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Altered circadian rhythms regulate growth vigor in hybrids and allopolyploids

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Abstract

Segregating hybrids and stable allopolyploids display morphological vigor^{1,2,3}, and Arabidopsis allotetraploids are larger than the parents *Arabidopsis thaliana* and *A. arenosa*^{1,4}. The mechanisms are unknown. Circadian clocks mediate metabolic pathways and increase fitness in animals and plants^{5,6,7,8}. Here we report that epigenetic modifications of the circadian clock genes *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* and *LATE ELONGATED HYPOCOTYL (LHY)*^{9,10} and their reciprocal regulators *TIMING OF CAB EXPRESSION 1 (TOC1)* and *GIGANTEA (GI)*^{10,11,12} mediate expression changes in downstream genes and pathways. During the day, epigenetic repression of *CCA1* and *LHY* induced expression of *TOC1*, *GI* and downstream genes that contain *CCA1* binding site (CBS)¹³ in chlorophyll and starch metabolic pathways in allotetraploids and F₁ hybrids, which produced more chlorophyll and starch than the parents in the same environment. Mutations in *cca1* and *cca1 lhy* and daily repression of *cca1* in *TOC1:cca1*-RNAi transgenic plants increased expression of downstream genes and chlorophyll and starch

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Author Contributions Z.N. examined gene expression, ChIP, chlorophyll, starch, and sugars in allotetraploids and hybrids. E.D.K. analyzed *CCA1* mRNA, protein, chlorophyll and starch in the transgenic plants and mutants. M.H. conducted statistical tests. E.L. performed EMSA. J.L. assayed Western and ChIP. Y.Z. assisted cloning. Q.S. discussed the experiments. Z.J.C. conceived the project, analyzed the data, and wrote the paper.

List of key genes: *CCA1*, *LHY*, *TOC1*, *GI*, *PORA*, *PORB*, *AMY*, *DPE1*, *GWD3*, *ELF4*

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content, whereas constitutively expressing *CCA1* or ectopically expressing *TOC1:CCA1* had the opposite effects. The causal effects of *CCA1* on output traits suggest that hybrids and allopolyploids gain advantages from the control of circadian-mediated physiological and metabolic pathways, leading to growth vigor and increased biomass.

Keywords

circadian clock; polyploidy; hybrid vigor; epigenetics; gene expression; biomass

Polyploidy (whole genome duplication) is an evolutionary innovation in many plants and some animals. Many important crops such as wheat, cotton, and canola are of allopolyploidy that contains two or more divergent genomes^{14,15}, and some plants and animals exist as interspecific hybrids¹⁶. The common occurrence of polyploids suggests an evolutionary advantage of having additional genetic materials for growth and adaptation. Moreover, heterozygosity and novel genomic interactions in allopolyploids induce phenotypic variation and growth vigor¹⁵.

In stable allotetraploids that were resynthesized by interspecific hybridization between *A. thaliana* and *A. arenosa* (Supplementary Fig. 1)⁴, over 1,400 genes (>5% and up to 9,800 genes or ~38%) were nonadditively expressed¹. Nonadditive expression indicates that the expression level of a gene in an allotetraploid is not equal to the sum of two parental loci ($1 + 1 = 2$), leading to activation (>2), repression (<2), dominance, or overdominance¹⁵. Many genes in energy and metabolism including photosynthesis and starch pathways are upregulated¹, coinciding with growth vigor in the allotetraploids. This morphological vigor is commonly observed¹⁷, and phenotypic variation among allotetraploids are related to genetic and epigenetic mechanisms¹⁵.

Among 128 genes upregulated in the allotetraploids, 86 (~67%) each contains at least one CBS (AAAAATCT) or evening element (EE, AAAATATCT)¹³ within the ~1,500-kbp upstream region (Supplementary Table 1), which is significantly higher than all genes containing putative EE and CBS (~15%, $\chi^2 = 157$ and $P = 2.2e^{-16}$). These EE- and CBS-containing genes are likely the targets of *CCA1* and *LHY*^{9,18,19}.

CCA1 and *LHY* are MYB-domain transcription factors and have partially redundant but incompletely overlapping functions^{9,10}. They negatively regulate *TOC1* and *GI* expression, whereas *TOC1* and *GI* positively regulate *CCA1* and *LHY* expression^{10,11,12}. This circular feedback regulation affects central oscillation as well as input and output pathways that maintain the rhythms, amplitude and/or phase of circadian clock in *Arabidopsis*²⁰. Disrupting oscillator control alters the expression of ~10% *Arabidopsis* genes¹³, while maintaining circadian clock regulation increases CO₂ fixation, growth, and fitness^{5,8}.

We found that *CCA1* and *LHY* were repressed, and *TOC1* and *GI* were upregulated at noon in the allotetraploids¹. As in the parents, both *CCA1* and *LHY* displayed diurnal expression patterns in the allotetraploids (Fig. 1a and Supplementary Fig. 2a and Table 2). Their expression peaked at dawn (ZT0), decreased 6 hours after dawn (ZT6), and continued declining until dusk (ZT15). Interestingly, *CCA1* and *LHY* were expressed 2–4-fold lower in

the allotetraploids than the mid-parent value (MPV) at ZT6–12 and higher than the MPV at dusk (ZT15). *TOC1* and *GI* expression was inversely correlated with *CCA1* and *LHY* expression (Fig. 1b and Supplementary Fig. 2b), suggesting feedback regulation in the allotetraploids as in the diploids^{10,11,12}. However, *TOC1* and *GI* expression fluctuated in the allotetraploids, indicating that other factors may be involved²⁰. The expression changes of these genes from noon to dusk in the allotetraploids may alter the amplitude but not the phase of circadian clock, as they quickly gained the expression levels similar to MPV after dusk (ZT18–24).

To determine how *CCA1* and *LHY* expression was repressed, we examined expression patterns of *A. thaliana* and *A. arenosa* loci in the allotetraploids using RT-PCR and cleaved amplified polymorphic sequence (CAPS) analyses¹ that are discriminative of locus-specific expression patterns (Supplementary Table 3). While *A. thaliana* and *A. arenosa* loci were equally expressed in respective parents, in two allotetraploids *A. thaliana CCA1* (*AtCCA1*) expression was down-regulated ~3-fold, and *A. arenosa CCA1* (*AaCCA1*) expression was slightly reduced (Fig. 1c). Similarly, *AtLHY* expression was dramatically reduced (~3.3-fold), whereas *AaLHY* expression was decreased ~2-fold in the allotetraploids. Conversely, *AtTOC1* and *AtGI* loci were upregulated in the allotetraploids. The data suggest that *A. thaliana* genes are more sensitive to expression changes in the allotetraploids probably through *cis*- and *trans*-acting effects and chromatin modifications as observed in other loci¹⁷.

We examined chromatin changes in the upstream regions (~250-bp) of *CCA1*, *LHY*, *TOC1*, and *GI* (Supplementary Table 4) using antibodies against histone H3-Lys9 acetylation (H3K9Ac) and H3-Lys4 dimethylation (H3K4Me2), two marks for gene activation²¹. H3K9Ac and H3K4Me2 levels in the *CCA1* and *LHY* promoters were 2–3-fold lower in the allotetraploids than that in *A. thaliana* and *A. arenosa* (Fig. 1d), consistent with *CCA1* and *LHY* repression. Likewise, *TOC1* and *GI* upregulation correlated with increased levels of H3K9Ac and H3K4Me2. Changes in H3K9Me2, a heterochromatic mark²¹, were undetectable (data not shown). These data suggest that diurnal expression changes of *LHY*, *CCA1*, *TOC1*, and *GI* are associated with euchromatic histone marks. Alternatively, autonomous pathways and other factors such as ELF4 may mediate *TOC1* and *GI* expression^{20,22}.

To test downstream effects of *CCA1* and *LHY* repression, we examined expression of two subsets of EE/CBS-containing genes (Fig. 2a). One subset consists of the genes encoding protochlorophyllide (pchlide) oxidoreductases a and b, *PORA* and *PORB*, that mediate the only light-requiring step in chlorophyll biosynthesis in higher plants²³. *PORA* and *PORB* are strongly expressed in seedlings and young leaves, and upregulation of *PORA* and *PORB* increases chlorophyll a and b content²⁴. Both *PORA* and *PORB* were upregulated in the allotetraploids (Fig. 2d). The total chlorophyll content in both allotetraploids was ~60% higher than in *A. thaliana* and ~15% higher than in *A. arenosa* (Fig. 2b). Chlorophyll a increased more than chlorophyll b, and the allotetraploids accumulated ~70% more chlorophyll a than *A. thaliana*.

The other subset of EE/CBS-containing genes encodes enzymes in starch metabolism and sugar transport^{25,26}, many of which show strong diurnal rhythmic expression patterns^{13,27}. Starch metabolism involves the genes encoding *AMY3*, *BAM1*, 2 and 3, *DPE1* and 2, *GTR*, *GWD1* and 3, *ISA1*, 2 and 3, *LDA*, *MEX1*, and *PHS1* and 2^{25,26} (Fig. 2c and Supplementary Table 5). Many contained EE/CBS (Fig. 2a) and were upregulated 1.5–4-fold in the allotetraploids (Fig. 2e), when *CCA1* and *LHY* were down-regulated (Fig. 1, a and c). *MTR* and *BAM3*, 4 lacking EE/CBS showed little expression changes, suggesting that their expression is independent of clock regulation or undergoes post-transcriptional regulation²⁶.

As a result, allotetraploids accumulated more starch than the parents in both mature and immature leaves using iodine-staining (Fig. 3a) and quantitative assays (Fig. 3b). In the mature leaves, allotetraploids accumulated starch 2-fold higher than *A. thaliana* and 70% higher than *A. arenosa*. In the immature leaves, allotetraploids contained 4-fold higher starch than *A. thaliana* and 50–100% higher sugar content than the parents (Fig. 3c), mainly due to increases in glucose and fructose content, suggesting high rates of starch and sugar accumulation in young leaves. The sucrose content in allotetraploids was similar to *A. arenosa* but higher than in *A. thaliana* in immature leaves and similar among all lines tested in mature leaves (data not shown), indicating rapid transport and metabolism of sucrose especially in the mature leaves. Together, chlorophyll, starch, and sugar amounts were consistently high in the allotetraploids.

We further tested if circadian clock regulation is altered in F₁ hybrids as in the interspecific hybrids and allopolyploids. At ZT6 (noon), *CCA1* and *LHY* were repressed ~2-fold, whereas *TOC1* was upregulated ~2-fold in the F₁ hybrids relative to the parents (C24 and Columbia) (Supplementary Fig. 3). At ZT15, *CCA1* and *LHY* were upregulated, whereas *TOC1* was repressed in the hybrids. The F₁ hybrids displayed morphological vigor (Fig. 3d) and contained ~12% more total chlorophylls and ~10% more starch than the higher parent (Fig. 3e).

To determine how *CCA1* regulates downstream genes and output traits, we examined *CCA1* function in the allotetraploids and their parents. *CCA1* protein levels in these lines were high at dawn (ZT0) and low at noon (ZT6) (Fig. 3f), corresponding to the *CCA1* transcript levels (Fig. 1a). *CCA1* levels were constantly high in *A. thaliana* constitutive *CCA1*-overexpression (*CCA1*-OX) lines¹⁸. Electrophoretic mobility shift assay (EMSA) indicated specific binding of recombinant *CCA1* to EE-containing fragments of the target genes *TOC1*, *PORB*, *PORA*, *DPE1*, and *GWD3* (Fig. 3g, Supplementary Fig. 4 and Table 6). Using antibodies against *CCA1* in chromatin immunoprecipitation (ChIP) assays¹⁷, we further demonstrated that endogenous *CCA1* in the *TOC1* promoter was ~2.5-fold lower at ZT6 (noon) than at ZT0 (dawn) (Fig. 3h), which is inversely correlated with *TOC1* expression levels that were higher at noon than at dawn (Fig. 1b).

These data collectively suggest that *CCA1* directly affects *TOC1* and downstream genes in clock regulation, photosynthesis, and starch metabolism. Clock dependent upregulation of output genes^{13,27} may lead to growth vigor. Indeed, overexpressing *PORA* and *PORB*

increases chlorophyll content, seedling viability, and growth vigor in *A. thaliana*²⁴, while mutants of starch metabolic genes display reduced starch content and growth vigor (ref.²⁶).

If *CCA1* repression promotes growth, *CCA1* overexpression would reduce growth vigor in diploids. Indeed, *TOC1:CCA1* transgenic plants expressing *CCA1* under the clock-regulated *TOC1* promoter (Supplemental Fig. 5) displayed 3-fold induction of *CCA1* expression at noon (Fig. 4a) and 1.5–30-fold repression of the downstream genes *PORA*, *PORB*, *AMY*, *DPE1*, and *GWD* (Supplementary Fig. 6a), resulting in ~14% and ~17% reduction of chlorophyll and starch contents, respectively (Fig. 4a). *CCA1-OX* had ~20% reduction of chlorophyll content in seedlings (Supplementary Fig. 5c) and may affect various regulators in clock and other pathways related to growth vigor. For example, *gi* mutants in *A. thaliana* increase starch content and flower late²⁸, but *GI* induction in the allotetraploids correlates with starch accumulation. *CCA1-OX* lines also flowered late¹⁸ and may increase chlorophyll and starch content in late stages.

To test whether *CCA1* repression has positive effects on growth vigor in diploids as in the hybrids and allotetraploids (Fig. 2b and Fig. 3, a-e), we examined starch content in *cca1* single and *cca1 lhy* double mutants^{9,22,29}. *CCA1* expression was not completely abolished in these mutants (Fig. 4b) probably because of the T-DNA insertion near the ATG codon²⁹. The five downstream genes examined were upregulated 1.5–12.5-fold in the mutants (Supplementary Fig. 6b), and the starch content was doubled in the *cca1* mutant (Fig. 4b). The starch content was lower in the double mutant than in *cca1*, indicating a metabolic penalty of severely lacking clock regulation⁵. Furthermore, to reduce *CCA1* expression during the day, we expressed *cca1*-RNAi driven by the *TOC1* promoter (Supplementary Fig. 6c). In the *TOC1:cca1*-RNAi transgenic plants, *CCA1* mRNA and protein levels were down-regulated 2–10 fold (Fig. 4c, left) and 1.4–3 fold (right), respectively. Consequently, four downstream genes examined were upregulated in the *TOC1:cca1*-RNAi lines (Supplementary Fig. 6e), and the starch content increased ~28% (Fig. 4d). Taken together, the data suggest a mechanistic role of *CCA1* repression in promoting downstream pathways, increasing chlorophyll and starch accumulation and growth vigor.

We propose a model that explains growth vigor and increased biomass in allotetraploids and hybrids (Fig. 4e). Correct circadian regulation enhances fitness and metabolism^{5,6,8}. In the allotetraploids the expression of clock regulators is altered through autonomous regulation²⁰ and chromatin modifications (Fig. 1d)¹⁵, including rhythmic changes in H3 acetylation in the *TOC1* promoter³⁰. During the day, *A. thaliana* *CCA1* (and *LHY*) is epigenetically repressed, leading to upregulation of EE- and CBS-containing downstream genes in photosynthesis and carbohydrate metabolism. As a result, the entire network is reset at a high amplitude during the day, increasing chlorophyll synthesis and starch metabolism. At night *CCA1* is derepressed and resumes normal oscillation. Although little is known about why the *A. thaliana* genes are repressed during the day¹⁵, the repression is likely associated with *cis*- and *trans*-acting effects on homoeologous loci in the allotetraploids, as observed in flowering-time genes¹⁷. Interestingly, modulation of circadian clock regulators in allopolyploids and hybrids is reminiscent of switching gene expression during dawn- and evening-phased rhythmic alternation^{13,20} that is required for properly maintaining homeostasis in clock-mediated metabolic pathways in diploids^{9,19}. Hybrids and

allopolyploids simply exploit epigenetic modulation of parental alleles and homoeologous loci of the internal clock regulators and use this convenient mechanism to alter the amplitude of gene expression and metabolic flux and gain advantages from clock-mediated photosynthesis and carbohydrate metabolism. Epigenetic regulation of a few regulatory genes induces cascade changes in downstream genes and physiological pathways and ultimately growth and development, which provides a general mechanism for growth vigor and increased biomass^{2,3,15} that are commonly observed in the hybrids and allopolyploids produced within and between species.

METHODS SUMMARY

Allotetraploids were resynthesized as previously described^{1,4}, and hybrids were made by crossing C24 with Columbia. Unless noted otherwise, 8–15 plants (grown under 22°C and 16-hour light/day) from each of 2–3 biological replications were pooled for the analysis of DNA, RNA, protein, chlorophyll, starch, and sugar. *TOC1:CCA1* and *TOC1:cca1*-RNAi transgenic plants were produced using pEarlygate303 (CD694) and pCAMBIA (CD3–447) derivatives, respectively. *cca1–11* (CS9378) and *cca1-11 lhy-21* (CS9380) mutants^{9,22,29} were obtained from ABRC. Protein blot, EMSA, and ChIP assays were performed as previously described^{17,18}.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

METHODS

Plant Growth

Plant materials included *A. thaliana* autotetraploid (At4, ABRC accession#CS3900), *A. arenosa* (Aa, CS3901), and two independently resynthesized allotetraploid lineages (Allo733 and Allo738) (CS3895–96) (F₇ to F₈). All plant materials were generated as previously described^{1,4}. Plants for 24-hour rhythm analysis were grown for 4 weeks in 16/8-hr (light/dark) cycles and harvested at indicated zeitgeber time (ZT0 = dawn)²⁰. For each genotype, mature leaves from five plants were harvested every 3 hrs for a period of 48 hrs and frozen in liquid nitrogen. The data from the first 24-hr period were shown because the second-period data were the same. Leaves were collected prior to bolting (6–8 rosette leaves in *A. thaliana*, 10–12 leaves in *A. arenosa*, and 12–15 leaves in allotetraploids) to minimize developmental variation among genotypes^{31,32}. Unless noted otherwise, analyses for gene expression, chlorophyll, starch, and sugars were performed at ZT6 (noon), 6, 9, and 15.

CCA1 transgenic plants

The constitutive *CCA1*-overexpression line (CCA1-OX) was kindly provided by Elaine Tobin at University of California, Los Angeles. We amplified a *TOC1* (At5g61380.1) promoter fragment using *A. thaliana* Columbia genomic DNA and the primer pair 5'-GGGAATTCCGTGTCTTACGGTGGATGAAGTTGA-3' (*Eco*RI) and 5'-GGGGATCCGTTTTGTCAATCAATGGTCAAATTATGAGACGCG-3' (*Bam*HI) and a full-length *CCA1* cDNA fragment using the primer pair: 5'-

GCGGCCGGATCCATGGAGACAAATTCGTCTGG AG-3' (*Bam*HI) and 5'-GGCCGCTCTAGATCATGTGGAAGCTTGAGTTTC-3' (*Xba*I). The *TOC1* promoter fragment was fused to *CCA1* cDNA and cloned into pBlueScript. The inserts were validated by sequencing and subcloned into pEarlyGate303 (CD694) using the primer pair 5'-GGGACAAGTTTGTACAAAA AAGCAGGCTTACGTGTCTTACGGTGGATGAAGTTGA -3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTGGAAGCTTGAGTTTCCAACCG -3'. The construct (*ProTOC1:CCA1*) was transformed into *A. thaliana* (Columbia) plants³³ (Supplementary Fig. 4b). One-week old T1 seedlings (two true leaves) were sprayed with basta solution (~100 mg/L), and the positive plants were genotyped (Supplementary Fig. 4). T2 transgenic plants (*TOC1:CCA1*) were subjected to chlorophyll, starch, and gene expression analysis.

To make the *TOC1:cca1*-RNAi construct, we amplified a *TOC1* promoter fragment (*ProTOC1*) using the primer pair: F-EcoRI-*ProTOC1* 5'-GGGAATTCGGTGTCTTACGGTGGATGAAGTTGA-3' and R-*ProTOC1*-NcoI 5'-GCGGCCCATGGGTTTTGTCAATCAATGGTCAAATTATGAGACGCG-3' and replaced 35S promoter with *ProTOC1* in pFGC5941 (CD3-447) (Supplementary Fig. 5c). A 250-bp *CCA1* fragment was amplified using the primer pair: F-RNAi *CCA1 XbaI AscI* 5'-GCGGCCTCTAGAGGCGCGCCTCTGAAAACGGTAATGAGCAAGGA-3' and R-RNAi *CCA1 BamHI SwaI* 5'-GGCCGCCCTAGGTAAATTTACACCACTAGAATCGGGAGGCCAAA-3'. We subcloned the *Bam*HI-*Xba*I fragment and then the *Asc*I-*Swa*I fragment into the same vector, generating two *CCA1* fragments in opposite orientations (*pTOC1:cca1*-RNAi) (Supplementary Fig. 5c). Four *TOC1:cca1*-RNAi T1 transgenic plants were used to analyze gene expression and starch content.

We obtained mutant seeds of *cca1-11* (CS9378) and *cca1-11 lhy-21* (CS9380)^{22,29} from ABRC. Gene expression, chlorophyll and starch assays were performed when the mutant plants were about 3-4 weeks old and had 6-8 true leaves under 16/8 hours of day/night before bolting⁹.

Note that *CCA1-OX* and *TOC1:CCA1* lines flowered late (Supplementary Fig. 4)¹⁸, whereas *cca1* and *cca1/lhy* mutants flowered early^{9,29}. A few *TOC1:cca1*-RNAi lines flowered early, whereas some flowered late (Supplementary Fig. 5d), which may be related to various secondary and systematic effects on the downstream genes related to flowering time. All assays in mutant and transgenic plants were performed before bolting.

DNA and RNA Analysis

Genomic DNA was extracted using a modified protocol³². Total RNA was extracted using RNeasy plantmini kits (Qiagen, Valencia, CA). The first-strand cDNA synthesis was performed using reverse transcriptase (RT) Superscript II (Invitrogen, Carlsbad, CA). An aliquot (1/100) of cDNA was used for quantitative RT-PCR (qRT-PCR) analysis using the primer pairs for *LHY*, *CCA1*, *TOC1*, and *GI* (Supplementary Table 2) in an ABI7500 machine (Applied Biosystems, Foster City, CA) as previously described³⁴, except that

ACT2 was used as a control to estimate the relative expression levels (R.E.L.) in three biological replications.

To distinguish locus-specific expression patterns, the RT-PCR products were amplified using the primer pairs (Supplementary Table 3) and subjected to cleaved amplified polymorphism sequence (CAPS) analysis³².

Semi-quantitative RT-PCR was used to determine the expression levels of the genes in chlorophyll a and b biosynthesis and starch metabolism (Supplementary Table 5).

Chlorophyll, starch and sugar contents

Chlorophyll was extracted in dark with 5 ml of acetone (80%) at 4°C from 300 mg 4-week-old seedlings. The chlorophyll content was calculated using spectrophotometric measurements at light wavelengths of 603, 645 and 663 nm and 80% acetone as a control³⁵ and shown as milligram of chlorophyll per gram of fresh leaves.

$$Ca \text{ (mg/g)} = 12.7 \times OD_{663} - 2.69 \times OD_{645} \text{ (Chlorophyll a)}$$

$$Cb \text{ (mg/g)} = 22.9 \times OD_{645} - 4.86 \times OD_{663} \text{ (Chlorophyll b)}$$

$$Ca+b \text{ (mg/g)} = 8.02 \times OD_{663} + 20.20 \times OD_{645} \text{ (Chlorophyll a+b)}$$

Starch content was measured from leaves of five plants (about 600 mg fresh weight). The leaves were boiled in 25 mL 80% (v/v) ethanol. The decolorized leaves were stained in an iodine solution or ground with a mortar and pestle in 80% ethanol^{36,37}. Total starch in each sample was quantified using 30 µL of the insoluble carbohydrate fraction using a kit from Boehringer Mannheim (R-Biopharm, Darmstadt, Germany).

To quantify soluble sugars, 600 mg fresh leaves were extracted with 80% ethanol according to a published protocol³⁸. The sugar concentration was determined enzymatically using Maltose/Sucrose/D-Glucose and D-Glucose/D-Fructose kits, respectively (Boehringer Mannheim, R-Biopharm) and shown as milligram of sugar per gram of fresh leaves.

Promoter motif analysis

DNA sequences from ~1,500-bp upstream of the transcription start sites of the upregulated genes identified in the allotetraploids¹ were extracted and searched for evening element (EE, AAAATATCT) or CCA1 binding site (CBS, AAAAATCT)^{10,18,39}. The same method was used to analyze motifs in all genes in Arabidopsis genome⁴⁰. The list of 128 upregulated genes and motif locations is provided in Supplementary Table 1.

Chromatin immunoprecipitation (ChIP)

The ChIP assays were performed using a modified protocol^{41,42}. We used 1/10 of chromatin solution as input DNA to determine DNA fragment sizes (0.3–1.0-kbp). The

remaining chromatin solution was diluted 10-fold and divided into two aliquots; one was incubated with 10 μ l of antibodies (anti-dimethyl-H3-Lys4, anti-dimethyl-H3-Lys9, anti-acetyl-H3-Lys9, all from Upstate Biotechnology, NY; or anti-CCA1), and the other incubated with protein beads. The immunoprecipitated DNA was amplified by semi-quantitative PCR using the primers designed from the conserved sequences of the *CCA1*, *LHY*, *TOC1*, and *GI* upstream of the ATG codon from both *A. thaliana* and *A. arenosa* loci (Supplementary Table 4). Two independent experiments were performed and analyzed.

Electrophoretic mobility shift assay (EMSA)

A *CCA1* full-length cDNA was amplified from *A. thaliana* cDNA using a primer pair ATTB1_CCA1_F_XHO: 5'-
GGGGACAAGTTTGTACAAAAAAGCAGGCTCCCTCGAGATGGAGACAAATTCGT
 CT-3' and CCA1-R-Avr2-AttB2: 5'-
GGGGACCACTTTGTACAAGAAAGCTGGGTCCCCTAGGTCATGTGGAAGCTTGAG
 TTTC-3'. The cDNA was cloned into pDONR221 and validated by sequencing. The resulting insert was transferred by recombination into pET300/NT-DEST expression vector (Invitrogen Corp., Carlsbad, CA) and expressed in *Escherichia coli* Rosetta-gami B competent cells (Novagen, Madison, WI). Recombinant CCA1 protein was purified and subjected to EMSA39 in 6% native polyacrylamide gels using rCCA1 (10 fmoles) and ³²P-labeled double-stranded oligonucleotides (10 fmoles, Supplementary Table 6). The cold probe (Cp) concentrations were 0 (-), 50 (5 \times), 100 (10 \times), 200 (20 \times), and 500 (50 \times) fmoles, respectively.

Western blot analysis

Protein crude extracts were prepared from fresh leaves as previously described¹⁸. The immunoblots were probed with anti-CCA1, and antibody binding was detected by ECL (Amersham, Piscataway, NJ).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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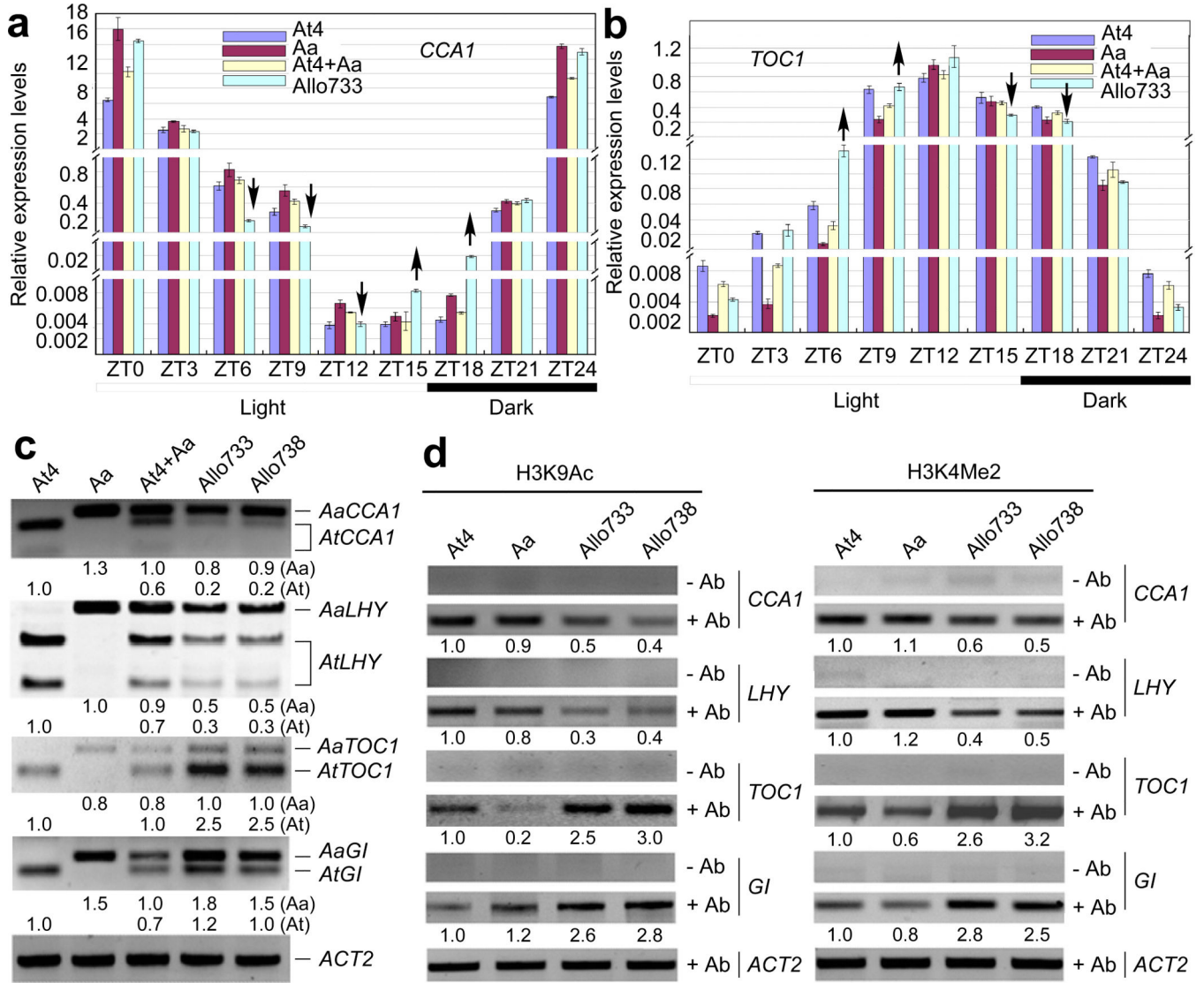


Figure 1. Locus-specific and chromatin regulation of circadian clock genes in the allotetraploids. **a.** qRT-PCR analysis of *CCA1* expression (n = 3, *ACT2* as a control) in a 24-hour period (light/dark cycles) starting from dawn (ZT0, 6 am) (arrows indicate up- and down-regulation, respectively). **b.** qRT-PCR analysis of *TOC1* expression (n = 3). **c.** Repression of *A. thaliana* *CCA1* and *LHY* and upregulation of *A. thaliana* *TOC1* and *GI* in the allotetraploids. RT-PCR products were digested with *Ava*II (*CCA1*), *Af*III (*LHY*), *Ssp*I (*TOC1*), and *Spe*I (*GI*). **d.** ChIP analysis of *CCA1*, *LHY*, *TOC1*, and *GI* using antibodies against H3K9Ac and H3K4Me2 (n = 2). -Ab: no antibodies.

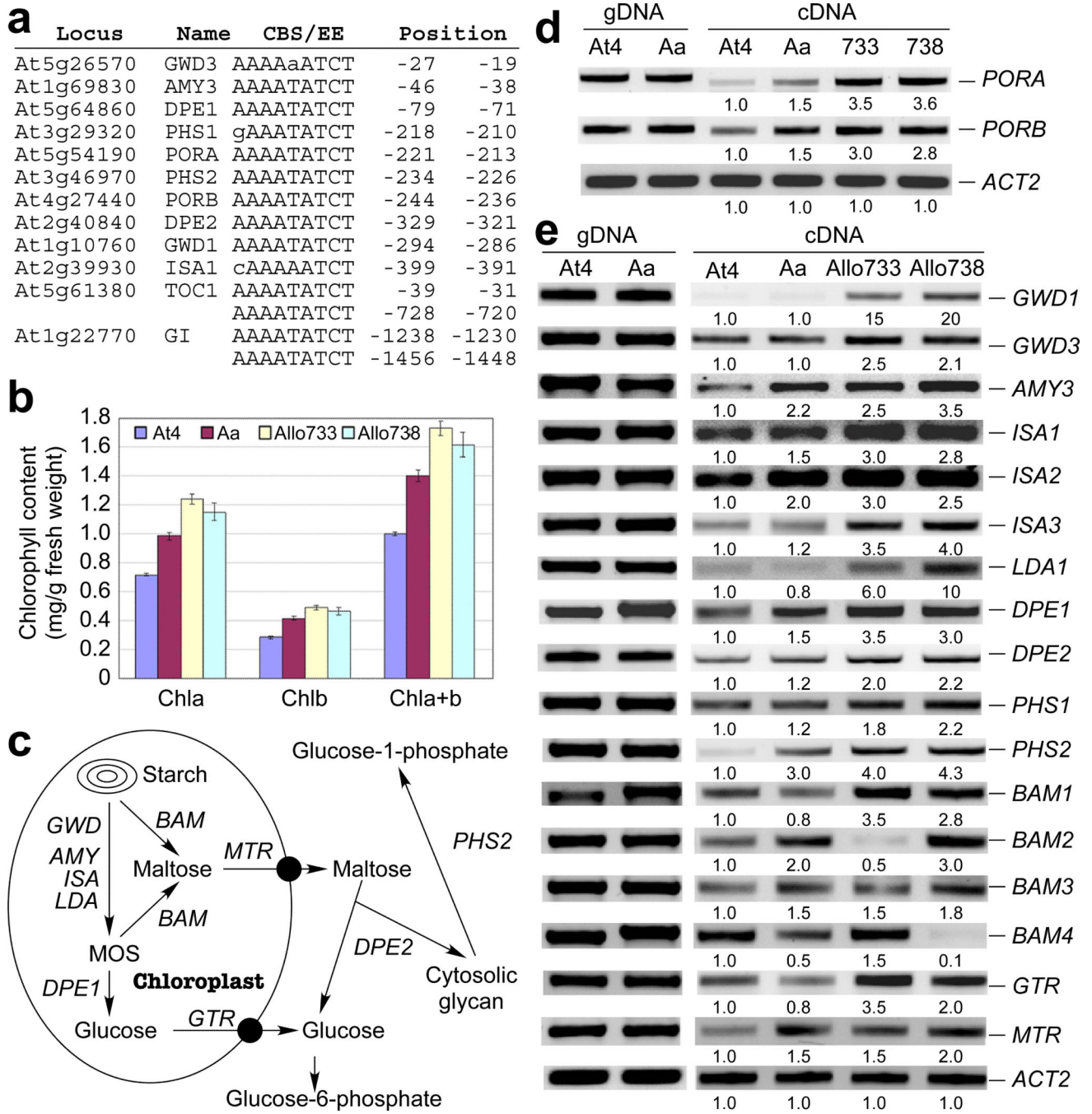


Figure 2. Increase in chlorophyll content and upregulation of the genes involved in chlorophyll and starch biosynthesis in allotetraploids. **a.** Locations of CCA1 binding site (CBS) or evening element (EE) in the downstream genes (Supplementary Table 1). Lower-case letter: nucleotide variation. **b.** Increase of chlorophyll (a, b, and total) content in the allotetraploids (n = 3). **c.** Starch metabolic pathways (modified from that of26) in the chloroplast (circled) and cytoplasm. **d.** Upregulation of *PORA* and *PORB* in the allotetraploids at ZT6 (n = 2).

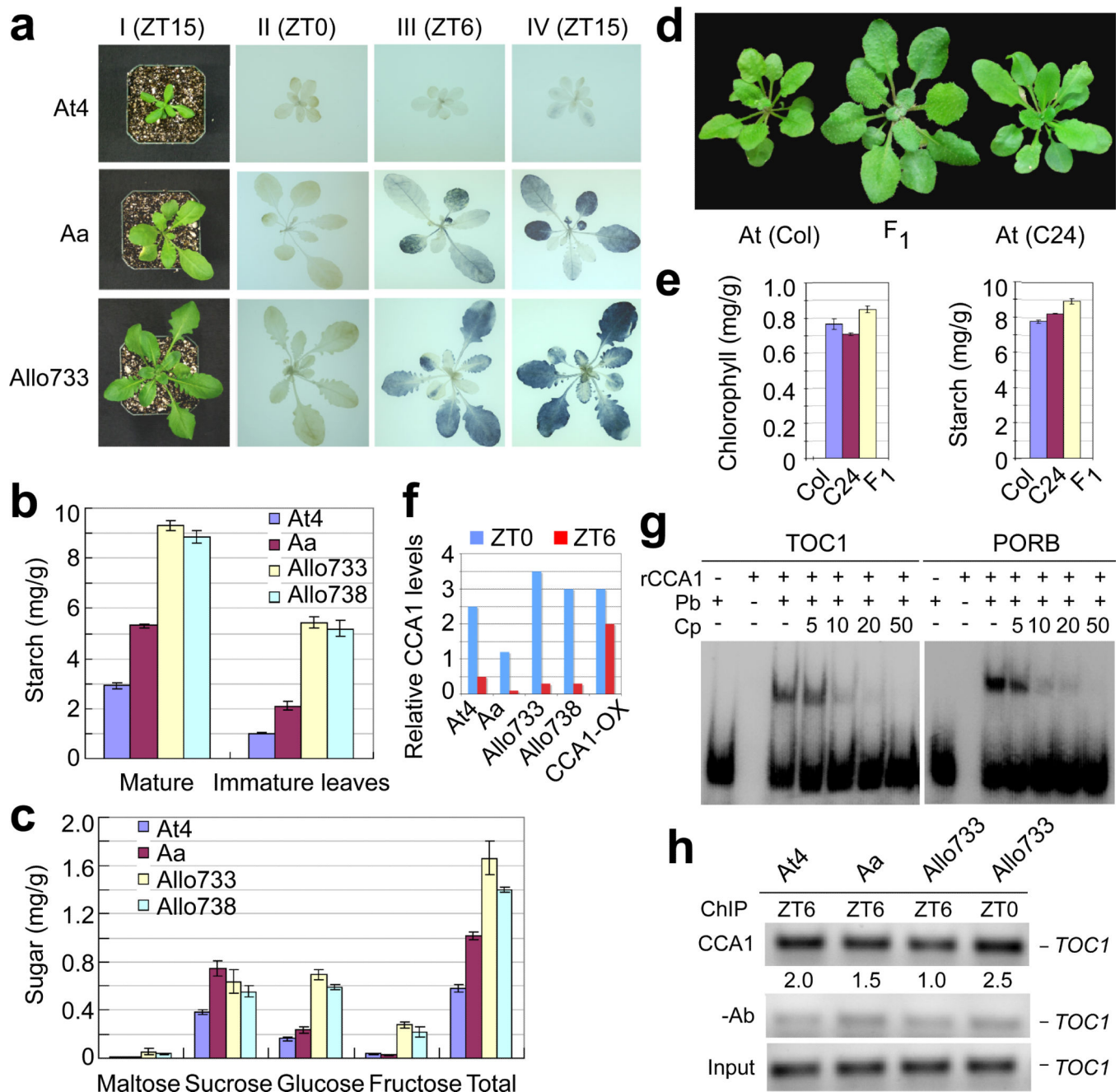
gDNA: Genomic PCR. e. Upregulation of starch metabolic genes in allotetraploids (n = 2) at ZT6. See Supplementary Table 5 for gene names.

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**Figure 3.**

CCA1 function and increased amounts of chlorophyll, starch and sugar in allotetraploids and F₁ hybrids. **a.** Starch staining in *A. thaliana* (At4), *A. arenosa* (Aa), and allotetraploid (Allo733) at ZT0, ZT6, and ZT15. **b.** Increased starch content in allotetraploids at ZT6. **c.** Increased sugar content in allotetraploids at ZT6. **d.** Morphological vigor in F₁ hybrids between *A. thaliana* Columbia (Col) and C24. **e.** Increased chlorophyll (ZT6, left) and starch (ZT15, right) accumulation in F₁. **f.** CCA1 protein levels changed at ZT6 and ZT0. **g.** Specific CCA1 binding activity to EE of downstream genes (*TOC1* and *PORB*) *in vitro*. Cp: cold probes; Pb:³²P-labeled EE-containing probes (Supplementary Table 6). **h.** CHIP assays

of endogenous TOC1 binding to the TOC1 promoter. The levels were normalized using input DNA (n = 2).

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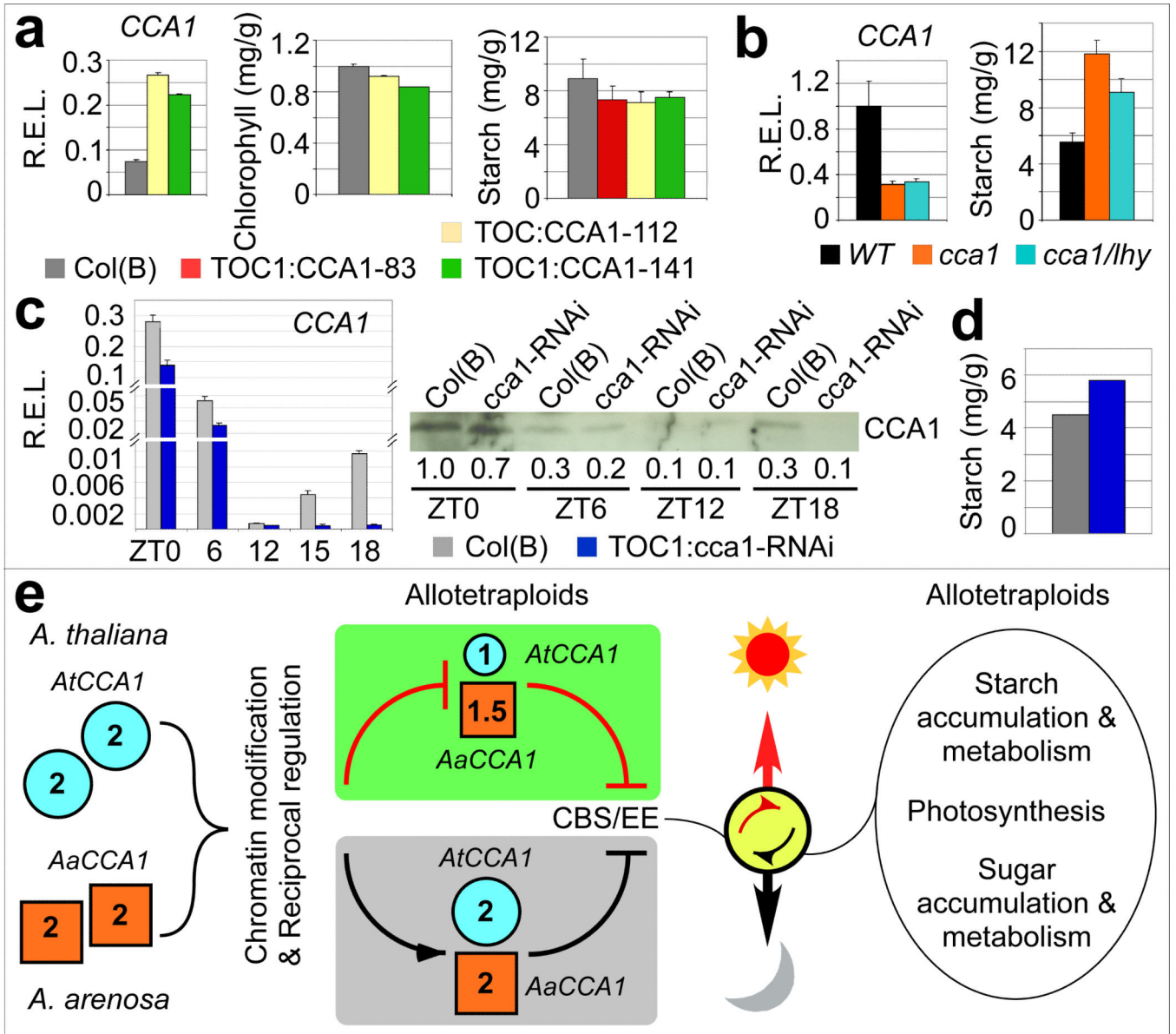


Figure 4. A role of CCA1 in growth vigor in allotetraploids and hybrids. **a.** Relative expression levels (R.E.L.) of *CCA1* (ZT6, left) and reduced chlorophyll (ZT9, middle) and starch (ZT15, right) accumulation in *TOC1:CCA1* lines (n = 3) (Supplementary Fig. 4). Col(B): Columbia transformed with basta gene. **b.** Reduced *CCA1* expression (ZT6, left) and increased starch content (ZT15, right) in *cca1-11* and *cca1-11 lhy-21* mutants^{9,29} (n = 3). WT: Wassilewskija (Ws) or Col. **c.** Decreased expression of *CCA1* mRNA (right, n = 3) and protein (right, n = 2) (ZT0–18, T2) in *TOC1:cca1*-RNAi transgenic plants. **d.** Increased starch content in *TOC1:cca1*-RNAi lines (ZT15, n = 2). **e.** A model for growth vigor and increased biomass. Chromatin-mediated changes in internal clock regulators (e.g., *AtCCA1*) in allotetraploids lead to up- and down-regulation (red and black arrows) and normal

oscillation (yellow circle) of gene expression and output traits (photosynthesis, starch and sugar metabolism) at noon (sun) and dusk (moon).

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