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RESEARCH PAPER

Meristem identity gene expression during curd proliferation and flower initiation in *Brassica oleracea*

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Abstract

The regulation of reproductive development in cauliflower (*Brassica oleracea* var. *botrytis* DC) and broccoli (*B. oleracea* L. var. *italica* Plenck) is unusual in that most enlargement occurs while development is arrested at a distinct stage. Cauliflower and broccoli curds are composed of inflorescence meristems and flower buds, respectively. To determine whether this arrest is maintained by altered expression of the genes that specify these steps in *Arabidopsis*, the expression of each copy of their homologues (MADS-box genes *BoAP1-a*, *BoAP1-c*, *BoCAL*, *BoFUL-a*, *BoFUL-b*, *BoFUL-c*, and *BoFUL-d*; and non-MADS-box genes *BoLFY*, *AP2*, *UFO*, and *BoTFL1*) and the cauliflower curd-specific genes *CCE1* and *BoREM1* were measured simultaneously in heads that were arrested at different developmental stages by varying temperature, but had a common genotype. Transcript abundance of *BoFUL* paralogues and *BoLFY* was highest at the cauliflower stage of arrest, consistent with these genes initiating inflorescence meristems. The expression of other genes was the same regardless of the developmental stage of arrest. The expected models can therefore be excluded, wherein maintenance of arrest at the inflorescence meristem is a consequence of suppression of *BoCAL*, *BoAP1-a*, or *BoLFY*, or failure to suppress *BoTFL1*. Floral primordia and floral buds were normal in *boap1-a boap1-c bocal* triple mutants; therefore, other meristem identity genes can specify floral initiation (A-function) in *B. oleracea*. *BoTFL1*, a strong repressor of flowering in *Arabidopsis*, did not suppress the formation of the floral primordium in *B. oleracea*. Initiation of floral primordia and enlargement of floral buds in broccoli and cauliflower is not controlled solely by homologues of the genes that do so in *Arabidopsis*.

Key words: *Brassica oleracea*, broccoli, cauliflower, developmental arrest, flowering, meristem identity genes, temperature.

Introduction

Brassica oleracea is a species with a remarkable variety of cultivars and a variety of edible forms. Reproductive development determines the value of the crop, yet key steps in this phase of growth remain physiologically and genetically poorly understood. The curd phenotype in cauliflower (*Brassica oleracea* var. *botrytis*) corresponds to inflorescence meristems that share characteristics of both the vegetative and reproductive apices (Sadik, 1962). In broccoli (*Brassica oleracea* var. *italica*), the arrest occurs before anthesis and the head is composed of flower buds (Fujime and Okuda, 1996). Several studies have tried to elucidate the genetic control of developmental arrest in *B. oleracea* by identifying and characterizing homologues of the *Arabidopsis* floral homeotic genes. In *Arabidopsis*, an increase in *LEAFY* (*LFY*) expression, and consequent suppression of *TERMINAL FLOWER 1* (*TFL1*), initiates flowering by up-regulation of *APETALA 1* (*AP1*) and *CAULIFLOWER* (*CAL*). The homologues in *B. oleracea* are not consistently expressed in a manner that directly parallels their *Arabidopsis* functions. The onset of *BoLFY* expression does not correlate with the initiation of the floral primordium in cauliflower (Anthony *et al*., 1993; Jordan *et al*., 1994).

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Abbreviations: Ct, threshold cycle; DH, double haploid; HSD, honestly significant difference; MIG, meristem identity gene; NG, normalizer gene; SEMs, standard error of the means; TG, target gene.

Several lines of evidence support a model for developmental arrest in which *BoAP1* and *BoCAL* are primary regulators. The effect of a mutant *CAL* allele is present in cauliflower and causes a cauliflower phenotype in *Arabidopsis ap1* mutants (Bowman *et al*., 1993; Kempin *et al*., 1995). Kempin *et al*. (1995) proposed that this mutation is responsible for the cauliflower phenotype in *B. oleracea*. The wild-type *BoCAL* allele was later found in broccoli (Carr and Irish, 1997), strengthening the parallel. *BoAP1-a* and *BoCAL* had additive roles in the stage of arrest in a segregating population of doubled haploid lines from a cross of broccoli and cauliflower (Smith and King, 2000). The phylogenetic distribution of functional and non-functional alleles is consistent with both *BoCAL* and *BoAP1-a* being necessary for floral development in *B. oleracea* (Lowman and Purugganan 1999). Finally, *BoAP1-a* accumulates at the site of floral initiation in broccoli (Anthony *et al*., 1995; Carr and Irish, 1997).

There is also evidence that the developmental arrest is more complex, suggesting that *BoCAL* and *BoAP1*, while involved in curding, do not predict the phenotype in other populations. The mutant allele at *BoCAL* is present in some broccoli and non-heading *Brassica* accessions (Purugganan *et al*., 2000; Smith and King, 2000), and the wild-type *BoCAL* allele can occur in cauliflower (Smith and King, 2000). A subsequent survey of broccoli and cauliflower accessions found only a weak association between the *boap1-a* and *bocal-a* mutant alleles and the cauliflower phenotype (Labate *et al*., 2006). Furthermore, curd-related traits, such as days to budding and days from budding to flowering, among others, are affected by as many as 86 quantitative trait loci (Lan and Paterson, 2000), suggesting that the cauliflower arrest is under multigenic control.

The aim of the present study was to identify genes that control the arrest of the reproductive development in heading *B. oleracea*. The thorough description of genetic control of flowering in *Arabidopsis thaliana* identified a set of candidate genes for the molecular basis of the arrest of curd and head formation in confamilial cauliflower and broccoli. Candidate genes selected for this study were meristem identity genes (MIGs) that in *Arabidopsis* are involved in time to flower, the transition from inflorescence meristems to floral primordium, and floral organ identity: *LEAFY* (*LFY*), *APETALA 2* (*AP2*), *UNUSUAL FLORAL ORGANS* (*UFO*), and the MADS-box genes *APETALA 1* (*AP1*), *CAULIFLOWER* (*CAL*), and *FRUITFULL* (*FUL*) (Bowman *et al*., 1993; Shannon and Meeks-Wagner, 1993; Ferrandiz *et al*., 2000), as well as *TERMINAL FLOWER 1* (*TFL1*), the floral repressor potentially responsible for maintaining arrest in cauliflower. Two other genes have been specifically associated with the cauliflower curd: *CAULIFLOWER CURD EXPRESSION 1* (*CCE1*) and *REPRODUCTIVE MERISTEM 1* (*BoREM1*). *CCE1* could potentially be a gene involved in maintaining the arrest (as *TFL1* does in *Arabidopsis*) (Palmer *et al*., 2001), while *BoREM1* could break the arrest (as *LFY* does in *Arabidopsis*) to allow continued reproductive development (Franco-Zorrilla *et al*., 1999). The candidate gene approach permits the use of quantitative real-time PCR, a technique sensitive enough to detect low-abundance genes such as *BoTFL1* and to distinguish paralogues with high sequence similarity.

This study is novel because it investigates which genes change expression as different stages of arrest are overcome by simultaneously measuring the expression of several genes having overlapping function using tissue of a single genotype arrested at different developmental stages. Furthermore, *B. oleracea* has several copies of the key MIGs, allowing determination of whether duplication was followed by subfunctionalization, degeneration, or retained redundant functions.

Curd development and arrest are affected by temperature. Cool temperatures promote flower development, while warm temperatures inhibit reproductive development from proceeding. In production of broccoli and cauliflower, these effects are a serious economic concern. Premature floral buds in cauliflower cause a 'ricy' head at low temperatures (Fujime and Okuda, 1996; Grevsen *et al*., 2003), whereas excessive heat inhibits flower bud development in broccoli and promotes bract and bracteole formation in both forms (Booij and Struik, 1990; Fujime and Okuda, 1996; Björkman and Pearson, 1998; Grevsen *et al*., 2003, Kop *et al*., 2003). Here, advantage was taken of temperature effects to induce arrest at specified developmental stages (Labate *et al*., 2006).

Studies in both *Arabidopsis* and *B. oleracea* have indicated that temperature interacts with MIGs. In *Arabidopsis*, expression of *AP1* and *LFY* transcripts increases with high temperature (Bowman *et al*., 1993). In cauliflower, heat causes a decline of *BoLFY* and *BoAP1* expression in the shoot apex (Anthony *et al*., 1996). Also, the *BoAP1-a* locus has been found to interact with temperature in bracting. Even though the allelic state of *BoAP1-a* had the greatest influence in bracting (the *boap1-a* allele increases bracting), high temperatures increased bracting in plants with the same genotype (Kop *et al*., 2003). This study clarifies whether MIG expression responds directly to temperature, or is associated with the developmental stage.

Materials and methods

Plant material and growth conditions

Experiment 1: gene expression at different developmental stages and the same genotype: Seeds of the F_1 hybrid cauliflower cv. Green Harmony (Known-You Seed Company Ltd, Taiwan) were sown into modular trays of 50 cells (4.5 cm × 4.5 cm) containing Cornell Mix A (Boodley and Sheldrake, 1977). Trays were placed

in a greenhouse at day/night temperatures of 22–24 °C/16–18 °C. Seedlings were transplanted into pots containing Cornell Mix A and Osmocote 14–14–14 (slow-release fertilizer) and subsequently fertilized as required with water-soluble fertilizer (EXCEL Cal-Mag 15–5–15, The Scotts Company, Marysville, OH, USA). The reproductive transition was considered to have occurred in the planting when the dissected apical meristem measured 500 μm in sample plants, and the apical meristem under observation presented cauline leaf primordium with incipient axillary meristems. At this time (~28 d after sowing), plants were moved into growth chambers (Conviron E15, Winnipeg, Manitoba, Canada) at three different day/night temperature regimes: 16 °C/12 °C, 22 °C/17 °C, and 28 °C/22 °C with 14 h photoperiod, 75% humidity, and light intensity of ~500 mmol m^{-2} s^{-1}. Plants were kept in the growth chambers until curds reached harvest maturity, right before the curd started to separate as a result of bolting.

Experiment 2: gene expression at different temperature regimes and at the same developmental stage: In this experiment, a long-term and a short-term temperature treatment were performed. For the long-term treatment, experiment 1 was repeated with a purple cauliflower (intermediate stage of arrest) from southern Italy (Cavolfiore Violetto di Sicilia), accession 'HRI 5295' [University of Warwick, Genetic Resources Unit of Warwick Horticulture Research International (HRI), UK], less sensitive to temperature changes than 'Green Harmony F_1'. Plants were transferred to the growth chambers right at the initiation of the reproductive stage (determined by dissection and measurement of the apical meristem as described for experiment 1), 39 d after sowing. For the short-term temperature treatment, 'Green Harmony F_1' plants were raised in the greenhouse (as indicated for experiment 1) until curd diameter was between 2 cm and 3 cm. At this time, plants arrested at the same developmental stage were transferred into growth chambers, under the same three temperature regimes and conditions described above, for a 24 h period. Before moving the plants into the growth chambers the following morphological traits were annotated: number of leaves, curd diameter, and curd characteristics. This information was used later to group the plants in three sets, each of them including three plants, one for each temperature treatment.

Experiment 3: effect of BoAP1 and BoCAL genotype on expression of BoFUL and BoTFL1: Seeds from the double haploid (DH) lines (Table 1) generated from anther culture of the F_1 from the cross between the DH cauliflower parent N (CA25: DH line produced from an F_1 cauliflower variety, Nedcha) and the recurrent inbred Calabrese broccoli parent B (BI87053), kindly provided by Graham King and Graham Teakle (Warwick HRI, Wellesbourne, Warwick, CV 35, UK), were kept in the greenhouse (the same conditions described for experiment 1) until curds reached ~2 cm in diameter, and tissue samples were taken. Plants were characterized phenotypically at the time of sampling. The arrest stages were coded into one of four phenotypic classes: broccoli, composed of fully developed floral buds; intermediate curd, composed of small floral buds and floral primordia; ricy cauliflower, composed of inflorescence meristem and some initial primordia; and cauliflower, composed of inflorescence meristems.

Table 1. *Genotype, phenotype, and N×B double haploid lines used in experiment 3*

Genotype	Line	Phenotype[a]
BoCAL, BoAP1-a[bc]	9	B
	475	Ic
	689	B
bocal, BoAP1-a[bc]	17	Ic
	41	B
	146	B
BoCAL, boap1-a[b]	473	Ic
	483	RC
bocal, boap1-a[b]	58	RC
	109	C
	478	C

[a] B, broccoli; Ic, intermediate curd; RC, ricy cauliflower; C. cauliflower
[b] Genotypes used for *BoFUL* expression.
[c] Genotypes used for *BoTFL1* expression.

RNA extraction and reverse transcription

Tissue was isolated in each case from the whole surface of individual curds. Total RNA of each sample (50 mg of tissue) was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the protocol described by the manufacturer. Contaminating genomic DNA was removed using a RNase-free DNase solution according to Ausubel *et al*. (1994). Total RNA quality was evaluated on a 1.3% formaldehyde gel, and concentration was determined by measuring A_{260} using a BioMate series 3 spectrophotometer (Thermo Spectronic, Waltham, MA, USA).

Reverse transcription-PCR (RT-PCR) was done in two steps. RNA reverse transcription was performed using the RETROscript kit (Ambion, Inc., Austin, TX, USA) with random decamers according to the manufacturer's protocol. The absence of contaminating genomic DNA was determined by PCR with a set of primers designed around an intron sequence that amplifies the constitutive α-subunit of the translation elongation factor 1 gene (*EF1-α*) (Vidal *et al*., 1996).

Gene expression

Quantitative real-time PCR was performed using SYBR GREEN in the Bio-Rad iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA). All reactions were run in triplicate, and a non-template control for the primer set under study was included in each run. Deviations of Ct (cycle threshold) values for replicates of the same cDNA were not larger than 0.2 cycles, and 18S rRNA was used as the normalizer gene. For each 20 μl of final reaction, the following were added: 10 μl of IQ SYBR Green Supermix 2X (Bio-Rad), 2 μl of cDNA (0.25 μg of initial RNA), 1.5 μl of reverse and forward primers (final concentration of 0.3 μM each), and 6.5 μl of distilled sterile water. For PCRs using 18S rRNA-specific primers, the cDNA sample was diluted in a 2:1000 ratio of which 1 μl was used in the reaction mix. Amplification of cDNAs involved a 3 min denaturation step at 95 °C, followed by 40 cycles with 95 °C denaturation for 15 s and 60 °C for 30 s. Reaction products were analysed by performing a 'melting curve': from 55 °C to 95 °C at 0.5 °C per 7 s (80 cycles). Ratio calculations were obtained using the mathematical model proposed by Pfaffl (2001) that includes efficiency correction for target and normalizer (reference) genes. All the data incorporated in this equation (Equation 1) were an average from two or three runs per sample and the corresponding biological replications done in each experiment. Efficiency of the PCR for each individual run was estimated based on Ramakers *et al*. (2003).

$$R = E_t^{\Delta Ct(TG)} / E_r^{\Delta Ct(NG)} \quad (1)$$

where R is the relative expression, E is the real-time efficiency for target (t) and reference (r) gene transcripts, and ΔCt is the Ct value difference between control or reference sample (gene expression at day

temperature 22 °C) and sample (gene expression at day temperatures 16 °C and 28 °C) of target (TG) and normalizer gene (or reference gene) (NG) transcripts. Ct is the point at which fluorescence increased above a fluorescence threshold above the background fluorescence. The same threshold was used for all samples.

Primer pairs were designed using Primer-Express Software 1.5, Primer3 Software (http://frodo.wi.mit.edu/) (Rozen and Skaletsky, 2000), or by hand after alignment of sequences of interest using Clustal W (1.82), EMBL-EBI, (http://www.ebi.ac.uk/clustalw/) (Chenna *et al*., 2003), following standards recommended for the real-time RT-PCR technique (Bustin, 2000) (Table 2). The specificity of the primers designed was evaluated in different ways. First, amplified PCR products for each set of primers were analysed by agarose electrophoresis, purified using a Mermaid Spin kit (Q-BIOgene BIO101 Systems, USA), cloned into a plasmid using the p-GEM-T Easy Vector kit (Promega, Madison, WI USA), and sequenced in the BioResource Center (BRC), Cornell University, using the Applied Biosystems Automated 3730 DNA Analyzer with Big Dye Terminator chemistry and AmpliTaq-FS DNA polymerase. Secondly, calculation of the melting temperature expected for each amplicon was performed and compared with melting peaks obtained in quantitative real-time PCR. Criteria for the choice of the best primer set included: no primer dimer (checked by melting curves), highest Ct value, and highest PCR efficiency.

Genotyping

Leaf disks 10 mm in diameter were taken from 'Green Harmony F_1', 'HRI 5295', and DH plants in order to genotype the following loci: *BoAP1-a*, *BoAP1-c*, and *BoCAL*. DNA was extracted using the protocol of Doyle and Doyle (1987). PCR assays included homozygous mutants (parent line CA25: DH cauliflower parent N), homozygous wild-type (parent line BI87053: inbred broccoli parent B), and heterozygous (F_1 progeny from N×B cross) controls.

The allelic variation of the *BoCAL* and *BoAP1-a* locus was assayed as described by Labate *et al*. (2006). *BoCAL* genotyping was based on the presence of the *Spe*I restriction enzyme recognition site in the mutant allele at *BobCAL* [premature stop codon (Kempin *et al*., 1995)]. *BoAP1-a* allelic variation was determined by an amplicon length polymorphism in the 100 bp upstream of the start codon (Smith and King, 2000). For genotyping the *BoAP1-c* locus, a simple sequence repeat (SSR) polymorphism assay was used [AP1cSSRF (5′-CGAGCTTATCACGCTGTTGT-3′) and AP1cSSRR (5′-CCTCTCATAGCGTTCCAGTA-3′)]. The assay detects a 12 bp difference between the mutant and the wild-type alleles given by the presence of a microsatellite at the 3′ end of intron 1 (Smith, 1999).

Statistical analysis

All statistical analyses were performed using SAS (version 8; SAS Institute Inc., Cary, NC, USA). Statistical analyses of differentially expressed genes for experiment 1 and experiment 2 were done using analysis of variance (ANOVA) with PROC GLM. Assumptions of normal distribution and equal variances were tested in each case, and transformation of the data (log base 10) was used when necessary. When the *F*-test was significant, multiple comparisons of

Table 2. *Primer sequence for genes of interest*

Gene	Accession GenBank	Forward Primer 5′— 3′	Reverse Primer 5′— 3′	Amplicon size (bp)
BoAP1-a	AJ505845	CGAGCCCTTCTTA	CATACTGAAGCAAAA	118
*Boi2AP1**	U67452	TCCAACTAATT	GAACTTGAGAAA	
BoAP1-c	AJ505846	TGGCTAGCTTCTT	CACATACTAGAACCA	114
*Boi1AP1**	U67451	TCTATCCAATTAA TA	AAAACTTACAAAGAG A	
BoFUL-a	AJ505841	CGCCCTACGACGA ATGAATAG	CACAAAAATGCTGAG ATACATTATGA	145
BoFUL-b	AJ505842	CGCCCTACCACGA AAGAATAA	CAGTAAATCCAGAAA AATGCTGATATACA	102
BoFUL-c	AJ505843	ACGTCCTGCTACC AATGAGTAAAAT	TACGTTCTTGACATTG TAATTCCGTC	148
BoFUL-d	AJ505844	TCGTCGTTGATTG AACCAAACT	AGTCACCAAAAAAGC TGATACATTATGA	129
BoFH	AJ505845	AGCGACTTTGGTT GGTGGTATT	AACCACAGCAACTCA TGAACTAATTAA	86
BoTFL1-1	AB017530	CGTGAATTTGCGA	TTTCTCTCTGAGCGTT	70
BoTFL1-2	AB017531	TCGAGAAT	GAAGAAGA	
BoCAL	L36926	AAACCGCAGCCAC	AAGGAGATGATGCCA	68
*BoiCAL**	U67454	CATGTA	TGTAAGGA	
CCE1	AF227978	TCGTTCCACCACC TTCCAAA	ACGAGCCTGAAATGG TCGTAAT	69
BoREM1	AF051772	CCACGTTAAGTTT CCTTTTCAGTATTT	TGAGCCATGGAACCG AACA	85
UFO	U97020	TTGCGGATATGAT CAAAGGAAA	ATTCAAAAGCCCATT GGTTCT	120
AP2	NM 126118	AGCTTCTAACCTT GAGGAGAATATAC CAGA	GTGCGTCGTTTAGAT CCCACAT	110
18S	AF513990	CGAGACCTCAGCC TGCTAACTAG	TCAAACTTCCTTGGCC TAAACG	93

* Same gene under different accession number.

the means among the three different stages of development (cauliflower, intermediate, and broccoli) in experiment 1, or between the different day/night temperature regimes (16 °C/12 °C, 22 °C/17 °C, and 28 °C/22 °C) in experiment 2 were done using Tukey's studentized range [honestly significant difference (HSD)]. For the short-term temperature experiment, every set of plants with similar morphological characteristics was considered a block. When block effect was not significant, it was removed from the model and the main effect (temperature) re-evaluated.

To estimate the assay variation, SEMs were calculated assuming statistical independence of the quantities using propagation of error formulae that allow the inclusion of experimentally determined errors for each efficiency and Ct value (Ku, 1966).

In experiment 3, the statistical analysis of differentially expressed genes was done with ANOVA using PROC MIXED. *BoAP1* and *BoCAL* genotypes were considered fixed effects, and plant line was considered a random effect. Assumption of normal distribution and equal variances were tested in each case, and transformation of the data was used when necessary (log base 10). A χ^2 test of independence was performed to test the association between the categorical variables: *BoAP1* and *BoCAL* genotype with respect to phenotype. This test was necessary to determine the variables that needed to be included in the model for the ANOVA.

Results

Genotyping results

Genotyping results showed that 'Green Harmony F_1' is homozygous mutant at *BoAP1-a*, *BoAP1-c*, and *BoCAL*, while 'HRI 5295' is homozygous mutant at *BoAP1-a*, and segregates for *BoAP1-c* and *BoCAL*. Genotyping results for N×B DH lines are shown in Table 1.

Experiment 1: gene expression at different developmental stages and the same genotype

'Green Harmony F_1' is highly sensitive to changes in temperature, thus, by using different temperature regimes at the time of reproductive initiation, it was possible to create phenotypic variation in the stage at which the curd arrested (Fig. 1).

The relative expression profiles of all the genes studied, between the reference sample (also called control sample: intermediate stage of arrest) and the other two samples (cauliflower and broccoli stages of arrest), normalized to 18S, are shown in Fig. 2. *BoAP1-a* and *BoAP1-c* transcripts accumulated significantly (P=0.0006 and P=0.0018, respectively) once the floral primordium initiated. In floral primordium, up-regulation of *BoAP1-a* and *BoAP1-c* transcripts increased by a factor of 23 and 21, correspondingly, with respect to transcript levels at the inflorescence meristem stage. Levels of expression were maintained at the floral bud stage of arrest (Fig. 2A). *BoCAL* had its maximum expression at the inflorescence meristem–floral primordium stages and thereafter declined significantly (P=0.02) by a factor of 2.3 as the reproductive meristem formed floral buds (Fig. 2A). *AP2* transcripts reached the highest expression levels at the floral bud stage. Abundance was higher by a factor of 2 (P=0.024) with respect to the intermediate and inflorescence meristem stages of arrest (Fig. 2A). *BoLFY* was expressed equally in inflorescence meristem and floral primordium stages. Expression was lower by a factor of 8.5 (P <0.0001) in the floral buds. *BoLFY* reached its maximum expression in the initial stage of reproductive development, the vegetative to reproductive transition (Fig. 2B). *BoTFL1* decreased expression by a factor of 2.1 (P=0.008) from floral primordium to floral bud (Fig. 2B).

'Green Harmony F_1' forms floral buds and complete flowers (Fig. 3), even though it is a *boap1-a boap1-c bocal* triple mutant. Candidate complementary genes specifying floral initiation are *FUL* or *UFO*. All four *BoFUL* paralogues were expressed at all stages of arrest.

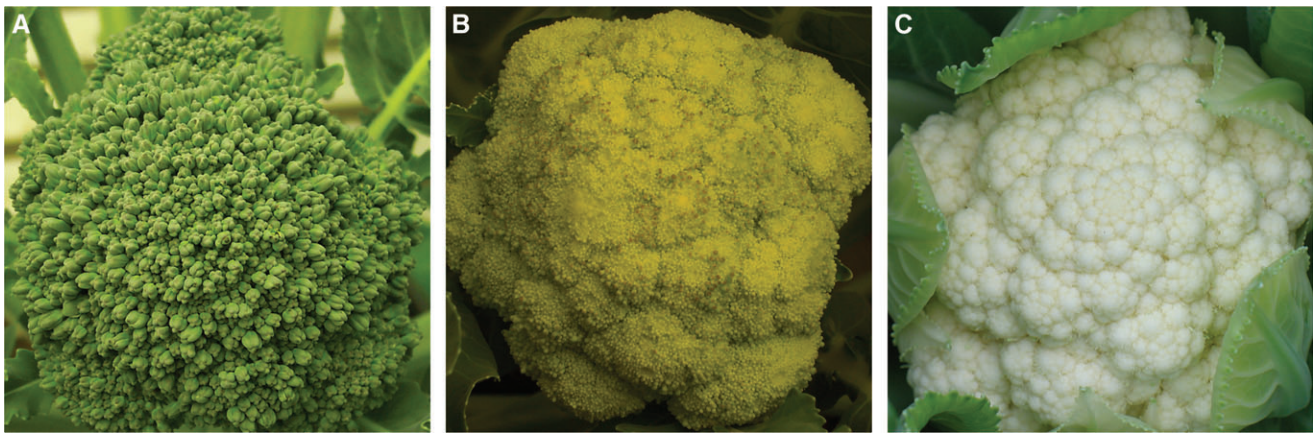

Fig. 1. Temperature effect on the stage of arrest of *Brassica oleracea* cv. Green Harmony F_1 grown under three different day/night temperature regimes during reproductive development. (A) At 16 °C/12 °C, curd arrested at floral bud stage (broccoli-like head). (B) At 22 °C/17 °C, curd arrested at floral primordium stage (intermediate curd). (C) At 28 °C/22 °C, curd arrested at inflorescence meristem stage (cauliflower-like curd).

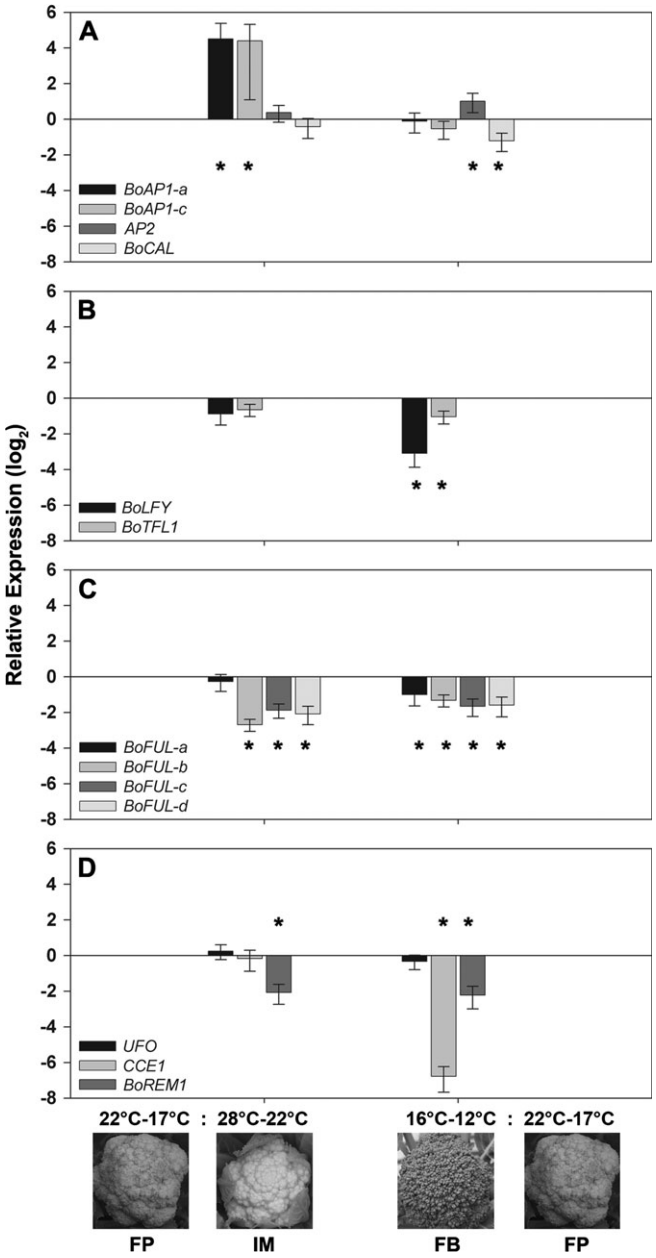

Fig. 2. Relative expression of genes in *Brassica oleracea* cv. Green Harmony F_1 plants arrested at three different developmental stages. Left: relative expression of genes at the intermediate stage of arrest [floral primordium (FP)] to the cauliflower stage [inflorescence meristem (IM)]; these are gene expression changes associated with the development of the floral primordium. Right: relative expression of genes at the broccoli stage of arrest [floral bud (FB)] to the intermediate stage [floral primordium (FP)]; these are gene expression changes associated with the subsequent enlargement of floral buds. Bars are means ±SE, n=6. Asterisks indicate a significant difference (P <0.05) between the reference sample [intermediate stage of arrest (22 °C/17 °C)] and the sample used in the ratio calculation.

BoFUL-c and *BoFUL-d* were the most abundant transcripts. *BoFUL-b*, *BoFUL-c*, and *BoFUL-d* had maximum expression at the inflorescence meristem stage. Their transcript levels decreased significantly (P= 0.0001) by a factor of 6.4, 3.66, and 4.23, respectively, from inflorescence meristem to floral primordium. From floral primordium to floral bud, the decrease in the transcript abundance was significant for the three genes [P=0.0007 (*BoFUL-b*), P=0.0004 (*BoFUL-c*), and P=0.0017 (*BoFUL-d*)] by a factor of 2.49, 3.15, and 3.01, respectively (Fig. 2C). *BoFUL-a* had a slightly different pattern. Its maximum transcript levels were reached at inflorescence meristem stage and it was maintained until plants developed floral buds. At this stage, the gene was down-regulated significantly (P=0.035) by a factor of 2 with respect to its expression levels at the intermediate stage of arrest (Fig. 2C). *UFO* was expressed at very low levels at all developmental stages, with no significant differences among them (Fig. 2D).

Two genes specifically associated with cauliflower curd phenotype were analysed. *CCE1* transcript levels were equally high in inflorescence meristem and floral primordium. However, it was lower by a factor of 110 (P <0.0001) in floral buds (Fig. 2D). *BoREM1* expression was highest at the inflorescence meristem stage, and transcripts were detectable in floral primordium and floral bud stages. *BoREM1* expression was 4.2-fold lower (P= 0.0006) in floral primordium than in inflorescence meristem, and a further 4.9-fold lower in floral buds (P= 0.0003) (Fig. 2D).

Experiment 2: gene expression at different temperature regimes and at the same developmental stage

As a control to determine whether the temperature exposure used to generate different developmental stages directly caused differences in gene expression, two tests were carried out. First, expression was measured in cauliflower plants from the accession 'HRI 5295', which had the same phenotype of floral primordia at all three temperature regimes used during reproductive growth (comparable with the intermediate stage represented in Fig. 1B). Most of the genes under study had no significant differences in expression (Fig. 4). Only *BoAP1-a* had a significant (P=0.0098) 2.4-fold increase in the abundance of transcripts between the 22 °C/17 °C and 16 °C/12 °C treatments (Fig. 4A). Second, 'Green Harmony F_1' was exposed to the three temperature regimes for 24 h after heads formed, and development arrested at floral primordia–floral bud stages (set 1, small size floral buds; set 2, very small floral buds; set 3, medium size floral buds.). There were no statistically significant differences in the expression of the genes (Fig. 5). While *BoAP1-a* transcript abundance increased ~4-fold in set 2 and set 3 between the 28 °C/22 °C and

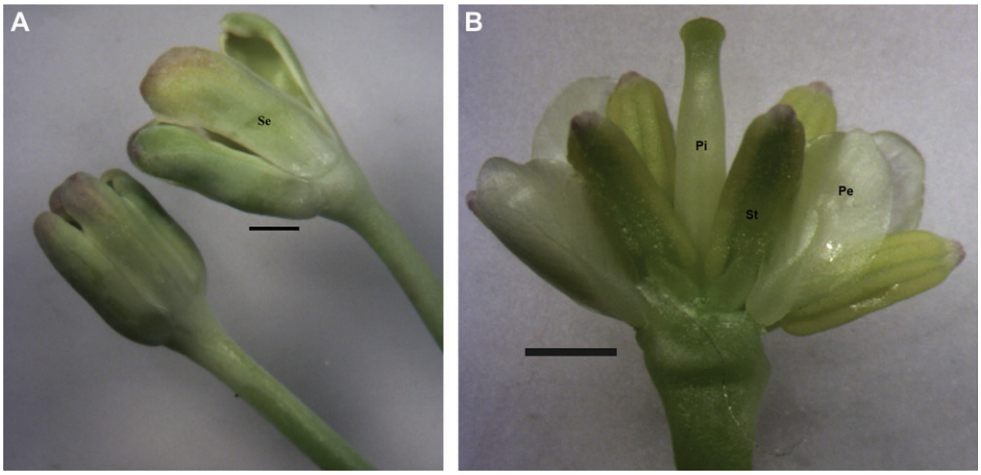

Fig. 3. Floral bud dissection in *bocal boap1-a boap1-c* triple mutant 'Green Harmony F_1' plants. (A) Intact floral bud before dissection showing four sepals (Se). (B) Floral bud partially dissected containing four petals (Pe), six stamens (St), and one pistil (Pi). Bar = 1 mm.

22 °C/17 °C treatments, this difference was not statistically significant.

Experiment 3: effect of BoAP1 and BoCAL genotype on expression of BoFUL and BoTFL1

This experiment was designed to test whether *BoFUL* accumulates to compensate for the absence of *BoCAL* and *BoAP1* or whether it is developmentally regulated independently of these genes. It also tests whether *BoTFL1* expression is greater when *BoCAL* is mutant, as would be expected if the primary role of *CAL* is to repress *TFL1*. Expression of the genes was measured in a DH population segregating for *BoAP1-a*, *BoAP1-c*, and *BoCAL*, where individual DH lines were selected for representative inflorescence phenotypes (Table 1).

The *BoCAL* genotype did not affect expression of the four *BoFUL* paralogues (Fig. 6). The observed variation was not statistically significant (Table 3). The phenotype was also independent of the *BoCAL* genotype (χ^2=2.598, df=1, P=0.1078). The *BoAP1* genotype, in contrast, was significantly correlated (Table 3) with increased expression of *BoFUL-b*, *BoFUL-c*, and *BoFUL-d* (Fig. 6). The variation in *BoFUL-b*, *-c*, and *-d* expression due to *BoAP1* genotype could not be distinguished from the developmental stage of the curd because the phenotype was dependent on the *BoAP1* genotype (χ^2=21.55, df=1, P <0.0001).

BoTFL1 transcript abundance was not associated with *BoCAL* genotype (Fig. 7). The two lines with the highest expression of *BoTFL1*, L475 (CCAA), and L17 (ccAA), were arrested at the floral primordium stage, while the other lines were arrested at the floral bud stage (Table 1).

Discussion

Conservation of the sequence in homeotic genes from several species does not necessarily indicate conservation in function. What should be expected from species in the same family that share flower morphology, such as *Arabidopsis* and *B. oleracea*? This study tested a model of arrest in *B. oleracea* that incorporated homologues of the key genes involved in the *Arabidopsis* floral transition: *AP1*, *CAL*, *FUL*, *LFY*, *UFO*, *AP2*, and *TFL1*, as well as the cauliflower curd-specific genes *CCE1* and *BoREM1*. Since some of these genes are present in multiple copies in *B. oleracea*, the expression pattern of all the genes and their paralogues was examined in combination and in tissue of the same genotype that was arrested at different developmental stages by varying the temperature regime.

Is the initiation of floral primordium development specified by an increase in BoLFY expression in B. oleracea? Does BoTFL1 maintain arrest in cauliflower by suppressing genes that may be involved in floral primordium specification (BoAP1, BoCAL, and BoLFY)?

Maintenance of the inflorescence meristem in cauliflower could be caused by *BoLFY* expression being below the threshold required to trigger floral primordium initiation, expression being repressed by high activity of *BoTFL1*. In *Arabidopsis*, *LFY* is responsible for the initial step in the reproductive transition specifying inflorescence meristem identity and floral primordium (Mandel *et al*., 1992; Kempin *et al*., 1995; Blazquez *et al*., 1997), *TFL1* is a repressor of flowering and maintainer of indeterminate shoot meristem (Shannon and Meeks-Wagner, 1993; Bradley *et al*., 1997; Ratcliffe *et al*., 1999), and their

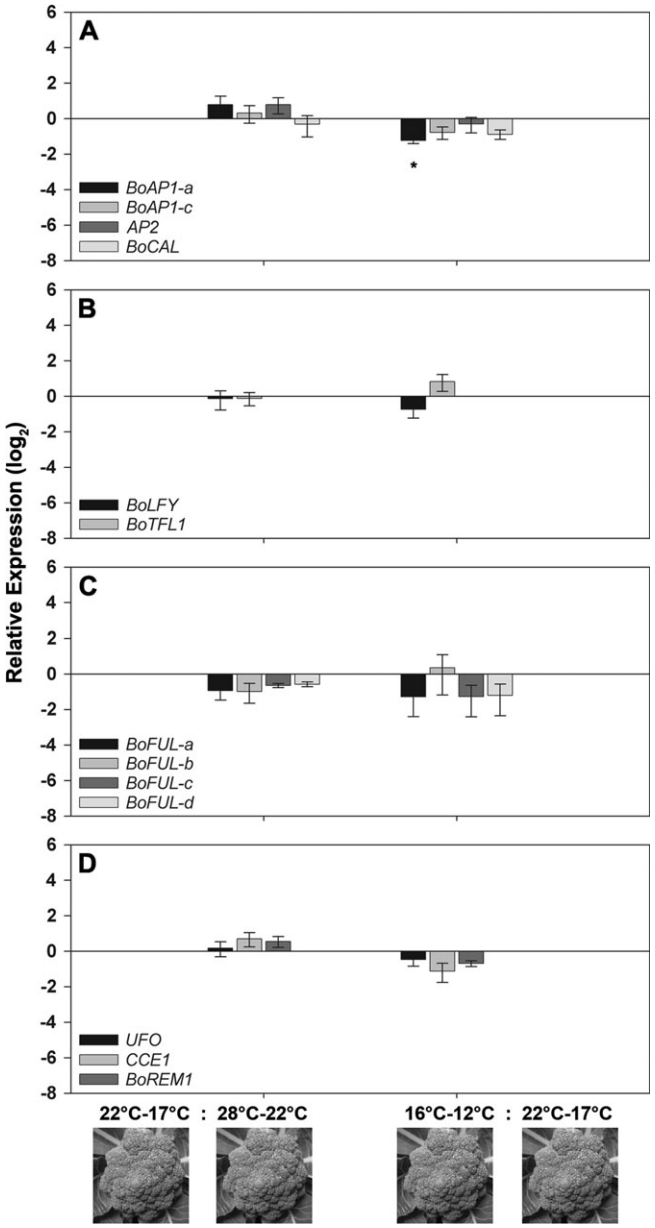

Fig. 4. Relative expression of genes in *Brassica oleracea* accession 'HRI 5295' grown under three different day/night temperature regimes. Curds at all temperatures were arrested at the intermediate stage and were composed of floral primordia. Left: relative expression of genes at 22 °C/17 °C to 28 °C/22 °C. Right: relative expression of genes at 16 °C/12 °C to 22 °C/17 °C. Bars are means ±SE, n=3. Asterisks indicate a significant difference (P <0.05) between the reference sample [intermediate stage of arrest (22 °C/17 °C)] and the sample used in the ratio calculation.

expression is restricted to different zones through mutual suppression. In *B. oleracea*, *BoLFY* expression did not increasee in concert with a decline in *BoTFL1* expression, as would be expected if mutual suppression resulted in the spatial separation observed in *Arabidopsis*. If the cauliflower stage of arrest was maintained by suppression of *BoLFY* expression, there would have been significantly higher *BoLFY* transcript levels in the intermediate stage, and if *BoTFL1* prevented floral identity genes from being expressed, then its transcript abundance should have been lower at the intermediate stage than in the inflorescence meristem stage. That model can therefore be rejected (Fig. 8). The presence of *BoLFY* transcript at all stages is consistent with *BoLFY* being necessary for inflorescence meristem initiation, floral primordium specification, and B- and C-function [stamen and carpel formation (Coen and Meyerowitz, 1991)], as it is in *Arabidopsis*. *BoTFL1* expression is consistent with a role in the enlargement of the curd rather than maintenance of the arrest.

Another model tested is whether the reproductive transition occurs because *BoTFL1* is repressed by *BoAP1* or *BoCAL*. In *Arabidopsis*, *AP1* and *CAL* suppress the repressor gene *TFL1* (Fig. 8) (Shannon and Meeks-Wagner, 1993; Bradley *et al*., 1997; Ratcliffe *et al*., 1999). If this regulation occurs in *B. oleracea*, the absence of BoCAL would allow greater *BoTFL1* expression, leading to the arrest in cauliflower curds. However, whether the developmental stage was varied by environment (Fig. 2B) or by genotype (Fig. 7), the pattern of expression of *BoTFL1* was consistently associated with the developmental stage of the curd and independently of the *BoCAL* genotype. Furthermore, the pathway that up-regulates *BoAP1-a* and initiation of floral primordia occurs, yet *BoTFL1* is expressed in all stages of development. None of these results is consistent with *BoTFL1* being regulated by *BoAP1* or *BoCAL*, or with the transition being a consequence of *BoTFL1* suppression.

The expression of *BoTFL1* also differs from that observed in *Brassica napus*, in which the gene is expressed predominantly in flowers (Mimida *et al*., 1999). In both *Brassica* species the expression pattern is inconsistent with *TFL1* being a repressor of flowering as it is in *Arabidopsis*.

Is the initiation of floral primordium development specified by an increase in BoAP1 and BoCAL expression in B. oleracea?

Genetic evidence for *BoAP1* and *BoCAL* in specifying floral primordium initiation is ambiguous. One element of such specification would be that conditions promoting floral initiation would cause up-regulation. In mutant genotypes in which the mutation affects the post-transcription of the gene, as in *BoAP1* and *BoCAL*, such up-regulation would be without direct effect, but indicative of the upstream regulatory pathways. In *B. oleracea*, the pattern of expression of *BoAP1-a* and *BoAP1-c* is consistent with a regulatory mechanism that triggers *BoAP1* expression to initiate floral primordia and

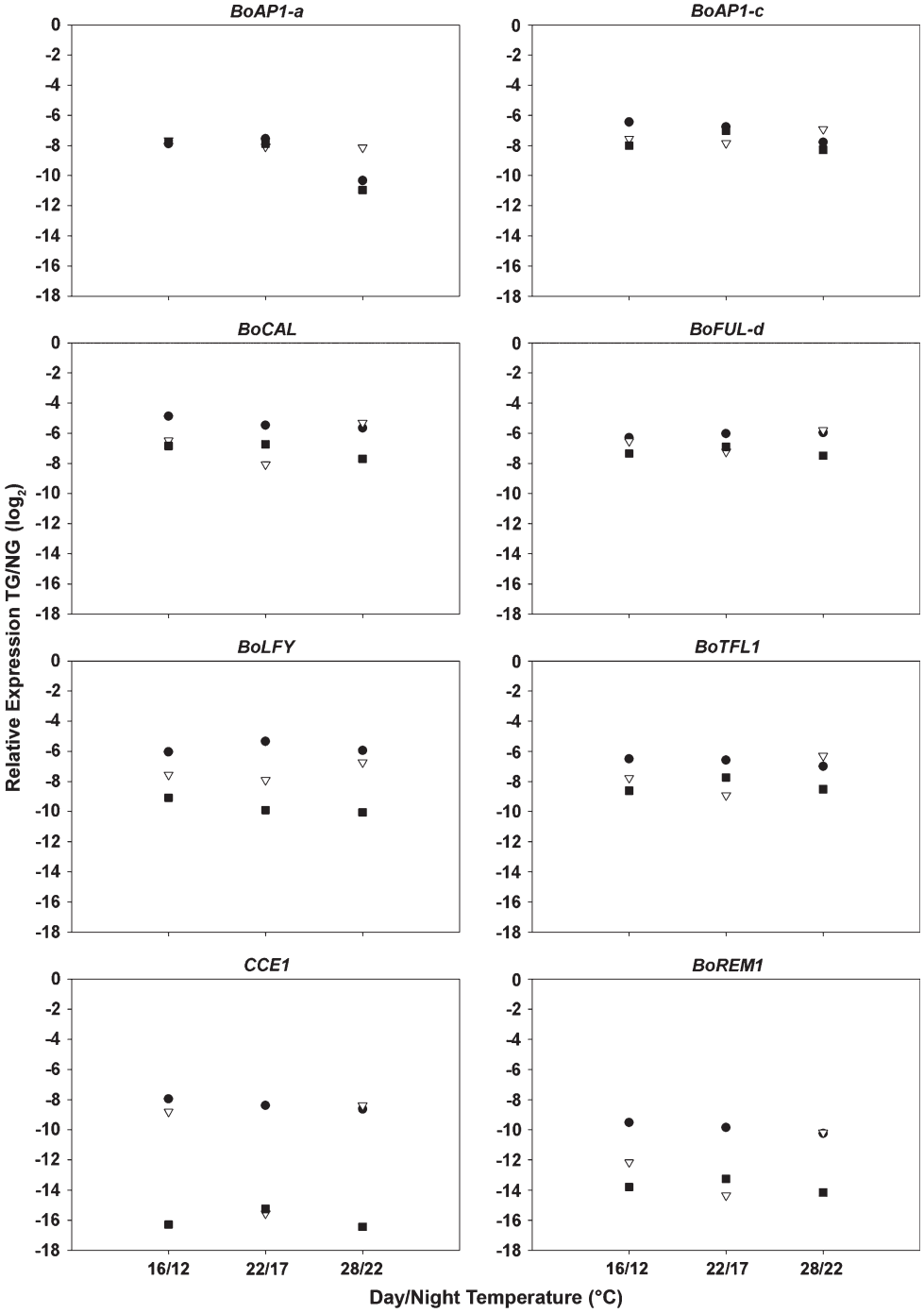

Fig. 5. Relative expression of genes in *Brassica oleracea* cv. Green Harmony F_1 after a 24 h temperature treatment under three different day/night temperature regimes: 16 °C/12 °C, 22 °C/17 °C, and 28 °C/22 °C. The amount of the target gene (TG) is quantified in proportion to the normalizer gene (NG) 18S. Plants were grouped in three sets of three plants each (one for each temperature treatment) based on the morphological characteristics of the curd. Circle, small size floral buds; triangle, very small floral buds; square, medium size floral buds.

with both paralogues being under the same regulatory mechanism. The presence of *BoAP1-c* transcripts in floral primordia is novel, since this gene has previously only been identified with bract formation, not with flower bud development (Smith and King, 2000; Kop, 2003). Expression of *BoCAL* (Fig. 2A) was not consistent with up-regulation to initiate floral primordium. The different expression patterns of *BoCAL* and *BoAP1* suggest that they are under independent regulatory control (Fig. 8).

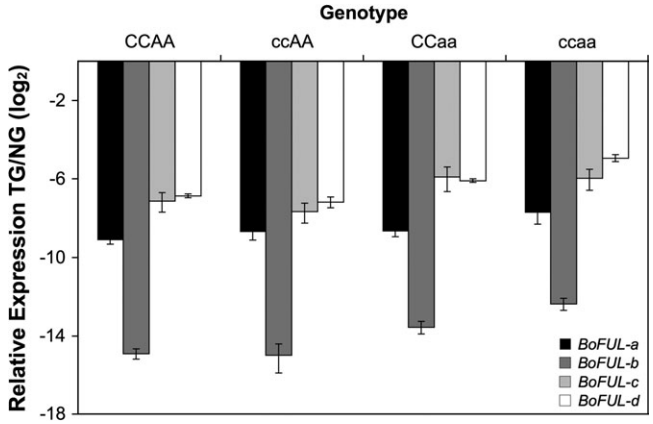

Fig. 6. Relative expression of *BoFUL* paralogue genes in different genotypic backgrounds for *BoCAL* and *BoAP1*.The amount of the target gene (TG) is quantified in proportion to the normalizer gene (NG) 18S. Analysis included three replications per line and three lines per genotype. The CCaa genotype only had two lines. Symbols are means ±SE. C, *BoCAL*; c, *bocal*; A, *BoAP1*; a, *boap1*.

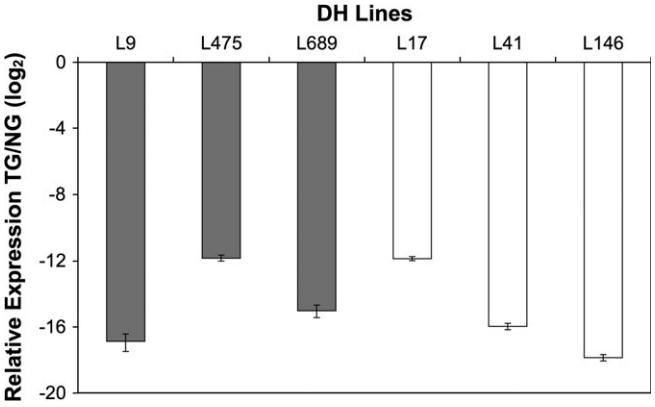

Fig. 7. Relative expression of the *BoTFL1* gene in different genotypic backgrounds for *BoCAL*. The amount of the target gene (TG) is quantified in proportion to the normalizer gene (NG) 18S. Analysis included three replications per line and three lines per genotype. Grey bars represent the *BoCAL BoAP1* genotype, and white bars represent the *bocal BoAP1* genotype. Symbols are means ±SE.

Table 3. *Probability values for the effect of BoAP1 and BoCAL genotype on the expression of the BoFUL paralogue genes*

Asterisks represent a significant effect ($P < 0.05$).

Varying locus	Responding gene			
	BoFUL-a	*BoFUL-b*	*BoFUL-c*	*BoFUL-d*
BoAP1	0.2564	0.014*	0.011*	0.0044*
BoCAL	0.5045	0.999	0.3889	0.6013

Is BoAP1 associated with an A-function gene in B. oleracea? Can floral primordia develop when both BoAP1 and BoCAL are mutant, presumably by the action of other redundant genes?

AP1 is an A-function gene controlling the differentiation of sepals and petals in the two outer whorls of new flowers (Irish and Sussex, 1990; Meyerowitz *et al*., 1991; Mandel *et al*., 1992; Liljegren *et al*., 1999). The presence of *BoAP1-a* and *BoAP1-c* transcripts in the inflorescence meristem is inconsistent with a classic A-function. In *Arabidopsis*, *AP1* is not expressed in the inflorescence meristem (Fig. 8). The A-function concept has been already questioned by several authors (Gutierrez-Cortines and Davies, 2000; Litt and Irish, 2003). *SQUA* in *Antirrhinum* (Huijser *et al*., 1992) and *VAP1* in grapevine (Calonje *et al*., 2004) do not have the A-function. Functional analysis of *BoAP1-a* and *BoAP1-c* in *Arabidopsis* is clearly warranted to determine whether these genes have A activity.

In *Arabidopsis*, *ap1* mutant flowers are partially converted to inflorescence shoots, and sepal and petal development is disrupted (Bowman *et al*., 1993). In the *boap1-a boap1-c bocal* triple mutant cultivar Green Harmony F_1, plants form floral primordia and floral buds (Fig. 3). The organ-specifying function of these genes must be fulfilled by some other MIGs. A prime candidate for the complementary MIG is *BoFUL*.

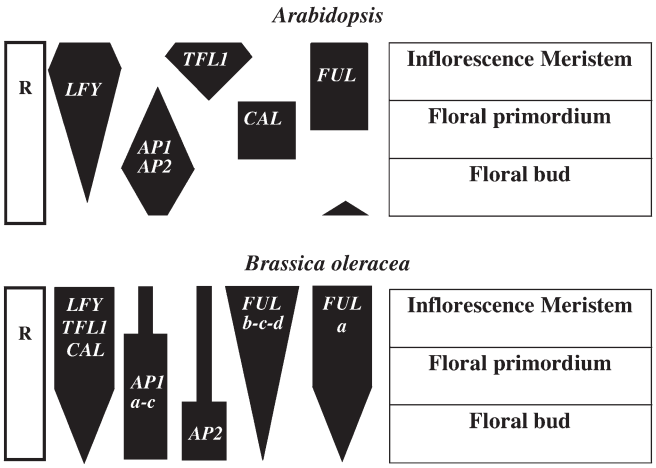

Fig. 8. Qualitative parallel representation of gene expression patterns between the proposed model of flowering in *Arabidopsis* and the expression patterns observed in *B. oleracea* cv. Green Harmony F_1 at three different developmental stages. Variations in the width of the shapes with developmental stage represent changes in gene expression.

Is the initiation of floral primordium development specified by an increase in BoFUL expression in B. oleracea? Are BoFUL paralogues redundant? Is BoFUL expression associated with BoAP1 or BoCAL genotype?

In *Arabidopsis*, *FUL* has a redundant role with *AP1*, *CAL*, and *LFY* in promoting flowering time and floral primordium initiation. It is also involved in cauline leaf morphology and carpel/fruit development (Gu *et al*., 1998; Ferrandiz *et al*., 2000). Transcripts are abundant in the organs where it is active (Fig. 8) (Mandel and

Yanofsky, 1995; Gu *et al*., 1998; Ferrandiz *et al*., 2000). Since *FUL* is a multifunctional gene in *Arabidopsis*, multiple copies of this gene in other species could increase specialization through subfunctionalization. The presence of four *BoFUL* paralogues could support even more. However, *BoFUL-b*, *BoFUL-c*, and *BoFUL-d* had identical patterns of expression, while *BoFUL-a* had a slightly different one (Figs. 2C, 8).

The steady decreases in expression from inflorescence meristem towards floral bud imply that these genes act in a threshold manner, as do *BoLFY* and *BoAP1*, requiring higher levels for inflorescence specification and lower levels for floral primordium development. For *BoFUL* to overcome the absence of BoAP1 and BoCAL, the level of expression should have been higher or equal in inflorescence meristem at the onset of floral primordium. The only paralogue with that pattern was *BoAP1-a*. The expression pattern for all four paralogues differed from the *Arabidopsis* model (Fig. 8) by being present in floral primordium and floral bud.

BoFUL expression may be suppressed by *BoAP1* or *BoCAL*. In *Arabidopsis*, *AP1* negatively regulates *FUL* in emerging flower primordia (Mandel and Yanofsky, 1995), and in proliferating meristems of *ap1 cal* double mutants *FUL* is ectopically expressed, allowing the plants to form flowers (Ferrandiz *et al*., 2000). In order to test this hypothesis, *BoFUL* expression was quantified in DH lines with different *BoAP1*/*BoCAL* genotypic combinations (Table 1). *BoCAL* genotype did not have any significant association with the expression of any of the four *BoFUL* paralogues. *BoAP1* genotype, on the other hand, was significantly correlated with the expression pattern observed for *BoFUL-b*, *BoFUL-c*, and *BoFUL-d* (Table 3); however, *BoAP1* genotype was highly correlated with the phenotype. In 'Green Harmony F_1' plants (Fig. 2C), *BoFUL* expression changed with the stage of the arrest, suggesting that expression of *BoFUL* paralogues was associated with the developmental stage of the curd and not with *BoAP1* or *BoCAL*.

Have AP2 and UFO conserved their roles as both floral primordium promoters and organ identity genes in B. oleracea as they do in Arabidopsis?

There are additional candidate genes for initiating floral primordium and specifying sepal identity. In *Arabidopsis*, *AP2* (Irish and Sussex, 1990; Bowman *et al*., 1993; Shannon and Meeks-Wagner, 1993) and *UFO* (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; Samach *et al*., 1999) act redundantly with the MADS-box gene *AP1* in the specification of floral primordium. If these genes have conserved this role in *B. oleracea*, then both genes should increase their expression at the floral primordium stage for the flower meristem to initiate (Fig. 8). However, the expression pattern observed in the mutant plants 'Green Harmony F_1' does not support *AP2* or *UFO* as alternative genes to specify floral meristem identity. In *B. oleracea*, the *AP2* pattern is consistent with a role for the gene in organ identity, but not in flower initiation (Fig. 2A).

Are CCE1 or BoREM1 associated with arrest at the inflorescence meristem stage?

A gene that prevents floral initiation and maintains the cauliflower curd would be expressed preferentially in the meristems of the curd, decreasing expression after resumption of floral development. This study shows clearly that *CCE1* is not the gene responsible for maintaining the cauliflower arrest since its down-regulation was observed later in development, when floral buds developed. *CCE1* could have a putative role in the suppression of class B- and C-function genes. How CCE1 fulfils this role is an interesting question since this protein belongs to a new, so far unidentified, family of transmembrane receptors (Palmer *et al*., 2001).

The *BoREM1* pattern (Fig. 2D) indicates that its function is probably threshold dependent, like *BoFUL* and *BoLFY*, and seems to be involved in both inflorescence meristem specification and floral primordium initiation. Like *LFY* in *Arabidopsis*, *BoREM1* may also be a transcription factor that up-regulates floral meristem genes since the protein sequence has a potential leucine zipper and a motif that resembles a localization signal found in proteins transported to the nucleus (Franco Zorilla *et al*., 1999).

It is possible that the changes in gene expression are not the result of regulatory processes downstream of the temperature-sensing process, but are direct temperature effects on these genes. To account for that possibility, expression was analysed after varying the temperature without variation in the developmental stage. Temperature was varied throughout reproductive growth in a non-responsive genotype (Fig. 4), or for 24 h in the same genotype (Fig. 5). There was no variation in the expression of the genes studied, resulting in only small significant differences in *BoAP1-a*. The major differences in gene expression observed in experiment 1 are therefore associated with the developmental stage of the curd.

The long-term exposure resulted in a small decrease in the abundance of *BoAP1-a* transcripts between the middle and cool temperature (Fig. 4A). Between the same temperatures when they caused a developmental change, there was no expression change to account for. 'Green Harmony F_1' is homozygous mutant at *BoAP1-a*, *BoAP1-c*, and *BoCAL*, while 'HRI 5295' is a homozygous mutant at *BoAP1-a*, but segregates for *BoAP1-c* and *BoCAL*. It is possible that one of these genes could mask a temperature-dependent pathway. In the short-term experiment, *BoAP1-a* transcript abundance did not change

significantly (Fig. 5). The numerical difference between the high and middle temperatures was only 20% of that between the same temperatures when they caused a developmental change. Therefore, even the largest change in this control fails to support direct temperature regulation.

In *B. oleracea*, bracteole initiation ('fuzzy' and 'leafy' heads) caused by high temperatures has been associated with a decline of *BoLFY* and *BoAP1* expression in the shoot apex (Anthony *et al.*, 1996) resembling the effect caused by *lfy* mutations in *Arabidopsis* plants (partial conversion of the floral primordium to inflorescence meristem accompanied by bracts and cauline leaves) (Weigel *et al.*, 1992; Bowman *et al.*, 1993; Kempin *et al.*, 1995; Liljegren *et al.*, 1999). In this study, *BoLFY* expression was not affected by high temperature.

Future work should determine with more certainty whether temperature directly regulates *BoAP1-a* but not *BoAP1-c*. If so, the regulation of these genes could provide a good model for discovering regulatory pathways sensitive to moderate temperatures.

Even though *Arabidopsis* and *B. oleracea* are members of the same family and share flower morphology, reproductive development in *B. oleracea* cannot be explained with the *Arabidopsis* model. In this model, *TFL1* maintains the indeterminate inflorescence fate, *LFY* specifies inflorescence meristem, the MADS-box genes *AP1*, *CAL*, and *FUL* redundantly with the non-MADS-box genes *LFY*, *AP2*, and *UFO* specify floral development, and *AP1* with *AP2* are A-function genes involved in formation of sepals and petals. All of these gene functions are inconsistent with the expression of the *Brassica* homologues (Fig 8). The model must be expanded to identify additional genes. Of particular interest as candidate genes for the control of floral primordium initiation are the recently discovered genes in *Arabidopsis*: *LATE MERISTEM IDENTITY* genes (*LMIs*) (Saddic *et al.*, 2006).

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