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Transcriptional repression of the APC/C activator CCS52A1 promotes active termination of cell growth

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Spatial and temporal control of cell growth is central for the morphogenesis of multicellular organisms. For some cell types that undergo extensive post-mitotic cell growth, such as neurons and hair cells, orchestrating the extent of post-mitotic cell growth with development is vital for their physiology and function. Previous studies suggested that the extent of cell growth is linked with an increase in ploidy by endoreduplication but how developmental signals control endocycling and cell growth is not understood in both animals and plants. In this study we show that a trihelix transcription factor, GT2-LIKE 1 (GTL1), actively terminates ploidy-dependent cell growth and its developmentally regulated expression is one of the key determinants of cell size in Arabidopsis leaf hair cells (trichomes). Through genome-wide chromatin-binding studies (ChIP-chip) coupled with transcriptional profiling, we further demonstrate that GTL1 directly represses the transcription of *CDH1/FZR/CCS52*, an activator of the anaphase-promoting complex/cyclosome (APC/C), to stop the endocycle progression and ploidy-dependent cell growth. Thus, our findings uncover a previously uncharacterised key molecular link between developmental programming and cell-size control, highlighting the central role of APC/C in post-mitotic cell growth.

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Introduction

Organ morphogenesis in multicellular organisms relies on the coordinated progression of cell proliferation and cell growth. Compared with animals, plants often undergo far more extensive post-mitotic cell growth, sometimes up to 1000-fold of original size, and an increase in cell size generally contributes to a larger extent to organ growth. It is also known that some cell types, such as neurons and various hair cells in insects, animals and plants, undergo massive cell growth during differentiation and this is vital for their specialised physiology and function (Sugimoto-Shirasu and Roberts, 2003). Since the final size of cells is often fairly constant under given conditions, the duration of post-mitotic cell growth is likely to be developmentally programmed. We still know surprisingly little about how cell size is determined and how developmental signals link to this control. Most of loss-of-function mutants with developmental defects display reduced cell-size phenotypes, pointing to the existence of robust mechanisms to promote cell growth (Breuer *et al*, 2010). Several mutations, on the contrary, cause larger cells (Perazza *et al*, 1999; Horiguchi *et al*, 2005; Imai *et al*, 2006; Lasorella *et al*, 2006; Breuer *et al*, 2009) but whether there are some mechanisms to actively restrain or terminate cell growth has not been extensively studied.

In various eukaryotes, cell size often positively correlates with the nuclear DNA content or ploidy (Nagl, 1976; Melaragno *et al*, 1993). This correlation was already proposed early last century, referred to as the 'nuclear–cytoplasmic ratio' theory (Wilson, 1925), but there have been also reports in which the ploidy level does not match with cell size (Beemster *et al*, 2002; Schnittger *et al*, 2003). The situation is also more complex in plants where the vacuoles constitute most of cell volume and the cytoplasm accounts very little for overall cell size (Sugimoto-Shirasu and Roberts, 2003). Although it is generally accepted that some proportion of cell growth is ploidy-dependent, how far this contributes to total growth and importantly, how cells grow in a ploidy-dependent and/or -independent manner still remains elusive.

One strategy to increase the ploidy level is to initiate an aberrant version of the mitotic cell cycle called endoreduplication cycle or endocycle. Endocycling cells skip the mitotic phase, thus they re-enter into the S phase without cytokinesis, resulting in the duplication of the nuclear DNA content (Nagl, 1976; Edgar and Orr-Weaver, 2001). Endoreduplication is widespread in plants and many mitotically dividing cells switch into the endocycle as they start to differentiate. Endocycling also occurs in specialised cell types of insects and mammals, potentially accounting for up to half of the biomass in the world (Zielke *et al*, 2011). Studies in Arabidopsis and Drosophila have revealed that some of the regulatory mechanisms governing the onset and progression of endocycles are shared between plants and animals, and an important step to promote the endocycle is to inactivate the mitotic cyclin (CYC)–cyclin-dependent kinase (CDK) complexes (Lee *et al*, 2009; Breuer *et al*, 2010; Komaki and Sugimoto, 2012). In Arabidopsis, for example, CDKB1;1, in complex with CYCA2;3, represses the mitotic-to-endocycle transition and its down-regulation results in an early endocycle onset (Boudolf *et al*, 2004, 2009). The abundance

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of active CYC–CDK complexes is controlled by several regulatory mechanisms, but one key event tightly connected to the endocycle is the proteolytic degradation of mitotic CYCs by the 26S proteasome (Hershko, 1997; Peters, 2002; De Veylder *et al*, 2011; Komaki and Sugimoto, 2012). The anaphase-promoting complex/cyclosome (APC/C) is the multisubunit E3 ubiquitin ligase complex that plays central roles in this control and its activation strongly depends on the presence of evolutionary conserved proteins, such as CELL DIVISION CYCLE 20 (CDC20)/Fizzy and CDC20 HOMOLOG 1 (CDH1)/CELL CYCLE SWITCH 52 (CCS52)/Fizzy-related (FZR) (Sigrist and Lehner, 1997; Visintin *et al*, 1997; Cebolla *et al*, 1999). The CDC20 and CDH1 activators interact with mitotic CYCs and target them to the core complex of the APC/C to facilitate their ubiquitination for prompt degradation. The Arabidopsis genome encodes five *CDC20* (*CDC20.1–CDC20.5*), and 3 CDH1 (*CCS52A1*, *CCS52A2* and *CCS52B*) homologues. Previous loss- and gain-of-function studies have uncovered strong association of CCS52A1 and CCS52A2 with the control of endocycle onset and progression (Lammens *et al*, 2008; Larson-Rabin *et al*, 2009; Kasili *et al*, 2010; Mathieu-Rivet *et al*, 2010). The function of CCS52B and the CDC20 homologues is less characterised but recent studies suggest that CCS52B also participates in this control (Iwata *et al*, 2011; de Almeida Engler *et al*, 2012).

With the tight connection between endocycling and cell growth or cell differentiation, its onset and progression are likely to be developmentally regulated. Accumulating evidence suggests that transcriptional regulation of the CDH1/CCS52/FZR activators is one common mechanism mediating this control in various organisms. For instance, endocycle establishment and progression during follicle cell development in the Drosophila ovary is controlled through the Notch signalling pathway (Deng *et al*, 2001). Several transcriptional regulators have been identified to modulate the *fzr* expression although whether they directly target the *fzr* locus is not known (Sun and Deng, 2005, 2007). The Notch pathway is not conserved in plants (Wigge and Weigel, 2001) but in contrast to flies, both *CCS52A1* and *CCS52A2* are under the direct transcriptional control of the E2FA-RETINOBLASTOMA-RELATED (RBR) complex and an atypical E2F transcription factor DEL1 (Lammens *et al*, 2008; Magyar *et al*, 2012). The E2FA–RBR complex transcriptionally represses expression of both *CCS52A1* and *CCS52A2*, whereas DEL1 represses only *CCS52A2* in mitotically dividing cells to suppress early developmental transition from the mitotic cycle into the endocycle.

Arabidopsis trichomes are unicellular and they serve as an excellent model system to study post-mitotic cell growth since they increase their cell volume by >100-fold in a developmentally specific window of time. Trichome cells also undergo several rounds of endocycle to increase their ploidy level on average up to 32C. Earlier molecular genetic studies have uncovered many regulators that participate in trichome differentiation and have shown that trichome cell growth is promoted by complex transcriptional networks (Ishida *et al*, 2008; Morohashi and Grotewold, 2009). Previously, we found that a mutation in *GTL1*, a GT-2 family trihelix transcription factor, results in extended endocycles and cell growth (Breuer *et al*, 2009) but the molecular mechanism underlying this control remains unknown. Here, we show that overexpression of GTL1 is sufficient to arrest the endocycle and cell growth in both trichome and other leaf epidermal cells. Furthermore, a combination of ChIP-chip and trichome-specific gene expression analysis revealed that GTL1 directly binds to the promoter of *CCS52A1* and represses its expression to terminate the endocycle. We also provide genetic evidence that trichome cells grow through both ploidy-dependent and -independent pathway and that transcriptional repression of *CCS52A1* by GTL1 accounts only for ploidy-dependent cell growth. Our data thus highlight that terminating cell growth is an active process and that plants accomplish this by programming the developmental timing to down-regulate the $APC^{CDH1/FZR/CCS52}$ activity.

Results

GTL1 is sufficient to terminate ploidy-dependent cell growth

GTL1 is expressed only at late stages of trichome development and is required to terminate its cell growth (Breuer *et al*, 2009). To investigate whether the expression of GTL1 is sufficient to block cell growth, we fused GTL1 to the green fluorescence protein (GFP) and expressed the GFP–GTL1 protein at an early stage of trichome differentiation using the *GLABRA2* (GL2) promoter (Szymanski *et al*, 1998; Schnittger *et al*, 2002). These GFP–GTL1 proteins are functional and complement the *gtl1-1* mutant phenotypes when expressed under its own promoter (Breuer *et al*, 2009). We confirmed the expression of GFP–GTL1 proteins in developing trichomes of *pGL2:GFP:GTL1* plants (Supplementary Figure S1A). Our scanning electron microscopy revealed that fully mature trichomes from 17-day-old wild-type (WT) plants have characteristic size and morphology with three branches (Figure 1A and B). In contrast, the *pGL2:GFP:GTL1* plants display severe defects in trichome development, ranging from an early arrest to complete loss of cellular outgrowth and branching (Figure 1A and B).

Previous studies have shown that *GTL1* is also expressed in non-trichome cells (Breuer *et al*, 2009; Yoo *et al*, 2010). To examine whether GTL1 regulates cell growth in a more general context, we expressed GFP–GTL1 proteins using the *ATML1* promoter that drives gene expression in cells at the outermost layer of shoot apical meristem and leaf epidermis (Sessions *et al*, 1999). The GFP–GTL1 proteins are expressed in both trichomes and other epidermal cells in *pATML1:GFP:GTL1* plants (Supplementary Figure S1A) and as expected, they repress cell growth in trichomes (Figure 1A and B). We occasionally saw some outgrowth of trichomes at the leaf margin but they hardly underwent branching and full growth. In addition, we found that epidermal pavement cells in *pATML1:GFP:GTL1* plants are small compared with WT cells that have undergone extensive cell growth (Figure 1E; Supplementary Figure S1E). Consequently, the final size of *pATML1:GFP:GTL1* leaves is also strongly reduced (Figure 1D; Supplementary Figure S1D) although overall number of adaxial epidermal cells is similar to that of WT (Supplementary Figure S1E and F).

We have previously shown that *gtl1* mutant trichomes undergo on average an additional round of endocycle, resulting in an increase of trichome ploidy (Breuer *et al*, 2009). To test whether overexpression of GTL1 also interferes with

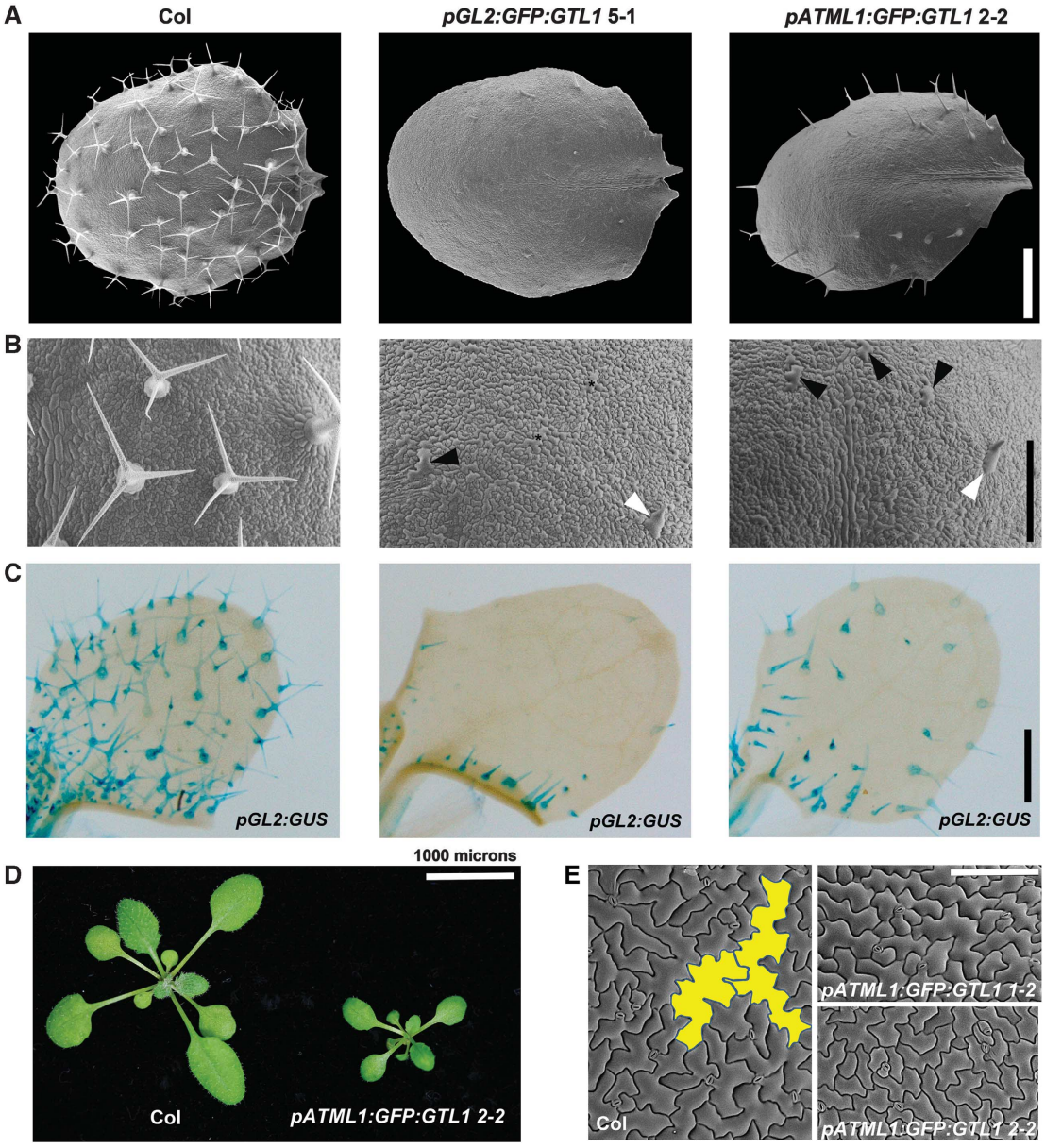

Figure 1 Overexpression of *GTL1* dramatically represses cell growth and cell differentiation. (**A**, **B**) Scanning electron micrographs of the fifth true leaves of 17-day-old WT (Col), *pGL2:GFP:GTL1* and *pATML1:GFP:GTL1* plants (**A**) and corresponding close-up views of their adaxial epidermis (**B**). White arrows mark out-grown, unbranched trichomes. Black arrows point to abnormally enlarged and pointed epidermal cells that may possess trichome identities. Another type of abnormally enlarged cells, marked with black asterisks, exhibit cellular morphology characteristic to epidermal pavement cells. (**C**) Expression of the trichome identity marker *pGL2:GUS* in the third true leaves of 10-day-old Col, *pGL2:GFP:GTL1* and *pATML1:GFP:GTL1* plants. (**D**, **E**) A light micrograph of 21-day-old Col and *pATML1:GFP:GTL1* plants (**D**) and scanning electron micrographs of their adaxial epidermis on fully expanded first true leaves (**E**). Two large pavement cells are highlighted in yellow in Col leaves. Epidermal cells of similar size are not found in *pATML1:GFP:GTL1* leaves. Scale bars: 300 μm in (**A**), 250 μm in (**B**), 500 μm in (**C**), 1 cm in (**D**), and 150 μm in (**E**).

the progression of endocycles, we first quantified the nuclear DNA content of individual trichomes isolated from 25-day-old WT and *pATML1:GFP:GTL1* plants. Compared with the average nuclear DNA content of WT trichomes, the average DNA content of *pATML1:GFP:GTL1* trichomes is strongly reduced (Supplementary Figure S1B), suggesting that excess levels of GTL1 delays the endocycle progression in trichomes. Because of the strong repression of trichome differentiation in *pGL2:GFP:GTL1* plants, we could not isolate trichomes for the quantification of their ploidy. Furthermore, flow cytometric analysis using the first pair of leaves from 21-day-old *pATML1:GFP:GTL1* plants shows an increase in 8C nuclei and reduction in 16C and 32C nuclei (Supplementary Figure S1C). These results establish that GTL1 is not only necessary but is also sufficient to repress ploidy-dependent cell growth and that temporally regulated transcription of GTL1 is one of the key determinants of final cell size in Arabidopsis trichomes.

The GTL1 expression interferes with trichome differentiation

We noticed that some of the leaf epidermal cells in the *pGL2:GFP:GTL1* plants are abnormally enlarged, yet they retain a relatively normal morphology similar to other

epidermal pavement cells (Figure 1B). To examine whether these cells possess trichome cell fate, we introduced a trichome identity marker, *pGL2:GUS*, into *pGL2:GFP:GTL1* plants. As shown in Figure 1C, early expression of GTL1–GFP proteins by the *GL2* promoter leads to a profound reduction of GUS-positive cells in *pGL2:GFP:GTL1* plants. Most of out-grown and unbranched cells are GUS positive while those that are abnormally enlarged but not out-grown are GUS negative although they must have had the *GL2* promoter active to express *GTL1* earlier in development. The impact of GTL1 expression under the *ATML1* promoter is less severe probably because of its weaker promoter activity during trichome development but compared with WT leaves, we also detected less *pGL2:GUS* positive cells in *pATML1:GFP:GTL1* leaves (Figure 1C). These results suggest that overexpression of GTL1 also interferes with full acquisition and/or maintenance of trichome cell fate.

Genome-wide identification of chromatin regions bound by GTL1

To understand how GTL1 regulates cell growth, we first identified its potential direct targets by the chromatin immunoprecipitation followed by the hybridisation on an Affymetrix Arabidopsis Tiling 1.0R array (ChIP-chip). To enrich the genomic region bound by GTL1 *in vivo*, we harvested whole aerial parts of 12-day-old *gtl1-1* plants complemented with the *pGTL:GTL1:GFP* constructs (Breuer *et al*, 2009) and immunoprecipitated the chromatin fragments associated with GTL1–GFP proteins using antibodies against GFP. After applying a cutoff *P*-values of 0.001, we identified a total number of about 3900 putative immediate target genes that showed consistent binding by GTL1 (Supplementary data S1). To assess the false discovery rate of these ChIP-chip experiments, we randomly chose 17 putative targets and subjected to validation by ChIP-qPCR. Out of 17 genes, 13 showed robust and reproducible binding of GTL1 within the predicted binding region (Supplementary Figure S2), suggesting a false discovery rate of <0.25. This is similar to previous studies that reported false discovery rates of 0.2 and 0.3 using the same experimental design (Morohashi and Grotewold, 2009). By examining the GTL1 binding within the 3-kb chromatin region of annotated transcriptional start sites (TSSs), we found that GTL1 preferentially interacts with the target promoter region within the first 0.5 kb upstream of TSSs (Figure 2A). Further gene structure analysis also supported that the binding of GTL1 is statistically enriched for the upstream region of predicted genes compared to 5′UTR, exons, introns and 3′UTR ($P<10^{-5}$; χ^2 test) (Figure 2B). GT transcription factors generally bind to GT elements (GT1 box, 5′-GGTTAA-3′; GT2 box, 5′-GGTAAT-3′; GT3 box, 5′-GGTAAA-3′) (Ni *et al*, 1996) and GTL1 was recently shown to bind to a GT3 box in the promoter of *STOMATA DENSITY AND DISTRIBUTION 1* (*SDD1*) (Yoo *et al*, 2010). Using a promoter element analysis tool MEME (Bailey *et al*, 2009), we found that a highly conserved GT3 box and an inverted partial GT3 box (5′-TTTAC-3′) are over-represented among the sequences identified by ChIP-chip (Figure 2C). Together, these results strongly suggest that the ChIP-chip experiments have been successful to identify potential targets of GTL1.

To investigate the biological function of these putative GTL1 target genes, we used the BiNGO analysis tool (Maere *et al*, 2005) and identified significantly enriched Gene Ontology (GO) categories. Among the enriched GO categories, we found 'negative regulation of cell cycle' ($P<0.01$) and 'DNA integrity checkpoint' are over-represented ($P<0.01$) (Figure 2D), suggesting that GTL1 may directly affect transcription of cell-cycle-related genes. Consistent with the role of GTL1 in trichome development, the enriched GO categories also included 'cell morphogenesis involved in differentiation' ($P<0.01$) and 'trichome morphogenesis' ($P<0.1$). In addition, we noticed that 'terpene metabolic process' ($P<0.01$), 'regulation of amino-acid transport' ($P<0.01$), 'oligosaccharide biosynthetic process' ($P<0.01$) are highly ranked, implying that GTL1 may target diverse biological processes linked to plant development.

Genome-wide identification of GTL1 response genes

Previous studies suggested that among putative direct target genes identified by ChIP-chip, only $<10\%$ respond transcriptionally to the misregulation of bound transcription factors and they include functional relevant targets (Busch *et al*, 2010). We therefore identified GTL1-responsive genes by microarray analysis. The *gtl1-1* mutants exhibit the extended cell growth phenotype at a late stage of trichome development (Figure 3A), thus we reasoned that changes in the expression of direct GTL1 target genes should be most prominent in trichome cells at the same developmental stage. To this end, we extracted total RNA from trichomes of fifth and sixth rosette leaves of 3-week-old WT and *gtl1-1* mutants (Figure 3B). With the significance threshold of $P<0.075$ (Hochberg and Benjamini, 1990), we found a total number of 1694 probes, corresponding to 1759 genes on the ATH1 chip that show differential expression of at least 1.3-fold. Out of these 1759 genes, 47.2% are positively regulated and 52.8% are negatively regulated by GTL1 (Figure 3C; Supplementary data S2). To our surprise, the significantly enriched GO category among positively regulated genes included 'photosynthesis' ($P<10^{-19}$), 'response to red light' ($P<10^{-7}$) and 'response to blue light' ($P<10^{-6}$) (Figure 3D). None of these processes has been previously linked to trichomes but it is worth mentioning that GT2, a close homologue of GTL1, was previously shown to bind to the promoter of *PHYTOCHROME A* (*PHYA*) gene (Kuhn *et al*, 1993; Ni *et al*, 1996). Our ChIP-chip experiments, however, suggest that none of the *PHYA* genes is directly bound by GTL1 (Supplementary data S1 and S2), implying that these transcriptional changes are indirect. Many of the highly ranked GO categories for genes negatively regulated by GTL1 were related to metabolic processes (Figure 3D), probably reflecting the transcriptional response accompanied with extended cell growth of *gtl1* trichomes.

GTL1 binds to the GT3 box within the CCS52A1 promoter

Among the 3900 genes identified by ChIP-chip, 2398 genes were represented on the ATH1 microarray chip used for the transcriptional analysis. By combining the list of these genes and 1759 GTL1-responsive genes, we narrowed down putative direct targets of GTL1 to 182 genes (Figure 4A) that represent about 7.6% of all putative targets. Considering that we used the whole aerial tissue for the ChIP-chip analysis and trichomes only for the microarray analysis, this is a reasonable overlap between these experiments. Out of these 182 genes, 79 genes are positively regulated by

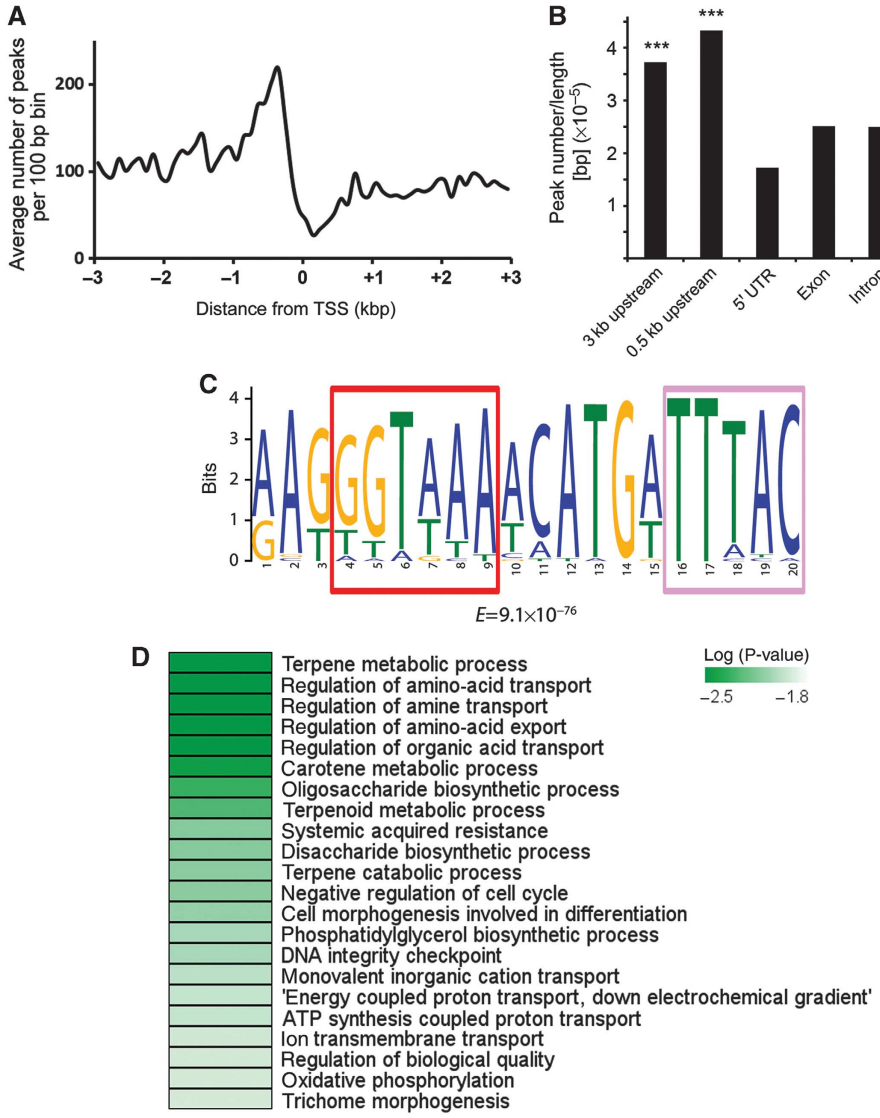

Figure 2 Genome-wide identification of chromatin regions bound by GTL1. (**A**) Distribution of GTL1 binding within the ± 3 kb region of the TSS. (**B**) Peak distribution of GTL1 binding to the structural elements of genes. Peak centres of individual GTL1-binding sites were used to score the distribution. The peak number was normalised by the length of the entire genome for the category of gene structure. $***P<10^{-5}$. The *P*-value was calculated based on the empirical null distribution with the numbers of 1000 permutations of randomly chosen from GTL1-binding peaks. (**C**) Putative GTL1 targets share a common DNA-binding motif that contains a GT3-box (5′-GGTAAA-3′) marked with red frame and a palindromic partial GT3 element (5′-TTTAC-3′) marked with light red frame. (**D**) Representation of the most enriched gene ontology (GO) categories among the putative GTL1 targets identified by ChIP-chip.

GTL1 while 103 genes are negatively regulated, suggesting that GTL1 may act as both a transcriptional activator and repressor. Given that GTL1 impacts on the endocycle, we first asked whether this gene list includes cell-cycle-related genes and found that two genes, *APC10* and an APC/C activator *CCS52A1*, associate with GTL1 and are down- and up-regulated, respectively, in *gtl1-1* (Supplementary data S3). Accumulating evidence suggests that modulation of CCS52A1 levels affects both endoreduplication and cell growth in various cell types (Lammens *et al*, 2008; Larson-Rabin *et al*, 2009; Vanstraelen *et al*, 2009; Kasili *et al*, 2010). In contrast, APC10 has not been directly linked to the endocycle and importantly, down-regulation of APC10 does not affect trichome development in Arabidopsis (Roodbarkelari *et al*, 2010), suggesting that CCS52A1 is a more likely functional target of GTL1 during trichome development. Using the Integrated Genome Browser (IGB), we indeed detected distinct peaks of GTL1 binding in the upstream region of *CCS52A1*, − 1,389 and − 765 base pairs upstream of the TSS. We also examined the upstream sequence of two other *CCS52* homologues, *CCS52A2* and *CCS52B*, but GTL1 seems to interact specifically with the *CCS52A1* promoter because we did not identify any significant signals around the loci of *CCS52A2* and *CCS52B* (Figure 4B).

To validate that GTL1 binds to the promoter sequence of *CCS52A1*, we performed ChIP-qPCR using several gene-specific primer sets. The promoter of *CCS52A1* contains three GT elements, a complete GT3 box at − 200 and − 800 bp, and a partial GT3 box at − 980 bp (Figures 4C and 5A). Using at least three independent biological replicates, we found strongest enrichment of GTL1 binding at the genomic region between − 800 and − 1000 bp (Figure 4C), suggesting that

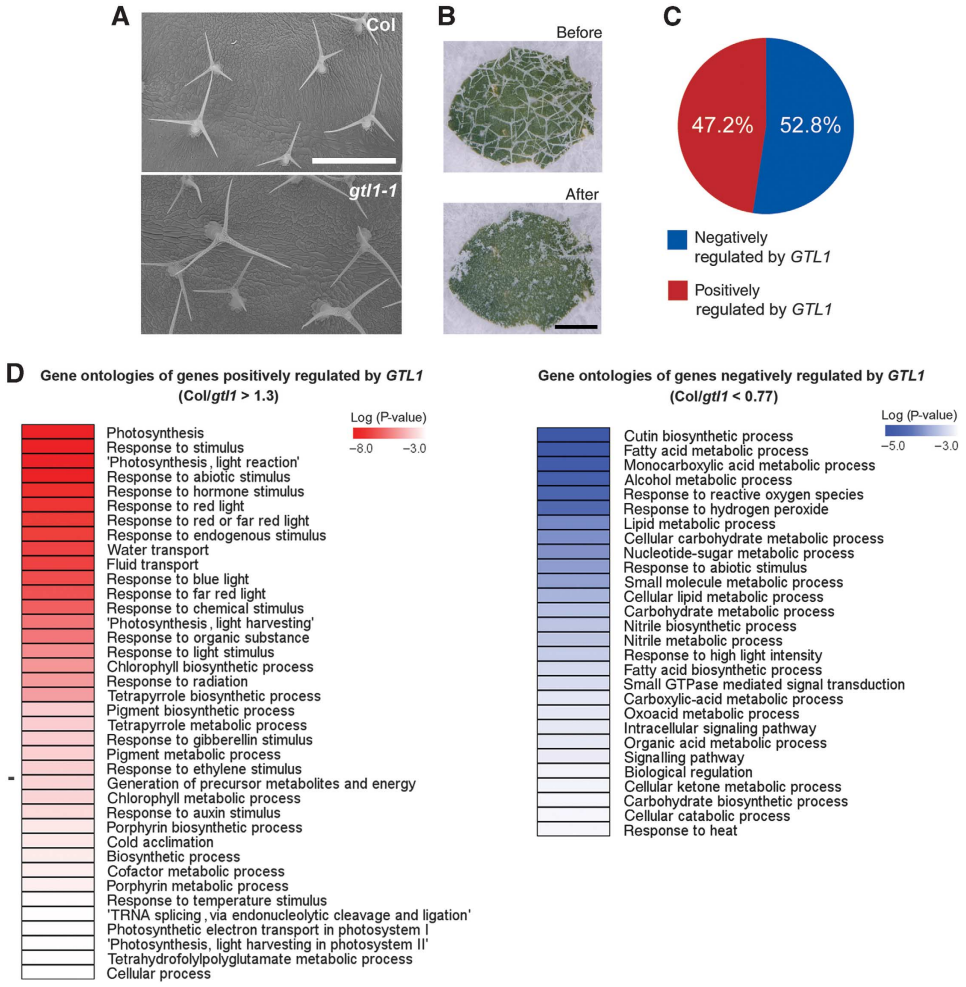

Figure 3 Genome-wide identification of GTL1-responsive genes. (**A**) Scanning electron micrographs of the adaxial epidermis of Col and *gtl1-1* leaves showing enlarged trichomes in *gtl1-1*. (**B**) Light micrographs of *gtl1-1* leaves before and after trichome removal. (**C**) A pie-chart showing the percentage of genes positively or negatively regulated by GTl1. (**D**) GO categories enriched for genes positively or negatively regulated by GTL1. Scale bars: 250 μm in (**A**), 500 μm in (**B**).

GTL1 preferentially binds to a GT3 box at −800 bp and/or −980 bp. To further confirm GTL1 binding to the *CCS52A1* promoter, we also carried out the yeast-one-hybrid assay and found that GTL1, but not GT-1 and GT-3, other trihelix family proteins carrying s single trihelix domain, interacts with a 1.5-kb fragment of the *CCS52A1* promoter in yeast cells (Figure 5B). To test whether any of the GT3 boxes is responsible for the GTL1 binding, we subsequently substituted nucleotides in the −800 bp box (5′-GGTAAAT-3′ to 5′-GATTCAG-3′) or the −980 bp box (5′-TTTAC-3′ to 5′-GCACG-3′) (Figure 5A). As shown in Figure 5B, nucleotide substitutions in the −980 bp box completely abolishes the GTL1 binding whereas mutations in the −800 box do not interfere with the binding. These results establish that GTL1 binds to the promoter sequence of the *CCS52A1* gene through preferential interaction with the GT3 box at −980 bp.

GTL1 represses the CCS52A1 expression at a late stage of trichome development

Our trichome-specific microarray data suggest that GTL1 negatively regulates the expression of *CCS52A1* (Supplementary data S2). To confirm this, we isolated trichomes from fourth and fifth leaves of WT and *gtl1-1* plants, and performed quantitative RT–PCR experiments using their total RNA. As shown in Figure 4D, the level of *CCS52A1* transcripts is significantly up-regulated in *gtl1-1* trichomes, supporting the view that GTL1 acts as a transcriptional repressor of *CCS52A1*. In our previous study we also recognised the elevated expression of *CCS52A2* in *gtl1-1* trichomes (Breuer *et al*, 2009) but *CCS52A2* was neither identified by ChIP-chip nor detected by the microarray experiment in this study.

The *GTL1* gene is not expressed at an early stage of trichome development when cells are still undergoing branching and its transcription starts while cells reach their maximum size (Figure 6A). We therefore hypothesised that this temporal transcription of GTL1 may result in the down-regulation of *CCS52A1*, and hence the cessation of endocycling, at a late stage of trichome development. To resolve the temporal activity of the *CCS52A1* promoter during trichome development, we generated a construct that drives the expression of nuclear targeted histone 2B (H2B) fused with GFP under the 1.5-kb *CCS52A1* promoter and introduced them into plants heterozygous for *gtl1-1*. We subsequently identified T2 plants homozygous for the *pCCS52A1:H2B:GFP* construct and heterozygous for *gtl1-1*, and assessed the expression of H2B:GFP fusion proteins in WT and *gtl1-1*

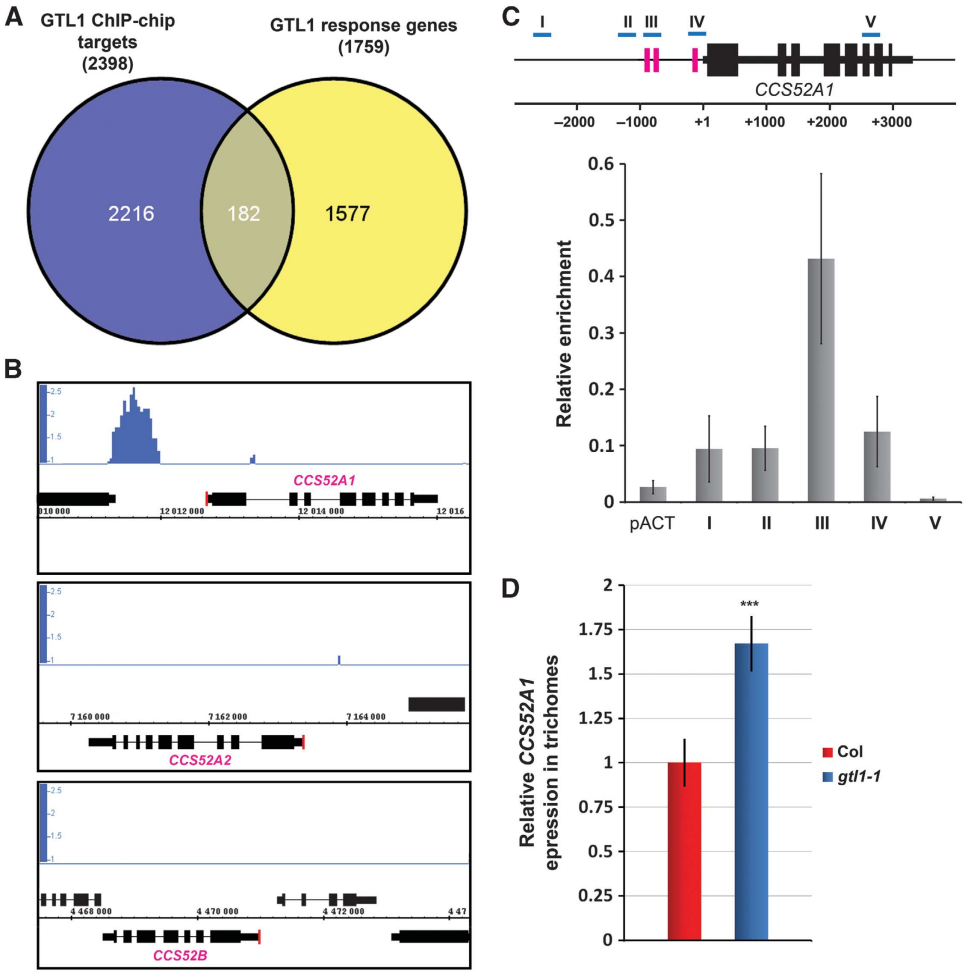

Figure 4 GTL1 directly binds to the promoter of the *CCS52A1* gene and represses its transcription. (**A**) Venn diagram analysis identifies 182 genes as a potential target of GTL1. (**B**) The IGB analysis of the *CCS52A1*, *CCS52A2* and *CCS52B* loci shows a clear signal enrichment in the 5′-region upstream of the *CCS52A1* open reading frame. Red lines mark the TSS. (**C**) (Upper panel) Schematic representation of the *CCS52A1* locus highlighting the position of GT3 boxes (pink) and primer sets used for ChIP-qPCR (I–V, blue). (Lower panel) ChIP-qPCR analysis reveals the strongest association of GTL1–GFP fusion proteins in the −850 bp region of the 5′UTR. The IP experiments were repeated three times with independent plant samples and the average enrichment of qPCR products from immunoprecipitated DNA is normalised against the corresponding input DNA. pACT shows signal enrichment in the promoter of actin (At3g18780) and error bars represent ±s.d. of the means ($n=3$). (**D**) Relative expression of *CCS52A1* in trichomes of fifth and sixth leaves from 17-day-old Col and *gtl1-1* plants. Errors represent ±s.d. of the means ($n=3$). Asterisks indicate a significant difference between Col and *gtl1-1* (Student's *t*-test, $P<0.009$).

mutants segregating at T3 generation. Our fluorescence microscopy analysis reveals that WT plants carrying *pCCS52A1:H2B:GFP* display clear nuclear accumulation of H2B–GFP proteins in young trichomes that have not completed the second branching while these signals are no longer detectable in older trichomes (Figure 6B). We detected background fluorescence signals in old trichomes but the nuclei we identified by bright-field microscopy never emitted the GFP fluorescence. In sharp contrast to this, we found that the nuclear expression of H2B:GFP proteins is extended into the late stage of development in *gtl1-1* (Figure 6B), indicating that loss of GTL1 allows prolonged activity of the *CCS52A1* promoter *in vivo*. These results confirm that GTL1 directly represses the *CCS52A1* expression in trichome cells.

CCS52A1 mediates ploidy-dependent cell growth governed by GTL1 but not ploidy-independent cell growth

The function of CCS52A1 as a positive regulator of the endocycle progression during early trichome development has been recently described (Larson-Rabin *et al*, 2009; Kasili *et al*, 2010; Heyman *et al*, 2011). Compared with WT, *ccs52a1* mutants develop smaller, under-branched and under-endoreduplicated trichomes while overexpression of *CCS52A1* under the *GL2* promoter leads to larger, overbranched and over-endoreduplicated trichomes. To test whether the prolonged expression of *CCS52A1* is responsible for the extended ploidy-dependent cell growth of *gtl1-1*, we generated *gtl1-1 ccs52a1-2* double mutants. As shown in Figure 7A, trichome cells in *gtl1-1 ccs52a1-2* plants are clearly smaller than those of WT or *gtl1-1* mutants and most of them are under-branched, similar to those in *ccs52a1-2* mutants. We quantified the average number of branching per trichomes and found that the *ccs52a1-2* mutation is indeed epistatic to *gtl1-1* for the trichome branching phenotype (Supplementary Figure S3). Consistently, the average ploidy level of *gtl1-1 ccs52a1-2* trichome nuclei is identical to that of *ccs52a1-2* (Figure 7B), indicating that the *ccs52a1-2* mutation completely suppresses the trichome ploidy phenotype of *gtl1-1*. Interestingly, however, a thorough analysis of

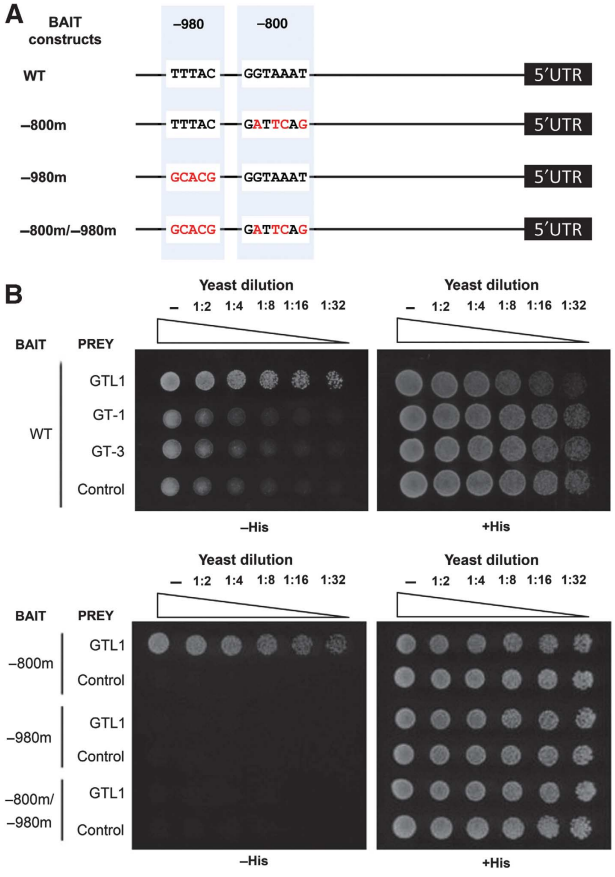

Figure 5 GTL1 binds to the GT3 box within the *CCS52A1* promoter. (**A**) Schematic representation of the bait constructs illustrating the two putative GT3 boxes, one at −980 bp and other at −800 bp upstream of 5′UTR, in the WT *CCS52A1* promoter. Substituted nucleotides are highlighted in red. (**B**) (Upper panel) Yeast cells transformed with the GAL4-fusions of trihelix transcription factor constructs (GTL1, GT-1, GT-3) and empty vector control were diluted and spotted on plates without (−His, +5 mM 3-AT, left) or with histidine (+His, right). GTL1, but neither GT-1 nor GT-3, binds to the *CCS52A1* promoter. (Lower panel) The same assay was carried out with the bait constructs harbouring the mutated *CCS52A1* promoter. Only the mutations in the GT3 box at −980 bp abolish the binding of GTL1 to the *CCS52A1* promoter.

trichome cell size, as represented by the length of individual branches, revealed that trichome cells in *gtl1-1 ccs52a1-2* are significantly larger than those in *ccs52a1-2* single mutants (Figure 7A and C), suggesting that the *ccs52a1-2* mutation does not completely suppress the cell growth phenotype of *gtl1-1*. These observations provide strong genetic evidence that CCS52A1 is a central regulator of the endocycle and cell growth acting directly downstream of GTL1. Our data also uncouple ploidy-dependent cell growth from ploidy-independent growth and predict the existence of additional targets acting downstream of GTL1 to control ploidy-independent cell growth.

Discussion

How cell size is controlled in multicellular organisms is an important question in biology but molecular mechanisms underlying this control remain poorly understood. Previous studies uncovered many regulators that promote cell growth but whether an active regulatory mechanism operates to cease cell growth was not known before. In this study we demonstrate that plants possess a genetic programme to actively terminate cell growth and that this is largely mediated through the transcriptional repression of *CDC20/FZR/CCS52*, an evolutionally conserved regulator of the endocycle. An involvement of the endocycle in cell-size control has been long debated but this study establishes that an increase in ploidy by the endocycle has major impacts on cell growth.

Developmental control of cell growth and cell differentiation

This study shows that a trihelix transcription factor GTL1 directly represses the endocycle and its developmentally programmed expression is one of the key determinants of final cell size in plants. Our model predicts that developmentally controlled expression of *GTL1* shuts down the transcription of *CCS52A1*, leading to the reduced activity of APC/C and thus cessation of the endocycle (Figure 8). While this CCS52A1-dependent pathway fully accounts for ploidy-dependent cell growth controlled by GTL1, our data hint that GTL1 also represses other pathways mediating ploidy-independent cell growth. An involvement of endoreduplication in the control of cell size has long been debated (Sugimoto-Shirasu and Roberts, 2003; Breuer *et al*, 2010) and the existence of ploidy-independent growth was previously proposed for trichome cells (Schnittger *et al*, 2003). Our results are consistent with this idea and strongly suggest that both ploidy-dependent and -independent cell growth contribute to the overall increase in cell size. We do not know the nature of other GTL1 targets acting in the ploidy-independent pathway but the remaining 181 genes identified by ChIP-chip and microarray analyses are the best candidates. Further validation of ChIP-chip results coupled with functional analyses of candidate genes should uncover how GTL1 coordinates ploidy-dependent and ploidy-independent cell growth.

The next important question is whether the same mechanism also governs general plant cell growth. Given that GTL1 is expressed in various cell types, for example, in leaves and roots, undergoing rapid cell expansion (Breuer *et al*, 2009; Yoo *et al*, 2010), we think this is very likely. Indeed our data show that overexpression of GTL1 represses growth of non-trichome leaf epidermal cells. Expressing a repressor of cell growth in expanding cells sounds counterintuitive but we speculate that such a mechanism may allow adjusting the pace and duration of cell growth in the context of development and/or in response to the environmental change. Although developmental defects of *gtl1* mutants are restricted to trichomes under our standard growth conditions, lack of other phenotypes might be due to the functional redundancies among other closely related GT-2 homologues. We should note that *gtl1* mutant trichomes are larger than those in WT but they do stop growing, hence additional mechanisms may repress further cell growth. Systematic identification of new mutations that permit extended growth should help uncovering other pathways involved in the repression of plant cell growth.

Our overexpression studies imply that GTL1 may also interfere with trichome cell differentiation either directly or indirectly. Our ChIP-chip experiment identified 10 putative GTL1 targets implicated in the control of trichome

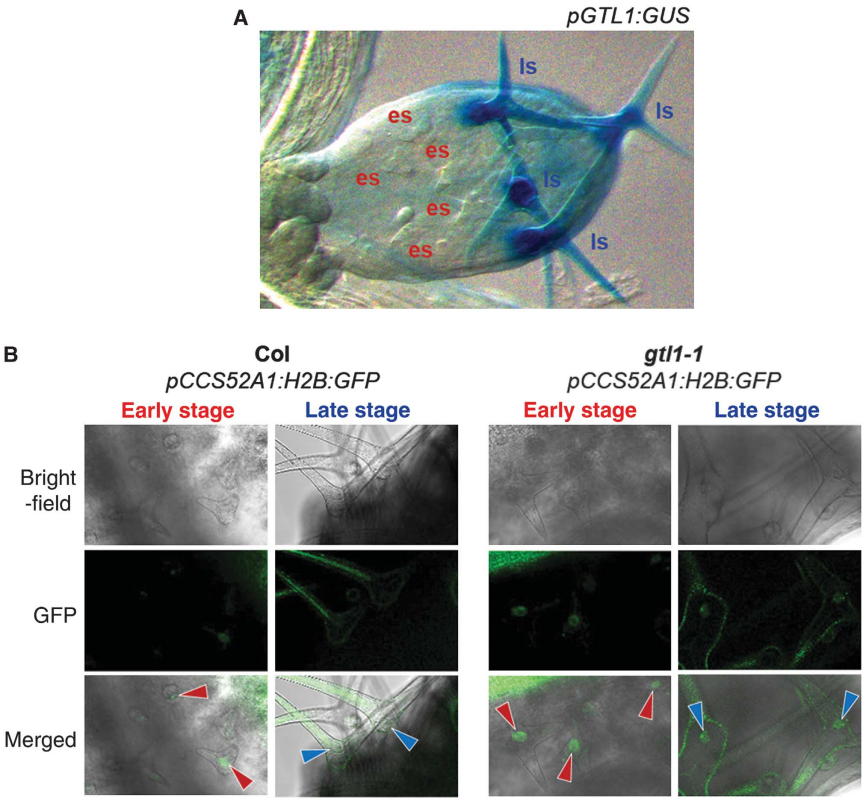

Figure 6 GTL1 represses the activity of the *CCS52A1* promoter in maturing trichomes. (**A**) The *pGTL1:GUS* reporter shows that the *GTL1* promoter activity is not detected at an early stage (es) of trichome development and it is only found during the late stage (ls) when trichome cells reach their maximum size. (**B**) The representative expression of *pCCS52A1:H2B:GFP* markers in trichomes from sixth leaves of 15-day-old Col and *gtl1-1* plants. Red and blue arrows mark trichome nuclei during early and late stage of development, respectively. Nuclear accumulation of H2B:GFP proteins is detected only at an early stage in Col but is extended to maturing trichomes in *gtl1-1*. Broad green signals found in the cytoplasm derive from autofluorecence in trichomes. Scale bars: 150 μm in (**A**), 50 μm in (**B**).

morphogenesis but none of them is directly linked to the acquisition and/or maintenance of trichome identities. Although GTL1 may target some uncharacterised genes functioning in these processes, an alternative hypothesis is that GTL1 indirectly influences trichome differentiation through the modulation of the endocycle. It is indeed shown that misregulation of endocycles has immediate consequences on trichome differentiation (Bramsiepe *et al*, 2010), thus it might be possible that the reduced level of endocycles in *pGL2:GFP:GTL1* trichomes is inhibitory to trichome cell differentiation.

Transcriptional control of the endocycle

Orchestrating the progression of the mitotic cycle and endocycle in space and time is vital for the optimum growth and development of multicellular organisms. In many plant species, cells proliferate through the mitotic cycle in the meristem and as soon as cells leave the meristem, they switch from the mitotic cycle to the endocycle. Temporal transcription of cell-cycle regulators is one core mechanism underpinning the timely mitotic-to-endocycle transition but how this is regulated is not well understood. Previous studies suggest that FZR/CDH1/CCS52 is a key regulator of this transition and that its expression is under the control of both typical and atypical E2F transcription factors (Lammens *et al*, 2008; Heyman *et al*, 2011; Magyar *et al*, 2012). In the Arabidopsis shoot meristem, an atypical E2F protein DEL1 prevents the premature transition into the endocycle by repressing the expression of *CCS52A2* (Lammens *et al*, 2008). In addition, a recent study shows that the E2FA–RBR complex represses the expression of both *CCS52A1* and *CCS52A2* in proliferating cells to inhibit an entry into the endocycle (Magyar *et al*, 2012). How the progression of consecutive endocycles is controlled is even less clear and an important discovery of this study is that at least in Arabidopsis the endocycle needs to be actively terminated through the transcriptional repression of *CCS52A1*. Trihelix transcriptional regulators are specific to plants and whether similar mechanisms operate in non-plant species is not currently known. In Drosophila, FZR/CDH1/CCS52 proteins control the mitotic-to-endocycle transition and the APC/C activity is necessary to sustain the endocycle progression (Sigrist and Lehner, 1997). Thus, it would not be surprising if transcriptional down-regulation of core APC/C submits or FZR/CDH1/CCS52 also triggers the endocycle cessation in Drosophila.

This study also highlights an involvement of transcription factors other than typical and atypical E2Fs in controlling the plant endocycle. Several developmental regulators are implicated in the transcriptional control of mitotic cycle genes (Sozzani *et al*, 2010; Xie *et al*, 2010; Vanneste *et al*, 2011) and our data extend this to the endocycle. Non-E2F transcription factors also regulate the endocycle initiation and maintenance in other organisms. For example, a Drosophila zinc-finger transcription factor escargot and its mouse homologue mSna prevent an entry into the endocycle and they are

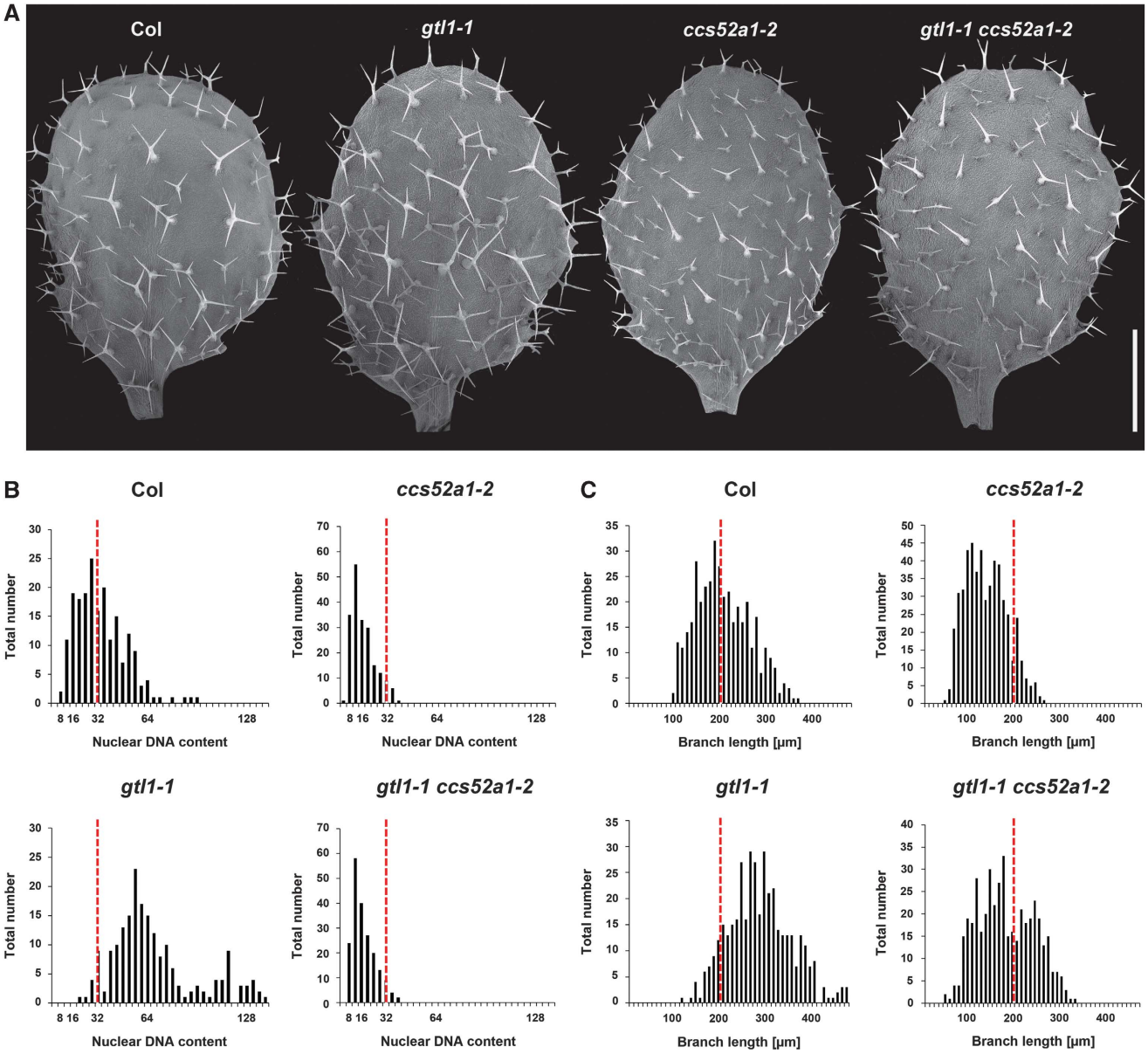

Figure 7 CCS52A1 functions downstream of GTL1 to mediate ploidy-dependent cell growth. (**A**) Scanning electron micrographs of adaxial epidermis from third leaves of 12-day-old Col, *gtl1-1*, *ccs52a1-2* and *gtl1-1 ccs52a1-2* double mutants. (**B**) Quantification of trichome nuclear DNA contents isolated from third and fourth leaves of 22-day-old plants. Values on the *y*-axis represent the number of trichomes counted for a given DNA content level. All ploidy measurements were normalised to the mean of 32C in WT trichome. (**C**) Quantification of the trichome branch lengths of the corresponding trichomes. Red lines in (**B**, **C**) represent the mean values of WT. Scale bar: 750 μm in (**A**).

required to maintain the Cdk1 activity (Hayashi, 1996; Nakayama *et al*, 1998). A basic helix-loop-helix transcription factor Hand1 promotes endoreduplication although its exact molecular function is not yet known (Martindill and Riley, 2008). Based on our overexpression studies, we speculate that GTL1 may also control earlier progression of endocycles in non-trichome cells potentially by modulating the *CCS52A1* expression. How E2F and non-E2F transcription factors cooperate during development for the spatio-temporal control of endocycles is an interesting question and should be further investigated in future studies.

Functional roles of the APC/C in post-mitotic cells

Recent studies are beginning to uncover previously uncharacterised roles of the APC/C in post-mitotic cells (Eguren *et al*, 2011). Several lines of evidence suggest an involvement of the APC/C in plant cell differentiation (Komaki and Sugimoto, 2012) and our data substantiate that sustained APC/C activity is the prerequisite for post-mitotic cell growth. Interestingly, previously reported *APC10* or *APC11* RNAi lines do not cause any defects in trichome cell growth and endocycle, proposing that at least those APC/C components are not required for the endocycle regulation in trichomes (Roodbarkelari *et al*, 2010). Loss- and gain-of-function lines of *CDH1/FZR/CCS52*, however, affect the endocycle progression in both animals and plants (Narbonne-Reveau *et al*, 2008; Larson-Rabin *et al*, 2009; Kasili *et al*, 2010; Heyman *et al*, 2011). Our results agree with these observations and support an involvement of the APC/C in the endocycle progression.

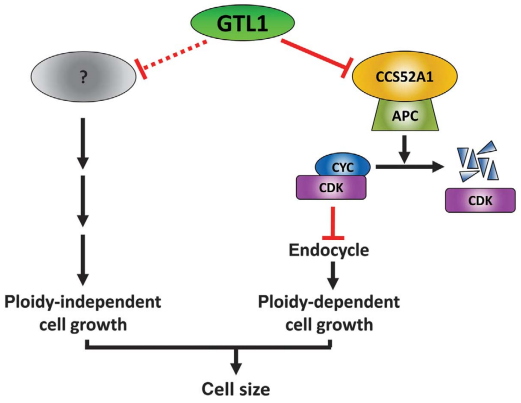

Figure 8 A schematic model illustrating how GTL1 regulates ploidy-dependent and -independent cell growth. During late trichome development, GTL1 represses the *CCS52A1* expression, resulting in a decline of the APC/C activity. Reduced APC/C activity then leads to stabilisation of active CYC/CDK complexes, which blocks further progression of the endocycle. This active repression of ploidy-dependent cell growth is the major pathway that determines final size of trichome hair cells. In addition, GTL1 is likely to act as a negative regulator of ploidy-independent cell growth by repressing the expression of other unknown genes.

The targets of the $APC^{CCS52A1}$ in trichome cells is not established but one good candidate is CYCA2;3 which together with CDKA;1, is required to terminate the endocycle in trichomes (Imai *et al*, 2006). In Arabidopsis roots, $APC^{CCS52A1}$ promotes the degradation of CYCA2;3, leading to the reduced activity of CYCA2;3–CDKB1;1 and thus an endocycle onset (Boudolf *et al*, 2009). If a similar mechanism operates in trichomes, the repression of $APC^{CCS52A1}$ towards the end of cell growth may increase the CYCA2;3–CDKA;1 activity which in turn triggers the endocycle cessation. According to this scenario, the repression of $APC^{CCS52A1}$ is lifted in *gtl1* trichomes, thus delaying the accumulation of CYCA2;3–CDKA;1 to terminate the endocycle.

Materials and methods

Plant material and growth conditions

The *gtl1-1*, *ccs52a1-2* and *pGL2:GUS* lines were previously described (Masucci *et al*, 1996; Lammens *et al*, 2008; Breuer *et al*, 2009). Plants were germinated and grown either on plates containing Murashige and Skoog salts, pH 5.7, 1% sucrose (w/v), and 0.5% phytogel (w/v) or on a 1:1 mixture of soil Supermix A (SAKATA, Japan) and vermiculite (VS Kako, Japan) in continuous light at 22°C.

Construction of binary plasmids for Arabidopsis transformation

The generation of *gtl1-1* plants carrying the *pGTL1:GTL1:GFP* constructs was described previously (Breuer *et al*, 2009). For the construction of the *pCCS52A1:H2B:GFP* plasmid, a 1977-bp fragment of the *CCS52A1* promoter was amplified with the oligonucleotides *attB4-proCCS52A1-Fw* 5′-GGGGACAACTTTGTATAGAAAAGTTGAAGAACTCGTCAGTCTTGTG-3′ and *attB1r-proCCS52A1-Rv* 5′-GGGGACTGCTTTTTTGTACAAACTTGCATTGGTTTTTTTTTTTGTTGACT-3′, and cloned into the vector pDONR-P4P1R by Gateway BP clonase II enzyme reaction (Invitrogen, Japan). Histone 2B cDNA was amplified from Arabidopsis cDNA derived from whole seedlings with the oligonucleotides *H2B-TOPO-F* 5′-CACCATGGCGAAGGCAGATAAGAAACC-3′ and *H2B-ΔSTOP-R* 5′-AGAACTCGTAAACTTCGTAACCGCC-3′ and cloned into pENTR-D-TOPO (Invitrogen, Japan). The CCS52A1 promoter and the H2B cDNA fragments were transferred into the R4pGWB504 destination vector by LR clonase II enzyme (Invitrogen, Japan) in a one-step tripartite cloning reaction (Nakagawa *et al*, 2008). Both *pATML1:GFP:GTL1* and *pGL2:GFP:GTL1* were generated by infusion into a previously generated pENTR-based *pGTL1:GFP:GTL1* construct (Breuer *et al*, 2009). First, the *pGTL1:GFP:GTL1* plasmid was amplified using the oligonucleotides *GFP–GTL1-INF-R1* 5′-AAGGGTGGGCGCGCCGACCCAG-3′ and *GFP–GTL1-INF-F1* 5′-ATGCCCGGGGGTGGCATGG-3′ and digested with *Dpn*I (Toyobo, Japan). For the infusion reaction a 3384-bp genomic fragment of the *ATML1* promoter and a 1748-bp genomic fragment of the *GL2* promoter were amplified from the BAC clones F17L22 and F19K16 using the oligonucleotide pair *ATML1p-F1* 5′-CGGCGCGCCCACCCTTAAGCTTATCAAAGAA-3′ and *ATML1p-R1* 5′-CCATGCCACCCCCGGGCATAACCGGTGGATTCAGGG-3′ and the pair *GL2p-F1* 5′-CGGCGCGCCCACCCTTACAATTCCCTAGGCCGTACGACG-3′ and *GL2p-R1* 5′-CCATGCCACCCCCGGGCATACAAATCCTGTCCCTAG-3′, respectively. The promoter fragments were then cloned into the amplified GFP:GTL1 backbone using the In-fusion Advantage PCR Cloning Kit (TAKARA, Japan). The fused fragments were then cloned by LR reaction into the binary vector pGWB1 (Nakagawa *et al*, 2007). All stable plant transformations were performed via floral dip (Clough and Bent, 1998).

RNA extraction and microarray analysis

Trichomes were removed with forceps from the fifth and sixth rosette leaves of 3-week-old WT and *gtl1-1* mutant plants according to a previously established protocol (Jakoby *et al*, 2008). Total RNA was extracted from three biological replicates by a Qiagen plant RNeasy kit, and treated with DNaseI (Takara Bio, Japan) following the manufacturer's instructions. RNA probes were labeled using an Affymetrix 3′ IVT Express Kit (Affymetrix, Japan) and hybridised on ATH1 chips. Microarray data analysis was performed using the R software with AffylmGUI of the Bioconductor package (Wettenhall *et al*, 2006). The expression ratio of WT versus *gtl1-1* mutants were subjected to the Benjamini Hochberg method (Hochberg and Benjamini, 1990), and statistical significance was considered with $P<0.075$, which is equivalent to an FDR <0.075.

Quantitative PCR

Total RNA from dissected trichomes was treated with DNaseI (Takara Bio, Japan) and reverse transcribed using the Invitrogen Superscript III kit. Transcript levels were determined by quantitative real-time PCR using the Toyobo SYBR Green Supermix kit and an Agilent Mx3000P QPCR system. The oligonucleotide pairs of *qCCS52A1-F2* 5′-CACGCTGCAAGAGAACAAGA-3′, *qCCS52A1-R1* 5′-ACCACTTGAGTCCGCATACC-3′ and of *ACT2F* 5′-CTGGATCGGTGGTTCCATTC-3′, *ACT2R* 5′-CCTGGACCTGCCTCATCATAC-3′ were used for PCR reaction to quantify the *CCS52A1* and *ACT2* expression, respectively.

ChIP-chip and ChIP-qPCR experiments

Whole aerial tissues of 2-week-old *gtl1-1* plants carrying *pGTL1:GTL1:GFP* were used for ChIP experiments as previously described (Morohashi *et al*, 2007; Morohashi and Grotewold, 2009). For amplification of precipitated and input DNA, the GenomePlex Whole Genome Amplification Kit (Sigma) was used. DNA fragmentation, labeling, hybridisation, washes and detection were performed following the Affymetrix 100K protocol (http://www.affymetrix.com/products/arrays/specific/100k.affx). CEL files were further analysed by MAT (Model-based Analysis of Tiling array; http://chip.dfci.harvard.edu/wli/MAT/) using the following parameters: BandWith = 300, MaxGap = 300, MinProbe = 10 and *P*-value = 0.001. Peaks consisting of continuous probes with significant MAT scores were evaluated using IGB (IGB, Affymetrix) with the additional criteria that the minimum gap should be <100 bp. We defined target genes as those for which 3 kb upstream regions contained at least one peak showing significant MAT scores (Morohashi and Grotewold, 2009). Sequences corresponding to peaks (FDR<0.1) were subjected to MEME (meme-maxsize 120000-nmotifs 5-minw 6-maxw 20). Computational analyses were performed using R (http://www.r-project.org/), BedTool (http://code.google.com/p/bedtools/) and custom made Perl scripts, which will be provided upon request.

ChIP-qPCR experiments were performed using immunoprecipitated and input DNA of WT and *gtl1-1* plants carrying *pGTL1:GTL1:GFP* as templates. Three independent ChIP replicates were used as material for the validation of targets by ChIP-chip. The qPCR reactions were run using an iQ SYBR Green Supermix

(Bio-Rad) in an iCycler thermocycler. Oligonucleotides used for individual genes are listed in Supplementary Table S1.

Yeast-one-hybrid assay

The *CCS52A1* promoter, containing 1490 bp fragment upstream of the start codon, was amplified with the oligonucleotides CCS52A1-1490F1 5′-ATGGCGGCCGCTTATCATTTGTTTTCTGATT-3′ and CCS 52A1-1490R1 5′-ATGGCGGCCGCTGGTTTTTTTTTTTTGTTGACT-3′, and cloned into the pINT1-HIS3NB vector (Lopato *et al*, 2006) resulting in the bait vector pINT1-HIS3-CCS52A1 that harbours a transcriptional fusion between the *CCS52A1* promoter and the *HIS3* gene. For making the yeast reporter strain, the reporter vector was introduced into the yeast strain AH109 (*MATa; trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ*, gal80*Δ*, LYS2::GAL1UAS-GAL1TATA-HIS3, MEL1, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ*; Clontech) and incubated at 30°C for 4 days. The open reading frame of *GTL1*, *GT-1*, and *GT-3* was fused to the GAL4 activation domain in the vector pGADT7 (Clontech) and transformed into the reporter yeast strain. Transformed yeast strains were gown to an $OD_{E600} = 0.1$ and spotted on plates in a dilution series of 1:2, 1:4, 1:8, 1:16 and 1:32 with or without histidine (+5 mM 3-AT). Transformation and selection of yeast were carried out according to the Yeast Protocol Handbook (Clontech).

To generate nucleotide substitutions in a GT3 box at the −800 or −980 bp regions of the *CCS52A1* promoter, the following oligonucleotide pairs were used to amplify the pINT1-HIS3-CCS52A1: -800mF1 5′-TTTTGATTCAGTTTCTTTTATATCTTTGATATT-3′, -800mR1 5′-AAGA AACTGAATCAAAAAAATATTCAAAAAATAATCC-3′ and -980mF1 5′-TC AATTGCACGTCATTTTAGTAAGGTTTTGT-3′, -980mR1 5′-AAAATGACG TGCAATTGATTGCAAGTTGCAATG-3′, respectively. The resulting PCR products were digested with *Dpn*I and propagated in *Escherichia coli* DH10B to produce plasmids we named pINT1-HIS3-CCS52A1-800m and pINT1-HIS3-CCS52A1-980m. For the construction of the bait construct pINT1-HIS3-CCS52A1-800m/-980m harbouring substitutions in both GT3 boxes, the pINT1-HIS3-CCS52A1-800m plasmid was amplified with the oligonucleotide pair -980mF1 and -980mR1, digested with *Dpn*I and propagated in *E. coli* DH10B.

Microscopy techniques

Confocal microscopy of young true leaves was carried out with a Zeiss 510 Meta confocal microscope. Recorded pictures were converted to JPEG format using the Zeiss LSM Image Browser version 4.0.0.157. For scanning electron microscopy of the adaxial epidermis of true leaves, a tabletop Hitachi T-1000 scanning electron microscope was used. Expression of the translational GFP-fusions in *pATML1:GFP:GTL1* and *pGL2:GFP:GTL1* lines was examined using a Leica MZ 16 FA dissection microscope equipped with a digital Leica DFC 500 camera. Contrast and brightness were adjusted with Adobe Photoshop CS3 version 10.0.

Ploidy measurements, histological tissue clearing and GUS staining

Nuclear ploidy level of whole leaves was measured with a ploidy analyser PA-I (Partec) as previously described (Sugimoto-Shirasu *et al*, 2002). To quantify ploidy levels of trichome nuclei, trichome cells were isolated from leaves (Marks *et al*, 2008) and their nuclei were stained with DAPI. DAPI-stained nuclei were visualised using an Olympus BX51 fluorescence microscope equipped with a digital Olympus DP70 camera and Olympus DP Manager software version 1.2.1.107 (Zhang and Oppenheimer, 2004), and fluorescent signals were measured with ImageJ version 1.37 as previously described (Schnittger *et al*, 1998). The average ploidy level of WT trichomes was set to 32C to normalise the ploidy level of mutant trichomes.

To quantify the area of epidermal pavement cells, fully expanded first true leaves were fixed overnight in glacial acetic acid: ethanol (1:1) at room temperature. The fixed plant material was de-hydrated by an ascending ethanol series of 30, 50, 75, 85 and 95% ethanol for 20 min in each concentration, and cleared in chloral hydrate: H_2O: glycerol (8:2:1) overnight at 4°C. The cleared tissue was unfurled on microscopy slides in clearing solution and used for differential interference contrast (DIC) microscopy using an Olympus BX51. ImageJ version 1.37 was used to quantify the cell area of individual epidermal pavement cells. GUS staining in young seedlings was performed as previously described (Breuer *et al*, 2009).

Accession numbers

Sequence data of genes from this publication can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers*: GTL1*, At1g33240; *ATML1*, At4g21750; *GL2*, At1g79840; *CCS52A1/FZR2*, At4g22910; *CCS52A2/FZR1*, At4g11920; *CCS52B/FZR3*, At5g13840. The microarray data from this publication have been submitted to the NCBI GEO database [http://www.ncbi.nlm.nih.gov/geo/] and assigned the accession number GSE40520.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: CB and KS conceived and designed the experiments. CB and AK performed most of the experiments at RIKEN. KM and EG carried out the ChIP-chip, ChIP-qPCR experiments and the computational analyses at OSU. NT and MU performed the yeast-one-hybrid assay at NAIST. TI contributed the *pCCS52A1:H2B:GFP* line. CB and KS wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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