CONSTANS and the Evolutionary Origin of Photoperiodic Timing of Flowering

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Abstract

A network of promoting and inhibiting pathways that respond to environmental and internal signals controls the flowering transition. The outcome of this regulatory network establishes, for any particular plant, the correct time of the year to flower. The photoperiod pathway channels inputs from light, day length and circadian clock to promote the floral transition. *CONSTANS* (*CO*) is a central regulator of this pathway, triggering the production of the mobile florigen hormone FT that induces flower differentiation. Because plant reproductive fitness is directly related to its capacity to flower at a precise time, the photoperiod pathway is present in all known plant species. Recent findings have stretched the evolutionary span of this photophase signal to unicellular algae, which show unexpected conserved characteristics with modern plant photoperiodic responses. In this review, a comparative description of the photoperiodic systems in algae and plants will be presented and a general role for the CO family of transcriptional activators proposed.

Key words: Photoperiod, floral transition, CONSTANS, florigen, evolution, Arabidopsis, Chlamydomonas, CrCO.

Introduction

Plants possess an extraordinarily well-adapted system to respond to external cues, mainly to temperature and light. Light is particularly important for a photosynthetic organism as it is the main source of energy to keep the rest of the physiological functions working and consequently has an enormous influence in plant development (Thomas, 2006). As a result, higher plants and algae have adopted several sophisticated methods to respond to light in a concerted way to gain an evolutionary advantage over other organisms that have not developed these traits. Light regulation is driven by many different mechanisms in plants but some are particularly important such as the redox (Buchanan and Balmer, 2005), photoreceptor-dependent (Quail, 2006), circadian clock (Dodd et al., 2005) and photoperiodic (Thomas and Vince-Pruce, 1997) regulatory systems. These mechanisms are not necessarily independent and often show a grade of interconnection between them that, arising from the conservation of the different components across phylogenetically diverse plants, is likely to have more importance than previously thought.

Light driven redox signalling is extremely important for plants as it coordinates, among other functions, whole metabolic rearrangements from starch-consuming catabolic reactions of the night phase to the light-driven anabolic synthesis of the day (Dietz, 2003). This regulatory level seems to have emerged very early in the evolution of photosynthetic organisms because a complex redox control system is already present in cyanobacteria (Li and Sherman, 2000). In plants, a role in flowering time for molecules involved in redox control such as glutathione, salicylic acid and ascorbic acid has been proposed before (Ogawa et al., 2001; Martínez et al., 2004; Barth et al., 2006). The role,

extent and association between ancient redox and photoperiod control of gene and protein expression is extremely interesting but beyond the scope of this review.

Another layer of control assures that transcription factors that activate photosynthetic genes are degraded during the night. This signal involves active proteasome-dependent protein degradation through a direct photoreceptor control and has been extensively reviewed elsewhere (Boccalandro et al., 2006, Strickland et al., 2006). The direct role of *CONSTITUTIVE PHOTOMORPHOGENIC 1* gene product COP1, an E3 ring-finger type ubiquitin ligase, in the control of flowering through the direct regulation of CO stability has been recently described (Jang et al., 2008). In this signalling, CRYPTOCHROME 2 through COP1 (Liu et al., 2008) and PHYTOCHROME B through another unknown ubiquitin ligase (Valverde et al., 2004) are involved in this process. A more detailed description of the control of CO protein stability by the proteasome will be provided below. Interestingly, the genomes of green eukaryotic algae possess homologues of cryptochromes, phytochromes and ring finger ubiquitin ligases similar to COP1 (Mittag et al., 2005; Riaño-Pachón et al., 2008) whose role in ancient control of light signalling is certainly worth investigating. Similarly, it has been recently reported that cell elongation occurs at a particular time of the night due to the gibberellin (GA)-dependent effect of DELLA proteins on bHLH transcription factors of the PHYTOCHROME INTERACTION (PIF) protein family (de Lucas et al., 2008). An interesting link between flowering and DELLA proteins, connected to both ethylene and gibberellin (GA) signalling, has been recently proposed (Achard et al., 2007) but these proteins appear in vascular plants and are absent in algae, so this mechanism is not as evolutionarily conserved as the photoperiodic signalling.

The circadian clock timekeeper is a major regulator of plant gene expression. The rotational movement of the earth around its axis determines a 24 h repetitive signal that is exploited by all photosynthetic organisms, as well as some fungi and animals, to precede external signals and provide a physiological advance (Dodd et al., 2005). The system is so critical and robust that in cyanobacteria three proteins, two modulators (KaiA, B) and the kinase/phosphatase KaiC, in the presence of ATP, can maintain a self-perpetuating clock with circa 24 h of autophosphorylation /dephosphoryation cycles when isolated *in vitro*, thus, in organisms that evolved very early, as some blue-green algae, the capacity was present to set time independently of transcriptional inputs (Ishiura et al., 1998; Nakajima et al., 2005). The influence of posttranslational modifications in clock proteins is a characteristic that is gaining more and more importance in the concept of circadian clocks (Mizoguchi et al., 2006; Mehra et al., 2009). The influence of the clock in the photoperiod response and other crucial developmental processes of plants (Mas and Yanovsky, 2009; Imaizumi, 2010) and algae (Schulze et al., 2010) has been recently reviewed. In this review some of the aspects that connect photoperiod and circadian regulation, common features that seem to have arisen very early in the lineage of the photosynthetic eukaryotes (Matsuo et al., 2008), will be briefly discussed.

The photoperiod pathway in Arabidopsis involves a number of genes that form its core, as well as several input and output genes (Reeves and Coupland, 2000). In this pathway *CONSTANS* (*CO*) is central in all plants analysed because it coordinates light and clock inputs in leaves to trigger the expression of *FLOWERING LOCUS T* (*FT*) whose protein, and possibly also its mRNA, can move from the phloem to the meristem (Corbesier et al., 2007; Tamaki et al., 2007). The CO-FT module is conserved in all known

120 plants but the final outputs of the signal diverge: whereas in *Arabidopsis thaliana*, a
121 facultative long-day (LD) plant, CO promotes the expression of *FT* under inducing long
122 days (Suárez-López et al., 2001), in rice, a short-day (SD) plant, the signals are different
123 and CO is a repressor in non-inductive long days (Hayama et al., 2003). These aspects
124 have been reviewed very recently (Hayama and Coupland, 2004; Song et al., 2010).

125 Another important aspect of CO regulation involves the spatial coordination of
126 the photoperiodic flowering signals due to the fact that light and photoperiod sensing
127 occurs in leaves and probably in other actively photosynthetic tissues, whereas the
128 developmental switch takes place in the non-photosynthetic meristem (Knott 1934;
129 Zeevart 2008). The movement of a developmental signal from the leaves to the
130 meristem was proposed early last century (Chailakhyan, 1936), but was only recently
131 attributed to the movement of FT from the companion cells of the phloem to the apical
132 meristem; this is probably one of the most important discoveries in recent plant biology
133 (Türck et al., 2008; Zeevart, 2008). Green microalgae such as *Chlamydomonas reinhardtii*
134 exhibit a strong photoperiod response that controls several important physiological
135 functions (Suzuki and Johnson, 2002). The presence of a gene in the Chlamydomonas
136 genome encoding a CO homologue and its connection with photoperiodic control of
137 growth and metabolism has been recently described (Serrano et al., 2009; Romero and
138 Valverde, 2009). The importance of this discovery and its confluences and divergences
139 with higher plant photoperiodism will be described herein.

140

141 **CONSTANS and the family of CO-like proteins**

142 Several mutagenesis experiments in Arabidopsis established a number of genes that
143 were affected in their capacity to flower in response to photoperiod (Rédei, 1962).

144 Among these the mutation called *constans* was particularly interesting because the
145 mutant was late flowering in long days but was not affected in short days, so it seemed
146 to have lost the capacity to discern the photophase (thus the name "*constans*" for
147 flowering in a "constant" manner regardless of photoperiod). *CONSTANS* encodes an
148 atypical transcription factor with three characteristic domains (Figure 1) which makes it
149 a unique kind of transcriptional regulator present only in the plant kingdom (Putterill et
150 al., 1995). It was soon found that a family of proteins closely similar to CO was present
151 in the Arabidopsis (Robson et al., 2001) and rice genomes (Sin et al. 2004) and that
152 representatives of this family could be identified in several EST databases from many
153 phylogenetically diverse plants (Griffiths et al., 2003). These CO-like or COL proteins
154 include homologues closely related to CO such as COL1, which is encoded in a gene next
155 to *CO* in the genome and seems to be the result of recent tandem duplication (Putterill et
156 al., 1995) and with which it shares an amino acid identity higher than 80%.
157 Nevertheless, overexpression of *COL1* under the 35S promoter in Arabidopsis does not
158 affect flowering so its function is not redundant with that of CO (Ledger et al., 2001).
159 Other COLs show a range of sequence identity with CO as illustrated in the tree in Figure
160 2, which includes proteins that lack complete protein regions, but keep a high grade of
161 identity in these domains, reflecting their importance for CO function. These domains
162 will be briefly described.

163 The amino terminal part of CO consists of two consecutive zinc finger domains
164 which are called b-boxes. These b-boxes are related to domains present in transcription
165 factors from animals and other organisms and are proposed to be involved in protein-
166 protein interaction rather than in DNA-binding functions (Khanna et al., 2009). In the Zn
167 finger domain, the cysteine and histidine residues that coordinate the binding of the Zn

168 atoms are strictly conserved. Mutants with amino acid alterations in conserved residues
169 of the b-boxes were late flowering (Robson et al., 2001). Employing tomato TCOL1 b-
170 boxes as baits in yeast two-hybrid assays, immunophilins and other b-box containing
171 proteins were identified (Ben-Naim et al., 2006). This strongly supports the idea that b-
172 boxes are involved in protein-protein interactions. Nevertheless, an interesting
173 suggestion involving a direct interaction of b-box proteins (BBXs) in a regulatory
174 complex with COP1 or other RING finger and coil-coil domain-containing proteins such
175 as in animal Tripartite Motif Proteins (TRIMs) has been proposed, widening the possible
176 functions of COL proteins (Datta et al., 2008).

177 The carboxy terminal part of CO consists of a span of 70-80 amino acids in which
178 a core of 40 amino acids is strictly conserved in a family of very distinct proteins
179 (Robson et al., 2001; Griffith et al., 2003). It was first described in CO, but has been found
180 since then in some other proteins which are central to the circadian clock such as
181 TIMING OF CAB EXPRESSION 1 (TOC1) and pseudoresponse regulators (PRRs). This
182 CCT domain of CO includes a nuclear import signal (Robson et al., 2001) and is the
183 domain of interaction with the ubiquitin ligase COP1 (Jang et al., 2008). Because it was
184 extremely difficult to demonstrate the DNA-binding function of CO, it was proposed that
185 CO was driven to the DNA by forming complexes through the CCT domain. Yeast two
186 hybrid analyses employing different CCT domains recovered a strong interaction with
187 several members of the family of HEME ACTIVATOR PROTEIN (HAP) of transcriptional
188 activators, specifically with HAP3 and HAP5 isoforms, but not with HAP2, both in tomato
189 and Arabidopsis (Ben-Naim et al., 2006, Wenkel et al., 2006). Overexpression of some
190 HAP2 or HAP3 isoforms from Arabidopsis strongly delayed flowering (Wenkel et al.,
191 2006) while in yeast TCOL1 was recruited to CCAAT motifs together with a

192 HAP2/HAP3/HAP5 recombinantly expressed complex (Ben-Naim et al., 2006). These
193 data strongly suggested that CO substituted the HAP2 isoform in a complex with HAP3
194 and HAP5 subunits and was thus recruited to the already described motif for HAP
195 complex, the CCAAT box, in Arabidopsis promoters. Very recently, CO was reported to
196 transiently access DNA directly through this CCT domain in DNA sequences different
197 from those reported for the HAP complex (Tiwari et al., 2010). This interaction was
198 reported to be exceptionally transient so it still remains a question whether it is
199 significant *in vivo* or depends on other protein factors.

200 The domain that shows a lower degree of conservation in amino acid sequence of
201 the COLs is the middle domain (Figure 1). This domain is enriched in acidic amino acids
202 and is reported to activate transcription in yeast-two hybrid assays (Ben-naim, 2006).
203 There has been no report in the literature of any amino acid change in this part that
204 affects flowering time, but there are fixed residues that show significant conservation
205 (Griffiths et al., 2003). The sizes of the closest homologues and orthologues of CO protein
206 are similar (around 350-400 amino acids), and these proteins always include a middle
207 domain with similar characteristics, further supporting the idea of the importance of the
208 middle domain in CO function. The real role of this domain in CO and COL activity
209 remains to be discovered.

210 COL proteins or, in a wider sense, b-box containing proteins (BBXs) constitute a
211 family of proteins in Arabidopsis with 32 members (Khanna et al., 2009) which, with a
212 varied number of components, is present in all higher plants sequenced to date. Many of
213 them have been reported to follow a circadian rhythm of expression (Ledger et al., 2001;
214 Shin et al., 2004; Kumagai et al., 2008) and they have been implicated in several different
215 regulatory pathways other than flowering time, such as tuberization in potato

(González-Schain and Suárez-López, 2008), red light signalling and growth in Arabidopsis (Datta et al., 2006; Datta et al., 2007) or dormancy in trees (Böhlenius et al., 2006). It was suggested that COL proteins could be evolving in the evolutionary direction of losing one of the Zn fingers in the b-box domain and thereby acquiring new functions (Griffiths et al., 2003). The fact that some microalgae already possess proteins with just one b-box (Matsuo et al., 2008; Serrano et al., 2009) and that they cluster in the same branch (group II) of the phylogenetic tree presented in Figure 2, with other modern COLs from rice and Arabidopsis, argue against this. It does, however, support the idea that COLs with just one Zn finger (such as AtCOL6) preceded those with two (such as CO or HD1), and that they have been retained during the evolution of photosynthetic organisms with distinct functions not yet deciphered, with some perhaps related to circadian control (Matsuo et al., 2008). A protein with a divergent carboxyl CCT domain and a putative single amino Zn finger, highly divergent from b-boxes, (VRN2) has a strong flowering repressing function in wheat (Yan et al., 2004). The relationship between protein function, circadian control and protein domain structure suggests that CCT and Zn finger domains may have an interesting association at the structure-function level worth investigating.

Control of CO function. The double external coincidence model.

CO regulation is complex and occurs at different levels, reflecting the importance for the plant to choose the exact time of the year the florigen signal should be released for a correct reproductive outcome. Several results suggest that plants detect the light input in photosynthetic tissues, mainly the leaves, and this signal has to be transmitted to the apex. Different experiments had elucidated this behaviour early last century (Garner and

240 Allar, 1925), but only very recently was the identity of the florigen signal revealed
241 (Turck et al., 2008). In fact, it is the exact regulation of CO protein expression, stability
242 and activity that triggers the expression of *FT* at the correct time of the year and it is the
243 transport of FT through the phloem to the meristem that triggers the differentiation of
244 the flower. Thus, not only is it the way *CO* expression is regulated that triggers flowering,
245 but also how it is controlled in localized cells at an exact season, growth stage and at
246 exactly the correct time of the day for its function to be correctly activated.

247 In fact, plants have traditionally been classified according to their photoperiodic
248 flowering behaviour. Briefly, plants are considered long day plants if they flower
249 preferentially during LD (for example 16 h light, 8 h dark); they are called short day
250 plants if they flower preferentially under SD (for example 8 h light, 16 h dark); whereas
251 they are considered day neutral if they flower independently of any photoperiod.
252 Observing the reproductive behaviour of several plant species, different models to
253 explain the photoperiodic induction of flowering were devised. These models contrasted
254 in the relative importance that external and internal signals were given in determining
255 the floral transition. By the beginning of this century, several laboratories working on
256 Arabidopsis came to the conclusion that the external coincidence model was the one
257 that could best explain photoperiodic flowering and in all these models CO occupied a
258 central position (Suárez-López et al., 2001; Yanovsky and Kay, 2002; Kobayashi and
259 Weigel, 2007). Because the regulation is complex it will be described in some detail.

260

261 . Control of *CO* mRNA levels.

262 The expression of *CO* mRNA is regulated by the circadian clock and the input of light
263 (Suárez-López et al., 2001). In this regulatory aspect the nuclear protein GIGANTEA (GI),

264 that is also regulated by the clock and light-induced degradation, plays a central role (Yu
265 et al., 2008). GI can interact with FLAVIN BINDING, KELCH REPEAT, F-BOX PROTEIN 1
266 (FKF1), an F-box protein with a LOV domain that upon perceiving blue light changes its
267 configuration, binds to GI and induces the ubiquitination and later degradation by the
268 proteasome of a subset of DOF transcription factors called CYCLING DOF FACTORS or
269 CDFs (Imaizumi et al., 2005; Sawa et al., 2007). The CDFs bind to the *CO* promoter
270 inhibiting its expression during the morning, so that their proteasome degradation due
271 to light-dependent GI-FKF interaction in the evening of a LD causes a strong induction of
272 *CO* expression during that time window and flowering. In fact, quadruple mutants of a
273 set of *CDFs* and *GI* are extremely early flowering (Fornara et al., 2009). As *GI* expression
274 is regulated by the clock and peaks at midday in LD (Fowler et al., 1999), this first
275 regulatory module explains why *CO* is expressed during the daytime but not why the
276 protein is absent during the night time, when the expression of *CO* reaches the highest
277 values. Complex as it is, this description does not fulfil the requirements of the external
278 coincidence model that explains flowering transition.
279
280 . Posttranslational modifications of CO
281 Although, as described above, the regulation of *CO* mRNA constitutes a first module of an
282 external coincidence model, for this model to function, not only an internal, clock-
283 mediated rhythm should exist, but also an external independent input should be
284 activated in order to trigger the flowering signal at exactly the correct time. The
285 production of the florigen at the correct season and in a precise daytime window is what
286 assures that flowering happens in a timely way for every particular plant adapted to a
287 particular environment. The complex regulation of *CO* expression in Arabidopsis

explains the presence of *CO* mRNA during the evening of a LD, but does not explain why CO protein is active only during a particular photophase. The answer has to come from modifications at the protein level. Posttranslational modifications of CO protein activity and stability are as important as its transcriptional regulation because the exact moment when CO is active does not necessarily coincide with the maximum peak of expression of its mRNA or even reflect the mRNA wave of expression (Suárez-López et al., 2001). Two clues from experiments in Arabidopsis had to be considered in order to solve the problem. Firstly, it was known that mutations in different photoreceptors affected flowering time, particularly *PHYTOCHROME B* (*phyB*) mutation that enhanced flowering and *CRYPTOCHROME 2* (*CRY2*) mutation that strongly delayed flowering (Mockler et al., 1999) and this effect was partially due to CO (Yanovsky and Kay, 2002). On the other hand, it was observed that overexpression of *CO* under the constitutive 35S promoter induced much higher levels of *FT* mRNA in LD than in SD, even though *CO* expression was equally high in both photoperiods and plants were equally early flowering in both conditions (Onouchi et al., 2000; Valverde et al., 2004). CO was demonstrated to be unstable in the dark and stable during the daytime by monitoring the presence of a GFP:CO fusion protein under the confocal microscope and by immunoblots employing specific antibodies (Valverde et al., 2004). This explained the differences in *FT* expression in 35S::*CO* plants under LD and SD photophases. The same work demonstrated that the stability of the protein was compromised by incubating plants with different monochromatic lights, so that CO stability was high in blue light (a light that induces flowering time by affecting both CRY2 and FKF) and low in red light or in *phyB* mutants (which activates PHYB activity and represses flowering). For similar reasons, 35S::*CO* plants showed modifications in flowering time when crossed to

312 photoreceptor mutant backgrounds: they showed a strong delay when crossed into a
313 *cry1cry2* double mutant background but flowered only slightly earlier in a *phyB*
314 background (Valverde et al., 2004).

315 It was further demonstrated that CO stability was controlled by the proteasome
316 because GFP:CO fusions were detected in nuclear speckles and because ubiquitination
317 assays in nuclear extracts employing recombinant protein demonstrated that CO was
318 ubiquitinated. In fact, there are two moments in the day when CO is degraded by the
319 proteasome, during the dark and during the morning and different photoreceptors are
320 implicated in this process (Valverde et al., 2004). In a similar way to other light-induced
321 processes the complex between the ubiquitin ligas SPA1 and COP1 was demonstrated to
322 be involved in CO dark stability (Laubinger et al., 2006; Jang et al., 2008; Liu et al., 2008).
323 Thus, there is a window of coincidence in which circadian and light control of *CO*
324 expression in the evening of a LD allowed by the GI-FKF-CDFs complex coincides with
325 the COP1-SPA1-CRY2 induced stability of the protein. Stable CO is then probably
326 activated by light-induced modifications and CO can then be recruited to the promoter of
327 *FT* to induce its expression (Turck et al., 2008).

328 It is peculiar that *FT* induction takes place in the phloem companion cells but *CO*
329 is expressed in diverse tissues (Simon et al., 1996; An et al., 2004). This observation
330 hints at a mechanism that restricts activation of *FT* to particular cell types. Several
331 experiments may give clues to explain these results. First, the expression of *PHYB* that
332 affects flowering occurs in mesophyll cells (Endo et al., 2005), so its effect must be
333 through a mobile signal (which could be PHYB itself moving actively cell to cell). Second,
334 expression of *CO* was very effective in promoting flowering time when confined to the
335 companion cells of the vascular tissue under the *SUC2* promoter, but absolutely

ineffective when expressed under the meristem specific promoter *KNAT1* (An et al. 2004). Furthermore, CRY2 expression, which was demonstrated to affect flowering through COP1 mediated degradation of CO (Liu et al., 2008) only affected flowering when expressed in the vascular tissues, but not when it was expressed in the mesophyll (Endo et al., 2007). So the question remains as to the mechanism that restricts the spatial expression of FT; this may well be a complex combination of signals that restrict its expression to the tissue where this photoperiod mechanisms exist (leaf phloem) and not in those not able to give a photoperiod dependant signal (meristem) (Corbesier et al., 2007). In this spatio-temporal control of flowering, the postranscriptional control of florigen production by circadian epigenetic mechanisms or protein modifications, or a combination of both, seems the most plausible explanation.

The photoperiod response in algae

Green algae possess metabolic, physiological and developmental characteristics that are conserved in the rest of the plant kingdom. Some of its most studied members, such as the Chlamydomonas and Volvox genera, have been used as models for photosynthetic physiology for many years (Gutman and Niyogi, 2004). Like Arabidopsis for higher plants, Chlamydomonas is a model for green algae because its genome has been sequenced and correctly annotated (Merchant et al., 2007), genetics are feasible (Rochaix, 1995) and sexual and vegetative growing conditions are versatile and optimal for scientific research (Harris, 1989). Some of the most important physiological processes of the green microalga *C. reinhardtii* are under circadian and/or photoperiod control, including phototaxia (Johnson et al., 1991), starch accumulation (Ral et al., 2006) and the synchronicity of cell cycle and growth that happens under specific

conditions (Lien and Knutsen, 1976). Nevertheless, there is no model described how the photoperiod response may work in algae.

. *COL* genes in algae.

Searching for mutants affected in the circadian control of a gene coding for a chloroplast protein, Matsuo et al. 2008, found a mutant called *ROC66* that had an altered clock rhythm and, as a result, a defect in growth. The gene mutated encoded a protein with similarities to COLs, including an uncommon internal CTT and two amino terminal b-boxes, only the first one showing the conserved features of a COL Zn-finger domain. The gene showed a distinct circadian expression pattern. The genome of *C. reinhardtii* contains genes coding for some other proteins with CCT domains, including a recently identified member of the *PRRs* family (Holm et al., 2010) and other b-boxes domains in uncharacterised proteins (Merchant et al., 2007). Strikingly, an annotated sequence (JGI protein ID: 159133) showed several characteristics of a typical *COL* gene, including size (around 1.2 kb) and a conserved domain structure (Figure 1). The coded protein presented two typical amino terminal b-boxes and a conserved CCT domain at the carboxy terminal part and even some conserved amino acid patches in the middle acidic domain (Serrano et al., 2009).

In a phylogenetic analysis similar to the one in Figure 2, constructed with all proximal Arabidopsis and rice COL proteins (Griffiths et al., 2003), and representatives from other algae and lower plants, *C. reinhardtii* CO (CrCO) appeared at the base of the tree indicating that it is in the origin of the separation of both main groups of sequences (group I and group II) (Serrano et al., 2009). Surprisingly CrCO and Volvox homologues, but not homologues from other green algae (Ostreococcus, Chorella) and red algae

384 (Galdieria) occurred in the tree close to CO and HD1 (Figure 2). Thus, it seems that the
385 algal lineage that gave rise to CO proteins is in the Volvocales order, curiously a group
386 including one of the first genera (Volvox) to show cellular differentiation in sexual
387 reproduction (Michod et al., 2007). Other distantly related algae like diatoms,
388 euglenoids, haptophytes or dinophytes do not show sequences similar to *COL* genes in
389 their genome drafts or extensive collection of ESTs. The fact that green microalgae, but
390 not earlier photosynthetic microorganisms, include *COL* genes in their genomes is
391 consistent with the idea that these genes appeared during, or just after, the
392 endosymbiotic event in the photosynthetic lineage. *COLs* have not been found outside
393 the plant evolutionary lineage.

394 In Chlamydomonas, the peak of *CrCO* mRNA abundance took place during the day
395 and was reduced during the night independently of the photoperiod the algae were
396 grown in. Nevertheless, the expression of *CrCO* showed a strong photoperiodic influence
397 in the sense that absolute levels of its mRNA were augmented as the day length of the
398 cycle was reduced. Thus, absolute levels of *CrCO* mRNA were much higher in SD than in
399 LD. The expression of *CrCO* was also circadianly regulated, maintaining a fairly stable
400 expression pattern after several days in LL or DD condition, although mRNA levels
401 suffered a drastic decrease (Serrano et al. 2009). The other B-box gene described in
402 Chlamydomonas, *ROC66*, also followed a circadian rhythm of mRNA expression, peaking
403 during the day time (Matsuo et al., 2008).

404 The pattern of production of CrCO protein followed closely that of the mRNA in
405 all photoperiods, so at first sight it seemed that the complex posttranscriptional
406 regulation of CO stability observed in Arabidopsis was missing in the alga. Nevertheless,
407 confirmation of this point needs further experimental data since, for example,

experiments employing different light qualities, which are crucial to identify posttranslational modifications of CO, were not reported in these studies (Serrano et al., 2009). On the other hand, the GI and FKF proteins that are involved in the first regulatory module that defines the expression of *CO* in Arabidopsis, have no detectable homologues in Chlamydomonas or other algae (Corellou et al., 2009).

When *CrCO* or *ROC66* were missexpressed in Chlamydomonas, the recombinant algae presented defects in growth. In the case of *ROC66*, the circadian rhythm of a chloroplast marker, as well as the growth rates of the alga, were accelerated in the mutant compared to wild type. For *CrCO* it was demonstrated that the expression of genes known to be regulated by the clock like *GBSSI*, involved in starch synthesis, and genes involved in cell cycle regulation like cyclins (*CYCA1*) or cyclin-dependent kinases (*CDKB1*) were affected when *CrCO* levels were reduced (Serrano et al., 2009). Overexpression of *CrCO* augmented *GBBS1*, *CYCA1* and *CDKB1* mRNA levels, affecting both the capacity of the algal cells to accumulate starch and to divide properly. Synchronous growth of Chlamydomonas, which reflects the capacity of some algae to coordinate growth and cell cycle under specific photoperiods, was completely disrupted in over- and miss-expressing *CrCO* recombinant lines. Thus, both augmenting and decreasing *CrCO* mRNA levels, severely affected growth, starch synthesis and algal cell cycle, often causing lethality. An immediate question then arises, as to the degree that these basic physiological functions are also conserved in CO or other members of the COL family in higher plants. This question is extremely important because if so, the contribution of the photoperiod response to basic metabolism and growth would have a stronger influence than reported to date and in this crucial physiological aspect the role of COL proteins would be central.

New roles for COL proteins

With the information we have today, the most plausible scenario is that COL proteins first appeared associated with the primary endosymbiotic event and their structure evolved from a single protein with one b-box and CCT domain with no defined size (Figure 2, group II) to the double b-box, middle and CCT domain of CO and HD1 with a strict protein size (Figure 2, group I). Other b-box proteins (BBXs) even lack the CCT domain and could not be considered '*bona fide*' COLs, because, although no biochemical study on its function has been performed to date, their lack of a CCT domain will prevent many of the functions attributed to COLs, such as nuclear localization, interaction with ubiquitin ligases, DNA binding or interaction with the HEME ACTIVATOR PROTEIN (HAP) complex. Still, a photoperiodic role through the interaction with other COLs employing their b-boxes as dimerization domains cannot be ignored, as the data from tomato ATCOL1 suggest (Ben-Naim et al., 2006).

It seems that from a single locus gene in algae, plants have developed a complex family of COLs (Zobell et al., 2005; Chia et al., 2008) that have adopted different functions throughout evolution but have kept some common characteristics: many of them are regulated in a circadian manner and many are involved in light-dependent processes. Furthermore, when COL proteins other than CO, are expressed in Arabidopsis they have either very little or no role on flowering time. A paradoxical case is *COL1* that cannot complement the *co* mutation, in spite of its extremely close evolutionary relationship to CO, whereas overexpression of the more divergent *CrCO* under a 35S promoter induced extremely early flowering, phenocopying CO function and even complementing the *co* mutation (Serrano et al., 2009). Expression of *CrCO* under a

specific phloem promoter also induced early flowering but not if the expression was under a meristem specific promoter. What does this tell us? First, and in an extremely surprising way, that CrCO function in algae and plants must be very similar at the biochemical level. Second, that it is the unique three-dimensional structures of every specific COL protein that determines its function and that CrCO and CO must be extremely close in this structure. Because it has been shown that CO is subjected to a complex posttranslational regulation involving phytochromes, cryptochromes, E3 ubiquitin ligases and HAP proteins it is probable that CrCO was also able to form complexes with these proteins in the CrCO overexpressing plants to perform CO function. This happened at a notoriously similar time and space frame.

CrCO function is essential in algae and severely reduced levels of *CrCO* decreased Chlamydomonas growth causing cellular instability and lethality (Serrano et al., 2009). The question then remains why, considering the degree of functional conservation between CO and CrCO, these extreme phenotypes have not been described for *col* mutants in Arabidopsis or other plants. Again we have to call on complexity and an evolutionary point of view to answer this question (Romero and Valverde, 2009).

As can be seen in Figure 3A, the current model for CO function and the photoperiod pathway is mainly centred on the flowering response. In our model, COL functions are more numerous but operate through the same or similar basic mechanistic processes (Figure 3B). If CO function is activated by light quality, day length, clock and probably other external signals, it seems possible that other COL proteins are regulated in a similar way. By the same reasoning, if the CCT domain and b-boxes of CONSTANS homologues from Arabidopsis and other plant species are able to interact with similar protein partners, it is highly likely that other COL proteins, particularly those closer to

CO, would also be able to interact with some of these partners. It is also plausible that through b-boxes different COLs could interact and modify each others function as has been demonstrated in other transcription factors, particularly in the MADs group (Davies et al., 1996). In this rational thinking, the complexity of the redundancy in their biological function and possible interaction and hetero-dimerization could explain the lack of particular information about the role of COLs. There is simply not enough information accumulated to answer these questions.

Conclusion

In the last fifteen years an extremely complex model of the photoperiod response has emerged (Amasino, 2010). In this model the flowering response in Arabidopsis has been crucial to describe how the signal is created in the photosynthetic tissues and how a mobile molecule (florigen) is transported to the apical meristem to change the tissue fate. This CO-FT module is now at the root of every photoperiod response in higher plants and has already been shown to be involved in different developmental processes such as tuberization in potato (Martínez-García et al., 2002; González-Schain and Suárez-López, 2008), bud dormancy (Bohlenius et al., 2006) or juvenile to adult phase change in Populus (Zhang et al., 2010).

The production of the florigen at the correct season and in a precise time window of the day is what ensures that flowering will happen in a timely fashion for every particular plant adapted to a particular environment. The mechanism has to be extremely precise but at the same time has to allow for certain plasticity because fluctuations in the seasonal temperature have to be counteracted with strong and reliable photoperiod and circadian inputs to assure the correct floral transition. In this

504 scenario, data coming from simple systems like unicellular algae could be extremely
505 useful to understand what the molecular mechanisms that activate CONSTANS are and
506 upon what particular illumination this activation takes place. It could also be extremely
507 useful to define molecular complex partners and find out in what possible metabolic and
508 cell cycle regulatory events are the different COL proteins involved.

509 Recent works in the photoperiod response employing diverse plant species are
510 illuminating a wider photoperiodic response than the one described in Arabidopsis. In
511 some species the CO-FT module could work as an inhibitory signal in non-inductive
512 conditions, such as rice, or could have a less important role than originally assumed, like
513 in *Solanum* species (Martínez-García et al., 2002). It is still unclear how the florigen
514 signal is transported, how it is produced in specific tissues and whether it is just FT or a
515 mixture of substances. All these differences reside, not so clearly in undiscovered crucial
516 genes, but in the complex relationship between a myriad of, often redundant, secondary
517 partners and the way they globally influence the photoperiod response. The potential
518 application of day length signalling to artificially modulate the photoperiodic response
519 of crops has an enormous agro-biotechnological interest. By modifying the photoperiod
520 response, we could alter at will not only flowering time but also other important traits
521 like dormancy, growth rates, or crop yield. This could be part of a new tailored strategy
522 to alter pinpoint aspects of physiological and developmental programs to produce next-
523 generation crops.

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Figure legends

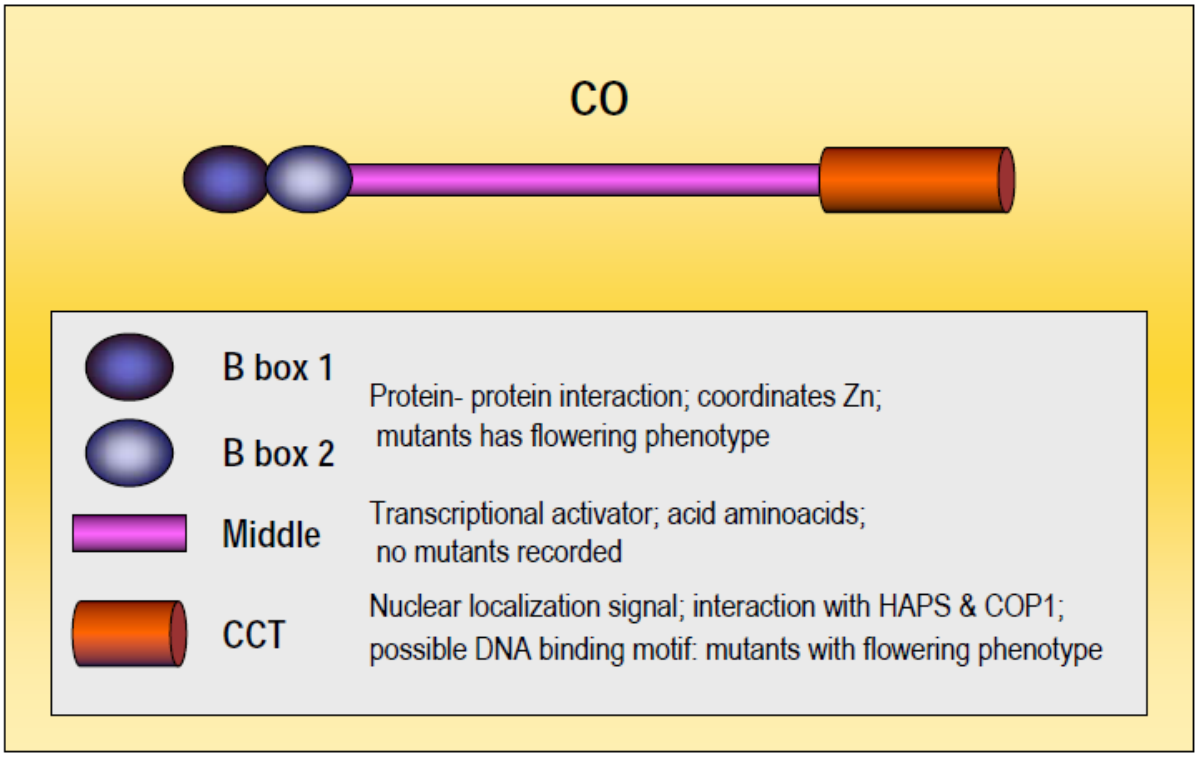
Figure 1. Graphic domain structure of CO. For each important domain of CONSTANS protein, which has been used as a model, a small representative picture is shown: In different shades of blue, the two amino terminal b-boxes; in magenta, the acidic middle domain and in red the CCT domain. A small legend with tips for molecular function and mutant effect is given in the square box below. The picture is not drawn to scale. HAPS: Heme Activated Proteins; COP1: COnstitutive Photomorphogenic 1.

Figure 2. **Phylogenetic trees of COL proteins from plants and algae.** The phylogenetic tree represents the evolutionary relationship between protein sequences of different COLs from Arabidopsis (AtCOL1-16); rice (OsCOL1-9); the moss Physcomitrella (PpCOL1-3); the spikemoss Selaginella and the microalgae Volvox, Galdieria, Chlorella, Ostreococcus and Chlamydomonas (CrCO). The tree is drawn to scale with branches representing more than 95% bootstrap marked with an asterisk. The tree defines roughly two groups of COL proteins: Group I comprises protein with domain structure as in Figure 1 and group II comprises COL proteins lacking one of the b-boxes. (Modified from Serrano et al., 2009). In group I, the genes demonstrated to affect flowering have been highlighted (FLOWERING), as well as the ones with a probable function in other light-dependent processes (LIGHT).

Figure 3. New model for the photoperiod response in plants. A. The picture on the left represents the currently accepted model from Arabidopsis, in which light-activated CO overcomes the temperature-dependent inhibition from FLC and induces the expression of FT in the phloem companion cells. FT is moved to the phloem and

channelled to the apical meristem where it binds to FD and the complex is recruited into the nucleus. FT-FD binds to the promoter of *SOC1* and other meristematic floral integrators changing the vegetative developmental program to the ABC program, eventually producing flowers. **B.** The model proposed here includes that depicted in A, but also recruits similar photoperiodic mechanisms to regulate other developmental programs and basic physiological processes. Yellow arrows represent external signals: day/night transition; circadian clock; light quality; and a metabolic signal represented by a fertilizer bottle. Black arrows indicate some of the outputs of the photoperiodic response.

Figures



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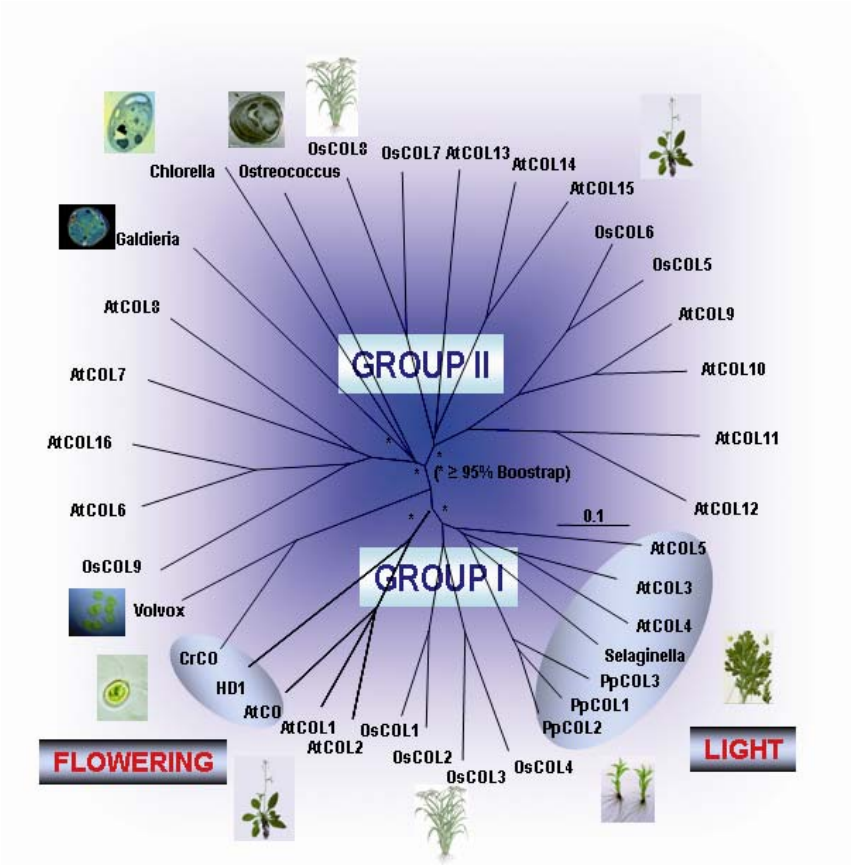

Figure 2. **Phylogenetic trees of COL proteins from plants and algae.** The phylogenetic tree represents the evolutionary relationship between protein sequences of different COLs from Arabidopsis (AtCOL1-16); rice (OsCOL1-9); the moss Physcomitrella (PpCOL1-3); the spikemoss Selaginella and the microalgae Volvox, Galdieria, Chlorella, Ostreococcus and Chlamydomonas (CrCO). The tree is drawn to scale with branches representing more than 95% bootstrap marked with an asterisk. The tree defines roughly two groups of COL proteins: Group I comprises protein with domain structure as in Figure 1 and group II comprises COL proteins lacking one of the b-boxes. (Modified from Serrano et al., 2009). In group I, the genes demonstrated to affect flowering have been highlighted (FLOWERING); as well as the ones with a probable function in other light-dependent processes (LIGHT).

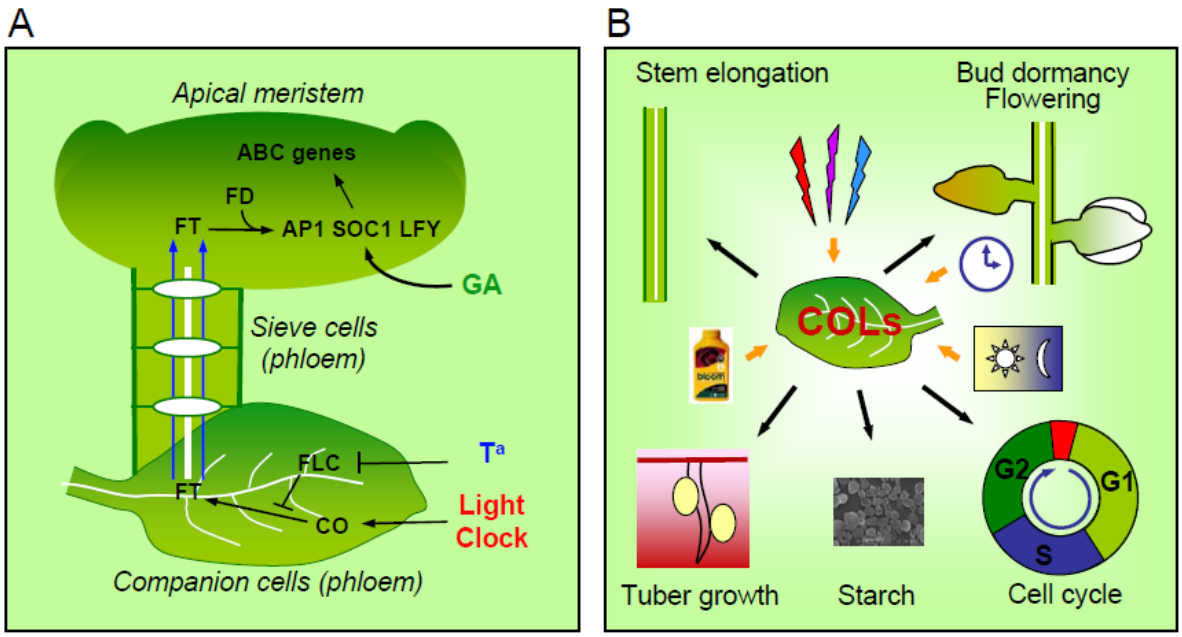

Figure 3. New model for the photoperiod response in plants. A. The picture on the left represents the currently accepted model from Arabidopsis, in which light-activated CO overcomes the temperature-dependent inhibition from FLC and induces the expression of FT in the phloem companion cells. FT is moved to the phloem and channelled to the apical meristem where it binds to FD and the complex is recruited into the nucleus. FT-FD binds to the promoter of *SOC1* and other meristematic floral integrators changing the vegetative developmental program to the ABC program, eventually producing flowers. **B.** The model proposed here includes that depicted in A, but also recruits similar photoperiodic mechanisms to regulate other developmental programs and basic physiological processes. Yellow arrows represent external signals: day/night transition; circadian clock; light quality; and a metabolic signal represented by a fertilizer bottle. Black arrows indicate some of the outputs of the photoperiodic response.