The Plant Journal (2006) **46**, 462–476 doi: 10.1111/j.1365-313X.2006.02706.x

The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA

Orna Ben-Naim[1], Ravit Eshed[2], Anna Parnis[1], Paula Teper-Bamnolker[2], Akiva Shalit[1], George Coupland[3], Alon Samach[2,*] and Eliezer Lifschitz[1,†]

[1]*Department of Biology, Technion–Israel Institute of Technology, Haifa 32000, Israel,*

[2]*The Robert H. Smith Institute for Plant Sciences and Genetics, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University, Rehovot 76100, Israel, and*

[3]*Max Planck Institute for Plant Breeding, D-50829 Cologne, Germany*

Received 28 September 2005; revised 28 December 2005; accepted 6 January 2006.
*For correspondence (fax +972 8 9489899; e-mail samach@agri.huji.ac.il).
†For correspondence (fax +972 4 8225153; e-mail lifs@tx.technion.ac.il).

Summary

CONSTANS-Like (COL) proteins are plant-specific nuclear regulators of gene expression but do not contain a known DNA-binding motif. We tested whether a common DNA-binding protein can deliver these proteins to specific cis-acting elements. We screened for proteins that interact with two members of a subgroup of COL proteins. These COL proteins were Tomato COL1 (TCOL1), which does not seem to be involved in the control of flowering time, and the *Arabidopsis thaliana* CONSTANS (AtCO) protein which mediates photoperiodic induction of flowering. We show that the C-terminal plant-specific CCT (CO, CO-like, TIMING OF CAB EXPRESSION 1) domain of both proteins binds the trimeric CCAAT binding factor (CBF) via its HAP5/NF-YC component. Chromatin immunoprecipitation demonstrated that TCOL is recruited to the CCAAT motifs of the yeast *CYC1* and *HEM1* promoters by HAP5. In Arabidopsis, each of the three CBF components is encoded by several different genes that are highly transcribed. Under warm long days, high levels of expression of a tomato *HAP5* (*THAP5a*) gene can reduce the flowering time of Arabidopsis. A mutation in the CCT domain of TCOL1 disrupts the interaction with THAP5 and the analogous mutation in AtCO impairs its function and delays flowering. CBFs are therefore likely to recruit COL proteins to their DNA target motifs *in planta*.

Keywords: CCT domain, NF-Y, tomato, Arabidopsis, DNA binding, protein–protein interaction.

Introduction

CONSTANS-Like (*COL*) genes encode proteins containing two highly conserved domains: an N-terminal zinc-finger B-box (Borden, 2000) domain which is also found in animals, and a plant-specific C-terminal CCT (CO, CO-like, TIMING OF CAB EXPRESSION 1, Strayer *et al.*, 2000) domain. *COL* genes have been identified in different plant species (Griffiths *et al.*, 2003) and each plant seems to host large families of them. In Arabidopsis there are 17 *COL* genes (Robson *et al.*, 2001), and there are at least 16 of them in the rice genome (Griffiths *et al.*, 2003).

The best-characterized function of a specific set of COL proteins, Arabidopsis CONSTANS (AtCO, Putterill *et al.*, 1995) and rice Heading Date 1 (HD1, Yano *et al.*, 2000), involve mediation of the photoperiodic induction of flowering. These proteins act by regulating transcription levels of floral integrators (Hayama and Coupland, 2004). One such common integrator belongs to the CETS (CEN, TFL1, SP) family (Pnueli *et al.*, 2001) and is encoded by *FLOWERING LOCUS T* (*FT*, Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999) in Arabidopsis and *Heading Date 3A* (*HD3A*, Kojima *et al.*, 2002) in rice.

Accumulation of FT or HD3A promotes flowering in both species, and accumulation of AtCO and HD1 occurs under long photoperiods (Suarez-Lopez *et al.*, 2001; Valverde *et al.*, 2004). AtCO activates *FT* expression (An *et al.*, 2004; Samach *et al.*, 2000; Takada and Goto, 2003) while HD1 seems to repress *HD3A* expression (Hayama *et al.*, 2003). As a result, extended photoperiods delay the transition to flowering in rice and promote it in Arabidopsis (Hayama and Coupland, 2004).

In contrast to Arabidopsis and rice, in tomato the timing of the transition to flowering is not affected by photoperiod, although other environmental factors such as light intensity and ambient temperatures do influence flowering time (Calvert, 1964). The transition to flowering in tomato terminates the shoot apical meristem. A secondary shoot arising from the axil of the youngest leaf, however, allows subsequent vegetative sympodial growth which eventually terminates when flowers are produced. The SELF PRUNING (SP) CETS protein regulates floral transitions of secondary sympodial meristems (Pnueli *et al.*, 1998). Using a yeast two-hybrid (Y2H) approach with SP, we previously identified a subfamily of Basic Region/Leucine Zipper (bZIP) transcription factors as likely candidates for mediating the action of CETS proteins (Pnueli *et al.*, 2001). The recent cloning of the flowering time gene *FD*, which encodes a bZIP transcription factor that interacts with FT and is required for its function (Abe *et al.*, 2005; Wigge *et al.*, 2005), has confirmed the validity of such screens.

No DNA-binding activity has been demonstrated for AtCO or for any other COL protein. Nevertheless, some of them function as transcription factors (Cheng and Wang, 2005; Samach *et al.*, 2000). One likely possibility is that binding to target promoters is mediated by adjunct DNA-binding factors (Hepworth *et al.*, 2002). We reasoned that, similar to CETS proteins, all COL proteins might be recruited to their targets by a common DNA-binding factor, despite their diverse effects in different plants.

To test our hypothesis, we isolated *COL* genes from tomato, a non-photoperiodic plant. One member of the tomato COL family, TCOL1, has no evident role in the control of flowering time, based on over-expression studies in tomato, tobacco and Arabidopsis. We asked whether TCOL1 and the flowering-time protein AtCO interact with a similar class of proteins. Using the Y2H system, we screened a library of tomato proteins and identified several different interacting proteins. After applying several filters and criteria, using both *in vitro* and *in vivo* binding assays on mutated and intact genes, and studying transgenic yeast and Arabidopsis, the major candidate emerging as a likely mediator of COL interaction with DNA is the CCAAT binding factor (CBF/HAP/NF-Y), a universal eukaryotic complex of three or more proteins (Maity and de Crombrugghe, 1998). There is only one gene representing each Hem Activator Protein (HAP) subunit in yeast and mammals. However, in plants, each member of this complex is encoded by a large family of genes, sharing conserved and non-conserved domains and specific expression patterns (Gusmaroli *et al.*, 2001, 2002; Kusnetsov *et al.*, 1999). There is thus great potential variety in content of CBF complexes formed within a cell. Together with the variation in the specificity of interactions with each COL protein, and the ability of COL proteins to interact with additional specific partners, these protein interactions provide a framework for understanding how this important family of plant proteins takes on diverse roles in plant development.

Results

Group 1A COL *genes from tomato display diurnal expression patterns*

Three Tomato *COL* (*TCOL*) genes were identified by screening, under low stringency, of a tomato genomic library with the *AtCO* gene as a probe. *TCOL2* and *TCOL3* are arranged in tandem while *TCOL1* is located in a different region of the genome (Figure 1a). All three genes contain sequences encoding both B-boxes and a CCT domain (Figure S1a), but a single base deletion in the *TCOL2* gene alters the open reading frame before the CCT domain (Figure S2a). COL proteins are classified into different subgroups (Griffiths *et al.*, 2003). We compared protein sequences encoded by *TCOL1–3*, eight additional *TCOL* expressed sequence tags (ESTs) [TIGR Tomato Gene index (http://www.tigr.org), *Solanaceae* Genomics network] and the Arabidopsis COL proteins. Our analysis suggested that *TCOL1*, *TCOL2* and *TCOL3* belong to Group 1A *COL* genes which are represented in Arabidopsis by *AtCO*, *COL1* and *COL2* (Figure S3a). Similar to Arabidopsis Group 1 *COL* genes, an intron is located before the sequence encoding the conserved C-terminal CCT domain. However, the three tomato genes have an additional intron at the 3′ end of the B-box domain (Figure 1a), creating a second exon that precisely defines the region encoding the activation domain (AD) of COL proteins (Figure 2 and below).

Several *COL* genes from different species display diurnal expression patterns and are under circadian-clock regulation (Cheng and Wang, 2005; Ledger *et al.*, 2001; Shin *et al.*, 2004; Suarez-Lopez *et al.*, 2001). Using reverse transcriptase (RT)-PCR with gene-specific primers, and the α-tubulin gene as a reference, we detected robust cycling of transcripts for both *TCOL1* and *TCOL3* in tomato plants entrained under either long or short days. Robust cycling continued when plants were moved to constant dark, suggesting circadian-clock regulation (Figure 1b–f). A strong effect of day length on peak expression time of these genes, as shown for other clock-regulated genes in Arabidopsis (Millar and Kay, 1996), was also observed.

High levels of expression of the TCOL3 *gene conferred reduced sensitivity to photoperiod in the flowering response of Arabidopsis*

Unlike Arabidopsis and rice, transition to flowering in tomato is not affected by photoperiod. Nevertheless, in all three species, flowering is accelerated by increased expression of *FT*-like genes (Hayama and Coupland, 2004; E. Lifschitz, unpublished data). We have generated transgenic tomato plants expressing the *TCOL1*, *TCOL3* or *AtCO*

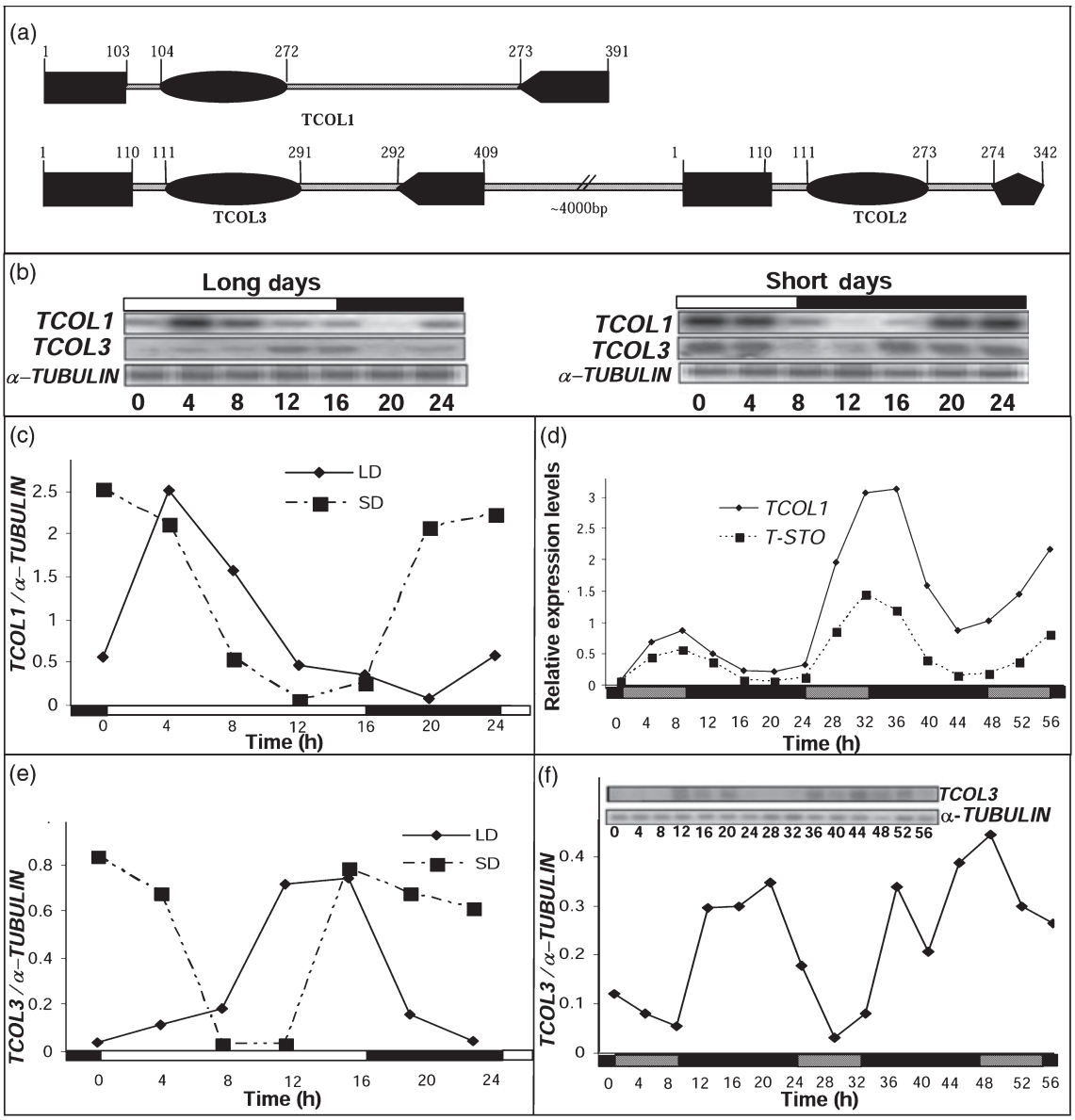

Figure 1. Genomic organization and expression profiles of Group 1A *COL* genes from tomato.
(a) Schematic genomic maps and intron–exon sites for the three tomato *COL* genes. The tomato genes contain two introns versus only one in Arabidopsis, and their second exon defines the complete COL activation domain (see Figure 2). Numbers above the lines indicate amino acids at the borders of the exons. The CCT domain of the *TCOL2* gene is missing due to a single bp deletion.
(b) Semi-quantitative RT-PCR analysis of expression profiles of *TCOL1* and *TCOL3* under long and short days. Tomato plants, 1 month old, with five expanding leaves were grown for 7 days under the indicated light regimes before sampling 20 apices. Bars above represent the light (white) or dark (black) periods of the day. Numbers represent hours from dawn.
(c) Quantification of *TCOL1* expression data presented in (b). Solid and dashed lines represent expression levels, relative to the tomato α-tubulin gene, under long days (LD) and short days (SD) respectively. Bars and numbers are as in (b).
(d) Quantification of *TCOL1* (solid line) and *TSTO* (dotted line) expression in continuous dark, based on semi-quantitative RT-PCR. Plants were moved to constant conditions after entrainment to short days. Grey bars represent subjective light periods. Other bars and symbols are as in (c).
(e) Quantification of *TCOL3* expression data presented in (b). Symbols as in (c).
(f) Quantification of *TCOL3* expression in constant dark, as in (d).

genomic clones under the control of the ubiquitous CaMV35S promoter. None of the transgenic lines showed significant early flowering. The inheritance of late-flowering phenotypes found in some of the T1 transformants containing 35S:*TCOL1* or 35S:*TCOL3* could not be tested because these plants were sterile. The same constructs were also introduced into day-neutral tobacco plants. Here we observed a delay in the flowering of transgenic tobacco

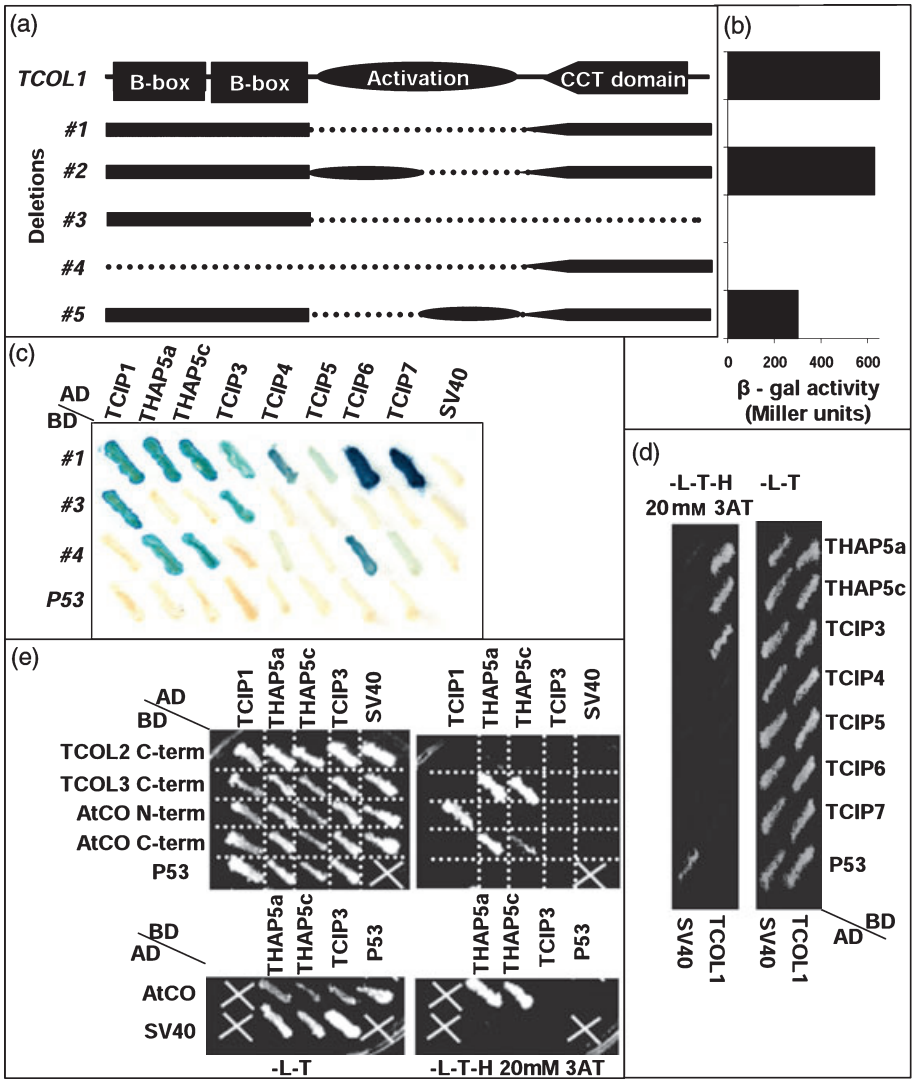

Figure 2. Identification and characterization of the tomato COL-interacting proteins.
(a) A scheme of the five deletion constructs of *TCOL1* tested for their transcriptional–activation potential in yeast cells.
(b) Quantitative β-galactosidase activity (expressed as Miller units) of five *BD-TCOL1* deletion constructs. The activation potential of TCOL1 resides along its second exon. Deletion 1 was chosen as bait in the two-hybrid screen.
(c) Interactions between specific domains of TCOL1 and the TCIP proteins. A Colony lift β-galactosidase test. TCIPs, identified by virtue of their interaction with deletion 1, were tested for their interactions with the N-terminal (deletion 3) and the C-terminal (deletion 4) domains of TCOL1. TCIP 1 and 3 interacted solely with the N-terminal domain of TCOL1, whereas the other TCIPs interacted specifically with the C-terminal domain of TCOL1.
(d) THAP5a, THAP5c and TFKBP12 (TCIP3) interact with the full-length TCOL1 protein. Seven TCIPs were tested, as BD fusions, for binding with the full-length TCOL1 protein. TSTO (TCIP1) could not be tested due to its strong activation potential. Growth of hybrid lines on non-selective (-L-T) and selective (-L-T-H 20-mM 3AT) plates is shown. Only THAP5a, THAP5c and TFKBP12 (TCIP3) interact with the full-length TCOL1 protein.
(e) Conserved interactions between TCIPs and specific COL domains. TSTO (TCIP1) interacts with the N-terminal domain of the AtCO protein. THAP5a and THAP5c interact with the C-terminal domains of TCOL3 and AtCO and with the full-length AtCO protein but not with TCOL2, which lacks a CCT domain. The interaction between TFKBP12 (TCIP3) and the N-terminal domain is specific to the TCOL1 protein. Yellow X indicates empty sites.

plants expressing high levels of *AtCO* (not shown); note that these plants were fertile. These results suggest that TCOL1 and TCOL3 have no obvious effect on flowering time, and that regulation of the tomato *FT* orthologue might not be linked to levels of Group 1A COL factors.

In addition to *AtCO*, over-expression of the *CONSTANS* orthologue from *Pharbitis nil* also promoted flowering in Arabidopsis (Liu *et al.*, 2001). We generated transgenic Arabidopsis plants expressing the 35S:*TCOL1* or 35S:*TCOL3* genes. Plants expressing *TCOL3*, but not *TCOL1*, consistently flowered slightly later under long days and earlier under short days (Table 1).

Photoperiodic flowering is an output of the circadian clock (Samach and Coupland, 2000). We tested whether

Table 1 Flowering time (number of leaves to flowering) of two homozygous transgenic Arabidopsis lines (independent) overexpressing the tomato TCOL3 gene

		Rosette				Cauline				Total				
	Genotype	Av	Rng	SD	SE	Av	Rng	SD	SE	Av	Rng	SD	SE	N
LDs	Wt (L.*er*)	7.83	6–10	0.924	0.218	3.39	2–4	0.608	0.143	11.28	10–13	0.890	0.210	18
+ Incandescent	*35S:TCOL3#1-1*	10.29	9–13	1.007	0.220	4.71	3–6	0.784	0.171	15.00	12–18	1.483	0.324	21
	35S:TCOL3#2-1	10.05	8–13	1.174	0.250	4.32	3–5	0.650	0.140	14.36	12–18	1.649	0.352	22
LDs	Wt (L.*er*)	8.93	8–11	0.780	0.140	3.10	2–4	0.550	0.102	12.03	10–15	1.130	0.210	30
	35S:TCOL3#1-1	12.62	10–16	1.780	0.399	4.10	3–6	0.850	0.190	16.45	14–21	2.200	0.500	20
	35S:TCOL3#2-1	11.90	11–14	0.890	0.110	4.14	3–5	0.480	0.150	16.05	15–18	0.970	0.210	21
SDs	Wt (L.*er*)	28.71	18–37	6.290	2.380	6.71	3–9	2.060	0.780	35.43	21–45	7.870	2.970	7
	35S:TCOL3#1-1	12.30	11–13	1.400	1.000	3.00	0	0.000	0.000	15.30	14–16	1.410	1.000	5
	35S:TCOL3#2-1	17.00	8–24	5.760	2.350	5.17	2–8	2.560	1.040	22.17	10–32	8.260	3.370	6

LDs, long days; SDs, short days; Av, average; Rng, range; SD, standard deviation; SE, standard error; N, number of plants.

Arabidopsis plants expressing the 35S:*TCOL3* gene exhibit an additional phenotype associated with clock dysfunction: inhibition of hypocotyl growth by light is gated by the circadian clock (Dowson-Day and Millar, 1999). Hypocotyls of 35S:*TCOL3* and wild-type Arabidopsis plants were measured under different light regimes (red, blue, white and dark) but no significant differences were found (data not shown). Thus, the effect of *TCOL3* on flowering time is unlikely to be through major disruption of clock function.

A screen for COL interacting proteins reveals domain-specific interacting proteins

Plants expressing 35S:*TCOL1* showed no obvious flowering-time phenotype, and *TCOL1* was thus chosen as 'bait' in the screen for common COL interacting proteins. Preliminary tests indicated that, as a binding-domain (BD) fusion, the complete TCOL1 protein is an extremely strong activator of transcription (Figure 2a,b) and could therefore not be used as bait, in Y2H screens. To identify and exclude the activation domain, five deletion constructs of *TCOL1* (Figure 2a) were examined for their transcriptional-activation potential. The results suggested that the activation region of TCOL1 occupies the entire second exon, the two halves of which conferred nearly equal upregulation of the reporter gene (Figure 2b). Deletion No. 1, containing the complete N-terminal B-box and the CCT C-terminal domains but missing the activation domain, was therefore chosen as bait. A cDNA library made from apices RNA (Pnueli *et al.*, 2001) and containing 4×10^6 clones was screened on histidine (HIS)-selective medium containing 2.5-mM 3amino-triazol (3AT). 127 positive clones, designated *TCIP* (Tomato CONSTANS Interacting Proteins), were validated (see Experimental procedures section), and found to represent eight genes (Table 2).

In pair-wise combinations, none of the fusion proteins interacted with the control p53 protein (Figure 2c) or with each other (data not shown). The eight TCIPs expressed as BD fusions were further tested for their binding to specific domains of TCOL1 (Figure 2c), or to the full TCOL1 protein expressed this time as AD fusions (Figure 2d). Four proteins

Table 2 Tomato genes encoding TCOL1-interacting proteins

						Similar protein				
Name	Renamed	No. of clones	Accession number	Minimal interaction domain	Chr #	Name	Sp.	Accession	Details	% & (length) of similarity
TCIP1	*TSTO*	45	AY490242	233 aa	6	Salt Tolerance	*A. t*	At1g06040	B-box	58% (146 aa)
TCIP2a	*THAP5a*	46	AY490243	232 aa	6	At-HAP5a	*A. t*	At3g48590	CBF factor	85% (175 aa)
TCIP2b	*THAP5c*	5	AY490244	182 aa	1	At-HAP5c	*A. t*	At1g08970	CBF factor	76% (122 aa)
TCIP3	*TFKBP12*	26	AY490245	112 aa	1	At-FKBP12	*A. t*	At5g64350	Immunophilin	83% (94 aa)
TCIP4		2	AY490246	198 aa		SBP1	*P.h*	AAR92230	S-ribonuclease binding protein	98% (310 aa)
TCIP5		1	AY490247	602 aa		Expressed protein	*A. t*	At3g48500	–	77% (421 aa)
TCIP6		1	AY490248	484 aa		Tropomyosin-related	*A. t*	At5g48160	Tropomyosin-related	66% (324 aa)
TCIP7		1	AY490249	473 aa		HIP2	*S. t*	CAD45375	Protease-interacting protein	94% (445 aa)

Sp., species; *A. t*, *Arabidopsis thaliana*; *P. h*, *Petunia X hybrida*; *S. t.*, *Solanum tuberosum*; Chr #, Tomato chromosome number, based on mapping to *Solanom pennellii* introgression lines.

(TCIP4–7) did not interact with full-length TCOL1 (Figure 2d) and they were not studied further.

B-box binding proteins

TCIP3 (26/127 clones recovered) encodes a protein similar to mammalian immunophilin FKBP12 (Siekierka *et al.*, 1989), and was therefore named *Tomato FKBP12* (*TFKBP12*). Expression of *TFKBP12* showed no clear diurnal pattern of expression (Figure S4). It interacted specifically with the N-terminal B-box domain of TCOL1 and with the full-length TCOL1 protein, but did not interact with the B-box domains encoded by *AtCO* (Figure 2e). For that reason it was not studied further.

TCIP1 (45/127 clones) encodes a protein with two zinc-finger B-box motifs typical of COL proteins, but contains no CCT domain. We renamed it TSTO because it showed the highest similarity in sequence to the Arabidopsis Salt Tolerance protein (Lippuner *et al.*, 1996). Similarly to *STO* (Smith *et al.*, 2004), *TSTO* expression showed diurnal fluctuations (Figure S4) and continued to cycle under constant dark conditions (Figure 1d). TSTO (TCIP1) interacted specifically with the N-terminal B-box containing domain of TCOL1 (Figure 2c) and AtCO (Figure 2e), but could not be tested against the full-length proteins because it acts as an activator itself when fused to the BD (not shown). *TSTO* does not encode a protein with a known DNA-binding motif and will not be considered further in this context here.

CCT-domain interacting proteins are functional components of the CBF

The two remaining genes, *TCIP2a* (46/127 clones) and *TCIP2b* (5/127 clones), encode proteins showing high sequence similarity to the yeast transcription factor HAP5 (McNabb *et al.*, 1995). HAP5, together with HAP2 and HAP3, forms the trimeric CBF to regulate transcription by direct binding to a common promoter motif in many eukaryotic genes. While yeast and mammals contain one gene for every component of the CBF complex, plant genomes encode several copies of each subunit (see Discussion section). *TCIP2a* and *TCIP2b* were renamed *THAP5a* and *THAP5c* based on their sequence similarities to two out of nine Arabidopsis *HAP* genes, *AtHAP5a* and *AtHAP5c* (Edwards *et al.*, 1998; Figure S3b). All subunits of the HAP complex are required for its function: yeast cells bearing a mutant allele in any of the subunits are unable to grow on non-fermentable carbon sources (Sinha *et al.*, 1995). This phenotype was used to test whether the two tomato genes coding for HAP5-like proteins can functionally replace HAP5 in yeast. The *Hap5* gene in the yeast haploid strain, Y1064, was disrupted with a *URA3* insertion (see Experimental procedures section). Both tomato *HAP5* genes successfully complemented the yeast mutation (Figure 3a).

The HAP5 component of CBF interacts with Group 1A COL proteins through the CCT domain

The two THAP5 proteins were shown to interact with the CCT-domain-containing C-terminal region of TCOL1 (Figure 2c), the CCT domains of TCOL3 and AtCO (Figure 2e), and the full-length TCOL1 protein (Figure 2d). The two THAP proteins did not interact with the full-length TCOL2 lacking a CCT domain (Figure 2e). Thus, THAP5 proteins interact specifically with the CCT domain of Group 1A COL proteins originated from different species and having distinct functions.

The THAP5–TCOL1 interaction was further verified in a non-yeast system. Anti-THAP5a antibody, prepared in rabbits (see Experimental procedures section), did not recognize the closely related THAP5c protein expressed in *Escherichia coli* or yeast cells (data not shown) but it specifically recognized the THAP5a protein expressed from the *35S:THAP5a* transgene in tobacco plants as a single *c.* 34-kDa band (Figure 3b). Using the affinity-purified anti-THAP5a antibody, we detected specific interaction between THAP5a expressed in tobacco and *in vitro*-translated TCOL1 (Figure 3c).

The THAP5 factor fails to interact with a mutant allele of the CCT domain that perturbs AtCO function

Two late-flowering alleles of *AtCO*, *co-5* and *co-7*, are caused by point mutations in the CCT domain (Robson *et al.*, 2001). The affected amino acids, P355 and R356, are conserved in the TCOL1 and TCOL3 proteins (Figure 4a), as well as in a broad range of CCT-domain proteins. We asked whether the reduced function of the two mutant alleles might be a result of reduced interaction with the HAP5 component. The two mutations, P355L and R356Q, and an additional mutation in a nearby conserved position, G359S, were introduced into the *TCOL1* gene, and the mutated proteins tested for binding to the THAP5 proteins in yeast. The *co-5* (P355L) mutation had no effect on binding (Figure 4b) and most likely affects other functions of the CCT domain. In contrast, *co-7* (R356Q), the other CCT-domain mutation that inactivates AtCO, and the adjacent novel G359S mutation, completely eliminated the interaction between TCOL1 and THAP5.

Elevated THAP5a levels affect flowering time of Arabidopsis in response to environmental conditions

No obvious diurnal cycling has been identified in the mRNA levels of the Arabidopsis *HAP5* genes (Smith *et al.*, 2004; Zimmermann *et al.*, 2004). Similarly, expression of the two *THAP5* genes was only moderately sensitive to day/night cycles (Figure S4). COL proteins are localized in the nucleus (Cheng and Wang, 2005; Robson *et al.*, 2001), as are the HAP proteins. We followed the distribution and intracellular localization of THAP5a in the tomato apex. Immunogold

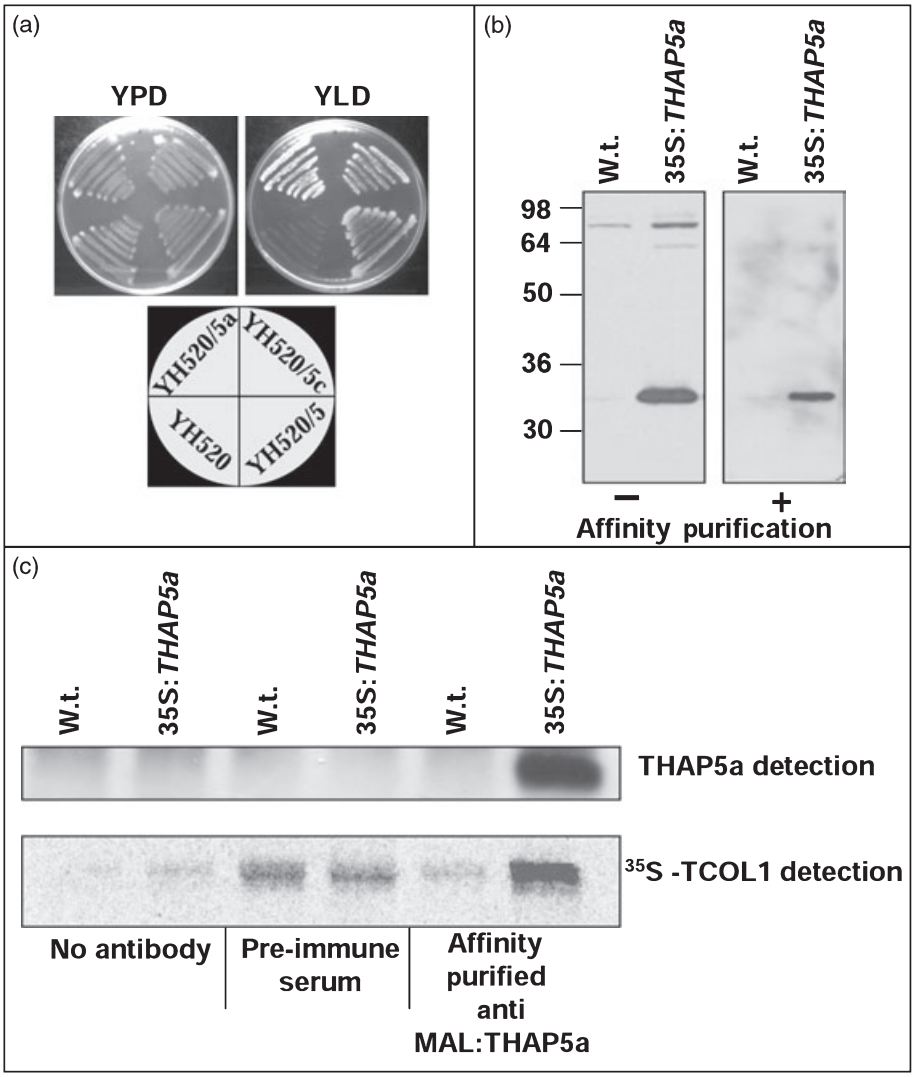

Figure 3. THAP5a complements HAP5, and interacts with TCOL1 independently of yeast factors.
(a) The *HAP5* gene of the yeast line Y1064 was inactivated by a *URA3* insertion (see Experimental procedures section). Mutant *hap5*⁻ cells, YH510, bearing BD fusions of the yeast *Hap5* gene (YH520/5), the tomato *THAP5a* (YH520/5a) and *THAP5c* (YH520/5b) genes, or an 'empty' BD (YH520) as a control, were tested for complementation on non-selective (left plate, YPD, glucose) and selective (right plate, YLD, lactate) media.
(b) Immunoblot detection of total protein extracts from wild-type and transgenic tobacco plants containing the 35S:*THAP5a* construct. The protein blot was probed with an anti-THAP5a antibody, before or after affinity purification (see Experimental procedures section). The THAP5a antibody recognized a single *c.* 34-kDa band which was strongly present in transgenic 35S:*THAP5a* plants.
(c) *In vitro* binding assays between *in vitro* translated ^{35}S-Met-TCOL1 and THAP5a from 35S:*THAP5a* transgenic tobacco plants. Protein extracts (1 mg) from wild-type or 35S:*THAP5a* plants were incubated with anti-THAP5a polyclonal antibody. The complex was bound and immobilized on protein A containing Dynabeads and challenged with *in vitro* translated ^{35}S-Met-TCOL1. After incubation, eluted fractions were resolved by SDS-PAGE, and probed for the presence of THAP5a with anti-THAP5a antibody, and for the presence of ^{35}S-Met-TCOL1 by exposing the blots to a Phosphorimager (model FLA-5000; Fujifilm, Tokyo, Japan) (see Experimental procedures section).

detection using the THAP5a specific antibody in longitudinal sections of a tomato apex (Figure 5a, see Experimental procedures section) localized THAP5a to cell nuclei (Figure 5a–c). The THAP5a antigen could be found in epidermal, meristematic, vascular and differentiated parenchyma cells.

To test whether the availability of HAP5a affects flowering time, the 35S:*THAP5a* construct was introduced into non-photoperiodic tomato and tobacco plants: no effect on flowering time was detected (data not shown).

Some of the nine *HAP5* genes are highly expressed in Arabidopsis (Gusmaroli *et al.*, 2001, 2002), suggesting that HAP5 levels in Arabidopsis might not be restrictive. No flowering-time effect was observed in transgenic 35S:*THAP5a* Arabidopsis plants grown under short-day conditions (Table S1). Under long-day conditions, only one or two of the transgenic lines showed a slight reduction in flowering time (Table S1). Analysis of publicly available microarray data [GENEVESTIGATOR web tool (https://www.genevestigator.

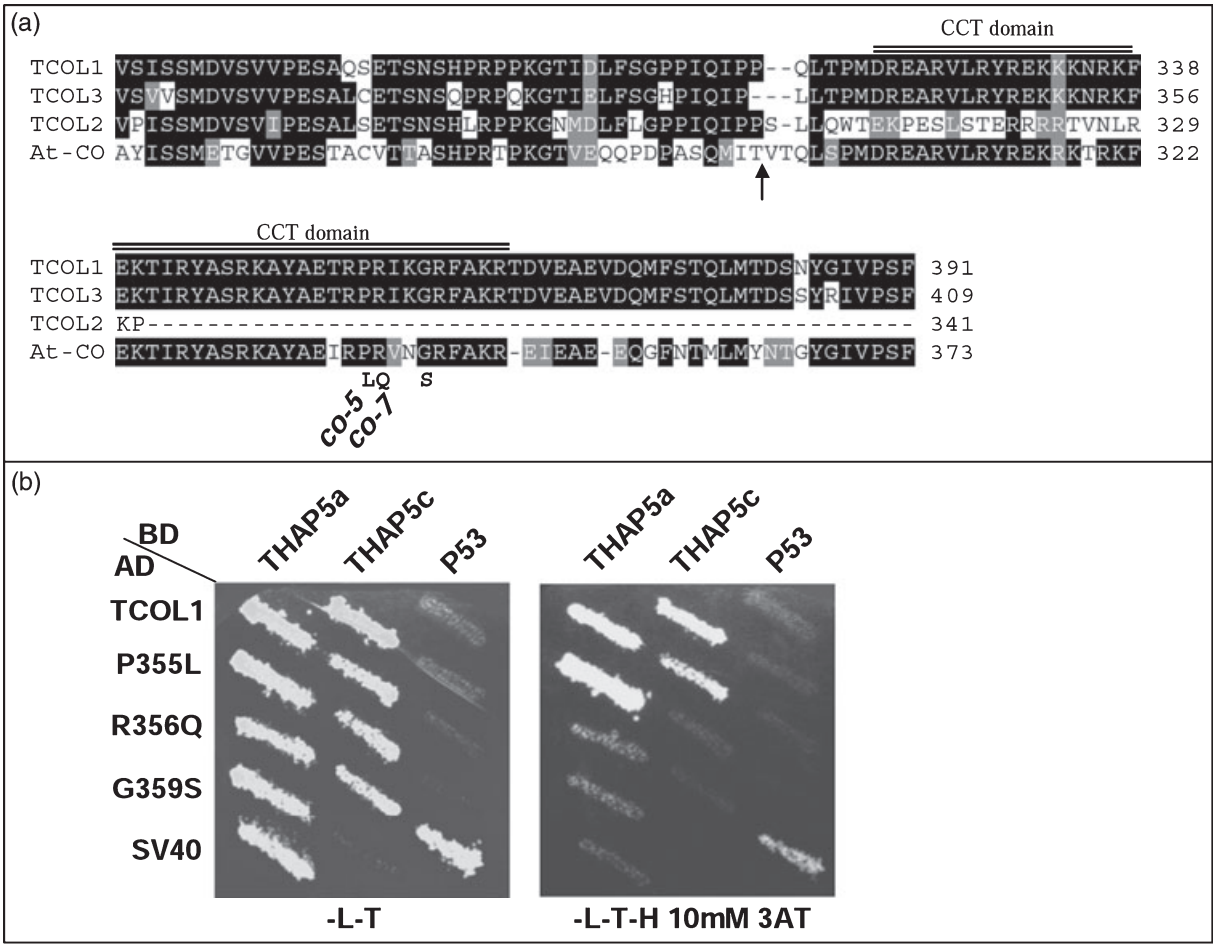

Figure 4. The conserved arginine 356 is required for both AtCO function and the interaction between the CCT domain and the THAP5 factors.
(a) Alignment of the predicted amino acid sequences of the C-terminal regions of the three TCOL and the AtCO proteins. The 43-residue-long CCT domain is underlined. The site of the shifted reading frame in the *TCOL2* gene is marked with an arrow. Altered amino acids are shown below, with the corresponding names of their *AtCO* alleles.
(b) Interactions, determined by Y2H tests, between the three mutant versions of the TCOL1 protein and the two THAP5 factors. Left and right panels: growth of tested colonies in the absence (left) or presence (right) of 10-mM 3AT. The R356Q and G359S mutations abolished the interactions with both THAP proteins.

ethz.ch/), Zimmermann *et al.*, 2004] showed that one of the most highly expressed *HAP5* genes in Arabidopsis, *At1g08970* (Gusmaroli *et al.*, 2002), exhibits reduced expression when plants are exposed to higher temperatures. Indeed, under warmer long-day conditions, all independent transgenic lines flowered significantly, albeit slightly earlier than wild-type plants (Figure 5d–f). This suggests that, under conditions in which its expression might be reduced, HAP5 might be a limiting factor for flowering. Further, this effect may be suppressed by over-expressing HAP5.

A functional HAP2/3/THAP5 complex recruits TCOL1 to the HAP-responsive CCAAT motifs of the yeast HEM1 *and* CYC1 *genes*

Transcription of the yeast *CYC1* and *HEM1* genes requires the binding of the trimeric CBF to their well-characterized CCAAT promoter motifs (Keng and Guarente, 1987; McNabb *et al.*, 1995; Olesen and Guarente, 1990). We used these motifs to test whether a functional CBF complex, containing the THAP5a protein, can actually recruit the TCOL1 factor to functional CCAAT sites. First, *hap5* mutant cells expressing the AD fusion of *TCOL1*, tagged with a 13Xmyc peptide, were prepared. The *hap5* mutation in these cells was successfully complemented by the yeast *Hap5*, and the *THAP5a* and *THAP5c* genes. Thus, the presence of the TCOL1 chimera protein did not compromise the ability of the three HAP5 factors to substitute for the endogenous disrupted hap5 factor. Furthermore, the TCOL1–13Xmyc protein interacted, in the Y2H test, with all three HAP5 variants (data not shown). Subsequently, Chromatin immunoprecipitation (ChIP) assays were used to detect possible binding of the tagged TCOL1 to the proven functional CCAAT motifs of the *CYC1* and *HEM1* promoters. Chromatin from each of the six tested lines (three HAP5 types, with or without the TCOL1–13Xmyc protein) was cross-linked and immunoprecipitated

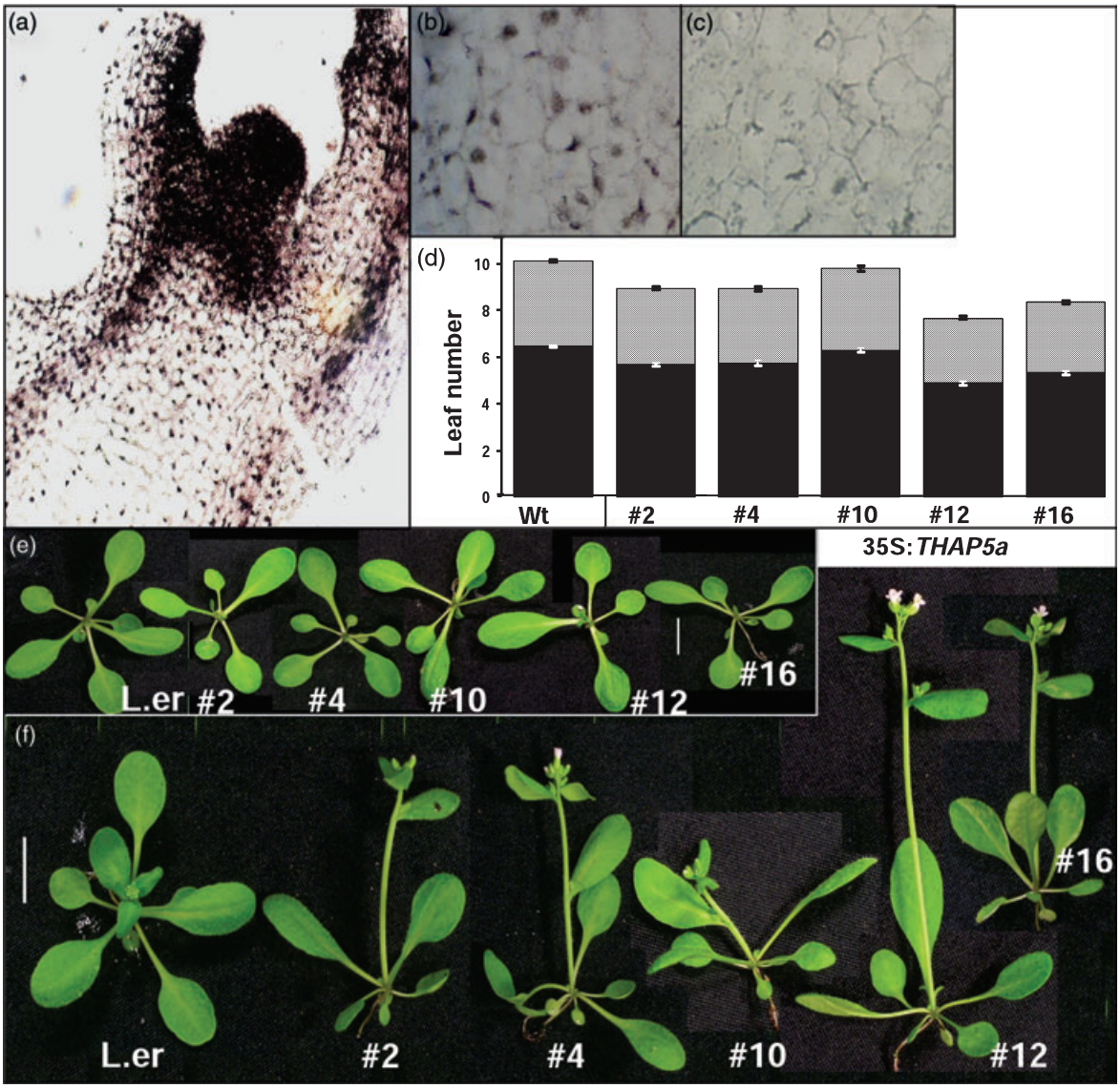

Figure 5. Increased levels of *THAP5a* accelerate the transition to flowering in Arabidopsis grown under warm long days.
(a) Immunogold localization of THAP5a in fixed longitudinal sections of the tomato apex, using an anti-THAP5a antibody. The intensive labelling of nuclei in the apex reflects the density of cells and the relative size of the nuclei.
(b) Enlarged section of labelled nuclei.
(c) Section treated with pre-immune serum (control).
(d) Flowering time of 35S:*THAPa* Arabidopsis plants grown under warm (28/22°C) day/night conditions under long days (16/8-h day/night photoperiods). Independent transformants were compared to the *Landsberg erecta* wild-type strain and most lines (except line 10) showed early flowering under these conditions. Flowering time was measured by counting rosette (grey) and cauline (black) leaves. Mean leaf number is shown ±SEM ($n = 5$ to 22).
(e) Photographs of 26-day-old representative plants from (d). The different genotypes were grown together under identical conditions. The inflorescence stem, including cauline leaves, was removed to visualize rosette leaf number easily. Plants were photographed separately at the same age.
(f) As (e), leaving the plants uncut. The scale bars in (e) and (f) are 1 cm.

with an anti-myc antibody. Using primers specific to sequences flanking the established CCAAT target sites of the *CYC1* or *HEM1* promoters or control *HAP2* gene sequences, CBF binding sites were found to be enriched in DNA immunoprecipitated along with the TCOL1–13Xmyc protein (Figure 6). Furthermore, no amplification of the target sequences was obtained after immunoprecipitation of cross-linked DNA from cell lines that acted as negative controls and did not contain pAD:TCOL1–13Xmyc (data not shown). A complex containing TCOL1 was thus shown to bind the CCAAT boxes of the yeast *CYC1* or *HEM1* genes.

Discussion

We have identified a protein complex that can recruit Group 1A COL proteins to DNA in eukaryotic cells. Flowering-

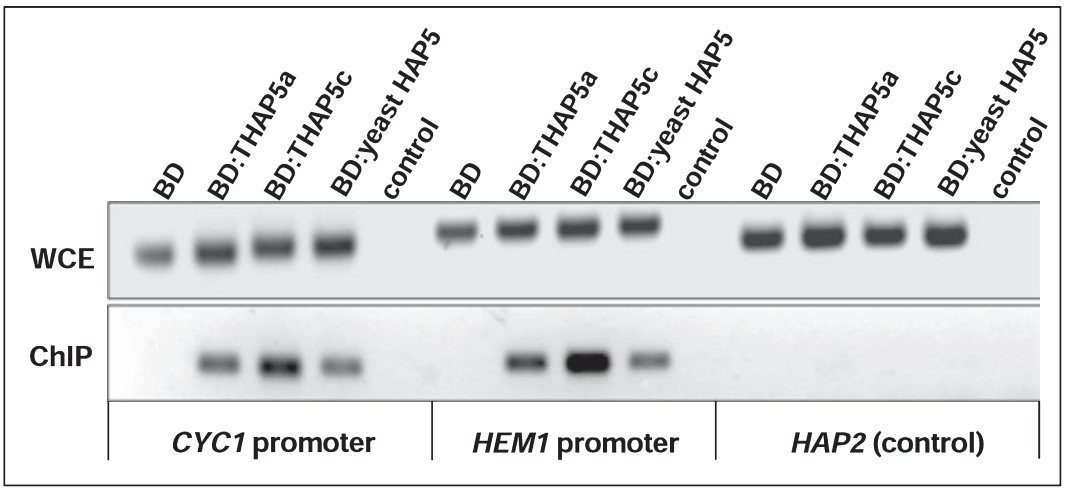

Figure 6. THAP5 recruits TCOL1 to HAP-binding CCAAT targets in yeast cells. Chromatin immunoprecipitation assays.
ChIP assays were performed on *hap5*$^{-}$ cells expressing the *TCOL1–13Xmyc* gene and either the parental BD vector or one of its derivative BD fusions with *HAP5*, *THAP5a* or *THAP5c* genes (Y1064, YH530, YH530a and YH530c respectively (see Experimental procedures section). Target DNA was detected with PCR using primers specific to the flanking sequences of the CCAAT motifs of the *CYC1* and *HEM1* promoters, or specific to the sequence of the *HAP2* gene as a control (Table S3). PCR reactions were performed before (WCE, input) or after (ChIP) immunoprecipitation with anti-myc antibody. No amplification of the target sequences after immunoprecipitation was obtained with the same cell lines lacking the *pAD:TCOL1–13Xmyc* gene.

related (AtCO) and non-flowering-related (TCOL1) members of the COL family can bind the HAP5 (NF-YC; CBF-C) subunit of the CBF through their CCT domain. The validity of this interaction has been shown by *in vitro* and *in vivo* approaches. Revealing the biological relevance of an association between proteins that are members of large families is not straightforward. Interactions between MADS-box transcription factors are still being determined using Y2H as a first step (de Folter *et al.*, 2005). Most *COL* genes are of unknown function and the abundance of gene copies for each component of the CBF increases the complexity. Nevertheless, when focusing on one biological function of a COL protein, the control of flowering in Arabidopsis by AtCO, our data showed premature flowering under specific conditions due to HAP5 over-expression. This, together with evidence for loss of HAP5 interaction in a mutation in AtCO that delays flowering, strongly supports a role for the CBF in *COL* gene function.

Do COL proteins regulate gene expression through CCAAT motifs?

In yeast, we have shown that TCOL1 is recruited by the HAP complex to promoters that contain CCAAT boxes. CCAAT motifs can be found within regulatory regions of known and potential targets of COL proteins (Table S2). In other systems, CBF interacts with other proteins directly (Taira *et al.*, 1999; Villard *et al.*, 2000), cooperates with other transcription factors that bind to independent sites (Dooley *et al.*, 1998) or competes with other factors for binding to overlapping motifs (el-Hodiri and Perry, 1995; Caruso *et al.*, 2002; van Heeswijck and Hynes, 1991). We cannot rule out the possibility that an interaction with COL proteins modifies or relaxes the DNA specificity of the CBF complex, such that this complex no longer binds only the CCAAT motif. Masiero *et al.* (2002), for example, reported that OsNF-YB1, a rice homologue of HAP3 capable of interacting with a rice MADS-box protein, does not bind a CCAAT motif, since it cannot form a trimeric CBF. A cis-element required for AtCO-dependent induction might contain a non-CCAAT motif to which additional, as yet unknown, components of the complex bind. Some of these components might be other interacting proteins identified in this screen.

Are COL–CBF interactions selective?

Previously an Arabidopsis *HAP2* gene was shown to complement yeast *Hap2* mutant (Edwards *et al.*, 1998). Here we show that two tomato *HAP5* genes complement a disrupted yeast *Hap5* gene. In Arabidopsis there are 11, 10 and nine genes encoding HAP2 (NF-YA; CBF-B), HAP3 (NF-YB; CBF-A) and HAP5 (NF-YC; CBF-C) subunits respectively (Edwards *et al.*, 1998; Gusmaroli *et al.*, 2001, 2002; Kwong *et al.*, 2003). Many redundant CBF variations are therefore possible. Only THAP5 was identified in the large two-hybrid screen, but whether other elements of the trimeric complex also interact with COL proteins remains an important question. Two members of the HAP3 family, *LEAFY COTYLEDON 1* (*LEC1*, Lotan *et al.*, 1998) and *LEC1-LIKE* (Kwong *et al.*, 2003), play a unique role in embryogenesis. In rice, a HAP3 isoform has been shown to play a role in chloroplast biogenesis (Miyoshi *et al.*, 2003). Although the *HAP2* and *HAP5* genes are

ubiquitously expressed (Edwards *et al.*, 1998; Gusmaroli *et al.*, 2001, 2002), the actual protein levels of the HAP2 component might be limited. Recently, eight of 10 genes encoding the HAP2 factors were found to contain a recognition site for the mir169 microRNA (Reinhart *et al.*, 2002) in their 3′ un-translated region, suggesting possible post-translational regulation of this component. Perhaps the ability of a certain CBF complex to bind a specific COL protein depends on the particular components of the CBF. Indeed, the two tomato HAP5 proteins seemed to differ in their interaction efficiencies.

Are COL *genes controlling flowering time in tomato?*

The *CO–FT* system in Arabidopsis and rice evolved to induce a single vegetative/reproductive transition in response to seasonal changes in day length. Tomato is one of many 'day-neutral' plants, in which photoperiod does not affect flowering time. Nevertheless, it contains *FT*-like genes that are involved in the transition to flowering (Carmel-Goren *et al.*, 2003; Pnueli *et al.*, 1998; Teper-Bamnolker and Samach, 2005; E. Lifschitz, unpublished data). Over-expression of *AtCO* or the *TCOL* genes did not delay flowering in tomato or tobacco, suggesting that the transition to flowering in these day-neutral plants does not involve Group IA COL proteins. Over-expression of *AtCO* in potato has no effect on flowering time but causes a reduction in the response of tuberization to photoperiod (Martinez-Garcia *et al.*, 2002). Since *TCOL1* and *TCOL* are under circadian regulation, they might mediate other photoperiodic or time-of-day-specific processes.

Expression of one *TCOL* gene delayed Arabidopsis flowering. High levels of TCOL3 might compete with endogenous AtCO and replace it in a transcriptional complex, thereby causing late or early flowering under long or short days respectively. Since Arabidopsis flowering is affected by high levels of AtCO and TCOL3, while tomato flowering is not, perhaps the tomato orthologue of *FT* has lost promoter motifs required for recognition by COL transcriptional complexes.

Conclusions

We provide substantial evidence, using a diverse array of approaches, that Group 1A COL proteins can use the CBF element to approach DNA and regulate transcription. Members of this group, isolated from different species, interact through the CCT domain with the HAP5 element of the CBF complex. We show that increasing levels of one of the CBF components modify flowering time in Arabidopsis. A yeast CBF complex, in which the tomato HAP5a replaces a dysfunctional endogenous HAP5, is biologically functional and can recruit a COL protein to two functional CCAAT motifs of yeast genes.

The interaction between COL proteins of Group 1A and the HAP factors requires a conserved amino acid which is essential for the biological function of the Arabidopsis CONSTANS protein. Members of the Arabidopsis pseudo-response regulators (APRR) protein family, many of which have been shown to be involved in circadian-clock function (Mizuno and Nakamichi, 2005; Strayer *et al.*, 2000), also contain a CCT domain. Another plant transcription factor, VRN2 which contains a remotely related CCT domain, regulates the vernalization response of wheat (Yan *et al.*, 2004). Replacement, in VRN2, of arginine 356, which is conserved in all CCT domains, with tryptophan (R356W), was sufficient to convert the growth habit of the DV92 accession of *Triticum monococcum* from a winter into a spring line. This mutation is analogous to the *co-7* mutation in *AtCO* (R356Q) which was shown here to disrupt specifically the interaction with THAP5.

Future studies are likely to reveal the details of the COL–CBF associations as well as the specific interactions between members of the COL and CBF families of transcription factors. More thorough understanding of the nature of the COL transcription complexes will require the analysis of other CCT-domain transcription factors and of the functional significance of the B-box domain and its interacting proteins.

Experimental procedures

Plant material and transgenic plants

Tomato line VF36 was used for cotyledon transformation (McCormick, 1991). Expression profiles of tomato genes were obtained from wild-type VFNT cherry plants.

Transformation and selection for transformants

Arabidopsis ecotype *Landsberg erecta* was transformed using the floral-dip method (Clough and Bent, 1998). Selection for kanamycin resistance was performed by spraying T1 seedlings with kanamycin 300 μg ml^{-1}, six times once every 3 days.

Growth conditions

Flowering time (rosette and cauline leaf number) was measured under several growth conditions. Short/long days: plants were exposed to 10/16 h of cool white fluorescent light. Long days + incandescent light: plants were exposed to 16 h of cool white fluorescent light, with the addition of incandescent light in the last 8 h of the light treatment.

Yeast strains

HF7c (Feilotter *et al.*, 1994) was the basic cell line used in the two-hybrid screen and tests (MATa ura3-52 his3-200 ade2-101 lys2-80 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3 URA::GAL4$_{17mers(\times3)}$-CyCl$_{TATA}$-lacZ). Y1064, a gift of Dr Y. Kassir, was used in the HAP5 complementation and ChIP experiments

(MATa, Ura3-52, leu2-3,112, trp1 (del), his3::hisG, ade 2-1, GAL+, CAN^S, met gal 80::his G, gal4::hisG).

Yeast two-hybrid procedures

HF7c was the basic host strain for the two-hybrid screen. The cDNA library representing 10^6 independent clones was prepared in the 'Hybrizap' AD vector (Stratagene, La Jolla, CA, USA) from shoot apices according to the procedures recommended by the manufacturer, and screening procedures were as in Pnueli *et al.* (2001). Initial selection was performed on -His medium with 2.5-mM 3-amino-1,2,4-triazole (3AT). Re-transformation of bait-expressing cells with DNA of positive clones was done on 5.0-mM 3AT. Positive and negative control plasmids were SV40 and p53 for the AD and BD respectively.

Cloning procedures

Full-length *TCOL1* cDNA clone in pAD was obtained as an *Eco*RI–*Xho*I fragment from the Y2H cDNA library. To prepare sense and antisense constructs for *TCOL1*, a 1409-bp cDNA clone was spliced in both orientations with the 35S promoter in the XbaI site of the pPZP111 vector. For sense expression of *TCOL3*, 1840-bp-long genomic clone was fused with the 35S promoter and cloned into the pCGN1458 vector. Sense and antisense fragments of 914 bp were also placed under the control of the 35S promoter and inserted into the XbaI site of pPZP111. To prepare the *AtCO* cDNA clone, a 2200-bp ClaI–BamHI genomic fragment containing the gene was cloned in pBS and the coding sequence was isolated using primers 13 and 14 (Figure S3) which flank the single *AtCO* intron.

Details of the constructions of the yeast plasmids for the Y2H binding experiments, and primers used for all cloning procedures, are available online in Table S3.

Construction of yeast lines for ChIP assays

For inactivation of HAP5 by homologous recombination, the *URA3* gene was inserted into XhoI–BglII sites of HAP5 to obtain a 2172-bp insert. Following transformation, positive clones were PCR-verified with primers 18 and 19 and tested for growth on lactate medium to give yeast line YH510. YH510 cells were transformed with the BD fusions of *HAP5, THAP5a* and *THAP5c* to give yeast line YH520/5, H520/5a and YH520/5c respectively, each tested for complementation on glucose and lactate media. Next, the AD fusion of a *TCOL1:13Xmyc* was introduced into each of the three YH520 strains to give YH530/5, 5a and 5c and shown to interact, in the two-hybrid test, with the corresponding HAP5 proteins but not to interfere with growth on a lactate medium and to support the regular level of TCOL1–HAP interaction.

ChIP assays

Cultures of each YH530 strain (50 ml) were grown overnight (SD, $\sim 1 \times 10^7$ cells ml^{-1}), then cells were washed and grown for an additional 2 h on lactate medium. Formaldehyde was added to a final concentration of 1% for 15 min at room temperature, and cross-linking was stopped by the addition of glycine to a final concentration of 140 mM. Cells were washed and lysed in the Lysis buffer (50-mM HEPES KOH pH 7.5, 140-mM NaCl, 1-mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate) using a bead-beater, dissolved in 400 μl Lysis buffer and sonicated in 15-sec rounds to obtain fragments of the average of 500 bp. Samples of the whole-cell extract [Input, whole cell extract (WCE)] were saved and the rest mixed with Dynabeads protein A (Dynal Biotech, Great Neck, NY, USA) beads for 2 h. The anti-myc antibody was anti-Human c-myc (clone 9E11; A3B2; Biosource, Camarillo, CA, USA). Beads were washed twice in Lysis buffer and once in Wash buffer (10-mM Tris-HCl pH 8.0, 250-mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1-mM EDTA) followed by overnight extraction at 65°C in TE (10-mM Tris-HCl pH 8.0, 1-mM EDTA), 1% SDS. Samples were treated with proteinase K in the presence of glycogen at 37°C. DNA was extracted, precipitated and dissolved in 30 μl of TE. Primers for the CCAAT-containing motifs were CYC1 primers 20 and 21, and primers for the HEM1 CCAAT motif were 22 and 23. As a negative control we used primers flanking upstream regulatory sequences of the HAP2 gene, 24 and 25 (Table S3).

Nucleic acids and protein procedures

RNA was extracted by the hot phenol/LiCl method (Verwoerd *et al.*, 1989). Poly(A)$^+$ RNA for semi-quantitative RT-PCR was isolated using Dynabeads (Dynal Biotech). RNA and DNA blots were performed according to established procedures.

Other procedures

Immunogold detection of THAP5a in fixed tissue sections was performed as in Parnis *et al.* (1997). Site-specific mutagenesis was according to Higuchi *et al.* (1988). ^{35}S-methionine-labelled TCOL1 was prepared using the TNT-coupled system (Promega, Southampton, UK).

Protein extracts of tobacco leaves

Fresh leaf tissue (0.2 g) was frozen in liquid nitrogen. The leaves were ground to a fine powder. After grinding, 1 ml of IP buffer was added (50-mM HEPES pH 8.0, 50-mM NaCl, 2-mM EDTA, 1-mM NaN_3, 5% glycerol). The mixture was vortexed and filtered through glass wool, its concentration was measured and proteins were stored at −20°C.

Preparation of anti-THAP5a antibody

Maltose binding protein (MAL) fusion of full-length *THAP5a* cDNA clone was expressed as in the pMAL-CRY plasmid. Protein extracts were fractionated on an amylose column (Kellermann and Ferenci, 1982), dialysed against 50-mM NaCl in phosphate buffer and loaded on to a monoQ column. MAL-THAP5a protein was eluted between 285- and 310-mM salt, brought to 1X PBS and used to immunize rabbits. Affinity-purified antiserum was tested against protein extracts of yeast cells expressing THAP5a or THAP5c and shown to be absolutely specific to THAP5a.

GeneBank accession numbers

AY490242: TCIP1 cDNA; AY49024: THAP5a cDNA; AY490244: THAP5c cDNA; AY490245: TCIP3 cDNA; AY490246: TCIP4 cDNA; AY490247: TCIP5 cDNA; AY490248: TCIP6 cDNA; AY490249: TCIP7 cDNA; AY490250: genomic TCOL1; AY490251: TCOL1 cDNA; AY490252: genomic TCOL2; AY490253: genomic TCOL3.

Acknowledgements

We thank Lilach Pnueli and Yona Kassir (Biology, Technion) for their indispensable advice on the ChIP experiments, the laboratory of

Dani Zamir (Hebrew University of Jerusalem, Rehovot, Israel) for the mapping experiments and Beni Horwitz (Biology, Technion) for his important comments on the manuscript. Thanks are due to Dana Hareven for the cytological work, and Ilana Nepomniashchi and Carmit Lifschitz for their excellent technical help. This work was supported by an Israel Science Foundation grant to AS and a joint German–Israeli Foundation (GIF) grant to AS and GC. The project on tomato flowering in the laboratory of EL was supported by grant No. QLK5-CT-2000-00357 from the European Commission, and by additional grants from the Human Frontier Science Program (HFSP) foundation and the Israel Science Foundation. O. Ben-Naim is a recipient of the Eshkol award from the Israeli Ministry of Science.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Alignment of putative amino acid sequences encoded by the tomato *TCOL* genes and the *AtCO* gene.

Figure S2. The *TCOL2* gene contains a frame-shift deletion and undergoes alternative splicing.

Figure S3. Estimates of phylogenetic distances between tomato and Arabidopsis COL and HAP5 proteins.

Figure S4. Diurnal expression profiles of *TCIP* genes.

Table S1 Flowering time of 35S:TCIP2a plants in long and short days at ambient temperatures of 20–22°C

Table S2 Sequence alignment of CCAAT motifs and flanking nucleotides in promoters of putative *CO* target genes

Table S3 List of primers used in the cloning procedures

This material is available as part of the online article from http://www.blackwell-synergy.com

References

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K. and Araki, T. (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science*, **309**, 1052–1056.

An, H., Roussot, C., Suarez-Lopez, P. *et al.* (2004) CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. *Development*, **131**, 3615–3626.

Borden, K.L.B. (2000) RING domains: master builders of molecular scaffolds. *J. Mol. Biol.* **295**, 1103–1112.

Calvert, A. (1964) Growth and flowering of the tomato in relation to natural light conditions. *J. Hort. Sci.* **39**, 182.

Carmel-Goren, L., Liu, Y.S., Lifschitz, E. and Zamir, D. (2003) The *SELF-PRUNING* gene family in tomato. *Plant Mol. Biol.* **52**, 1215–1222.

Caruso, M.L., Litzka, O., Martic, G., Lottspeich, F. and Brakhage, A.A. (2002) Novel basic-region helix-loop-helix transcription factor (AnBH1) of *Aspergillus nidulans* counteracts the CCAAT-binding complex AnCF in the promoter of a penicillin biosynthesis gene. *J. Mol. Biol.* **323**, 425–439.

Cheng, X.F. and Wang, Z.Y. (2005) Overexpression of *COL9*, a CONSTANS-LIKE gene, delays flowering by reducing expression of *CO* and *FT* in Arabidopsis thaliana. *Plant J.* **43**, 758–768.

Clough, S.J. and Bent, A.F. (1998) ''Floral dip'': a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* **16**, 735–743.

Dooley, K.A., Millinder, S. and Osborne, T.F. (1998) Sterol regulation of *3-hydroxy-3-methylglutaryl-coenzyme A synthase* gene through a direct interaction between sterol regulatory element binding protein and the trimeric CCAAT-binding factor/nuclear factor Y. *J. Biol. Chem.* **273**, 1349–1356.

Dowson-Day, M.J. and Millar, A.J. (1999) Circadian dysfunction causes aberrant hypocotyl elongation patterns in Arabidopsis. *Plant J.* **17**, 63–71.

Edwards, D., Murray, J.A. and Smith, A.G. (1998) Multiple genes encoding the conserved CCAAT-box transcription factor complex are expressed in Arabidopsis. *Plant Physiol.* **117**, 1015–1022.

el-Hodiri, H.M. and Perry, M. (1995) Interaction of the CCAAT displacement protein with shared regulatory elements required for transcription of paired histone genes. *Mol. Cell Biol.* **15**, 3587–3596.

Feilotter, H.E., Hannon, G.J., Ruddell, C.J.. and Beach, D. (1994) Construction of an improved host strain for two hybrid screening. *Nucleic Acids Res.* **22**, 1502–1503.

de Folter, S., Immink, R.G.H., Kieffer, M. *et al.* (2005) Comprehensive interaction map of the Arabidopsis MADS Box transcription factors. *Plant Cell*, **17**, 1424–1433.

Griffiths, S., Dunford, R.P., Coupland, G. and Laurie, D.A. (2003) The evolution of *CONSTANS-like* gene families in barley, rice, and Arabidopsis. *Plant Physiol.* **131**, 1855–1867.

Gusmaroli, G., Tonelli, C. and Mantovani, R. (2001) Regulation of the CCAAT-Binding NF-Y subunits in *Arabidopsis thaliana*. *Gene*, **264**, 173–185.

Gusmaroli, G., Tonelli, C. and Mantovani, R. (2002) Regulation of novel members of the Arabidopsis thaliana CCAAT-binding nuclear factor Y subunits. *Gene*, **283**, 41–48.

Hayama, R. and Coupland, G. (2004) The molecular basis of diversity in the photoperiodic flowering responses of Arabidopsis and rice. *Plant Physiol.* **135**, 677–684.

Hayama, R., Yokoi, S., Tamaki, S., Yano, M. and Shimamoto, K. (2003) Adaptation of photoperiodic control pathways produces short-day flowering in rice. *Nature*, **422**, 719–722.

van Heeswijck, R. and Hynes, M.J. (1991) The amdR product and a CCAAT-binding factor bind to adjacent, possibly overlapping DNA sequences in the promoter region of the *Aspergillus nidulans amdS* gene. *Nucleic Acids Res.* **19**, 2655–2660.

Hepworth, S.R., Valverde, F., Ravenscroft, D., Mouradov, A. and Coupland, G. (2002) Antagonistic regulation of flowering-time gene *SOC1* by CONSTANS and FLC via separate promoter motifs. *EMBO J.* **21**, 4327–4337.

Higuchi, R., Krummel, B. and Saiki, R.K. (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16**, 7351–7367.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J. and Weigel, D. (1999) Activation tagging of the floral inducer FT. *Science*, **286**, 1962–1965.

Kellermann, O.K. and Ferenci, T. (1982) Maltose-binding protein from *Escherichia coli*. *Methods Enzymol.* **90**, 459–463.

Keng, T. and Guarente, L. (1987) Constitutive expression of the yeast *HEM1* gene is actually a composite of activation and repression. *Proc. Natl Acad. Sci. USA*, **84**, 9113–9117.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. (1999) A pair of related genes with antagonistic roles in mediating flowering signals. *Science*, **286**, 1960–1962.

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T. and Yano, M. (2002) *Hd3a*, a rice ortholog of the Arabidopsis *FT* gene, promotes transition to flowering downstream of *Hd1* under short-day conditions. *Plant Cell Physiol.* **43**, 1096–1105.

Kusnetsov, V., Landsberger, M., Meurer, J. and Oelmuller, R. (1999) The assembly of the CAAT-box binding complex at a

photosynthesis gene promoter is regulated by light, cytokinin, and the stage of the plastids. *J. Biol. Chem.* **274**, 36009–36014.

Kwong, R.W., Bui, A.Q., Lee, H., Kwong, L.W., Fischer, R.L., Goldberg, R.B. and Harada, J.J. (2003) LEAFY COTYLEDON1-LIKE defines a class of regulators essential for embryo development. *Plant Cell*, **15**, 5–18.

Ledger, S., Strayer, C., Ashton, F., Kay, S.A. and Putterill, J. (2001) Analysis of the function of two circadian-regulated *CONSTANS-LIKE* genes. *Plant J.* **26**, 15–22.

Lippuner, V., Cyert, M.S. and Gasser, C.S. (1996) Two classes of plant cDNA clones differentially complement yeast calcineurin mutants and increase salt tolerance of wild-type yeast. *J. Biol. Chem.* **271**, 12859–12866.

Liu, J., Yu, J., McIntosh, L., Kende, H. and Zeevaart, J.A. (2001) Isolation of a *CONSTANS* ortholog from *Pharbitis nil* and its role in flowering. *Plant Physiol.* **125**, 1821–1830.

Lotan, T., Ohto, M., Yee, K.M., West, M.A., Lo, R., Kwong, R.W., Yamagishi, K., Fischer, R.L., Goldberg, R.B. and Harada, J.J. (1998) Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell*, **93**, 1195–1205.

Maity, S.N. and de Crombrugghe, B. (1998) Role of the CCAAT-binding protein CBF/NF-Y in transcription. *Trends Biochem. Sci.* **23**, 174–178.

Martinez-Garcia, J.F., Virgos-Soler, A. and Prat, S. (2002) Control of photoperiod-regulated tuberization in potato by the Arabidopsis flowering-time gene *CONSTANS*. *Proc. Natl Acad. Sci. USA*, **99**, 15211–15216.

Masiero, S., Imbriano, C., Ravasio, F., Favaro, R., Pelucchi, N., Gorla, M.S., Mantovani, R., Colombo, L. and Kater, M.M. (2002) Ternary complex formation between MADS-box transcription factors and the histone fold protein NF-YB. *J. Biol. Chem.* **277**, 26429–26435.

McCormick, S. (1991) Transformation of tomato with *Agrobacterium tumefacien*. *Plant Tissue Culture Manual*, **B6**, 1–9.

McNabb, D.S., Xing, Y. and Guarente, L. (1995) Cloning of yeast *HAP5*: a novel subunit of a heterotrimeric complex required for CCAAT binding. *Genes Dev.* **9**, 47–58.

Millar, A.J. and Kay, S.A. (1996) Integration of circadian and phototransduction pathways in the network controlling CAB gene transcription in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **93**, 15491–15496.

Miyoshi, K., Ito, Y., Serizawa, A. and Kurata, N. (2003) *OsHAP3* genes regulate chloroplast biogenesis in rice. *Plant J.* **36**, 532–540.

Mizuno, T. and Nakamichi, N. (2005) Pseudo-response regulators (PRRs) or true oscillator components (TOCs). *Plant Cell Physiol.* **46**, 677–685.

Olesen, J.T. and Guarente, L. (1990) The HAP2 subunit of yeast CCAAT transcriptional activator contains adjacent domains for subunit association and DNA recognition: model for the HAP2/3/4 complex. *Genes Dev.* **4**, 1714–1729.

Parnis, A., Cohen, O., Gutfinger, T., Hareven, D., Zamir, D. and Lifschitz, E. (1997) The dominant developmental mutants of tomato, Mouse-ear and Curl, are associated with distinct modes of abnormal transcriptional regulation of a *Knotted* gene. *Plant Cell*, **9**, 2143–2158.

Pnueli, L., Carmel-Goren, L., Hareven, D., Gutfinger, T., Alvarez, J., Ganal, M., Zamir, D. and Lifschitz, E. (1998) The *SELF-PRUNING* gene of tomato regulates vegetative to reproductive switching of sympodial meristems and is the ortholog of *CEN* and *TFL1*. *Development*, **125**, 1979–1989.

Pnueli, L., Gutfinger, T., Hareven, D., Ben-Naim, O., Ron, N., Adir, N. and Lifschitz, E. (2001) Tomato SP-interacting proteins define a conserved signaling system that regulates shoot architecture and flowering. *Plant Cell*, **13**, 2687–2702.

Putterill, J., Robson, F., Lee, K., Simon, R. and Coupland, G. (1995) The *CONSTANS* gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell*, **80**, 847–857.

Reinhart, B., Weinstein, E., Rhoades,, M., Bartel, B. and Bartel, D. (2002) MicroRNAs in plants. *Genes Dev.* **16**, 1616–1626.

Robson, F., Costa, M.M., Hepworth, S.R., Vizir, I., Pineiro, M., Reeves, P.H., Putterill, J. and Coupland, G. (2001) Functional importance of conserved domains in the flowering-time gene *CONSTANS* demonstrated by analysis of mutant alleles and transgenic plants. *Plant J.*, **28**, 619–631.

Samach, A. and Coupland, G. (2000) Time measurement and the control of flowering in plants. *Bioessays*, **22**, 38–47.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G. (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. *Science*, **288**, 1613–1616.

Shin, B.S., Lee, J.H., Jeong, H.J., Yun, C.H. and Kim, J.K. (2004) Circadian regulation of rice (*Oryza sativa* L.) *CONSTANS-like* gene transcripts. *Mol. Cells*, **17**, 10–16.

Siekierka, J.J., Staruch, M.J., Hung, S.H. and Sigal, N.H. (1989) FK-506, a potent novel immunosuppressive agent, binds to a cytosolic protein which is distinct from the cyclosporin A-binding protein, cyclophilin. *J. Immunol.* **143**, 1580–1583.

Sinha, S., Maity, S.N., Lu, J. and de Crombrugghe, B. (1995) Recombinant rat CBF-C, the third subunit of CBF/NFY, allows formation of a protein-DNA complex with CBF-A and CBF-B and with yeast HAP2 and HAP3. *Proc. Natl Acad. Sci. USA*, **92**, 1624–1628.

Smith, S.M., Fulton, D.C., Chia, T., Thorneycroft, D., Chapple, A., Dunstan, H., Hylton, C., Zeeman, S.C. and Smith, A.M. (2004) Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and post-transcriptional regulation of starch metabolism in Arabidopsis leaves. *Plant Physiol.* **136**, 2687–2699.

Strayer, C., Oyama, T., Schultz, T.F., Raman, R., Somers, D.E., Mas, P., Panda, S., Kreps, J.A. and Kay, S.A. (2000) Cloning of the Arabidopsis clock gene *TOC1*, an autoregulatory response regulator homolog. *Science*, **289**, 768–771.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F. and Coupland, G. (2001) CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. *Nature*, **410**, 1116–1120.

Taira, T., Sawai, M., Ikeda, M., Tamai, K., Iguchi-Ariga, S.M. and Ariga, H. (1999) Cell cycle-dependent switch of up-and down-regulation of human *hsp70* gene expression by interaction between c-Myc and CBF/NF-Y. *J. Biol. Chem.* **274**, 24270–24279.

Takada, S. and Goto, K. (2003) TERMINAL FLOWER2, an Arabidopsis homolog of HETEROCHROMATIN PROTEIN1, counteracts the activation of FLOWERING LOCUS T by CONSTANS in the vascular tissues of leaves to regulate flowering time. *Plant Cell*, **15**, 2856–2865.

Teper-Bamnolker, P. and Samach, A. (2005) The flowering integrator FT regulates *SEPALLATA3* and *FRUITFULL* accumulation in Arabidopsis leaves. *Plant Cell*, **17**, 2661–2675.

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A. and Coupland, G. (2004) Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science*, **303**, 1003–1006.

Verwoerd, T.C., Dekker, B.M. and Hoekema, A. (1989) A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.* **17**, 2362.

Villard, J., Peretti, M., Masternak, K., Barras, E., Caretti, G., Mantovani, R. and Reith, W. (2000) A functionally essential

domain of RFX5 mediates activation of major histocompatibility complex class II promoters by promoting cooperative binding between RFX and NF-Y. *Mol. Cell Biol.* **20**, 3364–3376.

Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U. and Weigel, D. (2005) Integration of spatial and temporal information during floral induction in Arabidopsis. *Science*, **309**, 1056–1059.

Yan, L., Loukoianov, A., Blechl, A., Tranquilli, G., Ramakrishna, W., SanMiguel, P., Bennetzen, J.L., Echenique, V. and Dubcovsky, J. (2004) The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. *Science*, **303**, 1640–1644.

Yano, M., Katayose, Y., Ashikari, M. *et al.* (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene *CONSTANS*. *Plant Cell*, **12**, 2473–2484.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L. and Gruissem, W. (2004) Genevestigator. Arabidopsis microarray database and analysis toolbox. *Plant Physiol.* **136**, 2621–2632.

