Translationally controlled tumor protein is a conserved mitotic growth integrator in animals and plants

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The growth of an organism and its size determination require the tight regulation of cell proliferation and cell growth. However, the mechanisms and regulatory networks that control and integrate these processes remain poorly understood. Here, we address the biological role of *Arabidopsis* translationally controlled tumor protein (AtTCTP) and test its shared functions in animals and plants. The data support a role of plant *AtTCTP* as a positive regulator of mitotic growth by specifically controlling the duration of the cell cycle. We show that, in contrast to animal *TCTP*, plant *AtTCTP* is not implicated in regulating postmitotic growth. Consistent with this finding, plant *AtTCTP* can fully rescue cell proliferation defects in *Drosophila* loss of function for *dTCTP*. Furthermore, *Drosophila dTCTP* is able to fully rescue cell proliferation defects in *Arabidopsis tctp* knockouts. Our data provide evidence that *TCTP* function in regulating cell division is part of a conserved growth regulatory pathway shared between plants and animals. The study also suggests that, although the cell division machinery is shared in all multicellular organisms to control growth, cell expansion can be uncoupled from cell division in plants but not in animals.

Arabidopsis | *Drosophila* | organ development | cell division

In both the animal and plant kingdoms, body size is a fundamental but poorly understood attribute of biological systems. It affects important fitness variables such as mate selection, predation, and tolerance to various biotic and abiotic stresses. Attaining the correct body size is one of the most rigorous demands of multicellularity because it requires the precise coordination of multiple developmental processes such as cell proliferation, cell growth, and programmed cell death to allow the ultimate differentiation into functional organs and tissues. Molecular and genetic studies in animals and plants are beginning to elucidate the basic growth machinery and its regulation that control and integrate these processes to generate the enormous variety in organ sizes and shapes in nature (1–3).

The translationally controlled tumor protein (TCTP) is ubiquitously found in all eukaryotes. Its expression is associated with many tumors (4, 5). Animal TCTP is involved in several cellular processes, such as cell proliferation, cell growth, malignant transformation, protection against various cellular stresses, and apoptosis (4, 6). In mice, knockout of *TCTP* leads to embryonic lethality associated with lack of proliferation and excessive cell death (7). In *Drosophila*, disruption of *dTCTP* expression in an organ-specific manner leads to size reduction of the targeted organ due to a reduction in cell numbers and defects in cell growth (8). In plants, *TCTP* mRNA expression correlates with mitosis in roots and is induced gradually in periods of darkness (9). Plant TCTP protein was also proposed to have a role in long-distance movement of phloem proteins and in pollen tube growth (10, 11). These published data suggest that TCTP not only regulates organismal growth but also asserts plant-specific functions. However, the molecular and biochemical mechanisms of TCTP function are not yet well characterized.

Plants, unlike animals, have the remarkable ability to continue organogenesis throughout their entire life cycle. We took advantage of this asset to generate a full knockout *Arabidopsis thaliana* for *TCTP* and used it to demonstrate a role of *TCTP* in regulating mitotic growth by controlling cell cycle progression. We further show that this particular function is conserved between plants and animals.

Results and Discussion

***AtTCTP* Is an Essential Gene in *Arabidopsis*.** In *Arabidopsis*, the gene At3g16640 (*AtTCTP*) encodes a protein that is homologous to TCTP (11, 12). We obtained two independent T-DNA insertion lines (*tctp*-1 and *tctp*-2) and studied their phenotypes. Whereas *Arabidopsis* plants homozygous for these T-DNA insertions were embryonic lethal, plants heterozygous for the T-DNA insertions (*AtTCTP*/*tctp* plants) were viable, fertile, and morphologically identical to WT plants. Analyses of developing siliques from *AtTCTP*/*tctp*-1 and *AtTCTP*/*tctp*-2 heterozygous plants revealed that one fourth of seeds were white in color and were arrested in their development (Fig. 1*A*). This ratio of seed viability corresponded to a 3:1 Mendelian segregation, suggesting that the $tctp^{-/-}$ mutation is a recessive lethal trait. Importantly, all white seeds contained endosperm and developing embryos, demonstrating that fertilization did take place between *tctp* haploid mutant gametes. Embryo development in white seeds was compared with that of embryos from seeds showing WT phenotype in the same silique and to that of embryos from WT plants of the same age (Fig. 1*B*). Homozygous *tctp* embryos went through early developmental stages, but their development was significantly delayed and never reached the cotyledon stage. In the same silique, all *tctp* embryos exhibited similar development delay compared with the WT. For example, when WT embryos were at heart stage, *tctp*-1 and *tctp*-2 embryos were at globular stage. These data suggest that embryonic lethality occurs in homozygous *tctp*-1 and *tctp*-2 mutants because the delayed development leads to abortion at the time when siliques dehisce.

To strengthen the conclusion that delayed development was the cause of abortion, we provided *tctp* embryos with nutrient supplements in vitro. Under such conditions, *tctp*-1 and *tctp*-2 embryos (white seeds produced by heterozygous plants) were able to complete their development on culture medium and *tctp* knockout plants could be obtained (Fig. 2*A*). Both *tctp*-1 and *tctp*-2 plants were null alleles of *AtTCTP* because no trace of *AtTCTP* mRNA or protein could be detected (Fig. 2 *B* and *C*). The nutrient-rescued homozygous *tctp* plants were sterile and released no pollen from the anthers of mature flowers (Fig. S1*A*). Application of WT pollen to the stigmas of homozygous *tctp* plants led to the formation of immature siliques that contained few developing seeds

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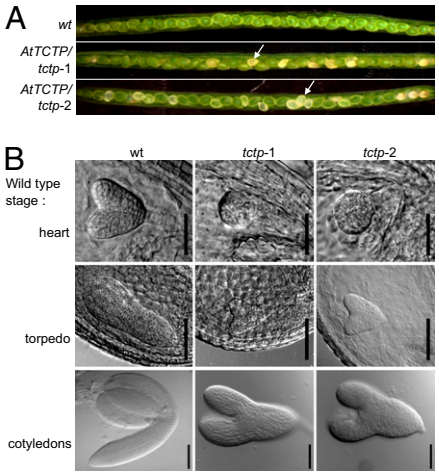

Fig. 1. Loss of function of *AtTCTP* results in delayed embryo development. (*A*) Siliques produced by WT or by heterozygous *AtTCTP*/*tctp*-1 and *AtTCTP*/*tctp*-2 plants. Arrows indicate *tctp* homozygous seeds. These seeds were white in color and segregated at a 3:1 ratio [χ^2(3:1) = 1.03, $P > 0.3$ for *tctp*-1 and χ^2(3:1) = 0.90, $P > 0.3$ for *tctp*-2]. (*B*) Embryos from white homozygous *tctp*-1 and *tctp*-2 seeds show delayed development compared with embryos of the same age from WT seeds. Developmental stages of WT embryo are denoted as "heart", "torpedo," and "cotyledons". [Scale bars, 50 μm (heart stage) and 100 μm (torpedo and cotyledons stages).]

(Fig. S1 *B–E*). These seeds contained *tctp*/*TCTP* heterozygous embryos that exhibited delayed development similar to that observed for *tctp* embryos, when compared with the WT, indicating

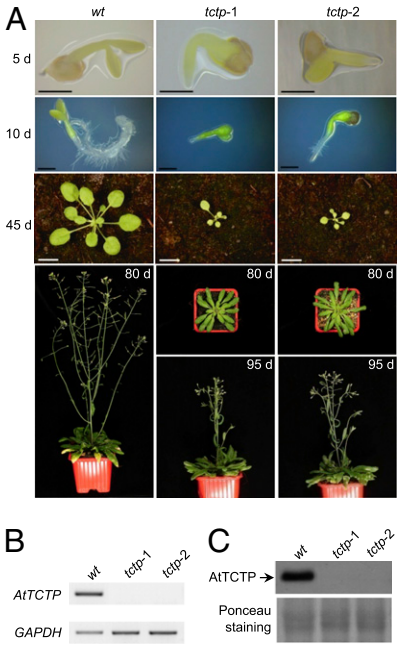

Fig. 2. Embryos homozygous for *tctp*-1 or *tctp*-2 mutations can be rescued by supplying nutrients in vitro. (*A*) Homozygous *tctp*-1 and *tctp*-2 embryos rescued by nutrient supplements in vitro develop into adult plants. WT plant development is used as a control. Days after germination are indicated as 5 d, 10 d, 45 d, 80 d, and 95 d. [Scale bars, 500 μm (5 and 10 d) and 1 cm (45 d).] (*B*) Semiquantitative RT-PCR analysis showing no expression of *AtTCTP* in leaves of both *tctp*-1 and *tctp*-2 plants. *GAPDH* was used as control. (*C*) Western blot analysis of AtTCTP accumulation in leaves of WT, *tctp*-1, and *tctp*-2 plants using anti-AtTCTP antibody. No AtTCTP protein accumulation is observed in *tctp*-1 and *tctp*-2 plants. Red Ponceau staining of total proteins is shown as control.

that heterozygous *tctp*/*TCTP* embryos were unable to develop in a homozygous *tctp* background. However, because heterozygous *tctp*/*TCTP* embryos could develop in *TCTP*/*tctp* background siliques, it is likely that homozygous *tctp* mutant plants are unable to adequately supply embryos with the necessary nutrients during their development. This observation and the results of our nutrient-supplement rescue experiment demonstrate that because *tctp* embryos do not terminate their growth at the silique dehiscence stage, they collapsed, leading to the observed lethality.

Expression of *AtTCTP* using *35S::AtTCTP*, *35S::YFP-AtTCTP* constructs or the full genomic construct (*AtTCTPg-GFP*) was able to complement loss of function of *AtTCTP* (Fig. S2), demonstrating that the embryonic lethality in *tctp*-1 and *tctp*-2 mutants is indeed associated with *AtTCTP* loss of function and that YFP-AtTCTP and AtTCTP-GFP fusion proteins are functional in plants.

Previous work reported that loss of function of *AtTCTP* (line *tctp*-1) was associated with impaired pollen tube growth that results in a lack of ovule fertilization, thus preventing the formation of homozygous mutant embryos (11). In contrast, we consistently observed fertilization in the two independent *tctp* knockout lines, *tctp*-1 and *tctp*-2, even when both *tctp*-1 and *tctp*-2 mutants were grown under different growth conditions (*SI Materials and Methods*). Perhaps under certain growth conditions fertilization can be blocked, as observed in Berkowitz et al. (11). However, our data support that the observed lethality in *tctp* knockout plants is a result of retarded development of the embryos that eventually collapse at the silique dehiscence stage. In animal studies using mouse and *Drosophila*, impaired *TCTP* function has been reported to lead to embryo lethality (7, 8), but the lack of a full knockout adult organism hampered the identification of the role of animal *TCTP* in developing organs. Our study generated full *TCTP* knockout adult organism and introduced *A. thaliana* as a model to study functions of *TCTP* in developing organs (13).

***tctp* Plants Display Growth Defects.** The *tctp*-1 and *tctp*-2 plants that were rescued by nutrient supplements showed a drastic reduction in size (Fig. 2*A*). These rescued mutant plants were severely delayed during development, flowering transition was 15 d later than in WT plants and no seed could be obtained. Similarly, plants that expressed an RNAi directed against *AtTCTP* (*RNAi-AtTCTP*) also showed retarded growth, size reduction (Fig. 3*A*), and 4–5 d delayed bolting when compared with the WT, a similar phenotype to that reported by Berkowitz et al. (11). *RNAi-AtTCTP* plants exhibited a reduction of rosette leaf size, ranging from 21% at 30 d postgermination (dpg) to 18% at 48 dpg (Fig. S3*A*), and a drastically reduced inflorescence stem size (Fig. 3*A*). However, in contrast to *tctp*-1 and *tctp*-2 knockouts, *RNAi-AtTCTP* lines were not embryonic lethal and could develop into adult plants. To explore the cause of such differences, we conducted RT-PCR experiments, which showed a significant reduction, but not full obliteration, of *AtTCTP* expression in *RNAi-AtTCTP* lines (Fig. S3*A*), suggesting that sufficient AtTCTP was produced, allowing embryos to develop. Altogether, these results demonstrate a role of *AtTCTP* as a ubiquitous regulator of plant growth, supporting the notion that *AtTCTP* may serve as a general regulator required for the development of the entire plant.

***AtTCTP* Is Involved in the Control of Cell Proliferation.** To explore the cause of the growth defects in the *tctp* mutants, we analyzed cell division and cell expansion profiles in *RNAi-AtTCTP* lines. The length of the medullar parenchyma cells in *RNAi-AtTCTP* inflorescence stems was similar to that of the WT (Fig. 3*B*; Fig. S4*A*), suggesting that the observed stem size reduction defect in *RNAi-AtTCTP* plants likely correlates with a reduction of cell numbers rather than cell size. Consistent with this observation, the cell density in the leaves and petals of *RNAi-AtTCTP* plants was de-

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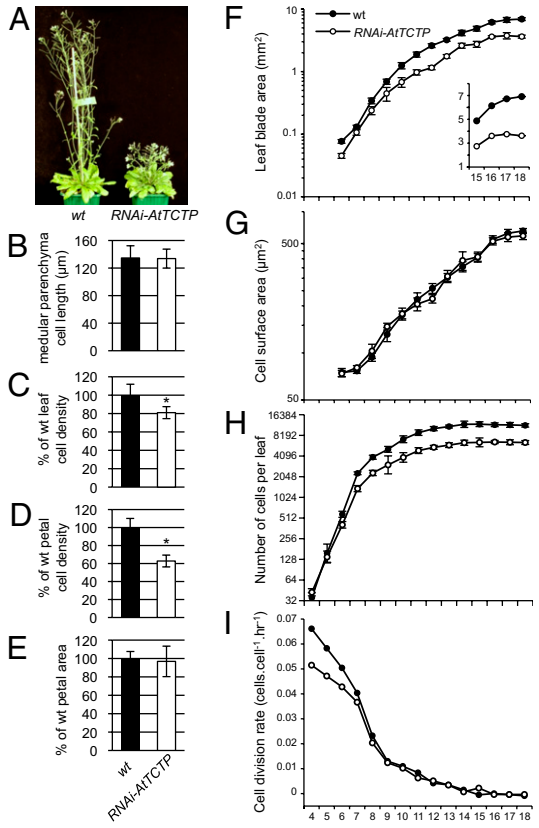

Fig. 3. *AtTCTP* loss of function affects cell division. (*A*) *RNAi-AtTCTP* plants exhibit severe dwarf phenotype compared with WT plants. (*B*) Inflorescence stem cell length measurements show no significant difference in medullar parenchyma cell length between WT and *RNAi-AtTCTP* (n = 120). (*C* and *D*) A significant decrease of leaf epidermal cell density (*C*) and conical petal cell density (*D*) is observed in *RNAi-AtTCTP* plants compared with the WT. Asterisks indicate significant differences with $P_{Student} < 0.001$. (*E*) Surface area measurements of WT and *RNAi-AtTCTP* petals show no significant change of petal size (n = 30). (*F–I*) Kinematic analysis of leaf growth performed on the first leaf pair of *RNAi-AtTCTP* and WT. Average of leaf blade area (*F*), cell area (*G*), cell numbers (*H*), and cell division rate (*I*) are presented. A linear scaled graph of leaf blade area is inserted in *F*. Note that leaves are twofold smaller in the *RNAi-AtTCTP* line compared with the WT.

creased about 20% and 37%, respectively, compared with that of the WT (Fig. 3 *C* and *D*). However, the size of *RNAi-AtTCTP* petals was similar to that of the WT because of an increase in cell size in *RNAi-AtTCTP* petals (Fig. 3*E* and Fig. S4 *C* and *D*). Similarly, *RNAi-AtTCTP* leaves also show a reduction in cell numbers and a partial increase in final cell size (Fig. 3*D* and Fig. S4*B*). These observations suggest the activation of a compensation mechanism that enhances cell expansion in the *RNAi-AtTCTP* leaves and petals, where cell proliferation is impaired. For the same reason, petals of *tctp*-1 and *tctp*-2 homozygous plants rescued by nutrient supplements also showed lower cell density and larger cells (Fig. S4*E*). In contrast, no compensation mechanism occurred in the inflorescence stems of *RNAi-AtTCTP* plants (Fig. 3*B* and Fig. S4*A*). Compensation mechanisms are commonly activated to maintain normal plant organ sizes when aberrant or deficient cell divisions occur during development (14, 15). The result is marked by an increase in cell volume when cell numbers decrease. However, examples in the literature have shown that a decrease in cell numbers does not always correlate with increased cell expansion (15). Specifically, compensation is usually seen in lateral organs with a determinate fate (e.g., leaves and petals) and not in organs with an indeterminate fate, such as roots (16). This probably explains the observed lack of compensation in *RNAi-AtTCTP* inflorescence stems, *Arabidopsis* organs with indeterminate growth. Therefore, we postulate that the cell size increase in *RNAi-AtTCTP* leaves and petals must be indirectly related to TCTP loss of function. Analysis of the DNA content of leaf cell nuclei by flow cytometry showed no significant modifications in *RNAi-AtTCTP* compared with that of the WT (Fig. S5), demonstrating that the cell size increase observed in leaves of the *RNAi-AtTCTP* line was not correlated with endoreduplication, a common landmark of cell expansion (17).

To test our hypothesis that *AtTCTP* controls cell division but not cell expansion, we conducted overexpression studies with lines *35S::AtTCTP* and *35S::YFP-AtTCTP*. The final adult plants overexpressing these constructs have a normal development, which is in agreement with previous work (11). However, *35S::AtTCTP* plantlets exhibited accelerated growth compared with the WT (Fig. S3*A*). For example, the growth in *35S::AtTCTP* plants was about 2 d ahead at 48 d postgermination. Petal surface area and cell density measurements showed no significant difference between *35S::AtTCTP* and WT (Fig. S3*B*), suggesting that accelerated cell proliferation is likely responsible for the observed accelerated growth in *35S::AtTCTP* plants, hence consistent with our proposed role of *AtTCTP* in regulating mitotic growth but not postmitotic growth.

To further strengthen the conclusion that cell division is impaired in *AtTCTP* loss-of-function and gain-of-function plants, we analyzed hypocotyl growth. In etiolated plants, elongation of the hypocotyl occurs solely via cell expansion and does not involve cell division (18). Measurements of hypocotyl length in *RNAi-AtTCTP* and *35S::AtTCTP* seedlings grown in the dark showed no significant difference with that of WT etiolated hypocotyls (Fig. S6), suggesting that cell elongation is likely not affected, thus further strengthening the role of *AtTCTP* as a positive regulator of mitotic growth but not cell expansion. Consistent with *AtTCTP*'s role in regulating cell proliferation, a higher accumulation of *Arabidopsis* AtTCTP protein was reported to be associated with young proliferating tissues such as the meristematic and division zones of the root or the embryo (11). In similar manner, we also observed that *AtTCTPg-GFP* plants, harboring the genomic sequence of *AtTCTP* containing the 5′ and 3′ UTRs, showed a strong GFP fluorescence in tissues undergoing active cell division, although low GFP fluorescence was observed in fully developed organs such as leaves or stems (Fig. S7 *A* and *B*). Interestingly, in *35S::YFP-AtTCTP* plants where the 5′ and 3′ UTRs are absent, we observed a fluorescence signal in all tissues (Fig. S7 *C* and *D*), suggesting that AtTCTP is translationally controlled and that the 5′ and 3′ UTRs are required to restrict the accumulation of TCTP protein to highly dividing tissues. Consistent with these data, AtTCTP protein accumulation did not always match its mRNA expression pattern (Fig. S8). Sequence analysis identified a conserved 5′-terminal oligopyrimidine tract (5′ TOP) motif in the 5′ UTR and two AUUUA motifs in the 3′ UTR of plant *AtTCTP* (Fig. S7 *E* and *F*). In animals, 5′ TOP- and CG-rich regions in the 5′ UTR or AUUUA motifs in the 3′ UTR have been reported as important for the control of TCTP translation (4).

***AtTCTP* Regulates the Duration of Cell Cycle.** To explore further the defects in cell proliferation due to the loss of AtTCTP, we used kinematic analysis of leaf growth using plantlets grown in vitro (19). A reduction in leaf area was already observed 6 d after sowing in the *RNAi-AtTCTP* mutant compared with WT plants. At day 10 after sowing, the *RNAi-AtTCTP* mutant exhibited an approximately twofold reduction in leaf area, and the size remained constant thereafter (Fig. 3*F*). The reduction was due to a decrease in cell numbers, which started at day 5 and continued until day 8 when it reached a maximum twofold reduction (Fig. 3 *G* and *H*). No reduction in cell size was observed (Fig. 3*G*). Therefore, the difference in leaf area between *RNAi-AtTCTP* and WT was directly proportional to the decrease in cell numbers.

From day 4 to day 9, the cell division rate was lower in *RNAi-AtTCTP* leaves, suggesting slower mitotic growth at early development stages compared with the WT (Fig. 3*I*). Average cell cycle duration in the whole leaf was estimated as the inverse of the relative cell division rate. Between day 4 and day 7 after sowing, when growth is mediated through cell division (19), the average cell cycle duration was about 4 h longer in the *RNAi-AtTCTP* leaf primordia compared with the WT. For example, at day 5, cell cycle durations were 17 h in WT and 21 h in *RNAi-AtTCTP* plants. It should be noted that the duration of leaf expansion and that of proliferative growth were similar between the WT and the *RNAi-AtTCTP* plants (Fig. 3 *F* and *H*). Therefore, the observed longer cell cycle in *RNAi-AtTCTP* plants is likely related to the observed cell number reduction and subsequent organ size reduction associated to the loss of function for *AtTCTP*. Furthermore, in contrast to *RNAi-AtTCTP* plants grown in soil, the reduction of cell numbers in the leaves of *RNAi-AtTCTP* grown in vitro was not compensated by an increase in the final cell size. It appears that in vitro culturing perturbed the compensation process observed in the *RNAi-AtTCTP* leaf. A comparable scenario has been observed for plants mutant for the *SWP* gene where compensation mechanisms were observed in *swp* plants grown in soil but not in *swp* plants grown in vitro (20). Hence, the in vitro data corroborate that the observed increase in cell expansion in leaves and petals of *RNAi-AtTCTP* and *tctp* knockout lines is likely independent of *TCTP* function.

To investigate the role of plant *TCTP* in cell cycle progression, we generated tobacco BY-2 cells expressing an RNAi targeted to *Nicotiana tabaccum NtTCTP* (line *RNAi-NtTCTP*). The real time quantitative PCR (qRT-PCR) and Western blot analyses showed very low levels of *NtTCTP* mRNA and protein accumulations in the *RNAi-NtTCTP* cells (Figs. 4 *A* and *D*). We synchronized BY-2 cells with aphidicholin and then determined their mitotic index (the percentage of mitotic cells) (Fig. 4*B*). After aphidicholin release (AAR), WT BY-2 cells exhibited a mitotic peak of about 47% at 9 h AAR, whereas *RNAi-NtTCTP* BY-2 cells exhibited a delayed mitosis 13 h AAR and only 10% of the mutant cells were in mitosis. This mitotic index state was maintained over time in *RNAi-NtTCTP* cells. DNA content measurements (Fig. 4*C*) showed a normal progression of cell cycle over time in WT BY-2 with a rapid reduction of G1 cells 2 h AAR and a concomitant increase of G2 cells. Mitosis occurred 8 h, 10 h and 13 h AAR with a rapid reduction of G2 cells and an increase in G1 cell numbers. As expected, *RNAi-NtTCTP* BY-2 cells were severely delayed in cell cycle progression. The number of *RNAi-NtTCTP* cells at G1 remained high 2 h AAR and continued as such 8 h, 10 h, 13 h and 22 h AAR. Cells entering G2 phase were also severely delayed in *RNAi-NtTCTP* BY-2 with a higher number of cells at 6 h, compared with 2 h in WT BY-2. Therefore, the *RNAi-NtTCTP* cells exhibit a prolonged G1 phase, which results in a delay of at least 4 h for cells to reach G2/M phase.

Analysis of cell cycle marker genes revealed that in the WT BY-2 cells, *proliferating cell nuclear antigen* (*PCNA*) (21) expression decreased rapidly during S/G2 transition (Fig. 4*D*), whereas in *RNAi-NtTCTP* cells *PCNA* expression decreased much more slowly compared with WT BY-2. These data are in agreement with the observed delay of cells leaving G1 phase in *RNAi-NtTCTP* cells. In these cells, the G2/M marker *cyclin A1.1* gene (21) exhibited lower levels of mRNA accumulation and delayed expression that reached a maximum between 10 and 14 h AAR. In WT BY-2, *cyclin A1.1* expression was highly induced until 8 h AAR followed by a rapid decrease in its expression. Similarly, the onset of the expression of the mitosis-specific marker *cyclin B1.2* (21) was delayed about 4 h in *RNAi-NtTCTP* BY-2. We also observed an approximately twofold reduction in *cyclin B1.2* level, and its expression was more spread out over time when compared with WT BY-2 (Fig. 4*D*). In WT BY-2, the expression of *cyclin B1.2* was at maximum 9 h AAR, which corresponds to the peak of mitotic index

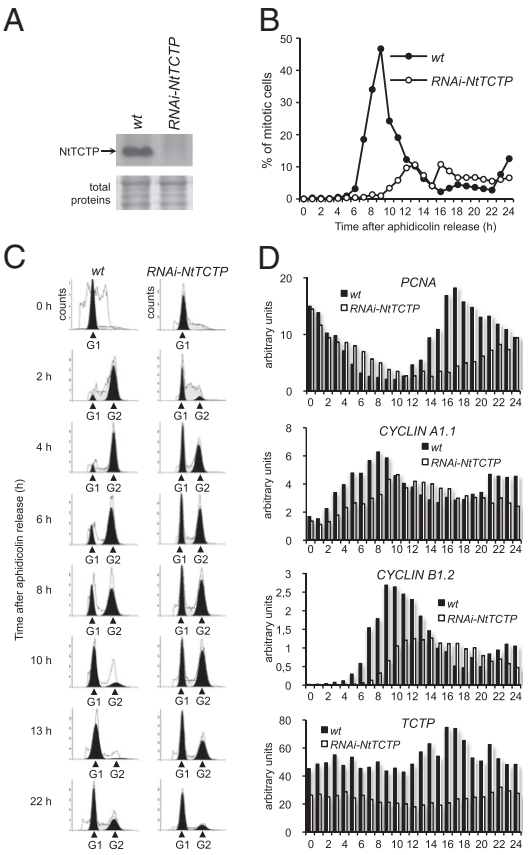

Fig. 4. Plant *TCTP* controls cell cycle duration (*A*) Western blot analysis of NtTCTP protein accumulation in WT and *RNAi-NtTCTP* BY-2 cells using anti-AtTCTP antibody. Coomassie staining of total proteins is shown as control (*Bottom*). (*B*) Mitotic index of synchronized WT and *RNAi-NtTCTP* BY-2 cells after aphidicholin release (AAR). Note the delay and the decrease of the mitotic peak in *RNAi-NtTCTP* cells compared with WT. (*C*) Flow cytometric analysis of DNA content in synchronized WT and mutant BY-2 cells. Note the constant G1 population after AAR in *RNAi-NtTCTP* cells. (*D*) Quantitative RT-PCR analysis of cell cycle marker genes (*PCNA*, *cyclin A1.1*, and *cyclin B1.2*) and *NtTCTP* in synchronized WT and mutant BY-2 cells.

(Fig. 4*B*). Therefore, the slowing of cell cycle progression in *RNAi-NtTCTP* cells was associated with delayed expression of A- and B-type cyclins. The aberrant prolonging of cell cycle duration in *RNAi-NtTCTP* cells and in the *Arabidopsis RNAi-AtTCTP* line (kinematics data) strongly indicates the role of plant TCTPs as mitotic growth regulators.

Plant *AtTCTP* and Animal *dTCTP* Have Conserved Function in Controlling Mitotic Growth. To explore whether *TCTP* function is conserved among plants and animals, we performed interspecies complementation experiments between *Arabidopsis* and *Drosophila*. *Drosophila dTCTP* was expressed under the control of the 35S constitutive promoter in *tctp*-2 plants. *dTCTP* rescued the embryonic lethality in *tctp*-2 and the cell proliferation defects associated with loss of function of plant *AtTCTP* (Fig. 5*A*), demonstrating that animal *dTCTP* performs similar function in plants and thus is involved in regulating mitotic growth.

Next, we examined whether the plant *AtTCTP* was able to complement *dTCTP* loss of function in *Drosophila*. We used flies in which the expression of *dTCTP* was silenced via the expression of an anti-*dTCTP* RNAi under the control of organ-specific promoters in the eye (line *ey* > *dTCTPi)*, in the wing (line *nub* > *dTCTPi*), or between the L3 and L4 wing veins (line *ptc* > *dTCTPi*). A GFP reporter confirmed the organ specificity of these promoters (Fig. S9). Interference with *dTCTP* led to a significant size reduction of the

targeted organ (8) (Fig. 5 *B* and *C*). In *dTCTPi* flies, wing organ size reduction is thought to be the result of the combined decrease in cell proliferation and cell expansion (8). *AtTCTP* expression driven by *nub-Gal4* (*nub* > *AtTCTP*) or by *ptc-Gal4* (*ptc* > *AtTCTP*) rescued the wing size reduction phenotype in *nub* > *dTCTPi* flies and the L3 and L4 vein distance reduction phenotype in *ptc* > *dTCTPi* flies (Fig. 5 *C* and *D*). However, the rescued fly wings were smaller than the WT wings, suggesting partial rescue of the cell proliferation and/or cell expansion. Comparison of cell numbers in the wings of *nub* > *dTCTPi* flies, of *nub* > *dTCTPi* flies expressing *nub* > *AtTCTP*, and of WT flies revealed that the partial restoration of wing size via expression of *AtTCTP* correlates with a full restoration of cell numbers (Fig. 5*E*). However, a partial restoration of cell size was also observed (Fig. 5*F*). These data suggest that plant *AtTCTP* is able to fully complement the cell division but not all cell expansion, defects associated with loss of function in *dTCTP* wings.

Silencing *dTCTP* in the eye using the *eyless* promoter (*ey* > *dTCTPi*) resulted in eye size reduction (8) (Fig. 5*B*). In *ey* > *dTCTPi* flies, the eye size reduction was associated with reduced ommatidia number but not ommatidia size (Fig. 5 *G* and *H*), suggesting that cell size in the eye was not affected in *ey* > *dTCTPi* flies and that eye size reduction was only associated with a decrease in cell numbers. This is in agreement with the fact that *eyless* promoter drives expression in the eye imaginal disk anterior to the furrow at the time when eye cell progenitors are actively dividing (22). Expression of *AtTCTP* in the eye (*ey* > *AtTCTP*), where *dTCTP* was silenced (*ey* > *dTCTPi*), completely rescued the eye size reduction phenotype (Fig. 5*B*). Furthermore, expression of *AtTCTP* (*ey* > *AtTCTP*) fully rescued the ommatidia number defects in *ey* > *dTCTPi Drosophila* (Fig. 5 *G* and *H*), supporting the model that *AtTCTP* regulates cell division.

These interspecies complementation experiments provide evidence that TCTP functions as a growth regulator by controlling cell division and that this function is conserved across the animal and plant kingdoms. In AtTCTP, 38% of its amino acids are identical to its *Drosophila* counterpart (Fig. S10). Many of the essential amino acids and domains known to be required for animal TCTP functions are conserved in AtTCTP. The rat TCTP C-terminal domain, shown to homodimerize in the yeast two-hybrid system (23), is conserved in AtTCTP (Leu122 to Cys168). Bimolecular fluorescence complementation (BiFC) experiments (Fig. S11*A*) demonstrated that AtTCTP or dTCTP were able to homodimerize in vivo. Furthermore, AtTCTP was able to interact with dTCTP, suggesting that despite the overall relatively divergent protein sequences, the homodimerization domains of AtTCTP and dTCTP are structurally and functionally conserved. In *Drosophila*, a substitution of Glu12 to Val renders dTCTP nonfunctional (8). We found that AtTCTP harboring such a mutation (AtTCTP^{E12V}) was unable to complement eye and wing size reduction phenotypes associated with *dTCTP* loss of function in flies (Fig. 5 *B–H*), demonstrating that the Glu12 is necessary for the correct function of TCTP in both plants and animals. In *Drosophila*, the dTCTP was reported to positively control the target of rapamycin (TOR) activity through interaction and activation of the Rab GTPase dRheb (8). TOR kinase is a part of a signaling complex that controls cell proliferation and growth in plants and animals (24, 25).

In *Arabidopsis*, at least 25 Rab GTPases share about 30–35% of their amino acids with dRheb. We found that AtTCTP is able to bind to four of these Rab GTPases (AtRABA4a, AtRABA4b, AtRABF1, and AtRABF2b) in vivo in BiFC experiments (Fig. S11*B*). In these experiments, AtTCTP also interacted with *Drosophila* dRheb, and is thus in agreement with interspecies complementation experiments demonstrating that plant and animal TCTPs act in the same regulatory pathway. Similarly, dTCTP was able to interact with dRheb and with plant AtRABA4a, AtRABA4b, AtRABF1, and AtRABF2b (Fig. S11*B*). GST-pull-down experiments confirmed these interactions in vitro (Fig. S11 *C* and *D*). These data suggest that, as in animals, plant TCTP may also act in the TOR pathway via Rab GTPases. Previously, structure-based modeling and analyses of genes in which expression is controlled by TOR also suggested that plant TCTP acts as a regulator of TOR (11).

In conclusion, the characterization of a viable *TCTP* knockout and the interspecies functional complementation studies demonstrate the function of plant *AtTCTP* as a cell division regulator. Although plants and animals diverged about 1.6 billion years ago, *TCTPs* have conserved their function in controlling mitotic organ growth. Nevertheless, the interspecies complementation experiments suggest that animal TCTP has diverged from that of plant TCTP to control cell growth. Determining and comparing the 3D

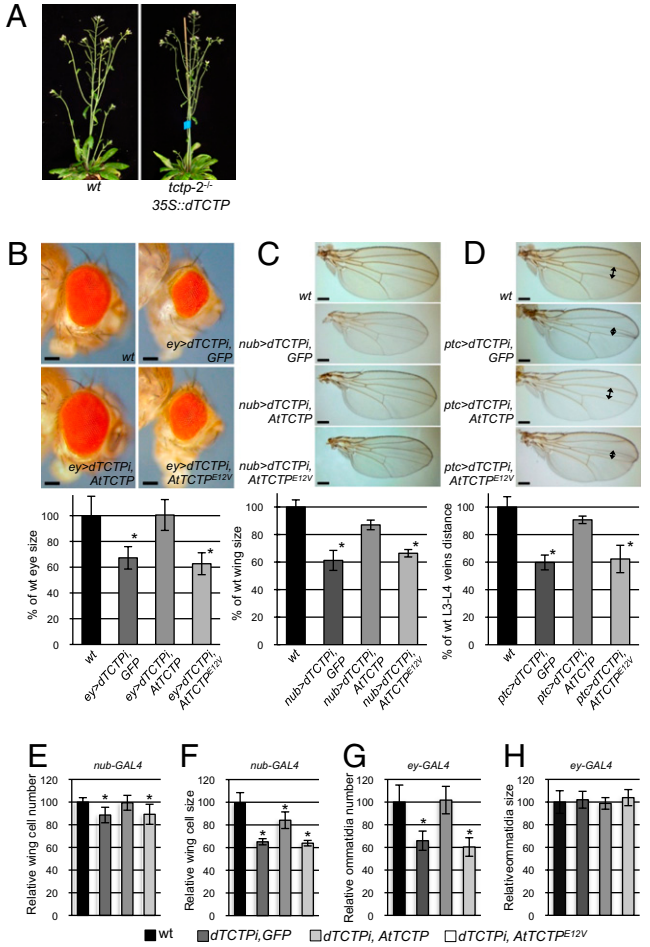

Fig. 5. *Drosophila dTCTP* complements *AtTCTP* loss of function in *Arabidopsis* and vice versa. (*A*) The *Drosophila dTCTP* (*35S::dTCTP*) is able to complement embryonic lethality in *tctp*-2 loss of function mutant. (*B–D*) *Arabidopsis AtTCTP* is able to complement loss of function of *dTCTP* in *Drosophila*. Comparison of eye surface (*B*), wing surface (*C*), and L3-L4 veins distance (*D*) in WT flies and in lines expressing an RNAi directed against *dTCTP* (*dTCTPi*) under the control of *eyless-GAL4* (*B*) (*ey*>), *nubbin-GAL4* (*C*) (*nub*>), or *patch-GAL4* (*D*) (*ptc*>) drivers, along with *GFP*, *AtTCTP*, or *AtTCTP^{E12V}*. Asterisks indicate significant differences with $p_{Student} < 0.001$; $n = 30$ for each condition. [Scale bars, 100 μm (*B*) and 200 μm (*C* and *D*).] (*E* and *F*) *AtTCTP* complements cell number defects fully (*E*) and cell size defects partially (*F*) in *nub* > *dTCTPi* fly wings. (*G* and *H*) The eye size reduction in *ey* > *dTCTPi* flies is associated with a reduction in ommatidia number (*G*) but not size (*H*). The defects in ommatidia number are fully complemented by expressing plant *AtTCTP* (*G*). Mutated *AtTCTP^{E12V}* is unable to complement cell number reduction in wing and ommatidia number reduction in eye of *dTCTPi* flies. Asterisks indicate significant differences with $p_{Student} < 0.001$; $n = 30$ for each condition.

structure of AtTCTP to its yeast and human counterparts (26, 27) and identifying the partners of TCTP will likely address the molecular mechanisms of the functional conservation and the differences between plant and animal TCTPs.

Materials and Methods

Constructions and Plant Lines. *A. thaliana* (Col-0 accession) T-DNA-insertion knockout lines *tctp*-1 (SAIL_28_C03) and *tctp*-2 (GABI_901E08) were obtained from the Nottingham *Arabidopsis* Stock Centre.

Anti-*AtTCTP* RNAi expressing plants, *35S::AtTCTP*, *35S::dTCTP* and *35S::YFP-AtTCTP* overexpression lines, and the *AtTCTPg-GFP* line (*AtTCTP* genomic sequence GFP-tagged) were obtained as described in *SI Materials and Methods*.

Embryo Rescue. Embryos homozygous for the *tctp* mutation (white seeds) were dissected from heterozygous *tctp*-1 and *tctp*-2 siliques at stage 17B (28) and then rescued by nutrient supplements in vitro as described previously (29). Developed plantlets were then transferred to soil. During all steps, embryos from WT siliques were used as control.

Cell Numbers, Size Measurements, and Kinematic Analysis of Leaf Growth. Leaves were cleared in a solution containing 80 g chloral hydrate/30 mL water and then mounted in clearing solution containing 20% glycerol. Petal surface area measurements were performed using digital images from cleared petals as described previously (12). Cell density was determined as the number of cells per surface unit from digital images (Nikon Optiphot 2 microscope with Nomarski optics) of cleared leaves and petals using ImageJ software (National Institutes of Health).

Kinematic analysis of leaf growth was performed on the two first-initiated leaves as previously described (19).

Transgene Constructions and *Drosophila* Lines. The constructs *pUAST::AtTCTP* and *pUAST::AtTCTP^{E12V}* that harbor WT or mutated *AtTCTP* cDNA, respectively (*SI Materials and Methods*), under the control of Upstream Activation Sequence (UAS) were used to generate transgenic *Drosophila* via embryo injections (BestGene Inc.). *Drosophila* lines *UAS::AtTCTP*, *UAS::AtTCTP^{E12V}* and *UAS::GFP* (used as control) were crossed with *UAS-dTCTP RNAi* line (8). The progenies were crossed with different lines expressing a GAL4-driver: *eyless-GAL4* (*ey-GAL4*), *patch-GAL4* (*ptc-GAL4*), and *nubbin-GAL4* (*nub-GAL4*) (Bloomington *Drosophila* Stock Center).

Drosophila eye and wing organ size was measured from digital images using ImageJ software. Wing cell numbers and cell size were calculated by counting of the number of hairs per surface area between the L3 and L4 veins (30). Ommatidia number and size in the eyes of *dTCTPi* flies expressing *AtTCTP* was assessed by measuring ommatidia density (number per surface area) and comparing it with that in WT and *dTCTPi* eyes.

Detailed procedures are provided as *SI Materials and Methods*.

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