

**Studies on Molecular Mechanisms Underlying
Photoperiodic Flowering Responses
in Circadian Clock Mutant**

January 2014

Miyuki HARA

**Studies on Molecular Mechanisms Underlying
Photoperiodic Flowering Responses
in Circadian Clock Mutant**

**A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Science
(Doctoral Program in Functional Biosciences)**

Miyuki HARA

Table of contents

Abstract	1
Abbreviations	4
General Introduction	7
Materials and Methods	15
1. Plant material and growth conditions	16
2. Measurement of flowering time	17
3. Sequence analysis	17
4. Gene expression analysis	18
Results	21
Part1: <i>CO-EXPRESSED WITH CLOCK GENES LHY AND CCA1 1 (CEC1)</i> is regulated by	
LHY and CCA1 and plays a key role in phase setting of <i>GI</i> in <i>Arabidopsis thaliana</i>.....	22
1. Accelerated flowering time in <i>cec1</i> and <i>cec2</i> mutants under LD and LL.....	22
2. Increased <i>CO</i> and <i>FT</i> mRNA levels in <i>cec1</i> and <i>cec2</i> mutants under LD	23
3. Phase shift of <i>GI</i> expression peaks in <i>cec1</i> under LD and LL.....	23

Abstract

Many biological processes, including the control of flowering time, are regulated by the circadian clock. Although a number of clock-associated genes have been characterized in *Arabidopsis thaliana* (Arabidopsis), the complete molecular mechanisms of the circadian clock remain unclear. To find a novel component that controls the photoperiodic pathway via the circadian clock, I screened mutants that showed altered photoperiodic flowering.

In Part1, I report that CO-EXPRESSED WITH CLOCK GENES *LHY* AND *CCA1* 1 (*CEC1*) plays an important role in circadian clock function in Arabidopsis. Three genes, *CEC1*, *CEC2*, and *CEC3*, are co-expressed with the clock genes *LATE ELONGATED HYPOCOTYL (LHY)* and *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*. The mutants, *cec1* and *cec2*, exhibited an early flowering phenotype under long-day (LD) and continuous-light (LL) conditions, possibly through an increase in *CONSTANS (CO)* and *FLOWERING LOCUS T (FT)* mRNA. In addition, rhythmic peaks of *GIGANTEA (GI)* expression were delayed in the *cec1* mutant plants, but the period length and amplitude of *GI* expression were not affected under LD and LL. These results suggest that *CEC1* might contribute to the determination of circadian phases.

In Part2, to understand molecular mechanisms, underlying the photoperiodic flowering regulated by circadian clock, genetic screening for the Arabidopsis mutant that showed late flowering under LD or LL and early flowering under SD was performed. I named the mutant as *short day plant (sdp)* mutant.

Abbreviations

Arabidopsis: *Arabidopsis thaliana*

bp: base pairs

CCA1: CIRCADIAN CLOCK ASSOCIATED 1

CDF1: CYCLING DOF FACTOR 1

CIB1: CRY2-interacting bHLH 1

CO: CONSTANS

COL: CONSTANS-LIKE 1

Col: Columbia

cDNA: complementary DNA

DNA: deoxyribonucleic acid

ELF3: EARLY FLOWERING 3

ELF4: EARLY FLOWERING 4

EMS: ethyl methanesulfonate

F₁: first filial generation

F₂: second filial generation

F₃: third filial generation

FLC: FLOWERING LOCUS C

FT: FLOWERING LOCUS T

gDNA: genomic DNA

GI: GIGANTEA

Hd1: HEADING DATE 1

Hd3a: HEADING DATE 3a

LCL5: LHY-CCA1 LIKE5

LD: long day

Ler: Landsberg *erecta*

LHY: LATE ELONGATED HYPOCOTYL

LL: continuous light

LNK1: NIGHT LIGHT INDUCIBLE AND CLOCK-REGULATED GENES 1

LUX: LUX ARRHYTHMO

OOP1: OUT OF PHASE 1

OsGI: *Oryza sativa* GIGANTEA

PCR: polymerase chain reaction

PHYB: PHYTOCHROME B

PRR: PSEUDO-RESPONSE REGULATOR

RVE8: REVEILLE 8

q-RT-PCR: Quantitative reverse transcription PCR

RNA: ribonucleic acid

TOC1: TIMING OF CAB EXPRESSION 1

SD: short day

SDP: SHORT DAY PLANT

SSLP: simple sequence length polymorphism

SVP: SHORT VEGETATIVE PHASE

WT: wild-type

Acknowledgements

It is with great pleasure that I thank many people who made this thesis possible.

I especially want to thank Professor Tsuyoshi Mizoguchi at the International Christian University, Japan for his continuous support during my PhD study and research.

I would like to thank my thesis supervisor, Professor Hiroshi Kamada (Tsukuba University) and my thesis committee; Associate Professor Michiyuki Ono (Tsukuba University) and Associate Professor Kenji Miura (Tsukuba University), for their encouragement, insightful comments, and advices.

This study was supported in part by the joint research program “Plant Transgenic Research Design, University of Tsukuba” of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) (to T.M.), the grants-in-aid for Scientific Research on Priority Areas and the grants-in-aid for Scientific Research (C) of the MEXT (to T.M.).

I am deeply grateful for my former and present lab mates who given me never ending help for my thesis I wish to thanks Yu Natsui (Tsukuba University), Dr. Nefissi Rim (Tsukuba University), Noriyuki Furuta (Tokyo University of Agriculture and Technology, Japan) and Kaito Kikuchi (International Christian University)

I also thanks to Professor. Hiroshi Kouchi (International Christian University, Japan), for his support and helpful comments during my work.

References

Boss PK, Bastow RM, Mylne JS, Dean C (2004) Multiple pathways in the decision to flower: enabling, promoting, and resetting. *Plant Cell* 16: S18-S31

Bunning E (1964) Circadian leaf movement in bean plants: Earlier reports. *Science* 146: 551

Carre IA (2002) *ELF3*: a circadian safeguard to buffer effects of light. *Trends Plant Sci* 1: 4-6

Covington MF, Panda S, Liu XL, Strayer CA, Wagner DR, Kay SD (2001) *ELF3* modulates resetting of the circadian clock in Arabidopsis. *Plant Cell* 6: 1305-1315

Engelmann W, Johnsson A (1978) Attenuation of the petal movement rhythm in *Kalanchoe* with light pulses. *Physiol Plant* 43: 68-76

Dixon LE, Knox K, Kozma-Bognar L, Southern MM, Pokhilko A, Millar AJ (2011) Temporal repression of core circadian genes is mediated through *EARLY FLOWERING 3* in *Arabidopsis*. *Curr Biol* 21: 120–125

Dowson-Day MJ, Millar AJ (1999) Circadian dysfunction causes aberrant hypocotyl elongation patterns in Arabidopsis. *Plant J* 17: 63–71

Dunlap JC (1999) Molecular bases for circadian clocks. *Cell* 96: 271–290

Farinas B, Mas P (2011) Functional implication of the MYB transcription factor

RVE8/LCL5 in the circadian control of histone acetylation. *Plant J* 66: 318-329

Folter S, Immink RGH, Kieffer M, Paenico L, Henz SR, Weigel D, Busscher M,

Kooiker M, Colombo L, Kater MM, Davies B, Angeneta GC (2005)

Comprehensive interaction map of the Arabidopsis MADS Box transcription factors.

Plant Cell 17: 1424-1433

Fornara F, Panigrahi KC, Gissot L, Sauerbrunn N, Ruhl M, Jarillo JA, Coupland

G (2009) *Arabidopsis* DOF transcription factors act redundantly to reduce *CONSTANS*

expression and are essential for a photoperiodic flowering response. *Dev Cell* 17: 75-86

Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G,

Putterill J (1999) *GIGANTEA*: a circadian clock-controlled gene that regulates

photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible

membrane-spanning domains. *EMBO J* 18: 4679-4688

Fujiwara S, Oda A, Yoshida R, Niinuma K, Miyata K, Tomozoe Y, Tajima T,

Nakagawa M, Hayashi K, Coupland G, Mizoguchi T (2008) Circadian clock proteins

LHY and CCA1 regulate SVP protein accumulation to control flowering in Arabidopsis.

Plant Cell 20: 2960–2971

Hall A, Bastow RM, Davis SJ, Hanano S, McWatters HG, Hibberd V, Doyle MR,

Sung S, Halliday KJ, Amashino RM, Millar AJ (2003) The *TIME FOR COFFEE*

gene maintains the amplitude and timing of Arabidopsis circadian clocks. *Plant Cell* 11:

2179-2129

Hassidim M, Hairi Y, Yakir E, Idon K, Green RM (2009) Over-expression of

CONSTANS-LIKE 5 can induce flowering in short-day grown Arabidopsis. *Planta* 230:

481-491

Hayama R, Yokoi S, Tamaki S, Yano M, Shimamoto K (2003) Adaptation of

photoperiodic control pathways produces short-day flowering in rice. *Nature* 422:

719-722

Helfer A, Nusinow DA, Chow BY, Gehrke AR, Bulyk ML, Kay SA (2011) *LUX*

ARRHYTHMO encodes a nighttime repressor of circadian gene expression in the *Arabidopsis* core clock. *Curr Biol* 21: 126–133

Hepworth SR, Valverde F, Ravenscroft D, Mouradov A, Coupland G (2002)

Antagonistic regulation of flowering-time gene *SOC1* by CONSTANS and FLC via separate promoter motifs. *EMBO J* 21: 4327-4337

Huq E, Tepperman JM, Quail PH (2000) GIGANTEA is a nuclear involved in

phytochrome signaling in *Arabidopsis*. *Proc Natl Acad Sci* 17: 9789-9794

Imaizumi T, Schultz TF, Harmon FG, Ho LA, Kay SA (2005) FKF1 F-box protein

mediates cyclic degradation of a repressor of *CONSTANS* in *Arabidopsis*. *Science* 309: 293–297

Imaizumi T, Kay SA (2006) Photoperiodic control of flowering: not only by

coincidence. *Trends Plant Sci* 11:1360-1385

Ishiura M, Kutsuna S, Aoki S, Iwasaki H, Anderson CR, Tanabe A, Golden SS,

Johnson CH, Kondo T (1998) Expression of a gene cluster *kaiABC* as a circadian

feedback process in cyanobacteria. *Science* 281: 1519–1523

Jackson FR, Bargiello TA, Yun SH, Young MW (1986) Products of *per* locus of *Drosophila* shares homology with proteoglycans. *Nature* 320: 185–188

Kim WY, Hicks KA, Somers DE (2005) Independent roles for *EARLY FLOWERING 3* and *ZEITLUPE* in the control of circadian timing, hypocotyl length, and flowering time. *Plant Physiol* 139: 1557-1569

Kolmos E, Nowak M, Werner M, Fischer K, Schwarz G, Mathews S, Schoof H, Nagy F, Bujnicki JM, Davis SJ (2009) Integrating *ELF4* into the circadian system through combined structural and functional studies. *HFSP J* 3: 350–366

Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 68: 2112–2116

Koornneef M, Alonso-Blanco C, Blankestijin de Vries H, Hanhart CJ, Peeters AJ (1998) Genetic interaction among late-flowering mutants of Arabidopsis. *Genetics* 2: 885-892

Ledger S, Strayer C, Ashton F, Kay SA, Putterill J (2001) Analysis of the function of two circadian-regulated *CONSTANS- LIKE* genes. *Plant J* 26: 15–22

Liu H, Yu X, Li K, Klejnot J, Yang H, Lisiero D, Lin C (2008) Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in *Arabidopsis*. *Science* 322:1535-1539

Liu Y, Li X, Liu H, Lin C (2013) Multiple bHLH protein form heterodimers to mediate CRY2-dependent regulation of flowering-time in *Arabidopsis*. *Plos* 9: 1-12

Locke JC, Southern MM, Kozna-Bognar L, Hibberd V, Brown PE, Turner MS, Millar AJ (2005) Extension of a genetic network model by iterative experimentation and mathematical analysis. *Mol Syst Biol* 0013: 1-9

Locke JC, Kozma-Bognar L, Gould PD, Feher B, Kevei E, Nagy F, Turner MS, Hall A, Millar AJ (2006) Experimental validation of a predicted feedback loop in the multi-oscillator clock of *Arabidopsis thaliana*. *Mol Syst Biol* 59: 1-6

McClung CR, Fox BA, Dunlap JC (1989) The *Neurospora* clock gene *frequency* shares a sequence element with the *Drosophila* clock gene *period*. *Nature* 339: 558–562

Mikkelsen MD, Thomashow MF (2009) A role for circadian evening elements in cold-regulated gene expression in *Arabidopsis*. *Plant J* 60: 328-339

Millar AJ, Straume M, Chory J, Chua NH, Kay SA (1995) The regulation of circadian period by phototransduction pathways in *Arabidopsis*. *Science* 267: 1163–1166

Miura K, Lee J, Jin JB, Yoo CY, Miura T, Hasegawa PM (2009) Sumoylation of ABI5 by the *Arabidopsis* SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. *Proc Natl Acad Sci USA* 106: 5418-5423

Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song HR, Carre IA, Couplant G (2002) *LHY* and *CCA1* are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. *Dev Cell* 2: 629-641

Mizoguchi T, Wright L, Fujiwara S, Cremer F, Lee K, Onouchi H, Mouradov A, Fowler S, Kamada H, Putterill J, Coupland G (2005) Distinct roles of *GIGANTEA* in promoting flowering regulating circadian rhythms in *Arabidopsis*. *Plant Cell* 17: 2255–2270

Nakamichi N, Kita M, Ito S, Sato E, Yamashino T, Mizuno T (2005) The *Arabidopsis* Pseudo-Response Regulators, *PRR5* and *PRR9*, coordinately play essential roles for circadian clock function. *Plant Cell Physiol* 46: 609–619

Nakamichi N, Kiba T, Henriques R, Mizuno T, Chua NH, Sakakibara H (2010)

PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the *Arabidopsis* circadian clock. *Plant Cell* 22: 594-605

Natsui Y, Nefissi R, Miyata K, Oda A, Hase Y, Nakagawa M, Mizoguchi T (2010)

Isolation and characterization of suppressors of the *early flowering 3* in *Arabidopsis thaliana*. *Plant Biotech* 27: 463-468

Nefissi R, Natsui Y, Miyata K, Hase Y, Nakagawa M, Ghorbel A, Mizoguchi T

(2011) Double loss-of-function mutation in *EARLY FLOWERING 3* and *CRYPTOCHROME 2* genes delays flowering under continuous light but accelerates in under long day and short days: an important role for *Arabidopsis* CRY2 to accelerate flowering time in continuous light. *JXB* 8: 2731-2744

Niwa Y, Ito S, Nakamichi N, Mizoguchi T, Niinuma K, Yamashino T, Mizuno T

(2007) Genetic linkages of the circadian clock-associated genes, *TOC1*, *CCA1*, and *LHY*, in the photoperiodic control of flowering time in *Arabidopsis thaliana*. *Plant Cell Physiol* 48: 925-937

Onai K, Ishiura M (2005) *PHYTOCLOCK 1* encoding a novel GARP protein essential for the *Arabidopsis* circadian clock. *Genes Cells* 10: 963–972

Park DH, Somers DE, Kin YS, Choy YH, Lim HK, Kim HJ, Kay SA, Nam HG (1999) Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis* GIGANTEA gene. *Science* 285: 1579-1582

Penfield S, Hall A (2009) A role for multiple circadian clock genes in the response to signals that break seed dormancy in *Arabidopsis*. *Plant Cell* 21: 1722-1732

Pokhilko A, Fernandez AP, Edwards KD, Southern MM, Halliday KJ, Millar AJ (2012) The clock gene circuit in *Arabidopsis* includes a repressilator with additional feedback loops. *Mol Syst Biol* 8: 574-587

Putterill J, Robson F, Lee K, Simon R, Coupland G (1995) The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell* 80: 847-857

Quail PH (2002) Phytochrome photosensory signaling networks. *Nat Rev Mol Cell Biol* 3: 85–93

Rugnone ML, Faigon Soverna A, Sanchez SE, Schlaen RG, Hernando CE,

Seymour DK, Mancini E, Chernomoretz A, Weigel D, Mas P, Yanovsky MJ (2013)

LNK genes integrate light and clock signaling networks at the core of the *Arabidopsis* oscillator. *Proc Natl Acad Sci USA* 110: 12120-12125

Salome PA, Michel TP, Kearns EV, Fett-Neto AG, Sharrok RA, McClung CR

(2002) The *out of phase 1* mutant defines a role for PHYB in circadian phase control in *Arabidopsis*. *Plant Physiol* 129: 1674-1685

Sawa M, Nusinow DA, Kay SA, Imaizumi T (2007) FKF1 and GIGANTEA complex

formation is required for day-length measurement in *Arabidopsis*. *Science* 318: 261-265

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carre IA, Coupland G

(1998) The *late elongated hypocotyl* mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* 93: 1219-1229

Sehgal A, Price JL, Man B, Young MW (1994) Loss of circadian behavioral rhythms

and *per* RNA oscillations in the *Drosophila* mutant *timeless*. *Science* 263: 1603–1606

Stanewsky R (2003) Genetic analysis of the circadian system in *Drosophila*

melanogaster and mammals. *J Neurobiol* 54: 111–147

Suarez-Lopez P, Wheatley K, Robsin F, Onouchi H, Valverde F, Coupland G

(2001) *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. *Nature* 410: 1116-1120

Sun ZS, Albrecht U, Zhuchenko O, Bailey J, Eichele G, Lee CC (1997) RIGUI, a

putative mammalian ortholog of the *Drosophila period* gene. *Cell* 90: 1003–1011

Thomas B, Vince-Prue D (1997) Photoperiodism in Plants. *New York: Academic Press*

Thain SC, Vandenbussche F, Laarhoven LJJ, Dowson-Day MJ, Wang ZY, Tobin

EM, Harren FJM, Millar AJ, Straeten DVD (2004) Circadian rhythms of ethylene emission in *Arabidopsis*. *Plant Physiol* 136: 3751–3761

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G (2004)

Photoreceptor regulation of *CONSTANS* protein in photoperiodic flowering. *Science* 303: 1003-1006

Wang ZY, Tobin EM (1998) Constitutive expression of the *CIRCADIAN CLOCK*

ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93: 1207-1217

Yanovsky MJ, Kay SA (2002) Molecular basis of seasonal time measurement in *Arabidopsis*. *Nature* 419: 308-312

Yanovsky MJ, Kay SA (2003) Living by the calendar: how plants know when to flower. *Nat Rev Mol Cell Biol* 4: 265-275

Yoshida R, Fekih R, Fujiwara S, Oda A, Miyata K, Tomozoe Y, Nakagawa M, Niinuma K, Hayashi K, Ezura H, Coupland G, Mizoguchi T (2009) Possible role of early flowering 3 (ELF3) in clock-dependent floral regulation by short vegetative phase (SVP) in *Arabidopsis thaliana*. *New Phytol* 182: 838-850

Zagotta MT, Hicks KA, Jacobs CI, Young JC, Hangarter RP, Meeks-Wagner DR (1996) The *Arabidopsis* *ELF3* gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering. *Plant J* 4: 691-702