

Supplementary Note 12

Genetic structure of the circadian clock in Petunia

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Abstract

The circadian clock is a complex network comprising three layers of transcriptional, post-translational and metabolic control. Together they play a role in integrating environmental cues and metabolism to create coherent outputs for growth, adaptation and fine-tuning of secondary metabolism. We surveyed the *Petunia* genomes and compared the genetic structure of the clock with *Arabidopsis* and with other *Solanaceae*. We found a complex situation whereby the general configuration of the circadian clock is conserved in *Solanaceae* compared to *Arabidopsis*. However, we did not find a complete set of duplicated clock genes that would have been expected based on the paleohexaploidy shared by *Solanaceae* and a gene dosage hypothesis. Instead we found gene duplications for some of the genes involved in posttranslational control such as *GI*, *ELF3*, *ELF4* or proteins with dual function in DNA-binding and protein complex formation such as *TOC1*, *PRR7* and *PRR5*. The transcription factors *LHY* and *LUX/PCL1* were retained mostly as a single copy gene in *Petunia* and the rest of the *Solanaceae*. When compared to genes from other families, *LHY*, *GI* and *LUX* show subclades grouping the *Solanaceae* genes indicating a family specific evolution of the complete pathway that occurs both by changes in gene copy and coding region. The set of genes taking part in the circadian clock seems to be species-specific, and might explain the diversity of ecological niches colonized by the *Solanaceae*.

Introduction

Day to day coordination of basic biological functions within the environment is achieved by the entrainment of an endogenous circuit of control known as the circadian clock. It comprises three different parts, one is formed by a group of genes involved in entrainment and coordination with the environment, a second one is the so-called core clock, and finally those genes that act in the output of the clock. Physical signals such as light and temperature act as inputs of the clock. Endogenous chemical signals such as sugar or nitrogen levels can also modify its function (Gutierrez et al., 2008; Haydon et al., 2013; Salomé et al., 2010; Jarillo et al., 2006). The core of the clock is a set of genes comprising three loops known as morning, midday or evening loop (Pokhilko et al., 2012). Some core clock has components that also play direct roles in activation and repression of target genes as the outputs of the clock (Egea-Cortines et al., 2013; Yakir et al., 2007; Mas et al., 2013). The major processes known to be controlled as outputs of the clock are flowering time, response to cold, growth and basic metabolic processes like photosynthesis, starch metabolism and secondary metabolism such as scent emission (Farré and Weise, 2012; de Montaigu et al., 2010; Graf and Smith, 2011). New evidence links clock outputs to a variety of important traits like adaptation to environment, pathogen resistance or fitness (Egea-Cortines et al., 2013).

Work in *Arabidopsis*, crops and non-model species has shown that adaptation to environmental signals might have occurred by modifications within the clock, and not in downstream processes (Mallona et al., 2011; Weller et al., 2012; Brachi et al., 2010; Zakhrabekova et al., 2012). This indicates that the fine-tuning and divergences found in the clock in different species might show their evolutionary history and will help us understand and improve plant adaptation. Indeed recent evidence shows that circadian clock tuning maybe important during plant domestication in tomato (Müller et al., 2016).

The clock structure and function in higher plants is best understood in *Arabidopsis*. Five members of the small gene family of *PSEUDORESPONSE REGULATOR* form part of the clock (Mas et al., 2013). The genes *PSEUDORESPONSE REGULATOR 9*, *7* and *5* form the morning loop, *PRR3* is apparently a vascular-specific component of the clock (Para et al., 2007). The core loop is formed in *Arabidopsis* by two paralogous *MYB* genes *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LONG ELONGATED HYPOCOTYL (LHY)* (Takata et al., 2010) together with *PRR1* known as *TIMING OF CAB1 (TOC1)* (Alabadi et al., 2001). Finally the evening loop comprises three genes *EARLY FLOWERING 3* and *4 (ELF3 and 4)* and *LUX ARRHYTHMO* (Nusinow et al., 2011).

Understanding the evolution of the plant circadian clock is becoming important as the major traits of relevance in agriculture are directly controlled or affected by the clock (Panda et al., 2002). A current hypothesis is that circadian clock genes may be under special selective pressure as they form a coordinated genetic network comprising mutual activation and repression via direct interactions. These are sometimes the result of several independent gene products that form protein complexes (McClung and Gutiérrez, 2010; Lou et al., 2012). As the genome of the *Solanaceae* lineage has undergone at least a paleohexaploidy event (Tomato and Consortium, 2012; Kim et al., 2014; Sierro et al., 2014; Xu et al., 2011), a starting

hypothesis would be that the complete set of genes may have undergone a duplication and we may find at least one additional paralog specific for the Solanaceae.

Petunia has been used in establishing the circadian clock as a master regulator of development in plants. Early work showed that the *CHLOROPHYL A/B BINDING PROTEIN* gene (*CAB*) has a rhythmic expression (Stayton et al., 1989). This confirmed previous works that identified rhythmic expression of *CAB* in tomato and tobacco plants (Paulsen and Bogorad, 1988; Piechulla, 1988). Amongst the outputs of the clock, the circadian emission of floral scent has been studied with detail in Petunia and Antirrhinum (Kolossova et al., 2001; Verdonk et al., 2003). Furthermore, the *MYB* transcription factor *ODORANT1*, involved in quantitative control of scent emission in Petunia, displays a rhythmic expression indicating a direct regulation of the clock on scent output in Petunia (Verdonk et al., 2005). Recent studies using artificial vision image analysis show that *Petunia x hybrida* flowers open in a short time frame right after dawn indicating that important aspects of morphogenesis, beyond hypocotyl elongation, might have a light and circadian clock control (Navarro et al., 2012). A recent work overexpressing the *Petunia x hybrida* *LHY* gene shows the control of scent emission timing by the clock (Fenske et al., 2015), confirming the role of the clock in controlling secondary metabolism.

We have performed a comprehensive search for the genes involved in the clock of *Petunia axillaris* and *P. inflata* using Arabidopsis as a template. As the circadian clock genes are not explored with detail in the *Solanaceae* and there is a genome duplication specific for the family, we have analysed and compared the findings in Petunia with those of other sequenced *Solanaceae* genomes. Our results indicate a basic structure similar to that of Arabidopsis, but the number of specific paralogs found for a given gene seems to be species specific and does not follow a simple rule, based on genome duplications, and maybe related to specific biological and molecular functions.

Results

We surveyed the Petunia genomes using proteins identified by BLAST in tomato corresponding to circadian clock components. We found seven genes in *P. axillaris* and *P. inflata* with high similarity to the *PRR9*, *7*, *5*, *3* and *TOC1* genes from Arabidopsis. This increased number was due to gene duplications in *PRR7* and *PRR5* resulting in two paralogs present in Petunia. As we found gene duplications for *PRR7* and *5* in different *Solanaceae*, we assumed that the complete set of clock related *PRR* genes has been duplicated (Table 1-list of genes). This would be in agreement with the genome duplications found in the *Solanaceae* (Tomato and Consortium, 2012; Xu et al., 2011). However this hypothesis was not correct. The gene *PRR3* appeared as single copy genes in the species surveyed (Figure 1).

Table 1: A list of genes of the circadian clock from *Petunia* and Solanaceae

Arabidopsis	<i>P. inflata</i>	<i>P. axillaris</i>	Other Solanaceae
PRR9	<i>P. inflata</i> PRR9	<i>P. axillaris</i> PRR9	Duplicated in <i>N. benthamiana</i>
PRR7	<i>P. inflata</i> PRR7a <i>P. inflata</i> PRR7b	<i>P. inflata</i> PRR7a <i>P. axillaris</i> PRR7b	Single or double copy
PRR5	<i>P. inflata</i> PRR5a <i>P. inflata</i> PRR5b	<i>P. axillaris</i> PRR5a <i>P. axillaris</i> PRR5b	Single or double copy
PRR3	<i>P. inflata</i> PRR3	<i>P. axillaris</i> PRR3	Single copy
TOC1	<i>P. inflata</i> TOC1	<i>P. axillaris</i> TOC1	Duplicated in <i>N. benthamiana</i>
LHY	<i>P. inflata</i> LHY	<i>P. axillaris</i> LHY	Single copy in Solanaceae
CCA1	Absent	Absent	Absent
ELF3	<i>P. inflata</i> ELF3 <i>P. inflata</i> ELF3a <i>P. inflata</i> ELF3b <i>P. inflata</i> ELF3c	<i>P. axillaris</i> ELF3 <i>P. axillaris</i> ELF3a <i>P. axillaris</i> ELF3b	Three to four copies in Solanaceae
ELF4	<i>P. inflata</i> ELF4a; <i>P. inflata</i> ELF4b	<i>P. axillaris</i> ELF4a; <i>P. axillaris</i> ELF4b	Duplicated in Solanaceae
LUX	<i>P. inflata</i> LUX	<i>P. axillaris</i> LUX	Duplicated in <i>S. lycopersicum</i> and <i>S. tuberosum</i>
GI	<i>P. inflata</i> GI1; <i>P. inflata</i> GI2; <i>P. inflata</i> GI3	<i>P. axillaris</i> GI1; <i>P. axillaris</i> GI2	Two to four copies in Solanaceae

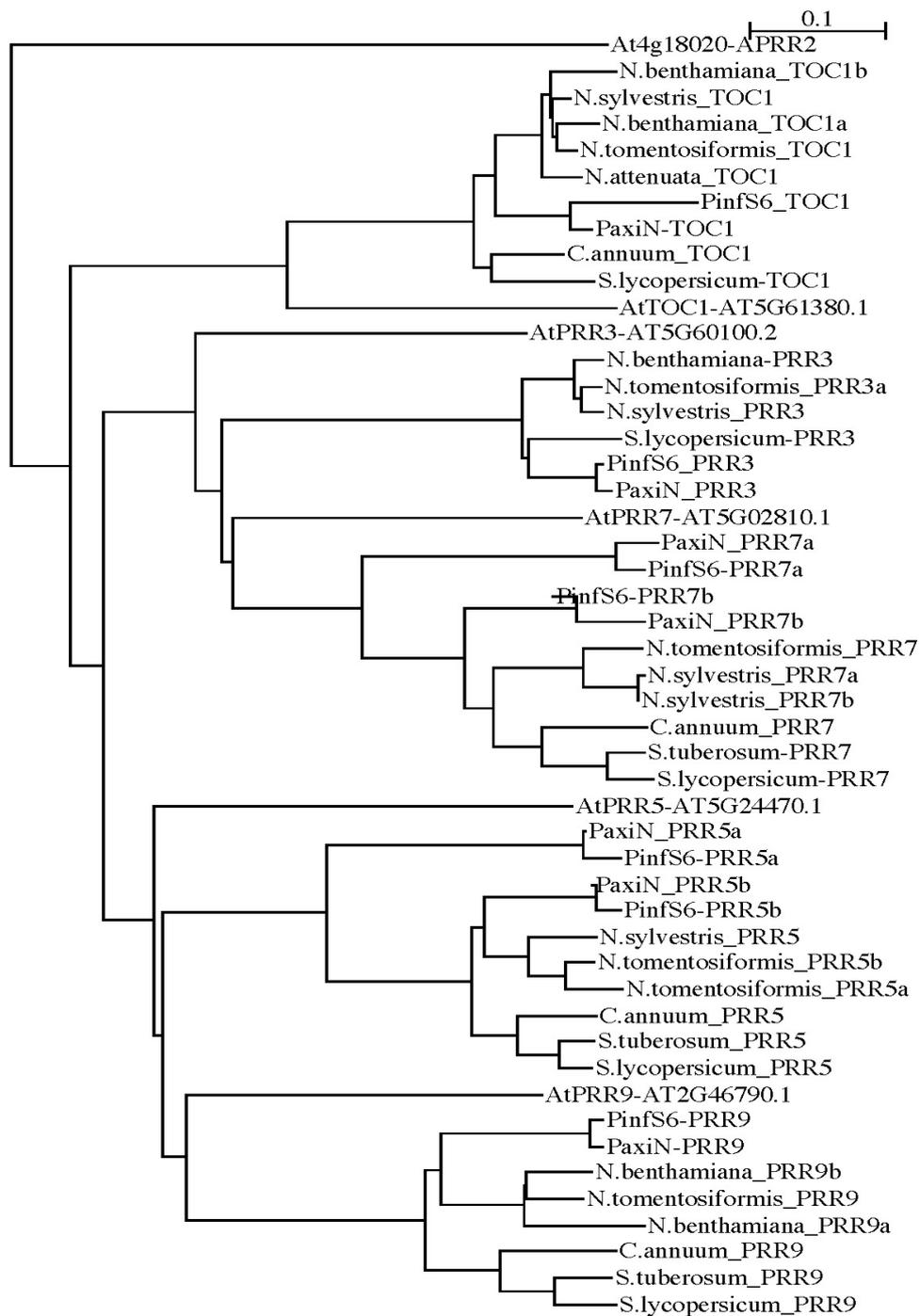


Figure 1. Phylogenetic analysis of *PSEUDORESPONSE REGULATOR* genes from *Petunia* and other *Solanaceae*. We used *Arabidopsis* genes to establish the families corresponding to PRR),7,5,3 and TOC1. Plant species correspond to: At, *Arabidopsis thaliana*; *Nicotiana sylvestris*; *Nicotiana tomentosiformis*; *Nicotiana benthamiana*; *Nicotiana attenuata*, *Solanum lycopersicum* and *Solanum tuberosum*.

The survey of the Petunia genomes for *LHY* and *CCA1* orthologs showed several genes with high degree of homology. Both Arabidopsis *CCA1* and *LHY* gave identical BLAST hits with one scaffold showing higher scores than the rest. This indicated a possible structure of midday loop that would comprise a single *LHY/CCA1* gene as compared to Arabidopsis that has *CCA1* and *LHY* (Figure 2). A comparative analysis with the rest of the *Solanaceae* sequenced genomes identified several *LHY/CCA1* homologous genes with different BLAST e values. However, the inclusion of the identified clones in the phylogenetic framework supported the previous preliminary finding as we identified a single *LHY* gene present in *Solanum tuberosum*, *Nicotiana benthamiana*, *Capsicum annuum*, *P. axillaris* and *P. inflata* (Figure 2). The other genes found clustered with the *REVEILLE* gene family of circadian clock related *MYB* genes (Rawat et al., 2009; Farinas and Mas, 2011; Rawat et al., 2011). Our current data indicates that there is a single *LHY* ortholog in the Solanaceae.

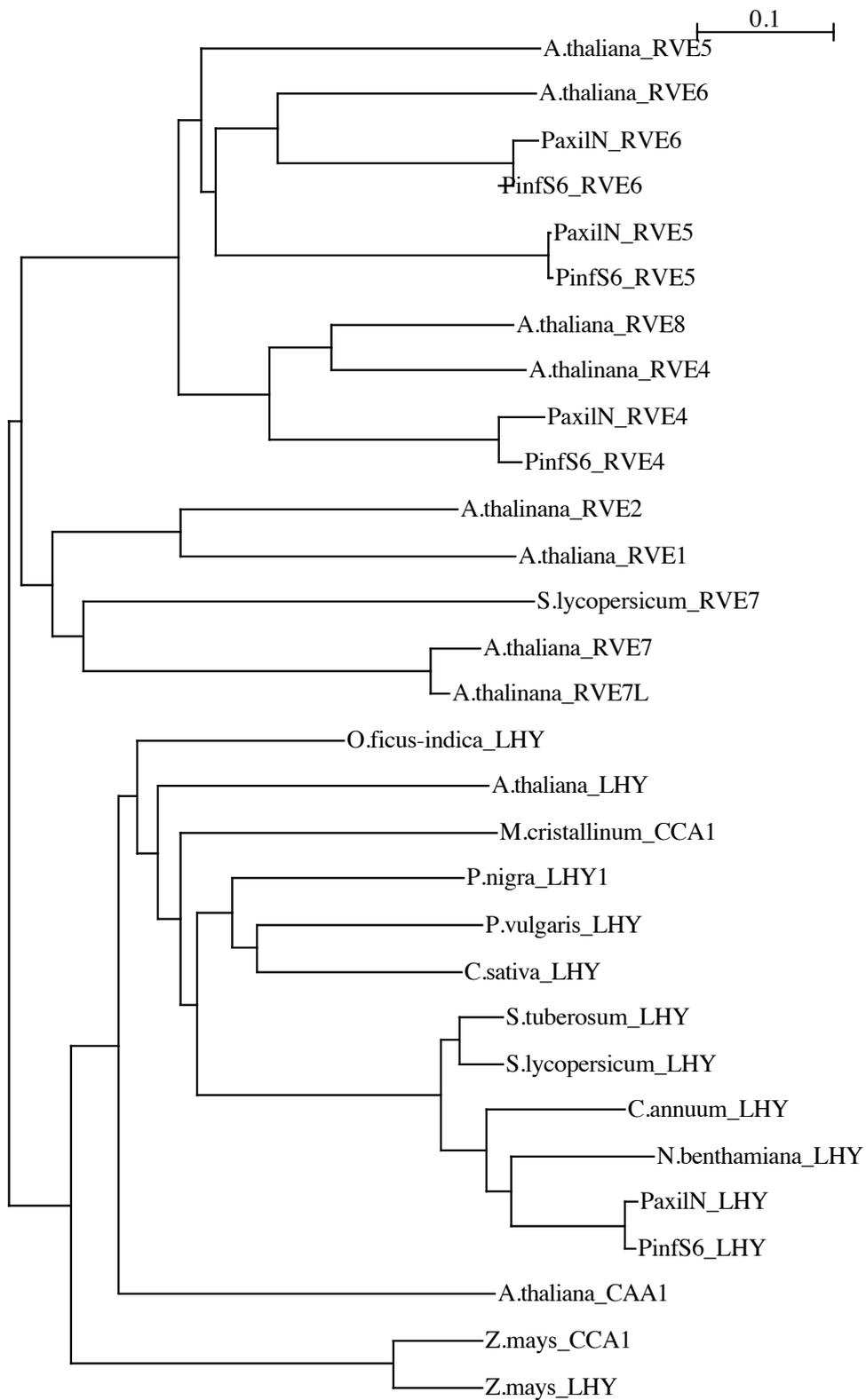


Figure 2. Phylogenetic analysis of MYB genes involved in circadian clock in *Petunia* and other *Solanaceae*. Species correspond to *Opuntia ficus-indica*; *Mesembryanthemum crystallinum*; *Populus nigra*, *Phaseolus vulgaris*; *Castanea sativa* and *Zea mays*.

EARLY FLOWERING 3 forms together with *ELF4* and *LUX ARRHYTHMO (LUX)* a protein complex involved in repression of the morning loop comprising *PRR9*, *7* and *5*. The *ELF3* gene is single copy gene in Arabidopsis. We identified three *ELF3* in *P. axillaris* and four in *P. inflata*, forming two distinct clades (Figure 4). One comprising a cluster of genes together with *S.lycopersicum* and *S.tuberosum*, showed a separate phylogenetic position between two paralogs from rice and the rest of the *ELF3* genes from Eudicots, including Arabidopsis. This indicates that this gene may be an ancient ortholog. The second clade comprises what appears to be a further gene duplication with two closely related paralogs in all the species except for *P. axillaris* that presents one gene. The *Physcomitrella patens* genome comprises three *ELF3*-like genes albeit with low homology (blast e-14) forming a distinct clade, indicating that the set of genes arose in different event.

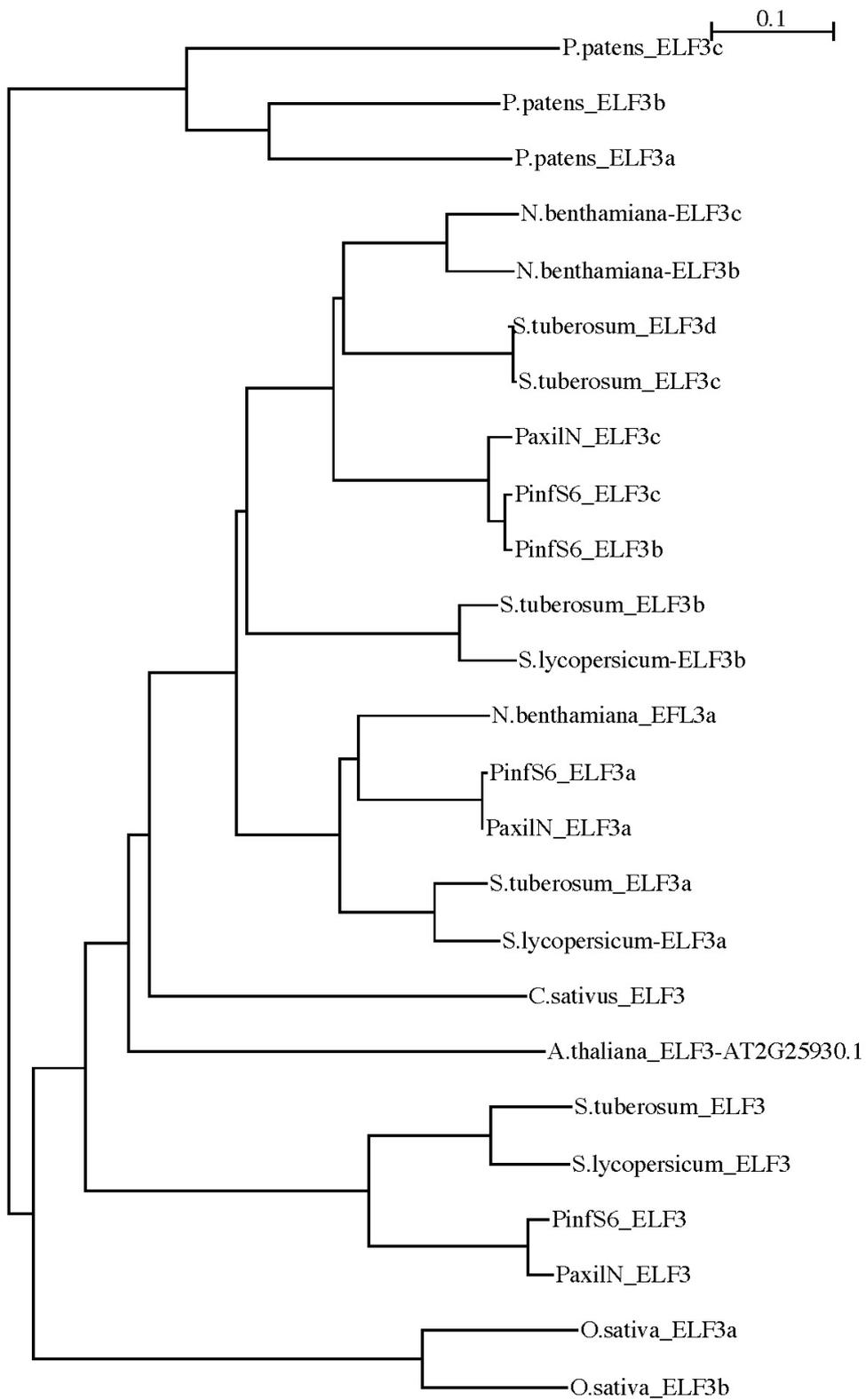


Figure 3. Phylogeny of ELF3 genes in *Petunia*, *Solanaceae*, *Physcomitrella patens* and *Oryza sativa*.

The *ELF4* gene belongs to a small gene family of five members in Arabidopsis (*ELF4*, *ELF4-L1*, *ELF4-L2*, *ELF4-L3* and *ELF4-L4*). *ELF4* encodes a nuclear-localized protein of 111 amino acids with unknown molecular function, and the protein family lacks introns. A biological function is known in detail only for *ELF4*. It is involved in photoperiod perception and flowering time (Doyle et al., 2002; Nusinow et al., 2011; Kikis et al., 2005). We found two genes that clustered with *ELF4* in *P. inflata* and *P. axillaris*, indicating a gene duplication of *ELF4* (Figure 4). We also found duplications in *S. lycopersicum* and *C. annuum* indicating a conservation of this duplication in the *Solanaceae*. There were additional ORFs in Petunia that were closer to the *ELF4-L2*, *ELF4-L3* and *ELF4-L4* from Arabidopsis of unknown biological function (Figure 4).

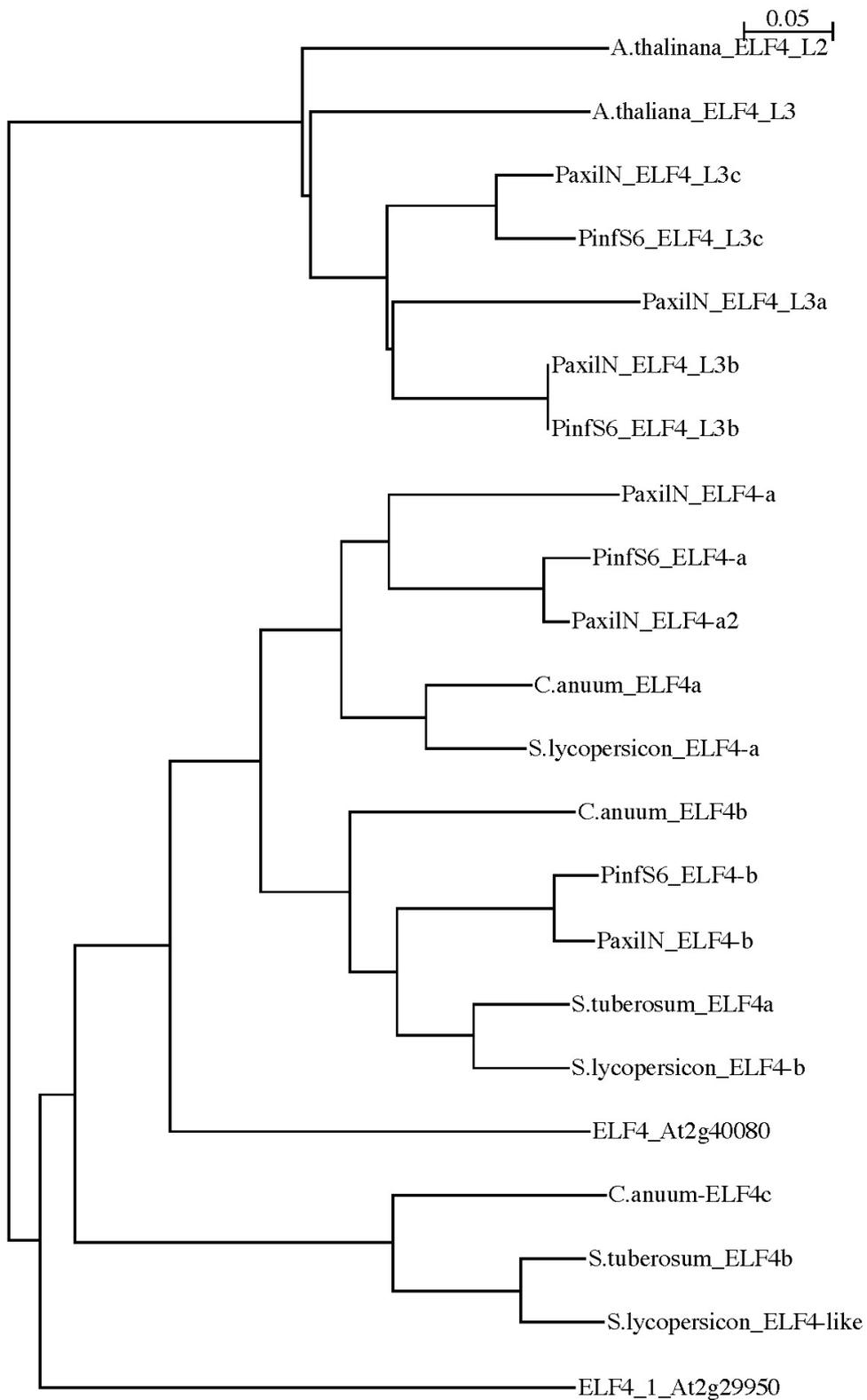


Figure 4. Phylogeny of *ELF4* and *ELF4*-like genes in *Solanaceae* and *Arabidopsis*.

The *LUX* gene also known as *PHYTOCLOCK1 (PCL1)* (Hazen et al., 2005; Onai and Ishiura, 2005) is a single *MYB* domain transcription factor of the GARP family (Dubos et al., 2010). The *Arabidopsis* genome has two copies, *LUX* and *BROTHER OF LUX ARRHYTHMO (BOA)*, also involved in circadian clock regulation (Dai et al., 2011). The so-called evening complex is formed by ELF3, ELF4 and LUX proteins whereby the LUX DNA-binding domain is thought to provide DNA binding capacity (Helfer et al., 2011). A survey of the *Petunia* genomes showed a single scaffold with high similarity to *LUX* (Figure 5). Additional BLAST searches found other genes but the phylogenetic analysis placed them outside the *LUX* and *BOA* clade. We also found that the *Solanaceae* genes clustered in a subclade while the *Fabaceae* genes formed a second subclade suggesting the acquisition of family specific features.

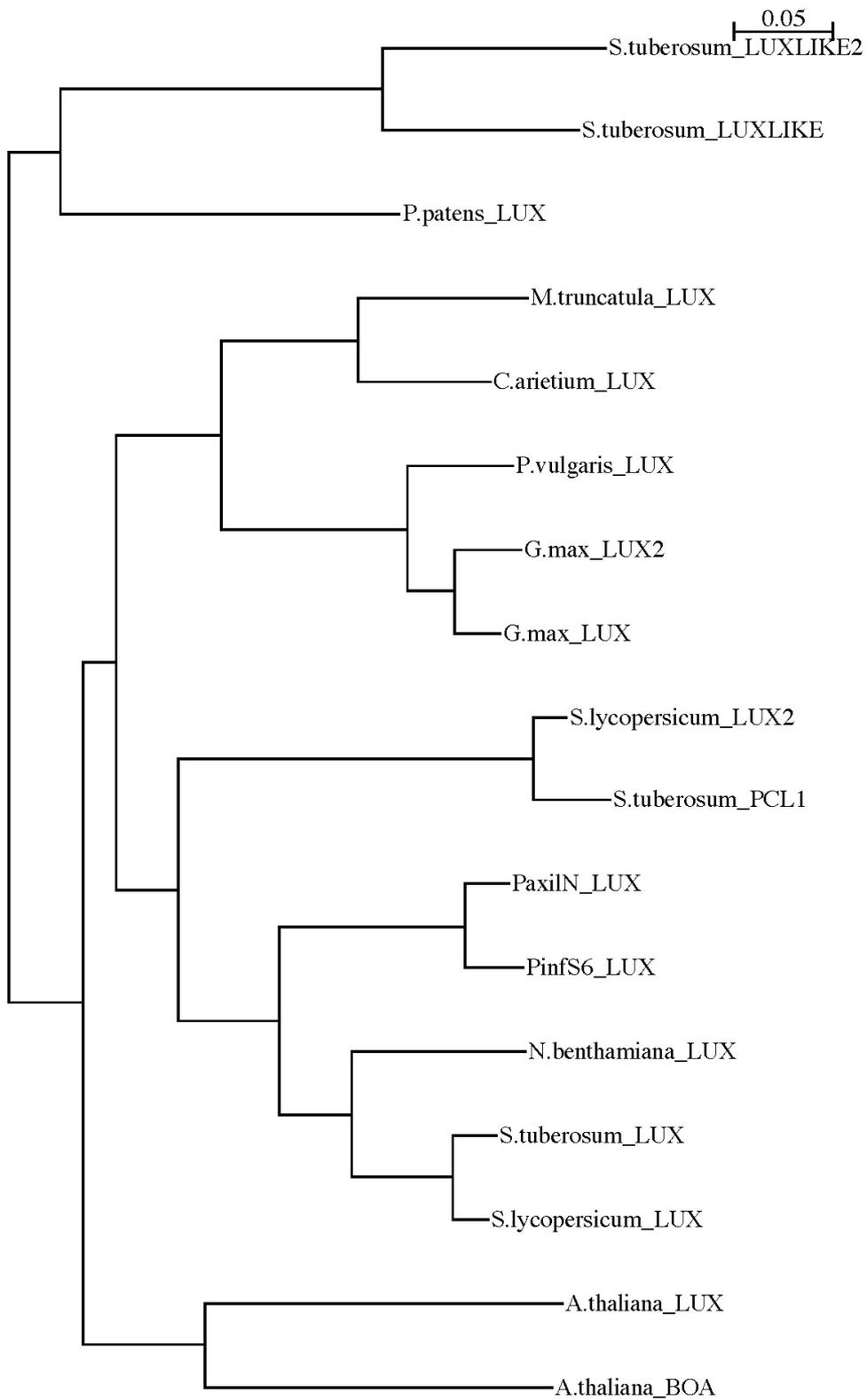


Figure 5. Phylogenetic analysis of *LUX ARRHYTHMO* in *Petunia* and *Solanaceae* and comparison to genes from *Leguminosae* and *Arabidopsis*.

The *Gl* locus is a single copy gene in *Arabidopsis*. An initial survey in the *Petunia* and other *Solanaceae* genomes indicated the presence of at least two copies of *Gl*. The *Solanaceae* genes clustered together, away from *Arabidopsis* separating the *Fabaceae* clade. The *Solanaceae* clade showed a middle split indicating a conserved genome duplication that seems to be maintained at the *Gl* loci. Further gene duplications within one of the subclades have occurred in *Pinfs6* and tomato that show an additional gene duplication whereas *N.benthamiana* shows duplications in both (Figure 6).

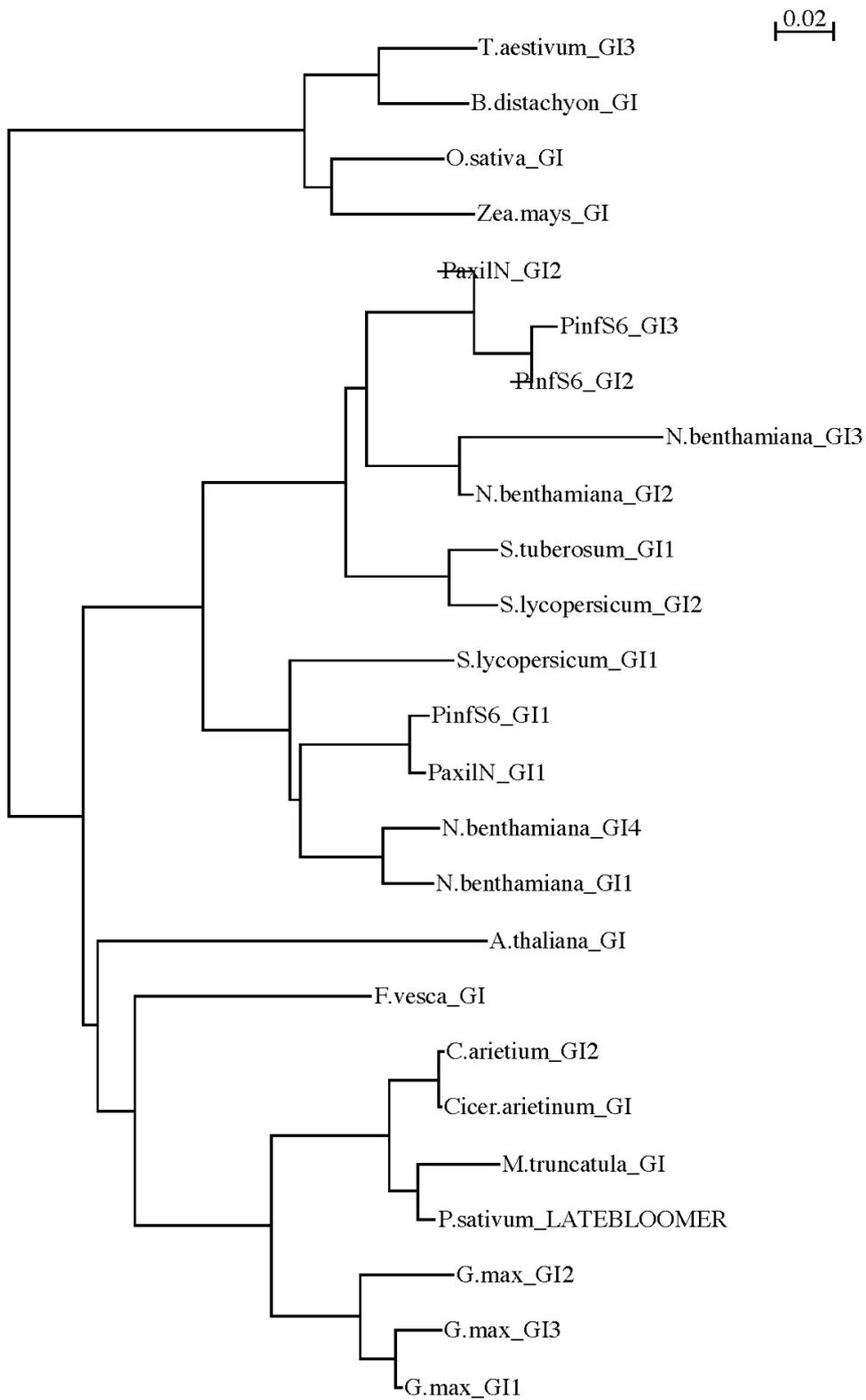


Figure 6. Phylogenetic analysis of GIGANTEA genes from Petunia.

Altogether the clock genes are conserved in *Petunia*, detailed analysis indicates a departure from the *Arabidopsis* structure, with some features that are probably common to the *Solanaceae*.

Discussion

The simplest known circadian clock gene regulatory network currently identified in plants is found in the single cell algae *Ostreococcus tauri* and it comprises a core loop formed by a *CCA1* and a *TOC1* ortholog (Corellou et al., 2009). An additional protein with a LOV-domain similar to ZTL-FKF-LKP2 is important for circadian control indicating further similarities in the structure (Djouani-Tahri et al., 2011). Three loops that are apparently conserved in *Arabidopsis* and other plants form the structure of the clock. Here we have studied the main genes of the morning, core and evening loop of *P. inflata* and *P. axillaris* and performed a comprehensive comparison with other *Solanaceae* that have sequenced genomes available. As the morning, core and evening loops function at the molecular level via protein complexes, a current hypothesis is that gene dosage and protein stoichiometry play a fundamental role in robust pacing (Kim and Forger, 2012; Lou et al., 2012). The *Solanaceae* genomes have undergone genome duplications and they have retained conserved duplicated genes in floral homeotic genes in tomato and *Petunia* (Quinet et al., 2014; Vandenbussche et al., 2003, 2004). We found that this is not the case for clock genes in *Petunia* and other *Solanaceae*. The morning loop comprises the *PRR* paralogs *PRR9*, 7 and 5. They form a protein complex with *TOPLESS* and sequentially repress the expression of the core loop genes *CCA1* and *LHY* (Wang et al., 2013). Our analysis shows that *Solanaceae* tend to have a single *PRR9*. In contrast, *PRR7* and *PRR5* are mostly duplicated in the *Solanaceae* analyzed. The *PRR3* gene, involved in selective degradation of *TOC1* (Para et al., 2007) was also found as a single copy gene. A single copy gene in *P. axillaris*, and *P. inflata* represents the core element *TOC1*. Our data indicates that the *PRR* genes, involved in control of the circadian clock, display a different type of evolution, with *PRR5* and 7 showing ubiquitous conserved duplications and *PRR9*, 3 and *TOC1* appearing mostly as single copy genes.

The *CCA1/LHY* belong to the *R1/R2* single *MYB* family of transcription factors (Dubos et al., 2010). From an evolutionary perspective, plants like *Lemna*, *Physcomitrella patens*, *Arabidopsis* or *Poplar* have two *CCA1/LHY* genes resulting from taxon specific genome duplications (Miwa et al., 2006; Dubos et al., 2010; Okada et al., 2009; Takata et al., 2009). In contrast the *Solanaceae* appear to have a single copy of *LHY/CCA1* in agreement with other families like *Oryza*, *Carica papaya* and *Vitis vinifera* (Lou et al., 2012; Murakami et al., 2007). Furthermore our results support the hypothesis that *CCA1* belongs to a set of genes that originated in the alpha and beta duplication from the Brassicales, as *CCA1* appears in this family but not in others (Lou et al., 2012).

The dose sensitive theory for clock genes may apply to *PRR3* and *LHY*, but may not be true for the rest of the genes analysed, *ELF3*, *ELF4*, *FKF* and *GI* that are involved in protein complexes. We found that overall the fate of the different gene families in the *Solanaceae* cannot be predicted based on this simple hypothesis. The *ELF3* gene is found as a single copy gene in *Arabidopsis* while we found three in the different *Solanaceae* genomes analysed. As a gene with high homology to *ELF3* is involved in heteromorphic self incompatibility in *Fagopyrum esculentum* (Yasui et al., 2012), we can assume a diverging biological function of the *ELF3* paralogs, but functional work is required to test this hypothesis.

The *Solanaceae* genomes apparently share a similar number of *ELF4* genes. However both *P. axillaris* and *P. inflata* have a gene duplication for the closest ortholog of *ELF4*. The *GI* gene plays a key role in adaptation to cold and temperature compensation (Gould et al., 2006; Cao et al., 2007). It is duplicated across the *Solanaceae* and *P. inflata* has three genes suggesting a possible role for *GI* in adaptation to local environments in *P. inflata*, or acquisition of new biological functions.

Altogether the data presented show that the *Solanaceae* has a common genetic structure of the circadian clock. However, the precise set-up in terms of genes present in each species seems to be diverse. This may explain the distinct ecological niches colonized by this family of plants.

Materials and methods

Identification of clock genes

We obtained the translated proteins from *Arabidopsis* of the clock genes using gene names and accession numbers from (www.arabidopsis.org). The *Arabidopsis* proteins were used to obtain putative orthologs from tomato using BLASTP (solgenomics.net). The tomato translated protein sequences were used to obtain contigs, scaffolds and proteins from *P. axillaris* and proteins. The contigs and scaffolds obtained were manually trimmed to fragments encompassing 1 kb up and downstream of the putative start and stop codons. The tomato protein and the *Petunia* genomic fragment were aligned using GeneWise, obtaining the predicted complete coding region and ORF (Birney et al., 2004). Correspondence of exon/intron number to the *Arabidopsis* genomic structure was visually inspected. We used a simple in-house software (KaraTekka), to manually annotate the exon and intron sequences of the genes analysed.

Phylogenetics

Phylogenetic analysis was performed using CLUSTALX using the Phylip algorithm for Neighbour joining tree using 1000 bootstraps (Larkin et al., 2007) and trees were rendered with NJplot. In

order to verify main trees, we used a pipeline comprising a sequence alignment set for maximum accuracy with MUSCLE (Edgar, 2004). Aligned sequences were automatically curated from poorly aligned positions and blocks with Gblocks (Castresana, 2000). The phylogenetic analysis was performed by PhyML that uses a Near Neighbour Interchange algorithm (Guindon and Gascuel, 2003) and trees were rendered by Treedyn (Chevenet *et al.*, 2006). Gene to gene differences were identified using ALIGN (www.ebi.ac.uk).

Gene ontology annotation

The Gene ontology (Ashburner *et al.*, 2000) descriptors of the genes involved in the clock of *Petunia* were manually annotated for GO terms using the Arabidopsis homepage (www.arabidopsis.org) and comparing it to GO terms annotated for orthologs of the annotated legume genomes of *Medicago truncatula*, *Glycine max* and *Lotus japonicus* (<http://plantgrn.noble.org/LegumeIP/>).

Identification of polymorphism

We identified DNA polymorphisms between *PaxilN* and *PinfS6* using KALIGN. Protein differences were identified using multiple alignments obtained with CLUSTALX.

Acknowledgments

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