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Research Article

In silico analysis of the endogenous time-keeping mechanism in citrus

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Abstract

The endogenous time-keeping mechanism is responsible for organizing plant physiology and metabolism according to periodic environmental changes, such as diurnal cycles of light and dark and seasonal progression throughout the year. In plants, circadian rhythms control gene expression, stomatal opening, and the timing component of the photoperiodic responses, leading to enhanced fitness due to increased photosynthetic rates and biomass production. We have investigated the citrus genome databases of expressed sequence tags (EST) in order to identify genes coding for functionally characterized proteins involved in the endogenous time-keeping mechanism in *Arabidopsis thaliana*. Approximately 180,000 EST sequences from 53 libraries were investigated and 81 orthologs of clock components were identified. We found that the vast majority of *Arabidopsis* circadian clock genes are present in citrus species, although some important components are absent such as SRR1 and PRR5. Based on the identified transcripts, a model for the endogenous oscillatory mechanism of citrus is proposed. These results demonstrate the power of comparative genomics between model systems and economically important crop species to elucidate several aspects of plant physiology and metabolism.

Key words: central oscillator, circadian clock, data mining, photoperiodic responses, regulatory feedback loop.

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Introduction

The rotation of the earth on its axis causes alternating cycles of light and dark at a period of approximately 24 h. The physiology, metabolism and behavior of most organisms are drastically affected by these daily environmental changes that lead to apparent biological oscillations known as diurnal rhythms. The conceptual model of circadian clocks consists of an entrainment or input pathway that synchronizes the pacemaker with its environmental surroundings, a central oscillator that generates and sustains rhythmicity, and multiple output pathways that link the oscillator to the organism's physiology and metabolism (Bell-Pedersen *et al.*, 2005).

In plants, the entraining stimulus is mainly light, with a smaller contribution from temperature (Salomé and McClung, 2005; Edwards *et al.*, 2006). Accordingly, the input pathway to the plant clock consists mostly of photoreceptor families (Millar, 2004), ubiquitously found in plants, including economically important species (Santelli and Sivieiro, 2001; Hecht *et al.*, 2005).

The central oscillatory mechanism of all known circadian clocks is dependent on autoregulatory feedback loops (Bell-Pedersen *et al.*, 2005). In plants, the *Arabidopsis* circadian clock mechanism has been partially elucidated. However, circadian control of metabolism and physiology is evident in most plant species, including citrus, where several economically important features are circadianly regulated, such as flavonoid and terpenoid metabolism regulation and essential oil composition (Lee and Castle, 2001; Lin *et al.*, 2002; Frizzo *et al.*, 2004). Moreover, gene expression and developmental processes in citrus have also been demonstrated to be under control of an endogenous time-keeping mechanism (Smith and Wareing, 1972; Abied and Holland, 1994).

The negative limb of the *Arabidopsis* clock consists of two highly similar single MYB-domain factors CCA1 (CIRCADIAN AND CLOCK ASSOCIATED1) and LHY (LATE ELONGATED HYPOCOTYL) (Wang and Tobin, 1998). The positive limb is the pseudo-response regulator TOC1 (TIMING OF CAB 1), also called PRR1 (*Arabidopsis thaliana* PSEUDO-RESPONSE REGULATOR 1), its orthologs PRR5, PRR7 and PRR9 (Makino *et al.*, 2000; Strayer *et al.*, 2000; Alabadí *et al.*, 2001; Mizoguchi *et al.*, 2002), the MYB transcription factor LUX ARRHYTHMO (LUX)/PHYTOCLOCK 1 (PCL1) (Hazen *et al.*, 2005, Onai and Ishiura, 2005) and the novel protein ELF4 (EARLY FLOWERING 4) (Kikis *et al.*, 2005).

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The oscillatory mechanism depends on CCA1- and LHY-binding to the evening element (EE, AAATATCT) in the *TOC1* promoter, which represses transcription of the response regulator-like gene (Alabadí *et al.*, 2001). During the day, the levels of CCA1 and LHY decrease, releasing their repression on *TOC1* that peaks at dusk when the levels of CCA1 and LHY are at their trough. Subsequently, the increasing levels of TOC1 during the evening induce its function as a positive regulator of *CCA1* and *LHY* expression through an as yet uncharacterized mechanism (Alabadí *et al.*, 2001, Matsushika *et al.*, 2002; Mizoguchi *et al.*, 2002; Harmer and Kay, 2005; Hazen *et al.*, 2005). Several other gene products are required for *CCA1* and *LHY* expression, including GIGANTEA (GI), ELF3, ELF4, and LUX (Park *et al.*, 1999; Doyle *et al.*, 2002; Mizoguchi *et al.*, 2002; Hazen *et al.*, 2005); thus, generating the interlocked feedback loops. The second proposed loop consists of *TOC1* transcriptional activation by GI, which in turn, is repressed by TOC1 (Locke *et al.*, 2005). A third autoregulatory loop is hypothesized to consist of CCA1 and LHY as positive regulators of TOC1-family members *PRR5*, *PRR7*, and *PRR9* (Farré *et al.*, 2005; Harmer and Kay, 2005). Currently, distinct lines of evidence support the idea that PRR5/7/9 are negative regulators of CCA1/LHY: (i) *CCA1* and *LHY* transcripts accumulate in *prr7* and *prr7 prr9* mutants (Farré *et al.*, 2005) and, (ii) *CCA1* is constitutively transcribed in the arrhythmic *prr5 prr7 prr9* triple mutant (Nakamichi *et al.*, 2005b). Moreover, PRR5/7/9 and TOC1 are thought to be mutually repressive and partially redundant (Farré *et al.*, 2005; Nakamichi *et al.*, 2005a; Salomé and McClung, 2005).

Post-transcriptional regulation is involved in several processes of circadian timing in plants. CCA1 and LHY are phosphorylated by casein kinase II (CK2) in a manner similar to the pace-control mechanism of the mammalian circadian clock (Sugano *et al.*, 1998, Sugano *et al.*, 1999). The robustness of LHY oscillations has been attributed to post-translational modifications and proteasome-mediated degradation (Kim *et al.*, 2003; Song and Carré, 2005). A second type of posttranslational modification, implied in clock function, is protein acetylation; mutants lacking the N-acetylglucosamine transferase activity of SPINDLY (SPY) exhibit altered leaf movement rhythms (Tseng *et al.*, 2004). Recent evidence has demonstrated the crucial role of light- and clock-controlled proteolysis for the plant endogenous clock. The novel family of putative photoreceptors ZEITLUPE (ZTL), LOV KELCH PROTEIN 2 (LKP2) and FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1) provides a direct link between the central oscillator and ubiquitin-mediated protein degradation (Nelson *et al.*, 2000; Somers *et al.*, 2000; Schultz *et al.*, 2001). ZTL is a component of the Skp1-Cullin-F-box (SCF) complex that recruits TOC1 for proteasomal degradation (Somers *et al.*, 2000; Más *et al.*, 2003b; Han *et al.*, 2004).

Day length is measured by the integration of circadian clock and light perception information at the level of CONSTANS (CO) protein expression and activity (Suárez-López, 2001; Yanovsky and Kay, 2002; Imaizumi *et al.*, 2003; Valverde *et al.*, 2004; Imaizumi *et al.*, 2005). *CO* directly regulates the expression of *FLOWERING LOCUS T* (*FT*) in *Arabidopsis* and of its homolog *HEADING DATE 3a* (*HD3a*) in rice (Putterill *et al.*, 1995; Kobayashi *et al.*, 1999; Samach *et al.*, 2000; Onouchi *et al.*, 2000; Izawa *et al.*, 2002; Kojima *et al.*, 2002), which promotes the expression of meristem-identity genes triggering the transition from vegetative to reproductive development at the shoot apical meristem. The expression and activity of CO is regulated by the internal circadian clock of the plant, so that *CO* mRNA transcription is restricted to 12 h after dawn until around dawn of the following day (Suárez-Lopez *et al.*, 2001; Valverde *et al.*, 2004). Under long day conditions, *CO* mRNA is therefore present during the day, and when the CO protein is translated it is stabilized in the nucleus of the cell and will activate the expression of other genes (An *et al.*, 2004; Valverde *et al.*, 2004). Under short day conditions, *CO* mRNA is only transcribed during the night because the days are shorter than 12-h long, and the dark translated CO protein is rapidly broken down, and unable to activate gene expression (Valverde *et al.*, 2004). Thus, early flowering of *Arabidopsis* occurs in the long days of spring and early summer through the coincidence of circadian clock control of *CO* mRNA expression and direct exposure of the plant to light (Yanovsky and Kay, 2002; Valverde *et al.*, 2004). In rice, *CO* gene homolog, called *HEADING DATE 1* (*HD1*) also has a key role in flower timing control. However, the function of *HD1* in rice is reversed, thus, promoting flowering on short days. The rice CO homolog protein is activated at the end of a long day; but instead of activating other genes as it does in *Arabidopsis*, HD1 represses gene activity and flowering under longer photoperiods (Yano *et al.*, 2001; Izawa *et al.*, 2002). In contrast, under short day conditions HD1 repression is absent, allowing HD3a to promote flowering (Onouchi *et al.*, 2000; Izawa *et al.*, 2002; Kojima *et al.*, 2002). In temperate forest trees, the *CO/FT* regulatory module has been demonstrated to control of flowering time in response to variations in daylength; and, unexpectedly, it also controls the short-day-induced growth cessation and bud set occurring in the fall (Böhlenius *et al.*, 2006). Thus, annual species and trees appear to have a common time keeping mechanism to control development during the progression of the seasons. The conserved functionality of key meristem identity genes between *Arabidopsis* and citrus has been demonstrated (Endo *et al.*, 2005; Pillitteri *et al.*, 2004; Pena *et al.*, 2001). However, the regulatory mechanisms of their induction, including light quality and photoperception, temperature and day length changes remain elusive.

Recent evidence has demonstrated that photoperiodic induction of synchronous vegetative bud break or flowering is also common in tropical plants (Borchert *et al.*, 2005). At tropical latitudes, changes in day length that are large enough to affect plant development occur around the equinoxes. Therefore, a single annual period of synchronous flowering after the autumn equinox indicates the induction of flowering in `short-day plants' in response to declining day length; whereas, `long-day plants' flowering and vegetative bud break is induced by an increasing day length that occurs after the spring equinox (Borchert *et al.*, 2005). The molecular mechanism leading to day length measuring in the tropics has been hypothesized to involve the integration of photoperception and the endogenous clock as observed in temperate species (Borchert *et al.*, 2005).

The present work was designed to investigate the circadian temporal programming and its integrative pathways to physiology and metabolism in citrus, employing combined *in silico* EST profiling and domain structural data analysis. The results presented here demonstrate that the vast majority of the gene products involved in circadian timing in *Arabidopsis* are present in citrus species. Moreover, genetic distance and domain structure analyses have uncovered extensive amino acid sequence conservation, providing indications of functional equivalence of *Arabidopsis* and citrus transcripts. Taken together, the results have demonstrated the feasibility of incorporating *in silico* analyses for gene discovery in non-model species.

Material and Methods

Database searches and alignments

Homologs of the *Arabidopsis thaliana* circadian clock were identified in BLAST searches (Altschul *et al.*, 1997) against citrus EST databases (CitEST), which consist of approximately 180,000 ESTs obtained from the sequencing of 53 specific libraries. Data validation was performed by tBLASTx and tBLASTn searches of the retrieved sequence against the locally built GenBank database at CitEST. Sequences failing to retrieve the original sequence used to query the database were eliminated from the projects. The resulting alignments were filtered by a threshold e-value of 1e-15 and the hits were further analyzed according to functional domain description. Validated sequences were translated and protein (deduced amino acid) alignments were performed using ClustalX (Thompson *et al.*, 1997). When necessary, alignments were manually adjusted using Lasergene MegAlign (DNASTAR, Madison, WI, USA).

Motif analysis and *in silico* characterization

The identified homologs were further investigated for the presence and sequence conservation of recognizable functional domains described in several protein analysis and gene function databases (European Bioinformatics Institute-European Molecular Biology Laboratory - EMBL-EBI; Expert Protein Analysis System - ExPaSy from Swiss Institute of Bioinformatics - SIB; Gene Ontology database - GO; Protein Families database - Pfam).

Phylogenetic analysis

The functionality of citrus genes in comparison to their *Arabidopsis* counterparts was assessed by genetic distance and phylogenetic studies. Phylogenetic analyses were performed using distance and parsimony methods in the software PAUP* 4.0b10, using the software default parameters. Resampling bootstrap trees containing 1000 random samples were constructed using PSIGNFIT software. Modular functional domains were employed for genetic distance studies for genes previously characterized as having divergent regions and conserved blocks.

In silico gene expression analysis

Qualitative gene expression profiling was performed by *in silico* analyses of the afore mentioned citru*s* EST databases using virtual northern blot analyses. The gene of interest was used in queries against reference sequence databases, generating an alignment of the input gene to its paralogs. The resulting alignment was then used to find sequences in the entire mRNA input that are specific to the gene (probe). The resulting alignments were collectively used to query the EST database again using BLAST. This heuristic attempts to avoid false-positives, or ESTs from a paralog of the input gene rather than the gene itself. The identity numbers of the ESTs matching the probes were recovered and CitEST was used to find the names of the libraries from which those ESTs were derived. The frequency of reads of each EST contig in a given library was calculated and normalized according to the total number of reads from the investigated library and the total number of reads in all libraries. A correlation matrix between EST contigs and libraries was then generated and gene expression patterns among ESTs and libraries were obtained by hierarchical clustering based on a Spearman Rank correlation matrix using Cluster v.2.11 software (Eisen *et al.*, 1998), by substituting the clusters by their average expression pattern. Graphic outputs were generated using Tree View v.1.6 software and presented in grayscale.

Results and Discussion

The essential components of the circadian clock input pathway, gating mechanism, central oscillator, the output and photoperiod integrative pathways from *Arabidopsis thaliana,* and to a lesser extent *Oryza sativa*, were compiled and the protein sequences of the main components were employed to search citrus EST databases. The identified hits were ranked according to the deduced amino acid sequence identity to the functionally characterized protein used as bait and further analyzed for their functionality.

This way, 81 citrus ESTs and EST contigs that are similar to components of the endogenous time-keeping mechanism and its ancillary regulation loops were identified and functionally assigned to gene ontology classes (Figure 1). From this total, 20 transcripts correspond to input pathway photoreceptors, proteins involved in the gating mechanism and in ancillary entrainment loops in plants (Table 1). Twenty-nine citrus sequences showed extensive sequence conservation to components of the central oscillator mechanism (Table 2) and 32 resemble proteins involved in the integration between the pacemaker and plant metabolism and physiology (Table 3). Interestingly, some important components of the time keeping-mechanism from *Arabidopsis* are absent from citrus sequence databases, such as the input gene *SRR1* (AT5G59560; Staiger *et al.*, 2003) and the TOC1/PRR1-quintet member *PRR5* (AT5G24470, Nakamichi *et al.*, 2005a). Moreover, citrus and *Arabidopsis* gene families, including the DOF transcription factor *CDF1* and MADS-box *CONSTANS*, appear to have undergone differential expansion events.

Input pathway

Light entrainment pathway

Four of the five *Arabidopsis* phytochromes (PHYA, PHYB, PHYD and PHYE) function in an additive manner in the red-light (R) input to the clock (Devlin and Kay, 2000). CRYPTOCHROME 1 (CRY1) acts as the main clock input photoreceptor under high and low fluences of blue light (B) whereas both CRY1 and CRY2 redundantly function at intermediate fluences of B (Devlin and Kay, 2000; Somers *et al.*, 1998). Surprisingly, clock entrainment under white light seems to be dependent on the physical interaction of CRY2 and PHYB and their localization in nuclear speckles (Más *et al.*, 2000). Moreover, *cry1* mutant plants also show altered entrainment under red light suggesting that CRY1 is required for PHYA signaling to the clock in both R and B (Devlin and Kay, 2000). Quadruple *Arabidopsis* mutants lacking *phyA, phyB, cry1* and *cry2* are able to maintain circadian rhythmicity (Yanovsky *et al.*, 2000), suggesting that additional components participate in the light input pathway to reset the clock. The rhythmic expression of *PHY* and *CRY* genes (Tóth *et al.*, 2001) has been hypothesized to contribute to the gating mechanism on the input to the clock, although the bulk PHY protein levels do not oscillate (Sharrock and Clack, 2002).

Loss-of-function *ztl* mutants show fluence-rate dependent period alteration, indicating that ZTL has a role in the light input pathway (Somers *et al.*, 2000). ZTL mRNA abundance is not clock regulated, but its protein levels peak around dusk and reach trough levels around dawn (Kim *et al.*, 2003). The rate of proteasome-mediated degradation of ZTL varies during the course of the day: ZTL is more stable at dusk, close to its peak value, and is more rapidly degraded at dawn when it reaches its trough. F-box proteins provide specificity to proteasomal degradation pathways by interaction and polyubiquitination-mediated degradation of determined targets. ZTL has been demonstrated to recruit the oscillator component TOC1 for degradation (Somers *et al.*, 2000; Más *et al.*, 2003b; Han *et al.*, 2004). In *ztl* mutants, TOC1 levels are elevated and only weakly rhythmic, demonstrating that ZTL is critical for TOC1 proteolysis and rhythmicity. Correspondingly, ZTL overexpression induces a dosage-dependent period shortening (Han *et al.*, 2004). Citrus photoreceptor families and the components of their proteolytic degradation pathway have been extensively discussed in the accompanying paper on photoperception and light signal transduction.

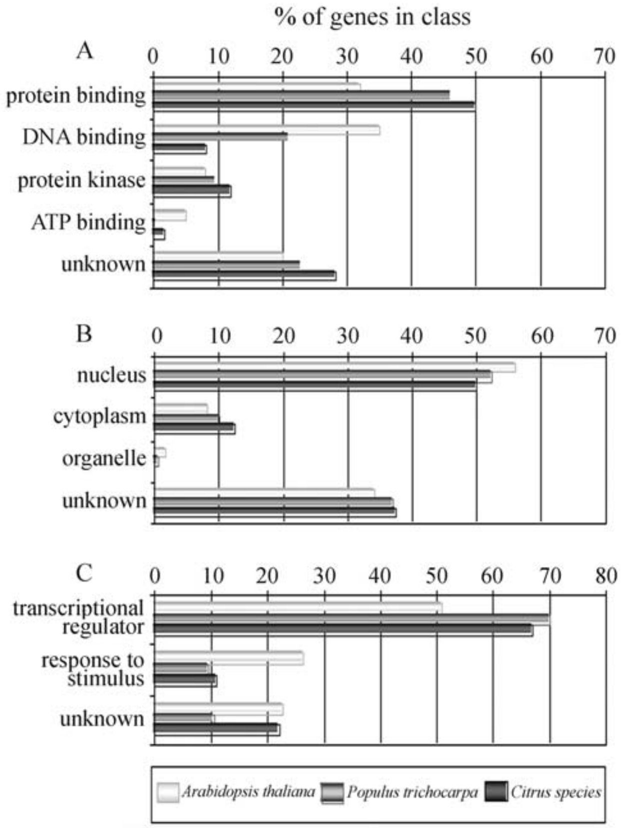

Figure 1 - Functional classification of citrus transcripts associated to the endogenous time-keeping mechanism using gene ontology. **A.** molecular function; **B.** cellular component; **C.** biological process. Assignments are based on the data available at the TIGR *Arabidopsis thaliana* Gene Index version 13.0.

Gating mechanism

In *Arabidopsis*, light-conditional arrhythmic behavior is observed in the absence of ELF3 (EARLY FLOWERING 3) and TIC (TIME FOR COFFEE) proteins (Hicks *et al.*, 1996; Hicks *et al.*, 2001; McWatters *et al.*, 2000; Covington *et al.*, 2001; Liu *et al.*, 2001; Hall *et al.*, 2003). Interestingly, *elf3* null mutants are arrhythmic at different phases of the oscillation when compared to *tic* mutants and double *elf3 tic* mutants lack rhythmicity under light and dark conditions (Hall *et al.*, 2003). Thus, these

Table 1 - *Citrus* ESTs with homology to genes involved in the input pathway to the circadian clock in *Arabidopsis thaliana*.

Arabidopsis thaliana		*Cit*EST			Protein motifs[d] and biological process	References
Name[a]	Gene	EST (n. reads)[b]	%[c]	e value		
Entrainment photoreceptors						
CRY1	AT4G08920	C10-CR (2) S1-CS	23.3 (P: 48%) 74.6 (FL)	3e-91 1e-106	FAD binding domain, DNA photolyase, B photoreceptor, circadian clock entrainment	Cashmore *et al.*, 1999
PHYA	AT1G09570	S2-CS S3 - CS	58.3 (FL) 34.7 (FL)	6e-86 4e-81	PAS1, PAS2, chromophore binding domain, HKL domain, R/FR photoreceptor	Sharrock and Quail, 1989
PHYB	AT2G18790	S4-CR S5-CS	15.3 (FL) 8.1 (P: 72%)	3e-75 3e-55	*PHY* family	Boylan *et al.*, 1994
FKF1	AT1G68050	C5-CS (2) C6-CR/CS (3)	76.3 (FL) 19.3 (FL)	1e-142 1e-46	Kelch repeats, F-box domain, LOV domain, putative photoreceptor, photoperiodic flowering control, circadian clock	Nelson *et al.*, 2000; Imaizumi *et al.*, 2005
LKP2	AT2G18915	S6-CS	22.0 (FL)	1e-76	*ZTL/FKF1/LKP2* family	Schultz *et al.*, 2001
ZTL	AT5G57360	C5-CS (2)	29.7 (FL)	8e-71	*ZTL/FKF1/LKP2* family	Somers *et al.*, 2000
Ancillary factors						
ELF3	AT2G25930	C1-CS/CR (2) C2- CR (2) S7-CA S8-PT	9.8 (FL) 10.6 (FL) 19.4 (FL) 7.5 (FL)	6e-24 1e-18 2e-20 1e-15	novel nuclear protein, hydroxyproline-rich glycoprotein family protein, zeitnehmer, rhythmic input to the clock	Carré, 2002
GI	AT1G22770	C1-CS (6) C2-CS/CR (2) C3-CG/CR/CS (4) S9-CS S10-CR	31.2 (FL) 25.4 (P: 73%) 25.4 (FL) 15.3 (P: 76%) 18.8 (P:73%)	5e-144 2e-45 1e131 2e-56 5e-55	putative membrane spanning domains, nuclear localization, phyB signal transduction, photoperiodic control of flowering time	Fowler *et al.*, 1999
PIF3	AT1G09530	C6-CG/LT (2)	25.5 (FL)	4e-27	transcriptional regulator, bHLH domain, photomorphogenesis	Ni *et al.*, 1999

[a] Gene name abbreviations: *CRY*: cryptochrome; DASH: *Drosophila*, *Arabidopsis*, *Synechocistis*, human; *ELF*: early flowering; *FKF1*: F-box, Kelch repeat, Flavin-binding protein1; *GI*: GIGANTEA; *LKP2*: LOV domain, Kelch repeat protein2; *PCL*: phytoclock; *PHY*: phytochrome; *PIF*: phytochrome interacting factor; *ZTL*: Zeitlupe.

[b] C : contig (number); S: singlet (number); CA: *Citrus aurantium*; CG: *Citrus aurantifolia*; CR: *Citrus reticulata*; CS: *Citrus sinensis;* LT: *Citrus latifolia;* PT: *Poncirus trifoliata*.

[c] Identity percentage at the amino acid level; (FL), full-length cDNA; (P), partial cDNA: percentage of *Arabidopsis* cDNA.

[d] Functional domain abbreviations: FAD: flavina adenosine dinucleotide; LOV: light, oxygen, voltage subtype PAS domain; PAS: Per, ARNT, Sym domain; HKL: histidine kinase-like.

proteins are likely to be responsible for the gating mechanism at different times of the day and are termed *Zeitnehmer* (from German, *time taker*). The ELF3 protein contains no recognizable domains and the *TIC* gene remains to be identified (Liu *et al.*, 2001; Hall *et al.*, 2003). In citrus transcriptome, two EST contigs from *Citrus sinensis* and *C. reticulata*, one from *C. aurantium* and one from *Poncirus trifoliata,* sharing moderate sequence similarity to ELF3, were identified (Table 2). The lack of functionally characterized domains in *Arabidopsis* ELF3 prevents further considerations about the role of the citrus homologs.

Temperature entrainment pathway

Temperature signaling to the clock is much less well defined; however, gene expression and cotyledon movement have been demonstrated to be effectively entrained by temperature cycles in *Arabidopsis* (Michael and McClung, 2002; Salomé *et al.*, 2002; Salomé and McClung, 2005). The pseudo-response regulators PRR7 and PRR9 are essential for temperature-mediated clock synchronization, as *prr7 prr9* double mutants fail to entrain to temperature cycles that are effective to entrain wild type plants (Salomé and McClung, 2005). Citrus genome has three cDNAs similar to PRR7, two from *C. sinensis* and one from *P. trifoliata,* and two EST contigs highly similar to PRR9 (Table 3). In *Arabidopsis* and rice, these proteins participate in the central oscillator mechanism as well (Farré *et al.*, 2005; Murakami *et al.*, 2005; Nakamichi *et al.*, 2005b). The citrus PRR homologs are discussed in-depth in the following section. Interestingly, the photoperiodic transcriptional activator, GIGANTEA (GI) and the MADS-box protein FLC have been assigned to a quantitative trait locus (QTL) responsible for a substantial portion of natural variation in temperature-mediated circadian clock entrainment (Ed-

Table 2 - *Citrus* ESTs with homology to genes involved in the central oscillator of the circadian clock in *Arabidopsis thaliana*.

Arabidopsis thaliana		*Cit*EST			Protein motifs[d] and biological process	References
Name[a]	Gene	EST (n. reads)[b]	%[c]	e value		
CCA1	AT2G46830	C3-CS (2) C7-CS/PT (8) C16-PT (4) C18-CS/CR (4) S11-CR	23.3 (FL) 24.6 (FL) 65.2 (FL) 23.4 (FL) 64.5 (FL)	9e-46 4e-35 2e-51 2e-26 2e-36	MYB-related transcription factor, circadian rhythms, central oscillator component, negative feedback loop	Green and Tobin, 1999; Wang and Tobin, 1998
CK2	AT3G60250	C2-CR/CS (6) S12-CG S13-CR S14-CS	26.2 (FL) 64.9 (FL) 16.9 (FL) 16.4 (FL)	1e-34 6e-43 3e-30 1e-32	regulatory (β) subunit of the protein kinase CK2, circadian rhythms regulator, transcription factor binding and phosphorylation	Sugano *et al.*, 1998; Sugano *et al.*, 1999
DET1/ FUS2	AT4G10180	S15-CS S16-LT	14.3 (FL) 12.5 (P: 87%)	3e-55 2e-57	nuclear localized photomorphogenesis repressor	Chory and Peto, 1990
ELF4	AT2G40080	S17-CR S18-CS S19-PT	40.9 (FL) 11.4 (FL) 11.4 (FL)	2e-32 3e-28 1e-27	no recognizable domain, circadian clock central oscillator ancillary loop, photomorphogenesis	Doyle *et al.*, 2004; Kikis *et al.*, 2005
LHY	AT1G01060	C11-CS/CR (22) C14-PT (2) S20-PT S21-CR	26.5 (FL) 16.9 (FL) 20.1 (FL) 16.8 (FL)	1e-55 6e-40 3e-36 5e-32	MYB-related transcription factor, circadian rhythms, central oscillator component, negative feedback loop	Schaffer *et al.*, 1998; Green and Tobin, 1999
LUX /PCL1 family[e]	AT3G46640	C1-CS (6) (LUX3) C2- CS/PT (3) (LUX/PCL) S22-CR (LUX5) S23-CR (LUX5)	12.9 (FL) 11.5 (FL) 14.2 (P: 45%) 30.7 (P: 75%)	4e-72 6e-54 8e-52 1e-54	single MYB DNA-binding domain (type SHAQKYF), putative activator of CCA1/LHY expression, repressed by CCA1/LHY	Hazen *et al.*, 2005; Onai and Ishiura, 2005
PRR1/ TOC1	AT5G61380	S24-CS	68.0 (P: 85%)	5e-66	pseudo-response regulator, response regulator receiver domain, circadian rhythm, photomorphogenesis	Alabadi *et al.* 2001
PRR3	AT5G60100	C3-CS/CR/LT (8) S25-CS S26-CS	72.0 (FL) 70.7 (FL) 64.5 (FL)	2e-37 1e-21 2e-22	*PRR* family	Matsushita *et al.*, 2002; Murakami *et al.*, 2004
PRR7	AT5G02810	S27-CS S28-CS S29-PT	68.1 (P: 73%) 59.2 (FL) 56.5 (FL)	3e-31 9e-22 6e-25	*PRR* family	Nakamichi *et al.*, 2005
PRR9	AT2G46790	C1-CS (2) C2-CS/CR (3)	80.0 (FL) 77.6 (FL)	2e-39 6e-29	*PRR* family	Nakamichi *et al.*, 2005
SPY	AT3G11540	C1-CS/CR (7) S30-CR S31-CS	27.4 (P: 91%) 25.0 (P: 75%) 15.5 (P: 77%)	2e-65 4e-43 3e-28	N-acetyl glucosamine transferase	Tseng *et al.*, 2004

[a] Gene name abbreviations: *PRR*: *Arabidopsis* pseudo response regulator; *CCA*: circadian and clock associated; *CK*: casein kinase II regulatory subunit; *ELF*: early flowering; *LHY*: long hypocotyl; *SPY*: spindly; *TOC*: timing of CAB.

[b] C: contig (number); S: singlet (number); CA: *Citrus aurantium*; CG: *Citrus aurantifolia*; CR: *Citrus reticulata*; CS: *Citrus sinensis;* LT: *Citrus latifolia;* PT: *Poncirus trifoliata*.

[c] Identity percentage at the amino acid level.

[d] Functional domain abbreviations: FAD: flavina adenosine dinucleotide; LOV: light, oxygen, voltage subtype PAS domain; PAS: Per, ARNT, Sym domain; HKL: histidine kinase-like.

[e] LUX/PCL1 family members in *Arabidopsis thaliana*: AT5G59570, AT5G05090, AT3G10760, AT2G40970.

wards *et al.*, 2005). Five citrus transcripts, whose deduced amino acid sequences share significant homology to GI and GI-like proteins, and three FLC homologs were identified (Table 2 and Table 3, respectively). Thus, the *Arabidopsis* components of the temperature-mediated clock entrainment pathway are present in citrus transcriptome databases.

Central oscillator

The simplified model for the transcriptional/translational negative-feedback loop of the *Arabidopsis* circadian clock consists of the reciprocal regulation between the highly similar MYB-type transcription factors CCA1/LHY in the negative limb and TOC1 and the novel protein ELF4

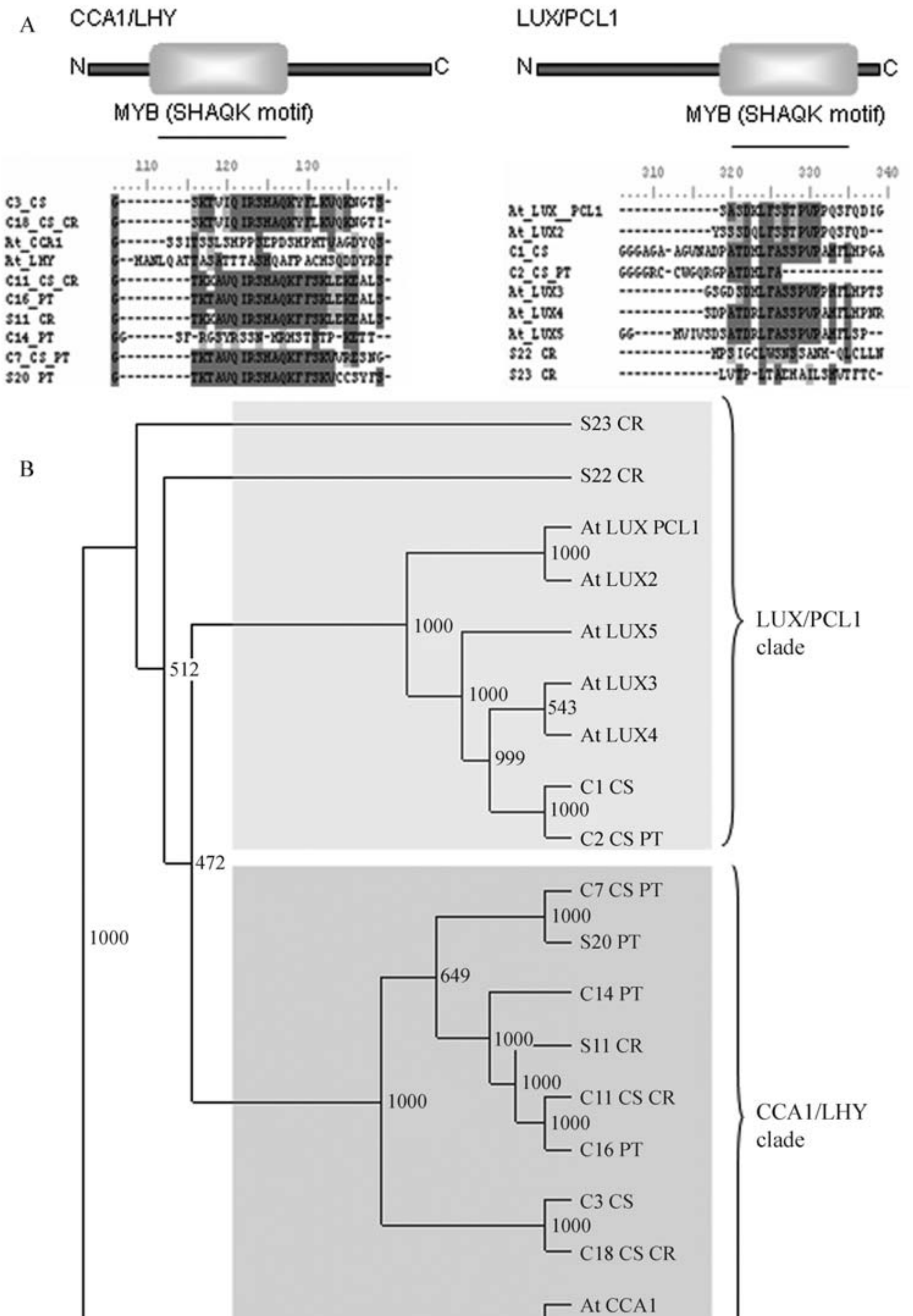

Figure 2 - MYB-type transcription factors involved in the circadian clock in citrus and *A. thaliana*. **A.** Schematic representation of CCA1/LHY and LUX protein domains and MYB domain alignment in citrus and *A. thaliana*. **B.** Phylogenic analysis of citrus and *Arabidopsis* CCA1/LHY and LUX protein families. Neighbor-joining tree for citrus deduced amino acid and *Arabidopsis* full length sequences were aligned with ClustalX. Bootstrap values are indicated above each branch. At, *Arabidopsis thaliana*; C Number, contig number; CS, *Citrus sinensis*; CR, *Citrus reticulata*; PT, *Poncirus trifoliata*.

in the positive limb (Strayer *et al.*, 2000; Kikis *et al.*, 2005; McClung, 2006). In citrus transcriptome databases, nine homologs to the TOC1/PRR1 family of pseudo-response regulators, nine cDNAs whose deduced amino acid sequences are similar to the MYB-type transcription factors CCA1 and LHY, and three similar to ELF4 were present (Table 3). Moreover, *in silico* analyses have identified four homologs of the LUX/PCL1 family, which are hypothesized to function on an interlocked loop to activate CCA1 and LHY transcription (Hazen *et al.*, 2005; Onai and Ishiura, 2005), four homologs of CK2, a casein kinase responsible for CCA1 phosphorylation (Daniel *et al.*, 2004)

Table 3 - *Citrus* ESTs with homology to genes involved in the integration of the circadian clock mechanism and the generation of output responses in *Arabidopsis thaliana*.

Arabidopsis thaliana		*Cit*EST			Protein motifs[d] and biological process	References
Name[a]	Gene	EST (n. reads)[b]	%[c]	e value		
CDF1 and *CDF* family[e]	AT5G62430	C4-CS/PT (3)	33.3 (FL)	3e-37	DOF-type zinc finger, H-protein promoter binding factor-2a, interacts with LKP2 and FKF1, repressor of CONSTANS	Imaizumi *et al.*, 2005
		C10- CA/CR (2)	36.9 (FL)	4e-46		
		S1-CS	43.2 (FL)	3e-56		
		S2-PT	33.3 (FL)	2e-45		
		S3-PT	16.7 (FL)	6e-31		
		S4-PT	16.7 (FL)	5e-46		
CO	AT5G15840	C5-CG/CS (4)	54.7 (FL)	3e-58	Zn finger C2HC5 B-box DNA binding motif, CCT motif	Putterill *et al.*, 1995, Suárez-López *et al.*, 2001
COL1 and *COL* family[f]	AT5G15850	C4-CS/CR (24)	62.9 (FL)	7e-83	*CONSTANS* family	Robson *et al.*, 2001, Griffiths *et al.*, 2003
		(COL2)	40.0 (FL)	1e-39		
		C1-CS (2)	38.4 (FL)	8e-39		
		(COL7)	29.3 (FL)	1e-25		
		C7-CR (2)	35.1 (FL)	9e-28		
		(COL16)	29.4 (FL)	2e-26		
		C13-PT(2)	57.0 (FL)	5e-79		
		(COL5)	50.7 (FL)	3e-70		
		C14-CR/CA (3)	48.4 (P: 87%)	2e-67		
		(COL15)	42.6 (FL)	6e-64		
		C15-LT (2)	37.5 (FL)	2e-31		
		(COL7)	31.6 (P: 77%)	4e-30		
		S1-CS (COL1)	29.3 (FL)	1e-25		
		S2-CS (COL7)	27.8 (FL)	7e-24		
		S3-PT (COL1)				
		S4-CR (COL13)				
		S5-CL (COL4)				
		S6-CS (COL4)				
		S7-CS (COL2)				
		S8-PT (COL7)				
ELF7	AT1G79730	C1-CR/LT (3)	19.7 (FL)	1e-26	PAF1 homolog (yeast complex associated to RNA pol II, FLC and FLM regulator	He *et al.*, 2004
ELF8	AT2G06210	C1-CS (4)	34.4 (FL)	1e-43	CTR9 homolog (yeast PAF1 complex), FLC expression induction	He *et al.*, 2004
FD	AT4G35900	S1-CG	17.8 (FL)	5e-16	bZIP transcription factor, flowering promoter, interacts with FT	Abe *et al.*, 2005; Wigge *et al.*, 2005
FLC	AT5G10140	C1-CS (4)	40.8 (FL)	7e-33	MADS-box protein, K-box transcription factor, floral transition repressor, temperature integration to circadian clock	Edwards *et al.*, 2006
		S1-CS	37.8 (FL)	7e-25		
		S2-CS	39.3 (P: 83%)	8e-23		
FT/	AT1G65480	S1-CS	23.5 (FL)	2e-51	phosphatidylethanolamine-binding protein motif, induced by long days in *Arabidopsis*, flowering promoter, target of CO and acts upstream of SOC1	Izawa *et al.*, 2002, Halliday *et al.*, 2003
TSF	AT4G20370		21.7 (FL)	2e-51		
PFT1	AT1G25540	C2-CS/CR (6)	68.6 (P: 53%)	1e-148	von Willebrand factor type A, glutamine-rich C-terminal	Cerdán and Chory, 2003
SOC1	AT2G45660	S1-PT	22.4 (P: 75%)	1e-51	K-box region, SRF-type transcription factor, bZIP DNA binding motif, plant DUF1319	Onouchi *et al.*, 2000
		S2-PT	22.5 (P: 77%)	1e-51		

[a] Gene name abbreviations: *CDF*: cycling DOF factor; *CO*: CONSTANS; *COL*: CONSTANS-like, *ELF*: early flowering; *FD*: flowering locus D; *FLC*: flowering locus C; *FT*: flowering locus T; *PFT*: phytochrome and flowering time; *SOC*: suppressor of CONSTANS; *TSF*: twin sister of FT.

[b] C : contig (number); S: singlet (number); CA: *Citrus aurantium*; CG: *Citrus aurantifolia*; CR: *Citrus reticulata*; CS: *Citrus sinensis;* LT: *Citrus latifolia;* PT: *Poncirus trifoliata*.

[c] Identity percentage at the amino acid level; (FL), full-length cDNA; (P), partial cDNA: percentage of *Arabidopsis* cDNA.

[d] Functional domains abbreviations: bZIP: basic leucine zipper; CCT: *CONSTANS*, *CONSTANS*-like and *TOC1*; CTR: calcitonin receptor, DOF: DNA binding with one finger, DUF: domain of function unknown; MADS:, MCM1, AGAMOUS, DEFICIENS and SRF; PAF: platelet-activating factor; SRF: serum response factor; Zn finger: zinc finger DNA binding domain.

[e] CDF1 family members in *Arabidopsis thaliana*: AT3G47500, AT5G39660.

[f] COL family members in *Arabidopsis thaliana*: AT5G15850 , AT3G02380 , AT1G04500 , AT3G07650 , AT1G07050 , AT1G49130 , AT2G24790, AT2G33350, AT3G12890, AT3G21150, AT3G21890, AT4G15248 , AT5G24930 , AT5G48250, AT5G53420, AT1G06040.

and two SPY-like sequences (Table 3). Thus, at least two interlocked feedback regulatory transcription/ translation loops of the *Arabidopsis* central oscillator and two post-translational modification systems are present and likely to be functional in a citrus endogenous time-keeping mechanism.

Citrus transcriptome analyses revealed the presence of 13 cDNAs corresponding to *Arabidopsis* MYB-type of transcriptional regulators involved in the circadian clock central oscillator mechanism; namely, CCA1, LHY and the LUX/PCL1 family (Table 3, Figure 2). CCA1/LHY-like sequences are highly similar in *C. sinensis*, *C. reticulata* and *P. trifoliata*, sharing up to 62.2% of sequence identity (Figure 2A). Moreover, the sequences are highly similar to their *Arabidopsis* counterparts (Table 2, Figure 2A). Interestingly, the single-MYB LUX/PCL1 family in citrus is more divergent and appears to have undergone a differential expansion process (Figure 2B), as the highest sequence identity to *Arabidopsis* LUX proteins was of 30.7% and only four members were found, in contrast to the five members identified in *Arabidopsis* (Hazen *et al.*, 2005). *In silico* transcriptional analysis of citrus MYB-like genes with similarities to clock components demonstrated a high frequency of *CCA1/LHY* and *LUX* homologs in developing fruits and a similar expression pattern (Figure 2). However, the normalized levels of *LUX* transcripts were approximately 1.5 times smaller than the levels observed for *CCA1/LHY* (Figure 3).

The *Arabidopsis* PRR family consists of TOC1/PRR1 and four other members (PRR3, PRR5, PRR7 and PRR9) whose structural designs are very similar (Matsushika *et al.*, 2000) and include a receiver-like (or pseudo-receiver) domain at their N-terminus followed by a long specific intervening sequence, followed by another motif of about 50 amino acids at the very C-terminal end that is shared by the PRR family, termed the CCT motif (CONSTANS, CONSTANS-like and TOC1) (Figure 4A) (Mizuno and Nakamichi, 2005). In citrus, eight transcripts sharing extensive sequence similarity to *Arabidopsis* and *Oryza sativa* PRR were identified (Table 3). The identified transcript sequences are highly conserved at the CCT region and more divergent at the response regulator domain (Figure 4B, Figure 4C). Citrus PRR family is highly represented in fruit developmental stage libraries and shows an expression profile similar to CCA1/LHY and LUX (Figure 3). Interestingly, the transcripts of *PRRs* begin to accumulate after subjective dawn in the following order *PRR9-PRR7-PRR5-PRR3-PRR1*, separated by 2-3 h intervals (Matsushika *et al.*, 2000). In *Oryza sativa*, *PRR* family also consists of 5 members that present sequential transcriptional induction from subjective dawn and with approximately 2-3 h interval between

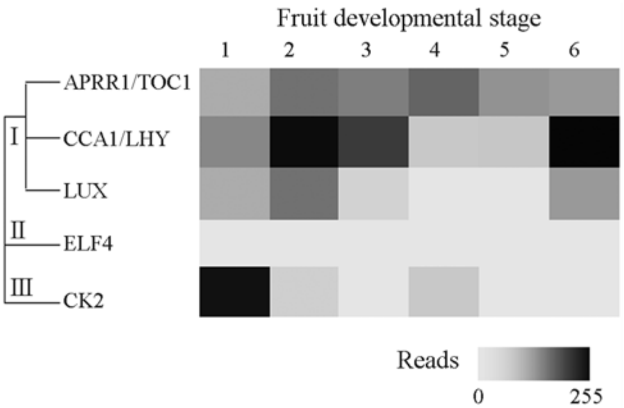

Figure 3 - *In silico* expression profile of the components of the central oscillator mechanism of the endogenous clock in citrus. The normalized number of reads for the transcripts in each library is represented as a grayscale. Fruit developmental stage libraries are represented as columns. Hierarchical clustering of the expression patterns is represented by roman numerals.

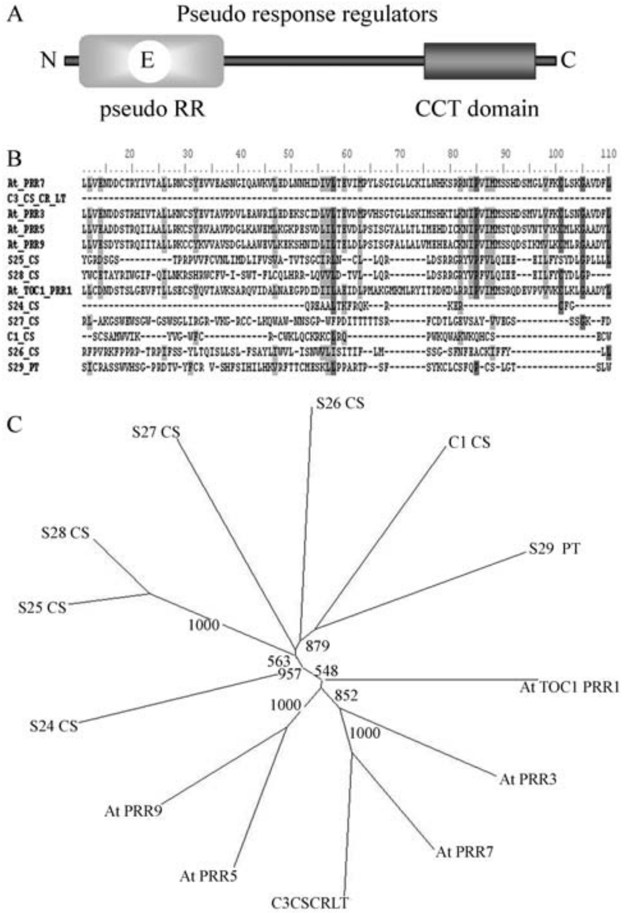

Figure 4 - Pseudo response regulator family of circadian clock components in citrus. **A.** Schematic representation of the domain structure of PRR proteins lacking the invariant phospho-accepting asparagine (replaced by a glutamate residue). **B.** Amino acid alignment of the pseudo-response regulator domain of circadian clock PRR proteins. **C.** Phylogenetic analysis of citrus and *A. thaliana* PRR family. Neighbor-joining unrooted tree for citrus deduced amino acid and *Arabidopsis* full length sequences were aligned with ClustalX. Bootstrap values are indicated inside each branch. At, *Arabidopsis thaliana*; C Number, contig number; S Number, singlet number; CS, *Citrus sinensis*; CR, *Citrus reticulata*; LT, *Citrus latifolia*, PT, *Poncirus trifoliata*.

them (Murakami *et al.*, 2005). Such sequential induction is preceded by *CCA1* transcriptional activation, resulting in waves of sequential expression of *CCA1-PRR9/7/5/3/1* (Mizuno and Nakamichi, 2005). These phenomena were referred to as "circadian waves of the PRR quintet"; however, the biological function of the coordinated induction of *CCA1* and *PRR* transcription remains elusive. The presence of the "circadian waves of the PRR quintet" in citrus remains to be investigated. TOC1/PRR1 and PRR9 have been demonstrated to participate in phytochrome-mediated photomorphogenesis regulation (Makino *et al.*, 2001; Ito *et al.*, 2003; Más *et al.*, 2003a; Ito *et al.*, 2005). The expression of citrus PRR in developing fruits suggests that they might be involved in light-regulated secondary metabolism, although further functional analysis is required.

Post-translation modification inducers

The post-translational modifications involved in the circadian mechanism in *Arabidopsis* are protein phosphorylation, proteolytic degradation and acetylation (McClung, 2006). Four *CK2*-homologous transcripts have been identified in citrus transcriptome (Table 3). As observed for other oscillator components investigated, CK2 homologs are frequent in libraries obtained from fruit developmental stages and present a unique expression pattern in comparison to other putative pacemaker components; they are highly induced in developing fruits in the first and second developmental stages (Figure 3). The ZTL/FKF1/LKP2 family, responsible for TOC1 ubiquitin-mediated degradation and regulation of photoperiodic responses, is also represented in citrus, as discussed in the accompanying paper on photoperception. Two singlets highly similar to the N-acetylglucosamine transferase SPY protein were identified in citrus (Table 3). Thus, it is likely that the post-translational mechanisms involved in circadian clock regulation in *Arabidopsis* are functional in citrus.

Clock outputs and integrative pathways

The endogenous time-keeping mechanisms allow organisms to adjust the timing of crucial developmental transitions to occur at the most favorable time of the year, leading to seasonal adaptation. The mechanism of day length measuring in higher plants is based on the integration of circadian clock and light perception, at the level of CO protein expression and activity (Suárez-López *et al.*, 2001; Yanovsky and Kay, 2002; Imaizumi *et al.*, 2003; Valverde *et al.*, 2004; Imaizumi *et al.*, 2005).

Arabidopsis CO and its rice homolog HD1 directly regulate the expression of *FT* and *HD3a*, respectively (Putterill *et al.*, 1995; Kobayashi *et al.*, 1999; Samach *et al.*, 2000; Onouchi *et al.*, 2000; Izawa *et al.*, 2002; Kojima *et al.*, 2002), which promote the expression of meristem-identity genes triggering the transition from vegetative to reproductive development at the shoot apical meristem (Komeda, 2004). Fifteen *CO*/*COL* homologs were identified in citrus transcriptome analysis (Table 3). The characteristic bipartite B-box Zn finger DNA binding domain is present at the N terminus of all citrus *CO* homologs (Figure 5A), although sequence divergence is observed in IIIa and IIIb sub-groups (Figure 5). A single EST contig from citrus lacked the B1 sub-domain and was clustered in the sub-group where the second B-box is replaced by a more divergent zinc-finger domain (Griffiths *et al.*, 2003) (Figure 5B). Similarly, the signature CCT domain is highly conserved in groups I, II and IIIa and more divergent in group IIIb (Figure 5B).

Flowering requires the integration of temporal and spatial information to restrict its initiation to specific locations. Recently, Abe *et al.* (2005) and Wigge *et al.* (2005) have demonstrated that the interaction between FT expressed in the leaves and the transcription factor FLOWERING LOCUS D (FD) occurs exclusively in the shoot apex and triggers the expression of floral identity genes in the new primordial. Moreover, Huang *et al.* (2005) have shown that upon CO-mediated photoperiodic activation, *FT* mRNA is transported from the leaves to the shoot meristem via the plant vascular tissue. Therefore, CO action in the phloem activates *FT* expression in leaves in a cell-autonomous manner, according to the observation that CO activates *FT* expression and promotes flowering only when expressed under the control of phloem-specific promoters in the leaf, but not under control of apex-specific promoters in the shoot (An *et al.*, 2004; Ayre and Turgeon, 2004; Valverde *et al.*, 2004). These results indicate that the activity of CO is crucial for the generation of the mobile signal that originates in the leaf and is perceived in the apex at onset of flowering. Thus, leaf-produced FT activates the transcription of floral identity genes, such as *APETALA1* (*AP1*), at the shoot apex through its interaction with a new bZIP transcription factor, FD that is expressed preferentially at the shoot apex in the region where new primordia are being generated (Abe *et al.*, 2005; Wigge *et al.*, 2005). Multiple lines of evidence from these studies suggest a model by which FD provides the spatial framework or timely activation of flowering by FT (Blázquez, 2005). FT and FD have been shown to physically interact *in vitro* and *in vivo* and are hypothesized to act together to activate downstream targets (Abe *et al.*, 2005). Ectopic expression of FD caused up-regulation of *AP1* expression in leaves only under conditions that increase *FT* expression, such as transfer of plants from short to long days (Wigge *et al.*, 2005). The presence of transcripts sharing extensive sequence homology to *Arabidopsis* flowering integrators suggest that a similar mechanism may operate in citrus as demonstrated for other woody species (Böhlenius *et al.*, 2006).

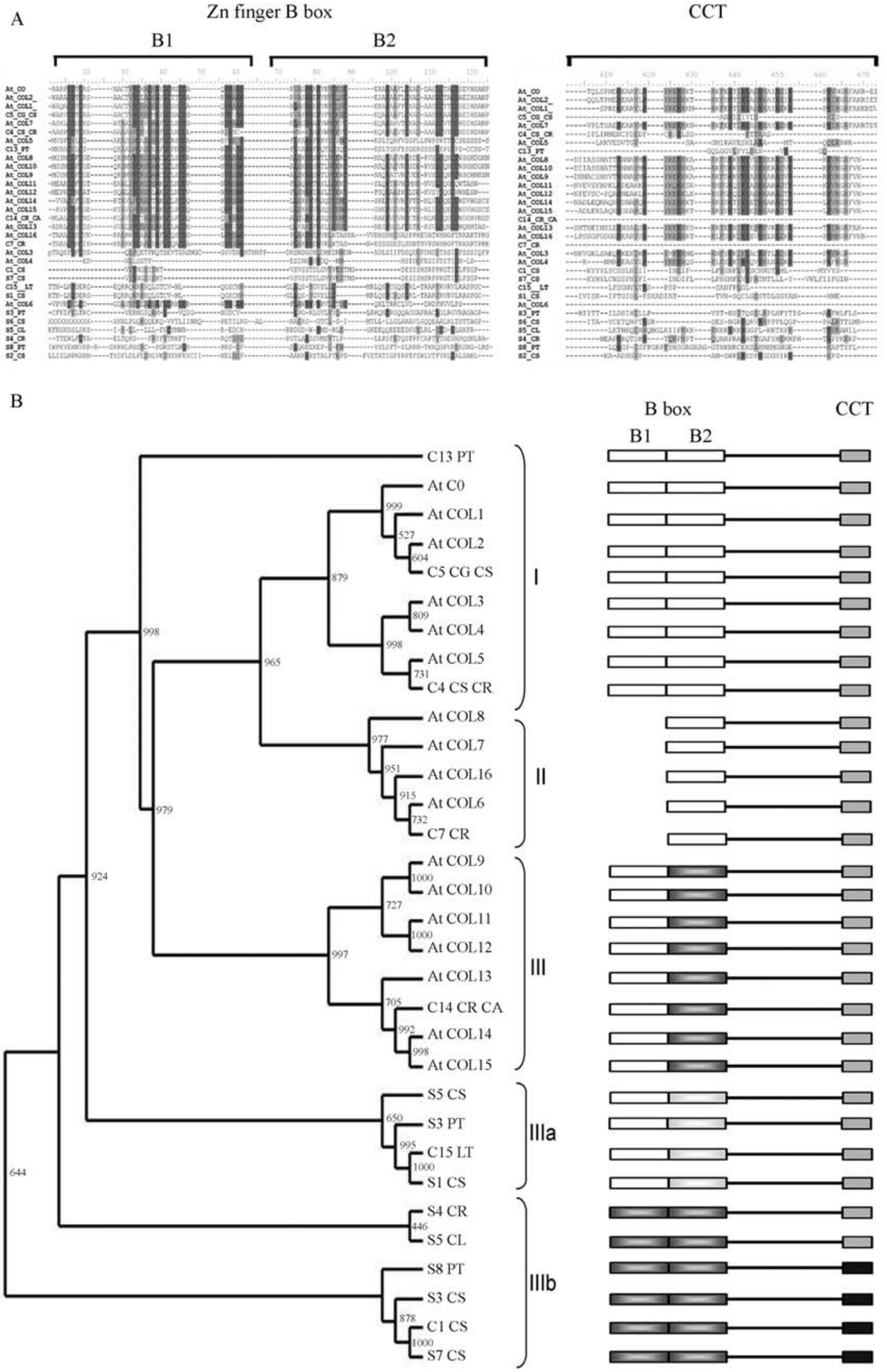

Figure 5 - CONSTANS family in citrus. **A.** Alignment of predicted B-box and CCT peptide domains of *CONSTANS* and related genes from *Arabidopsis* and citrus. The shaded residues represent conserved C residues and consensus spacing defining B-box domains.

Concluding Remarks

A model for the endogenous oscillatory mechanism in citrus, based on comparative transcriptome and sequence conservation analysis, is proposed (Figure 6). This preliminary survey of citrus circadian clock-associated genes has provided basic information for in-depth studies relating to the endogenous time-keeping mechanism in this important plant group. Further studies of the genes identified here will also give a new perspective on the circadian regulation of economically important metabolic processes, such as secondary metabolism control. Moreover, functional characterization of the identified pathways will help to uncover the molecular basis for seasonal responses in tropical tree species. The results highlight the potential and the power of comparative genomics and *in silico* transcriptional profiling in perennial species.

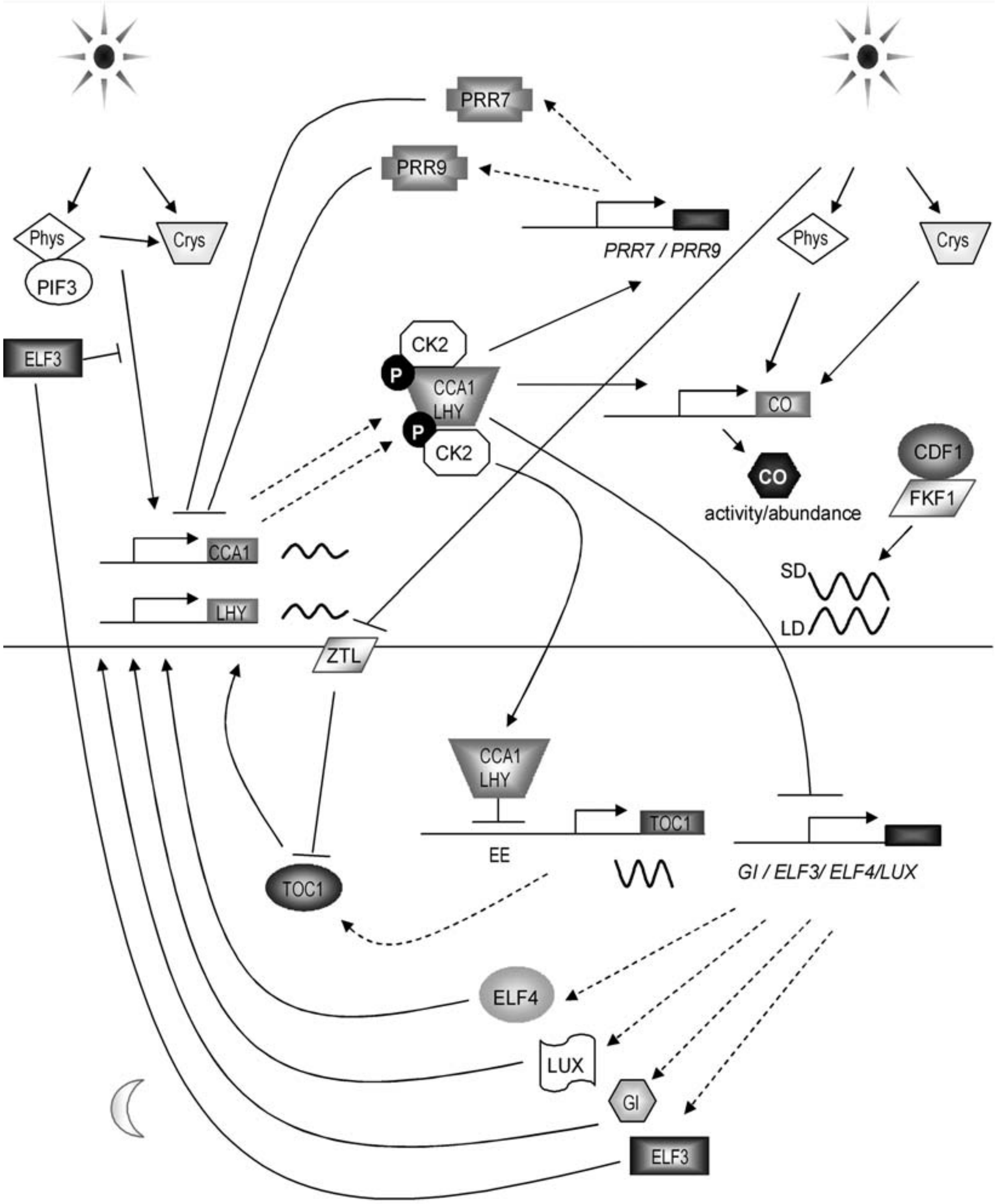

Figure 6 - Schematic overview of the endogenous time-keeping mechanism in citrus based on comparative genomic analysis. Solid boxes with italics lettering represent genes. Proteins are represented by capital letters inside oval or oblong shapes. Transcription and translation proteins are indicated by dashed lines. Protein activity is indicated by solid lines with arrowheads for positive action and perpendicular lines for negative action. The core CCA1/LHY/TOC1 feedback loop is represented by the innermost cycle whereas the ancillary loops are represented as outer loops. Phosphorylation of LHY and CCA1 by CK2 is indicated with solid circled Ps. The lower half of the figure represents activities peaking during the subjective night (moon schematic representation), whereas the superior part indicates activities peaking during the subjective day (sun schematic representation).

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Supplementary Material

Table 1S- *Citrus* reads constituting contigs and singlets with homology to genes involved in the input pathway to the circadian clock in *Arabidopsis thaliana*.

Table 2S - *Citrus* reads constituting contigs and singlets with homology to genes involved in the central oscillator of the circadian clock in *Arabidopsis thaliana*.

Table 3S - *Citrus* reads constituting contigs and singlets with homology to genes involved in the integration of the circadian clock mechanism and the generation of output responses in *Arabidopsis thaliana*.

This material is available as part of the online article from http://www.scielo.br/gmb.

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Table 1S - *Citrus* reads constituting contigs and singlets with homology to genes involved in the input pathway to the circadian clock in *Arabidopsis thaliana*.

Gene	Contig / Singlet	Read(s)
CRY1	C10-CR	CR05-C3-702-066-D11-CT , CR05-C3-702-061-F10-CT
	S1-CS	CS00-C1-100-038-A12-CT
PHYA	S2-CS	CS00-C3-701-101-C11-CT
	S3 - CS	CS00-C3-705-056-G06-CT
PHYB	S4-CR	CR05-C1-102-036-H07-CT
	S5-CS	CS12-G8-000-003-D03-CT
FKF1	C5-CS	CS00-C1-102-025-G12-CT, CS00-C1-102-019-A06-CT
	C6-CR/CS	CR05-C3-700-019-F11-EU, CS00-C1-102-053-E02-CT, CS00-C2-003-018-C11-CT
LKP2	S6-CS	CS00-C3-702-030-D01-CT
ZTL	C5-CS	CS00-C1-102-025-G12-CT, CS00-C1-102-019-A06-CT
ELF3	C1-CS/CR	CS00-C3-702-072-C10-CT, CR05-C1-100-007-E05-CT
	C2- CR	CR05-C3-701-025-F10-CT, CR05-C1-102-098-H10-CT
	S7-CA	CA26-C1-002-082-B12-CT
	S8-PT	PT11-C1-900-009-D09-CT
GI	C1-CS	CS00-C3-703-068-A06-CT, CS00-C1-102-030-G09-CT, CS00-C3-702-018-C07-CT, CS00-C1-102-024-G11-CT, CS00-C3-705-013-A05-CT, CS00-C3-705-019-F09-CT
	C2-CS/CR	CS00-C3-703-056-C05-CT, CR05-C1-100-083-B11-CT
	C3-CG/CR/CS	CG32-C1-003-008-F11-CT, CG32-C1-003-008-F10-CT, CR05-C3-701-28-12-CT, CS00-C2-003-033-G06-CT
	S9-CS	CS00-C1-102-069-D09-CT
	S10-CR	CR05-C3-702-072-F10-CT
PIF3	C6-CG/LT	CG32-C1-003-054-B09-CT, LT33-C1-003-071-A08-CT

Table 2S - *Citrus* reads constituting contigs and singlets with homology to genes involved in the central oscillator of the circadian clock in *Arabidopsis thaliana*.

Gene	Contig / Singlet	Read(s)
CCA1	C3-CS	CS00-C3-700-046-B06-CT, CS00-C3-702-034-H03-CT
	C7-CS/PT	CS00-C3-702-101-A04-CT, CS12-C1-001-007-F05-CT, CS12-C1-001-012-C11-CT, PT11-C1-900-055-F12-CT, CS00-C1-100-122-C11-CT, CS00-C1-100-103-A02-UV, PT11-C1-901-048-D05-CT, PT11-C1-900-043-B06-CT
	C16-PT	PT11-C1-900-047-B09-CT, PT11-C1-901-054-F01-CT, PT11-C1-900-080-D04-CT., PT11-C1-900-048-E05-CT
	C18-CS/CR	CS00-C1-650-031-A10-CT, CS00-C3-701-030-A11-CT, CR05-C1-100-043-F03-CT, CR05-C1-102-049-H08-CT
	S11-CR	CR05-C1-103-085-D10-CT
CK2	C2-CR/CS	CR05-C3-701-006-F08-CT, CS00-C1-100-096-D01-UV, CS00-C3-700-002-H11-CT, CS00-C3-700-006-G03-CT, CS00-C3-700-008-H05-CT, CS00-C3-700-074-F07-CT
	S12-CG	CG32-C1-003-100-G07-CT
	S13-CR	CR05-C1-103-023-A01-CT
	S14-CS	CS00-C3-700-023-H06-CT
DET1/FUS2	S15-CS	CS00-C3-700-016-F11-CT
	S16-LT	LT33-C1-003-085-H03-CT
ELF4	S17-CR	CR05-C1-102-100-G07-CT
	S18-CS	CS13-C1-001-024-G11-CT
	S19-PT	PT11-C2-300-083-B03-CT
LHY	C11-CS/CR	CS00-C1-650-047-H07-CT, CS00-C1-100-085-E03-EU,

		CR05-C3-701-026-B05-CT, CS00-C3-705-032-D04-CT, CS00-C1-102-032-E11-CT, CR05-C3-701-082-D12-CT, CS00-C1-100-042-E09-CT, CS00-C1-100-042-E10-CT, CS00-C3-700-001-D05-CT, CS00-C1-100-081-H04-CT, CS00-C3-702-046-G10-CT, CS00-C1-100-016-G11-CT, CS00-C1-100-115-F08-CT, CS00-C1-100-041-D04-CT, CS00-C3-704-082-A10-CT, CS00-C3-705-032-E11-CT, CS00-C3-702-074-D03-CT, CR05-C3-701-001-G09-CT, CR05-C3-701-030-A10-CT, CS00-C3-702-052-G12-CT, CR05-C1-100-076-B12-CT, CS00-C1-101-009-H10-CT
	C14-PT	PT11-C1-901-005-C05-CT, PT11-C1-901-061-A10-CT
	S20-PT	PT11-C1-900-008-A04-CT
	S21-CR	CR05-C1-102-100-G07-CT
LUX /PCL1 family	C1-CS	CS00-C3-701-070-D03-CT, CS00-C3-702-058-D08-CT, CS00-C3-700-045-C11-CT, CS00-C3-705-096-B11-CT, CS00-C3-705-096-H07-CT, CS00-C3-701-031-F06-CT
	C2- CS/PT	CS00-C1-100-065-D11-CT, PT11-C1-901-020-E04-CT, CS00-C1-100-105-A06-EU
	S22-CR	CR05-C3-700-068-H07-CT
	S23-CR	CR05-C3-701-021-A12-CT
PRR1/ TOC1	S24-CS	CS00-C1-100-073-A05-CT
PRR3	C3-CS/CR/LT	CR05-C3-701-012-G04-CT, CS00-C3-703-079-A01-CT, CS00-C3-703-079-F01-CT, LT33-C1-003-018-F05-CT.F, CS00-C3-705-007-F07-CT, CR05-C3-701-082-C01-CT,

		CS00-C3-705-002-H03-CT, CS00-C3-702-052-A12-CT
	S25-CS	CS00-C1-102-110-C02-CT
	S26-CS	CS00-C3-701-087-D07-UV
PRR7	S27-CS	CS00-C3-702-097-G04-CT
	S28-CS	CS00-C3-703-091-H07-CT
	S29-PT	PT11-C1-901-059-F10-CT
PRR9	C1-CS	CS00-C3-702-035-D05-CT, CS00-C3-700-053-E01-CT
	C2-CS/CR	CS00-C3-704-006-B05-CT, CS00-C3-704-009-E12-CT, CR05-C1-102-033-A01-CT
SPY	C1-CS/CR	CR05-C3-702-099-H11-CT, CS00-C2-003-046-H03-CT, CR05-C1-100-064-E08-CT, CR05-C3-700-108-H02-CT, CR05-C1-100-007-B05-CT, CS00-C3-705-041-D11-CT , CS00-C3-702-023-B02-CT
	S30-CR	CR05-C1-103-015-E09-CT
	S31-CS	CS00-C1-102-059-G10-CT

Table 3S - *Citrus* reads constituting contigs and singlets with homology to genes involved in the integration of the circadian clock mechanism and the generation of output responses *Arabidopsis thaliana*.

Gene	Contig / Singlet	Read(s)
CDF1 and *CDF* family	C4-CS/PT	CS00-C1-100-122-G01-CT, PT11-C1-900-024-E10-CT, CS00-C1-100-014-F05-CT
	C10- CA/CR	CA26-C1-002-099-H09-CT, CR05-C1-102-048-C08-CT
	S1-CS	CS00-C1-100-077-E09-CT
	S2-PT	PT11-C1-900-023-D03-CT
	S3-PT	PT11-C1-900-057-F03-CT
	S4-PT	PT11-C1-901-002-A06-CT
CO	C5-CG/CS	CS00-C1-100-086-A06-CT, CS00-C1-100-008-H03-CT, CS12-C1-001-026-C04-CT, CS12-C1-001-027-D01-CT
COL1 and *COL* family	C4-CS/CR	CS00-C1-101-087-B11-UV, CS00-C1-101-065-A09-CT, CS00-C1-100-123-E03-CT, CS00-C1-100-045-C06-CT, CS00-C1-100-011-G06-CT, CS00-C3-700-072-G04-CT, CS00-C1-101-102-D07-EU, CS00-C1-101-031-H07-CT, CS00-C1-100-041-B09-CT, CS00-C1-101-006-F05-UV, CS00-C1-650-027-B06-CT, CS00-C1-650-027-E11-CT, CS00-C1-100-061-E06-UV, CR05-C3-701-035-A11-CT, CS00-C3-702-013-B06-CT, CS00-C1-650-002-C12-CT, CS00-C1-650-021-F04-CT, CS00-C1-100-099-A01-UV, CR05-C3-701-056-E06-CT, CR05-C1-103-084-D07-CT, CS00-C3-705-019-C05-CT, CS00-C3-705-022-G05-CT, CS00-C1-102-088-F03-CT, CR05-C1-103-066-E12-CT

	C1-CS	CS13-C1-001-040-D02-CT, CS00-C1-100-008-C08-CT
	C7-CR	CR05-C3-701-028-A11-CT, CR05-C1-100-028-C11-CT
	C13-PT	PT11-C9-005-035-E08-CT, PT11-C1-901-063-G03-CT
	C14-CR/CA	CR05-C3-701-040-A06-CT, CR05-C3-700-021-A02-UV, CA26-C1-002-073-H02-CT
	C15-LT	LT33-C1-003-105-G01-CT, LT33-C1-003-106-C12-CT
	S1-CS	CS00-C1-101-022-A10-CT
	S2-CS	CS00-C1-101-004-F12-CT
	S3-PT	PT11-C1-900-069-G04-CT
	S4-CR	CR05-C1-102-087-D12-CT
	S5-CL	CL06-C4-500-018-F06-CT
	S6-CS	CS00-C1-102-043-C11-CT
	S7-CS	CS13-C1-001-040-D02-CT
	S8-PT	PT11-C1-901-084-D12-CT
ELF7	C1-CR/LT	CR05-C3-701-022-H04-CT, CR05-C1-103-040-G06-CT, LT33-C1-003-056-B09-CT
ELF8	C1-CS	CS00-C1-101-036-F06-CT, CS00-C1-101-013-H07-CT, CS00-C1-102-019-G06-CT, CS00-C3-700-074-H01-CT
FD	S1-CG	CG32-C1-003-009-B06-CT
FLC	C1-CS	CS12-C1-001-012-H03-CT, CS00-C1-102-067-G11-CT, CS00-C1-102-066-G08-CT, CS00-C1-102-065-H11-CT, CS00-C1-101-023-C09-CT
	S1-CS	CS00-C1-101-021-F12-CT
	S2-CS	CS12-C1-001-009-G11-CT
FT/	S1-CS	CS00-C3-704-020-B11-CT

TSF		
PFT1	C2-CS/CR	CS00-C1-101-049-D12-CT, CS00-C3-701-019-D08-CT, CS00-C1-650-028-B01-CT, CS12-G8-000-105-E11-CT, CR05-C1-103-095-D04-CT, CR05-C1-103-074-G03-CT
SOC1	S1-PT	PT11-C1-900-082-A11-CT
	S2-PT	PT11-C1-900-063-G11-CT