufficient to promote significantly faster curding; plants vernalised for seven days were observed to curd slightly faster, but 28 days of vernalisation were needed before a statistically significant hastening of visible curd formation was measured.

The only vernalisation length treatment in which physiological age at visible curd formation was affected by post-vernalisation temperature was the 2V treatment, with devernalised plants slightly slower to form curds than those grown in 'optimal' conditions. This is possibly because these plants had received the least vernalisation, and were therefore more prone to devernalising effects than plants from the other treatments. The continuous gradient of heteroblastic leaf morphologies present, particularly in the 2V and 7V treatments, made it difficult to accurately count leaf number. As such, the leaf number method of determining physiological plant age was deemed misleading. The faster curd formation observed in 2D plants should therefore be regarded with circumspection.

Differences in the ontogenetic degree of development at the point of curd arrest between 28D and 28O treatments were noted. 28D plants exhibited bract-like vegetative structures subtending the floral stalks of the curds, and 28O had well-developed, distinct floral buds. Plants exposed to shorter vernalisation treatments did not display noticeable differences in curd morphology in response to post-vernalisation temperature. It is likely that this is because 28V plants formed curds just one week after the vernalisation treatment, meaning that the developing curd was exposed to two weeks of 22°C or 32°C before transfer to

glasshouse conditions, whereas the 2V and 7V plants had not formed visible curds at the time of post-vernalisation treatment.

The developmental stage of curd arrest has been shown to be temperature-dependent in some cauliflower varieties (Fujime 1983; Fujime & Okuda 1996; Duclos & Björkman 2008), with hotter temperatures resulting in earlier stages of curd arrest. For example, Fujime & Okuda (1996) noted that when young 'Snow Queen' cauliflower cultivars were exposed to temperatures above 30°C, bracty curds showing signs of floral reversion were produced; temperatures of 20 – 25°C resulted in normal curds arrested at the inflorescence meristem stage; temperatures below 15°C caused 'ricy' curds with mature floral buds. Clearly, parent line 50 is strongly influenced by temperature during curd development, with 32°C inducing floral reversion, and supposedly 'optimal' conditions of 22°C evidently sub-optimal for this particular cultivar, which is a *boflc2* mutant and is probably adapted to warmer climates. Besides differences in the stage of development at curd arrest, the rate of growth, in terms of curd size, was much slower in devernalised plants than in optimal plants, especially in the 28O and 28D treatments. The effect was probably less noticeable in 2V and 7V plants because curds were not produced until they were transferred to glasshouse conditions.

Once again, flowering generally occurred at a younger physiological age in plants that had been vernalised for longer. Although there was very little difference in physiological age at visible curd formation between plants grown in the two post-vernalisation conditions, devernalised plants consistently flowered when they were physiologically older than plants grown at optimal temperatures. This suggests that in parent line 50, an important effect of hot post-vernalisation temperatures is slowed curd growth and development, as distinct from delayed curd initiation. Both the susceptibility of different brassica genotypes to devernalisation and their sensitivity to post-vernalisation temperature effects on the ontogenetic stage of curd arrest may be a significant source of flowering time variation between parent lines in seed crops.

In this experiment, 28D plants flowered on average 2.5 days later than 28O plants. This contrasts with the shorter vernalisation treatments, where 7D plants flowered only 0.75 days later than 7O, and 2D plants actually flowered earlier than 2O, by two days. It is suspected that the larger difference between 28D and 28O treatments is primarily due to the fact that the developing curd was exposed to hot temperatures, leading to delayed growth and floral

reversion. In the other treatments, the smaller difference between treatments D and O are more likely to be due to 'true' devernalisation, with the 2V treatment more susceptible to devernalisation than the 7V treatment. Although the difference was not statistically significant, the average physiological age at flowering was greater in 2D plants than in UV plants, suggesting that hot post-vernalisation temperatures in plants that have received only short periods of vernalisation may result in slower flowering than if the plant was never vernalised at all.

In order to assess whether these differences in curd growth and development are due specifically to vernalisation, or whether general stress factors associated with high temperatures are involved, the expression of genes throughout the floral induction pathway are now considered.

2. Expression of Genes in the Vernalisation Pathway

This experiment was conducted before the contribution of *BoFLC2* functionality to cauliflower flowering time had been established. Parent line 50 was selected for examination to complement concurrent brassica research, but in retrospect, the selection of a *boflc2* line was unfortunate because the expression behaviour of *boflc2* is unlikely to be directly contributing to flowering behaviour. Although designated by Rijk Zwaan breeders as a 'medium late'-flowering line, these results indicate that curding occurs in the absence of vernalisation, meaning that there was less opportunity to test whether vernalisation length reduced the propensity for devernalisation.

Two days of vernalisation did not significantly affect the expression of either of *BoFLC2* or *BoFLC3*, but more sustained periods of vernalisation had the effect of downregulating both genes, with the degree of downregulation proportional to the length of vernalisation. A novel finding of this study was that the vernalisation-induced repression of both *BoFLC* genes was unstable, returning to pre-vernalisation levels or higher upon return to warmer conditions, regardless of vernalisation duration. This is in contrast to the Arabidopsis model, in which *FLC* is stably downregulated. In recent years, several authors have provided examples of unstable *FLC* downregulation in other members of the Brassicaceae: for example, Lin (2005) found that the downregulation of functional *BoFLC4-1* in transgenic Arabidopsis by seed vernalisation was unstable and incomplete. In white mustard (*Sinapis alba*; also known as

Brassica hirta (Primard et al. 1988)), *SaFLC* in two-week-old seedlings was unstably downregulated by one week of vernalisation at 7°C, with stable downregulation produced by longer vernalisation (D'Aloia, Tocquin & Périlleux 2008). Wang et al. (2009) described an *FLC* orthologue (*PEP1*) whose vernalised, downregulated state was not stable in *Arabis alpina*, where uninterrupted cold exposure is required for the cold-promoted transition to flowering to occur. However, this study identifies the first known example of unstable downregulation of *FLC* in a member of *B. oleracea*.

Bernier, Kinet & Sachs (1981) have noted that the physiological manifestations of devernalisation are most evident when the heat treatment is applied immediately after cold treatment, but that this effect declines with increased duration of vernalisation. Hence, whilst a return to pre-vernalisation *BoFLC* expression might have at least been consistent with the expected physiological effects of devernalisation following the 2V treatment, it is surprising to find that 28 days of vernalisation at 5°C did not affect a stable repression of *BoFLC*. It would be interesting to see whether *BoFLC* repression is stable in plants that are gradually returned to warm conditions.

BoFLC3 expression was consistently higher in plants returned to optimal temperatures than in those returned to devernalising temperatures, and *BoFLC2* was higher in all optimal post-vernalisation treatments except in the 28V plants. However, these differences were not statistically significant, with considerable variation within the two replicates. We had initially expected devernalised plants to exhibit higher levels of both *BoFLC* transcripts, but the gene expression findings are consistent with the minimal differences observed in age at visible curd formation between optimal and devernalised plants.

Given the status of *FLC* downregulation as a molecular marker of the vernalised state (Michaels & Amasino 2000), the discovery that *BoFLC* is upregulated in plants exposed to warm temperatures following vernalisation is important in that it potentially localises the genes regulated by the devernalisation process upstream of *BoFLC*, as opposed to alternatives downstream (for example, at the *BoFT* promoter). Although the homologue *boflc2* was non-functional in this particular line, its expression profile would be expected to reflect upstream processes, even if it is not itself playing a role in the vernalisation pathway. Furthermore, the almost identical behaviour of *BoFLC3* is consistent with an upstream process resulting in reversal of the vernalised state in response to high temperatures. Nonetheless, it would be

valuable to observe whether the same degree of *BoFLC2* instability is present in functional lines. In order to fully understand the role of this gene in devernalisation, further studies of vernalisation treatments of the effect of devernalisation on both *BoFLC2* and *boflc2* lines would be necessary. Stable repression of functional *BoFLC2* associated with reduced devernalisation sensitivity would support the idea that maintenance of the vernalised state (i.e. by *VRN1* and *VRN2*) is dependent on functionality of the *BoFLC2* gene, providing a target for plant breeders seeking to develop varieties suitable for warmer, drier production conditions.

Based on our current knowledge of the behaviour of the genes upstream of *FLC*, it appears possible that the instability of *BoFLC3* and (despite its non-functionality) *BoFLC2* in this particular cauliflower parent line may possibly be due to incomplete or reversed methylation by *VRN1/VRN2*, raising the question of whether this mechanism may underpin the process of devernalisation in general. For example, Gendall et al. (2001) and Levy et al. (2002) both reported that *FLC* returned to pre-vernalisation levels when returned to warm conditions in *vrn1* and *vrn2* Arabidopsis mutants, and Trap-Gentil et al. (2011) recently showed that devernalisation increased DNA methylation levels in the SAM of *Beta vulgaris.* The mechanisms of epigenetic silencing are currently receiving a great deal of attention, and studies published at the time of writing have provided evidence that the epigenetic silencing and stable repression of *AtFLC* depends on COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR), an antisense transcript encoded at the *FLC* locus, that, along with PcG protein complexes, is involved in *FLC* chromatin remodelling (Heo & Sung 2011). A similar antisense transcript called COOLAIR (Swiezewski et al. 2009) is also proposed to play an important role in the epigenetic downregulation of *FLC.*

There was little change in *BoFT* expression after two days or one week of vernalisation. After four weeks of vernalisation, *BoFT* had increased significantly (P=0.005), reflecting the younger curding and flowering age in 28V plants. After return to warm conditions, increases in *BoFT* expression proportional to vernalisation length were noted in all plants; there was no reversal of *BoFT* expression, such as was observed in the *BoFLC* genes. Plants that were returned to optimal conditions always evinced greater upregulation than those returned to devernalising conditions. The difference in *BoFT* expression between the two post-vernalisation treatments increased as the vernalisation length increased. These findings are consistent with the observed physiological behaviour (most notably, later curding and

flowering in 28D as opposed to 28O plants) and with the expectation that longer vernalisation durations would assuage the effects of devernalisation.

Unlike other gene expression studies contained in this thesis, which typically show an inverse relationship between *BoFLC* and *BoFT*, these findings reinforce that *BoFT* is regulated by temperature via other genes besides *BoFLC*. A prolonged period of vernalisation clearly promotes reproductive development, despite the non-functionality of *boflc2*, causing *BoFT* to be upregulated. This may be via other *BoFLC* genes not investigated here, elements of the autonomous induction process or an unknown *BoFLC*-independent pathway that is vernalisation-responsive. Furthermore, it is possible that an interaction between temperature and photoperiod may influence *BoFT* expression through genes such as *CO*; for example, increases in responsiveness to LD photoperiods have been observed when LDs immediately follow vernalisation, with sub-optimal vernalisation treatments capable of inducing flowering if they occur when the photoperiod is lengthening, as in Spring (D'Aloia, Tocquin & Périlleux 2008).

Vernalisation for two or seven days had little effect on *BoVIN3* expression. This is in contrast to results from the previous chapter which showed that even short vernalisation treatments were capable of upregulating *BoVIN3* in ten-week-old plants. Given the evidence that *BoVIN3* responsiveness to vernalisation increases with plant age, it is possible that one reason for the absence of upregulation seen here was that the plants were just seven weeks of age. The 28V treatment increased *BoVIN3* expression in one replicate, consistent with the strong downregulation of *BoFLC* observed in this treatment. Differences between optimal and devernalising treatments were unclear due to high variability. As it seems that *BoFLC* is of less consequence to flowering time in this parent line, it also seems that the importance of *BoVIN3* is reduced in this line. Subsequent studies could be improved by defining the conditions and genotypes in which the devernalisation effect is strongest before attempting to analyse gene expression. For example, the use of *BoFLC2* parent lines known to be susceptible to devernalisation and floral reversion (such as lines 4, 12 and 46: see Chapter VII), the application of a broader range of vernalisation treatments, and greater replication to allow for variability in gene expression would enable a more definitive assessment of both the molecular basis of devernalisation, and physiological aspects, such as the effect of vernalisation length on the capacity of plants to be devernalised.

3. *BoAP1-c* Isolation

The role of *BoAP1* in curd development remains of considerable practical interest, with the possibility that this gene could be used as an indicator of bolting in those varieties with curds that remain arrested for extended periods. It may also serve as an early indicator for floral reversion or devernalisaion. For example, Anthony, James & Jordan (1996) reported that *BoAP1* is responsive to post-vernalisation temperatures, with expression decreasing in devernalising conditions (the opposite of what occurs in Arabidopsis). Smith & King (2000) have suggested that the differences in developmental stage at curd arrest between heading brassicas are due primarily to the combination of *BoCAL-a* and *BoAP1-a* alleles, with temperature effects also influencing curd arrest stage. Kop et al. (2003) proposed that *BoAP1-a* allele may have a role in bract suppression and reversion, and Carr & Irish (1997) note that *AP1* expression is strongly associated with floral determination, indicating that the *Boap1-a* allele present in many cauliflower varieties may serve to undermine floral commitment and the capacity for maintenance of floral identity. More recently, the importance of the allelic combination of these two genes in determining developmental stage of arrest has been questioned, following evidence that other genetic mechanisms contribute more to this process (Labate, Robertson & Bjorkman 2004; Labate et al. 2006; Duclos & Björkman 2008). Investigation of the expression of members of the *BoAP1* gene family in response to a broad range of vernalising/devernalising conditions in parallel with evaluation of other key genes in the brassica flowering pathway would help to clarify its role in floral determination and curd arrest.

E. Conclusion

Cultivation of vernalisation-responsive brassicas (including cauliflower) as annual seed crops in warm or dry areas exposes growers to the risk of unexpected high temperatures during the floral induction phase which may lead to uneven or delayed flowering and poor seed yields. Warmer environments associated with possible climate change also threaten to exacerbate this problem in growing environments that are currently marginal. By establishing the expression patterns of key flowering genes in response to temperatures shown to delay flowering, this study has confirmed the potential for mRNA transcript levels to serve as indicators of reproductive status.

BoFLC2 has been shown to make a strong contribution to flowering time (Chapter III), and this is reflected in the expression dynamics of functional alleles (Chapter V). In this study, unstable downregulation of *FLC* has been demonstrated for the first time in *B. oleracea*, indicating that the expression of both *BoFLC3* and the non-functional *boflc2* is affected by warm post-vernalisation temperatures, and that it is potentially the basis of devernalisation problems encountered in the production of seed crops. The finding that *BoFT* expression continues to rise in warm post-vernalisation environments reinforces the idea that other genes besides *BoFLC* are involved in its regulation. Differences in *BoFT* expression between 28D and 28O treatments are likely to be due the influence of temperature-regulated genes not investigated here.

Chapter VII

USING GENE EXPRESSION TO PREDICT FLOWERING TIME

A. Introduction

In the previous chapters, several key genes in the cauliflower flowering pathway were identified and characterised. This information has the potential to form the basis of genetic neural networks that may be used to model flowering behaviour in brassica crops. For example, Welch, Roe & Dong (2003) studied flowering time in a range of *A. thaliana* strains with loss-of-function mutations at key flowering network loci in an early attempt to convert the existing detailed knowledge of the qualitative aspects of genetic flowering networks into quantitative modelling tools that could be used to predict the overall integrated effects of these networks on flowering time. More recently, attempts have been made to develop genetically-informed, computational phenology models in agriculturally-significant crops such as soybean and pea (Messina et al. 2006; Wenden et al. 2009). It is expected that such models, which bridge the laboratory and field by integrating both genetic and environmental data, will become increasingly effective tools for predicting the timing of key biological events. Such crop models, however, are a long-term goal requiring detailed understanding of the genetic and molecular processes being modelled. A more immediate application for knowledge of the regulation of genes involved in cauliflower flowering pathways concerns the use of expression data as a means of predicting floral initiation in production environments in a way that would improve hybrid seed producers' ability to apply management strategies in a timely manner.

Although the molecular basis of vernalisation-dependent flowering time regulation in Arabidopsis has been intensively studied under controlled conditions, comparatively little is known about flowering gene expression control in complex natural environments. Recently, it has been shown that gene regulation in a fluctuating environment is substantially different to that observed in the homogenous and controlled environment of the laboratory (Richards et

al. 2009). Subsequent to the work described in this chapter, Aikawa et al. (2010) reported results from a two-year census of the seasonal expression of *FLC* in an perennial population of *Arabidopsis halleri* grown outdoors, finding that seasonal trends of temperature were detected at the level of *FLC* regulation. *FLC* was slowly downregulated throughout autumn and winter before being gradually upregulated in spring and summer. Fluctuations in temperature throughout these seasons had little effect on expression, suggesting the involvement of a 'buffering' mechanism in *FLC* regulation that allows seasonal cues to be extracted from the background noise of fluctuating temperatures.

Apart from the study of *FLC* expression in Arabidopsis (Aikawa et al. 2010), there are very few reports of the measurement of seasonal/temporal changes in gene expression under natural conditions. Certainly, there does not appear to be any published research into the seasonal expression of flowering genes in field crops. Consequently, it is important that this issue is addressed in cauliflower if the goal of using gene expression to predict flowering in dynamic and complex production environments is to be realised. Although interest in the development of genetically-informed models of progression towards flowering has increased in recent years (Uptmoor et al. 2008; Wilczek et al. 2009), the concept of using gene expression data to generate real-time feedback as to a plant's developmental status is highly novel. In this chapter, the first steps are taken towards understanding flowering gene expression throughout the growth and development of cauliflowers exposed to natural, fluctuating temperature conditions. This understanding will be essential to determine whether the correlations between gene expression and reproductive state seen in the previous chapters are also valid under field conditions, and will be used in an initial evaluation of the feasibility of using gene expression data to predict curd induction and flowering in a production environment.

B. Materials & Methods

The following experiments were conducted in the first and second years of this project in order to take advantage of existing plant material and field trials. In each of the experiments, tissue harvest, RNA extraction, RT-PCR and qPCR were performed according to the methods described in Chapter II. RT-qPCR primer details and annealing temperatures for *BoFLC2*, *BoFLC3*, *BoFT*, *BoVIN3* and reference gene *BoACT* are listed in Chapter V. All gene expression data presented in this chapter are based on one biological replicate only, and the fine details of the expression profiles should therefore be interpreted with care; nonetheless, the presence of internal replicates (i.e. multiple data points collected within a time series, or multiple lines from the same genotype) permits confident interpretation of general trends. All pot-grown plants were fertilised with Hoagland's solution (Hoagland & Broyer 1936) as required.

1. Pot-Based Outdoors Trial

Fifty seedlings of parent line 51 (*BoFLC2*) and 50 seedlings of parent line 48 (*boflc2*) were raised by Hills Transplants (Devonport, Tasmania) in a vermiculite-based seed raising mix. In order to facilitate normal plant growth and development and to synchronise flowering between the two lines, the earlier-flowering line 48 was sown later than the late-flowering line 51. Line 51 seeds were sown on the 22nd of February 2007 and seedlings were transplanted into 0.5 L and 4.5 L pots containing general purpose potting mix on the 4th of April and 2nd of May, respectively. Line 48 seeds were sown on the 7th of March, and seedlings were potted into 0.5 L and 4.5 L pots on the 27th of April and 30th of May, respectively. Plants were grown on outdoor terraces at the Horticultural Research Centre (Sandy Bay, Tasmania). Average maximum and minimum temperatures for the months during which the plants were grown are shown in Figure 43. Plants were fertilised with additional slow-release Osmocote 5-6 month, 11:4.8:14.9 (Scotts Australia, Baulkham Hills, NSW). Observations of plant development and harvesting of leaf and apex samples for RNA extraction were conducted every fourteen days.

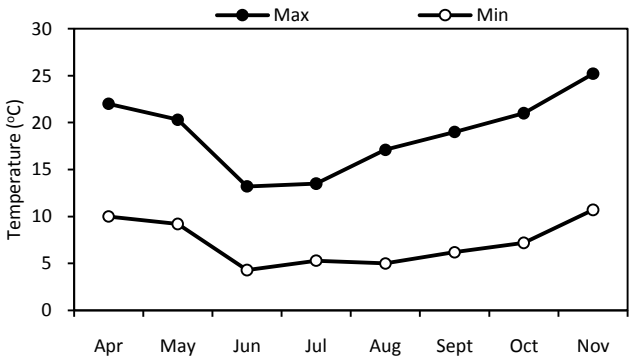


Fig. 43. Average 2007 maximum and minimum temperatures for the months April – November at the Horticultural Research Centre, Sandy Bay Tasmania.

2. Pot-Based Glasshouse Trial

Seeds from 25 different parent lines (Table 14) were sown into seed raising mix (Grow Better Garden Products Pty Ltd, Bayswater VIC), and 11 seedlings from each line were transplanted into 4.5 L pots containing general purpose potting mix when 37 days old. Plants were grown under glasshouse conditions, where, during the 91 weeks for which this trial was conducted, temperatures ranged from a minimum of 4.9°C in winter months, to a maximum of 43.7°C in the summertime. When aged 41 days, tissue was harvested from three replicates for RNA extraction; at this sample point, plants were too small to yield sufficient apical tissue for RNA extraction, so apex tissue was combined with young leaf tissue (c.f. Lin et al. 2005) based on previous observations that the genes being investigated were expressed similarly in shoot apices and young leaves. Parent lines 1 and 49 germinated poorly, meaning that there were insufficient replicates to allow RNA extraction at this sample point. When plants were 49 days old, five of the eight replicates were subjected to a mild vernalisation treatment at 7°C for 12 hours per night for three weeks. The developmental stage of plants was recorded on a weekly basis, and when visible curds were first observed, leaf and apical tissue was harvested for RNA extraction.

Table 14. Cauliflower parent lines in the pot-based glasshouse trial.

FLOWERING CLASS	LINES
Early	10, 32, 38, 40, 54
Med Early	6, 23, 36, 47, 48
Med Late	21, 41, 44, 49, 52
Late	1, 13, 17, 46, 51
Very Late	2, 4, 7, 11, 12

3. Field Trial

Fifteen cauliflower lines with flowering behaviour ranging from Early to Very Late, were raised by Hills Transplants (Devonport, Tasmania) in a vermiculite-based seed raising mix. Time of sowing was varied according to flowering behaviour in an attempt to achieve uniform curd initiation and flowering between lines; later-flowering lines were sown in mid-summer, and earlier-flowering lines were sown at the end of summer (details shown in Table 15). Seedlings were transplanted into field conditions when aged between 41 and 51 days.

Table 15. Cauliflower parent lines used in the Forthside field trial.

LINE	FLOWERING CLASS	DATE SOWN	DATE TRANSPLANTED
1	Late	5th Feb 07	18th Mar 07
2	Very Late	29th Jan 07	11th Mar 07
3	Very Late	29th Jan 07	11th Mar 07
7	Very Late	29th Jan 07	11th Mar 07
9	Late	5th Feb 07	18th Mar 07
21	Med-Late	12th Feb 07	1st Apr 07
25	Late	5th Feb 07	18th Mar 07
35	Early	26th Feb 07	18th Apr 07
36	Med-Early	19th Feb 07	8th Apr 07
37	Early	26th Feb 07	18th Apr 07
38	Early	26th Feb 07	18th Apr 07
41	Med-Late	12th Feb 07	1st Apr 07
44	Med-Late	12th Feb 07	1st Apr 07
47	Med-Early	19th Feb 07	8th Apr 07
48	Med-Early	19th Feb 07	8th Apr 07

The field site at the Forthside Vegetable Research Station, Northern Tasmania (41°12'15"S, 146°15'42"E, WGS84; climatic data shown in Figure 44) was sprayed with Roundup CT (450 g/L Glyphosate; 3 L/ha), and Mycrobor (21% boron; 10 kg/ha) and sodium molybdite (400 g/ha) were applied. The site was ploughed and fertilised with 1 ton/ha of Bejo proprietary fertiliser (3:5:14 + trace elements) and 1.6 m wide raised beds were formed with two rows per bed, each 800 mm apart. Prior to planting, the site was sprayed with Goal (240 g/L oxyfluorfen; 3 L/ha), Stomp440 (440 g/L pendimethalin; 2 L/ha) and Roundup CT (450 g/L Glyphosate; 2.5 L/ha). Experimental plots were arranged in a randomised complete block design, and plants were transplanted by hand into the basalt-derived red ferrosol soil at a spacing of approximately 450 mm. There were three replicates of each parent line, with approximately ten plants per replicate. Leaf and apex tissue was harvested for RNA extraction on the 13th of June. Observations of curd initiation and development were made every 7-11 days from the 31st of October until the 10th of December. Both curding and

flowering time were expressed as the average number of days from the 13th of June sampling date.

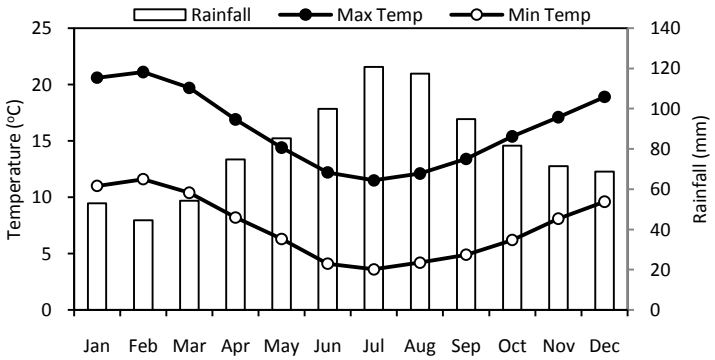


Fig. 44. Long-term mean monthly minimum and maximum temperature and rainfall data for Forthside Research Station weather station (Australian Bureau of Meteorology 2011).

C. Results

1. Pot-Based Outdoors Trial

Staggering the sowing time of late-flowering parent line 51 and early-flowering line 48 successfully synchronised the transition from vegetative to reproductive growth in the two lines, with both forming visible curds around the 3rd of August, and with subsequent reproductive development occurring at a similar rate. Comparison of changes in gene expression dynamics throughout plant growth between two different parent lines that are developing at a similar rate, and under the same environmental conditions, made it possible to attribute such changes to the reproductive transition, rather than to differing and unrelated external factors, with greater confidence.

Levels of *BoFLC2* mRNA in apices of the late flowering line 51 were initially high during early vegetative growth in late autumn (Figure 45). *BoFLC2* expression was rapidly downregulated throughout early winter, reaching minimum values by the 23rd of June, nearly a month before a swollen apical bud was detected. Expression remained low throughout early curd development, eventually rising slightly in curds with distinct floral buds. It has been previously established that the level of *BoFLC2* expression is usually much lower in *boflc2* lines than in *BoFLC2* lines (see Chapter V). Consistent with this, *boflc2* expression was greatly diminished in the non-functional line 48, but the same basic profile was recorded, albeit with more pronounced upregulation as curds elongated and flowered during the spring months.

Figure 45 shows that initial *BoFT* expression levels in line 51 were very low throughout May, June and July. A consistent, but slight upregulation representing a thirteenfold increase in mRNA levels between the 19th of April and the 20th of July may be seen when the data is viewed at a smaller scale, but it is not until the 3rd of August, when visible curds were first detected, that upregulation of *BoFT* accelerates substantially. However, these changes in *BoFT* expression are minor compared to the dramatic upregulation which occurs during curd expansion, and the subsequent upregulation associated with the development of floral buds throughout spring. The main *BoFT* upregulation occurred nearly a month after the *BoFT*-inhibitor *BoFLC2* bottomed out. In the younger, early-flowering parent line 48, a similar profile was generated, with a dramatic increase in *BoFT* expression also observed

immediately following curd formation, before mRNA levels taper off during later curd development. The absolute level of *BoFT* mRNA is much higher in the *boflc2* line, reflecting its lack of suppression by a functional *BoFLC2* gene, but because of a significant elapse of time between line 48 and line 51 RT-qPCRs and the fact that only one replicate was processed, this finding should be treated circumspectly.

BoFLC3 expression in parent 51 generally decreased throughout winter, and was low when curds were first observed (Figure 45). During curd elongation in spring, it increased slightly. In line 48, a similar profile was evident; initial *BoFLC3* expression levels were slightly higher than in parent 51 (c.f. differences in *BoFLC3* expression between *BoFLC2* and *boflc2* lines in Figure 36). *BoVIN3* expression in line 51 generally increased throughout autumn/winter before a peak on the 20th of July, when 'fat' apices were first noted. After this point, *BoVIN3* expression trended downwards. In the *boflc2* line, *BoVIN3* expression was highly erratic from week to week, and there does not appear to be any connection between the stage of reproductive development and *BoVIN3* expression.

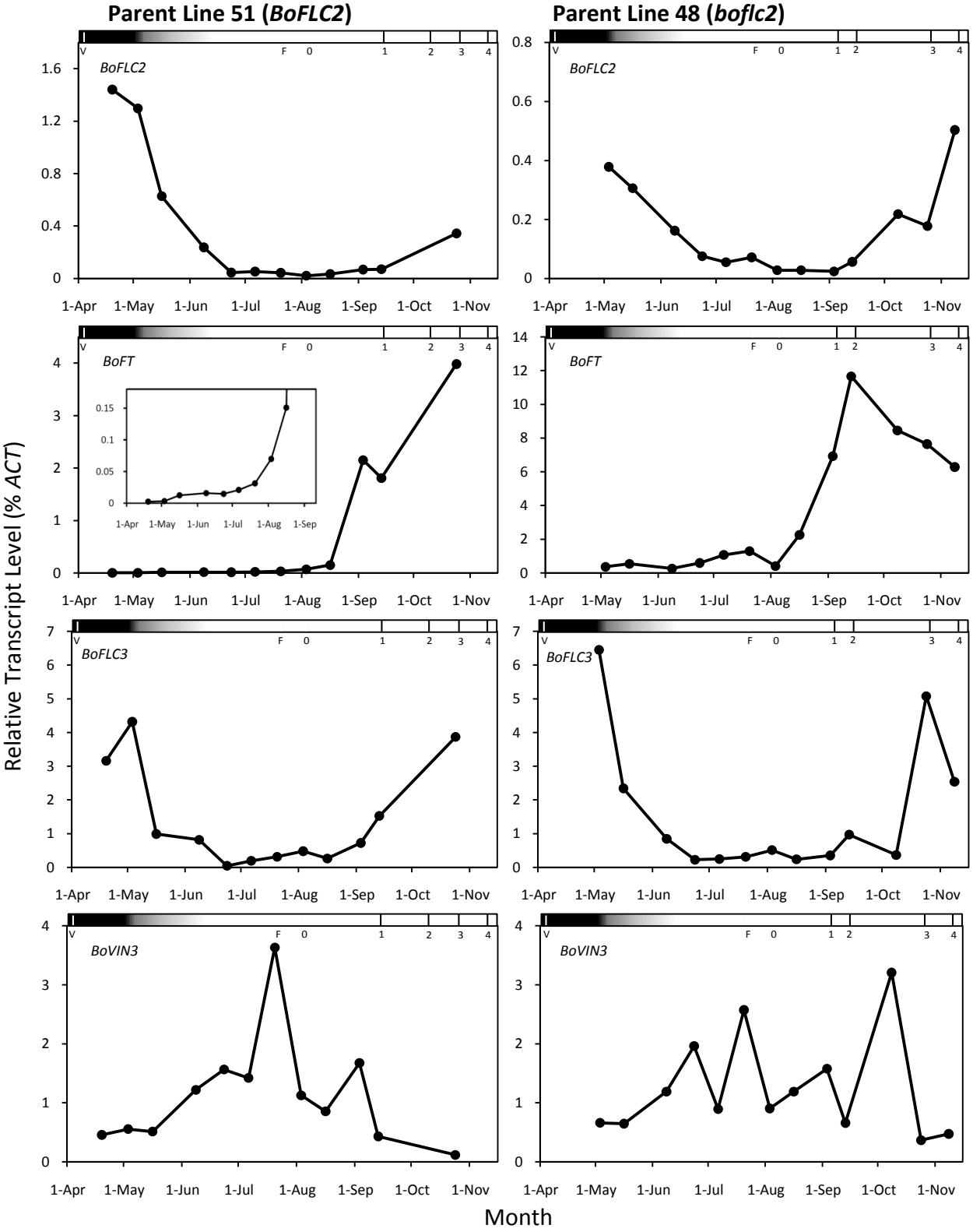


Fig. 45. Expression of *BoFLC2*, *BoFT*, *BoFLC3* and *BoVIN3* throughout autumn, winter and spring in apical tissue of parent lines 51 (*BoFLC2*; left) and 48 (*boflc2*; right). Inset: detail of *BoFT* expression between 19th April and 16th August. Values are based on one replicate only. The developmental stage of both parent lines is shown above each main graph, where V = vegetative, F = fat, expanded apex without any discernable curd structure, and 0-4 = developmental stages according to Figure 3.

2. Pot-Based Glasshouse Trial

The curd initiation behaviour observed in the 25 lines grown in the pot-based glasshouse trial was consistent with the data displayed in Figure 22; *boflc2* lines produced curds the earliest, followed by *BoFLC2* romanesco varieties, *boflc2* 'bunchy' lines and *BoFLC2* genotypes (Figure 46a). There was no significant difference in the number of days to curd formation between the five vernalised plants and the three unvernalised plants across the 25 lines (P=0.625), possibly because of the relative mildness and short duration of the vernalisation treatment relative to the cool night temperatures experienced in the glasshouse. Consequently, the two treatments were disregarded, and curding data for the five vernalised plants and three unvernalised plants were pooled to generate values that were the average of eight replicates.

A clear difference in *BoFLC2* transcript level during vegetative growth can once again be seen between *BoFLC2* and *boflc2* lines (Figure 46b). Because the *BoFLC2* mutation causes a loss of gene function, the relationship between *BoFLC2* expression and timing of reproductive development is disrupted in *boflc2* lines. Therefore, any correlation between *boflc2* expression and curding time is unlikely to be meaningful. When non-functional *boflc2* lines were disregarded and lines with functional *BoFLC2* were considered separately, a moderate, statistically significant correlation (r=0.557, P= 0.047) was seen between *BoFLC2* expression at the fixed vegetative stage, and the number of days until visible curd formation. For example, lines 2, 4 and 7 have the highest level of *BoFLC2* expression at this point, and are also among the slowest plants to form curds. Lines 51 and 46 are the two non-romanesco *BoFLC2* lines with the lowest level of *BoFLC2* expression, and are also the earliest-curding, non-romanesco *BoFLC2* parent lines. These results suggest that a single measurement of *BoFLC2* mRNA transcript prior to curd induction could potentially be used to predict time until curd formation. There was no correlation between pre-curding *BoFLC3* expression and the number of days until curd initiation. There were many late-curding lines that had very low pre-curding *BoFT* expression, and a number of early-curding parents that had high pre-curding *BoFT* expression. However, there were also a number of lines where this relationship is not maintained, and no significant correlation (r=-0.324, P=0.066) between pre-curding *BoFT* expression and the number of days from sowing until visible curd formation was found across the 25 lines.

The intention in measuring the level of gene expression at the point of curd formation was to identify molecular variation between parent lines and to establish whether there were key differences in the regulation and behaviour of flowering time genes between early- and late-flowering genotypes during the reproductive transition. Figure 46c shows that of the three genes investigated, the greatest expression variability between lines at curd formation is in *BoFLC2*, consistent with the emerging hypothesis that this gene is the single most important source of variation in cauliflower flowering time. It is noteworthy that two of the parents with the highest level of *BoFLC2* expression at visible curd formation – lines 4 and 12 – were also lines that reverted to vegetative growth (Figure 47a) following the formation of a small curd with numerous vegetative structures. It is possible that increased *BoFLC2* expression in these lines was associated with reversion (as noted previously in Chapter VI, Figure 42). Furthermore, *BoFLC2* expression in lines 2, 7 and 11 was also high, possibly reflecting the fact that these lines never actually formed curds (Figure 47b). However, the level of *BoFLC2* expression seems somewhat incongruous in several of the parent lines; for example, *BoFLC2* expression of the romanesco line 44 appears to be relatively high considering the fact that it was amongst the earliest-curding *BoFLC2* lines, and parent line 46 also exhibited floral reversion similar to lines 4 and 12, and yet, *BoFLC2* expression is relatively low in this line.

BoFLC3 and *BoFT* expression was fairly uniform between the lines at the point of curd initiation, excepting the high level of *BoFT* expression at the point of curd initiation in parent lines 54 and 10. Throughout all experiments in this thesis, these two lines are consistently among the fastest-flowering, and it is possible that the maintenance of *BoFT* upregulation post-curd induction is somehow involved in the rapid transition to flowering exhibited by these two lines. There are several lines with lower *BoFT* expression at visible curd formation than those lines which never formed curds. It is possible that *BoFT* transcript would have decreased immediately prior to curding in these lines, but measurement ceased before this could be confirmed.

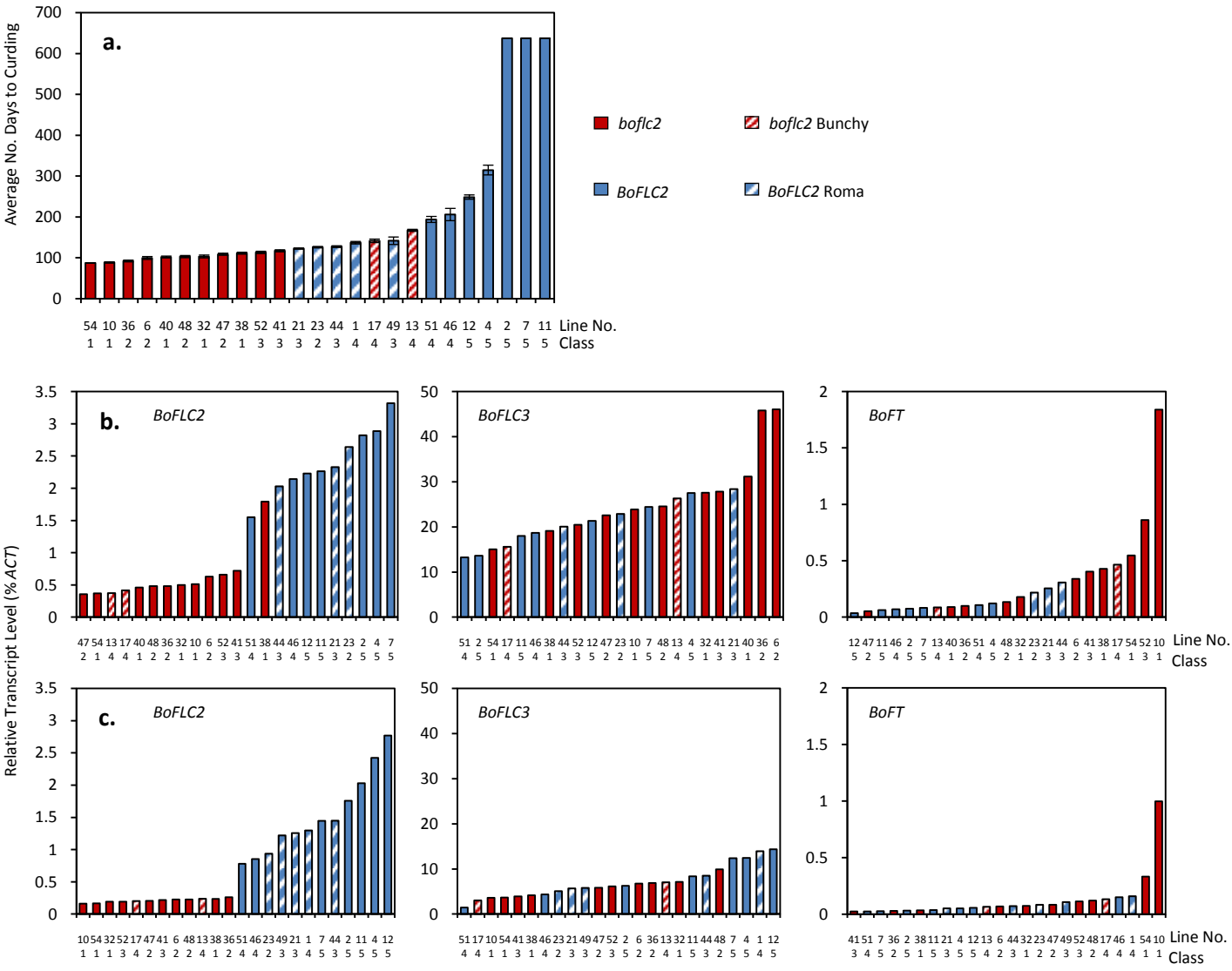


Fig. 46. Average number of days to curding in cauliflower parent lines grown at under glasshouse conditions (a), and relative *BoFLC2*, *BoFLC3* and *BoFT* expression in leaves at (b) a fixed time point during the vegetative phase (41 days old) and (c) at visible curd formation. Values for the average number of days to curding are counted from sowing and are the average of up to eight plants, with dead or missing plants excluded from the analysis. Plants that had not curded by the end of the observation period were assigned a maximum score of 637 days. For the expression data, only one replicate is shown. For those lines that never formed curds, the second tissue sample for RNA isolation was harvested at day 637, when apices were still vegetative. Red and blue columns denote mutant (*boflc2*) and functional (*BoFLC2*) parent lines, respectively. Columns with thick stripes represent romanesco parent lines, and columns with thin stripes denote *boflc2* lines that formed a tightly-bunched 'head' of leaves prior to curd formation. The flowering class of each parent line (as described in Table 2) is shown beneath the line number, where class 1 = Early; 2 = Med Early; 3 = Late Early; 4 = Late; 5 = Very Late. Bars represent the standard error of the mean.

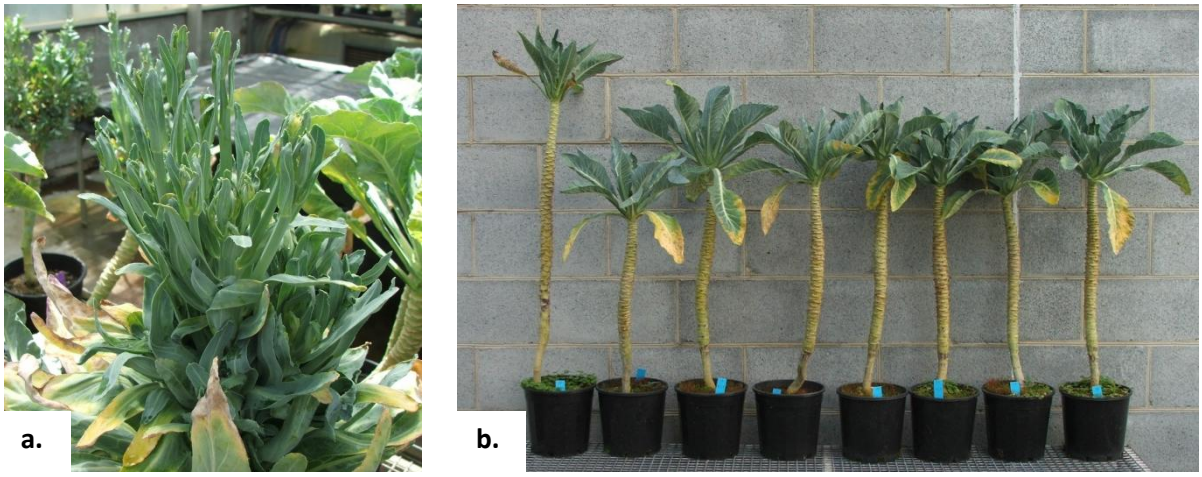


Fig. 47. Floral reversion in a glasshouse-grown line 4 plant (a) and vegetative line 11 plants grown in a glasshouse for over 80 weeks (b).

3. Field Trial

Although attempts were made to synchronise reproductive development to some degree by staggering the sowing and transplanting of cauliflower parent lines at the Forthside trial site, differences in both curding and flowering time between lines were noted (Figure 48a and 47b). Parent lines 37 and 41 had already formed curds by the first observation date, and because observations were infrequent during the period when parent lines 1, 9, 21 and 25 began to form curds, the figures presented for these lines are approximate only (Figure 48a). Despite the fact that they formed the earliest curds under warmer glasshouse conditions, curd formation was normally slowest in the *boflc2* lines from the earliest two flowering classes (e.g. lines 35, 36, 37, 47 and 48) under field conditions. Although some delay in curding due to slower accumulation of the necessary GDD was expected when these lines were grown in a colder environment, it seems likely that the drastically delayed curding in this trial is primarily because these varieties are adapted to warm, or even tropical environments, and were grossly unsuited, in terms of whole-plant biology, to the cold field conditions, leading to ill-health and suppressed growth and development. *BoFLC2* lines were better-suited to the growing conditions, and of these lines, parents 2, 3 and 7 were the slowest to form curds. The romanesco varieties 1, 21 and 44, along with parent line 25, formed curds much more rapidly.

Flowering behaviour was closely related to curd formation, with *boflc2* lines 25, 26, 38 and 48 slowest to flower due to poor growth (Figure 48b). The early-curding line 41 was also early-flowering in this environment, but the curds of early-curding line 37 were susceptible to

fungal infection, resulting in many plant deaths, and generally delaying flowering time. Once again, *BoFLC2* lines 2, 3 and 7 were later to flower than the romanesco lines. These were in turn later than line 25, which was slow to progress from the curd stage to the flowering stage. The shoot apex gene expression data displayed in Figure 48c are based on one replicate only. Additional replicates were also processed, revealing very similar relative expression, but different apparent absolute levels of expression. Where gene expression for a parent line differed between replicates, this was invariably consistent with differences in the stage of development of the individual harvested plants. Consequently, only one replicate is presented for the sake of clarity.

Consistent with previous findings that *BoFLC2* expression is greatly reduced in *boflc2* lines, measurement of apex *BoFLC2* mRNA transcript levels on the 13th of June revealed that *BoFLC2* was, in a majority of cases, substantially higher in *BoFLC2* parent lines than in *boflc2* parent lines (Figure 48c). *BoFLC2* lines 2, 3 and 7 had the highest levels of *BoFLC2* expression, consistent with the fact that they were the slowest curding and flowering *BoFLC2* plants. Romanesco lines 1, 21 and 44 were clustered together as the group of lines with the second-highest level of *BoFLC2* expression, once again consistent with their curding and flowering behaviour. *BoFLC2* expression levels in line 25 plants were similar to those measured in *boflc2* lines, and were notably lower than the typical expression levels measured in other *BoFLC2* lines, consistent with line 25 being the earliest-flowering parent line investigated in this study. Within the *BoFLC2* parent lines, there was a strong positive correlation between *BoFLC2* expression on the 13th of June and both the average number of days to curding ($r=0.892$, $P=0.003$) and the average number of days to flowering ($r=0.880$, $P=0.004$). These results provide strong evidence in support of the idea that *BoFLC2* expression in vegetative plants is a reliable indicator of curding and flowering time in *BoFLC2* parent lines.

By contrast, *BoFLC3* expression in apices prior to the transition from vegetative to reproductive development bears no discernable relation to either curding or flowering time across the range of parent lines (Figure 48c), with no strong or significant correlation. Although some early-curding and flowering lines (e.g. lines 25 and 37) had low levels of *BoFLC3* expression, and some late-curding and flowering lines (e.g. lines 36, 35 and 33) had relatively high levels of *BoFLC3* expression, there were many parent lines whose *BoFLC3* expression appeared to be inconsistent with the observed flowering time, and the status of

BoFLC3 as a putative contributor to the vernalisation/flowering time response, supporting the idea that *BoFLC2* the chief regulator of this process in cauliflower.

BoFT expression levels in apices on the 13th of June sampling date were consistent with subsequent reproductive behaviour. A moderate negative correlation was found between *BoFT* expression and both curding time (r=-0.514, P=0.025) and flowering time (r=-4.40, P=0.05) across both *BoFLC2* and *boflc2* genotypes. Of the eight lines with the lowest level of *BoFT* expression, seven (2, 3, 7, 35, 36, 38 and 47) were represented in the eight most late-curding lines (Figure 48c). The four individual harvested plants representing the four lines with the highest level of *BoFT* expression (37, 41, 44 and 48) each had a small 'buttoned' curd, possibly accounting for the discrepancies between the expression data and the observed average curding/flowering time of that group of plants as a whole (e.g. parent line 48). Romanesco lines 1 and 21, which had the next-highest *BoFT* mRNA transcript levels formed curds very shortly afterwards. Therefore, notwithstanding some unexpected results (such as the early-flowering line having a relatively low level of *BoFT* mRNA), it seems that *BoFT* expression, like *BoFLC2* expression, appears to be useful for predicting flowering time of cauliflower plants in a production environment. Unlike *BoFLC2* however, *BoFT* may potentially be used as an indication of curding and flowering time for both *BoFLC2* and *boflc2* varieties.

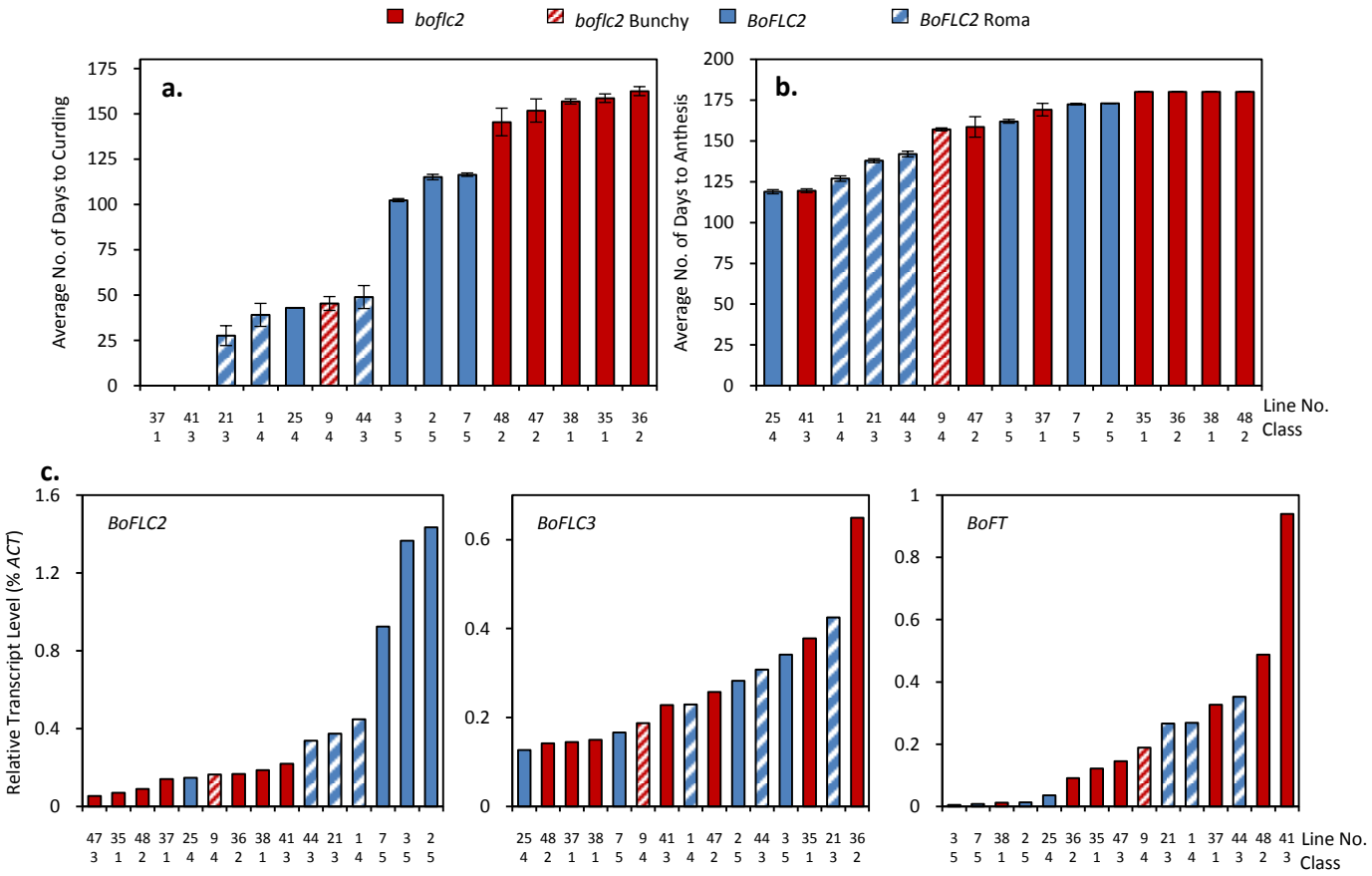


Fig. 48. Average number of days to curding (a) and anthesis (b) in cauliflower parent lines grown at the Forthside field site and relative *BoFLC2, BoFLC3* and *BoFT* expression in apical tissue at a fixed time point (c). Values for the average number of days to both curding and anthesis are counted from the 13th of June and are the average of up to 30 plants (three replicates with 10 duplicates in each replicate), with dead or missing plants excluded from the analysis. Plants that had not curded or flowered by the end of the observation period were assigned a maximum score of 180 days. Gene expression data was collected on the 13th of June. Only one replicate is shown here. Red and blue columns denote mutant (*boflc2*) and functional (*BoFLC2*) parent lines, respectively. Columns with thick stripes represent romanesco parent lines, and columns with thin stripes denote *boflc2* lines that formed a tightly-bunched 'head' of leaves prior to curd formation. The flowering class of each parent line (as described in Table 2) is shown beneath the line number, where class 1 = Early; 2 = Med Early; 3 = Late Early; 4 = Late; 5 = Very Late. Bars represent the standard error of the mean.

D. Discussion

The problem of asynchronous flowering between parent lines in cauliflower hybrid seed crops could potentially benefit from a reliable method of predicting curd initiation and flowering. Even with thorough knowledge of a cultivar's reproductive physiology, seasonal variability and vagaries of the local environment often result in unexpected flowering behaviour. Having isolated several key flowering genes for the first time in cauliflower, and having demonstrated both conserved and unique aspects of their behaviour, there exists an opportunity to apply knowledge of flowering gene regulation in a practical manner that may be used to accurately predict upcoming reproductive transitions and provide growers with early warning of delayed or premature flowering. Specifically, the measurement of mRNA levels of *BoFLC2* and *BoFT* during vegetative growth, and the timely analysis of this data could provide real-time feedback as to the plant's developmental status.

The three experiments in this chapter were conducted prior to identification of the previously-described *BoFLC2* mutation, and before the full importance of the functionality of *BoFLC2* to flowering time was understood. Consequently, the *boflc2* lines grown in these experiments were selected purely for their early-flowering behaviour, without any knowledge of their *BoFLC2* genotype. As a result, the *boflc2* expression of these mutant lines is not especially informative in terms of using *BoFLC2* expression to predict flowering time. However, the use of these non-functional lines emphasises that the primary source of flowering-related molecular variation between late- and early-flowering parent lines is *BoFLC2* functionality, with no major differences in expression of other genes between *boflc2* and *BoFLC2* parent lines (apart from the reduction in *BoFT* mRNA levels, which is likely to be a direct result of the *BoFLC2* mutation, and potentially indicates both a general and vernalisation-dependent component to *BoFLC2* repression of *BoFT*).

The data in this chapter show that many of the molecular responses to vernalisation observed in controlled-environment experiments are also present in 'real-world' plant growth environments. For example, it was shown that 'natural' vernalising temperatures are capable of downregulating *BoFLC2* and upregulating *BoFT*. All previous experiments have involved 'artificial' vernalisation treatments, where plants grown in warm growth cabinets were abruptly transferred to vernalising temperatures to elicit changes in the expression of *BoFLC2* and *BoFT*. In Figure 45, *BoFLC2* is shown to be downregulated by cool autumn

temperatures over the space of several months. *BoFT* is also upregulated slowly throughout autumn. A dramatic and sudden increase in *BoFT* mRNA levels is evident as curds form and develop, despite there being no 'sudden' vernalisation. *BoVIN3* also increases slowly in the *BoFLC2* line, with expression peaking at a point just prior to curd initiation. These long-term gene expression studies provide the first realistic profile of gene expression under natural conditions in cauliflower, and are an essential link between controlled-environment experiments and 'real-world' application. Importantly, they also indicate robust regulation of the long-term control of *BoFLC* and *BoFT* in both lines, with episodic warm temperatures throughout the autumn and winter months seeming to have little influence on the overall direction of gene regulation. This suggests that the epigenetic memory of winter in these two lines is sufficiently long to buffer against short-term temperature fluctuations (c.f. Aikawa et al. 2010). It would be informative to observe whether this is also the case in the parent line utilised in the previous chapter, where downregulation of *BoFLC2* was not stable upon exposure to warm temperatures.

Figure 45 also shows that gene expression may be used to predict curd and floral development. For example, downregulation of *BoFLC2* occurs over a month prior to curd initiation. By assaying this gene in seed crop parent lines throughout early growth, it could be determined whether this critical downregulation had occurred, and whether a plant is competent to flower in this respect. Figure 45 also indicates, somewhat counter-intuitively, that both *BoFLC2* and *BoFLC3* expression increases slightly as curds break and progress to flowering. This could potentially be useful in cultivars where curds remain at the arrested stage for extended periods of time. However, given that this figure is based on just one replicate, further investigation is necessary to validate this behaviour. Furthermore, it is certainly possible that gradual upregulation of *BoFLC2* in both lines during bolting, and the gradual downregulation of *BoFT* during bolting of the *boflc2* parent, is simply a seasonal response to warmer spring temperature, rather than an important component of the bolting process. Indeed, recent research by Aikawa et al. (2010) described the cyclic nature of *FLC* expression in perennial *A. halleri* plants grown outdoors over two seasons. Thus, while it appears that the memory of winter in these two lines is sufficiently long to buffer short-term temperature fluctuations as noted above, it also appears that the epigenetic memory of past temperatures is short enough to enable responses to a seasonal trend. Evaluation over multiple seasons would be necessary to verify this. Genes further downstream that are known to confer inflorescence identity such as *BoAP1* and *BoFH* (*LFY*) could potentially be

more useful and relevant for the purpose of predicting bolting and flowering, and would be worthy subjects of future research in this area. The extreme upregulation of *BoFT* did not appear until curds had already formed, making it an unsuitable indicator of curding time; however, the slight upregulation seen prior to curd initiation has potential to indicate the onset of curding.

Results from the pot-based glasshouse trial (Figure 46) revealed a positive correlation between *BoFLC2* expression at a fixed time point and curd initiation timing across a range of *BoFLC2* parent lines. Similarly, results from the field-based trial (Figure 48) showed that *BoFLC2* expression at a fixed time point was correlated with both curding and flowering time across *BoFLC2* parent lines. In the field trial especially, a clear relationship between *BoFLC2* expression and flowering time was evident, with the flowering time of *BoFLC2* lines arranged into three groups (late-flowering lines 2, 3, 7; romanesco lines 1, 21 and 44; and the early-flowering line 25) corresponding to three distinct groups of *BoFLC2* expression. However, because of the disrupted relationship between *BoFLC2* expression and flowering in *boflc2* plants, this gene cannot be used to predict flowering within the *boflc2* parent lines. *BoFT* expression, by contrast, was negatively correlated with both curding and flowering time across the full range of *BoFLC2* and *boflc2* parent lines, suggesting potential as an early molecular indicator of flowering across all cauliflower types. This correlation is maintained despite the development of several parent lines being limited by environmental constraints.

While these findings dovetail to support the basic principle that curd initiation and flowering time could be predicted with timely analysis of flowering gene expression, a more detailed study carried out in multiple growth environments, with a larger number of expression replicates, and potentially with the inclusion of floral markers such as *BoAP1* would be needed to validate this theory and gauge its practical potential. Although this project commenced with the field work described in this chapter, the focus was ultimately turned to the development of our understanding of the molecular aspects of cauliflower vernalisation in more controlled environments as a foundation for subsequent work that others may now undertake, rather than attempting to commence more comprehensive field trials without the basic understanding of the molecular processes involved. The commercial adoption of a fully-developed flowering time molecular assay would not only depend on the reliability of predictions made using this system, but would also require a cost-benefit analysis to

determine whether the benefits of improved prediction (i.e. potentially increased management capacity and higher seed yields) outweigh the costs associated with sampling and processing a sufficient number of replicates and time points throughout the season to enable accurate and reliable prediction.

E. Conclusions

At the broadest level, *BoFLC2* genotype indicates whether or not a cauliflower plant will be early- or late-flowering, assuming temperature conditions that do not adversely affect plant health and normal plant growth. It has previously been shown *BoFLC2* expression is reduced in cauliflowers in response to artificial vernalisation, and that it is lower in plants that are approaching curd induction. The results from this chapter show that within those plants that have functional *BoFLC2*, it is possible to predict when curds will form and when flowering will occur based on the level of *BoFLC2* expression at a single time point during vegetative growth of naturally-vernalised plants. Furthermore, there is a moderate correlation between *BoFT* expression during vegetative growth and curding/flowering in field-grown cauliflowers that indicates potential for this gene to serve as a reproductive indicator of both *BoFLC2* and *boflc2* lines. There exists considerable commercial interest in being able to predict curd induction and flowering in new environments, or in situations where environmental conditions have varied from the norm. An understanding of the genetic and molecular regulation of the flowering process, combined with techniques for rapid assessment of expression patterns may provide the answer to a problem that has plagued the hybrid seed production industry.

Chapter VIII

GENERAL DISCUSSION

A. Summary of Main Outcomes

Management of the flowering process is critical to commercial hybrid vegetable seed production. Coincident flowering between parent lines is needed to ensure pollen transfer from pollen donor inbred parent lines to male sterile genetic lines in order to produce hybrid seed. Asynchronous flowering between parent lines, or incomplete bolting of one or both lines is often responsible for yield reductions or even total crop failures (Verdial et al. 2001). Prediction of flowering times for different parent lines under different environments and crop management conditions is currently based on extensive planting date x location trials. Models have been developed to predict curd initiation in cauliflower based on field observations of temperature response and, to an extent, juvenility length (Wurr et al. 1993), but such models naturally have their limitations, and commercial hybrid seed parent lines do not always conform to these models (C. Spurr 2007, pers. comm).

The primary goal of this research, as broadly defined in the first chapter, was to address this lack of ability to efficiently and reliably predict the requirements for, and timing of reproductive development in cauliflower by investigating and clarifying the environmental control of the flowering process at the molecular level in crop species of the *Brassica* genus. This was achieved through an integrated physiological and molecular approach.

A key aspect of this study was the identification of the flowering inhibitor *BoFLC2* as a critical determinant of flowering time (Chapter III). For the first time, a functional *BoFLC2* allele was identified in annual *B. oleracea* cultivars (Figure 7), and a mutated *boflc2* allele was recorded in a biennial *B. oleracea* cultivar. Allelic variation in this gene was strongly associated with curd formation, curd growth rate and flowering time in a diverse range of unrelated *B. oleracea* inbred parent lines, indicating that it is an important contributing factor

to the annual/biennial flowering habit (Chapter IV). The importance of *BoFLC2* functionality to flowering time within the annual brassica sub-group was demonstrated by analysis of a segregating population derived from a cross between two closely-related cauliflowers (Figures 9-12). The *BoFLC2* gene explained approximately 65% of the genetic variance in this population, and behaved in a dosage-dependent, incompletely dominant manner, contrasting with previous reports by Okazaki et al. (2007).

Having established the fundamental importance of the *BoFLC2* gene to cauliflower flowering time, the vernalisation-based regulation of *BoFLC2* and several other flowering time candidate genes was investigated in the leaves and shoot apices of a range of cauliflower parent lines (Chapter V). Results revealed for the first time that the basic downregulation response of *BoFLC2* and *BoFLC3* to vernalisation is conserved in cauliflower, and that the upstream regulator *BoFT* is upregulated by vernalisation (e.g. Figure 31). The isolation of a novel *B. oleracea* gene whose sequence and transcriptional dynamics suggest that it is homologous to the Arabidopsis *FLC*-regulator *VIN3* was also reported. *BoFLC* genes were downregulated by vernalisation regardless of plant age, but *BoFT* was less responsive to vernalisation at very young ages, suggesting the involvement of upstream genes other than *BoFLC* in regulating juvenility length (Figure 32). Several differences in the expression of these genes between cauliflower and cabbage were described, but no fundamental sources of genetic variation for factors such as vernalisation responsiveness or length of juvenile period were identified between *BoFLC2* and *boflc2* cauliflowers. Rather, the primary differences seen between the two flowering classes were related to the overall transcript level of *BoFLC2* and *BoFT* genes (Figure 36).

High temperatures which coincide with the temperature-sensitive phase of cauliflower development may cause devernalisation or floral reversion, problems known to contribute to asynchronous flowering in cauliflower seed crops (Chapter VI). Consequently, growers are forced to avoid warm and dry areas that would otherwise be conducive to high-yielding crops (Björkman & Pearson 1998). In contrast to findings in Arabidopsis, this research shows that downregulation of cauliflower *BoFLC* is unstable under some circumstances, with expression returning to pre-vernalisation levels after return to warm conditions (Figure 42). Such expression behaviour may be directly related to devernalisation problems encountered in the production of hybrid cauliflower seed. *BoFT*, by contrast, was stably upregulated, which may suggest involvement of a *BoFLC*-independent process.

In the final research chapter of this thesis (Chapter VII), evidence was provided to support the idea that the transcript levels of flowering genes may have potential as a molecular assay to predict flowering time in commercial seed crops. 'Natural' outdoor vernalising temperatures reduced *BoFLC2* expression and upregulated *BoFT* in a range of cauliflower parent lines (Figure 45), and a moderate correlation was found between the expression of both of these genes during vegetative growth, and curding/flowering time (Figures 46 and 48).

B. Applications

Investigation of the molecular mechanisms governing flowering is, in one sense, a form of basic, explanatory research carried out to advance understanding of a fundamental process. However, the knowledge and understanding gained from this research clearly has numerous direct and practical applications, given the commercial significance of cauliflower, and the essential importance of flowering to both its natural and agricultural reproduction. In particular, results from this research project may find application in three areas: QTL-based approaches to predicting flowering, plant breeding, and expression-based prediction of developmental changes.

1. QTL-Based Approaches to Predicting Flowering

In recent decades, researchers in the field of theoretical production ecology have expended considerable energy developing crop growth models which may be used to predict the growth and performance of cultivars under varying environmental conditions (Yin et al. 2000). These models enable plant breeders to test hypotheses and conduct 'virtual experiments' that would otherwise take years in field conditions (Fourcaud et al. 2008). Several attempts have been made to incorporate QTL inputs into such models in order to predict environmental effects on complex plant traits that interact strongly with the environment and are influenced by multiple genes. Flowering time is one such complex trait. In a study of QTL-based prediction of flowering time, Yin et al. (2005) identified multiple flowering time QTLs in glasshouse-grown spring barley (*Hordeum vulgare* L.). QTL data were incorporated into an ecophysiological model of flowering time, and this model was employed to predict flowering time in a number of different field environments with a moderate degree of success.

In this thesis, *BoFLC2* was characterised and identified as a key contributor to cauliflower flowering time, representing an important first step in defining the genes that are responsible for major variation in both the timing of *B. oleracea* reproductive development, and plant responsiveness to the environmental signals which regulate it. By beginning to define the physiological and genetic basis of flowering time variation and vernalisation-response variation, the potential to use QTL-based approaches for the development of cultivars that are 'best fits' for a particular environment is improved. However, the results presented here, particularly those from Chapter III, indicate that other genetic factors make significant contributions to flowering time variability and vernalisation responsiveness in cauliflower. The next step towards the development of an effective QTL-based system of predicting flowering time is to define and comprehensively characterise these genetic factors. One recent attempt to do so was made by Uptmoor et al. (2011), who combined an ecophysiological model of flowering time with QTL-based model parameters in order to predict flowering time in a *B. oleracea* population. These authors found that flowering time variability across genotypes was primarily due to differences in vernalisation responsiveness, but given the absence of functional *BoFLC2* in the population studied, the scope for application of this model to a broader range of *B. oleracea* flowering types is likely to be limited.

The 'natural' population of unrelated *B. oleracea* inbred parent lines used in this study was extremely diverse in terms of flowering time. This potentially makes the collections of both cauliflower and cabbage accessions very useful for the identification of flowering time QTLs using an association mapping approach (also known as linkage disequilibrium [LD] mapping). Such an approach has several key advantages over the use of specially-designed segregating populations derived from controlled crossing of two parental lines. For example, in mapping populations derived from a cross between two parents, only QTLs for which parents differ will be identified, whereas in genetically diverse collections of unrelated genotypes, evaluation of the numerous alleles available enables greater breadth of QTL identification (Zhao et al. 2007). In general, the mapping resolution possible with an association mapping approach that relies on historic recombination events is greater than that possible with small, typical F_1-derived mapping populations where the amount of recombination that can occur is meiotically limited (Flint-Garcia, Thornsberry & Buckler 2003). However, in this study, the number of accessions available was relatively small, possibly placing a limit on its suitability for fine-mapping and identifying the specific genes

responsible for QTLs. The cost and time required to establish segregating populations is an additional factor leading to greater interest in association mapping in plants, and as marker identification technology develops, genome-wide association mapping is likely to become an increasingly important tool in crop breeding programmes (Cowling & Balázsa 2010). Recently, Zhao et al. (2010a) developed a *B. rapa* core collection for association mapping studies and used this approach to map *BrFLC* flowering time candidate genes.

2. Plant Breeding

The development of novel cauliflower cultivars that flower predictably under the local environments for which they are bred is an important goal of plant breeding efforts. Marker-assisted selection (MAS) is proving to be an increasingly useful means of achieving such goals (see Collard et al. 2005 for a review). Molecular markers may be used to rapidly screen plant populations for polymorphisms, and the locations of these markers may then be used to construct linkage maps based on recombination frequencies. Such linkage maps may then be used to identify chromosomal regions which contain QTLs associated with phenotypic traits. Knowledge of the physical location of a QTL makes it possible to identify the actual genes that contribute to the specification of a trait. Once high-resolution QTL maps have been constructed and markers have been validated in multiple populations grown under multiple environments, they may be used to select plants for use in breeding programmes. An MAS approach saves time by minimising the need for field trials that would normally need to be conducted at specific times of year in specific locations. Furthermore, genotypes may be selected at seedling stage, and multiple genes may be selected at once. MAS also eliminates the possibility of inaccurate phenotypic evaluation associated with environmental influences.

The CAPS marker developed in this study for the key regulator of floral transition, *BoFLC2*, has been validated in several populations. It has the advantage of being a 'functional' marker that is completely linked with trait locus alleles (Andersen & Lübberstedt 2003). Furthermore, the contribution of this gene to flowering time appears to be large in the populations examined in this study. Because of these qualities, this marker could be usefully employed by brassica breeders who wish to screen germplasm or segregating populations for favourable cauliflower and cabbage genotypes without the need for time-consuming trials under multiple growth conditions to visually assess flowering phenotype and vernalisation responsiveness. Similarly, this marker could be used for quality control of seed lots, as

demonstrated by the identification of unexpected genotypes in the inbred parent lines studied in Chapter III. The number of crosses required for doubled haploid (DH) breeding programmes could be also be minimised by using this marker to select parent lines with suitable combinations of alleles.

It is necessary to moderate and balance comments on the helpfulness of molecular markers in plant breeding by noting that many complex traits show strong genotype x environment (GxE) effects. Such traits tend to have 'unstable' QTLs that are detectable in one environment, but not another, complicating the design of useful markers (Crossa 2012). For such traits with large GxE, association studies carried out across a range of environments are a necessary part of the plant breeding process. In the case of *BoFLC2*, substantial differences in reproductive behaviour were observed between glasshouse- and field-grown parent lines. This was particularly the case in *boflc2* lines, which formed curds later than expected when grown outdoors. Nonetheless, we suggest that these differences in flowering behaviour between growth environments do not diminish the suitability of this gene as a marker of flowering time for three reasons. First, the observed differences in curd initiation time between environments are thought to be overwhelmingly due to the poor growth of the *boflc2* tropical-adapted varieties, which were drastically unfit in terms of whole-plant biology for the growth conditions imposed, rather than due to any interaction of the *boflc2* gene *in particular* with the environment. Secondly, the effect of *BoFLC2* allele on flowering time within trials was obvious despite the considerable diversity and genetic 'background noise' of the unrelated parent lines used in this study. Thirdly, the biological function of *FLC* in both Arabidopsis (Michaels & Amasino 1999; Sheldon et al. 1999) and brassica (Tadege et al. 2001; Lin et al. 2005) is well-established. Gene expression behaviour reported in this study also supports the established view of *FLC* as a key regulator of flowering time; therefore, the 'functional' CAPS marker described here is potentially very useful as an indicator of flowering time.

In some cases, direct modification or transformation of *BoFLC2* could be made to alter timing of floral induction without significant negative pleiotropic effects on yield or general fitness. For example, (Kim et al. 2007) reported a substantial delay in Chinese cabbage (*B. rapa* ssp. *pekinensis*) floral induction by over-expression of *FLC*. In *B. oleracea* cultivars where delayed curd induction or flowering is sought (e.g. in certain hybrid seed production

situations or in production of cauliflowers for the fresh market, where early bolting can spoil the product), such an approach may find a similar application.

3. Expression-Based Prediction of Flowering Time

The concept of collecting flowering gene expression data in commercial seed crops to enable the prediction and management of flowering time is highly novel. In this study, the expression response to vernalisation was characterised in several genes exposed to multiple controlled vernalisation and devernalisation treatments. The basic patterns observed in controlled vernalisation experiments were maintained under natural vernalisation conditions, and moderate to strong correlations were found between expression levels and curd induction and flowering time in a field environment. These findings represent a small first step towards the practical adoption of a molecular method of predicting flowering in 'real-time' and although results are promising, expectations should be balanced with the realisation that much work remains to validate the usefulness of this method of predicting flowering time. Identification of a correlation between expression and flowering time is one matter; it is another matter altogether to be sufficiently confident of the robustness and meaning of gene expression data to rely upon it to execute a management strategy that could potentially compromise crop yields if inappropriately performed. Furthermore, the practical usefulness of such a method of predicting flowering time is also uncertain; in many cases, an agronomist who is moderately experienced with cauliflower seed crops could estimate curd induction or flowering with a greater level of accuracy than could be achieved through the use of gene expression analysis. This technique would potentially be more useful in cabbage crops, where vernalisation requirements are greater and are less likely to be satisfied, and where flowering time is often more difficult to accurately predict.

C. Future Research

Although this research represents a solid contribution to the foundational knowledge of flowering time regulation in brassica crops, there is ample scope for future development and extension of this work. For example, a more extensive effort to identify additional flowering time QTLs in the F_2 cauliflower populations described in Chapter III would be beneficial. Previous authors have identified a number of flowering time QTLs in other brassica populations including *B. napus* (e.g. Osborn et al. 1997; Long et al. 2007; Mei et al. 2009;

Wang et al. 2011a) and *B. rapa* (e.g. Osborn et al. 1997; Lou et al. 2007; Zhao et al. 2010b), with QTLs mapping to regions homologous to Arabidopsis genes such as *CO*, *FLC*, *FY* and *FRI*. *B. oleracea* flowering time QTLs have been identified in crosses between cabbage and broccoli (Kennard et al. 1994; Carmago & Osborn 1996; Okazaki et al. 2007) and Chinese kale x calabrese (Bohuon et al. 1998; Rae, Howell & Kearsey 1999). Similarly, it would be desirable to map QTLs in the closely-related cauliflower x cauliflower crosses utilised in this project. Detailed flowering time data exists for these populations (especially the CD population), and despite being a relatively 'close' cross (i.e. between two members of the same cultivar), a wide range of flowering behaviour was observed, making it a suitable population for further investigation. While the *BoFLC2* genotype was found to explain up to 65% of the genetic variance in the CD population, identification of QTLs associated with the remaining variance would be highly valuable. In this study, a candidate gene approach was adopted in order to identify polymorphisms in specific Arabidopsis flowering gene homologues. Very few polymorphisms were identified using this method, and greater success would be possible in future studies with use of high-throughput marker identification techniques (such as Amplified Fragment Length Polymorphism, or AFLP). The generation of a high-resolution linkage map using such high-throughput techniques would enable fine-mapping of the QTLs associated with flowering time variation (although the size of the population would limit map density), possibly enabling these QTLs to be targeted in cauliflower breeding programmes, incorporated into flowering time models, and potentially leading to the identification of the genes responsible for these traits. It would also be interesting to assess the expression of such genes in order to fully characterise and describe their molecular identities, in the same way that *BoFLC2* was characterised here.

Extension of the *BoFLC2* results reported here to cabbage would also be revealing. In this thesis, a mutant *boflc2* allele was reported in a cabbage variety for the first time. It would be highly interesting to determine whether progeny resulting from inbreeding of this line segregate, and what affect *BoFLC2* allele combination has on flowering time.

It has been clearly shown that the basic vernalisation response of *BoFLC*, *BoFT* and *BoVIN3* is conserved in cauliflower. When expression of these genes was evaluated in field conditions, mRNA levels were found to be correlated with both curd initiation and flowering time. If this research is to be applied in a production environment, it is essential that a more thorough study be carried out to assess the robustness of this method for predicting

reproductive events. This would involve a consistent system for the measurement of gene expression and flowering time in a range of different *BoFLC2* parent lines grown under multiple outdoor environments. *B. oleracea* homologues have been identified for numerous Arabidopsis meristem identity genes including *LFY* (*BoFH*), *AP1*, *AP3*, *CAL*, *FUL*, and *TFL1* (Anthony, James & Jordan 1993; Carr & Irish 1997; Mimida et al. 1999; Kop 2002). Given their relevance to the later-stage aspects of reproductive development, incorporation of such floral markers would be likely to be valuable in expression-based predictions of bolting and flowering. In particular, *AP1* is well-recognised as a reporter gene for identifying the floral meristem (Mandel & Yanofsky 1995). As such, the isolation and characterisation of this gene should be seen as a priority for future research into molecular assays for the prediction of the timing of the transition from curding to flowering. In *B. oleracea* cultivars such as cabbage, where the growing season is longer and greater potential for variability in flowering time exists, this technique for predicting flowering time would be especially useful, and subsequent research would benefit from assessment of the potential to use flowering gene expression to predict flowering time in cabbage seed crops.

The subject of devernalisation has only been very briefly investigated here, and much remains to be discovered about the molecular processes responsible for this phenomenon. Principally, it would be interesting define the temperatures and conditions where devernalisation is known to occur in order to determine whether or not *BoFLC2* expression in functional *BoFLC2* parent lines is stably downregulated in such conditions. Preliminary results indicate that changes in *BoFLC2* and *BoFT* expression may be reversed during the transition from winter to spring in both *BoFLC2* and *boflc2* cauliflowers grown outdoors (Chapter VII). Although a more detailed study is needed, this appears to suggest that even in *BoFLC2* parent lines, the downregulation of *BoFLC2* is not completely stable. Therefore, further experimentation in both controlled and natural conditions is needed to specifically define the length of the epigenetic memory of downregulation of this gene (i.e. how long a plant must be vernalised before the molecular effects are maintained even in the absence of the stimulus), and to determine if this corresponds to the plant's physiological susceptibility to devernalisation (i.e. how long the plant must be vernalised before return to warm conditions results in symptoms of devernalisation.

The ability to predict flowering time is of relatively little use if flowering time cannot be manipulated. Therefore, further research is needed to investigate practical methods of

delaying or promoting curd induction and flowering in brassica crops. The research reported in this thesis was complemented by agronomic trials which have already begun to address this issue by trialling the use of pre-planting artificial vernalisation and plant growth regulators as a means of manipulating flowering time in cauliflower. Preliminary results from field trials examining flowering responses to the application of GA biosynthesis inhibitors and exogenous GA_3 have confirmed that Paclobutrazol, a GA biosynthesis inhibitor, can significantly delay flowering in cauliflower (c.f. Guo et al. 2004). GA_3 applied during early curd development promoted curd break and early flowering, but reduced flower quality and seed yield, suggesting limited commercial value. Given that gibberellins are expected to promote flowering via regulation of genes such as *LFY*, *SOC1* and *FT* in Arabidopsis (Blázquez et al. 1998; Moon et al. 2003; Mutasa-Göttgens & Hedden 2009), expression analyses of these genes would potentially be useful in assessing the affects of such treatments.

D. Concluding Remarks

Cultivated members of the *B. oleracea* species are highly domesticated, with their use in hybrid seed production systems requiring intensive management, particularly with regard to flowering time. By extending the molecular model proposed for the control of flowering in Arabidopsis to these closely-related crop species, the potential of molecular tools to be applied by brassica breeders and growers of high-value hybrid seed crops seeking to predict or modify flowering time has been demonstrated. Our verification of the fundamental importance of *BoFLC2* to flowering time variability and vernalisation responsiveness is of particular relevance. Much work remains to be done at the fundamental level in order to add depth and clarity to these findings, and at the applied level, considerable research and validation is required to develop our understanding to a level where molecular tools can be used to form the basis of new strategies for the control of brassica flowering and to improve the efficiency of current management practices.

REFERENCES

Abe, M, Kobayashi, Y, Yamamoto, S, Daimon, Y, Yamaguchi, A, Ikeda, Y, Ichinoki, H, Notaguchi, M, Goto, K & Araki, T 2005, 'FD, a bZIP Protein Mediating Signals from the Floral Pathway Integrator FT at the Shoot Apex', *Science*, vol. 309, pp. 1052-1056.

Ahn, JH, Miller, D, Winter, VJ, Banfield, MJ, Lee, JH, Yoo, SY, Henz, SR, Brady, RL & Weigel, D 2005, 'A Divergent External Loop Confers Antagonistic Activity on Floral Regulators *FT* and *TFL1*', *EMBO*, vol. 25, pp. 605-614.

Aikawa, S, Kobayashi, MJ, Satake, A, Shimizu, KK & Kudoh, H 2010, 'Robust Control of the Seasonal Expression of the Arabidopsis *FLC* gene in a Fluctuating Environment', *Proceedings of the National Academy of Sciences*, vol. Advance Access, doi:10.1073/pnas.0914293107.

Alabadí, D, Oyama, T, Yanovsky, MJ, Harmon, FG, Ma, P & Kay, SA 2001, 'Reciprocal Regulation Between *TOC1* and *LHY/CCA1* Within the *Arabidopsis* Circadian Clock', *Science*, vol. 293, pp. 880-883.

Amasino, RM 2004, 'Vernalization, Competence, and the Epigenetic Memory of Winter', *The Plant Cell*, vol. 16, pp. 2553-2559.

---- 2010, 'Seasonal and Developmental Timing of Flowering', *The Plant Journal*, vol. 61, pp. 1001-1013.

Andersen, JR & Lübberstedt, T 2003, 'Functional Markers in Plants', *Trends in Plant Science*, vol. 8, pp. 554-560.

Anthony, RG, James, PE & Jordan, BR 1993, 'Cloning and Sequence Analysis of a *flo/lfy* Homologue Isolated from Cauliflower (*Brassica oleracea* L. var. *botrytis)*', *Plant Molecular Biology*, vol. 22, pp. 1163-1166.

---- 1995, 'The cDNA Sequence of a Cauliflower *apetala-1/squamosa* Homolog', *Plant Physiology*, vol. 108, pp. 441-442.

---- 1996, 'Cauliflower (*Brassica oleracea* var. *botrytis* L.) Curd Development: the Expression of Meristem Identity Genes', *Journal of Experimental Botany*, vol. 47, pp. 181-188.

Atherton, JG, Hand, DJ & Williams, CA 1987, 'Curd initiation in the Cauliflower (*Brassica oleracea* var. *botrytis* L.)', in JG Atherton (ed.), *Manipulation of Flowering*, Butterworths, London, UK.

Australian Bureau of Meteorology 2011, Australian Bureau of Meteorology, Melbourne, VIC, viewed 4th February 2011, <http://www.bom.gov.au/>.

Axelsson, T, Shavorskaya, O & Lagercrantz, U 2001, 'Multiple Flowering Time QTLs Within Several *Brassica* Species Could be the Result of Duplicated Copies of One Ancestral Gene', *Genome*, vol. 44, pp. 856-864.

Ayele, M, Haas, BJ, Kumar, N, Wu, H, Xiao, Y, Van Aken, S, Utterback, TR, Wortman, JR, White, OR & Town, CD 2005, 'Whole Genome Shotgun Sequencing of *Brassica oleracea* and its Application to Gene Discovery and Annotation in *Arabidopsis*', *Genome Research*, vol. 15, pp. 487-495.

Bastow, RM, Mylne, JS, Lister, C, Lippman, Z, Martienssen, RA & Dean, C 2004, 'Vernalization Requires Epigenetic Silencing of *FLC* by Histone Methylation', *Nature*, vol. 427, pp. 164-167.

Bernier, G 1988, 'The Control of Floral Evocation and Morphogenesis', *Annual Review of Plant Physiology and Plant Molecular Biology*, vol. 39, pp. 175-219.

Bernier, G, Kinet, J-M & Sachs, RM 1981, *The Physiology of Flowering* vol. II, CRC Press, Inc., Boca Raton, Florida, USA.

Bernier, G & Perilleux, C 2005, 'A Physiological Overview of the Genetics of Flowering Time Control', *Plant Biotechnology Journal*, vol. 3, pp. 3-16.

Björkman, T & Pearson, KJ 1998, 'High Temperature Arrest of Inflorescence Development in Broccoli (*Brassica oleracea* var. *italica* L.)', *Journal of Experimental Botany*, vol. 49, pp. 101-106.

Blackman, BK, Rasmussen, DA, Strasburg, JL, Raduski, AR, Burke, JM, Knapp, SJ, Michaels, SD & Rieseberg, LH 2011, 'Contributions of Flowering Time Genes to Sunflower Domestication and Improvement', *Genetics*, vol. 187, pp. 271-287.

Blázquez, M, Green, R, Nilsson, O, Sussman, MR & Weigel, D 1998, 'Gibberellins Promote Flowering of Arabidopsis by Activating the *LEAFY* Promoter', *Plant Cell*, vol. 10, pp. 791-800.

Bohuon, EJR, Ramsay, LD, Craft, JA, Arthur, AE, Marshall, DF, Lydiate, DJ & Kearsey, MJ 1998, 'The Association of Flowering Time Quantitative Trait Loci with Duplicated Regions and Candidate Loci in *Brassica oleracea*', *Genetics*, vol. 150, pp. 393-401.

Booij, R 1990, 'Effects of Gibberellic Acids on Time of Maturity and on Yield and Quality of Cauliflower', *Netherlands Journal of Agricultural Science*, vol. 38, pp. 641 – 651.

Booij, R & Struik, PC 1990, 'Effects of Temperature on Leaf and Curd Initiation in Relation to Juvenility of Cauliflower', *Scientia Horticulturae*, vol. 44, pp. 201-214.

Boss, PK, Bastow, RM, Mylne, JS & Dean, C 2004, 'Multiple Pathways in the Decision to Flower: Enabling, Promoting, and Resetting', *The Plant Cell*, vol. 16, pp. S18-S31.

Bowman, JL, Alvarez, J, Weigel, D, Meyerowitz, EM & Smyth, DR 1993, 'Control of Flower Development in *Arabidopsis thaliana* by *APETALA1* and Interacting Genes', *Development*, vol. 119, pp. 721-743.

Brown, J & Caligari, PDS 2008, *An Introduction to Plant Breeding*, Blackwell Publishing, Oxford, UK.

Brunner, AM & Nilsson, O 2004, 'Revisiting Tree Maturation and Floral Initiation in the Poplar Functional Genomics Era', *New Phytologist*, vol. 164, pp. 43-51.

Canola Council of Canada 2011, Canola Council of Canada Winnipeg, MB, Canada, viewed 4 August 2011, <http://www.canolacouncil.org/canola_the_official_definition.aspx>.

Carmago, LEA & Osborn, TC 1996, 'Mapping Loci Controlling Flowering Time in *Brassica oleracea*', *Theoretical and Applied Genetics*, vol. 92, pp. 610-616.

Carr, SM & Irish, VF 1997, 'Floral Homeotic Gene Expression Defines Developmental Arrest Stages in *Brassica oleracea* L. vars. *botrytis* and *italica*', *Planta*, vol. 201, pp. 179-188.

Chang, Y-F, Imam, S & Wilkinson, MF 2007, 'The Nonsense-Mediated Decay RNA Surveillance Pathway', *Annual Review of Biochemistry*, vol. 76, pp. 51-74.

Chew, YH, Wilczek, AM, Williams, M, Welch, SM, Schmitt, J & Halliday, KJ 2012, 'An Augmented Arabidopsis Phenology Model Reveals Seasonal Temperature Control of Flowering Time', *New Phytologist*, vol. 194, pp. 654–665.

Cockram, J, Jones, H, Leigh, FJ, O'Sullivan, D, Powell, W, Laurie, DA & Greenland, AJ 2007, 'Control of Flowering Time in Temperatre Cereals: Genes, Domestication, and Sustainable Productivity', *Journal of Experimental Botany*, vol. 58, pp. 1231-1244.

Collard, BCY, Jahufer, MZZ, Brouwer, JB & Pang, ECK 2005, 'An Introduction to Markers, Quantitative Trait Loci (QTL) Mapping and Marker-Assisted Selection for Crop Improvement: The Basic Concepts', *Euphytica*, vol. 142, pp. 169-196.

Cowling, WA & Balázsa, E 2010, 'Prospects and Challenges for Genome-Wide Association and Genomic Selection in Oilseed *Brassica* species', *Genome*, vol. 53, pp. 1024-1028.

Crisp, P 1982, 'The Use of an Evolutionary Scheme for Cauliflowers in the Screening of Genetic Resources', *Euphytica*, vol. 31, pp. 725-734.

Crisp, P & Walkey, DGA 1974, 'The Use of Aseptic Meristem Culture in Cauliflower Breeding', *Euphytica*, vol. 23, pp. 305-313.

Crossa, J 2012, 'From Genotype x Environment Interaction to Gene x Environment Interaction', *Current Genomics*, vol. 13, pp. 225-244.

Curtis, WM & Morris, DI 1993, *The Student's Flora of Tasmania: Part 1*, 2nd edn, 4 vols., St David's Park Publishing, Hobart, Tasmania.

D'Aloia, M, Tocquin, P & Périlleux, C 2008, 'Vernalization-Induced Repression of *FLOWERING LOCUS C S*timulates Flowering in *Sinapis alba* and Enhances Plant Responsiveness to Photoperiod', *New Phytologist*, vol. 178, pp. 755-765.

Dahanayake, SR & Galwey, NW 1998, 'Effects of Interactions Between Low and High Temperature Treatments on Flowering of Spring Rape (*Brassica napus* var. *annua*)', *Annals of Botany*, vol. 81, pp. 609-617.

Dixon, GR 2007, *Vegetable Brassicas and Related Crucifers*, CAB International, Wallingford, UK.

Doyle, MR, Davis, SJ, Bastow, RM, McWatters, HG, Kozma-Bognár, L, Nagy, F, Millar, AJ & Amasino, RM 2002, 'The *ELF4* Gene Controls Circadian Rhythms and Flowering Time in *Arabidopsis thaliana*', *Nature*, vol. 419, pp. 74–77.

Duclos, DV & Björkman, T 2008, 'Meristem Identity Gene Expression During Curd Proliferation and Flower Initiation in *Brassica oleracea*', *Journal of Experimental Botany*, vol. 59, pp. 421-433.

Engelen-Eigles, G 1996, 'Irradiance and Light Quality Interact to Affect Vernalization in *Raphanus sativus* L.', Masters thesis, University of Minnesota.

Erwin, J 2007, 'Factors Affecting Flowering in Ornamental Plants', in NO Anderson (ed.), *Flower Breeding and Genetics: Issues, Challenges and Opportunities for the 21st Century*, Springer, Dordrecht, pp. 7-44.

Fellows, JR, Reader, RJ & Wurr, DCE 1997, 'A Model of Leaf Production and Apex Development in Calabrese', *Journal of Horticultural Science*, vol. 72, pp. 327-337.

Fink, GR 1998, 'Anatomy of a Revolution', *Genetics*, vol. 149, pp. 473-477.

Finley, JW 2003, 'The Antioxidant Responsive Element (ARE) May Explain the Protective Effects of Cruciferous Vegetables on Cancer', *Nutrition Review*, vol. 61, pp. 250-254.

Flint-Garcia, SA, Thornsberry, JM & Buckler, ES 2003, 'Structure of Linkage Disequilibrium in Plants', *Annual Review of Plant Biology*, vol. 54, pp. 357-374.

Fourcaud, T, Zhang, X, Stokes, A, Lambers, H & Körner, C 2008, 'Plant Growth Modelling and Applications: The Increasing Importance of Plant Architecture in Growth Models', *Annals of Botany*, vol. 101, pp. 1053-1063.

Franco-Zorrilla, JM, Fernández-Calvín, B, Madueño, F, Cruz-Alvarez, M, Salinas, J & Martínez-Zapater, JM 1999, 'Identification of Genes Specifically Expressed in Cauliflower Reproductive Meristems. Molecular Characterization of *BoREM1*', *Plant Molecular Biology*, vol. 39, pp. 427-436.

Friend, DJC 1985, *Brassica*, vol. II, V vols., CRC Handbook of Flowering, CRC Press, Inc., Boca Raton.

Fujime, Y 1983, 'Studies on Thermal Conditions of Curd Formation and Development in Cauliflower and Broccoli, with Special Reference to Abnormal Curd Development', *Memoirs of the Faculty of Agriculture, Kagawa University*, vol. 40, pp. 117-123.

Fujime, Y & Okuda, N 1996, 'The Physiology of Flowering in *Brassicas*, Especially About Cauliflower and Broccoli', *Acta Horticulturae*, vol. 407, pp. 247-254.

Gazzani, S, Gendall, AR, Lister, C & Dean, C 2003, 'Analysis of the Molecular Basis of Flowering Time Variation in Arabidopsis Accessions', *Plant Physiology*, vol. 132, pp. 1107-1114.

Gendall, AR, Levy, YY, Wilson, A & Dean, C 2001, 'The *VERNALIZATION 2* Gene Mediates the Epigenetic Regulation of Vernalization in Arabidopsis', *Cell*, vol. 107, pp. 525-535.

George, RAT 1999, *Vegetable Seed Production (2nd ed.)*, CABI Publishing, Wallingford, Oxfordshire, UK.

Gray, AR 1982, 'Taxonomy and Evolution of Broccoli (*Brassica oleracea* var. *italica*)', *Economic Botany*, vol. 36, pp. 397-410.

Griffiths, J, Murase, K, Rieu, I, Zentella, R, Zhang, ZL, Powers, SJ, Gong, F, Phillips, AL, Hedden, P, Sun, TP & Thomas, SG 2006, 'Genetic Characterization and Functional Analysis of the GID1 Gibberellin Receptors in *Arabidopsis*', *The Plant Cell*, vol. 18, pp. 3399-3414.

Gultormsen, G 1981, 'Climatic Effects in Chinese Cabbage for Early Production', *Acta Horticulturae*, vol. 122, pp. 77-83.

Guo, DP, Shah, GA, Zeng, GW & Zheng, SJ 2004, 'The Interaction of Plant Growth Regulators and Vernalization on the Growth and Flowering of Cauliflower (*Brassica oleracea* var. *botrytis*)', *Plant Growth Regulation*, vol. 43, pp. 163-171.

Hancock, JF 2004, *Plant Evolution and the Origin of Crop Species*, CAB International, Oxford, UK.

Hazen, SP, Schultz, TF, Pruneda-Paz, JL, Borevitz, JO, Ecker, JR & Kay, SA 2005, '*LUX ARRHYTHMO* Encodes a Myb Domain Protein Essential for Circadian Rhythms', *Proceedings of the National Academy of Sciences*, vol. 102, pp. 10387-10392.

He, Y & Amasino, RM 2005, 'Role of Chromatin Modification in Flowering-Time Control', *Trends in Plant Science*, vol. 10, pp. 30-35.

Hecht, V, Foucher, F, Ferrándiz, C, Macknight, R, Navarro, P, Morin, J, Vardy, ME, Ellis, N, Beltrán, JP, Rameau, C & Weller, JL 2005, 'Conservation of Arabidopsis Flowering Genes in Model Legumes', *Plant Physiology*, vol. 137, pp. 1420-1434.

Hecht, V, Laurie, RE, Vander Schoor, JK, Ridge, S, Knowles, CL, Liew, LC, Sussmilch, FC, Murfet, IC, Macknight, RC & Weller, JL 2011, 'The Pea *GIGAS* Gene Is a *FLOWERING LOCUS T* Homolog Necessary for Graft-Transmissible Specification of Flowering but Not for Responsiveness to Photoperiod', *The Plant Cell*, vol. 23, pp. 147-161.

Heide, OM 1970, 'Seed-Stalk Formation and Flowering in Cauliflower. I. Daylength, Temperature and Time Relationships', *Meldinger fra Norges Landbrukshogskole*, vol. 49 pp. 1 – 21.

Helliwell, CA, Wood, CC, Robertson, M, Peacock, WJ & Dennis, EA 2006, 'The Arabidopsis FLC Protein Interacts Directly *in vivo* with *SOC1* and *FT* Chromatin and is Part of a High-Molecular Weight Protein Complex', *The Plant Journal*, vol. 46, pp. 183-192.

Hempel, FD, Weigel, D, Mandel, MA, Ditta, G, Zambryski, PC, Feldman, LJ & Yanofsky, MF 1997, 'Floral Determination and Expression of Floral Regulatory Genes in Arabidopsis', *Development*, vol. 124, pp. 3845-3853.

Heo, JB & Sung, S 2011, 'Vernalization-Mediated Epigenetic Silencing by a Long Intronic Noncoding RNA', *Nature*, vol. 331, pp. 76-79.

Hepworth, SR, Valverde, F, Ravenscroft, D, Mouradov, A & Coupland, G 2002, 'Antagonistic Regulation of Flowering-Time Gene *SOC1* by *CONSTANS* and *FLC* Via Separate Promoter Motifs', *EMBO*, vol. 21, pp. 4327-4337.

Hicks, KA, Millar, AJ, Carre, IA, Somers, DE, Straume, M, Meeks-Wagner, DR & Kay, SA 1996, 'Conditional Circadian Dysfunction of the Arabidopsis *Early Flowering 3* Mutant', *Science*, vol. 274, pp. 790–792.

Hoagland, DR & Broyer, TC 1936, 'General Nature of the Process of Salt Accumulation by Roots with Description of Experimental Methods', *Plant Physiology*, vol. 11, pp. 471–507.

Imaizumi, T, Schultz, TF, Harmon, FG, Ho, LA & Kay, SA 2005, '*FKF1* F-Box Protein Mediates Cyclic Degradation of a Repressor of *CONSTANS* in *Arabidopsis*', *Science*, vol. 309, pp. 293-297.

Irwin, JA, Lister, C, Soumpourou, E, Zhang, Y, Howell, EC, Teakle, G & Dean, C 2012, 'Functional Alleles of the Flowering Time Regulator *FRIGIDA* in the *Brassica oleracea* Genome', *BMC Plant Biology*, vol. 12, no. 21.

Izawa, T, Takahashi, Y & Yano, M 2003, 'Comparative Biology Comes Into Bloom: Genomic and Genetic Comparison of Flowering Pathways in Rice and Arabidopsis', *Current Opinion in Plant Biology*, vol. 6, pp. 113-120.

Jung, C & Müller, AE 2009, 'Flowering Time Control and Applications in Plant Breeding', *Trends in Plant Science*, vol. 14, pp. 563-573.

Kardailsky, I, Shukla, VK, Ahn, JH, Dagenais, N, Christensen, SK, Nguyen, JT, Chory, J, Harrison, MJ & Weigel, D 1999, 'Activation Tagging of the Floral Inducer *FT*', *Science*, vol. 286 pp. 1962-1965.

Katari, M, O'Shaughnessy, A, Palmer, L, Bahret, A, Baker, J, Balija, V, Bell, M, Cunnius, DM, King, L, Kirchoff, K, Kuit, K, Miller, B, Nascimento, L, Preston, R, Rodriguez, S, Santos, L, Shah, R, Vil, MD, Zutavern, T, Bal, H, Dedhia, N & McCombie, WR 2001, 'Whole Genome Shotgun Reads from *Brassica oleracea*', *Unpublished: NCBI reference sequence BH008415*.

Kempin, SA, Savidge, B & Yanofsky, MF 1995, 'Molecular Basis of the *cauliflower* Phenotype in *Arabidopsis*', *Science*, vol. 267, pp. 522-525.

Kennard, WC, Slocum, MK, Figdore, SS & Osborn, TC 1994, 'Genetic Analysis of Morphological Variation in *Brassica oleracea* Using Molecular Markers', *Theoretical and Applied Genetics*, vol. 87, pp. 721-732.

Kieffer, M, Fuller, MP & Jellings, AJ 1998, 'Explaining Curd and Spear Geometry in Broccoli, Cauliflower and 'Romanesco': Quantitative Variation in Activity of Primary Meristems', *Planta*, vol. 206, pp. 34-43.

Kim, DH, Zografos, BR & Sung, S 2010, 'Vernalization-Mediated *VIN3* Induction Overcomes the LIKE-HETEROCHROMATIN PROTEIN1/POLYCOMB REPRESSION COMPLEX2-Mediated Epigenetic Repression', *Plant Physiology*, vol. 154, pp. 949-957.

Kim, KW, Shin, JH, Moon, J, Kim, M, Lee, J, Park, MC & Lee, I 2003, 'The Function of the Flowering Time Gene *AGL20* is Conserved in Crucifers', *Molecules and Cells*, vol. 16, pp. 136-141.

Kim, SY, Park, BS, Kwon, SJ, Kim, J, Lim, MH, Park, YD, Kim, DY, Suh, SC, Jin, YM, Ahn, JH & Lee, YH 2007, 'Delayed Flowering Time in *Arabidopsis* and *Brassica rapa* by the Overexpression of *FLOWERING LOCUS C* (*FLC*) Homologs Isolated From Chinese Cabbage (*Brassica rapa* L. ssp. *pekinensis*)', *Plant Cell Reports*, vol. 26, pp. 327-336.

Kimber, DS & McGregor, DI 1995, 'The Species and Their Origin, Cultivation and World Production', in DS Kimber & DI McGregor (eds), *Brassica Oilseeds: Production and Utilization*, CAB International Wallingford, UK, pp. 1-9.

Kole, C, Quijada, P, Michaels, SD & Amasino, RM 2001, 'Evidence for Homolgy of Flowering-Time Genes *VFR2* from *Brassica rapa* and *FLC* from *Arabidopsis thaliana*', *Theoretical and Applied Genetics*, vol. 102, pp. 425-430.

Koornneef, M, Hanhart, CJ & Van der Veen, JH 1991, 'A Genetic and Physiological Analysis of Late Flowering Mutants in *Arabidopsis thaliana*', *Molecular and General Genetics*, vol. 229, pp. 57-66.

Koornneef, M & Van der Veen, JH 1980, 'Induction and Analysis of Gibberellin Sensitive Mutants in *Arabidopsis thaliana* (L.) Heynh.', *Theoretical and Applied Genetics*, vol. 58, pp. 257-263.

Koornneef, M, van Eden, J, Hanhart, CJ, Stam, P, Braaksma, FJ & Feenstra, WJ 1983, 'Linkage Map of *Arabidopsis thaliana*', *The Journal of Heredity*, vol. 74, pp. 265-272.

Kop, EP 2002, 'MADS-box genes and the genetics of cauliflower curd development', PhD thesis, The University of Warwick.

Kop, EP, Teakle, GR, McClenaghan, ER, Lynn, JR & King, GJ 2003, 'Genetic Analysis of the Bracting Trait in Cauliflower and Broccoli', *Plant Science*, vol. 164, pp. 803-808.

Kowalski, SP, Lan, TH, Feldmann, KA & Paterson, AH 1994, 'Comparative Mapping of *Arabidopsis thaliana* and *Brassica oleracea* Chromosomes Reveals Islands of Conserved Organization', *Genetics*, vol. 138, pp. 499-510.

Kwon, SJ, Park, BS, Kim, SY, Choi, HS, Lee, MC, Kim, JS, Lee, SI, Lim, KB, Kim, JA, Lee, MR, Jin, YM, Kim, D & Kim, HI 2004, 'Brassica rapa Kwonsim VRN1 mRNA', *Unpublished: NCBI reference sequence AY517929*.

Labate, JA, Robertson, LD, Baldo, AM & Björkman, T 2006, 'Inflorescence Identity Genes are Poor Predictors of Inflorescence Type in Broccoli and Cauliflower', *Journal of the American Society for Horticultural Science*, vol. 131, pp. 667-673.

Labate, JA, Robertson, LD & Bjorkman, T 2004, 'Utility of *BoCAL-a* and *BoAP1-a* Genotypes in Identifying Broccoli and Cauliflower Accessions', *Hortscience*, vol. 39, pp. 773-773.

Lagercrantz, U 1998, 'Comparative Mapping Between *Arabidopsis thaliana* and *Brassica nigra* Indicates That Brassica Genomes Have Evolved Through Extensive Genome Replication Accompanied by Chromosome Fusions and Frequent Rearrangements', *Genetics*, vol. 150, pp. 1217-1228.

Lagercrantz, U & Lydiate, DJ 1996, 'Comparative Genome Mapping in *Brassica*', *Genetics*, vol. 144, pp. 1903-1910.

Lagercrantz, U, Putterill, J, Coupland, G & Lydiate, DJ 1996, 'Comparative Mapping in *Arabidopsis* and *Brassica*, Fine Scale Genome Colinearity and Congruence of Genes Controlling Flowering Time', *The Plant Journal*, vol. 9, pp. 13-20.

Laibach, F 1943, '*Arabidopsis thaliana* (L.) Heynh als Objekt für genetische und entwicklungsphysiologische Untersuchungen', *Botanisches Archiv*, vol. 44, pp. 439-455.

Lan, TH & Paterson, AH 2000, 'Comparative Mapping of Quantitative Trait Loci Sculpting the Curd of *Brassica oleracea*', *Genetics*, vol. 155, pp. 1927-1954.

Lee, J & Lee, I 2010, 'Regulation and Function of *SOC1*, a Flowering Pathway Integrator', *Journal of Experimental Botany*, vol. 61, pp. 2247–2254.

Leshem, Y & Steiner, S 1968, 'Effect of Gibberellic Acid and Cold Treatment on Flower Differentiation and Stem Elongation of Cauliflower (*Brassica oleracea* var. *botrytis*)', *Israel Journal of Agricultural Research*, vol. 18, pp. 133-134.

Levy, YY, Mesnage, S, Mylne, JS, Gendall, AR & Dean, C 2002, 'Multiple Roles of Arabidopsis *VRN1* in Vernalization and Flowering Time Control', *Science*, vol. 297, pp. 243-246.

Li, Z, Zhao, L, Cui, C, Kai, G, Zhang, L, Sun, X & Tang, K 2005, 'Molecular Cloning and Characterization of an Anti-Bolting Related Gene (*BrpFLC*) from *Brassica rapa* ssp. *Pekinensis*', *Plant Science*, vol. 168, pp. 407-413.

Liew, LC, Hecht, V, Laurie, RE, Knowles, CK, Vander Schoor, JK, Macknight, RC & Weller, JL 2009, '*DIE NEUTRALIS* and *LATE BLOOMER 1* Contribute to Regulation of the Pea Circadian Clock', *Plant Cell*, vol. 21, pp. 3198–3211.

Lifschitz, E, Eviatar, T, Rozman, A, Shalit, A, Goldshmidt, A, Amsellem, Z, Alvarez, JP & Eshed, Y 2006, 'The Tomato *FT* Ortholog Triggers Systemic Signals that Regulate Growth and Flowering and Substitute for Diverse Environmental Stimuli', *Proceedings of the National Academy of Sciences*, vol. 103, pp. 6398-6403.

Lin, SI, Wang, JG, Poon, SY, Su, CL, Wang, SS & Chiou, TJ 2005, 'Differential Regulation of *FLOWERING LOCUS C* Expression by Vernalization in Cabbage and Arabidopsis', *Plant Physiology*, vol. 137, pp. 1037-1048.

Lin, XY, Kaul, SS, Rounsley, S, Shea, TP, Benito, MI, Town, CD, Fujii, CY, Mason, T, Bowman, CL, Barnstead, M, Feldblyum, TV, Buell, CR, Ketchum, KA, Lee, J, Ronning, CM, Koo, HL, Moffat, KS, Cronin, LA, Shen, M, Pai, G, Van Aken, S, Umayam, L, Tallon, LJ, Gill, JE, Adams, MD, Carrera, AJ, Creasy, TH, Goodman, HM, Somerville, CR, Copenhaver, GP, Preuss, D, Nierman, WC, White, O, Eisen, JA, Salzberg, SL, Fraser, CM & Venter, JC 1999, 'Sequence and Analysis of Chromosome 2 of the Plant *Arabidopsis thaliana*', *Nature*, vol. 402, pp. 761-768.

Liu, C, Chen, H, Ling, H, Soo, HM, Kumar, PP, Han, JH, Liou, YC & Yu, H 2008, 'Direct Interaction of *AGL24* and *SOC1* Integrates Flowering Signals in *Arabidopsis*', *Development*, vol. 135, pp. 1481-1491.

Locke, JCW, Kozma-Bognár, L, Gould, PD, Fehér, B, Kevei, E, Nagy, F, Turner, MS, Hall, A & Millar, AJ 2006, 'Experimental Validation of a Predicted Feedback Loop in the Multi-Oscillator Clock of *Arabidopsis thaliana*', *Molecular Systems Biology*, vol. 2, no. 59.

Long, Y, Shi, J, Qiu, D, Li, R, Zhang, C, Wang, J, Hou, J, Zhao, J, Shi, L, Park, BS, Choi, SR, Lim, YP & Meng, J 2007, 'Flowering Time Quantitative Trait Loci Analysis of Oilseed Brassica in Multiple Environments and Genomewide Alignment with Arabidopsis', *Genetics*, vol. 177, pp. 2433-2444.

Lou, P, Zhao, J, Kim, JS, Shen, S, Del Carpio, DP, Song, X, Jin, M, Vreugdenhil, D, Wang, X, Koornneef, M & Bonnema, G 2007, 'Quantitative Trait Loci for Flowering Time and Morphological Traits in Multiple Populations of *Brassica rapa*', *Journal of Experimental Botany*, vol. 58, pp. 4005-4016.

Lowman, AC & Purugganan, MD 1999, 'Duplication of the *Brassica oleracea APETALA1* Floral Homeotic Gene and the Evolution of Domesticated Cauliflower', *The Journal of Heredity*, vol. 90, pp. 514-520.

Lukens, L, Zou, F, Lydiate, DJ, Parkin, I & Osborn, T 2003, 'Comparison of a *Brassica oleracea* Genetic Map With the Genome of *Arabidopsis thaliana*', *Genetics*, vol. 164, pp. 359-372.

Macknight, R, Bancroft, I, Page, T, Lister, C, Schmidt, R, Love, K, Westphal, L, Murphy, G, Sherson, S, Cobbett, C & Dean, C 1997, '*FCA*, a Gene Controlling Flowering Time in Arabidopsis, Encodes a Protein Containing RNA-Binding Domains', *Cell*, vol. 89, pp. 737-745.

Mandel, MA, Gustafson-Brown, C, Savidge, B & Yanofsky, MF 1992, 'Molecular Characterization of the Arabidopsis Floral Homeotic gene *APETALA1*', *Nature*, vol. 360, pp. 273-277.

Mandel, MA & Yanofsky, M 1995, 'A Gene Triggering Flower Formation in Arabidopsis', *Nature*, vol. 377, pp. 522-524.

Martynov, VV & Khavkin, EE 2004, 'Two Homologs of the *FLOWERING LOCUS C* Gene from Leaf Mustard (*Brassica juncea*)', *Russian Journal of Plant Physiology*, vol. 51, pp. 234-240.

Mayer, K, Schuller, C, Wambutt, R, Murphy, G, Volckaert, G, Pohl, T, Dusterhoft, A, Stiekema, W, Entian, KD, Terryn, N, Harris, B, Ansorge, W, Brandt, P, Grivell, L, Rieger, M, Weichselgartner, M, de

Simone, V, Obermaier, B, Mache, R, Muller, M, Kreis, M, Delseny, M, Puigdomenech, P, Watson, M, Schmidtheini, T, Reichert, B, Portatelle, D, Perez-Alonso, M, Boutry, M, Bancroft, I, Vos, P, Hoheisel, J, Zimmermann, W, Wedler, H, Ridley, P, Langham, SA, McCullagh, B, Bilham, L, Robben, J, Van der Schueren, J, Grymonprez, B, Chuang, YJ, Vandenbussche, F, Braeken, M, Weltjens, I, Voet, M, Bastiaens, I, Aert, R, Defoor, E, Weitzenegger, T, Bothe, G, Ramsperger, U, Hilbert, H, Braun, M, Holzer, E, Brandt, A, Peters, S, van Staveren, M, Dirkse, W, Mooijman, P, Lankhorst, RK, Rose, M, Hauf, J, Kotter, P, Berneiser, S, Hempel, S, Feldpausch, M, Lamberth, S, Van den Daele, H, De Keyser, A, Buysshaert, C, Gielen, J, Villarroel, R, De Clercq, R, Van Montagu, M, Rogers, J, Cronin, A, Quail, M, Bray-Allen, S, Clark, L, Doggett, J, Hall, S, Kay, M, Lennard, N, McLay, K, Mayes, R, Pettett, A, Rajandream, MA, Lyne, M, Benes, V, Rechmann, S, Borkova, D, Blocker, H, Scharfe, M, Grimm, M, Lohnert, TH, Dose, S, de Haan, M, Maarse, A, Schafer, M, Muller-Auer, S, Gabel, C, Fuchs, M, Fartmann, B, Granderath, K, Dauner, D, Herzl, A, Neumann, S, Argiriou, A, Vitale, D, Liguori, R, Piravandi, E, Massenet, O, Quigley, F, Clabauld, G, Mundlein, A, Felber, R, Schnabl, S, Hiller, R, Schmidt, W, Lecharny, A, Aubourg, S, Chefdor, F, Cooke, R, Berger, C, Montfort, A, Casacuberta, E, Gibbons, T, Weber, N, Vandenbol, M, Bargues, M, Terol, J, Torres, A, Perez-Perez, A, Purnelle, B, Bent, E, Johnson, S, Tacon, D, Jesse, T, Heijnen, L, Schwarz, S, Scholler, P, Heber, S, Francs, P, Bielke, C, Frishman, D, Haase, D, Lemcke, K, Mewes, HW, Stocker, S, Zaccaria, P, Bevan, M, Wilson, RK, de la Bastide, M, Habermann, K, Parnell, L, Dedhia, N, Gnoj, L, Schutz, K, Huang, E, Spiegel, L, Sehkon, M, Murray, J, Sheet, P, Cordes, M, Abu-Threideh, J, Stoneking, T, Kalicki, J, Graves, T, Harmon, G, Edwards, J, Latreille, P, Courtney, L, Cloud, J, Abbott, A, Scott, K, Johnson, D, Minx, P, Bentley, D, Fulton, B, Miller, N, Greco, T, Kemp, K, Kramer, J, Fulton, L, Mardis, E, Dante, M, Pepin, K, Hillier, L, Nelson, J, Spieth, J, Ryan, E, Andrews, S, Geisel, C, Layman, D, Du, H, Ali, J, Berghoff, A, Jones, K, Drone, K, Cotton, M, Joshu, C, Antonoiu, B, Zidanic, M, Strong, C, Sun, H, Lamar, B, Yordan, C, Ma, P, Zhong, J, Preston, R, Vil, D, Shekher, M, Matero, A, Shah, R, Swaby, I, European Union Arabidopsis, G, Washington, U & Sequenci, PEBA 1999, 'Sequence and Analysis of Chromosome 4 of the Plant *Arabidopsis thaliana*', *Nature*, vol. 402, pp. 769-777.

McMaster, GS & Wilhelm, WW 1997, 'Growing Degree-Days: One Equation, Two Interpretations', *Agricultural and Forest Meteorology*, vol. 87, pp. 291-300.

McWatters, HG, Bastow, RM, Hall, A & Millar, AJ 2000, 'The *ELF3* Zeitnehmer Regulates Light Signalling to the Circadian Clock', *Nature*, vol. 408, pp. 716-720.

Mei, DS, Wang, HZ, Hu, Q, Li, YD, Xu, YS & Li, YC 2009, 'QTL Analysis on Plant Height and Flowering Time in *Brassica napus*', *Plant Breeding*, vol. 128, pp. 458-465.

Mendham, NJ & Salisbury, PA 1995, 'Physiology: Crop Development, Growth and Yield', in DS Kimber & DI McGregor (eds), *Brassica Oilseeds: Production and Utilization*, CAB International, Wallingford, UK, pp. 11-64.

Messina, CD, Jones, JW, Boote, KJ & Vallejos, CE 2006, 'A Gene-Based Model to Simulate Soybean Development and Yield Responses to Environment', *Crop Science*, vol. 46, pp. 456-466.

Meyerowitz, EM 2001, 'Prehistory and History of Arabidopsis Research', *Plant Physiology*, vol. 125, pp. 15-19.

Michaels, SD & Amasino, RM 1999, 'FLOWERING LOCUS C Encodes a Novel MADS Domain Protein That Acts as a Repressor of Flowering', *The Plant Cell*, vol. 11, pp. 949-956.

---- 2000, 'Memories of Winter: Vernalization and the Competence to Flower', *Plant, Cell and Environment*, vol. 23, pp. 1145-1153.

---- 2001, 'Loss of *FLOWERING LOCUS C* Activity Eliminates the Late-Flowering Phenotype of *FRIGIDA* and Autonomous Pathway Mutations but Not Responsiveness to Vernalization', *The Plant Cell*, vol. 13, pp. 935-941.

Michaels, SD, Bezerra, IC & Amasino, RM 2004, '*FRIGIDA*-Related Genes are Required for the Winter-Annual Habit in *Arabidopsis*', *Proceedings of the National Academy of Sciences*, vol. 101, pp. 3281-3285.

Michaels, SD, Himelblau, E, Kim, SY, Schomburg, FM & Amasino, R 2005, 'Integration of Flowering Signals in Winter-Annual Arabidopsis', *Plant Physiology*, vol. 137, pp. 149-156.

Millar, AJ 2003, 'A Suite of Photoreceptors Entrains the Plant Circadian Clock', *Journal of Biological Rhythms*, vol. 18, pp. 217-226.

Mimida, N, Sakamoto, W, Murata, M & Motoyoshi, F 1999, '*TERMINAL FLOWER 1*-Like Genes in *Brassica* Species', *Plant Science*, vol. 142, pp. 155-162.

Mizoguchi, T, Wheatley, K, Hanzawa, Y, Wright, L, Mizoguchi, M, Song, HR, Carre, IA & Coupland, G 2002, '*LHY* and *CCA1* are Partially Redundant Genes Required to Maintain Circadian Rhythms in Arabidopsis', *Developmental Cell*, vol. 2, pp. 629-641.

Molinero-Rosales, N, Jamilena, M, Zurita, S, Gomez, P, Capel, J & Lozano, R 1999, '*FALSIFLORA*, the Tomato Orthologue of *FLORICAULA* and *LEAFY*, Controls Flowering Time and Floral Meristem Identity', *The Plant Journal*, vol. 20, pp. 685-693.

Moon, J, Lee, H, Kim, M & Lee, I 2005, 'Analysis of Flowering Pathway Integrators in *Arabidopsis*', *Plant Cell Physiology*, vol. 46, pp. 292-299.

Moon, J, Suh, SS, Lee, H, Choi, KR, Hong, CB, Paek, NC, Kim, SG & Lee, I 2003, 'The SOC1 MADS-box Gene Integrates Vernalization and Gibberellin Signals for Flowering in Arabidopsis', *Plant Journal*, vol. 35, pp. 613-623.

Murphy, DJ 2007, *People, Plants & Genes*, Oxford University Press, Oxford, UK.

Mutasa-Göttgens, E & Hedden, P 2009, 'Gibberellin as a Factor in Floral Regulatory Networks', *Journal of Experimental Botany*, vol. 60, pp. 1979-1989.

Nieuwhof, M 1969, *Cole Crops: Botany, Cultivation, and Utilization*, World Crops Books, Leonard Hill, London, UK.

Okamuro, JK, denBoer, BGW, LotysPrass, C, Szeto, W & Jofuku, KD 1996, 'Flowers into Shoots: Photo and Hormonal Control of a Meristem Identity Switch in Arabidopsis', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, pp. 13831-13836.

Okazaki, K, Sakamoto, K, Kikuchi, R, Saito, A, Togashi, E, Kuginuki, Y, Matsumoto, S & Hirai, M 2007, 'Mapping and Characterization of *FLC* Homologs and QTL Analysis of Flowering Time in *Brassica oleracea*', *Theoretical and Applied Genetics*, vol. 114, pp. 595-608.

Osborn, TC 2004, 'The Contribution of Polyploidy to Variation in *Brassica* Species', *Physiologica Plantarum*, vol. 121, pp. 531-536.

Osborn, TC, Kole, C, Parkin, IAP, Sharpe, AG, Kuiper, M, Lydiate, DJ & Trick, M 1997, 'Comparison of Flowering Time Genes in *Brassica rapa*, *B. napus* and *Arabidopsis thaliana*', *Genetics*, vol. 146, pp. 1123-1129.

Palmer, JE, Dikeman, DA, Fujinuma, T, Kim, B, Jones, JI, Denda, M, Martínez-Zapater, JM & Cruz-Alvarez, M 2001, 'A *Brassica oleracea* Gene Expressed in a Variety-Specific Manner May Encode a Novel Plant Transmembrane Receptor', *Plant Cell Physiology*, vol. 42, pp. 404-413.

Pearson, S, Hadley, P & Wheldon, AE 1994, 'A Model of the Effects of Temperature on the Growth and Development of Cauliflower (*Brassica oleracea* L. *botrytis*)', *Scientia Horticulturae*, vol. 59, pp. 91-106.

Pfaffl, MW 2001, 'A New Mathematical Model for Relative Quantification in Real-Time RT-PCR', *Nucleic Acids Research*, vol. 29, pp. 2002-2007.

Pires, JC, Zhao, J, Schranz, ME, Leon, EJ, Quijada, P, Lukens, L & Osborn, T 2004, 'Flowering Time Divergence and Genomic Rearrangements in Resynthesized *Brassica* Polyploids (Brassicaceae)', *Biological Journal of the Linnean Society*, vol. 82, pp. 675–688.

Primard, C, Vedel, F, Mathieu, C, Pelletier, G & Chévre, AM 1988, 'Interspecific Somatic Hybridization Between *Brassica napus* and *Brassica hirta* (*Sinapis alba* L.)', *Theoretical and Applied Genetics*, vol. 116, pp. 179-192.

Purvis, ON & Gregory, FG 1952, 'Studies in Vernalization. XII. The Reversability by High Temperature of the Vernalized Condition in Petkus Winter Rye', *Annals of Botany*, vol. 16, pp. 1-21.

Putterill, J, Laurie, R & Macknight, R 2004, 'It's Time to Flower: the Genetic Control of Flowering Time', *BioEssays*, vol. 26, pp. 363-373.

Putterill, J, Robson, F, Lee, K, Simon, R & Coupland, G 1995, 'The *CONSTANS* Gene of Arabidopsis Promotes Flowering and Encodes a Protein Showing Similarities to Zinc Finger Transcription Factors', *Cell*, vol. 80, pp. 847-857.

Rae, AM, Howell, EC & Kearsey, MJ 1999, 'More QTL for Flowering Time Revealed by Substitution Lines in *Brassica oleracea*', *Heredity*, vol. 83, pp. 586-596.

Rahman, MH, Bennett, RA, Yang, R-C, Kebede, B & Thiagarajah, MR 2011, 'Exploitation of the Late Flowering Species *Brassica oleracea* L. for the Improvement of Earliness in *B. napus* L.; an Untraditional Approach', *Euphytica*, vol. 177, pp. 365-374.

Rajandran, V 2008, 'Propensity for Devernalization in Cauliflowers', Honours thesis, University of Tasmania.

Rakow, G 2004, 'Species Origin and Economic Importance of *Brassica*', in EC Pua & CJ Douglas (eds), *Brassica*, Springer-Verlag, Heidelberg, vol. 54, pp. 3-11.

Ramin, AA & Atherton, JG 1994, 'Manipulation of Bolting and Flowering in Celery (*Apium graveolens* L. var. dulce): Effect of Photoperiod and Irradiance', *Journal of Horticultural Science*, vol. 69, pp. 861-868.

Razi, H, Howell, EC, Newbury, HJ & Kearsey, MJ 2008, 'Does Sequence Polymorphism of *FLC* Paralogues Underlie Flowering Time QTL in *Brassica oleracea?*', *Theoretical and Applied Genetics*, vol. 116, pp. 179-192.

Richards, CL, Hanzawa, Y, Katari, M, Ehrenreich, IM, Englemann, KE & Purugganan, MD 2009, 'Perspective on Ecological and Evolutionary Systems Biology', *Annual Review of Plant Biology*, vol. 35, pp. 331-351.

Sadik, S 1962, 'Morphology of the Curd of Cauliflower', *American Journal of Botany*, vol. 49, pp. 290-297.

Saito, H & Saito, T 2003, 'Effects of High Temperature Interruption During Vernalization on the Inflorescence Formation in Turnip Plants', *Journal of the Japanese Society for Horticultural Science*, vol. 72, pp. 329-334.

Salanoubat, M, Lemcke, K, Rieger, M, Ansorge, W, Unseld, M, Fartmann, B, Valle, G, Blocker, H, Perez-Alonso, M, Obermaier, B, Delseny, M, Boutry, M, Grivell, LA, Mache, R, Puigdomenech, P, De Simone, V, Choisne, N, Artiguenave, F, Robert, C, Brottier, P, Wincker, P, Cattolico, L, Weissenbach, J, Saurin, W, Quetier, F, Schafer, M, Muller-Auer, S, Gabel, C, Fuchs, M, Benes, V, Wurmbach, E, Drzonek, H, Erfle, H, Jordan, N, Bangert, S, Wiedelmann, R, Kranz, H, Voss, H, Holland, R, Brandt, P, Nyakatura, G, Vezzi, A, D'Angelo, M, Pallavicini, A, Toppo, S, Simionati, B, Conrad, A, Hornischer, K, Kauer, G, Lohnert, TH, Nordsiek, G, Reichelt, J, Scharfe, M, Schon, O, Bargues, M, Terol, J, Climent, J, Navarro, P, Collado, C, Perez-Perez, A, Ottenwalder, B, Duchemin, D, Cooke, R, Laudie, M, Berger-Llauro, C, Purnelle, B, Masuy, D, de Haan, M, Maarse, AC, Alcaraz, JP, Cottet, A, Casacuberta, E, Monfort, A, Argiriou, A, Flores, M, Liguori, R, Vitale, D, Mannhaupt, G, Haase, D, Schoof, H, Rudd, S, Zaccaria, P, Mewes, HW, Mayer, KFX, Kaul, S, Town, CD, Koo, HL, Tallon, LJ, Jenkins, J, Rooney, T, Rizzo, M, Walts, A, Utterback, T, Fujii, CY, Shea, TP, Creasy, TH, Haas, B, Maiti, R, Wu, DY, Peterson, J, Van Aken, S, Pai, G, Militscher, J, Sellers, P, Gill, JE, Feldblyum, TV, Preuss, D, Lin, XY, Nierman, WC, Salzberg, SL, White, O, Venter, JC, Fraser, CM, Kaneko, T, Nakamura, Y, Sato, S, Kato, T, Asamizu, E, Sasamoto, S, Kimura, T, Idesawa, K, Kawashima, K, Kishida, Y, Kiyokawa, C, Kohara, M, Matsumoto, M, Matsuno, A, Muraki, A, Nakayama, S, Nakazaki, N, Shinpo, S, Takeuchi, C, Wada, T, Watanabe, A, Yamada, M, Yasuda, M, Tabata, S,

European Union Chromosome, A, Inst Genomic, R & Kazusa, DNARI 2000, 'Sequence and Analysis of Chromosome 3 of the Plant *Arabidopsis thaliana*', *Nature*, vol. 408, pp. 820-822.

Samach, A, Onouchi, H, Gold, SE, Ditta, GS, Schwarz-Sommer, Z, Yanofsky, MF & Coupland, G 2000, 'Distinct Roles of *CONSTANS* Target Genes in Reproductive Development of *Arabidopsis*', *Science*, vol. 288, pp. 1613-1616.

Sawa, M & Kays, SA 2011, 'GIGANTEA Directly Activates *Flowering Locus T* in *Arabidopsis thaliana*', *Proceedings of the National Academy of Sciences*, vol. 108, pp. 11698-11703.

Sawa, M, Nusinow, DA, Kay, SA & Imaizumi, T 2007, '*FKF1* and *GIGANTEA* Complex Formation Is Required for Day-Length Measurement in *Arabidopsis*', *Science*, vol. 318 pp. 261-265.

Schlappi, MR 2006, '*FRIGIDA LIKE 2* is a Functional Allele in Landsberg erecta and Compensates for a Nonsense Allele of *FRIGIDA LIKE 1*', *Plant Physiology*, vol. 142, pp. 1728-1738.

Schönrock, N, Bouveret, R, Leroy, O, Borghi, L, Kohler, C, Gruissem, W & Hennig, L 2006, 'Polycomb-Group Proteins Repress the Floral Activator *AGL19* in the *FLC*-Independent Vernalization Pathway', *Genes & Development*, vol. 20, pp. 1667-1678.

Schranz, ME, Quijada, P, Sung, S, Lukens, L, Amasino, RM & Osborn, TC 2002, 'Characterization and Effects of the Replicated Flowering Time Gene *FLC* in *Brassica rapa*', *Genetics*, vol. 162, pp. 1457-1468.

Schultz, EA & Haughn, GW 1991, '*LEAFY*, a Homeotic Gene that Regulates Inflorescence Development in Arabidopsis', *The Plant Cell*, vol. 3, pp. 771-781.

Sheldon, CC, Burn, JE, Perez, PP, Metzger, J, Edwards, JA, Peacock, WJ & Dennis, ES 1999, 'The *FLF* MADS Box Gene: A Repressor of Flowering in Arabidopsis Regulated by Vernalization and Methylation', *The Plant Cell*, vol. 11, pp. 445-458.

Simpson, GG 2004, 'The Autonomous Pathway: Epigenetic and Post-Transcriptional Gene Regulation in the Control of Arabidopsis Flowering Time', *Current Opinion in Plant Biology*, vol. 7, pp. 570–574.

Simpson, GG & Dean, C 2002, 'Arabidopsis, the Rosetta Stone of Flowering Time?', *Science*, vol. 296, pp. 285-289.

Simpson, GG, Dijkwel, PP, Quesada, V, Henderson, I & Dean, C 2003, '*FY* is an RNA 3' End-Processing Factor that Interacts with *FCA* to Control the Arabidopsis Floral Transition', *Cell*, vol. 113, pp. 777-787.

Simpson, GG, Gendall, AR & Dean, C 1999, 'When to Switch to Flowering', *Annual Review of Cell Developmental Biology*, vol. 99, pp. 519-550.

Smith, HMS, Ung, N, Lal, S & Courtier, J 2010, 'Specification of Reproductive Meristems Requires the Combined Function of *SHOOT MERISTEMLESS* and Floral Integrators *FLOWERING LOCUS T* and *FD* During *Arabidopsis* Inflorescence Development', *Journal of Experimental Botany*, vol. 62, pp. 583-593.

Smith, LB 1999, 'The Molecular Genetics of Curd Morphology and Domestication of Cauliflower (*Brassica oleracea* L. var. *botrytis* L.)', PhD thesis, University of Warwick.

Smith, LB & King, GJ 2000, 'The Distribution of *BoCAL*-a Alleles in *Brassica oleracea* is Consistent with a Genetic Model for Curd Development and Domestication of the Cauliflower', *Molecular Breeding*, vol. 6, pp. 603-613.

Snogerup, S 1980, 'The Wild Forms of the *Brassica oleracea* Group (2n=18) and their Possible Relations to the Cultivated Ones', in S Tsunoda, K Hinata & C Gomez-Campo (eds), *Brassica Crops and Wild Allies, Biology and Breeding*, Japan Scientific Societies Press, Tokyo, Japan, pp. 151-163.

Snowdon, RJ, Friedrich, T, Friedt, W & Kohler, W 2002, 'Identifying the Chromosomes of the A- and C-Genome Diploid *Brassica* Species *B. rapa* (syn. *campestris*) and *B. oleracea* in Their Amphidiploid *B. napus*', *Theoretical and Applied Genetics*, vol. 83, pp. 602-607.

Song, KM, Osborn, TC & Williams, PH 1990, '*Brassica* Taxonomy Based on Nuclear Restriction Fragment Length Polymorphisms (RFLPs). 3. Genome Relationships in *Brassica* and Related Genera and the Origin of *B. oleracea* and *B. rapa* (syn. *campestris*)', *Theoretical and Applied Genetics*, vol. 79, pp. 497-506.

Spalding, EP & Folta, KM 2005, 'Illuminating Topics in Plant Photobiology', *Plant, Cell and Environment*, vol. 28, pp. 39-53.

Spurr, C & Geard, A 2010, *Improving Reliability of Flowering in Bolting Resistant Carrot Seed Crops*, Final Report, Horticulture Australia Limited Project VGO6155.

Suárez-López, P, Wheatley, K, Robson, F, Onouchi, H, Valverde, F & Coupland, G 2001, '*CONSTANS* Mediates Between the Circadian Clock and the Control of Flowering in *Arabidopsis*', *Nature*, vol. 410, pp. 1116-1120.

Sung, S & Amasino, RM 2004a, 'Vernalization and Epigenetics: How Plants Remember Winter', *Current Opinion in Plant Biology*, vol. 7, pp. 4-10.

---- 2004b, 'Vernalization in *Arabidopsis thaliana* is Mediated by the PHD Finger Protein VIN3', *Nature*, vol. 427, pp. 159-164.

Suwabe, K, Morgan, C & Bancroft, I 2008, 'Integration of Brassica A Genome Genetic Linkage Map Between *Brassica napus* and *B. rapa*', *Genome*, vol. 51, pp. 169-176.

Swiezewski, S, Liu, F, Magusin, A & Dean, C 2009, 'Cold-Induced Silencing by Long Antisense Transcripts of an Arabidopsis Polycomb Target', *Nature*, vol. 462, pp. 799-802.

Tabata, S, Kaneko, T, Nakamura, Y, Kotani, H, Kato, T, Asamizu, E, Miyajima, N, Sasamoto, S, Kimura, T, Hosouchi, T, Kawashima, K, Kohara, M, Matsumoto, M, Matsuno, A, Muraki, A, Nakayama, S, Nakazaki, N, Naruo, K, Okumura, S, Shinpo, S, Takeuchi, C, Wada, T, Watanabe, A, Yamada, M, Yasuda, M, Sato, S, de la Bastide, M, Huang, E, Spiegel, L, Gnoj, L, O'Shaughnessy, A, Preston, R, Habermann, K, Murray, J, Johnson, D, Rohlrng, T, Nelson, J, Stoneking, T, Pepin, K, Spieth, J, Sekhon, M, Armstrong, J, Becker, M, Belter, E, Cordum, H, Cordes, M, Courtney, L, Courtney, W, Dante, M, Du, H, Edwards, J, Fryman, J, Haakensen, B, Lamar, E, Latreille, P, Leonard, S, Meyer, R, Mulvaney, E, Ozersky, P, Riley, A, Strowmatt, C, Wagner-McPherson, C, Wollam, A, Yoakum, M, Bell, M, Dedhia, N, Parnell, L, Shah, R, Rodriguez, M, See, LH, Vil, D, Baker, J, Kirchoff, K, Toth, K, King, L, Bahret, A, Miller, B, Marra, M, Martienssen, R, McCombie, WR, Wilson, RK, Murphy, G, Bancroft, I, Volckaert, G, Wambutt, R, Dusterhoft, A, Stiekema, W, Pohl, T, Entian, KD, Terryn, N, Hartley, N, Bent, E, Johnson, S, Langham, SA, McCullagh, B, Robben, J, Grymonprez, B, Zimmermann, W, Ramsperger, U, Wedler, H, Balke, K, Wedler, E, Peters, S, van Staveren, M, Dirkse, W, Mooijman, P, Lankhorst, RK, Weitzenegger, T, Bothe, G, Rose, M, Hauf, J, Berneiser, S, Hempel, S, Feldpausch, M, Lamberth, S, Villarroel, R, Gielen, J, Ardiles, W, Bents, O, Lemcke, K, Kolesov, G, Mayer, K, Rudd, S, Schoof, H, Schueller, C, Zaccaria, P, Mewes, HW, Bevan, M, Kazusa, DNARI, Cold Spring Harbor Washington, U & European Union Arabidopsis, G 2000, 'Sequence and Analysis of Chromosome 5 of the Plant *Arabidopsis thaliana*', *Nature*, vol. 408, pp. 823-826.

Tadege, M, Sheldon, CC, Helliwell, CA, Stoutjesdijk, P, Dennis, ES & Peacock, WJ 2001, 'Control of Flowering Time by *FLC* Orthologues in *Brassica napus*', *The Plant Journal*, vol. 28, pp. 545-553.

Taylor, A, Massiah, AJ & Thomas, B 2010, 'Conservation of *Arabidopsis thaliana* Photoperiodic Flowering Time Genes in Onion (*Allium cepa* L.)', *Plant Cell Physiology*, vol. 51, pp. 1638–1647.

Theologis, A, Ecker, JR, Palm, CJ, Federspiel, NA, Kaul, S, White, O, Alonso, J, Altafi, H, Araujo, R, Bowman, CL, Brooks, SY, Buehler, E, Chan, A, Chao, QM, Chen, HM, Cheuk, RF, Chin, CW, Chung, MK, Conn, L, Conway, AB, Conway, AR, Creasy, TH, Dewar, K, Dunn, P, Etgu, P, Feldblyum, TV, Feng, JD, Fong, B, Fujii, CY, Gill, JE, Goldsmith, AD, Haas, B, Hansen, NF, Hughes, B, Huizar, L,

Hunter, JL, Jenkins, J, Johnson-Hopson, C, Khan, S, Khaykin, E, Kim, CJ, Koo, HL, Kremenetskaia, I, Kurtz, DB, Kwan, A, Lam, B, Langin-Hooper, S, Lee, A, Lee, JM, Lenz, CA, Li, JH, Li, YP, Lin, XY, Liu, SX, Liu, ZA, Luros, JS, Maiti, R, Marziali, A, Militscher, J, Miranda, M, Nguyen, M, Nierman, WC, Osborne, BI, Pai, G, Peterson, J, Pham, PK, Rizzo, M, Rooney, T, Rowley, D, Sakano, H, Salzberg, SL, Schwartz, JR, Shinn, P, Southwick, AM, Sun, H, Tallon, LJ, Tambunga, G, Toriumi, MJ, Town, CD, Utterback, T, Van Aken, S, Vaysberg, M, Vysotskaia, VS, Walker, M, Wu, DY, Yu, GX, Fraser, CM, Venter, JC & Davis, RW 2000, 'Sequence and Analysis of Chromosome 1 of the Plant *Arabidopsis thaliana*', *Nature*, vol. 408, pp. 816-820.

Thomas, D & Vince-Prue, B 1997, *Photoperiodism in Plants*, 2nd edn, Academic Press, New York.

Thompson, KF 1979, 'Cabbages, Kales, etc.: *Brassica oleracea* (Cruciferae)', in NE Simmonds (ed.), *Evolution of Crop Species*, Longman, London, UK, pp. 49-52.

Tiwari, SB, Shen, Y, Chang, H-C, Hou, Y, Harris, A, Ma, SF, McPartland, M, Hymus, GJ, Adam, L, Marion, C, Belachew, A, Repetti, PP, Reuber, TL & Ratcliffe, O 2010, 'The Flowering Time Regulator CONSTANS is Recruited to the *FLOWERING LOCUS T* Promoter Via a Unique Cis-Element', *New Phytologist*, vol. 187, pp. 57-66.

Tooke, F, Ordidge, M, Chiurugwi, T & Battey, N 2005, 'Mechanisms and Function of Flower and Inflorescence Reversion', *Journal of Experimental Botany*, vol. 56, pp. 2587-2599.

Town, CD, Cheung, F, Maiti, R, Crabtree, J, Haas, BJ, Wortman, JR, Hine, EE, Althoff, E, Arbogast, TS, Tallon, LJ, Vigouroux, M, Trick, M & Bancroft, I 2006, 'Comparative Genomics of *Brassica oleracea* and *Arabidopsis thaliana* Reveal Gene Loss, Fragmentation and Dispersal after Polyploidy', *Plant Cell*, vol. 18, pp. 1348-1359.

Trap-Gentil, MV, Hébrard, C, Lafon-Placette, C, Delaunay, A, Hagège, D, Joseph, C, Brignolas, F, Lefebvre, M, Barnes, S & Maury, S 2011, 'Time Course and Amplitude of DNA Methylation in the Shoot Apical Meristem are Critical Points for Bolting Induction in Sugar Beet and Bolting Tolerance Between Genotypes', *Journal of Experimental Botany*, vol. Advance Access, doi:10.1093/jxb/erq433.

Tseng, T-S, Salomé, PA, McClung, CR & Olszewskia, NE 2004, 'SPINDLY and GIGANTEA Interact and Act in *Arabidopsis thaliana* Pathways Involved in Light Responses, Flowering, and Rhythms in Cotyledon Movements', *The Plant Cell*, vol. 16, pp. 1550-1563.

Turck, F, Fornara, F & Coupland, G 2008, 'Regulation and Identity of Florigen: *FLOWERING LOCUS T* Moves Center Stage', *Annual Review of Plant Biology*, vol. 59, pp. 573–594.

U, N 1935, 'Genomic Analysis in *Brassica* with Special Reference to the Experimental Formation of *B. napus* and Peculiar Mode of Fertilization', *Japanese Journal of Botany*, vol. 7, pp. 389-452.

United States Department of Agriculture 2011, *Oilseeds: World Market and Trade*, March 2011 edn, viewed 1 April 2011, <http://www.fas.usda.gov/oilseeds/circular/2011/March/oilseeds.pdf>.

Uptmoor, R, Li, J, Schrag, T & Stützel, H 2011, 'Prediction of Flowering Time in *Brassica oleracea* Using a Quantitative Trait Loci-Based Phenology Model', *Plant Biology*, vol. Advance Access, doi:10.1111/j.1438-8677.2011.00478.x.

Uptmoor, R, Schrag, T, Stützel, H & Esch, E 2008, 'Crop Model Based QTL Analysis Across Environments and QTL Based Estimation of Time to Floral Induction and Flowering in *Brassica oleracea*', *Molecular Breeding*, vol. 21, pp. 205-216.

Van Poppel, G, Verhoeven, DT, Verhagen, H & Goldbohm, RA 1999, 'Brassica Vegetables and Cancer Prevention: Epidemiology and Mechanisms', *Advances in Experimental Medicine and Biology*, vol. 472, pp. 159-168.

Vasanthi, HR, Mukherjee, S & Das, DK 2009, 'Potential Health Benefits of Broccoli - A Chemico-Biological Overview', *Mini-Reviews in Medicinal Chemistry*, vol. 9, pp. 749-759.

Verdial, MF, Cardoso, AII, de Lima, MS & Chaves, FCM 2001, 'Coincidence of Flowering Time and the Productivity and Quality of Hybrid Cauliflower Seeds', *Scientia Agricola*, vol. 58, pp. 533-539.

Vorobiev, VA, Pankin, AA & Khavkin, EE 2006, 'Polymorphism of FRIGIDA gene in Brassica genus', *Unpublished: NCBI reference sequence DQ503574*.

Wang, J, Tian, L, Lee, H-S & Chen, J 2006, 'Nonadditive Regulation of *FRI* and *FLC* Loci Mediates Flowering-Time Variation in Arabidopsis Allopolyploids', *Genetics*, vol. 173, pp. 965–974.

Wang, N, Qian, W, Suppanz, I, Wei, L, Mao, B, Long, Y, Meng, J, Müller, AE & Jung, C 2011a, 'Flowering Time Variation in Oilseed Rape (*Brassica napus* L.) is Associated with Allelic Variation in the *FRIGIDA* Homologue *BnaA.FRI.a*', *Journal of Experimental Botany*, vol. Advance Access, doi:10.1093/jxb/err249.

Wang, R, Farrona, S, Vincent, C, Joecker, A, Schoof, H, Turck, F, Alonso-Blanco, C, Coupland, G & Albani, MC 2009, '*PEP1* Regulates Perennial Flowering in *Arabis alpina*', *Nature*, vol. 459, pp. 423-428.

Wang, XW, Wang, HZ, Wang, J, Sun, RF, Wu, J, Liu, SY, Bai, YQ, Mun, JH, Bancroft, I, Cheng, F, Huang, SW, Li, XX, Hua, W, Wang, JY, Wang, XY, Freeling, M, Pires, JC, Paterson, AH, Chalhoub, B, Wang, B, Hayward, A, Sharpe, AG, Park, BS, Weisshaar, B, Liu, BH, Li, B, Liu, B, Tong, CB, Song, C, Duran, C, Peng, CF, Geng, CY, Koh, CS, Lin, CY, Edwards, D, Mu, DS, Shen, D, Soumpourou, E, Li, F, Fraser, F, Conant, G, Lassalle, G, King, GJ, Bonnema, G, Tang, HB, Wang, HP, Belcram, H, Zhou, HL, Hirakawa, H, Abe, H, Guo, H, Wang, H, Jin, HZ, Parkin, IAP, Batley, J, Kim, JS, Just, J, Li, JW, Xu, JH, Deng, J, Kim, JA, Li, JP, Yu, JY, Meng, JL, Wang, JP, Min, JM, Poulain, J, Hatakeyama, K, Wu, K, Wang, L, Fang, L, Trick, M, Links, MG, Zhao, MX, Jin, MN, Ramchiary, N, Drou, N, Berkman, PJ, Cai, QL, Huang, QF, Li, RQ, Tabata, S, Cheng, SF, Zhang, S, Zhang, SJ, Huang, SM, Sato, S, Sun, SL, Kwon, SJ, Choi, SR, Lee, TH, Fan, W, Zhao, X, Tan, X, Xu, X, Wang, Y, Qiu, Y, Yin, Y, Li, YR, Du, YC, Liao, YC, Lim, Y, Narusaka, Y, Wang, YP, Wang, ZY, Li, ZY, Wang, ZW, Xiong, ZY & Zhang, ZH 2011b, 'The Genome of the Mesopolyploid Crop Species *Brassica rapa*', *Nature Genetics*, vol. 43, pp. 1035-1039.

Welch, SM, Roe, JL & Dong, Z 2003, 'A Genetic Neural Network Model of Flowering Time Control in *Arabidopsis thaliana*', *Agronomy Journal*, vol. 95, pp. 71-81.

Wenden, B, Dun, EA, Hanan, J, Andrieu, B, Weller, JL, Beveridge, CA & Rameau, C 2009, 'Computational Analysis of Flowering in Pea (*Pisum sativum*)', *New Phytologist*, vol. 184, pp. 153–167.

White, JW & Hoogenboom, G 1996, 'Simulating Effects of Genes for Physiological Traits in a Process-Oriented Crop Model', *Agronomy Journal*, vol. 88, pp. 416–422.

Wiebe, H, Habegger, R & Liebig, H 1992, 'Quantification of Vernalization and Devernalization Effects for Kohlrabi (*Brassica oleracea* convar. acephala var. gongylodes L.)', *Scientia Horticulturae*, vol. 50, pp. 11-20.

Wien, HC & Wurr, DCE 1997, 'Cauliflower, Broccoli, Cabbage and Brussel Sprouts', in HC Wien (ed.), *The Physiology of Vegetable Crops*, CAB International, Wallingford, UK.

Wigge, PA, Kim, MC, Jaeger, KE, Busch, W, Schmid, M, Lohmann, JU & Weigel, D 2005, 'Integration of spatial and temporal information during floral induction in Arabidopsis', *Science*, vol. 309, pp. 1056–1059.

Wilczek, AM, Roe, JL, Knapp, MC, Cooper, MD, Lopez-Gallego, C, Martin, LJ, Muir, CD, Sim, S, Walker, A, Anderson, J, Egan, JF, Moyers, BT, Petipas, R, Giakountis, A, Charbit, E, Coupland, G, Welch, SM & Schmitt, J 2009, 'Effects of Genetic Perturbation on Seasonal Life History Plasticity', *Science*, vol. 232, pp. 930-934.

Wilkinson, MD & Haughn, GW 1995, '*UNUSUAL FLORAL ORGANS* Controls Meristem ldentity and Organ Primordia Fate in Arabidopsis', *The Plant Cell*, vol. 7, pp. 1485-1499.

Willcox, JK, Catignani, GL & Lazarus, S 2003, 'Tomatoes and Cardiovascular Health', *Critical Reviews of Food Science and Nutrition*, vol. 43, pp. 1-18.

Wilson, RN, Heckman, JW & Somerville, CR 1992, 'Gibberellin is Required for Flowering in *Arabidopsis thaliana* Under Short Days', *Plant Physiology*, vol. 100, pp. 403-408.

Wurr, DCE, Fellows, JR, Phelps, K & Reader, RJ 1993, 'Vernalization in Summer/Autumn Cauliflower (*Brassica oleracea* var. *botrytis* L.)', *Journal of Experimental Botany*, vol. 44, pp. 1507-1514.

---- 1995, 'Vernalization in Calabrese (*Brassica oleracea* var. *italiaca*) - a Model for Apex Development', *Journal of Experimental Botany*, vol. 46, pp. 1487-1496.

Yamaguchi, A, Kobayashi, Y, Goto, K, Abe, M & Araki, T 2005, '*TWIN SISTER OF FT* (*TSF*) Acts as a Floral Pathway Integrator Redundantly with *FT*', *Plant and Cell Physiology*, vol. 46, pp. 1175-1189.

Yant, L 2012, 'Genome-Wide Mapping of Transcription Factor Binding Reveals Developmental Process Integration and a Fresh Look at Evolutionary Dynamics', *American Journal of Botany*, vol. 99, pp. 277-290.

Yin, X, Chasalow, SD, Dourleijn, CJ, Stam, P & Kropff, MJ 2000, 'Coupling Estimated Effects of QTLs for Physiological Traits to a Crop Growth Model: Predicting Yield Variation Among Recombinant Inbred Lines in Barley', *Heredity*, vol. 85, pp. 539-549.

Yin, X, Struik, PC, van Eeuwijk, FA, Stam, P & Tang, J 2005, 'QTL Analysis and QTL-Based Prediction of Flowering Phenology in Recombinant Inbred Lines of Barley', *Journal of Experimental Botany*, vol. 56, pp. 967-976.

Yu, LX, Parthasarathy, MV & Nasrallah, J 1998, 'A cDNA Clone Encoding Actin from *Brassica oleracea* Anthers', *Unpublished: NCBI reference sequence AF044573*.

Yu, X, Li, W, Jiang, X, Liu, D & Zhang, Y 2010, 'Changes of Endogenous Hormone During Devernalization in Cabbage', *China Vegetables*, no. 20, pp. 38-41.

Zeilinger, MN, Farre, EM, Taylor, SR, Kay, SA & Doyle, FJ 2006, 'A Novel Computational Model of the Circadian Clock in *Arabidopsis* that Incorporates PRR7 and PRR9', *Molecular Systems Biology*, vol. 2, no. 58.

Zhao, J, Artemyeva, A, Del Carpio, DP, Basnet, RK, Zhang, N, Gao, J, Li, F, Bucher, J, Wang, X, Visser, RG & Bonnema, G 2010a, 'Design of a *Brassica rapa* core collection for association mapping studies', *Genome*, vol. 53, pp. 884-898.

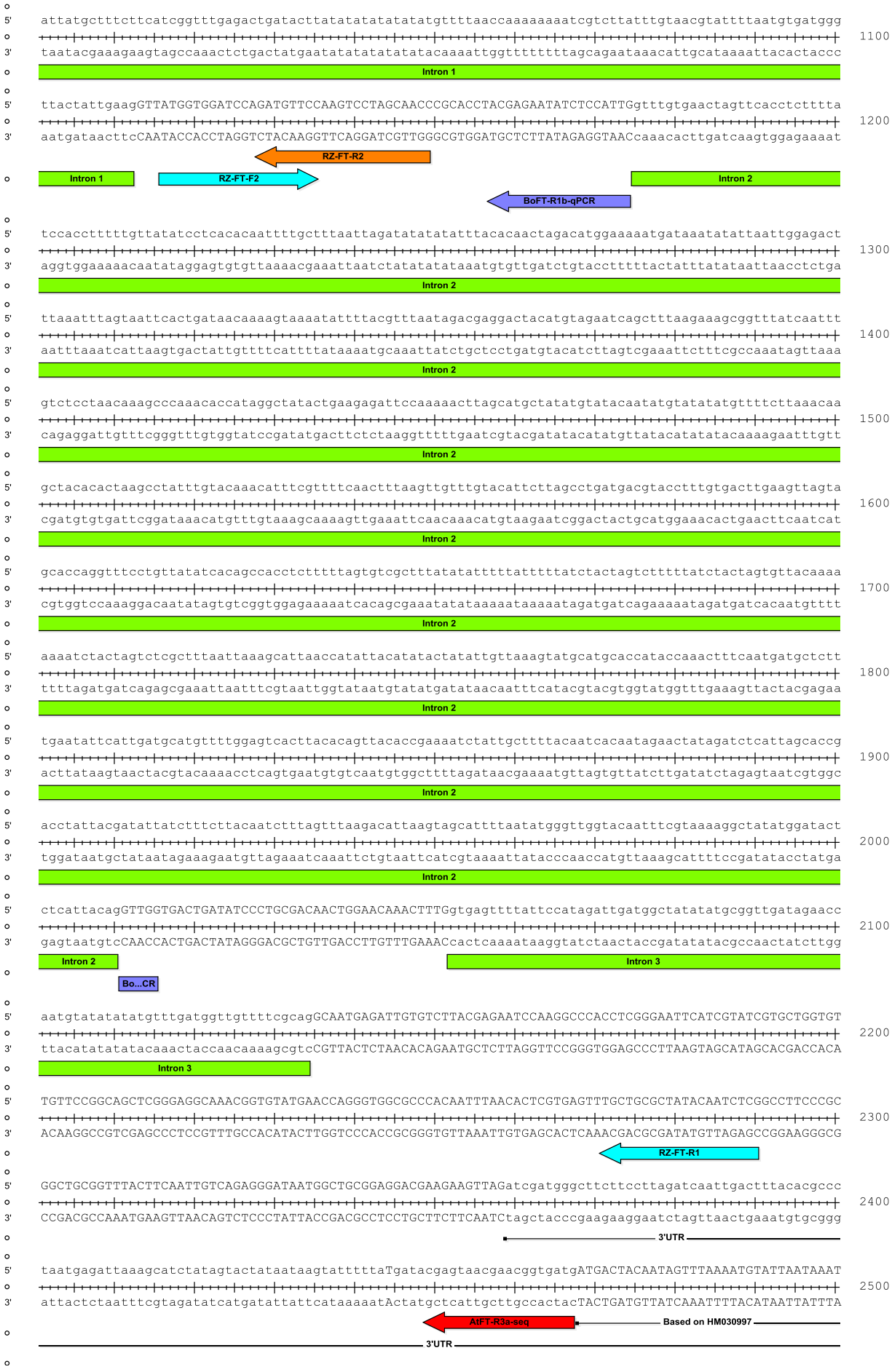
Zhao, J, Kulkarni, V, Liu, N, del Carpio, DP, Bucher, J & Bonnema, G 2010b, '*BrFLC2* (*FLOWERING LOCUS C*) as a Candidate Gene for Vernalization Response QTL in *Brassica rapa*', *Journal of Experimental Botany*, vol. 61, pp. 1817-1825.

Zhao, J, Paulo, M-J, Jamar, D, Lou, P, van Eeuwijk, F, Bonnema, G, Vreugdenhil, D & Koornneef, M 2007, 'Association Mapping of Leaf Traits, Flowering Time, and Phytate Content in *Brassica rapa*', *Genome*, vol. 50, pp. 963-973.

Appendix I

ANNOTATED *BoFT* gDNA SEQUENCE

Regions of cDNA are based on RNA extracted from an uncharacterised late-flowering cauliflower. Intron sequences are based on the consensus sequence of gDNA from cauliflower parent lines A and B (see Chapter III). Note that the reverse primer BoFT-R1b-qPCR (purple) spans intron 2.

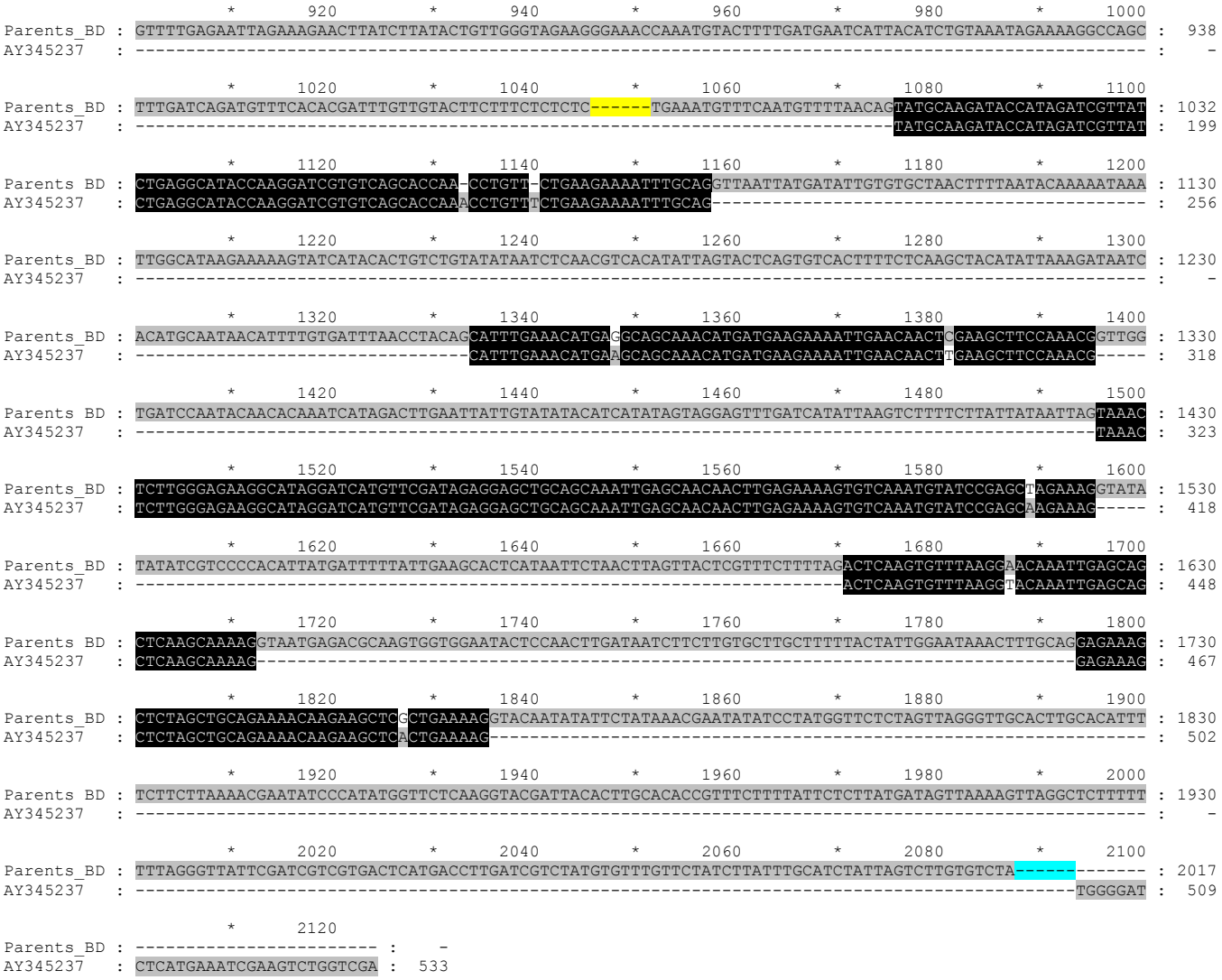

Intron 1
RZ-FT-R2
Intron 1
RZ-FT-F2
Intron 2
BoFT-R1b-qPCR
Intron 2
Intron 2
Intron 2
Intron 2
Intron 2
Intron 2
Intron 2
Intron 2
Intron 2
Intron 3
Bo...CR
Intron 3
RZ-FT-R1
3'UTR
AtFT-R3a-seq
Based on HM030997
3'UTR

Appendix II

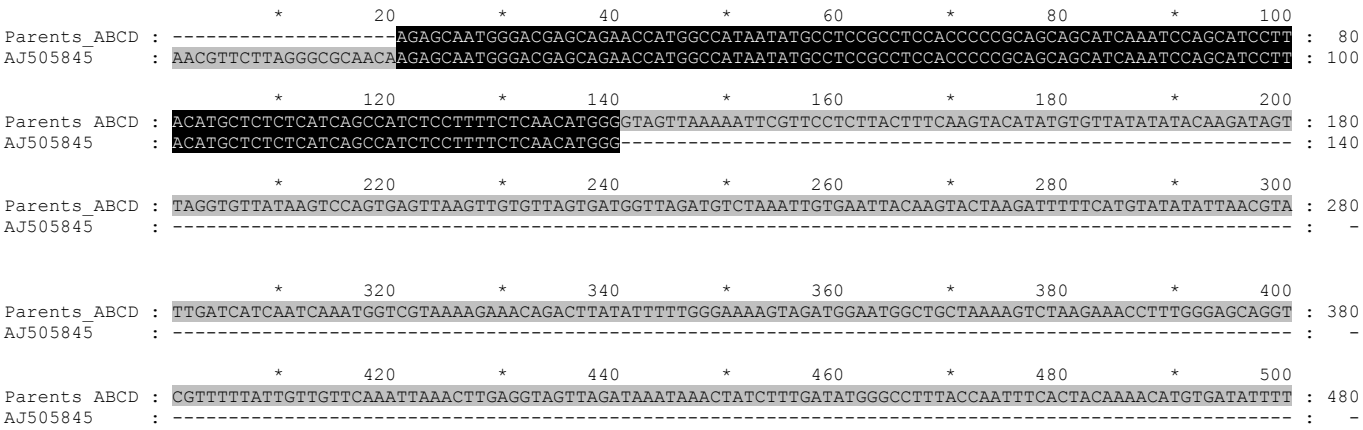
SEQUENCES AND ALIGNMENTS OF FLOWERING TIME CANDIDATE GENE PCR PRODUCTS IN PARENT LINES A, B, C, AND D

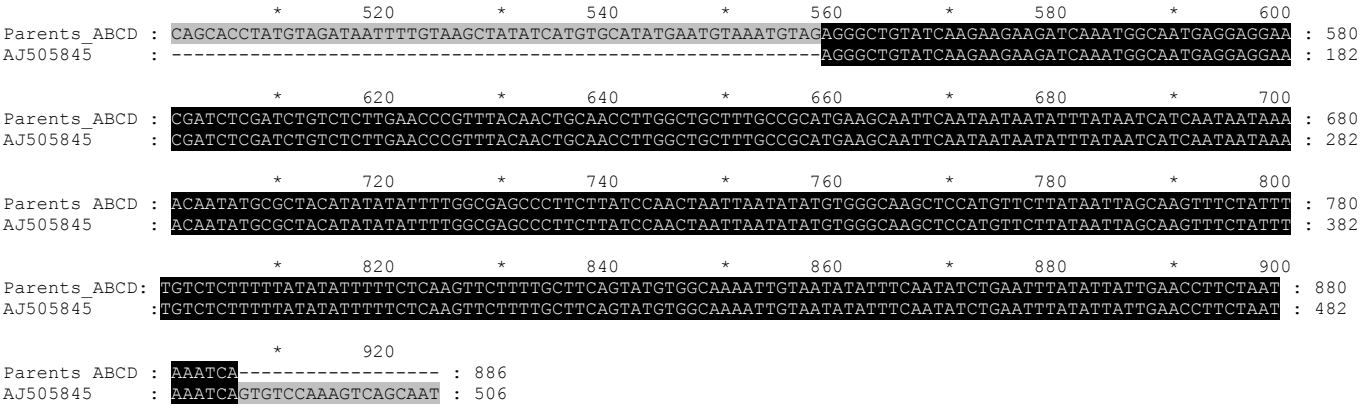
Of the 49 PCRs listed in Table 7 of Chapter III, 25 yielded fragments that were able to be sequenced. Five of these sequences were illegible, and of the remaining 20 sequences, four have already been shown in Chapter III (PCRs 25, 23, 33 and 46). *BoFLC5* sequences 13-15 are not shown here, as 13 and 14 are identical to reference sequence AM231519, and 15 differs only in one SNP (an A in the reference sequence becomes a T in the PCR fragment). *CCE1* (PCR 14) is also not shown here because it is identical to reference sequence AF227978. The remaining 12 sequences, which are presented here, either differ from their corresponding reference sequence or between the four parent lines.

```
                  *        20        *        40        *        60        *        80        *       100
Parents_BD : ------------------------------------------------------------GAAGGAATGGTTTGTTGAAAAAAGCCTTTGAGCTCTCA :   38
AY345237   : GGGCAAAACTCAGATGAAGCGAATAGAGAATGCAACAAGCAGACAAGTGACTTTCTCTAAGCGAAGGAATGGTTTGCTGAAAAAAGCCTTTGAGCTCTCA :  100

                  *       120        *       140        *       160        *       180        *       200
Parents_BD : GTCCTTTGTGATGCTGAAGTTTCTCTGATCATCTTCTCTCCTAAGGCAAAACTTTATGAATTCGCCAGCTCCAAGTACGTTCTCCTTTTTATACTATATA :  138
AY345237   : GTTCTTTGTGATGCTGAAGTTTCTCTGATCATCTTCTCTCCTAAGGCAAAACTTTATGAATTTGCCAGCTCCAA------------------------ :  174

                  *       220        *       240        *       260        *       280        *       300
Parents_BD : TATATAAATCGTCTACAGATAAAAGAGATAAATTTTTTTGTTTATATACCTTGGTTTATATATGGATCTGTTGGCGGTTTACTGAATCATATCTCTTTGA :  238
AY345237   : ------------------------------------------------------------------------------------------------- :    -

                  *       320        *       340        *       360        *       380        *       400
Parents_BD : CGTTTGCTTAGAGAGAGTGTTAACTGTTAAGTGAGTTTCTTGGTTTCTACAATTGTGTCTATTATTAAAGTAAAACCAAGTACATCATCTTATTTATTTC :  338
AY345237   : ------------------------------------------------------------------------------------------------- :    -

                  *       420        *       440        *       460        *       480        *       500
Parents_BD : TTGTTAACTACTACATCATCTTGTTTAATCCTATTAGATGTCTCTTTCTCATATTTAGTTTATGAGTTATGATAAACGTTAAAACACAAAAGAATTCTCT :  438
AY345237   : ------------------------------------------------------------------------------------------------- :    -

                  *       520        *       540        *       560        *       580        *       600
Parents_BD : GGCAAGTACTTTGCACTTGTTATATGTATGTATGTACAATTGATCATCTTGGATTGTTTTAGAGATCATAATCACACACTGGACAACTCCTTGAGTTTTG :  538
AY345237   : ------------------------------------------------------------------------------------------------- :    -

                  *       620        *       640        *       660        *       680        *       700
Parents_BD : TATTAAACTCTTTCTTGGTTCGTTCAAAAATGATATTTATGTTGCAATTAGCATTCTTTTGTTTTGACATGATACACAATCTGGTTGAACCAATCTGCCT :  638
AY345237   : ------------------------------------------------------------------------------------------------- :    -

                  *       720        *       740        *       760        *       780        *       800
Parents_BD : TGTCATTGCTTAAAAAAATTATAGATTGCTCTAACTGATAATTGGGAACTTTTTTTTATATCAGTCCTGAAAGATAAATCAGGCACGTTTTATTTCATGC :  738
AY345237   : ------------------------------------------------------------------------------------------------- :    -

                  *       820        *       840        *       860        *       880        *       900
Parents_BD : TCATTCCTAATATCGTTCAGTTAAATTGCATGTTTTGTTCCTACTGTTTCTCTACCTAATTTTTTGCTGTGAAACTCAAACCAGAATCTAAATGTAGATT :  838
AY345237   : ------------------------------------------------------------------------------------------------- :    -
```

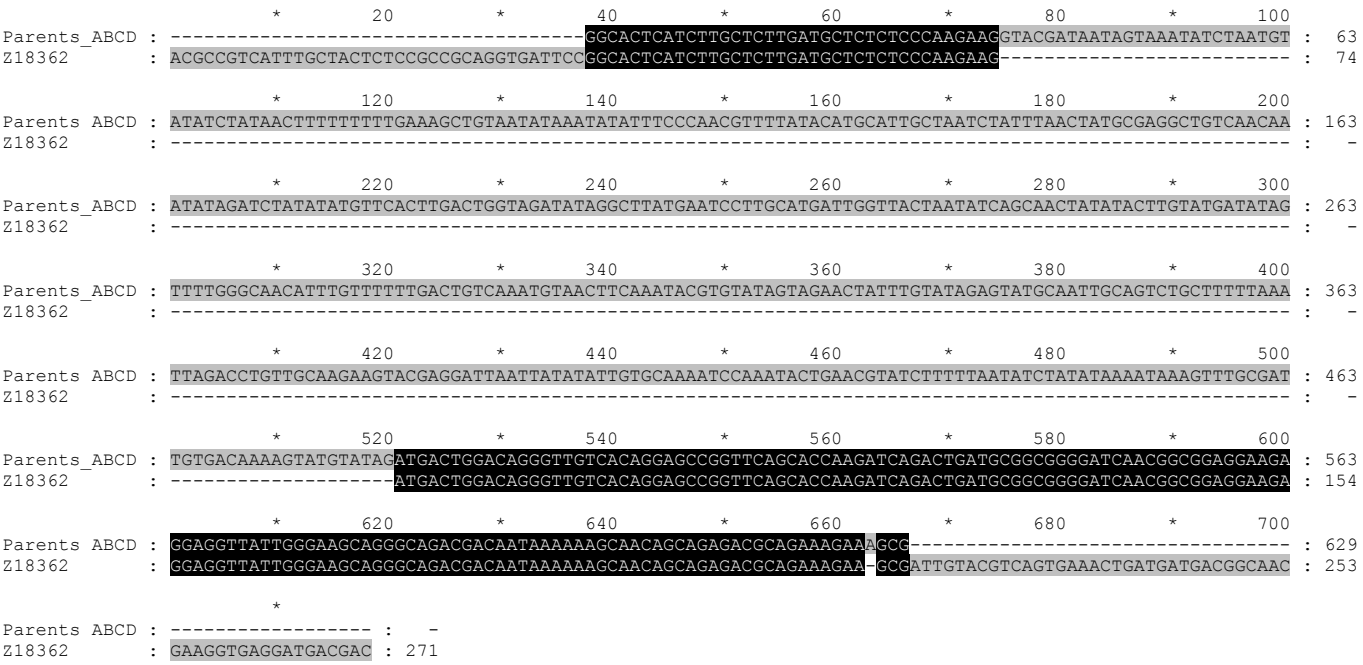


PCR 1: ***AGL20*** Sequences of parents A and C were illegible. B and D sequences are identical, and are aligned against the reference sequence AY345237 (cDNA) so that polymorphisms and intron sequences/locations may be seen. Note that a portion of the sequence near the end of intron 1 (position 1046; highlighted yellow), is missing due to insufficient overlap between forward and reverse sequences. A second portion of the sequence near the end of intron 6 (position 2088; highlighted blue), is missing for a similar reason.

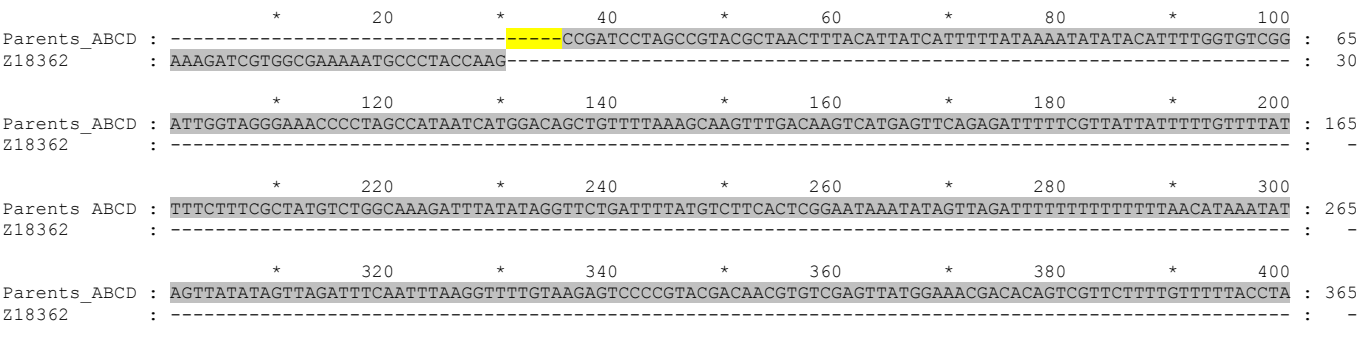


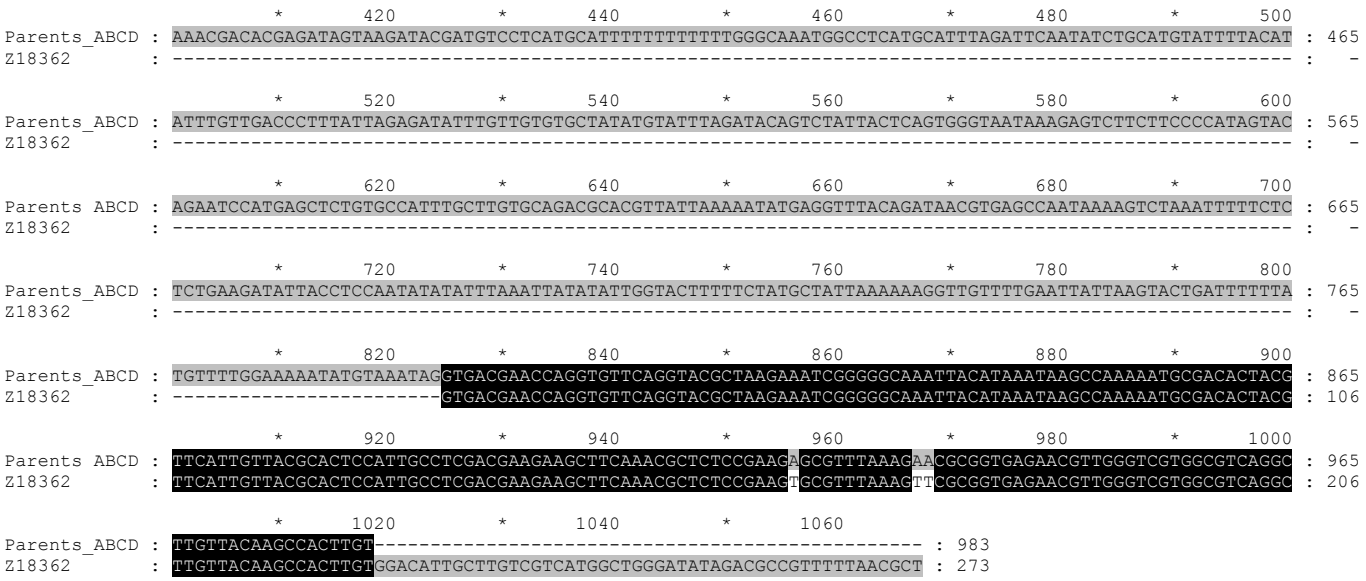


PCR 9: ***AP1*** Parents A/B/C/D are identical. Sequence is aligned to reference sequence AJ505845 (cDNA) to show intron location. Note that the sequence of primer AP15PF (Smith & King 2000) is not present in this reference sequence; to find its relative location, reference sequence Z37968 may be consulted.

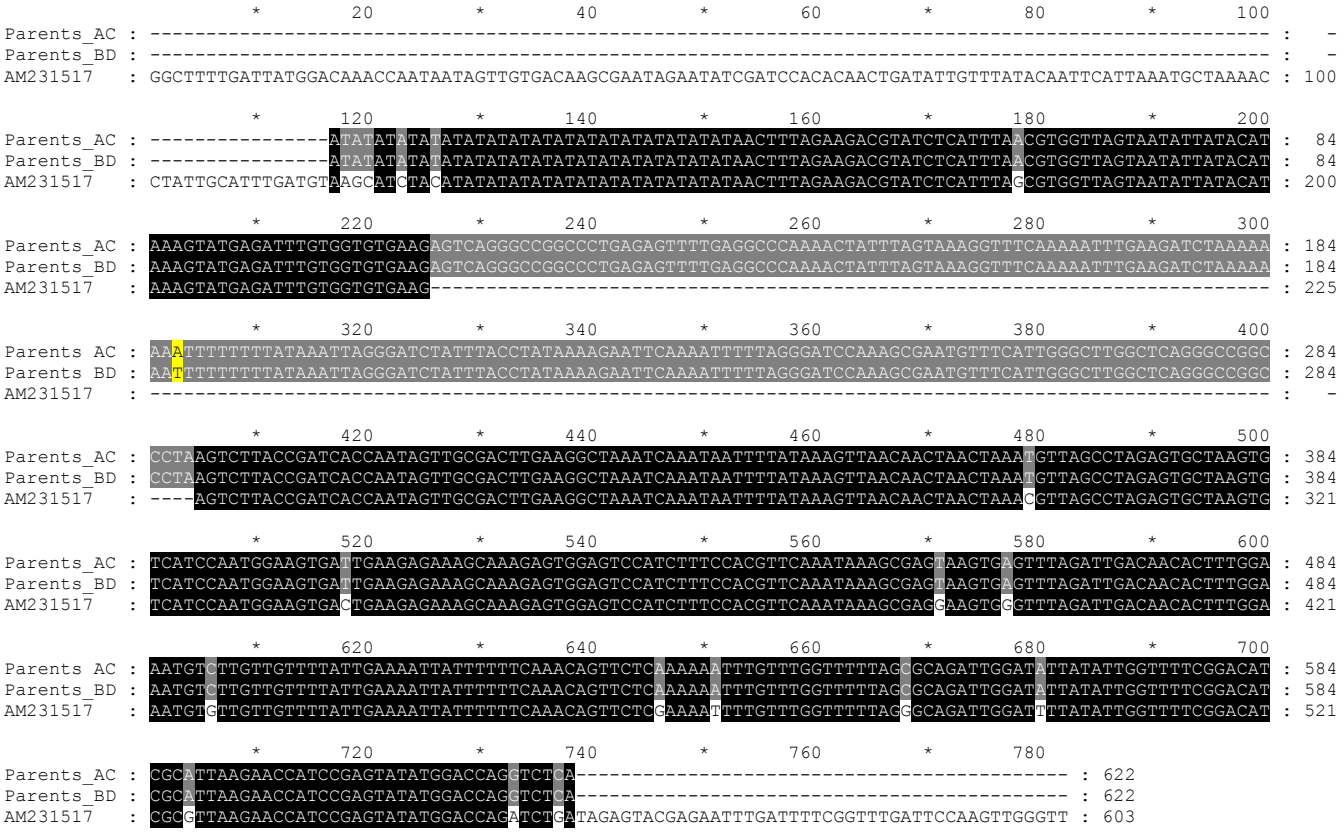


PCR 16: ***BoFH*** Parents A/B/C/D are identical. Sequence is aligned to reference sequence Z18362 (cDNA) to show intron locations.

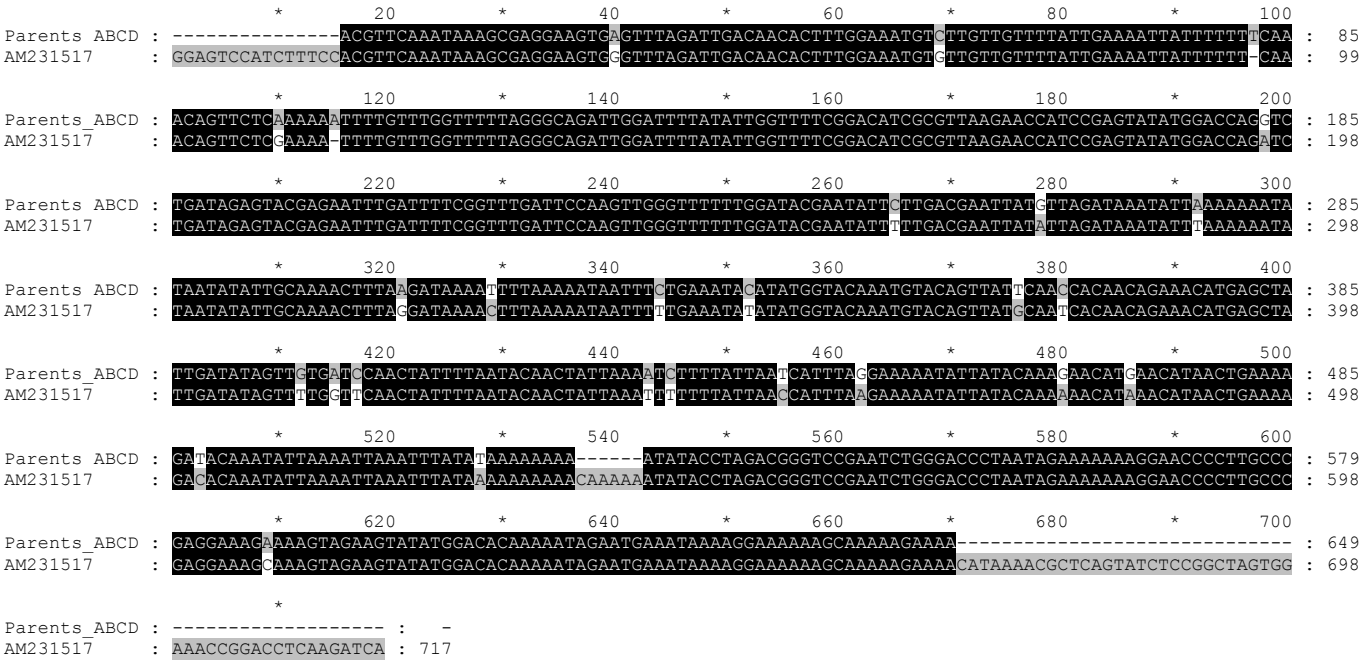




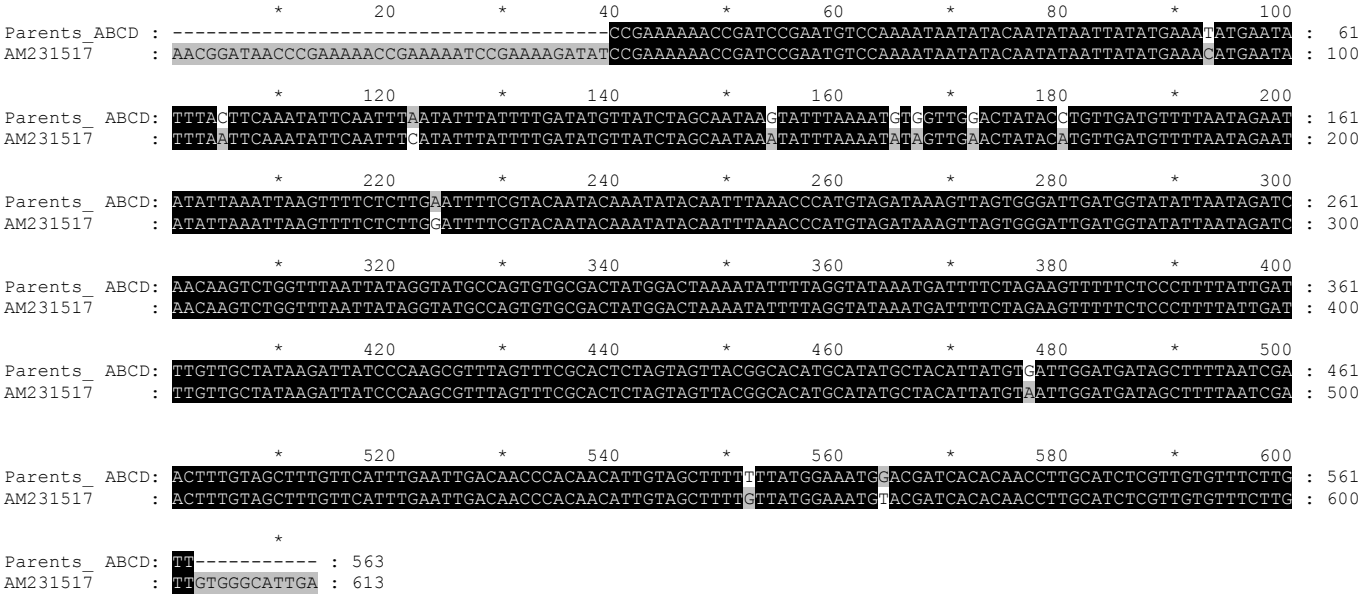
PCR 18: ***BoFH*** Parents A/B/C/D are identical. Sequence is aligned to reference sequence Z18362 (cDNA) to show intron locations. An unknown length of intron 2 sequence data is missing from the Parents ABCD sequence (highlighted in yellow).



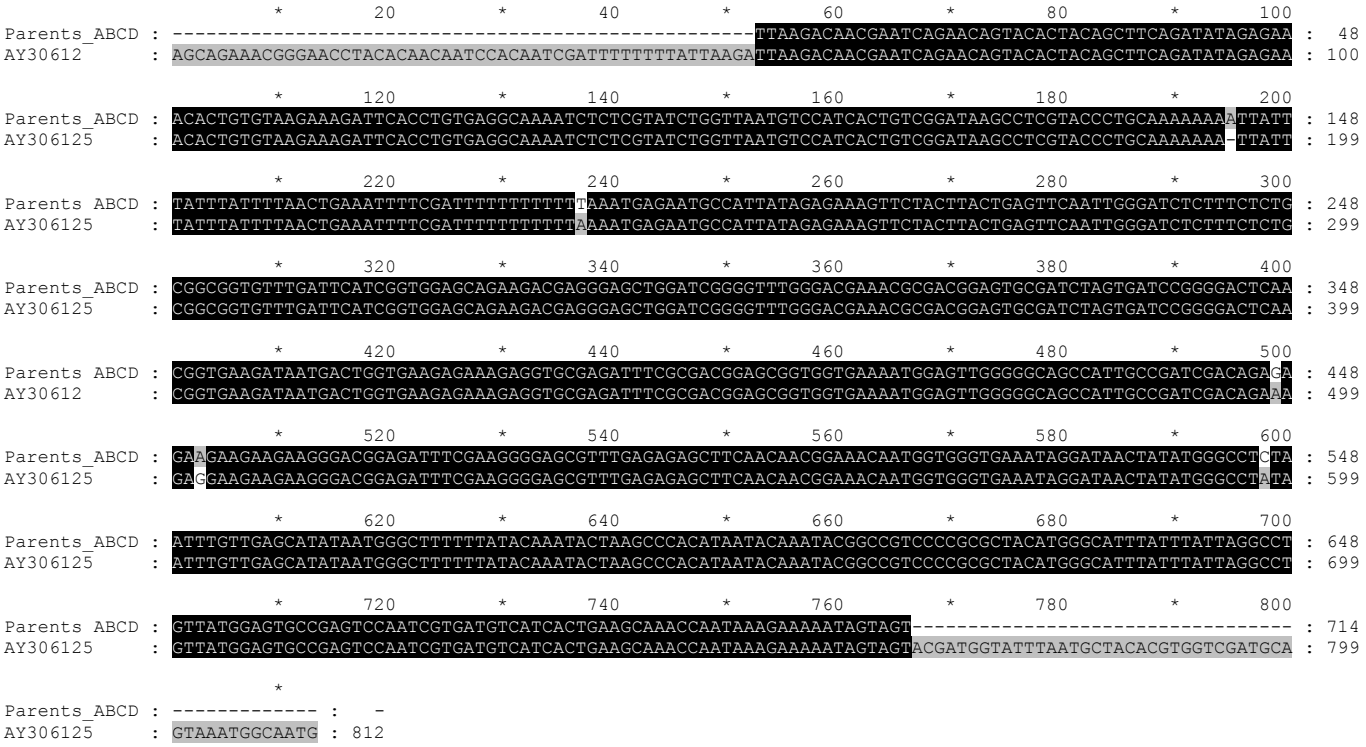
PCR 22: ***BoFLC1*** Parents A/B/C/D are identical except for one SNP between A/C and B/D parents (highlighted in yellow). All parents contain numerous differences compared to the reference sequence AM231517, including a 179 bp insertion.



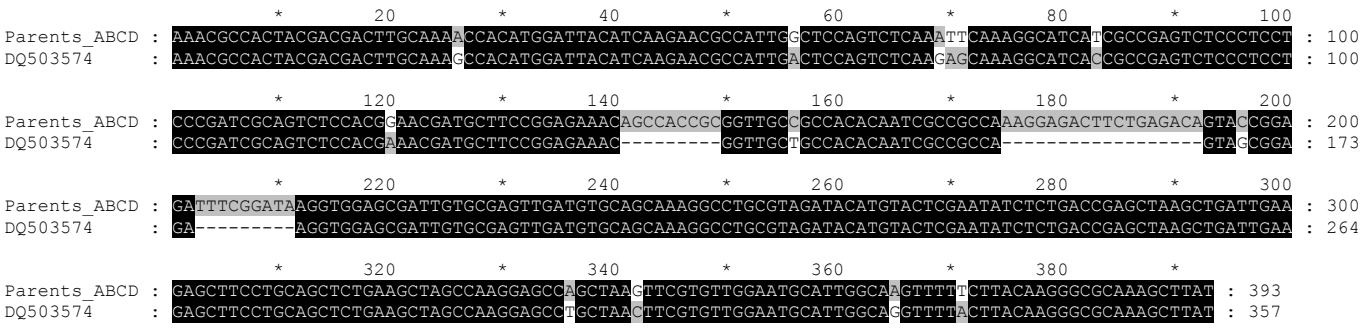
PCR 23: ***BoFLC1*** Parents A/B/C/D are identical. Numerous SNPs and some multiple base pair deletions may be observed when compared to AM231517.



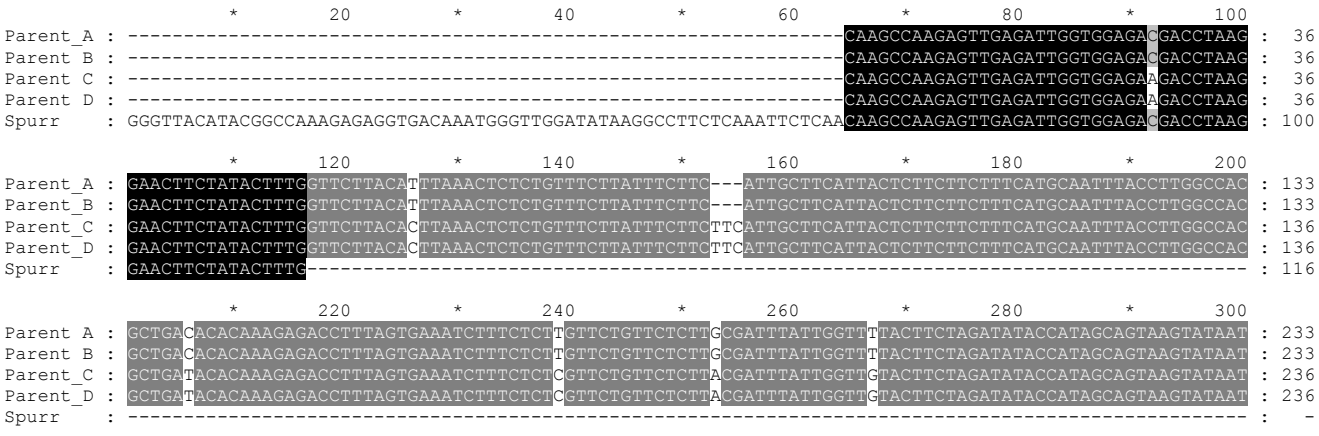
PCR 24: ***BoFLC1*** Parents A/B/C/D are identical. Numerous SNPs may be observed when compared to AM231517.

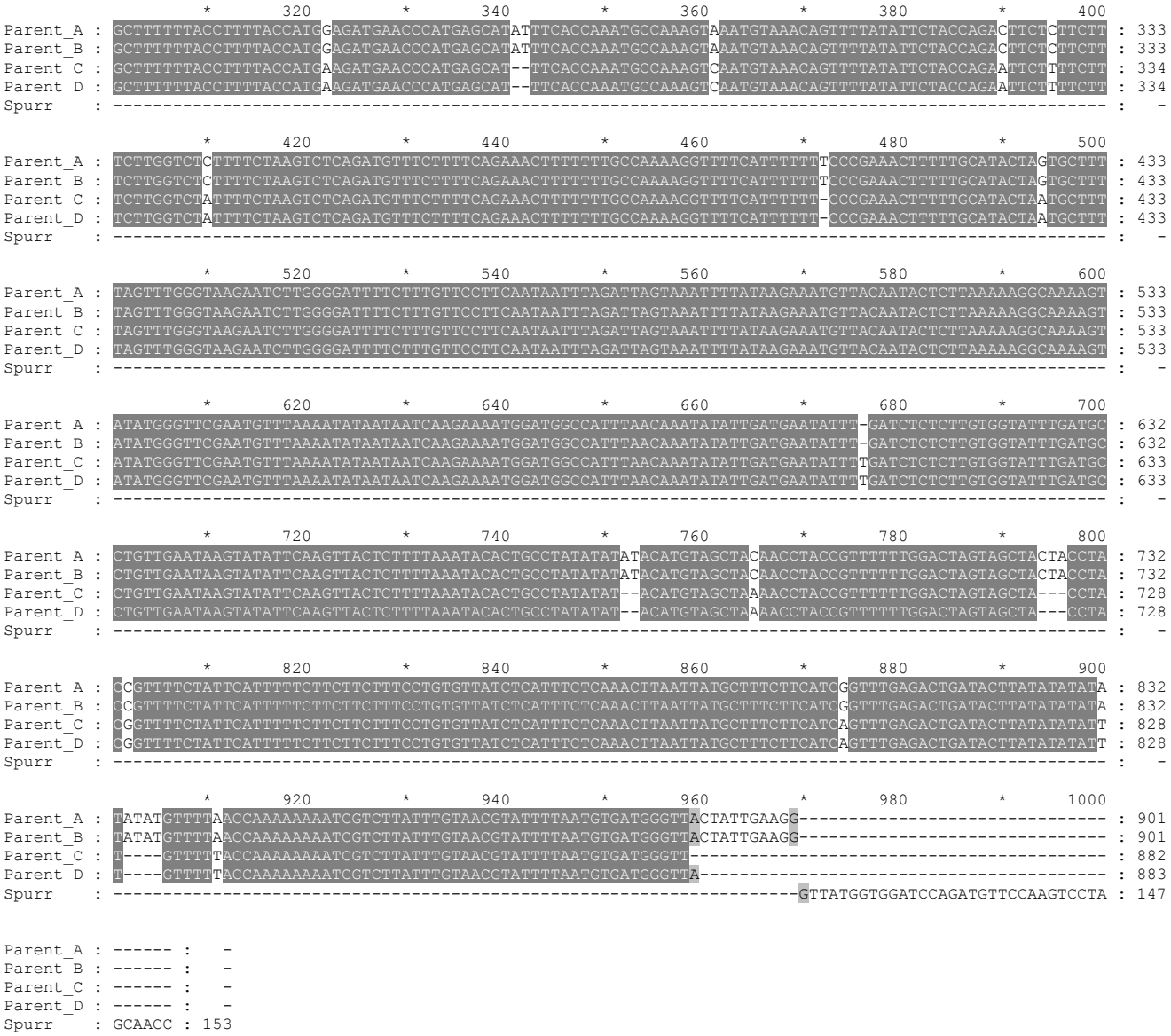


PCR 31: ***BoFLC3-2*** Parents A/B/C/D are identical. There are several ambiguous SNPs in comparison with AY306125.

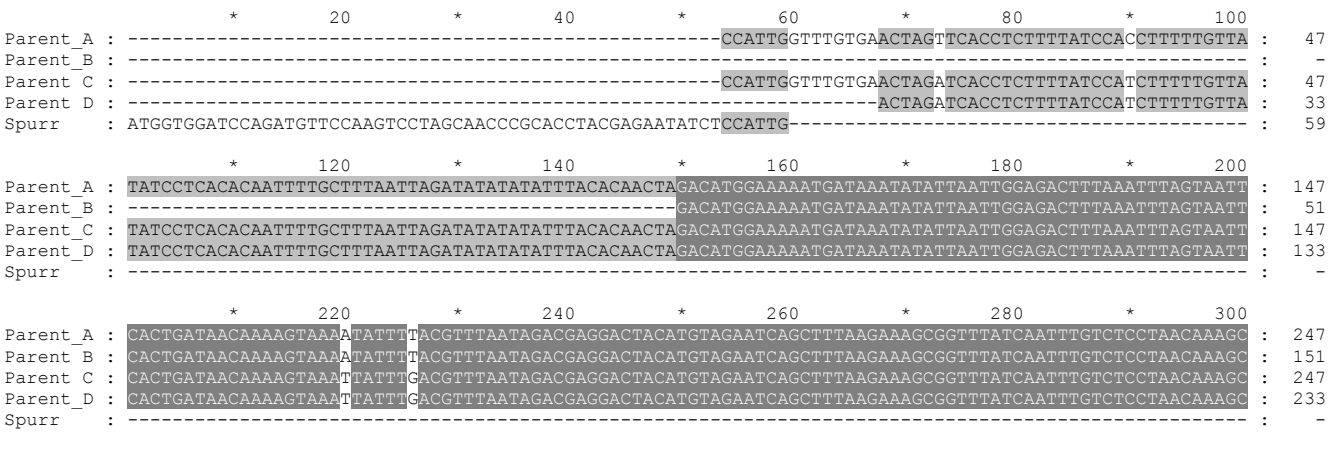


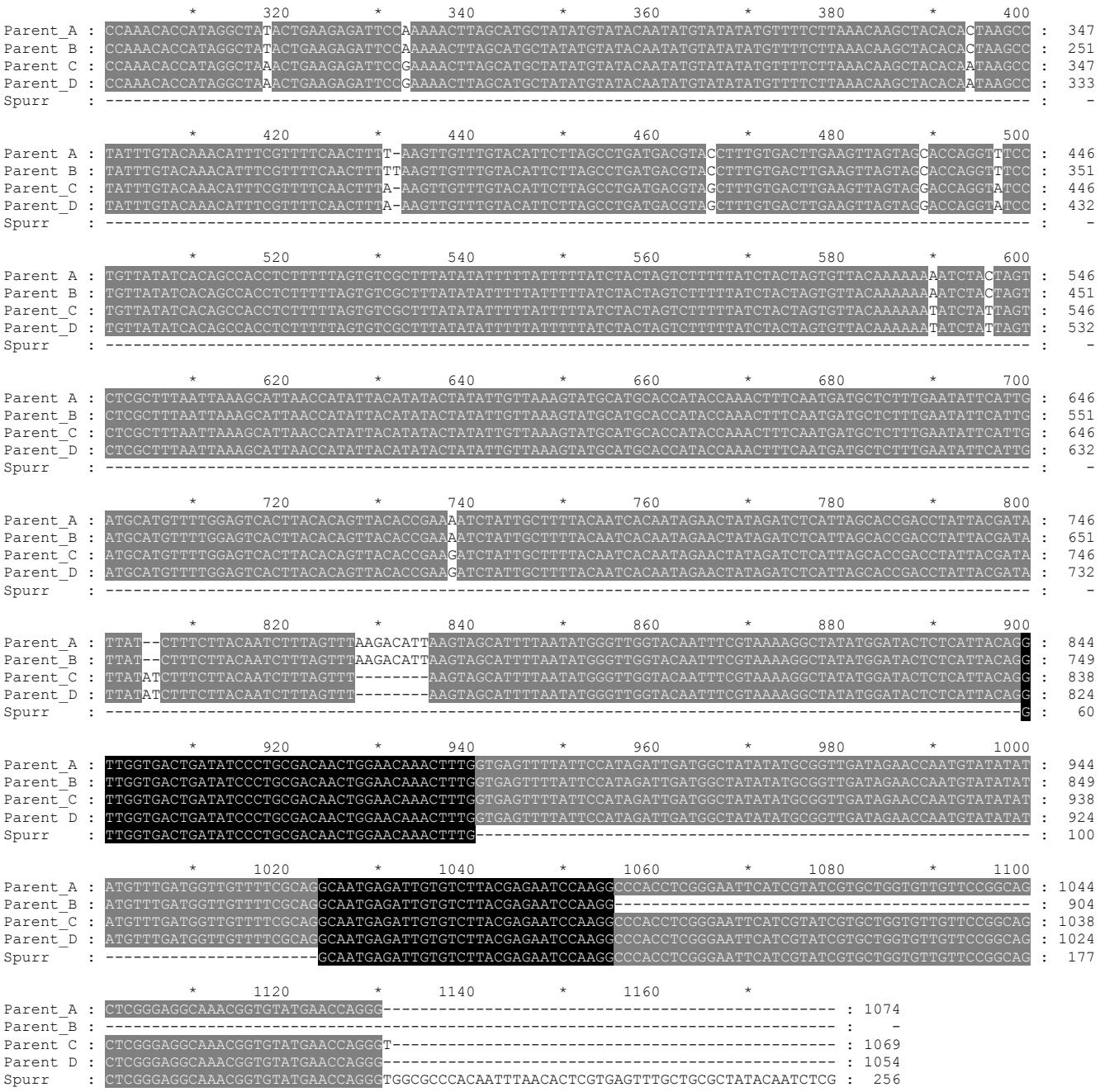
PCR 38: ***BoFRI*** Parents A/B/C/D are identical. There are numerous SNPs and three short (9-18) insertions in comparison to DQ503574.



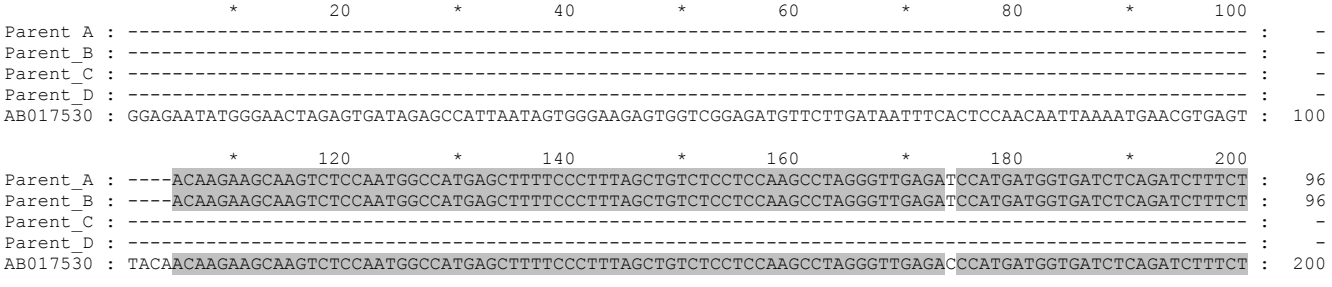


PCR 42: ***BoFT*** Unusually, parents A and B are identical to each other, and parents C and D are identical to each other, but there are differences between A/B and C/D sequences. The reference sequence "Spurr" is cDNA; this was sequenced by the author, and the full sequence is given in Appendix I.





PCR 43: ***BoFT*** Parents A and B are identical to each other (except for one ambiguous base where parent B differs from all other sequences) and parents C and D are identical to each other. Again, unusually, the main points of difference lie between A/B and C/D sequences. The reference sequence "Spurr" is cDNA; this was sequenced by the author, and the full sequence is given in Appendix I.



PCR 48: ***BoTFL1*** Parents A and B are identical to each other, but show differences compared to parents B and D. Parents B and D are identical except for one unique region in the C sequence that differs from all other lines. There are multiple differences between AB017530 and all other lines.

Appendix III

Bo VIN3 PARTIAL cDNA SEQUENCE

The following sequence is a contig of three sequenced PCR products using parent line 2 cDNA as a template; see Rajandran (2008) for additional details.

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TGTGGGCCCAGAAGAAGACATGGATCCTTCTTCATTTCAACGTGTCGT :  48
GTGCACGAGTTTCCTAAAAAGAAGGGTTTGAGTGTAAGCGAGAGAAGA :  96
GAACTGATCCACGCATTGTCCAAGCAGCCAGAAGAAGCTTCGGAGCTT : 144
TTAAACTCGTGGAGCCGGGACGAGATAATGAAGATCATATGCGCGGAG : 192
ATGGGTAAAGAGAGAAAGTACACTGGCCTAGCCAAACCAAAACTCATT : 240
GAAAACCTACTCAATCTCGTCTCTCGTCCTCTCGGTGAAACCTCGTGT : 288
CCTAACCGTAAAAGCTCGAGGAAGAAACAGAAAACGACTACTAGTTAC : 336
ATCATCTGCTGCGAGAATTTAGCTTGTAGAGCAGCGCTCGGAAGTGAA : 384
GATACCTTTTGTAGAAGATGTTCTTGCTGTGTTTGTCAGAACTTTGAT : 432
GAGAATAAAGATCCGAGTCTATGGATTGCTTGTGAGGGTTGTGGTTTG : 480
TCTTGTCATTTGGAGTGTGCTTTGAAGCAAGATGGGTATGGGATTGGG : 528
TATAATGATGGTAGTTTTCACTGCGTGTTTTGCGGCAAAGATAGTGAT : 576
CTTCTCGGATGCTGGAGGAAGCAAGTGAAGGTTGCGAAAGAGACTGGG : 624
CGTGTGGATGTACTTTGTTACCGTGTTTCTCTAGGACAGAAGCTGTTG : 672
CGAGGTACAAGGAGGTATCAGAATCTGTTGGAACTCATGGATGAGGCG : 720
GTGAAGAAGCTGGAGGGTGATATGGGTCCGTTAGTGAGTTGGGATATG : 768
AAGATGGCTCGGGAGATTGTCAATAGACTTGCTTCGGGATCACAAGTT : 816
CAGAAGCTGTGTCTA-------------------------------- : 831
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