Chapter 4

PRR1/TOC1, a key component of the *Arabidopsis* clock system, does not show a diurnal expression pattern in poplar

Summary

• The plant clock model developed for *Arabidopsis thaliana* consists of multiple interlocked feedback loops. However, current evolutionary analyses suggest that the types and number of clock-related genes in *Populus* differ from those of *Arabidopsis*, and that these differences may result in alterations to the regulation mechanism of its clock system. In the present study, I examined the regulation mechanism of the *Populus* clock system by determining the function of its circadian clock-related genes.

• The diurnal expression patterns of clock-related genes in *Populus* and *Arabidopsis* were compared using available microarray data. To investigate functional redundancy between *Populus* and *Arabidopsis PRR1/TOC1* genes, complementation analyses were performed using an *Arabidopsis PRR1/TOC1* null mutant.

• Most clock-related genes in *Populus* exhibited diurnal rhythms and showed peak expressions similar to their respective *Arabidopsis* orthologs. However, the *Populus PRR1/TOC1* gene did not show the typical diurnal expression pattern, nor did the *Populus PRR1/TOC1* fully complement the function of *Arabidopsis PRR1/TOC1* in the null mutant.

• The results in the present study indicate that the main feedback loop in the *Populus* clock system may be regulated differently from that of *Arabidopsis*.

Introduction

Circadian clock systems are found in many organisms, and enable them to respond to environmental day/night cycles. In the model plant *Arabidopsis thaliana*, the transcript levels of many genes are regulated by a circadian clock system (Harmer *et al.*, 2000; Michael & McClung, 2003; Schaffer *et al.*, 2001). These transcriptional changes influence a wide variety of diurnal and seasonal photoperiodic responses, such as photomorphogenic processes, floral transition, leaf movements, stomatal conductance, photosynthetic capacity and volatile emissions (reviewed in Yakir *et al.*, 2007). Thus the circadian clock system acts as a master controller of many photoperiodic responses under complex circumstances.

A model of the plant clock system has been developed for *A. thaliana* using a variety of genetic and systems biological methods (reviewed in McClung, 2006; Más, 2005). The first clock system proposed for *A. thaliana* was a simple positive-negative feedback loop composed of two single Myb transcriptional factors [*Late Elongated Hypocotyl* (*LHY*) and *Circadian Clock Associated 1* (*CCA1*)] and a pseudo-response regulator [*Pseudo-Response Regulator 1/Timing of CAB2 Expression 1* (*PRR1/TOC1*)] (Alabadí *et al.*, 2001). In this simple model, the morning-acting *LHY* and *CCA1*, which are partially redundant genes in their function, are induced indirectly by the evening-acting PRR1/TOC1. In turn, LHY and CCA1 directly repress the expression of the *PRR1/TOC1* gene (Alabadí *et al.*, 2001). This regulatory network was considered to be the core system of the plant circadian clock. However, this simple network does not support all the experimental evidence in the literature. For example, it can not explain the long delay between *PRR1/TOC1* transcription in the evening and *LHY/CCA1* activation the following morning (Salomé & McClung, 2004).

Additional genetic and mathematical analyses have refined the clock model, which is now thought to be composed multiple interlocked feedback loops (Locke *et al.,* 2006; Zeilinger *et al.,* 2006). In the new clock model, two additional feedback loops (morning and evening loops) are connected to the main loop (*LHY/CCA1-PRR1/TOC1*). In the morning loop, *LHY* and *CCA1* positively regulate *PRR7* and *PRR9*, which are orthologs of *PRR1/TOC1*, but are conversely repressed by these *PRR* genes (Farré *et al.*, 2005). In the evening loop, *Gigantea*

(*GI*) and *PRR5* construct the feedback loop with evening-expressed *PRR1/TOC1*, although the functions of *GI* and *PRR5* in this loop are still under debate (Kawamura *et al.*, 2008). In spite of the vast evidence accumulated to date, it is still unclear whether the complex regulation mechanism of the *Arabidopsis* clock model also exists in clock systems in other plant species.

Nevertheless, circadian clock-related genes composed of multiple feedback loops are widely conserved in monocots and eudicots (Murakami *et al.*, 2007; Takata *et al.*, in press; Chapter 3 in this thesis). Phylogenetic analyses of these genes have revealed their evolutionary relationships among angiosperms and, consequently, have led us to propose the evolutionary process of the plant clock system itself (Chapter 3 in this thesis). These evolutionary approaches demonstrate that the fundamental mechanism of the *Arabidopsis* clock model may have been organized before the radiation of rosids species in eudicots. On the other hand, the clock-related genes in *Populus* differ in their compositions and copy numbers because of several ancient evolutionary events, gene deletions, and gene duplications that have occurred in *Populus* lineages. Thus it is hypothesized that these evolutionary changes in *Populus* clock genes may have modified the regulation mechanism of its clock system.

Among the circadian clock-related genes of *A. thaliana*, *PRR1/TOC1* is one of the best characterized in terms of gene expression, posttranslational regulation, and physiological function. The *A. thaliana PRR1/TOC1* gene has a diurnal expression pattern that peaks around dusk. This diurnal expression pattern is also found in *PRR1/TOC1* genes in other angiosperms (Murakami *et al.*, 2003; Ramos *et al.*, 2005). The expression of *PRR1/TOC1* in *A. thaliana* is co-regulated by the modulation of chromatin structure and the direct binding of the morning-acting repressors, LHY and CCA1, to the specific *cis*-element (evening element; AAAATATCT) of the *PRR1/TOC1* promoter (Alabadí *et al.*, 2001; Perales & Más, 2007). The PRR1/TOC1 protein accumulates early in the night and interacts with phosphorylated PRR3 protein to stabilize its function (Fujiwara *et al.*, 2008). However, the PRR1/TOC1 protein gradually dissociates from PRR3 around and after midnight, and is subsequently posttranslationally degraded through the proteasome pathway mediated by Zeitlupe (ZTL) (Fujiwara *et al.*, 2008; Más *et al.*, 2003). In addition, genetic analyses demonstrate that the *PRR1/TOC1* gene

regulates photoperiodic responses, hypocotyl elongation, and floral induction (Más *et al.,* 2003; Somers *et al.,* 1998). It is suggested that *PRR1/TOC1* regulates floral transition in a *LHY/CCA1*-dependent manner in the main feedback loop, whereas *PRR1/TOC1* controls hypocotyl elongation downstream of the *LHY/CCA1* function (Ding *et al.*, 2007). These results imply that the *PRR1/TOC1* gene has dual roles; one in regulating expression of the *LHY/CCA1* gene in the feedback loop, and the other in controlling output genes' function in the photomorphogenic process.

To clarify the regulation mechanism of the *Populus* clock system, I examined the expression pattern and the function of *Populus PRR1/TOC1* gene. The protein product and genomic structures of the *Populus PRR1/TOC1* gene were highly similar to those of other angiosperms' *PRR1/TOC1* genes. In addition, the *Populus* PRR1/TOC1 protein was nuclear localized. However, *Populus PRR1/TOC1* did not show a diurnal expression pattern in either controlled or natural conditions. Furthermore, *Populus PRR1/TOC1* did not fully complement the function of *Arabidopsis PRR1/TOC1* in the *PRR1/TOC1* null mutant. These results indicate that the regulation of *PRR1/TOC1* expression and the function of its translated protein differ between *Populus* and *Arabidopsis*.

Materials and Methods

Plant materials and growth conditions

Hybrid aspens (*Populus tremula* x *tremuloides*) were grown aseptically on agar medium containing Murashige and Skoog basal salts (Murashige & Skoog, 1962), Murashige and Skoog vitamins, and 0.8% (w/v) agar at 21°C under 16 h light/8 h dark conditions (100–130 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Microarray data analysis

Expression data of the circadian clock genes [*LHY/CCA1*, *PRR*s, *GI*, *Early Flowering 3* (*ELF3*), *Early Flowering 4* (*ELF4*) and *Lux Arrhythmo/Phytoclock 1* (*LUX/PCL1*)] were retrieved using the Diurnal Search Tool (http://diurnal. cgrb.oregonstate.edu/). *A. thaliana* microarray data were based on results reported previously (Bläsing *et al.*, 2005; Smith *et al.*, 2004). Identification of clock-related genes in *A. thaliana* is shown in Table 4-1. To examine the microarray data of

Populus clock-related genes, probe Set IDs of the *Populus* clock-related genes were identified from the database in Affymetrix (http:// www.affymetrix.com/). Expression data were retrieved using the Probe Set IDs as shown in Table 4-1.

RNA extraction and real-time PCR

Leaves of *P. tremula* x *tremuloides* grown on agar medium were sampled at 1-h intervals to investigate the expression pattern of *Populus PRR1/TOC1* and *LHY2* genes. To further analyze the expression of *PRR1/TOC1*, leaves and stems of *P. nigra* grown under natural conditions on the campus of Iwate University were collected at 3-h intervals from 9:00 AM on July 7, 2006 to 9:00 AM on July 9, 2006. The samples were immediately frozen in liquid nitrogen and stored at –80°C until use.

Total RNA was isolated from samples using a NucleoSpin® RNA plant kit (Macherey-Nagel, Düren, Germany) or an RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) with in-column DNase I digestion. First-strand cDNA was synthesized using ReverTra Ace-α® (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Real-time PCRs were performed using a Thermal Cycler Dice® Real Time System (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The gene-specific primers for real-time PCR were as follows: *PRR1/TOC1* (forward, 5'-ATATCGACCTGATGTGCCAGGGAT-3'; reverse, 5'-GCTCGCCACATACTTGAAGTTTCTCT-3'), *LHY2* (forward, 5'-CT-CCATTGAGCTGCCTGAAACA-3'; reverse, 5'-CGACCGCATAGACTCCAAT-TC-3') and *ubiquitin 11* (*UBQ*) (forward, 5'-GGTTGATTTTTGCTGGGAAGC-3'; reverse, 5'-GATCTTGGCCTTCACGTTGT-3'). The *UBQ* gene was used as a normalization control. Each RNA sample was assayed in triplicate.

Isolation of full-length cDNA and genomic DNA encoding PRR1/TOC1

The *Populus PRR1/TOC1* gene was isolated from a full-length enriched cDNA library constructed from mRNA of *P. nigra* (Nanjo *et al.*, 2007). The *PRR1/TOC1* gene was subjected to dideoxy-nucleotide sequencing using a primer-walking method and nucleotide sequences were assembled by ATGC software (Genetyx, Tokyo, Japan).

The full-length genomic region of *P. nigra PRR1/TOC1* (*PnPRR1/TOC1*) was

isolated from genomic DNA using PCR. Genomic DNA was extracted using the CTAB (hexadecyltrimethyl-ammonium bromide) method from leaves of 1-month-old *P. nigra* plants maintained on agar medium (Murray & Thompson, 1980). PCR was performed using Takara LA Taq® polymerase (Takara Bio) according to the manufacturer's instructions and with the primer set 5'-CGCTTATATGCTCAATCAACCCC-3' and 5-CACCCTGGATGAGAAGAA-TTCAGG-3'. The PCR conditions were as follows: 94°C for 1 min; 30 cycles of 98°C for 15 s, 68°C for 10 min; and 72°C for 10 min. Amplified PCR fragments were subcloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA) and subjected to dideoxy-nucleotide sequencing using a primer-walking method. Nucleotide sequences were assembled by ATGC software (Genetyx).

Analysis of subcellular localization of Populus *PRR1/TOC1*

Protoplast transient expression assays were used to investigate the subcellular localization of *Populus* PRR1/TOC1. To increase the green fluorescent protein (GFP) signal intensity in these experiments, the *sGFP* (S65T) gene driven by the CaMV 35S promoter (Chiu *et al*., 1996) was replaced with the *EmGFP* gene (Invitrogen, Carlsbad, CA, USA). The cloning sites (*Xba*I and *Bam*HI) between the 35S promoter and *EmGFP* were altered into unique restriction sites (*Spe*I and *Kpn*I) by PCR-based site-directed mutagenesis (Sambrook & Russell, 2001) using the following primer set: forward, 5'-TACAGTCGACACTAGTGGTACCATGG-TGAG-3'; reverse, 5'-CTCACCATGGTACCACTAGTGTCGACTGTA-3'. The coding sequence (CDS) of *PnPRR1* (without stop codon) was amplified by PCR using KOD plus (Toyobo) according to the product manual with the following primer set: forward, 5'-CACCACTAGTATGGAGGGAGA-3'; reverse, 3'-GGT-ACCAGATCCTGAAGCAT-3'. The PCR product of *PnPRR1* was digested with *Spe*I and *Kpn*I and was subsequently introduced into the *Spe*I/*Kpn*I site between the CaMV 35S promoter and *EmGFP* in the expression vector.

Protoplasts were enzymatically isolated from leaves of *P. tremula* x *tremuloides* and *A. thaliana* (Columbia ecotype) as described by Uemura *et al.* (1995). *Populus* and *Arabidopsis* leaves were cut into rectangles and incubated in an enzyme solution consisting of 2.4% (w/v) cellulase "Onozuka" R-10 (Yakult Pharmaceutical Industry, Tokyo, Japan), 0.8% (w/v) macerozyme R-10 (Yakult),

0.05% (w/v) pectolyase Y-23 (Kyowa Chemical Products, Osaka, Japan), 0.4 M sorbitol, 10 mM MES-KOH (pH 5.6) and 1 mM $CaCl_2$. After leaves were incubated in the enzyme solution for 3 h at 27°C in the dark, the undigested leaf sections were removed by passing the solution through a nylon filter. The filtrate was centrifuged at 50 × *g* for 20 min at room temperature to collect the protoplasts. Isolated protoplasts were suspended in a 0.4 M sorbitol solution containing 1 mM MES-KOH (pH 5.6) and 1 mM $CaCl_2$.

Using a standard polyethylene glycol method (Sheen, 2001), 10 μg plasmid DNA of each construct was transfected into isolated *Populus* and *Arabidopsis* protoplasts in a solution consisting of 4% (w/v) polyethylene glycol 4000 (Sigma-Aldrich, St. Louis, MO, USA), 0.1 M $Ca(NO_3)_2$ and 0.4 M sorbitol. After incubation overnight, GFP expression was observed using a fluorescence microscope (BX61; Olympus, Tokyo, Japan) fitted with a cooled CCD camera (UB-7010; Keyence, Osaka, Japan).

Generation of transgenic Arabidopsis

The *Arabidopsis* T-DNA insertion mutant of *PRR1/TOC1* (*toc1-21*) in the Wassilewskija (Ws) ecotype was kindly provided by Dr. Seth J. Davis (Max Planck Institute for Plant Breeding Research, Germany) (Ding *et al.*, 2007). To perform complementation analyses using the *toc1-21* mutant, transgenic plants were generated as described below.

The *PRR1/TOC1* promoter region of *A. thaliana* (*AtPRR1pro*; 1558 bp upstream of the translation start site; Perales & Más, 2007), CDS of the *PRR1/TOC1* in *A. thaliana* (*AtPRR1*; with stop codon), and *PnPRR1* (with stop codon) were amplified by PCR using KOD plus (Toyobo) according to the product manual. The following primer sets were used: *AtPRR1pro* (forward, 5'-CACCGGATCCCGTGTCTTACGGTGGATGAAGTTG-3'; reverse, 5'-AGA-TACTAGTGATCAGATTAACAACTAAACCCACAC-3'), *AtPRR1* (forward, 5'-TGATCACTAGTATCTATGGATTTGAACGGTGAGTG-3'; reverse, 5'-TTG-GCGCGCCCTCAAGTTCCCAAAGCATCATCC-3') and *PnPRR1* (forward, 5'-TGATCACTAGTATCTATGGAGGGAGAGGTAGATGAG-3'; reverse, 5'-TT-GGCGCGCCCTTAAGATCCTGAAGCATCGTCCTC-3'). The *AtPRR1pro* PCR product was cloned into the pENTR™/D-TOPO® vector (Invitrogen). The

fragments of *AtPRR1* and *PnPRR1* were digested with *Spe*I and *Asc*I and were subsequently introduced into the *Spe*I/*Asc*I site downstream of *AtPRR1pro* in the pENTR™/D-TOPO® vector. These entry vectors harboring *AtPRR1pro::AtPRR1* and *AtPRR1pro::PnPRR1* were subjected to dideoxy-nucleotide sequencing and were used in the LR-Gateway reaction (Invitrogen) with pGWB1 (Nakagawa *et al.*, 2007). These constructs were introduced into the *toc1-21* mutant by *Agrobacterium tumefaciens* (Clough & Bent, 1998). Seeds of T1 plants were selected using appropriate antibiotics and transformed plants containing the constructs were checked by genomic PCR to amplify the foreign *AtPRR1* and *PnPRR1* fragments.

Hypocotyl length analysis

To measure hypocotyl length, seeds of Ws (wild-type), *toc1-21*, *pAtPRR1::AtPRR1* (T3 plants) and *pAtPRR1::PnPRR1* (T3 plants) were stratified on Murashige and Skoog plates with 3% (w/v) sucrose. Plates were incubated in the dark at 4°C for 3 days and transferred to a growth chamber at 23°C under short-day growth conditions (8 h light/16 h dark). Light was provided by white fluorescent lamps at 50–70 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Seedlings were grown for 7 days and then scanned at 600 dpi (GT-X700, Epson, Nagano, Japan). Hypocotyl length was measured using ImageJ software (http:// rsbweb.nih.gov/ij/). Genomic insertions in the constructs were checked by genomic PCR to amplify the foreign *AtPRR1* and *PnPRR1* fragments. Mean value ± SE was calculated for each genotype (*n*=20–35).

Measurement of flowering time

To measure flowering time, seeds of Ws, *toc1-21*, *pAtPRR1::AtPRR1* (T3 plants) and *pAtPRR1::PnPRR1* (T3 plants) were planted on soil and stratified in the dark at 4°C for 3 days. Plants were then transferred to short-day growth conditions (8 h light/16 h dark) at 23°C. Light was provided by white fluorescent lamps at 50–70 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Flowering time was measured by counting the number of rosette leaves and cauline leaves on the main stem. Mean value ± SE was calculated for each genotype (*n*=15–20).

Results

Comparison of diurnal expression patterns of circadian clock-related genes in Arabidopsis *and* Populus

Recent evolutionary analyses have implied that the changes in composition and number of *Populus* clock-related genes could result in modifications to the *Populus* clock system. I first compared the diurnal expression patterns of the clock-related genes *LHY/CCA1*, *PRR*s, *GI*, *ELF3*, *ELF4,* and *LUX/PCL1* in *Populus* and *Arabidopsis*. Expression data for these genes were retrieved from an available microarray database (Diurnal Search Tool; http://diurnal.cgrb. oregonstate.edu/). The microarray data showed that all *Arabidopsis* clock genes have typical diurnal expressions, which are similar to the patterns of expression observed in many other studies (Fig. 4-1a; Doyle *et al.*, 2002; Fowler *et al.*, 1999; Hicks *et al.*, 2001; Matsushika *et al.*, 2000; Onai & Ishiura, 2005; Schaffer *et al.*, 1998; Wang & Tobin, 1998). Likewise, most clock-related genes in *Populus* showed diurnal rhythms and had peak expressions similar to their respective *Arabidopsis* orthologs (Fig. 4-1b). However, two genes in *Populus* (*PRR1/TOC1* and *ELF3a*) had short-term fluctuations during the day and did not retain typical diurnal expressions. This finding suggests that this part of the *Populus* clock system differs from the model clock system in *Arabidopsis*.

Expression pattern of Populus PRR1/TOC1

PRR1/TOC1 and the partners *LHY* and *CCA1* play key roles in the main feedback loop of the *Arabidopsis* clock system. To further investigate the regulation mechanism of the main loop in *Populus*, expression patterns of *Populus PRR1/TOC1* and *LHY*s were analyzed in *P. tremula* x *tremuloides* and *P. nigra.* In leaves of *P. tremula* x *tremuloides* sampled at 1-h intervals, the ortholog of *LHY/CCA1* (*PttLHY2*) exhibited rhythmicity with peak expression around dawn, which is consistent with expression patterns of *LHY/CCA1* genes in other plant species (Fig. 4-2a; Izawa *et al.*, 2002; Ramos *et al.*, 2005; Schaffer *et al.*, 1998; Wang & Tobin, 1998). On the other hand, *PttPRR1/TOC1* did not show a typical diurnal expression pattern. In addition, the altered expression pattern of *Populus PRR1/TOC1* was also observed in both leaves and stems of *P. nigra* grown under field conditions (Fig, 4-2b). In *P. nigra*, *LHY*s had typical diurnal expressions

(Takata *et al.*, in press). These results indicate that the expression pattern of *Populus PRR1/TOC1* differs from that of other plant species (Murakami *et al.*, 2003; Ramos *et al.*, 2005; Strayer *et al.*, 2000) and that the altered expression of *PRR1/TOC1* is consistent among *Populus* species.

Characterization of Populus PRR1/TOC1 *gene*

To further characterize the *Populus PRR1/TOC1* gene, a cDNA clone of *PRR1/TOC1* (*PnPRR1/TOC1*) was isolated from a full-length enriched cDNA library of *P. nigra* (Nanjo *et al.*, 2007). The deduced amino acid sequence of *PnPRR1/TOC1* retains a conserved pseudo-receiver (PR) domain at the N-terminus and CONSTANS, CONSTANS-LIKE and TOC1 (CCT) motifs at the C-terminus. In addition, the genomic organization of *PnPRR1/TOC1* was similar to that of the *PRR1/TOC1* genes in *A. thaliana* and *Oryza sativa* (Fig. 4-3a).

Several conserved regions and amino acid residues are crucial for the functions of the PRR1/TOC1 protein (Kevei *et al.*, 2006; Hazen *et al.*, 2005; Strayer *et al.*, 2000). In the PRR1/TOC1 protein in *A. thaliana*, proline residues at positions 96 and 124 in the PR domain and the alanine residue at position 562 in the CCT motif are required to maintain the correct clock system (Hazen *et al.*, 2005; Somers *et al.*, 1998). In addition, *PRR* genes retain the unique glutamate residue in the PR domain that replaces the phospho-accepting aspartate residue in the Response Regulator (RR) domain of the RR superfamily (Mizuno, 2005). These amino acid residues were highly conserved in the deduced PRR1/TOC1 proteins of *P. nigra* (*PnPRR1/TOC1*), *P. trichocarpa* (*PtPRR1/TOC1*), *Castanea sativa* (*CsPRR1/TOC1*), *Vitis vinifera* (*VvPRR1/TOC1*), *O. sativa* (*OsPRR1/TOC1*) and *Sorghum bicolor* (*SbPRR1/TOC1*) (Figs. 4-3b and 4-3d). In addition, the region enriched in acidic amino acid residues immediately downstream of the CCT motif, which is a feature common to a number of transcriptional activators, was conserved among all PRR1/TOC1 proteins (Fig. 4-3e; Cress & Triezenberg, 1991). The direct repeat of 47 amino acid residues in the center of the PRR1/TOC1 protein in *A. thaliana*, the function of which is unknown, was conserved neither in *Populus* nor among other angiosperm species (Fig. 4-3c; Kolmos *et al.*, 2008). The typical specific regions and amino acid residues that are essential for the functionality of PRR1/TOC1 are highly

conserved in the *Populus* PRR1/TOC1 protein.

Subcellular localization of Populus *PRR1/TOC1*

Arabidopsis PRR1/TOC1 is localized in the nucleus (Strayer *et al.*, 2000). To determine whether *Populus* PRR1/TOC1 is also nuclear localized, PnPRR1/TOC1 fused to GFP under the control of the 35S promoter (*35S::PnPRR1/TOC1-EmGFP*) was expressed transiently in protoplasts isolated from *Arabidopsis* and *Populus*. GFP fluorescence was observed in the nuclei in both *Arabidopsis* and *Populus* protoplasts, whereas GFP fluorescence was restricted to the cytoplasm in protoplasts expressing the *35S::EmGFP* construct (Fig. 4-4). Thus the *Populus* PRR1/TOC1 appears to be nuclear localized in *Populus*.

Complementation analyses

To examine functional similarities between *Populus* and *Arabidopsis PRR1/TOC1* genes, complementation analyses were performed with a T-DNA insertion *Arabidopsis* mutant (*toc1-21*; Ws ecotype). This mutant lacks *PRR1/TOC1* expression and shows long hypocotyl and early flowering phenotypes (Ding *et al.*, 2007). Complementation lines included *toc1-21* mutants harboring *AtPRR1* or *PnPRR1* under the control of the *AtPRR1* promoter (*AtPRR1pro*). Hypocotyl length and flowering time of these plants were measured under short-day growth conditions (8 h light/16 h dark).

The *toc1-21* mutant had a longer hypocotyl than the wild-type plants (Ws) under short-day conditions (Fig. 4-5a). As expected, ectopic expressions of *AtPRR1* and *PnPRR1* in the *toc1-21* mutant complemented the long hypocotyl phenotype in three independent transformants (*pAtPRR1::AtPRR1*; #13, #15, and #19: *pAtPRR1::PnPRR1*; #1, #2, and #13). The hypocotyl length in these complemented transformants was almost equal to that of the wild-type. With respect to floral transition, the *toc1-21* mutant showed an early flowering phenotype compared with the wild-type. Two independent *pAtPRR1::AtPRR1* lines of the *toc1-21* mutant showed delayed flowering (i.e., an increase in the number of leaves before flowering) (Fig. 4-5b). On the other hand, ectopic expression of *PnPRR1* did not fully complement the early flowering phenotype of the mutant. These results imply that *Populus PRR1/TOC1* only partially

complements the function of *Arabidopsis PRR1/TOC1* in *Arabidopsis*.

Discussion

Current models of the *Arabidopsis* circadian clock are comprised of multiple interlocked feedback loops (reviewed in McClung, 2008). The *Arabidopsis* clock model greatly facilitates investigations of clock systems in other plant species. Recent evolutionary analyses of the plant clock system have revealed that circadian clock-related genes are widely conserved among angiosperms, and that the fundamental mechanism of the *Arabidopsis* clock model may have been established before the radiation of rosids species (Takata *et al.*, in press; Chapter 3 in this thesis). However, these analyses also show that clock-related genes differ in make-up and number in the *Populus* lineage, compared with those in the *Arabidopsis* system. These differences may result in alterations to the regulation mechanism of the *Populus* clock system. To elucidate how the *Populus* clock system differs from that of *Arabidopsis*, the regulation mechanism of the main feedback loop (*LHY/CCA1-PRR1/TOC1*) in *Populus* was examined. The results of this study provide evolutionary evidence that the *Populus* clock system differs from the *Arabidopsis* clock model.

Microarray data showed that the clock-related genes *LHY/CCA1*, *PRR*s, *GI*, *ELF3*, *ELF4,* and *LUX/PCL1* in *A. thaliana* show typical diurnal rhythmicity (Fig. 4-1a). These expression patterns are consistent with those reported previously (Doyle *et al.*, 2002; Fowler *et al.*, 1999; Hicks *et al.*, 2001; Matsushika *et al.*, 2000; Onai & Ishiura, 2005; Schaffer *et al.*, 1998; Wang & Tobin, 1998). In *Populus*, clock-related genes also showed diurnal patterns of expression, except for *PRR1/TOC1* and *ELF3b* (Fig. 4-1b). The peak expression of most clock-related genes in *Populus* resembled that of their respective orthologs in *A. thaliana*. For example, transcript levels of *GI*, *ELF4,* and *LUX/PCL1* in both *Populus* and *A. thaliana* peak around dusk. In addition, the *Populus LHY*s exhibited typical diurnal rhythmicity with peak expression around dawn, as does *LHY/CCA1* in *A. thaliana*. This pattern of expression has also been observed for *P. nigra LHY*s (Takata *et al.*, in press). On the other hand, the result that *Populus PRR1/TOC1* did not show diurnal expression is unexpected (Fig. 4-1b), because the *PRR1/TOC1* genes in other plant species clearly exhibit rhythmicity

(Murakami *et al.*, 2003; Ramos *et al.*, 2005). Therefore, these results imply that the make-up, number, and expression patterns of some *Populus* clock-related genes have altered through evolution.

PRR1/TOC1 genes in angiosperms exhibit typical diurnal rhythmicity with peak expression at dusk (Murakami *et al.*, 2003; Ramos *et al.*, 2005; Strayer *et al.*, 2000). The results of the present study clearly show that *Populus PRR1/TOC1* has not retained this typical expression pattern, whereas *LHY* genes exhibit diurnal expressions similar to those of *LHY/CCA1* genes in other plant species (Fig. 4-2; Takata *et al.*, in press). This is the first report of a plant lacking diurnal expression of the key clock component *PRR1/TOC1*.

The *Populus* PRR1/TOC1 protein has specific regions and essential amino acids that are highly conserved among angiosperms (Fig. 4-3). In addition, *Populus* PRR1/TOC1 is nuclear localized (Fig. 4-4). These observations may indicate that the *PRR1/TOC1* gene encodes a functional protein in *Populus*. Indeed, in the complementation analysis, *Populus* PRR1/TOC1 recovered the long hypocotyl phenotype of the *toc1-21* mutant as with PRR1/TOC1 in *A. thaliana* (Fig. 4-5a). However, the early flowering phenotype of the *toc1-21* mutant was not complemented by *Populus* PRR1/TOC1, while the plants expressing *Arabidopsis PRR1/TOC1* exhibited late-flowering phenotypes similar to the wild-type plants (Fig. 4-5b). It is difficult to explain why *Populus PRR1/TOC1* can recover the long hypocotyl phenotype but not the early flowering phenotype. *PRR1/TOC1* and *LHY/CCA1* are regulated in the negative-positive transcriptional feedback loop (Más, 2005). In this loop, PRR1/TOC1 regulates the floral transition in a LHY/CCA1-dependent manner while LHY/CCA1 functions upstream of PRR1/TOC1 in regulating hypocotyl elongation (Ding *et al.*, 2007). In addition, PRR1/TOC1 in *A. thaliana* directly interacts with Phytochrome-Interacting Factor 4 (PIF4) and PIF5, which are basic helix-loop-helix transcription factors that promote plant growth (Nozue *et al.*, 2007; Yamashino *et al.*, 2003). Thus our results imply that *Populus* PRR1/TOC1 successfully regulates the photomorphogenic process by interacting with PIF4 and PIF5 (Fig. 4-5a). On the other hand, the non-recovered floral phenotypes in *AtPRR1pro::PnPRR1* may be because *Populus PRR1/TOC1* can not positively regulate *LHY* and *CCA1* genes in the main feedback loop (Fig. 4-5b). Therefore,

the function of *Populus PRR1/TOC1* might differ from that of *Arabidopsis PRR1/TOC1* in the main feedback loop of the plant clock system.

In conclusion, most circadian clock-related genes in *Populus* exhibit diurnal expression patterns similar to those in *A. thaliana*. However, the *PRR1/TOC1* gene, a key component of the *Arabidopsis* clock system, did not show a diurnal expression pattern in *Populus*. In spite of this, the partners of *PRR1/TOC1* in the main feedback loop, the *LHY* genes, retained a typical rhythmic expression in poplar. Furthermore, the *Populus PRR1/TOC1* gene did not complement the floral phenotype, which *Arabidopsis PRR1/TOC1* regulates in a LHY/CCA1-dependent manner (Ding *et al.*, 2007). These results may indicate that the regulation mechanism of the main loop in *Populus* differs to that in *Arabidopsis*. Further studies are required to clarify this regulation mechanism; for example, expression analyses of *Populus* circadian clock-related genes in *Populus* transformants repressing/overexpressing *Populus LHY* or *PRR1/TOC1*.

Table 4-1. Gene IDs and Affymetrix probe set IDs of *Arabidopsis thaliana* and *Populus trichocarpa* used for microarray data search

Arabidopsis thaliana		*Populus trichocarpa*			Gene family or features
Gene	Gene ID[a]	Gene	Gene ID[b]	Affymetrix probe set ID[c]	
AtLHY	At1g01060	*PtLHY1*	eugene3.00021683	PtpAffx.125536.1.S1_at	Single MYB
AtCCA1	At2g46830	*PtLHY2*	estExt_Genewise1_v1.C_LG_XIV1950	Ptp.4550.1.S1_at	Single MYB
AtPRR1/TOC1	At5g61380	*PtPRR1/TOC1*	fgenesh4_pg.C_scaffold_129000038	PtpAffx.215744.1.S1_at	Pseudo-response regulator
AtPRR3	At5g60100				Pseudo-response regulator
AtPRR5	At5g24470	*PtPRR5a*	fgenesh4_pm.C_LG_XII000134	Ptp.2304.1.A1_s_at	Pseudo-response regulator
		PtPRR5b	eugene3.00150024	PtpAffx.37666.1.S1_at	Pseudo-response regulator
AtPRR7	At5g02810	*PtPRR7a*	estExt_fgenesh4_pm.C_LG_VIII0151	PtpAffx.207664.1.S1_x_at	Pseudo-response regulator
		PtPRR7a	estExt_fgenesh4_pm.C_LG_VIII0151	PtpAffx.29932.2.S1_s_at	Pseudo-response regulator
AtPRR9	At2g46790	*PtPRR9a*	fgenesh4_pg.C_LG_II001656	PtpAffx.202306.1.S1_at	Pseudo-response regulator
		PtPRR9b	estExt_fgenesh4_pg.C_LG_XIV0468	PtpAffx.18265.1.A1_at	Pseudo-response regulator
AtGI	At1g22770	*PtGI*	eugene3.00020603	Ptp.4849.1.S1_s_at	Nuclear protein
AtLUX/PCL1	At3g46640	*PtLUX/PCL1a*	gw1.I.1170.1	PtpAffx.200774.1.S1_at	GARP
		PtLUX/PCL1b	gw1.IX.4105.1	PtpAffx.158360.1.S1_s_at	GARP
AtELF4	At2g40080	*PtELF4*	fgenesh4_pg.C_LG_VIII000583	PtpAffx.12097.1.A1_at	Nuclear protein
AtELF3	At2g25930	*PtELF3a*	estExt_fgenesh4_pm.C_LG_VI0700	PtpAffx.206700.1.S1_at	Nuclear protein
		PtELF3b	eugene3.00030174	PtpAffx.202861.1.S1_at	Nuclear protein

[a]Gene IDs obtained from TIGR (http://www.tigr.org/tdb/e2k1/ath1/).
[b]Gene IDs obtained from JGI (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html).
[c]Affymetrix probe set IDs seen at the Affymetrix NetAffx™ Analysis Center (http://www.affymetrix.com/analysis/index.affx).

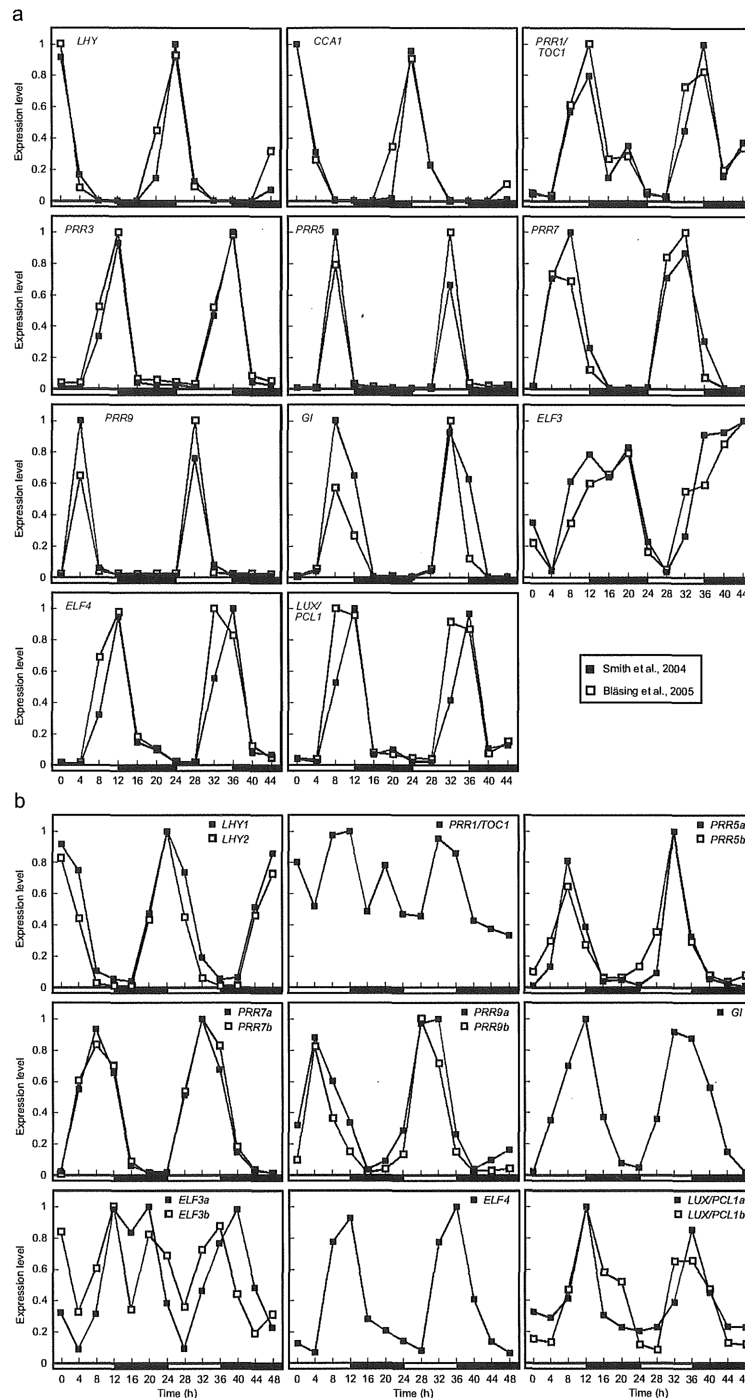

Figure 4-1. Expression patterns of circadian clock related-genes in *A. thaliana* (a) and *Populus* (b). Microarray data were retrieved from the Diurnal Search Tool (http://diurnal.cgrb.oregonstate.edu/). White and black boxes indicate the results from Bläsing *et al.* (2005) and Smith *et al.* (2004), respectively. (a) Expression data of the *Arabidopsis* clock related-genes (*LHY*, *CCA1*, *PRR*s, *GI*, *ELF3*, *ELF4* and *LUX/PCL1*) were provided from the results (Bläsing *et al.*, 2005; Smith *et al.*, 2004) and retrieved using gene IDs shown in Table 4-1. (b) Affymetrix probe set IDs of the *Populus* circadian clock genes (*LHY*s, *PRR*s, *GI*, *ELF3*s, *ELF4* and *LUX/PCL1*s) were identified from the database in Affymetrix (http://www.affymetrix.com/) (Table 4-1). Using these Probe Set IDs, their expression data were retrieved from the Diurnal Search Tool.

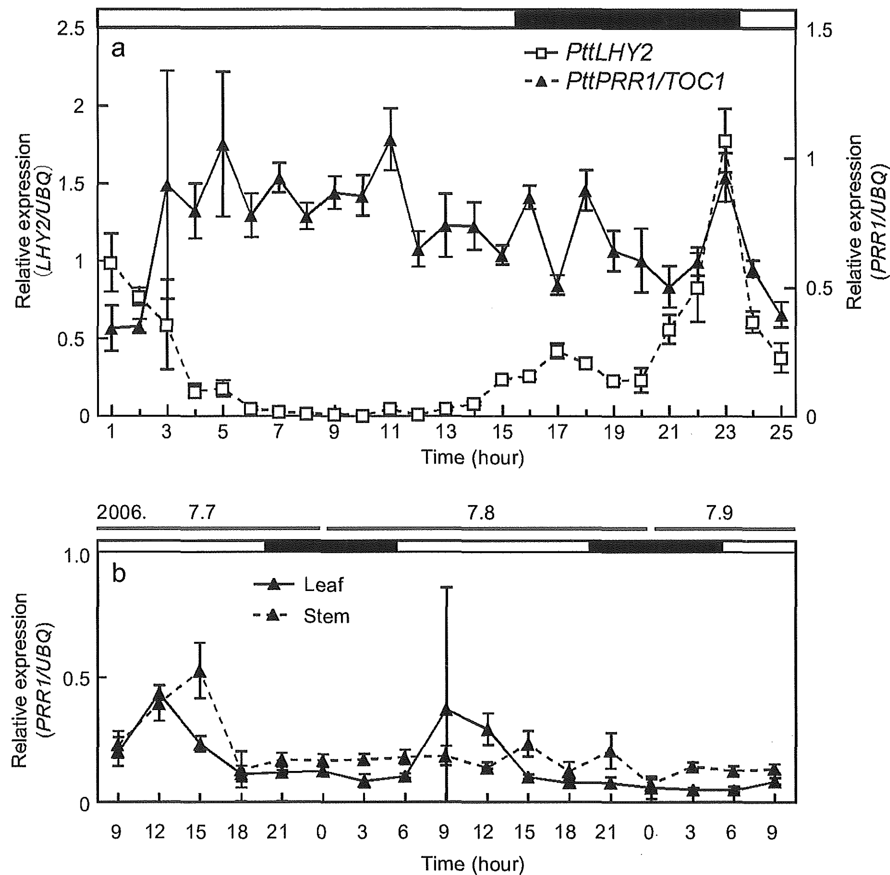

Figure 4-2. Expression pattern of the circadian clock related-genes in *Populus* plants. (a) Expression analyses were performed using leaves of *P. tremula* x *tremuloides* grown on agar medium. (b) Expression analyses were performed using leaves and stems of *P. nigra* grown under field conditions. With total RNA isolated from the samples, transcript accumulation of each gene was investigated by real-time PCR using gene-specific primers and normalized to that of *ubiquitin 11* (*UBQ*). The date and time of collection of the samples are shown above and below, respectively. White and black bars indicate day and night, respectively. Each RNA sample was assayed in triplicates. Values are means ± SD.

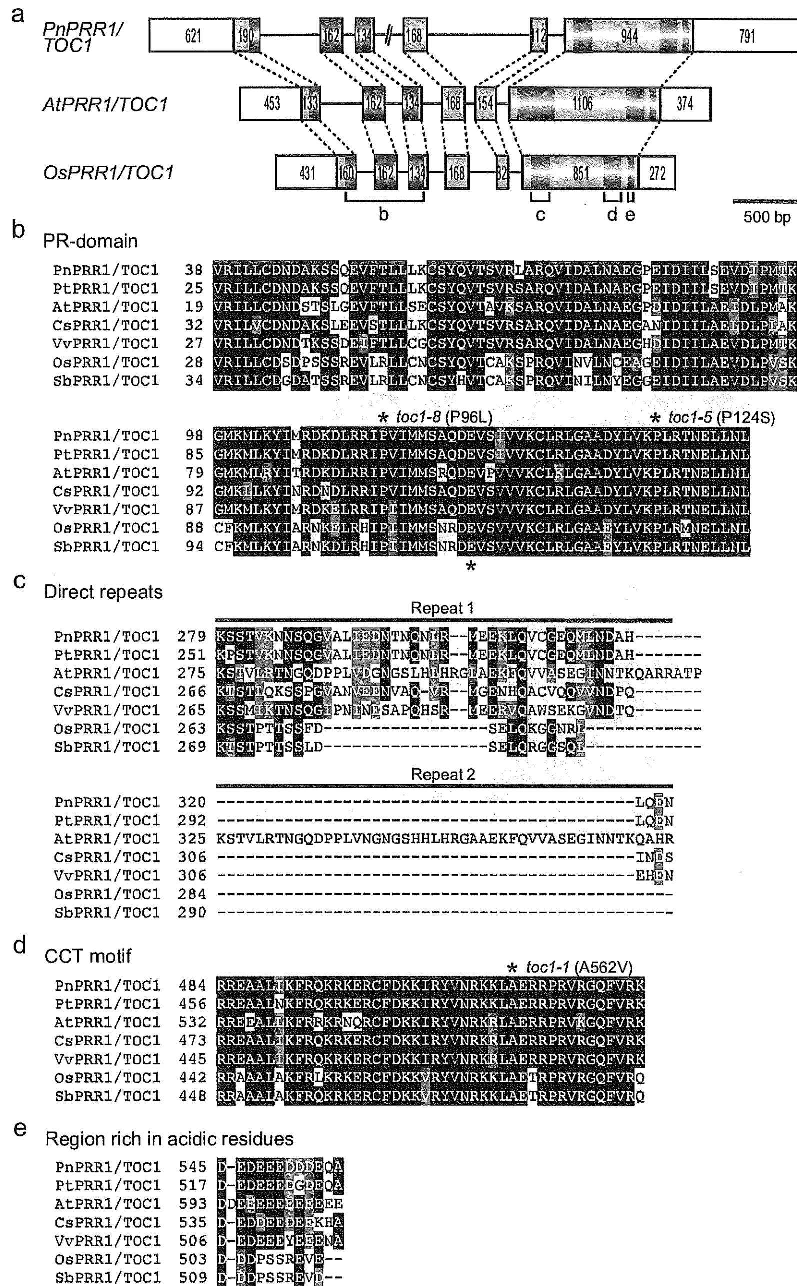

Figure 4-3. Characterization of *Populus PRR1/TOC1*. (a) Schematic diagram of the exon-intron structures of *PnPRR1/TOC1*, *AtPRR1/TOC1* and *OsPRR1/TOC1*. White, gray and black boxes indicate UTRs, CDSs and conserved regions, respectively. Length of nucleotide sequences indicated within the boxes. (b to e) Alignments of several unique domains in *PRR1/TOC1*. PR-domain (b), direct repeats (c), CCT motif (d) and region enriched in acidic residues (e). Amino acids sequences encoded by *PnPRR1/TOC1*, *PtPRR1/TOC1*, *AtPRR1/TOC1*, *CsPRR1/TOC1*, *VvPRR1/TOC1*, *OsPRR1/TOC1* and *SbPRR1/TOC1* are aligned using the TCoffee program. Identical and similar amino acid residues are highlighted with black and gray backgrounds, respectively. Asterisks indicate essential amino acids for the function indentified within AtPRR1/TOC1.

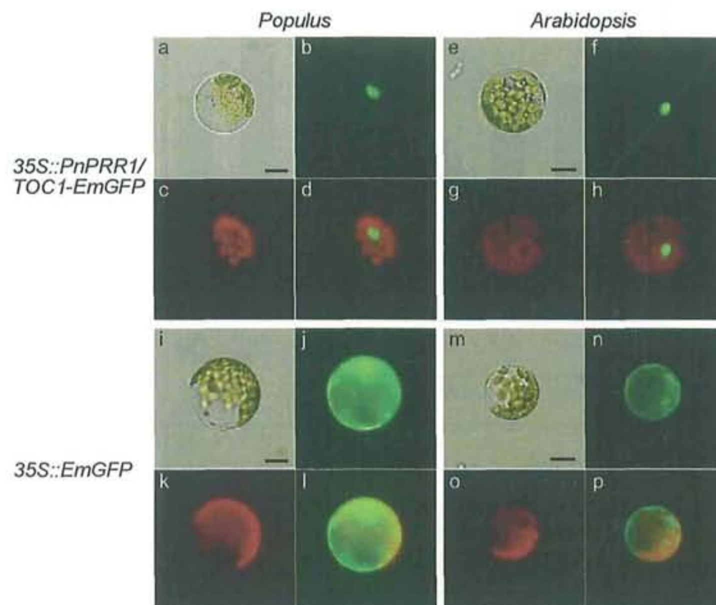

Figure 4-4. Subcellular localization of PnPRR1/TOC1 in *Populus* (a-d) and *Arabidopsis* (e-h). Plasmid DNA constructs containing *PnPRR1/TOC1-EmGFP* (a-h) or *EmGFP* (i-p) under CaM35S promoter were transfected into *Populus* (a-d, i-l) and *Arabidopsis* (e-h, m-p) protoplasts using polyethylene glycol. Light field image (a, e, l and m), GFP signal (b, f, j and n) and auto fluorescence of chloroplasts (c, g, k and o) were captured. GFP signal and auto fluorescence of chloroplasts were merged (d, h, l and p). All pictured were shown in the same scale and bar represents 20 μm.

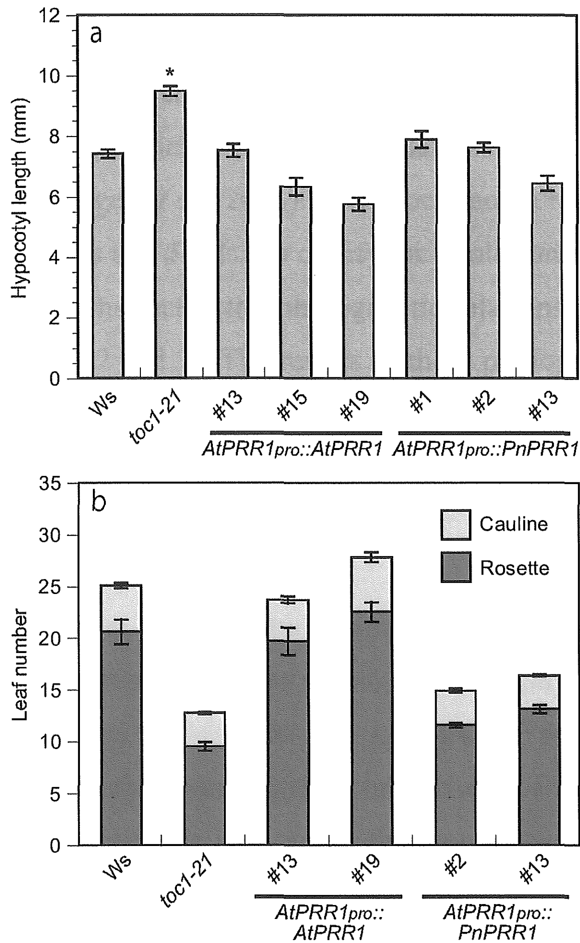

Figure 4-5. Complementation analyses of *Populus PRR1/TOC1* in *Arabidopsis toc1-21* mutant. (a) Hypocotyl elongation of Ws, *toc1-21*, *AtPRR1pro::AtPRR1* and *AtPRR1pro::PnPRR1* was measured after 7 days of growth under a short-day growth condition (8 h-light/16 h-dark). The mean value ± SE was calculated (n=20–35 for each genotype). (b) Flowering time of Ws, *toc1-21*, *AtPRR1pro::AtPRR1* and *AtPRR1pro::PnPRR1* was measured under a short-day growth condition (8 h-light/16 h-dark). The number of rosette and cauline leaves was counted to measure the time of floral transition. The mean value ± SE was calculated (n=15–20 for each genotype).

Chapter 5

General discussion

Evolutionary process of the plant circadian clock system

The plant clock system model consists of "multiple interlocked feedback loops", and was developed from genetic and biological analyses of *Arabidopsis thaliana* (Locke *et al.,* 2006; Zeilinger *et al.,* 2006). This clock model consists of two main gene families, *LHY/CCA1*s and *PRR*s. To clarify the evolutionary process of plant clock systems, I first reconstructed the phylogenetic relationships of *LHY/CCA1* and *PRR* genes (Chapters 2 and 3). The results of these phylogenetic analyses led to a hypothesis on the evolutionary process of the plant clock system in angiosperm evolutionary lineages.

Methodology of this study

The methodological feature of this study was to use information about the syntenic relationships to "fine tune" the topology of the phylogenetic trees. To reconstruct the molecular phylogenies of *LHY/CCA1* and *PRR* genes in angiosperms, three approaches were taken: comparison of genomic structures, reconstruction of the phylogenetic tree, and estimation of syntenic relationships. The first two are conventional and versatile methods, whereas the last approach has resulted from the rapid expansion of draft genome sequences available for angiosperm species (Sampedro *et al.*, 2005; Bocock *et al.*, 2008). The phylogenetic trees of *LHY/CCA1*s and *PRR*s showed ambiguous evolutionary patterns, for example, the gene duplication events of *LHY/CCA1* and *PRR5/PRR9* in *A. thaliana* (Figs. 2-2, 3-6 and 3-7). One reason for these ambiguous divergences appears to be the different evolutionary rates among plant species, which affects the topology of phylogenetic trees. In fact, it has been reported that rates of molecular evolution are consistently low in woody plants that have relatively long generation times, as compared with related herbaceous plants that generally have shorter generation times (Smith & Donoghue, 2008; Tuskan *et al.*, 2006; Van de Peer *et al.*, 1996). However, the methodological weakness of

analyses with phylogenetic trees can be overcome by using information about syntenic relationships across plant species. This is because the relationships are reconstructed by comparative genomics with extensive plant genome sequences and also by comparative analyses of chromosomal rearrangements and homologies, which are less dependent on molecular evolutionary rates (Blanc & Wolfe, 2004; Bowers *et al.*, 2003; De Bodt *et al.*, 2005; Jaillon *et al.*, 2007; Tang *et al.*, 2008; Tuskan *et al.*, 2006). Consequently, in this study, the estimation of syntenic relationships refines the ambiguous evolutionary patterns within the phylogenetic trees and also shows gene deletion events in angiosperms (Figs. 2-3 and 3-10). This methodology is valid for the molecular phylogeny of every gene in model plants but is limited to those species for which draft genome sequences are completely available. In future, the extension of plant genomic analyses will allow us to determine further details of the phylogenetic relationships of every plant gene.

Plant circadian clock system in angiosperms

The circadian clock-related genes *LHY/CCA1* and *PRR* are widely conserved among angiosperm evolutionary lineages (Tables 2-2 and 3; Ming *et al.*, 2008; Murakami *et al.*, 2007). This study revealed the molecular phylogenies of these genes (Figs. 2-7 and 3-10) and showed that copy numbers of almost all genes increased via ancient whole-genome duplication events (Figs. 2-3 and 3-10). After these polyploidy events, one or both of the duplicated genes have undergone various evolutionary fates. For example, after the β and α polyploidy events, gene losses occurred among duplicated-*PRR* genes, whereas duplicated-*LHY/CCA1* genes (*LHY* and *CCA1*) were retained and generated functional diversity during the evolutionary process (Figs. 2-7 and 3-10; Gould *et al.,* 2006; Schaffer *et al.*, 1998). These findings imply that the regulatory network of the plant clock system retained a degree of organization throughout the dynamic changes of copy numbers and functions of the circadian clock-related genes.

The phylogenetic analyses of *LHY/CCA1*s and *PRR*s uncovered the evolutionary process of the plant clock system in monocots and eudicots (Fig. 3-10). Genetic and systems biological studies on *A. thaliana* have resulted in a computational model of the plant clock system that consists of multiple

interlocked feedback loops (Locke *et al.,* 2006; Zeilinger *et al.,* 2006). On the basis of this clock model, the present analyses raise the possibility that the prototypic clock system, main feedback loop (*LHY/CCA1-PRR1/TOC1*), and the declined morning loop (*LHY/CCA1-PRR3/7*) had already developed before the divergence of monocots and eudicots 120–150 million years ago (Fig. 3-10; Soltis *et al.*, 2008). This hypothesis is also supported by the results that *LHY/CCA1*, *PRR1/TOC1*, *PRR37*, *73*, *59,* and *95* in *O. sativa* retain typical diurnal expression patterns, which are similar to the expression pattern of their respective orthologs in *A. thaliana* (Matsushika *et al.*, 2000; Murakami *et al.*, 2003; Wang & Tobin, 1998). *PRR37* and *73,* and *PRR59* and *95* are paralogous pairs within monocots (Figs. 3-9 and 3-10) and the expression patterns of *O. sativa PRR37/73* and *PRR59/95* are similar to those of *A. thaliana PRR7* and *PRR5*, respectively (Ito *et al.*, 2003; Matsushika *et al.*, 2000; Murakami *et al.*, 2003). This suggests that the ancestral clock system may not have included posttranslational regulation of PRR1/TOC1 by PRR3 and the transcriptional feedback loop (*LHY/CCA1-PRR9*) in the morning loop. In the lineage leading to rosids in eudicots, the γ triplication event was a significant turning point in the organization of the fundamental regulatory network of the multiple interlocked feedback loops. Before this event, the prototypic clock system had been organized after the divergence of monocots and eudicots (Fig. 3-10). However, after the γ triplication event, the ancestral *PRR3/7* and *PRR5/9* were duplicated into *PRR3* and *PRR7* and into *PRR5* and *PRR9*, respectively, and these genes have subsequently played different roles in the clock system (Fujiwara *et al.*, 2008; Makino *et al.*, 2001; Salomé & McClung, 2005). However, it remains unclear when the functional divergences between *PRR3* and *7* and between *PRR5* and *9* occurred. These findings indicate that the fundamental mechanism of the multiple interlocked feedback loops was established before the radiation of rosids species 90–120 million years ago (Soltis *et al.*, 2008). Further functional analyses using other plants in the rosids, for example *Vitis* and *Carica*, are needed to clearly understand the evolutionary process and functional diversity of the plant clock system.

Circadian clock system in *Populus*

Modification of regulatory network in the Populus *clock system*

Both evolutionary and functional studies suggested that the regulatory network of the *Populus* clock system differs from the model clock system in *A. thaliana.* Evolutionary analyses revealed that the composition and the number of clock-related genes in *Populus* differed to that in *A. thaliana*, and that these differences arose after the divergence of the *Populus* (eurosids I) and *Arabidopsis* (eurosids II) (Fig. 3-10). In particular, the *Populus PRR3* gene was lost after the speciation of *Populus* (eurosids I) and *Arabidopsis* (eurosids II), whereas *LHY/CCA1* and *PRR7* and *9* were retained and duplicated via the Salicoid polyploidy event (Figs. 2-7 and 3-9). Functional analyses were then used to clarify whether these evolutionary changes modified the regulation network of the *Populus* clock system. As expected, these analyses revealed that the expression pattern and the function of the *PRR1/TOC1* gene is modified in *Populus* plants, in that it did not show the typical diurnal rhythmicity with peak expression at dusk (Figs. 4-2). Furthermore, in the complementation analyses using the *Arabidopsis PRR1/TOC1* null mutant (*toc1-21*), *Populus PRR1/TOC1* complemented the long hypocotyl phenotype but did not recover the early flowering phenotype (Fig. 4-5). It is likely that the inability to complement the floral phenotype is because *Populus PRR1/TOC1* can not positively regulate *LHY* and *CCA1* genes in the main feedback loop. In *Arabidopsis*, PRR1/TOC1 regulates floral transition in a LHY/CCA1-dependent manner (Ding *et al.*, 2007). Therefore, these results suggest that the function of *Populus PRR1/TOC1* differs from *Arabidopsis PRR1/TOC1* in the main feedback loop of the plant clock system.

It is widely known that the *PRR1/TOC1* gene plays a key role in the plant clock system. In the regulatory network of the *Arabidopsis* clock system, the *PRR1/TOC1* loss-of-function mutant showed arrhythmic and decreased expressions of *LHY* and *CCA1* genes, and expressed *GI* genes at an earlier phase under constant light conditions (Alabadí *et al.*, 2001; Ito *et al.*, 2008). Likewise, the clock-related genes *LHY/CCA1*, *GI*, *PRR3*, *5*, *7,* and *9* showed arrhythmic expression in transgenic *Arabidopsis* overexpressing *PRR1/TOC1* in continuous light conditions (Makino *et al.*, 2002). Furthermore, the *Arabidopsis PRR3* null mutant expressed the *LHY* gene earlier than the wild-type under continuous light conditions (Salomé & McClung, 2005). In *Populus*, most circadian clock-related genes exhibited diurnal expression patterns similar to those in *A. thaliana* (Fig.

4-1), even though *Populus* has lost the *PRR3* gene and the *Populus PRR1/TOC1* gene did not show the typical diurnal rhythmicity. In particular, *LHY*s, the partner genes of *PRR1/TOC1* in the main loop, exhibited typical morning expression patterns under controlled and natural conditions (Figs. 2-4 and 4-2; Takata *et al.*, in press). Together, these results imply that *PRR1/TOC1* may not have a "major" role in the regulation network of the *Populus* clock system. However, *PRR1/TOC1* may have a function in the regulation of photoperiodisms because the ectopic expression of *Populus PRR1/TOC1* recovered the long hypocotyl phenotype of the *Arabidopsis PRR1/TOC1* null mutant (Fig. 4-5a). Further studies are required to characterize the *Populus PRR1/TOC1* gene with regard to protein function and regulation of gene expression in the clock system and photoperiodic responses, and to clarify the regulation network of the *Populus* clock system.

Circadian clock system and dormancy induction in Populus

The *Populus* clock system shows a degree of organization, because almost all of its clock-related genes have typical diurnal expressions (Fig. 4-1). One feature of clock systems is the ability to entrain environmental cues such as daily changes in light and temperature (reviewed in Yakir *et al.*, 2007). In the plant clock system, *LHY/CCA1* entrains the light signal via the phytochrome B (PhyB) and Phytochrome-Interacting Factor 3 (PIF3) signaling pathway, which results in their morning expressions (Martínez-García *et al.*, 2000). *Populus LHY* genes also showed typical rhythmicity with peak expression around dawn under controlled and natural conditions (Figs. 2-4 and 4-2; Takata *et al.*, in press). This result implies that *LHY* genes entrain light signals through the PhyB/PIF3 pathway in the *Populus* clock system.

In the plant clock system, *Gigantea* (*GI*) may be involved in loop II (the "evening loop") (Fig. 1; Locke *et al.*, 2006; Zeilinger *et al.*, 2006). The *GI* gene in *A. thaliana* exhibits typical diurnal rhythmicity with peak expression around dusk (Fig. 4-1a; Fowler *et al.*, 1999). Likewise, the *Populus GI* gene showed a typical evening expression pattern (Fig. 4-1). Furthermore, the *GI* gene has a key role in the regulation of the genes downstream from the plant clock system, *CONSTANS* (*CO*) and *Flowering Locus T* (*FT*) (Searle & Coupland, 2004). It is well known that *FT* is downstream of the *CO* gene in angiosperms (Searle & Coupland, 2004)

and is a candidate for the floral hormone, florigen (Corbesier *et al.*, 2007; Tamaki *et al.*, 2007). Interestingly, in *Populus* plants, the *CO/FT* regulatory module is required for the seasonal endodormancy transition (Böhlenius *et al.*, 2006). Collectively, the conservation of *GI* expression patterns implies that the role of *GI* in the *Populus* clock system is to regulate downstream genes such as *CO* and *FT*.

Consequently, it is likely that the *Populus* clock system entrains the external light/dark cycle and regulates downstream genes. Future studies should address how the plant clock system measures the seasonal fluctuation of natural daylength and how it controls expressions of *CO* and *FT,* which regulate induction of seasonal dormancy in trees (Böhlenius *et al.*, 2006).

Chapter 6

Conclusion remarks and perspectives

The aim of this study was to uncover molecular mechanisms of seasonal dormancy induction in boreal and temperate trees. To do this, I focused on the *Populus* clock system—the master controller of photoperiodic responses. Evolutionary and functional approaches revealed that the expression and function of the *PRR1/TOC1* gene, which is a key component of the *Arabidopsis* clock system, is modified in the *Populus* clock system. These results imply that *Populus PRR1/TOC1* does not have a major role in its clock system. However, this alteration does not significantly change the *Populus* clock system because most other circadian clock-related genes in *Populus* show typical diurnal expression patterns. Consequently, this study provides a perspective to reconstruct the fundamental mechanism of the *Populus* clock system. In particular, *LHY* genes may play a key role in both light entrainment through the phytochrome signaling pathway and regulation of the downstream genes that control plant photoperiodisms. The next step of this study is to confirm how the *Populus* clock system is regulated, and to uncover the role of this system in inducing seasonal dormancy of boreal and temperate trees. Resolution of these issues may resolve why trees have a long life span and why they can grow to be quite large.

Acknowledgments

I sincerely thank my major supervisor, Professor Matsuo Uemura, for giving me the chance to study at his creative laboratory and for being an inspiring mentor, insightful researcher, and efficient group leader during my studies.

I am very grateful to my co-supervisors Professor Masumi Okada and Professor Kiyoaki Kato for their encouragement, wise advice, and valuable discussions.

Special thanks to Professor Takayoshi Koike and Professor Shinji Akada for their co-supervision at the final stage of this thesis.

I wish to thank Professor Seizo Fujikawa, Professor Keita Arakawa, and Professor Daisuke Takezawa for leading me into the exciting field of cold hardiness in trees.

I wish to express my gratitude to Dr. Shigeru Saito and Claire Tanaka Saito, who taught me the fundamentals of molecular evolution and phylogenetics and kindly reviewed the manuscript.

I also wish to thank Dr. Yasunori Ohmiya for providing aspartic *Populus tremula* x *tremuloides* plants, and for his help and interest in exciting research areas concerning trees.

I thank Dr. Abidur Rahman for critically reading the manuscript and Dr. Yuzo Sano for his guidance with the mysteries of plant phylogeny.

I also thank Professor Malcolm M. Campbell and Professor Steven H. Strauss for helpful advice on poplar microarray data.

I am grateful to Dr. Tokihiko Nanjo and Dr. Kenji Shinohara for providing aspartic *Populus nigra* plants and full-length cDNAs, Dr. Yasuo Niwa for providing the expression vector for transient expression assays, Professor

Tsuyoshi Nakagawa for providing the pGWB1 binary vector and Dr. Seth J. Davis for the kind gift of seeds of the *Arabidopsis PRR1/TOC1* null mutant.

I am grateful to all current and previous members of the group, especially Dr. Tomokazu Yamazaki, Dr. Yukio Kawamura, Dr. Anzu Minami, Dr. Yutaka Sasaki and Dr. Yoshihiko Onda. It has been a pleasure to work with them.

Finally, I would like to thank my parents for their patience and support during this process.

This work was supported in part by a Grant-in-Aid for the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan (K-3 to MU) and by a grant from the Japan Society for the Promotion of Science (19·9498 to NT).

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