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Research Article

A genetic framework for flowering-time pathways in *Citrus* spp.

Marcelo Carnier Dornelas[1], Raquel Luciana Boscariol Camargo[2], Luciana Harumi Morimoto Figueiredo[2] and Marco Aurélio Takita[2]

[1]*Departamento de Fisiologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Cidade Universitária Zeferino Vaz, Campinas,SP, Brazil.*
[2]*Centro APTA Citrus Sylvio Moreira, Instituto Agronômico de Campinas, Cordeirópolis, SP, Brazil.*

Abstract

Floral transition is one the most drastic changes occurring during the life cycle of a plant. The shoot apical meristem switches from the production of leaves with associated secondary shoot meristems to the production of flower meristems. This transition is abrupt and generally irreversible, suggesting it is regulated by a robust gene regulatory network capable of driving sharp transitions. The moment at which this transition occurs is precisely determined by environmental and endogenous signals. A large number of genes acting within these pathways have been cloned in model herbaceous plants such as *Arabidopsis thaliana*. In this paper, we report the results of our search in the *Citrus* expressed sequence tag (CitEST) database for expressed sequence tags (ESTs) showing sequence homology with known elements of flowering-time pathways. We have searched all sequence clusters in the CitEST database and identified more than one hundred *Citrus* spp sequences that codify putative conserved elements of the autonomous, vernalization, photoperiod response and gibberelic acid-controlled flowering-time pathways. Additionally, we have characterized *in silico* putative members of the *Citrus* spp homologs to the *Arabidopsis* CONSTANS family of transcription factors.

Key words: reproductive development, flowering-time genes, reproduction, photoperiod, CONSTANS.

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Introduction

When grown from seeds, *Citrus* seedlings progress through a developmental ontogeny typical for woody perennials, eventually producing a moderately sized tree. After a juvenile period, typically lasting several years, *Citrus* trees enter the adult phase in which they are capable of continuously producing flowers in addition to vegetative shoots (Krajewski and Rabe, 1995). Flowers can potentially be produced throughout the year, but in most oranges and mandarins grown in temperate environments, the majority of flowers are produced during the spring flush. Thousands of flowers are usually produced on established trees, but only a relatively small proportion develops into fruit. In some varieties, pollination, fertilization and seed development are required for fruit set, while in others, parthenocarpic fruit development can occur. In some cases this is stimulated by pollination (Koltunow *et al.*, 2000).

For a given *Citrus* species and/or variety, the number of fruit on an individual tree is negatively correlated with final fruit size. Consequently, the tendency for *Citrus* to exhibit a biennial bearing pattern of different flowering intensities has a significant impact on fruit size at harvest. In "on" years a relatively large number of flowers are produced (and thus small fruits), while in "off" years relatively few flowers are formed as well as fewer, but bigger fruits (Garcia-Luis *et al.*, 1992; Garcia-Luis and Kanduser, 1995; Garcia-Luis *et al.*, 1995). Because of this effect, trees of a particular variety within a geographical area tend to become synchronized in their biennial bearing pattern. While this simplifies management to some extent, it greatly exacerbates the overproduction of small fruit in "on" years. Thus, the understanding of the molecular regulation of the flowering process is crucial for controlling fruit production in *Citrus*.

The rapid advances made in understanding *Arabidopsis* flowering have allowed researchers to begin similar investigations in perennial crops. This knowledge is greatly accelerating flowering research in perennial trees because, at least in a general sense, the same genes appear to be involved in flower initiation, flower formation, and fruit development in all of the important flowering plants. Using the DNA sequence of flowering genes from model plants as a starting point, flowering genes have been successfully isolated from several agriculturally important tree

Send correspondence to Marcelo C. Dornelas. Departamento de Fisiologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Cidade Universitária Zeferino Vaz, Caixa Postal 6109, 13.083-970 Campinas, SP, Brazil. E-mail: dornelas@unicamp.br.

crops, including apple (Yao *et al.*, 1999; Sung *et al.*, 1999; Sung *et al.*, 2000; Kotoda *et al.*, 2000), *Citrus* (Pillitteri *et al.*, 2004), grape (Boss *et al.*, 2001; Boss *et al.*, 2002), and *Eucalyptus* (Kyozuka *et al.*, 1997; Southerton *et al.*, 1998; Dornelas *et al.*, 2004; Dornelas and Rodriguez, 2005).

Here we concentrated on the characterization of genes involved in the pathways that lead to the transition from vegetative to reproductive development in *Citrus* species. With this goal, we have used the sequences of the key proteins of the different developmental pathways involved in the regulation of flowering-time available from *Arabidopsis* as bait to search the *Citrus* database of expressed sequence tags (CitEST) showing sequence homology with known elements of flowering-time pathways. Additionally, we have undertaken an extensive *in silico* characterization of the putative *Citrus* homologues of the CONSTANS gene family, which, in *Arabidopsis*, mediate the cross-talk between the circadian clock and the genes controlling reproductive meristem identity. We have identified *Citrus* sequences that codify putative conserved elements of the vernalization, photoperiod response, autonomous and gibberellic acid-controlled flowering-time pathways. We expect that our results will contribute to further studies describing how these pathways function in controlling the induction to flowering and thus the biennial fruit bearing pattern in *Citrus*.

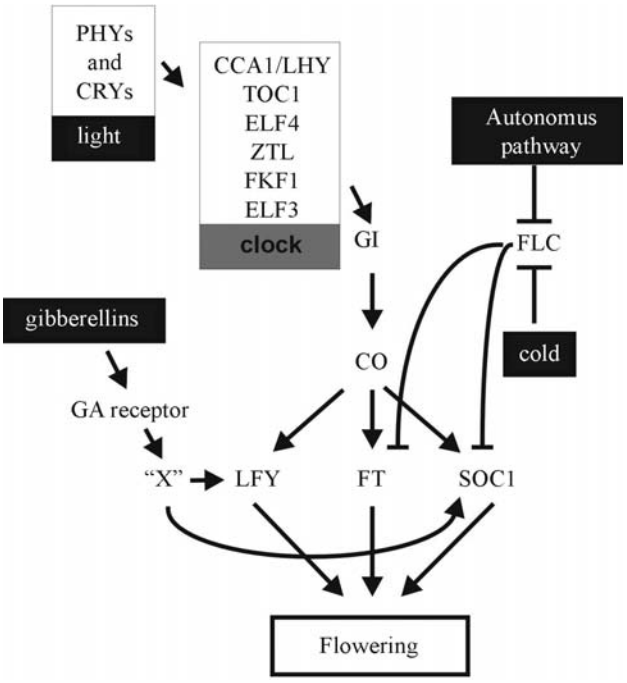

Figure 1 - Overview of the relationships among the elements involved in the flowering-time pathways in the model plant *Arabidopsis thaliana* (after Mouradov *et al.*, 2002 and Izawa *et al.*, 2003). The data underlying the model and the corresponding homologs in *Citrus* are presented in Table 1 and in the text. For abbreviations and gene names see Table 1.

Material and Methods

Searching *Citrus* ESTs homologs to *Arabidopsis* flowering-time genes

The overall goal of this study was to retrieve from the CitEST data set, *Citrus* spp homologs to all genes described to be involved in the control of flowering time, according to the processes showed in Figure 1. In order to achieve this, data mining in the CitEST database was carried out using published plant gene sequences as bait, as well as keyword searches in the CitEST home page (http://citest.centrodecitricultura.br/). Plant gene sequences used as bait were retrieved from public gene databases (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) using their corresponding accession numbers or by the use of keyword-oriented searches (Mouradov *et al.*, 2002; Izawa *et al.*, 2003). Protein (deduced amino acid) sequences from the retrieved bait sequences were compared to *Citrus spp* clustered EST sequences using a combination of different Blast algorithms (Altschul *et al.*, 1997), with the BLOSUM62 scoring matrix, with a threshold of $e < 10^{-10}$ for positive hits. The identity (in terms of donor cDNA library) and number of sequence read composition of each individual candidate cluster were checked to access their potential expression pattern.

For the results presented in Table 1, we have obtained e-values using the BLASTp algorithm (Altschul *et al.*, 1997) as described above. The identity and the similarity were calculated at the amino acid level, relative to the corresponding *Arabidopsis* putative homolog, within the extension of the successful sequence alignment produced by their pair-wise comparison.

In silico characterization of the *Citrus* homologs belonging to the *CONSTANS* gene family

The *Arabidopsis CONSTANS* (*CO*) gene family codifies putative transcription factors defined by two conserved domains (Putterill *et al.*, 1995; Griffiths *et al.*, 2003). The first is a zinc finger region near the amino terminus that resembles B-boxes, which regulate protein-protein interactions in several animal transcription factors (Putterill *et al.*, 1995). The second is a region of 43 amino acids near the carboxy terminus termed the CCT (CO, CO-like, TOC1) domain (Robson *et al.*, 2001). We have identified *Citrus* homologs to the *Arabidopsis CO* gene family by using the *Arabidopsis* sequences as bait and the BLAST algorithms (Altschul *et al.*, 1997) as described above. Only comparisons that produced an e-value better than e^{-50} were considered highly significant. In the cases where the obtained e-values were between e^{-50} and e^{-5}, a re-clusterization of all reads identified was performed using the CAP3 algorithm from the BioEdit Software (Hall, 1999). The novel cluster consensus sequences obtained were re-submitted to BLAST and frequently better e-values were obtained. We analyzed these using the CDD algorithm (Marchler-Bauer *et al.*, 2005) to identify the presence of conserved domains in the deduced protein sequence.

Table 1 - *Citrus* ESTs that share homology to flowering-time genes of *Arabidopsis*.

Category	*Arabidopsis*[a]	MIPS code	*Citrus*[b]	e-value[c]	ID/SIM[d]	ext[e]
Photoreceptor	PHYA	At1g09570	CS00-C3-701-101-C11	6e-86	87/93	70
			CR05-C3-701-030-B06	1e-39	55/65	65
			CG32-C1-003-003-A11	4e-32	68/88	66
	PHYB	At2g18790	CS12-G8-000-003-D03	5e-55	62/78	76
			CR05-C1-102-036-H07	4e-64	66/81	62
	PHYC	At5g35840	CS00-C3-705-056-G06	6e-82	67/83	63
			CA26-C1-002-046-B05	4e-38	47/66	68
	CRY1	At4g08920	CS00-C3-702-004-H06	1e-145	90/94	61
			CR05-C3-700-072-D08	1e-108	75/85	72
			CA26-C1-002-076-H02	2e-67	82/91	75
			PT11-C1-900-077-C09	1e-124	86/94	82
			LT33-C1-003-039-G09	3e-18	35/40	52
	CRY2	At1g04400	CR05-C3-702-066-D11	1e-113	77/89	73
Circadian clock	CCA1	At2g46830	CS00-C3-702-052-G12	3e-42	71/98	68
			CR05-C3-701-001-G09	4e-32	71/75	62
			CG32-C1-003-066-G07	1e-33	64/65	64
			CA26-C1-002-004-G09	3e-29	61/68	65
			PT11-C1-900-096-C04	9e-38	72/74	72
	LHY	At1g01060	CS00-C1-650-038-E07	4e-72	86/88	72
			CR05-C1-102-049-H08	2e-58	85/87	71
			CG32-C1-003-085-A11	6e-47	79/81	65
			CA26-C1-002-004-G09	3e-29	74/80	60
			PT11-C1-900-084-H09	2e-67	71/76	59
	GI	At1g22770	CS00-C3-705-019-F09	1e-111	88/89	65
			CR05-C1-100-075-E08	1e-121	84/87	68
			CG32-C1-003-008-F10	1e-105	79/80	78
			PT11-C1-900-095-D02	1e-44	81/82	87
			LT33-C1-003-095-A04	5e-74	66/75	65
	TOC1/APRR1	At5g61380	CS00-C3-702-097-G04	2e-69	78/85	64
			Cr05-c1-100-016-f10	1e-75	77/78	68
			CG32-C1-003-006-A02	2e-60	64/68	71
			CA26-C1-002-085-D04	3e-36	62/65	70
			PT11-C1-901-054-G04	1e-76	68/69	69
			LT33-C1-003-021-B10	1e-28	62/65	69
	ELF3	At2g25930	CS00-C3-702-072-C10	6e-10	65/68	65
			CR05-C1-100-007-E05	2e-20	68/69	65
			CA26-C1-002-082-B12	3e-35	56/64	78
			PT11-C1-900-009-D09	1e-15	55/57	71
	ZTL	At5g57360	CS00-C3-704-061-B11	1e-116	84/85	56
			CR05-C3-702-002-G04	1e-103	75/76	65
			CL06-C4-500-040-H10	5e-10	75/79	59
			CG32-C1-003-015-G02	1e-29	78/79	67
			CA26-C1-002-073-A02	4e-55	69/71	68
			PT11-C2-300-054-C06	1e-85	72/80	63
			LT33-C1-003-029-B10	1e-109	76/82	61
	LKP2	At2g18910	CS00-C3-704-061-B11	1e-85	56/84	59
			CR05-C3-702-002-G04	1e-103	56/71	72
			CL06-C4-500-040-H10	5e-10	46/56	56
			CG32-C1-003-072-B01	1e-116	76/77	70
			CA26-C1-002-015-B02	2e-96	72/74	64
			PT11-C2-300-054-C06	5e-72	76/79	68
			LT33-C1-003-029-B10	1e-109	66/68	64

Table 1 (cont.)

Category	*Arabidopsis*[a]	MIPS code	*Citrus*[b]	e-value[c]	ID/SIM[d]	ext[e]
Circadian clock mediator	FKF1	At1g68050	CS00-C1-102-053-E02	3e-95	68/78	62
			CR05-C3-700-019-F11	7e-62	85/89	64
			PT11-C1-900-027-F07	4e-10	77/88	62
	CO	At5g15840	CS00-C1-100-086-A06	6e-52	75/78	71
			CL06-C4-501-017-G07	1e-64	74/76	75
			CG32-C1-003-018-D09	1e-66	57/66	70
			CA26-C1-002-061-D07	2e-63	52/64	69
			LT33-C1-003-096-C01	2e-20	53/65	64
			PT11-C1-901-085-G05	2e-64	55/62	66
Floral pathway integrator	FT	At1g65480	CS00-C3-704-020-B11	5e-54	68/74	68
			CL06-C4-501-024-H01	1e-17	61/64	64
			PT11-C9-005-004-G03	1e-28	71/72	63
	LFY	At5g61850	not found (see text)			
	SOC1	At2g45660	CS00-C3-705-050-G08	1e-59	65/68	67
			CR05-C3-700-098-B05	7e-92	63/64	65
			CG32-C1-003-007-A12	9e-96	69/70	66
			CA26-C1-002-079-C12	3e-87	69/71	63
			PT11-C1-900-073-F02	2e-45	67/69	64
			LT33-C1-003-056-A03	2e-66	65/67	67
Vernalization pathway	FLC	At4g18280	CS00-C3-705-050-G08	9e-96	71/76	69
			CR05-C3-700-098-B05	7e-92	82/86	68
			CG32-C1-003-007-A12	1e-59	82/94	75
			CA26-C1-002-079-C12	2e-45	85/94	62
			PT11-C1-900-073-F02	3e-87	75/85	69
			LT33-C1-003-056-A03	2e-66	66/71	65
Chromatin-related	EMF2	At5g51230	CS00-C3-703-014-A10	8e-34	60/77	62
			CR05-C3-702-101-D11	7e-62	64/71	87
			CG32-C1-003-068-D09	3e-23	84/91	94
			CA26-C1-002-103-B01	9e-44	54/67	72
			PT11-C1-901-070-F02	6e-59	52/64	66
	FIE	At3g20740	CS00-C3-703-047-A03	2e-82	74/82	55
			CR05-C1-100-078-H01	1e-105	66/79	76
			CG32-C1-003-068-D09	3e-23	52/64	66
	LHP	At5g17690	CS00-C3-703-058-E10	4e-28	42/54	82
			CR05-C3-702-033-H07	7e-16	36/53	83
			CA26-C1-002-100-G04	9e-26	83/89	67

[a]Abbreviations: APRR1: *Arabidopsis* PSEUDO RESPONSE REGULATOR1; CCA1: CIRCADIAN CLOCK ASSOCIATED 1; CK2: casein kinase2; CO: CONSTANS; CRY: CRYPTOCHROME; ELF3: EARLY FLOWERING3; EMF2: EMBRYONIC FLOWERING2; FIE: FERTILIZATION INDEPENDENT ENDOSPERM; FKF1: FLAVIN-BINDING, KELCH-REPEATS, F-BOX1; FLC: FLOWERING LOCUS C; FT: FLOWERING LOCUS T; LFY: LEAFY; LHP1: LIKE HETEROCHROMATIN PROTEIN1; LHY: LATE ELONGATED HYPOCOTYL; GI: GIGANTEA; PHY: PHYTOCHROME; SOC1: SUPPRESSOR OF OVEREXPRESSION OF CO1; TOC1: TIMING OF CAB EXPRESSION1.
[b]Species identification code is CA: *Citrus aurantium*; CG: *C. aurantifolia*; CR: *C. reticulata*; CS: *C. sinensis*, LT: *C. latifolia*; PT: *Poncirus trifoliata*.
[c]Using the BLASTp algorithm (Altschul *et al.*, 1997).
[d]ID = identity; SIM = similarity; both based on the amino acid sequence, relative to the putative *Arabidopsis* homologs.
[e]ext = extension of the successful alignment including eventual insertion/deletion events.

Comparative and phylogenetic analysis of *CONSTANS* gene family homologs

To examine the relationships between the *Citrus* CO-like genes and their putative *Arabidopsis* homologs in more detail, their nucleotide and predicted peptide sequences were used to determine genetic distances and to construct phylogenetic trees. Because the middle regions of the genes were the most divergent, they could not be aligned with confidence. Therefore, neighbor-joining (NJ) and maximum parsimony (MP) trees were constructed using B-box (and CCT domain sequences when available) following the alignments obtained using the CLUSTALX software (Thompson *et al.*, 1994). The alignments were eventually corrected by hand. Phylogenetic trees were ob-

tained using parsimony and/or genetic distance calculations. Neighbor-joining (Saitou and Nei, 1987) and Bootstrap (with 1000 replicates) trees were built using the MEGA software (http://www.megasoftware.net).

Results

Identifying *Citrus* ESTs related to flowering-time pathway genes

Genetic analyses in model plants such as *Arabidopsis* identified a whole set of flowering-time genes that were subsequently assigned to four major genetic pathways according to their response to the exposure of a period of cold (vernalization) or to day length (photoperiod) (Simpson *et al.*, 1999; Araki, 2001; Mouradov *et al.*, 2002; Simpson and Dean, 2002; Bastow and Dean, 2003; Amasino, 2004; Boss *et al.*, 2004). The field of flowering time has thus been organized around these four pathways, with the photoperiod and vernalization pathways mediating the response to environmental cues and the autonomous and the gibberellin (GA) pathways acting largely independently of these external signals (Figure 1). Based on the systematic search in the CitEST database using *Arabidopsis* sequences as bait, we have identified 109 *Citrus* spp. EST clusters representing putative *Citrus* spp homologs to flowering-time genes. Some of these genes are required for the day length response, and some encode regulatory proteins specifically involved in the control of flowering, while others encode components of light signal transduction pathways or are involved in circadian clock function. A representation of the relationships among these processes is shown in Figure 1 and the putative homologs of the key players in *Citrus* spp are presented in Table 1. The role of each of these elements in the flowering-time pathways and their implication for the understanding of *Citrus* spp flowering processes are presented in the Discussion section.

Two genes play a prominent role at the "bottom" of the flowering promotion cascades: *CONSTANS* (*CO*) and *FLOWERING LOCUS C* (*FLC*). The *FLC* gene is the point of convergence of the autonomous and vernalization pathways (Figure 1). Ultimately and in part through CONSTANS (CO) and FLC, the flowering signals lead to the induction of a set of genes called floral meristem identity (FMI) genes and responsible for the fate change of the meristems emerging on the flanks of the shoot apex (Long and Barton, 2000). This group of genes includes the *LEAFY* (*LFY*) gene, expressed in early floral stages and responsible for their floral fate (Lohmann and Weigel, 2002). We could not find any putative homolog to LFY in the CitEST database, but Citrus homologs to this gene have already been identified (Pena *et al.*, 2001), thus indicating an underrepresentation of flowering-time sequences in the CitEST dataset.

The *CO* gene is probably the most downstream actor, specific for the photoperiod pathway (Figure 1) and both the light and the internal clock precisely regulate CO protein accumulation (Valverde *et al.*, 2004). Due to their importance to the regulation of flowering-time, the CO-like sequences found in the CitEST database were studied in greater detail and these results are presented separately in a separate section below.

Elements of the *Citrus CONSTANS-like* gene family

We have identified a total of 244 *Citrus* spp EST sequences showing significant (e-value lower than e-^{10}) similarity to the *Arabidopsis CO-like* (*COL*) genes, by means of a combination of BLAST algorithms and keyword searches in the CitEST database (Table 2). When submitted to the CAP3 algorithm, these sequences were initially organized into 75 clusters. With further comparison of their deduced amino acid sequences, the number of valid clusters was reduced to 27.

Based on previous studies on *Eucalyptus* (Dornelas and Rodriguez, 2005) and sugarcane (Dornelas and Rodriguez, 2006) COL proteins, we concluded that this gene family evolves rapidly, particularly in the middle regions (see also Lagercrantz and Axelsson, 2000). Thus our analysis focused on the B-box sequences only and we excluded putative homologs to the related *Arabidopsis STO* (*SALT TOLERANCE*) gene. *ST*O-like genes have B-boxes but no CCT domain. Additionally, we excluded the related *ZIM* gene from our analysis, which contains an additional ZIM motif. This short motif is found in a variety of plant transcription factors that contain GATA domains and its conserved amino acids form the pattern TIFF/YXG (Lagercrantz and Axelsson, 2000; Griffiths *et al.*, 2003). We thus restricted our analysis to *Citrus* spp sequences showing the conserved B-box and CCT domains, according to the definition of the COL family provided by Griffiths *et al.* (2003). These assumptions explain the reduced number of

Table 2 - *Citrus* putative homologs to the *CONSTANS*-like genes of *Arabidopsis*.

Species	Number of ESTs[a]	Clusters[b]	Putative homologs[c]
Citrus aurantifolia	14	8	4
Citrus aurantium	12	7	3
Citrus latifolia	5	3	0
Citrus limonia	5	3	1
Citrus reticulata	49	18	7
Citrus sinensis	130	23	7
Poncirus trifoliata	29	13	5

[a]When using the BLASTp algorithm (Altschul *et al.*, 1997) and considering an e-value of e^{-10}. All *Arabidopsis* CO-like proteins were used as alternative bait sequences.
[b]Number of clusters formed by the given number of ESTs when using CAP3 assembling algorithm (Huang and Madan, 1999).
[c]Number of clusters, after eliminating redundancy and after parsimony analysis.

true putative *Citrus* spp homologs of COL members shown in Table 2.

As most of the CCT domain sequences are not available for the *Citrus* spp COL proteins, we produced alignments of the predicted peptides of the conserved B-box region for all *Arabidopsis* AtCO and AtCOL proteins and their putative *Citrus* spp homologs (Figure 2A).

Variation within the B-box domain suggested that the *CO*-like genes could be further subdivided. To further examine the relationship between the putative *Citrus* spp COL homologs and their *Arabidopsis* counterparts in more detail, the sequence alignment shown in Figure 2A was used to determine genetic distances and to construct phylogenetic trees. Therefore, neighbor-joining (Figure 2B) and maximum parsimony trees (data not shown) were constructed, giving similar results. The proteins were consistently grouped into three principal clades (Figure 2B). These three groups were identified previously and are thought to have evolved prior to the divergence of monocots and dicots (Griffiths *et al.*, 2003; Dornelas and Rodriguez, 2005; 2006). Group III genes comprised *Arabidopsis* and *Citrus* spp proteins with two zinc finger domains, the second of which was diverged from the *C*O-type B-box. Group II genes comprised *Arabidopsis* and *Citrus* spp proteins with a single B-box. Group I comprised the most *CO*-like genes and included *Citrus* spp putative CO ortologs. Sequence comparisons showed that the clusters CS00-C1-100-086-A06, CL06-C4-501-017-G07, CG32-C1-003-018-D09, CA26-C1-002-061-D07 and LT33-C1-003-096-C01 presented significant similarity (e-value lower than e^{-10}) to CO (Table 1), but only CS00-C1-100-086-A06, CG32-C1-003-018-D09, and PT11-C1-901-085-G05 had complete B-box sequences; and thus only these were considered for the phylogenetic analysis. All these three *Citrus* spp sequences were consistently maintained in the same cluster together with AtCO (Figure 2B).

There were subdivisions within Group I, but these had low bootstrap values (Figure 2B). CS00-C1-100-038-C06 and CR05-C1-103-024-B09 had the most diverged B-Box domain of the *Citrus* spp genes and the phylogenetic analysis placed them, together with PT11-C1-901-054-A04 and CR05C3-701-033-C01, on the same clade of the related *Arabidopsis* proteins AtCOL16 and AtCOL6-8, within Group II.

Discussion

The flowering pathway regulated by gibberellins

Because of the importance of crop load, methods for reducing the extent of biennial bearing in *Citrus* have been investigated for use in commercial production. Winter sprays with gibberellic acid (GA) are one management tool that can be used to regulate flowering, and minimize the effect of biennial bearing. In *Citrus*, as in many other perennial crops, GA application during bud development can inhibit flower production (Monselise and Halevy 1964; Guardiola *et al.*, 1982; Lord and Eckard, 1987), and in the following spring lead to a greater proportion of single terminal flowers on leafy shoots, which tend to produce the larger fruits. On the other hand, in many annual plants such as *Arabidopsi*s, GA has a promoting effect on flowering. Thus, either GA has contrasting roles in the flowering of different species, or abnormally high GA levels in woody perennials such as *Citrus*, but not in annuals such as *Arabidopsi*s. This prevents normal flower formation, presumably by disrupting essential developmental events.

The *Arabidopsis ga1* biosynthetic mutant flowers extremely late (sometimes never) in SD (Blazquez *et al.*, 1998; Wilson *et al.*, 1992). GA acts, at least in part, by upregulating the *LEAFY* (*LFY*) gene. *LFY* expression is dramatically reduced in *ga1* mutant in short days and constitutive expression of *LFY* is sufficient to rescue the late flowering of this mutant (Blazquez *et al.*, 1998). A cis-element has been found in the *LFY* promoter that abolishes its response to GA without affecting *LFY* induction by photoperiod, indicating that the two different pathways are integrated at the level of *LFY* promoter (Blazquez and Weigel, 2000). GA is also involved in inducing *SOC1* expression (Moon *et al.*, 2003) and may also be the *FLOWERING TIME* (*FT*) gene. We have found *Citrus* putative homologs for *SOC1* and *FT*, but no clear homolog sequences to *LFY* were found within the CitEST database. Nevertheless, it is clear that the *Citrus* genome contains orthologs to *LFY* (Pena *et al.*, 2001; Pillitteri *et al.*, 2004). Accordingly, overexpressing the *Arabidopsis LFY* sequence in transgenic *Citrus* plants dramatically altered the flowering behavior and the transgenic plants flowered in a few months rather than several years (Pena *et al.*, 2001).

Autonomous and vernalization pathways

Plants require not only external (environmental) factors but also internal (developmental) factors to promote flowering. Although the ecotypes used in the laboratory of *Arabidopsis thaliana* flower earlier, many ecotypes flower very late or require a cold treatment, vernalization. The *FRIGIDA* (*FRI*) gene is responsible for the differences of the lateness of flowering among *Arabidopsis* ecotypes, as all known early-flowering ecotypes have mutations in the *FRI* gene (Johanson *et al.*, 2000). The *FRI* codes for a protein with 619 amino acids that has coiled-coil domain in two positions (Johanson *et al.*, 2000). No putative homolog could be assigned to *FRI* among the *Citrus* spp. EST clusters. The FRI protein is a positive regulator of the *Flowering Locus C* gene, which is a repressor for flowering (Michaels and Amasino, 1999). The *FLC* gene encodes a MADS-box protein (Michaels and Amasino, 1999; Peacock and Dennis, 1999). Despite the fact that no FRI homolog could be found among *Citrus* ESTs, we found putative homologs to *FLC* in six *Citrus* species (Table 1). Additionally, no sequence was found within the CitEST data set

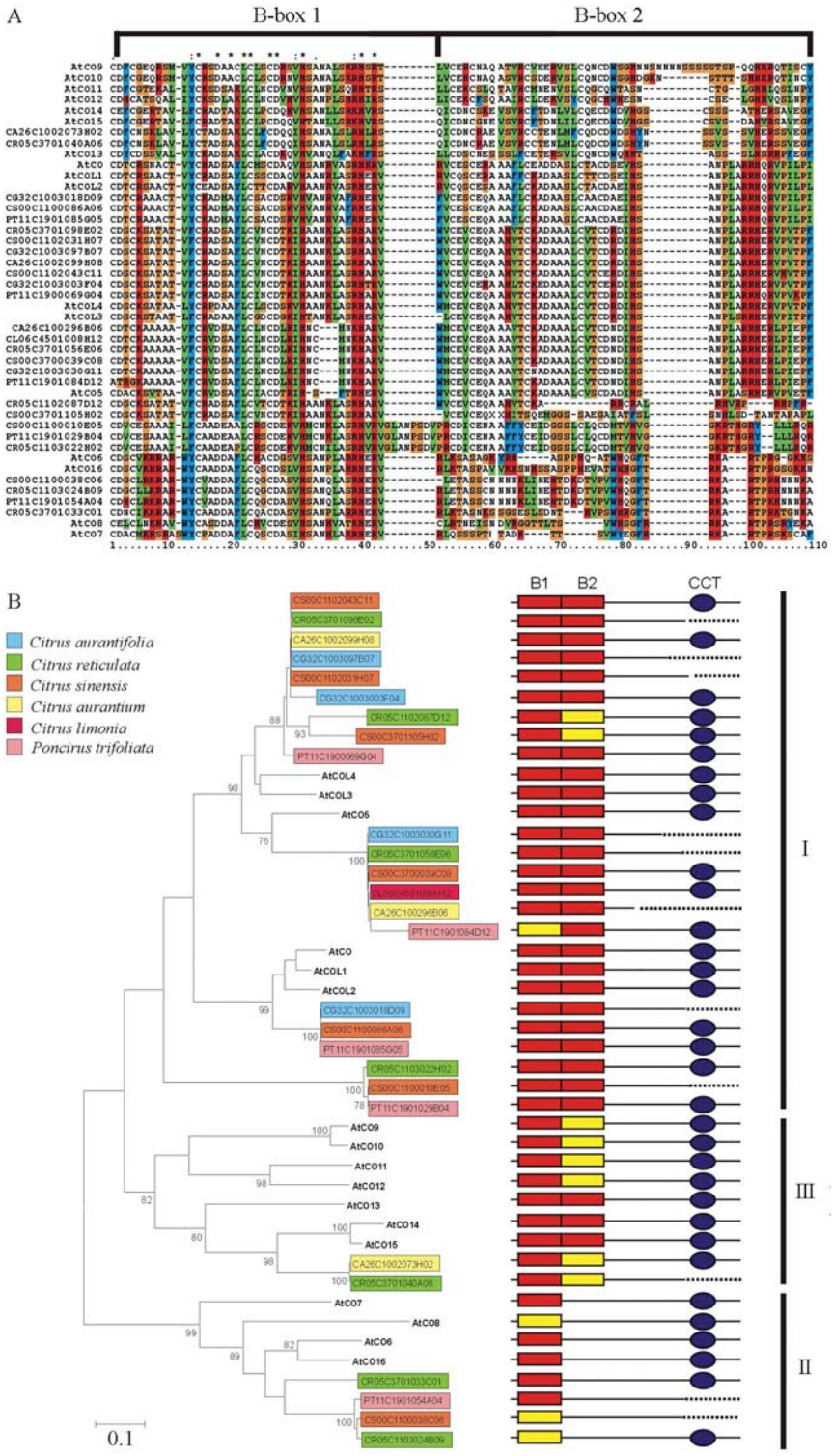

Figure 2 - Characterization of the putative *CONSTANS* gene family in *Citrus*. **A**. Alignment of predicted peptides of *Citrus CO*-like putative homologs and related genes from *Arabidopsis*. The region of the proteins aligned corresponds to the conserved B-box domains of the CO-like family (Robson *et al.*, 2001; Griffiths *et al.*, 2003). Amino acid colors are default of CLUSTAL software. **B**. Phylogenetic analysis of *CO*-like genes. A Neighbour-Joining tree was built based on the of B-box domain alignment shown in A. The *Citrus* deduced protein names are given in colored boxes. Genetic distances are shown at the given scale. Bootstrap values from 1,000 replicates were used to assess the robustness of the trees. Only bootstrap values above 75% are shown. The domain structures of each protein is also shown to the right side of their names. B1 and B2 are *CO*-like B-boxes (white rectangles) or derived zinc finger domains (solid rectangles). CCT is the conserved CCT carboxy-terminus domain (Robson *et al.*, 2001). The dotted lines represent incomplete sequences. *Arabidopsis* MIPS codes are as follows: AtCO (At5g15840); AtCOL1 (At5g15850); AtCOL2 (At3g02380); AtCOL3 (At2g24790); AtCOL4 (At5g24930); AtCOL5 (At5g57660); AtCOL6 (At1g68520); AtCOL7 (At1g73870); AtCOL8 (At1g49130); AtCOL9 (At3g07650); AtCOL10 (AB023039); AtCOL11 (At4g15250); AtCOL12 (At3g21880); AtCOL13 (At2g47890); AtCOL14 (At2g33500); AtCOL15 (At1g28050); AtCOL16 (At1g25440).

that would code for the other elements of the vernalization pathway: *VRN1* and *VRN2* (Chandler *et al.*, 1996) or for the *VIP1-7* genes. *VRN2* has a repressible role over the expression of *FLC* and codes for a protein with homology to PcG proteins (Sheldon *et al.*, 2000). *VIP4* was cloned and encodes another PcG protein (Zhang and van Nocker, 2002), and is a repressor of the *FLC* gene as well. These results indicate that the autonomous branch of the vernalization pathway may be present, in *Citrus*, but that the connection with cold-sensing may have been lost during evolution. One strong argument in favor of this speculation is that the elements of the vernalization pathway have not been found in any tropical plant for which genomic resources are available including rice, for which the genome is completely sequenced (Izawa *et al.*, 2003), *Eucalyptus* (Dornelas and Rodriguez, 2005) and sugarcane (Dornelas and Rodriguez, 2006).

Light-dependent pathway and the role of CONSTANS-like proteins

Red light is accepted by phytochrome proteins, which are encoded by *PHYA* through *E* genes in *Arabidopsis* (Reed *et al.*, 1993; Briggs *et al.*, 2001; Ohto *et al.*, 2001). We found putative *Citrus* spp homologs to PHYA, PHYB and PHYC, but similar to what was observed for other woody species (Dornelas and Rodriguez, 2005), we were not able to find significant similarities among *Arabidospsis* PHYD and PHYE within the CitEST data set (Table 1).

Blue light receptors are named as cryptochrome proteins, which are encoded by *CRY1* and *CRY2* in Arabidopsis (Ahmad and Cashmore, 1993; Lin *et al.*, 1998). We found a putative homolog to CRY2 only among *C. reticulata* sequences, but CRY1 homologs could be found in five different *Citrus* species (Table 1). *Arabidopsis* cryptochrome gene *CRY1* cooperatively functions with the *CRY2* gene to repress the function of *CO* and *GIGANTEA* (*GI)* (Mockler *et al.*, 1999).

The functions of genes *LH*Y, *CCA*1, *ELF*3, and *TOC1* are related to the circadian clock that processes the light signals and converts them into periodic information (Hicks *et al.*, 2001; Doyle *et al.*, 2002). The processed signal is transmitted to the *GI* gene, whose product activates the *CO* gene (Suarez-Lopez *et al.*, 2001). *Citrus* spp putative homologs to all these circadian clock elements were found (Table 1), suggesting that the molecular elements of the circadian clock may be conserved among herbaceous and woody plants, despite their divergent reproductive behavior. This has also been observed for other woody species such as *Eucalyptus* (Dornelas and Rodriguez, 2005). These results thus indicate that the observed differences in the reproductive development between herbaceous and woody plants are likely to be the product of different interactions among clock elements rather than differences in the clock components themselves.

We have paid special attention to the characterization of the putative Citrus spp homologs to the *Arabidopsis* CO-like family members. The *CO* and *CO*-like genes encode nuclear zinc finger-containing proteins, suggesting potential transcription factor function, but the precise mechanism of CO action is not yet understood (Parcy, 2005). In particular, CO has not been shown to bind DNA and is, therefore, assumed to be tethered to regulatory sequences through interaction with other transcription factors (Hepworth *et al.*, 2002). Recently, evidence has accumulated indicating that CCAAT binding factors can mediate interactions between CONSTANS-like proteins and DNA (Ben-Naim *et al.*, 2006). The members of the CO-family are very conserved and can be found among diverse angiosperm species and even in *Physcomitrella* (Zobell *et al.*, 2005), suggesting that the function of these proteins in controlling reproductive development may be conserved as well.

The precise analysis of *CO* expression pattern has recently led to new and exciting questions regarding CO mode of action (Takada and Goto, 2003; An *et al.*, 2004). Indeed, the photoperiodic signal was known to be perceived in leaves and somehow transmitted to the apex by the unknown florigen signal (Zeevaart, 1976; Bernier *et al.*, 1993; Colasanti and Sundaresan, 2000). The discovery that CO is expressed in the vascular system of the leaves (in the phloem companion cells) and induces *FT* in this tissue, suggests that the florigen signal is downstream or at the same level as *CO* (Takada and Goto, 2003; An *et al.*, 2004). Expression of *CO* from different promoters showed that CO triggers early flowering when expressed in the leaf phloem but not in the apex (An *et al.*, 2004, Ayre and Turgeon, 2004). These experiments convincingly suggested that CO acts from the leaves and that the florigen is downstream of *CO*. Accordingly, all *Citrus* spp. contigs that showed significant similarity to CO (Table 1; Figure 2) are formed exclusively by leaf-derived ESTs (with the exception of a *C. limonia* EST, CL06-C4-501-017-G07, which is derived from root tissues). As opposed to *C*O, its target gene *FT* can trigger early flowering when expressed either from the leaves or from the apex, suggesting either that FT itself is the florigen or that FT can induce the florigen synthesis both from leaves and the apex. Knowing that CO acts from the leaves to induce *FT* also raises many questions about the induction of *SOC1* and *LF*Y. In *Arabidopsis*, both *LFY* and *SOC1* expression increase at the apex during the floral transition (*SOC1* in the apex itself and *LFY* in the flower anlagen). To date, the function of other CO-like family members is largely unknown. Nevertheless, there is evidence that COL proteins may directly interact with CO to provide the correct control of flowering time mediated by light (Martin *et al.*, 2004). It will be interesting to access the expression patterns of the different *Citrus CO*-like family members to see if their transcription correlates with the transition to the reproductive phase.

Conclusions and Perspectives

There are physical, chemical, and biological signals that contain information for the onset of flowering. The four known pathways that respond to these signals have been characterized in *Arabidopsis* and some herbaceous model plants. The genetic-based framework of these pathways in these model plants can now be assessed by molecularly cloning each member. This task is generally much more difficult and time-consuming in woody plants due to their extended life cycles. Here we present the initial construction of a genetic framework containing the molecular elements which putatively control the flowering pathways in seven different *Citrus* species. Precise characterization of the *in situ* expression patterns of all these *Citrus* spp putative flowering-time genes will be important to understanding their roles in the flowering process, opening the way for the manipulation of their expression patterns in the future. The function of these elements can now be tested in heterologous systems, such as *Arabidopsis*, via transgenic approaches. We believe our results will be a valuable source for future research on the control of flowering and of biennial fruit bearing patterns in *Citrus*.

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Internet Resources

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