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Kotak, Jenna; Saisana, Marina; Gegas, Vasilis ; Pechlivani, Nikoletta ; Kaldis, Anthanasios; Papoutsoglou, Panagiotis; Makris, Athanasios; Burns, Julia; Kendig, Ashley L.; Sheikh, Minna; Kuschner, Cyrus E.; Whitney, Gabrielle; Caiola, Hanna; Doonan, John; Vlachonasios, Konstantinos; McCain, Elizabeth R.; Hark, Amy T.

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# The Histone Acetyltransferase GCN5 and the Transcriptional Coactivator ADA2b Affect Leaf Development and Trichome Morphogenesis in *Arabidopsis*

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## Main conclusion:

The histone acetyltransferase GCN5 and associated transcriptional coactivator ADA2b are required to couple endoreduplication and trichome branching. Mutation of *ADA2b* also disrupts the relationship between ploidy and leaf cell size.

**Keywords:** endoreduplication, epigenetics, chromatin, histone acetyltransferase

## ABSTRACT

Dynamic chromatin structure has been established as a general mechanism by which gene function is temporally and spatially regulated, but specific chromatin modifier function is less well understood. To address this question, we have investigated the role of the histone acetyltransferase GCN5 and the associated coactivator ADA2b in developmental events in *Arabidopsis thaliana*. *Arabidopsis* plants with T-DNA insertions in *GCN5* (also known as *HAG1*) or *ADA2b* (also known as *PROPORZI*) display pleiotropic phenotypes including dwarfism and floral defects affecting fertility. We undertook a detailed characterization of *gcn5* and *ada2b* phenotypic effects in rosette leaves and trichomes to establish a role for epigenetic control in these developmental processes. ADA2b and GCN5 play specific roles in leaf tissue, affecting cell growth and division in rosette leaves often in complex and even opposite directions. Leaves of *gcn5* plants display overall reduced ploidy levels,

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while *ada2b-1* leaves show increased ploidy. Endoreduplication leading to increased ploidy is also known to contribute to normal trichome morphogenesis. We demonstrate that *gcn5* and *ada2b* mutants display alterations in the number and patterning of trichome branches, with *ada2b-1* and *gcn5-1* trichomes being significantly less branched while *gcn5-6* trichomes show increased branching. Elongation of the trichome stalk and branches also vary in different mutant backgrounds, with stalk length having an inverse relationship with branch number. Taken together, our data indicate that in *Arabidopsis* leaves and trichomes ADA2b and GCN5 are required to couple nuclear content with cell growth and morphogenesis.

## INTRODUCTION

In eukaryotic genomes, DNA is wrapped around octamers of histone proteins and the resulting nucleosomes are further folded into higher order chromatin structures. The state of chromatin packaging is controlled during growth and development and can regulate the temporal and spatial expression of genes (Jarillo et al. 2009; Pfluger and Wagner 2007; Reyes 2006). One mode of control is through the covalent modification of histone proteins by the addition of acetyl groups, usually at the histone's N-terminal domain (Chen and Tian 2007). Histone acetylation is controlled by transcriptional coactivator complexes including a histone acetyltransferase (HAT). The well-characterized histone acetyltransferase GCN5 physically interacts with the transcriptional adaptor protein ADA2; the essential nature of their interaction is indicated by the lack of acetylation when ADA2 is absent (Candau et al. 1997). In metazoans, it has been shown that GCN5 and ADA2b are both members of SAGA and SAGA-like (SLIK, STAGA) complexes and there is also evidence for the presence of GCN5 in a separable ATAC complex (Spedale et al. 2012). While biochemical or structural evidence has not been used to define these transcriptional coactivator complexes in plants, a SAGA complex(es) in which both GCN5 and ADA2b would function together has been predicted (Srivastava et al. 2015). Our previous work suggests that GCN5 and ADA2b also play separable roles and that the paralog ADA2a has minor phenotypic impact in *Arabidopsis* (Hark et al. 2009; Vlachonasios et al. 2003).

Both the *GCN5* and *ADA2* genes play a critical role in regulating metazoan growth and development. In *Drosophila*, loss of *GCN5* results in defects in oogenesis and metamorphosis (Carré et al. 2005), while in *Mus musculus*, disruption of *GCN5* leads to embryonic lethality (Xu et al. 2000; Yamauchi et al. 2000). In *Arabidopsis*, disruption of the *GCN5* and *ADA2b* genes affects the development and growth of stems, roots, and leaves (Bertrand et al. 2003; Vlachonasios et al. 2003). Specifically, T-DNA insertional mutations in *GCN5* (also known as *HAG1*; Pandey et al. 2002; Table 1) result in dwarfism, folded and serrated leaves, loss of apical dominance, and flower abnormalities underlying reduced fertility (Bertrand et al. 2003; Cohen et al. 2009; Vlachonasios et al. 2003). *ADA2b* (also known as *PROPORZI*) disruption alleles have also been described in the literature as exhibiting a similar overall phenotype including dwarfism, altered leaf morphology, and floral defects (Sieberer et al. 2003; Vlachonasios et al. 2003).

While it was previously noted that the *gcn5-1* and *ada2b-1* rosette leaves were modified in morphology and had smaller palisade mesophyll cells (Vlachonasios et al. 2003), effects on trichomes, the single-celled hairs, have not been previously described. Trichomes are an attractive developmental model, with their accessibility on the leaf surface and the well-characterized genetics underlying their development. A trichome originates from specification of an epidermal cell on rosette and cauline leaves, the stem, and even the flower's sepals in *Arabidopsis* (Hülkamp et al. 1994). Post-specification, four key processes (endoreduplication, branch formation, directional growth by cell expansion, and differentiation) contribute to morphogenesis. Specifically, three rounds of karyokinesis occur prior to increased cell size and stalk elongation. The incipient pair of branches mark

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5 the primary branch point and appear just as the stalk begins to elongate. Further increase  
6 in cell size takes place after the fourth endoreduplication event and this is followed by  
7 secondary branching off the distal pointing branch and final differentiation of a mature 3-  
8 branched trichome (Hülkamp 2004; Hülkamp et al. 1994; Schellmann and Hülkamp  
9 2005)  
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12 There is a considerable amount of literature describing genes involved in various stages of  
13 trichome development, including cell fate determination (patterning) and morphogenesis.  
14 The activator-inhibitor, lateral inhibition model for the genetic control of trichome  
15 patterning requires positive and negative regulators, most of which are initially expressed  
16 in all cells during leaf growth and expansion (Hülkamp 2004). *GLABRA3* (*GL3*; Payne  
17 et al. 2000) acts as part of a trimer of positive regulators and triggers the transcription  
18 of negative regulators, including *TRIPTYCHON* (*TRY*) (Schellmann et al. 2002;  
19 Schnittger et al. 1999). These inhibitors translocate to adjacent cells and make the  
20 activator complex nonfunctional, leading to a non-trichome fate. In the cells destined to  
21 become trichomes, the activator complex turns on the *GLABRA2* (*GL2*) and *SIAMESE*  
22 (*SIM*) genes among others (Bramsiepe et al. 2010; Grebe 2012; Morohashi and Grotewold  
23 2009). The former initiates the pathway towards trichome differentiation (Rerie et al.  
24 1994), while the latter triggers endoreduplication by inhibiting *CDKA* and *CYCD*  
25 (Churchman et al. 2006).  
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31 Endoreduplication, the process in which cells duplicate their DNA but do not undergo  
32 mitosis, is seen in many eukaryotes but is most common in plants. There is significant  
33 evidence that plant endoreduplication correlates with an increase in cell size (Breuer et al.  
34 2007). For example, constitutively active *CDKA*;1 kinase leads to significantly reduced  
35 levels of endoreduplication, trichome size, and number of trichome branches (Dissmeyer et  
36 al. 2009). Other factors outside the cyclin/CDK family have also been shown to impact  
37 endoreduplication as well as trichome patterning and morphogenesis. *TRY* encodes for a  
38 MYB transcription factor that is known to impact both pattern formation of trichomes on  
39 the leaf and trichome branching (Hülkamp et al. 1994; Pesch and Hülkamp 2011;  
40 Schellmann et al. 2007). As noted above, *TRY* normally appears to act through a  
41 repression pathway, blocking endoreduplication and additional branching (Hülkamp et  
42 al. 1994; Schellmann et al. 2002). *KAKTUS* (*KAK*) encodes an E3 ubiquitin ligase (HECT  
43 protein) and loss of function results in increased trichome branching as well as enhanced  
44 endoreduplication events in trichomes (El Refy et al. 2004; Hülkamp et al. 1994). There  
45 are also other genes implicated in trichome differentiation that may have a less direct role  
46 in cell cycle control. For example, *ZWICHEL* (*ZWI*) encodes a member of the kinesin  
47 superfamily of motor proteins that binds to microtubules in a calmodulin-dependent  
48 manner. *ZWI* is involved in cell expansion/growth of the trichome stalk and, indirectly, in  
49 secondary branching, thus all *zwi* mutants have very short stalks and only primary  
50 branches (Oppenheimer et al. 1997).  
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57 Given the extensive study of trichomes as a model for cell differentiation, it is striking that  
58 the role of epigenetic factors in rosette leaf and especially trichome development has been  
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5 largely unreported. To determine how specific chromatin modifiers, GCN5 and ADA2b,  
6 function in concert with other transcriptional regulators to execute these developmental  
7 pathways, we used multiple approaches to identify the cellular and morphological defects  
8 of leaves and trichomes in the *gcn5* and *ada2b* mutants. We show that while disruption of  
9 either of these transcriptional coactivators leads to a reduction in the size of leaf mesophyll  
10 and pavement cells, *ada2b* mutants have reduced cell number and disruption of GCN5  
11 tends to increase the number of cells in the mesophyll. *gcn5* and *ada2b* mutants also  
12 display differences in trichome branching and have opposite effects on endoreduplication.  
13 Taken together, our data supports the idea that these chromatin modifiers are required for  
14 the established relationship between ploidy levels with cell size and trichome branching.  
15 This work also contributes to the larger picture of how individual chromatin factors impact  
16 specific developmental pathways.  
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## 22 **MATERIALS and METHODS**

### 23 ***Plant growth and genotypes***

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25 Plants were germinated in soil and grown at 20-25°C under continuous light conditions.  
26 Plants were watered twice a week with half-strength Hoagland's solution. Unless  
27 otherwise noted, data was obtained from the first five true leaves (not cotyledons) between  
28 3-5 weeks of age. *Ws-2*, *Col-0*, and *Ler* were used as wild type comparisons as  
29 appropriate. *ada2b-1* and *gcn5-1* (Vlachonasios et al. 2003) are T-DNA disruption alleles  
30 isolated from the University of Wisconsin (Madison, WI, USA) collection. *gcn5-5*  
31 (SALK\_048427) and *hag1-6/gcn5-6* (SALK\_150784) are from the Salk Institute  
32 collection (La Jolla, CA, USA); *gcn5-6* seeds were kindly provided by Jeff Long. *zwiA*  
33 (SALK\_031704) seeds were graciously provided by Henrik Buschmann (uni-  
34 osnabrueck.de). Seeds harboring mutations in *TRIPTYCHON* (*try-EMI*; (Hülkamp et al.  
35 1994) and *KAKTUS* (*kak1*; Perazza et al. 1999) were kindly donated by Martin Hülkamp.  
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41 The effects of three recessive *gcn5* T-DNA insertion alleles in different ecotype  
42 backgrounds were examined (Table 1). *gcn5-1* (*Ws-2* ecotype) is a loss-of-function  
43 mutation, arising from a 3' T-DNA insertion disrupting sequence encoding the  
44 bromodomain (Vlachonasios et al. 2003; Benhamed et al. 2008), which binds acetyl-lysine  
45 residues and has been shown to play a critical role in GCN5's acetylation of nucleosomes  
46 in other species (Li et al. 2009; Cieniewicz et al. 2014). *gcn5-5* is a weak loss-of-function  
47 allele created by a T-DNA insertion in intron 10 in the *Col-0* background (Cohen et al.  
48 2009). *gcn5-6* in the *Col-0* ecotype is most likely the strongest disruption allele, with a T-  
49 DNA insertion at the beginning of intron 1. *ada2b-1* results from T-DNA insertions in the  
50 middle of the *ADA2b* coding sequence (Vlachonasios et al. 2003).  
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### 54 ***Leaf analysis***

#### 55 Differential interference contrast (DIC) microscopy

56 Leaves were harvested and fixed immediately in ethanol: glacial acetic acid (3:1) for 12  
57 hours at 4 °C. After fixation, leaves were dehydrated in a series of ethanol (30%, 50%,  
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70%, 80% and 100%) for 20 min each. Subsequently, the leaves were immersed in the clearing solution, chloral hydrate: glycerol: H<sub>2</sub>O (8:2:1). Samples remained in the clearing solution for at least 12 hours at 4°C. The proximal and distal part of the leaf was removed and the lamina was dissected out along the mid vein. The two parts of the lamina were mounted onto a glass slide with *ca* 100 µl of the clearing solution, covered with coverslip (No 1) and sealed with ENTELLAN® (Merck, UK).

#### Imaging and data extraction

Slides were observed with a Nikon MicroPhot-SA microscope using DIC optics and images were captured with a Nikon CoolPix 990 digital camera. For determining the density and size of the pavement cells, images were taken at approximately half distance along the proximodistal and mediolateral axes. On average, 4 images per leaf were taken, *i.e.* 2 consecutive images per lamina side. Images of the mesophyll layer (palisade cells) were taken by focusing along the z-axis. The images were converted to TIFF files and imported into Image J 1.3' v software for further analysis. For cell size measurements, the scale was set according to a 100 µm scale that was photographed under the same conditions as the rest of the slides. Subsequently, the cell circumference was traced with the Image J tracing tool application and the cell area and perimeter were calculated. Cell density was determined by counting all the cells included in a fixed image area. Four images were taken per leaf and five leaves, each from a different plant, were used for each genotype. Cell density (number of cells per unit area) was calculated. Total leaf area was also calculated. Leaves were scanned on a flatbed scanner (HP Scanjet 8200) at 300dpi resolution. A ruler was also scanned for scale reference. The images were transferred into ImageJ in order to calculate area, perimeter, width and length of individual leaves using the “analyse particles” function. Images were processed using the Adobe Photoshop 7.0 package. The total number of cells within a cell layer of a whole leaf was then determined. Statistical significance was assessed using a T-test.

#### ***Flow cytometry (FCM)***

##### Sample preparation: Nuclei extraction and DAPI staining

The amount of tissue used for FCM varied depending on the type and age of the tissue and the type of analysis. For determining the endopolyploidy level, whole leaf tissue was taken from 3 plants (only the 5<sup>th</sup> leaf of true leaves) at 15 days old. The tissue was placed in a petri dish, chopped finely with a razor blade in 500 µl of extraction buffer (Partec, Germany) and filtered through a 30 µm mesh (Partec). At this stage, the sample was either analyzed soon after preparation or it was frozen in liquid N<sub>2</sub> and stored at -70°C. Prior to analysis, frozen samples were left to thaw at room temperature and then 1 ml Cystain UV staining solution (Partec) was added.

##### Endopolyploidy analysis: Instrument setting and analysis

Endopolyploidy analysis was performed with a PAS II Ploidy analyzer (Partec, Germany) using an arc-lamp. The instrument was calibrated using 3 µm calibration beads (Ex: 488 nm, Partec, Germany) for the alignment of the argon ion laser and using trout erythrocytes for the arc-lamp (excitation for UV < 420 nm). Coefficients of variation (CV values) of

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6 <4.0 for the calibration beads (main peaks for both FCS and SSC) and <2.0 for the trout  
7 erythrocytes were considered satisfactory for analysis. 20,000 events were counted in each  
8 run at an average speed of 50 events / sec. The CV value for most of the peaks obtained  
9 was <4, subject to the type and age of the tissue. The left cut-off point was set at 150 and  
10 the right cut-off at 999.9. All the data was acquired on a logarithmic amplification (Log3)  
11 scale unless otherwise stated. Endoreduplication index (EI) was calculated as described  
12 before (Barow and Meister 2002; Gegas et al. 2014).  
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#### 15 Data analysis: Data extraction and statistics

16 The number of counts of each peak was obtained by manual gating. The mean number of  
17 counts for each peak and the standard deviation was calculated and their significance was  
18 tested by performing a Student's t-test (t-test).  
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#### 21 ***Trichome analysis***

##### 22 Trichome dimensions

23 For experiments in the Ws-2 background, trichomes were isolated from first and second  
24 pair of leaves based on protocol by Zhang and Oppenheimer (2004). The leaves were  
25 incubated in 10:1 ethanol:acetic acid for 2 hours followed by three washes with PBST.  
26 Leaves were then placed under air vacuum for 15 minutes and incubated at 50°C for 3-4  
27 hours. The trichomes were removed with a paintbrush and put into an Eppendorf tube.  
28 Prior to microscopy the trichomes were washed three times with PBST and glycerol was  
29 added. Photographs were taken and analyzed with Image J. Statistical analysis of the  
30 results were made using t-test.  
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35 Analysis of Col and *gcn5-6* used a protocol adapted from Marks et al. (2008). Rosette  
36 leaves (first through fifth) were placed in a centrifuge tube with 50 mgs RiboPure Zirconia  
37 beads (Ambion; 50 mgs/ 1 gram of leaves) and a solution (15 ml/1 gram of leaves)  
38 containing 50 mM EGTA and 1X PBS. The trichomes were dislodged from the leaves by  
39 vortexing the tube for 30 seconds and then keeping on ice for 30 seconds, for sixteen  
40 cycles. The material was poured through two layers of screen door mesh (Lowes). The  
41 trapped material was washed off the mesh with PBS and then poured through a 100µm  
42 sieve to capture the trichomes. The fluid that passed through the screen door mesh was  
43 also passed over the sieve. Trichomes trapped in the sieve were rinsed off with 1X PBS  
44 and stored in a tube at 7°C. Small samples of trichomes in PBS were placed on  
45 microscope slides and covered with clay-footed cover slips. The clay was depressed until  
46 the trichomes were flattened and then the coverslip edges were sealed with nail polish to  
47 prevent dehydration. Undamaged trichomes were photographed at 40X with a Motic  
48 camera attached to a Nikon Eclipse microscope. Motic Images Plus 2.0 ML software was  
49 used to measure trichome stem and branch length; stem length was defined as the distance  
50 from the base to the center of the trichome, while branch length was from the tip of the  
51 longest trichome branch to the center of the trichome. The t-test was used to compare the  
52 stem, branch, and total lengths (stem + branch lengths) between different genotypes.  
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##### 58 Trichome branching

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6 Rosette leaves (second and third, from four to five-week-old plants) were fixed in 3%  
7 glutaraldehyde with 0.1 M phosphate buffer (pH 6.8), and followed by fixation in 1%  
8 OsO<sub>4</sub> with 0.1 M phosphate buffer. After processing through ethanol dehydration series,  
9 the leaves were critically point dried with liquid carbon dioxide. Carbon adhesive (CCC  
10 by EMS) held the leaves to the stubs, which were then sputter coated with gold. Samples  
11 were examined at 15 kV with the ABT-60 scanning electron microscope.  
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### 13 ***Double mutants***

14 The *gcn5-1; ada2b-1* double mutant was created using pollen from *gcn5-1* to fertilize  
15 *ada2b-1* heterozygous plants, since homozygous *ada2b-1* mutants display flower  
16 infertility. The resulting F1 generation was self-fertilized and the segregating F2  
17 population was genotyped using KAPA2GTM Fast PCR (Kapa Biosystems) with the  
18 primers listed in Online Resource 1. Progeny that were homozygous for mutant alleles at  
19 both loci were identified by PCR.  
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23 The *gcn5-1; try-EM1* and *gcn5-1; zwiA* double mutants were created using pollen from  
24 *gcn5-1* to fertilize *try* or *zwi* mutants. The progeny were then backcrossed to Ler and Col-0  
25 backgrounds respectively for at least four generations. The F2 population was evaluated  
26 for *try* mutant phenotype and genotyped for *zwiA* and *gcn5-1* mutations by PCR with gene  
27 and T-DNA specific primers.  
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### 31 ***Gene expression analysis***

32 Leaf tissue was isolated at ~4 weeks post planting and flash frozen in liquid nitrogen.  
33 Tissue was ground under liquid nitrogen and RNA prepared using a RNeasy Plant Mini Kit  
34 (Qiagen). RNA concentrations were determined using a Nanodrop and between 0.5-1 µg  
35 RNA was used in cDNA reactions with High Capacity RNA to cDNA Kit (Applied  
36 Biosystems) or Maxima First Strand cDNA synthesis (Thermo Scientific). qPCR was  
37 carried out using a comparative Ct approach on an Applied Biosystems StepOne Plus  
38 instrument. Reactions were set up using the FAST Universal Master Mix 2X protocol  
39 (Applied Biosystems). TaqMan assays (Applied Biosystems) used include GCN5  
40 (At0228249\_g1), ZWI (At02187047\_g1), TRY (At02321066\_g1), KAK  
41 (At02170143\_g1), and ACT8 (At02270958\_gH). Three replicates were performed for  
42 each data point per experiment (biological replicates), with allowable standard deviations  
43 of Ct < 5%. RQ or fold change values for each experiment were log<sub>2</sub> transformed and  
44 averaged for each experimental condition. Semi-quantitative RT-PCR was conducted using  
45 various primers (Online Resource 1) directed at portions of the GCN5 cDNA and at a  
46 housekeeping gene (At4g26410).  
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## 53 **RESULTS**

### 54 ***Transcriptional coactivators are required for correct specification of leaf cell number*** 55 ***and size***

56 Development of lateral organs such as leaves involves spatially and temporally controlled  
57 changes in cell proliferation, growth, and differentiation that are mediated in part by  
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6 alterations in chromatin structure (Bertrand et al. 2003; Kalve et al. 2014; Servet et al.  
7 2010; Vlachonasios et al. 2003). GCN5 and ADA2b play an essential role in leaf  
8 development and have been implicated in specifying correct cell size in mesophyll tissue  
9 (Vlachonasios et al. 2003). A more detailed microscopic analysis allowed us to extend  
10 these findings and assess possible effects on cell number. The *gcn5-1* mutant displayed  
11 smaller mesophyll and pavement cell size, but there was no significant difference  
12 between cell number in *gcn5-1* and Ws-2 leaves (Fig. 1a, b). Plants homozygous for the  
13 *GCN5* hypomorphic allele *gcn5-5* displayed increased cell number in the mesophyll but  
14 still showed reduced cell size (Fig. 1a, b). Loss of ADA2b resulted in a decreased number  
15 of mesophyll and pavement cells per leaf as well as reduced leaf cell size, resulting in  
16 smaller leaf area (Fig. 1a, b, c). Double mutant plants with disruption of both *GCN5* and  
17 *ADA2b* show some reduction in leaf area but not to the degree seen in *ada2b-1* single  
18 mutants (Fig. 1c).  
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### 22 ***GCN5 and ADA2b have opposite effects on ploidy***

23 Because final cell size is often correlated with the number of rounds of endoreduplication  
24 (Gegas et al. 2014; Sugimoto-Shirasu and Roberts 2003; Traas et al. 1998), we  
25 investigated ploidy levels using flow cytometry analysis. We found that 50% of nuclei  
26 isolated from the fifth rosette leaf of 15-days old Ws-2 seedlings give rise to 2C or 4C  
27 peaks (Fig. 2a), suggesting that the bulk of this population of cells do not enter the  
28 endocycle (4C nuclei can represent mitotic cells in G2 or cells that have endocycled once).  
29 Ws-2 leaves also possess approximately 10% of 16C and 32C peaks. Nuclear content  
30 profiles for *gcn5-1* and *gcn5-5* were similar to wild type controls (Fig. 2a). *gcn5-6*  
31 displayed a higher proportion of 4C nuclei than wild type plants and a lower proportion of  
32 16C and 32C nuclei (Fig. 2b). In contrast, *ada2b-1* plants displayed a reduced proportion  
33 of 2C and 4C peaks and notably higher 16C and 32C peaks (Fig. 2a). Nuclei counts were  
34 also used to calculate the Endoreduplication Index values (Barow and Meister 2003;  
35 Online Resource 2).  
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41 To extend these findings, we analyzed the ploidy levels in the *ada2b-1; gcn5-1* double  
42 mutant. The double mutant plant is dwarf (smaller than *gcn5-1*) and has characteristics of  
43 both *gcn5-1* and *ada2b-1* (Fig. 3a; Online Resource 3). In this experiment, the wild type  
44 plants showed slightly higher overall ploidy levels although the percentage of cells with  
45 less than 8C was similar between Ws-2 and *gcn5-1*, as shown in Fig. 2. The double  
46 mutant, however, shows reduced ploidy levels (Fig. 3b; Online Resource 2), indicative of  
47 fewer endocycles as compared to wild type plants.  
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### 50 ***Transcriptional coactivators impact trichome branch number, pattern, and expansion***

51 Trichomes provide a readily accessible single cell system in which the role of chromatin  
52 modifiers in defining cell shape and size can be investigated. Development of *Arabidopsis*  
53 trichomes has been described in a series of stages (Mathur 2006; Szymanski et al. 1998,  
54 2000) involving initially a radial expansion of the committed epidermal cell and then a  
55 vertical expansion, initiating the formation of the stalk. At the primary branch point, two  
56 branches arise and, after an increase in cell size, secondary branching takes place off the  
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6 distal branching point (Folkers et al. 1997; Szymanski et al. 1999). Changes in trichome  
7 branching were observed in plants harboring disruptions in *ADA2b* and *GCN5*. There were  
8 significant differences in the branch number of trichomes in *ada2b-1* plants, with an  
9 increase in unbranched and 2-branched trichomes and a decrease in 3-branched trichomes  
10 compared to wild type plants (Fig. 4a, b, g). A similar effect with another hypomorphic  
11 *ada2b* allele (*prz1-1*) was noted by Sieberer et al. (2003). A decrease in trichome branch  
12 number was also seen in *gcn5-1* mutants (Fig. 4c, h) and a trend towards decreasing branch  
13 number was observed in the *gcn5-5* background (Online Resource 4). Interestingly, plants  
14 homozygous for the likely null allele, *gcn5-6*, showed a significantly increased number of  
15 4- (or more) branched trichomes (Fig. 4d, e, f). Comparison of 4-branched trichomes of  
16 Col-0 vs. *gcn5-6* plants uncovers a difference in patterning: *gcn5-6* predominantly has two  
17 primary branches that each undergo a secondary branching event while in Col, the most  
18 common pattern is one secondary branch undergoing an additional bifurcation (Fig. 5).  
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23 To examine whether *GCN5* and *ADA2b* affect the overall outgrowth of the trichome from  
24 the leaf surface, we measured the length of the trichome stem (stalk) as well as total  
25 trichome length. Both parameters were longer in *gcn5-1* and *ada2b-1* mutants than in wild  
26 type controls (Fig. 6a, b). In the *gcn5-6* background, the dimensions of 3- and 4-branched  
27 trichomes were examined separately. Three-branched *gcn5-6* trichomes had a statistically  
28 significant longer branch length than that of wild type trichomes and therefore a longer  
29 total trichome length (Fig. 6c). In 4-branched trichomes, *gcn5-6* mutants have a  
30 statistically shorter stem length (and shorter overall length) than that of Col-0 4-branched  
31 trichomes (Fig. 6d). There were no significant differences in the trichome area and  
32 perimeter in *gcn5-1* and *ada2b-1* plants in comparison with wild type controls (Online  
33 Resource 5). However, *gcn5-5* trichome area and perimeter were significantly smaller than  
34 the wild type plants (Online Resource 5).  
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### 38 ***GCN5 may define an additional circuit in the trichome developmental pathway***

39 As our knowledge of details effects of chromatin modifiers increases, it will be interesting  
40 to understand how these factors act and interact with other cellular regulators to effect  
41 development. To test whether the histone acetyltransferase *GCN5* genetically interacts  
42 with known regulators of trichome branching, we examined the phenotypes of two double  
43 mutants, created by crossing *gcn5-1* plants with mutants of *ZWICHEL* (*ZWI*), which is a  
44 positive regulator of trichome branching, and the negative regulator *TRIPTYCHON* (*TRY*).  
45 *gcn5-1; zwiA* plants primarily displayed unbranched trichomes, a more severe phenotype  
46 than either single mutant (Figure 7 and 4h). Two branched trichomes seen in *gcn5-1; zwiA*  
47 double mutants displayed an underdeveloped, short second branch with a blunt tip, rather  
48 than an extended branch (Figure 7c). Trichomes of the *gcn5-1; zwiA* double mutant also  
49 showed a swollen base, as in seen with *zwi* mutants in the *Ws-2* background (Oppenheimer  
50 et al. 1997), and a very shortened trichome stalk characteristic of *zwi* mutants (Figure 7b,  
51 c). The double mutant plants display other characteristics of the overall *gcn5* mutant  
52 phenotype, e.g. dwarfism, leaf morphology differences (data not shown). *gcn5-1; try-EMI*  
53 double mutants displayed a degree of trichome branching that is similar to wild type  
54 controls (Figure 8). The overall plant phenotype resembled those of *gcn5* single mutants  
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5 (Figure 8).  
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8 To study whether possible interactions impact gene transcription, we assessed the  
9 expression levels of *ZWI* and *TRY* as well as the negative regulator *KAKTUS* in *gcn5-1* and  
10 *gcn5-6* mutants. Previous microarray analysis had shown that there was no change in *ZWI*  
11 expression and a slight increase (less than 3-fold) in *KAK* expression in *gcn5-1* mutants  
12 (Vlachonasios et al. 2003). We used qRT-PCR to assay *TRY* expression in a *gcn5-1*  
13 background and documented an average 4-fold decrease in expression (Figure 9).  
14 However, analysis of these three candidate genes in the *gcn5-6* mutant background did not  
15 reveal any consistent significant changes in gene expression (Figure 9). In addition, we  
16 analyzed expression levels of *GCN5* in *zwi*, *try*, and *kak* mutants. *GCN5* expression was  
17 increased in some individual mutant samples while the level remained similar to wild type  
18 in other isolates (Figure 9).  
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## 24 DISCUSSION

25 Our work provides valuable insights into how the transcriptional coactivators GCN5 and  
26 ADA2b impact leaf development at the tissue and cellular level, contributing to our  
27 understanding of how chromatin modifiers contribute to specific developmental processes.  
28 First, analysis of rosette leaves suggests that ADA2b is involved in both cell division and  
29 cell size (Fig. 1). A reduced rate of cell division in *ada2b-1* plants would be expected to  
30 result in fewer number of cells and smaller organs (Vlachonasios et al. 2003). Indeed,  
31 several members of the *KIP RELATED PROTEIN (KRP)* family have been shown to be  
32 misexpressed in an *ada2b* mutant background, which might contribute to deregulated cell  
33 proliferation upon auxin treatment (Anzola et al. 2010). The *gcn5-1* mutant displayed a  
34 wild type rate of division but showed reduced cell expansion. A weak GCN5 mutant  
35 allele, *gcn5-5*, displayed increased cell number, suggesting that GCN5 could also have an  
36 effect on cell proliferation depending on the allele or the genetic background (Fig. 1).  
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41 Secondly, our data show that GCN5 plays a role in trichome branching and that ADA2b  
42 acts specifically to promote trichome branch initiation. *ada2b-1* and *gcn5-1* trichomes were  
43 underbranched while *gcn5-6* plants displayed increased numbers of trichomes with four or  
44 more branches (Fig. 4). *gcn5-1* and *gcn5-5* are hypomorphs in the Ws-2 and Col-0  
45 backgrounds respectively while *gcn5-6* (in Col-0 background) results from a T-DNA  
46 insertion after the first exon. Semi-quantitative RT-PCR analysis indicates that the full-  
47 length transcript is disrupted in both the *gcn5-1* and *gcn5-6* backgrounds (Vlachonasios et  
48 al. 2003; Online Resource 6), although the site of disruption in *gcn5-1* impacts only the  
49 end of the bromodomain (which binds acetyl-lysine) while in *gcn5-6*, the disruption  
50 impacts the catalytic HAT domain. The differences in *gcn5* mutant phenotypes may be  
51 interpreted in light of the varying underlying molecular lesions and/or genetic  
52 backgrounds; we have previously documented different allelic effects on flower  
53 morphology (Cohen et al. 2009).  
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58 Our data also revealed a reciprocal relationship between trichome stem length and branch  
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6 number (Fig. 6). *ada2b-1* and *gcn5-1* trichomes with reduced branching have a longer  
7 stem and overall trichome length, while trichome area and perimeter remain unchanged.  
8 Conversely, 4-branched trichomes seen in the *gcn5-6* background have a shorter stem and  
9 overall length. These results support the idea that by later stages of trichome  
10 differentiation the overall size of the cell is determined, such that decreased branching  
11 results in an extended stem and increased branching imposes a shorter stem. There may  
12 also be temporal control. If the primary branch point is established later, the trichome stem  
13 may extend and the number of branches that can form would be limited. In contrast,  
14 earlier establishment of the primary branch may shorten the stem and facilitate additional  
15 branching.  
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19 It is interesting to consider the specific morphology of the *gcn5-6* 4-branched trichomes  
20 (Fig. 5). The shorter stem length here is reminiscent of *zwichel* mutants, which show a  
21 more pronounced version of this effect. The predominant pattern of one primary and two  
22 secondary branching events seems both regular and similar to that seen in another trichome  
23 overbranching mutant, *kak-1* (Perazza et al. 1999). These observations supported the  
24 hypothesis that the transcriptional coactivators work in concert with transcription factors  
25 and other known regulators of trichome development. TRIPTYCHON (TRY) acts to block  
26 trichome branching such that loss-of-function mutations show increased branch number  
27 (Hülkamp et al. 1994). *gcn5-1; try-EMI* plants show a degree of trichome branching  
28 similar to wild type, showing that the two mutations offset each other and that GCN5 and  
29 TRY operate in independent pathways (Figure 8). Our data also suggests that GCN5  
30 works in a separate pathway from ZWICHEL in its effects on trichome morphogenesis  
31 (Figure 7). Expression analysis (Figure 9) also generally supports the idea that GCN5 acts  
32 in a parallel pathway(s) to these established transcriptional regulators as well as the  
33 ubiquitin ligase KAKTUS to impact trichome development.  
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39 Benhamed et al. (2008) took a genome wide approach to identifying GCN5 interacting  
40 partners, by assessing promoter occupancy of GCN5 via a ChIP-chip approach. Relevant  
41 to the genes explored here, they detected binding of GCN5 at the ZWI promoter in wild  
42 type *Arabidopsis* seedlings but not in a *gcn5-1* mutant line. However, we have previously  
43 shown that there is no change in expression of ZWI in *gcn5-1* plants (Vlachonasios et al.  
44 2003), supporting Benhamed et al.'s findings that not all promoters bound by GCN5 show  
45 resulting transcriptional effects. While we see modest effects on TRY and KAK in *gcn5-1*  
46 (Fig. 9; Vlachonasios et al. 2003), there was no reported change in promoter binding or  
47 expression of TRY and KAK in the *gcn5-1* mutant background in Benhamed et al. 2008.  
48 We confirmed this lack of interaction in the stronger *gcn5-6* allele. There is no support for  
49 other known regulators of trichome branching (e.g., GLABRA3, STICHEL,  
50 ANGUSTIFOLIA) interacting with GCN5 or ADA2b (Benhamed et al. 2008;  
51 Vlachonasios et al. 2003). Neither our current work nor these genome wide studies  
52 investigate gene expression in trichome cells specifically, in part due to limitations of the  
53 mutant plants' leaf mass.  
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58 Endoreduplication in plant leaves has been shown to impact cell size and trichome  
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6 differentiation (Breuer et al. 2007; Bramsiepe et al. 2010). *ada2b-1* mutants display  
7 increased ploidy levels, suggesting an earlier transition to the endocycle and increased  
8 rounds of endoreduplication (Fig. 2). Leaves of *gcn5* mutants tend to have reduced ploidy  
9 (Fig. 2), indicating another distinct function between ADA2b and GCN5 (Vlachonasios et  
10 al. 2003). It may be worth noting that Kim et al. (2015) reported that *gcn5-6* plants show  
11 an overall delay in leaf development, which might impact ploidy (and less trichomes on the  
12 abaxial surface). ADA2b appears to act as a negative regulator of endoreduplication  
13 independent of GCN5, such that plants lacking ADA2b alone show increased ploidy.  
14 *ada2b-1* plants would be predicted to lack functional SAGA complex but retain other  
15 GCN5 functions. The ploidy analysis of *gcn5* single and double mutants (Fig. 3) suggests  
16 that GCN5 acts to promote endoreduplication and is epistatic to ADA2b functions. The  
17 double mutant phenotype also suggests that ADA2b acts as an enhancer of GCN5 function  
18 in endoreduplication.  
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23 As *ada2b-1* plants display increased ploidy but decreased cell size, loss of ADA2b  
24 uncouples the established correlation between ploidy and leaf cell size (Melaragno et al.  
25 1993). Increased endoreduplication is often associated with increased morphological  
26 complexity in a cell-type specific manner (Bramsiepe et al. 2010; Sugimoto-Shirasu and  
27 Roberts 2003). It is notable that in *ada2b* mutant leaves both pavement and trichome cells  
28 show reduced complexity (cell differentiation and trichome branching). Taken together  
29 our results suggest that ADA2b impinges on the transition between cell proliferation and  
30 differentiation and/or that ADA2b may mediate an endoreduplication-dependent  
31 mechanism for cell morphogenesis.  
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35 *gcn5* and *ada2b* mutants also disrupt the link between endoreduplication and trichome  
36 branching (Hülkamp et al. 1999). *ada2b-1* plants show increased ploidy and yet have  
37 decreased trichome branch points. The strong mutant allele *gcn5-6* displays reduced  
38 ploidy in leaves but increased branching of some trichomes. While there are examples of  
39 mutant lines in which trichome branching is impacted while ploidy remains unchanged  
40 (e.g., *STICHEL* as described by Ilgenfritz et al. 2003 and *BRANCHLESS TRICHOMES* as  
41 described by Kasili et al. 2011), in our study we see the *opposite* of the predicted effect, i.e.  
42 ploidy levels are changed but not in a way that correlates with the established effect of  
43 endoreduplication promoting increased branch number. We acknowledge that our ploidy  
44 analysis is not of trichome cells specifically; however, others have observed similar levels  
45 of DNA content in leaves analyzed by flow-cytometry and trichomes assessed by DAPI  
46 staining (Churchman et al. 2006; Hamdoun et al. 2016; Kasili et al. 2011; Li et al. 2012).  
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51 In summary, we demonstrate developmental impacts on rosette leaf cell division and  
52 growth and trichome morphogenesis by the histone acetyltransferase GCN5 and the  
53 transcriptional coactivator ADA2b. This work contributes to the ultimate goal of linking  
54 these chromatin modifiers to specific developmental pathways within cells and tissues, for  
55 which we still have a limited number of examples (Benhamed et al. 2006). It is also  
56 interesting to note that loss of these transcriptional coactivators uncouples established  
57 connections between endoreduplication and other developmental events in rosette leaves.  
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6 In particular, ADA2b is required to promote the positive correlations between ploidy and  
7 cell size as well as between rounds of endoreduplication and trichome branching. In  
8 general, our results support the overlapping and distinct developmental functions for these  
9 chromatin modifiers and highlight ADA2b's role as a cellular regulator beyond  
10 participation as a partner with GCN5.  
11

#### 12 13 14 **AUTHOR CONTRIBUTION**

15 Substantial contributions to the conception or design of the work (JK, JD, KV, EM, AH),  
16 data acquisition (JK, MSa, VG, NP, AK, PP, AM, MSh, JB, ALK, CK, GW, HC, KV, EM,  
17 AH), and/or interpretation of data (JK, VG, MSh, JB, ALK, CK, GW, HC, JD, KV, EM,  
18 AH) were made by all authors. Authors participated in drafting the work (JK, MSa, VG,  
19 NP, AK, PP, AM, MSh, JB, ALK, CK, GW, HC, JD, KV, EM, AH) and/or revising it  
20 critically for important intellectual content (JK, JD, KV, EM, AH). All authors have given  
21 final approval of the manuscript and agree to be accountable for all aspects of the work.  
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#### 40 41 42 43 44 45 46 47 48 **FIGURE CAPTIONS**

49 **Fig. 1** Analysis of (a) mean cell number per leaf, (b) mean cell area of mesophyll and  
50 pavement cells, and (c) mean leaf area in wild type (Ws-2 and Col-0) and mutant plants  
51 (n=5 leaves). A statistically significant difference between mutant lines and the relevant  
52 wildtype background is denoted p<0.001 with \*\*\* and p<=0.05 with \*. Error bars  
53 represent standard error.  
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56 **Fig. 2** Analysis of DNA content in wild type and mutant leaves. (a) The mean percent  
57 nuclei exhibiting specific ploidy levels in Ws-2 and Col-0 and the single mutants *gcn5-1*,  
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6 *ada2b-1*, and *gcn5-5* (n=3). Note that there were no <sup>32</sup>C nuclei detected in Ws, *gcn5-1*, or  
7 *gcn5-5* backgrounds. (b) The mean percent nuclei exhibiting specific levels of DNA  
8 content in Col-0 and *gcn5-6* (n=7) cells. Error bars represent standard error.  
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10 **Fig. 3** Phenotype of the Ws-2, single mutants *ada2b-1* and *gcn5-1*, and the double mutant  
11 *ada2b-1; gcn5-1*. (a) Plant growth 30 and 57 days post planting. (b) DNA content analysis  
12 of Ws-2, double mutant and *gcn5-1* (n=3). Error bars represent standard error.  
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15 **Fig. 4** Trichome branch arrangements and numbers on wild type and mutant leaves.  
16 Scanning electron micrographs illustrate predominant branching pattern for (a) 3-branched  
17 Ws, (b) 2-branched *ada2b-1*, (c) 2-branched *gcn5-1*, (d) 3-branched Col-0, (e) 4-branched  
18 *gcn5-6* and (f) 5-branched *gcn5-6*. Bar = 100  $\mu$ m. (g-i) The mean percentage of trichomes  
19 with 0 (unbranched), 2, 3, or 4+ branches. The number of trichomes having 0, 2, 3 or 4+  
20 branches (x axis) is represented as a percentage of the total number of trichomes on a given  
21 second rosette leaf. Error bars represent standard error. (g) Comparison between *ada2b-1*  
22 leaves (n=32) and Ws-2 leaves (n=10). (h) Comparison between *gcn5-1* leaves (n=16) and  
23 Ws (n=19). (i) Comparison between *gcn5-6* leaves (n=16) and Col-0 (n=23). All  
24 branching categories in the mutants are significantly different from wildtype plants, with p  
25 values equal to or much less than  $< 0.05$ . Error bars represent standard error.  
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30 **Fig. 5** Trichome anatomy and the three most common 4-branch patterns observed in Col-0  
31 and *gcn5-6*. (a) A typical 3-branched *Arabidopsis* trichome measured from a center point  
32 on the trichome; secondary branch (dashed line), primary branch (dotted line) and stem  
33 (solid line) are shown. Bar = 100  $\mu$ m. (b) The most common patterns for 4-branched  
34 trichomes (adapted with permission from Folkers et al., 1997. *Development*.124, 3781.  
35 <http://dev.biologists.org/content/124/19/3779.long>), which we refer to as types A, B, and  
36 C. Yellow circles denote primary branch point and blue circles denote secondary branch  
37 point. (c-e) Representative scanning electron microscope images of (c) type A, (d) type B,  
38 and (e) type C. Bar = 100  $\mu$ m. (f) The percent of surveyed Col-0 (n=43) and *gcn5-6* (n=43)  
39 trichomes from seven leaves that exhibit specific branching patterns.  
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43 **Fig. 6** Measurements of trichome dimensions ( $\mu$ m). (a) Mean trichome stem length. (b)  
44 Mean trichome total length. n=50 for all genotypes. (c) Mean stem, branch and total  
45 trichome length of 3-branched trichomes in Col-0 (n=171) and *gcn5-6* (n=169). (d) Mean  
46 stem, branch, and total trichome length of 4-branched trichomes in Col-0 (n=65) and *gcn5-*  
47 *6* (n=56). Statistical significance of the difference between wildtype and mutant plants is  
48 denoted with \*p<0.1, \*\*p<0.01, and \*\*\*p<0.001. Error bars represent standard error.  
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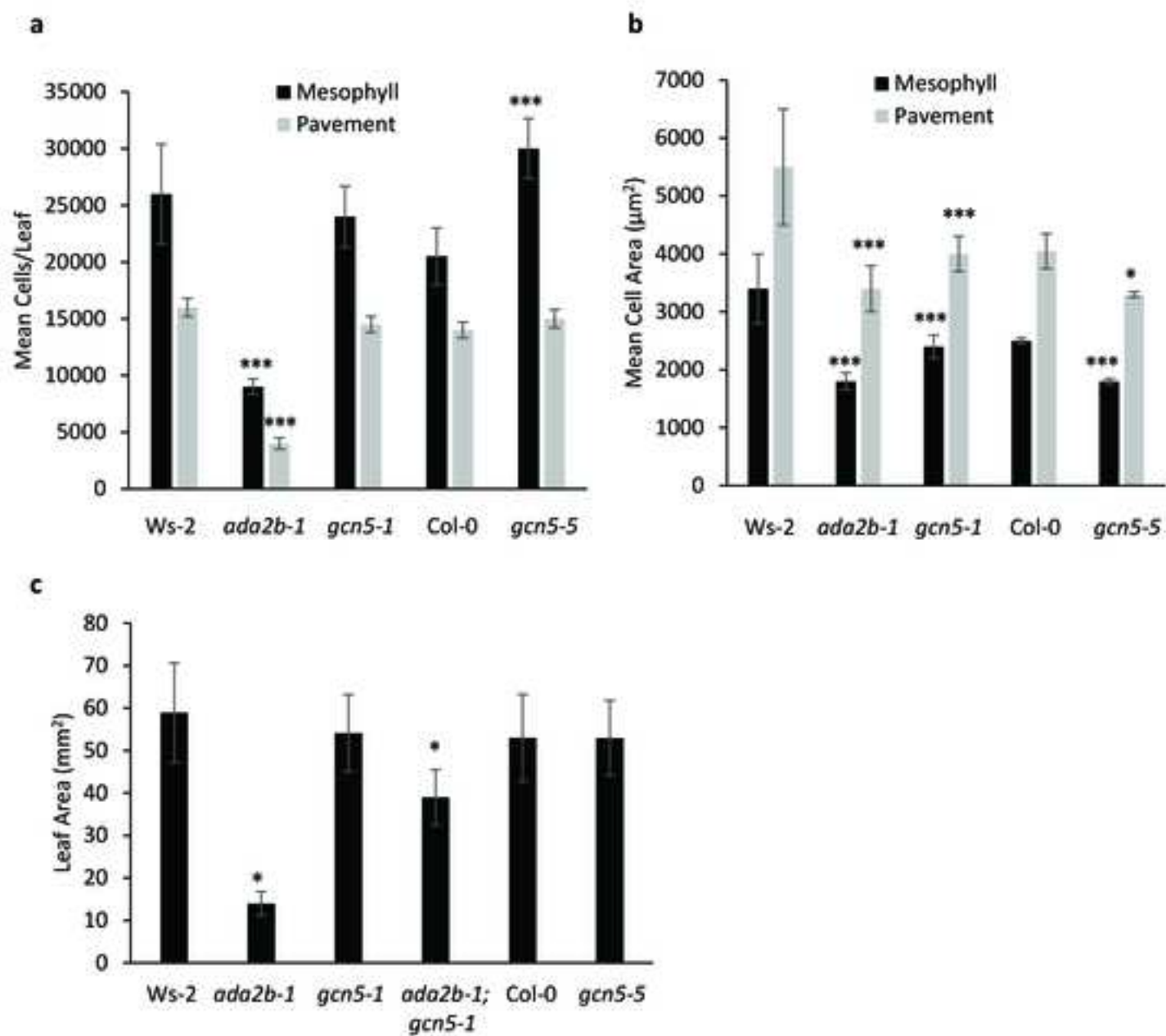
51 **Fig. 7** *zwiA* single and *gcn5-1; zwiA* double mutants. (a) Light microscopy of double  
52 mutant. (b) Scanning electron microscopy of *zwi-A* single mutant revealing the two types  
53 of 2-branched trichomes. Bar =100  $\mu$ m. (c) Scanning electron microscopy of *gcn5-1; zwiA*  
54 double mutant illustrating many trichomes with the strong *zwi* phenotype. Bar = 100  $\mu$ m.  
55 (d) Quantitative analysis of trichome branch number (compiled using SEM) in double  
56 mutants (n=12 leaves) and *zwiA* (n=17 leaves). The numbers of unbranched trichomes and  
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6 trichomes having 2 branches are represented as a percentage of the total number of  
7 trichomes on a given leaf. Error bars represent standard error; p values are  $\ll 0.05$  for  
8 comparison of both unbranched and two-branched trichomes between the *zwiA* single  
9 mutant and the double mutant genotype. Col (n=8 leaves, 298 trichomes) displayed no  
10 unbranched trichomes and only two 2-branched trichomes.  
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13 **Fig. 8** Analysis of Ler (a, d), the single mutants *try-EMI* (b, f), *gcn5-1* (c, e), and the  
14 double mutant *try-EMI; gcn5-1* (b, g). Plant growth 50 (a) or 40 (b, c) days after seed  
15 planting of genotypes as marked. (d-g) Gross leaf morphology and trichome distribution.  
16 (h) Branching patterns of each plant type were determined from eight to fourteen plants, 4  
17 leaves per plant, and for a total of 108-148 trichomes.  
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20 **Fig. 9** Gene expression changes as revealed by qRT-PCR. The average fold difference in  
21 expression is represented as its log<sub>2</sub> value, such that values between 1.0 and -1.0 indicate  
22 less than a 2-fold difference. Positive values indicate increased expression of the target  
23 gene in the mutant background. Error bars indicate standard deviation. n indicates the  
24 number of different plants (biological replicates) used.  
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Figure 1



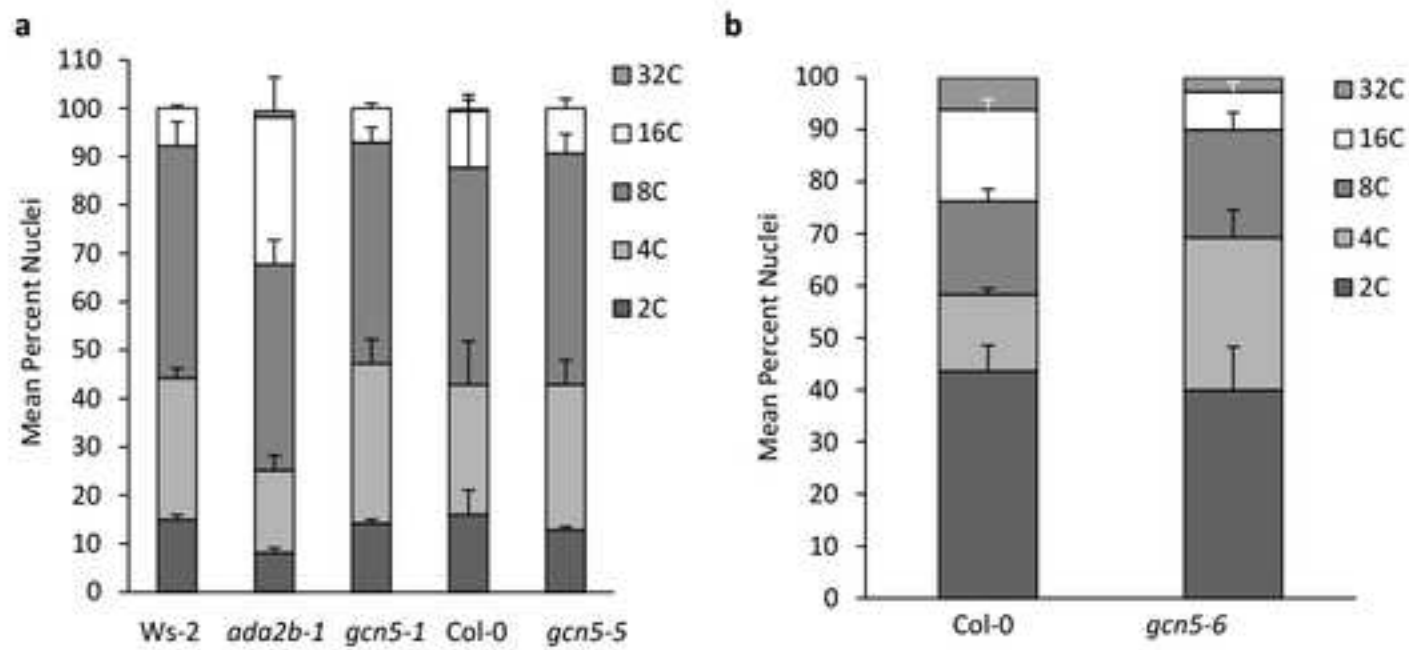


Figure 2



Figure 3

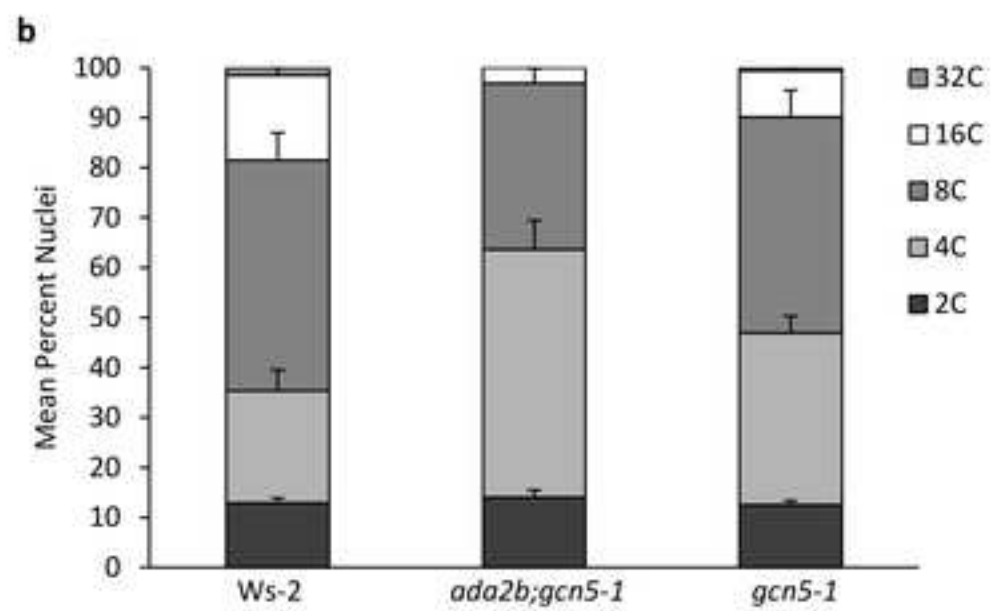
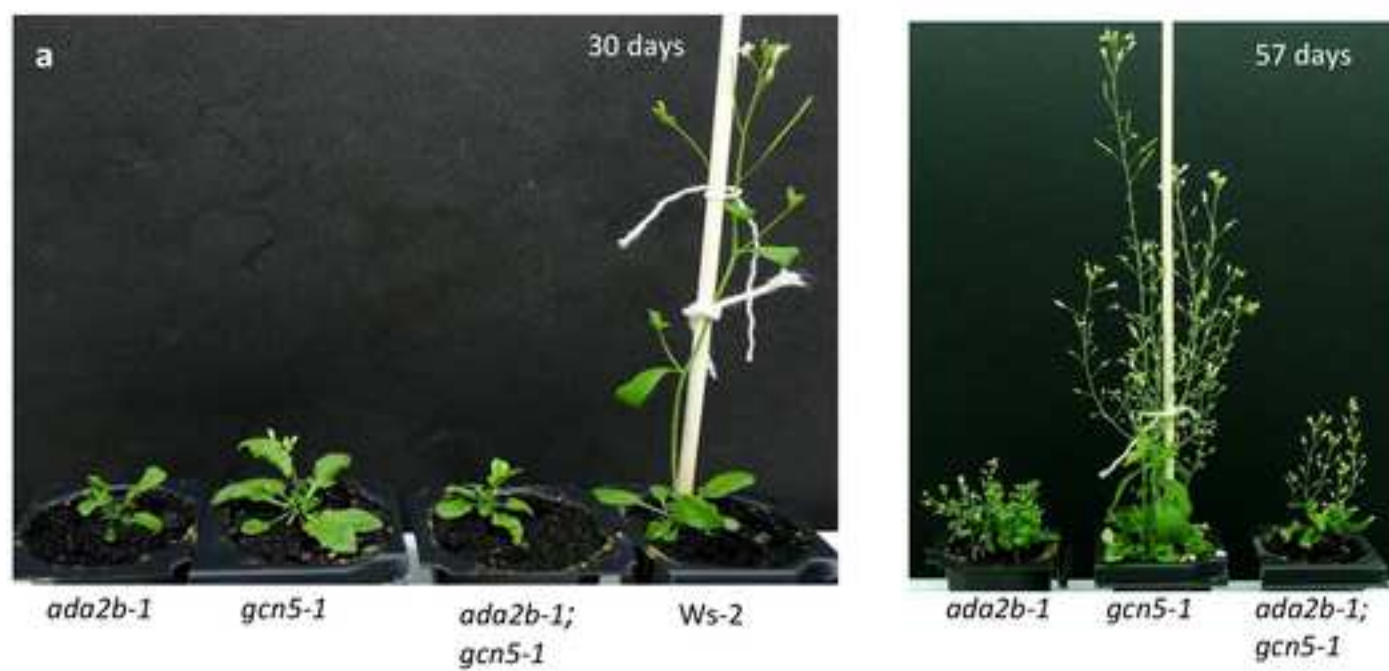
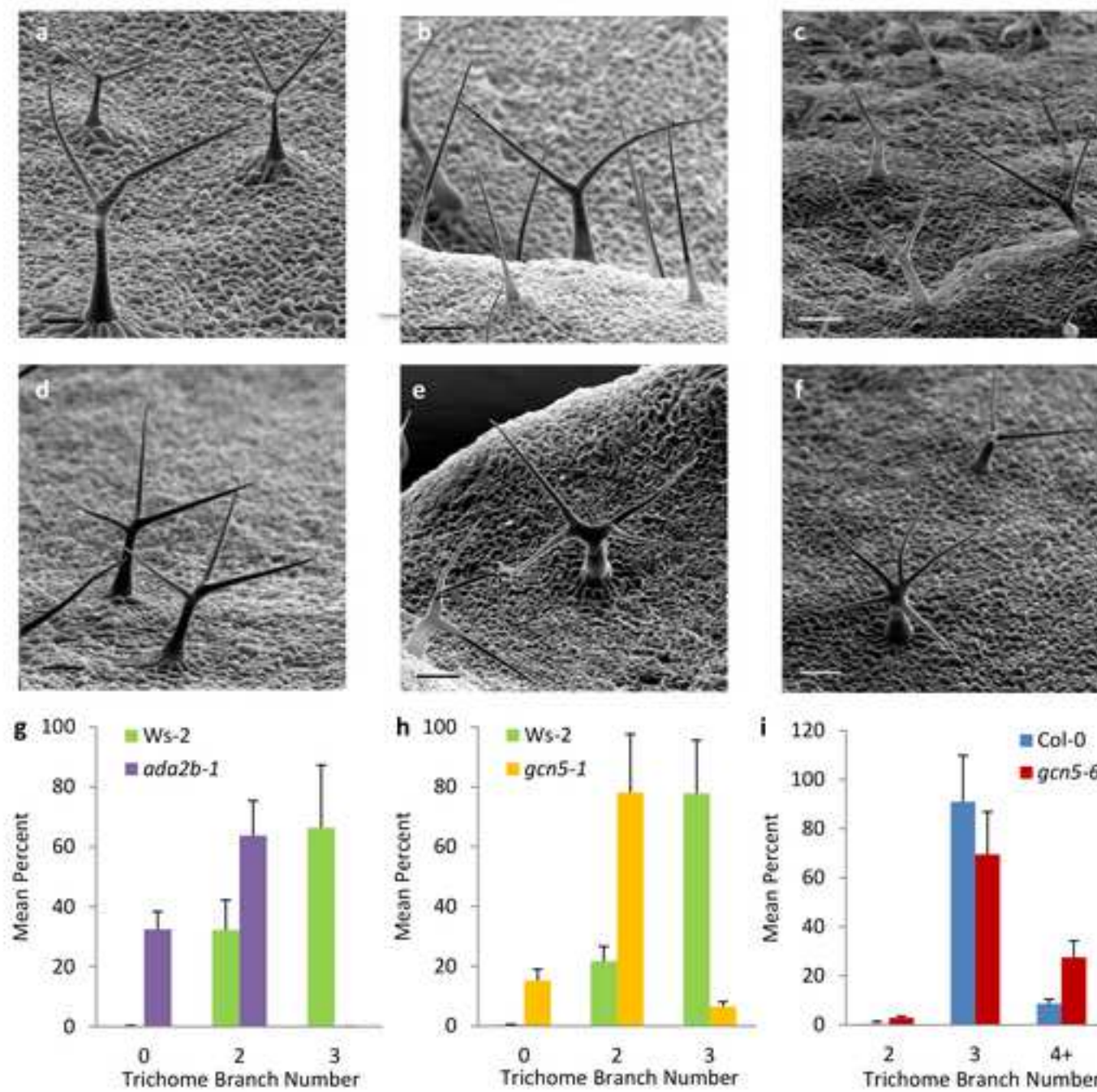


Figure 4



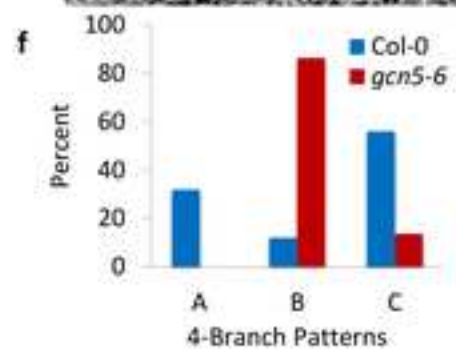
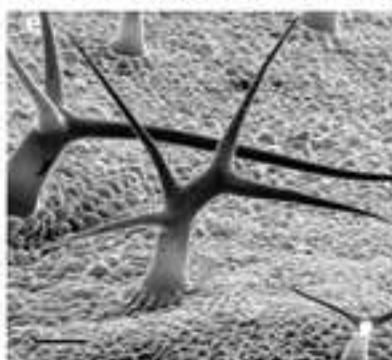
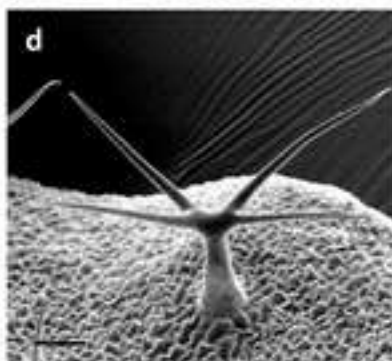
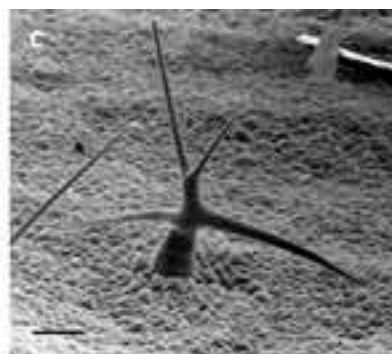
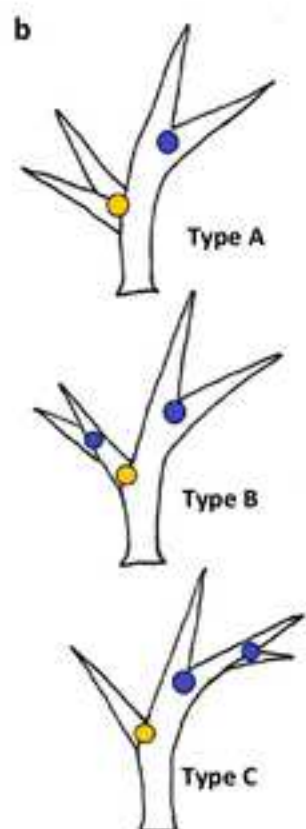
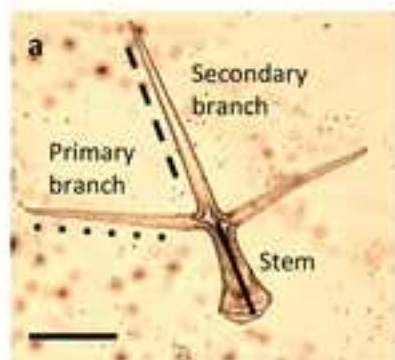


Figure 5

Figure 6

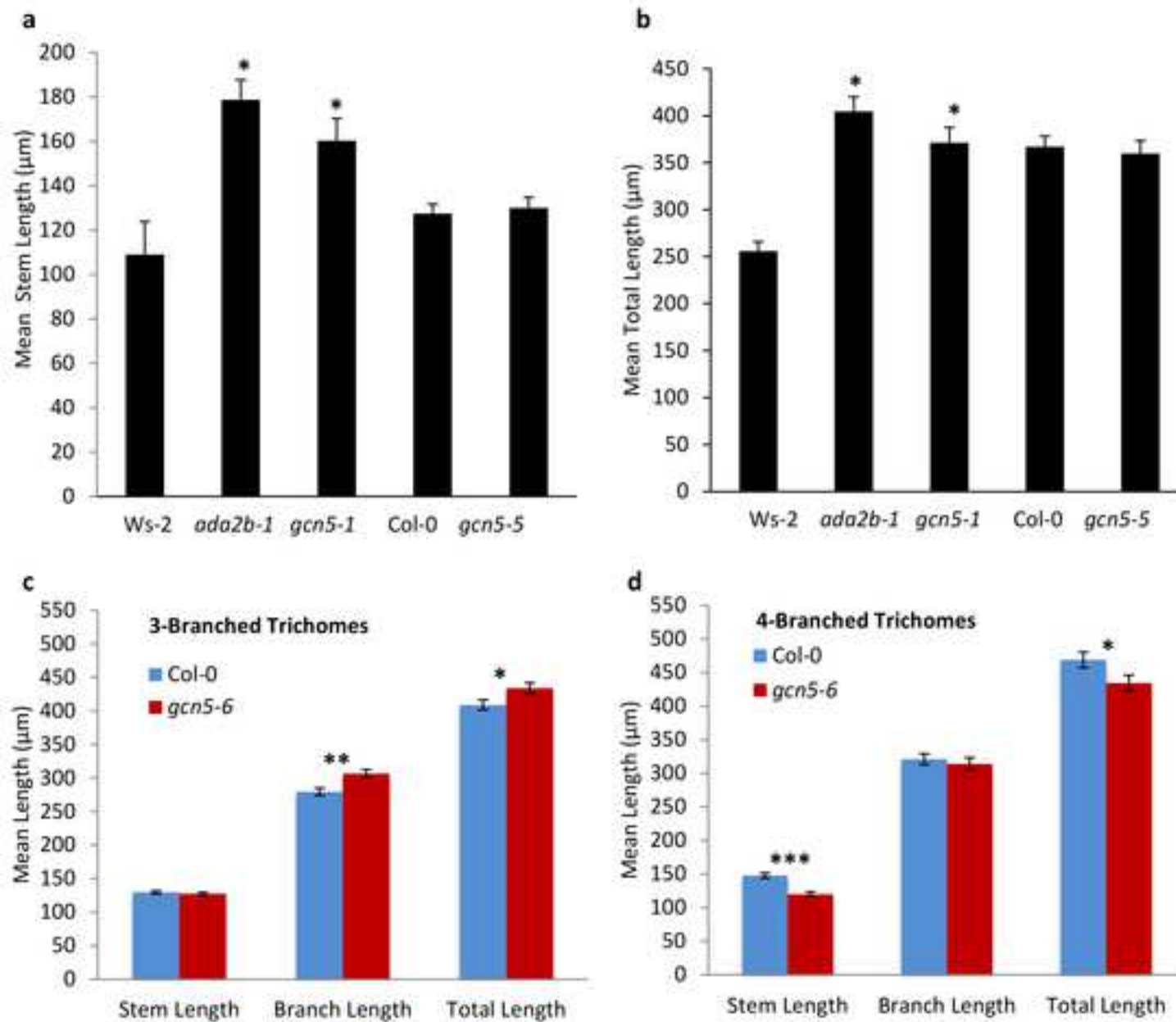
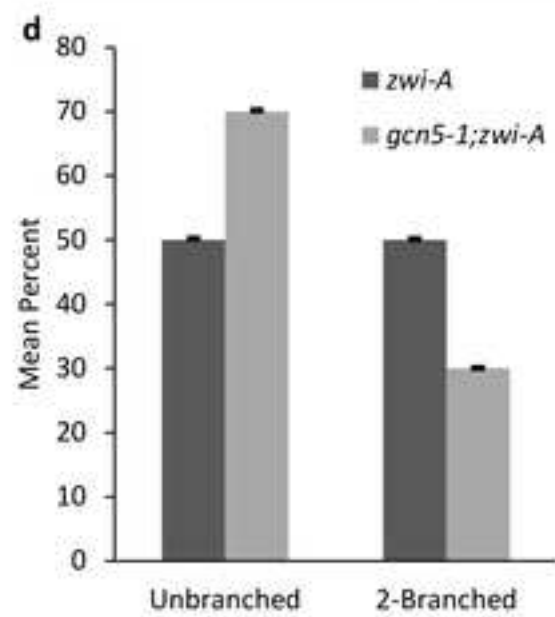
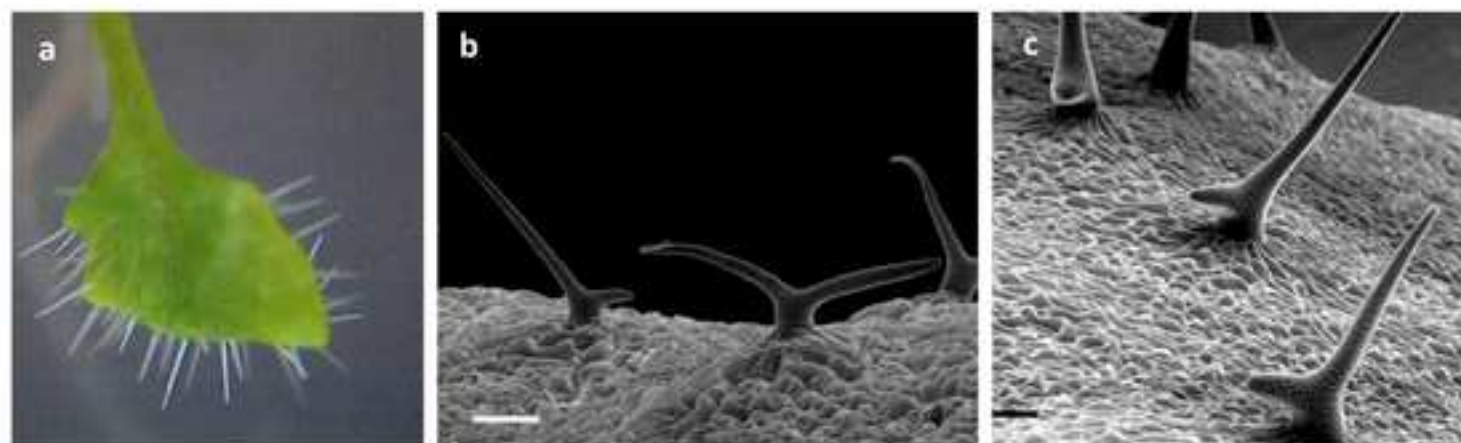




Figure 7



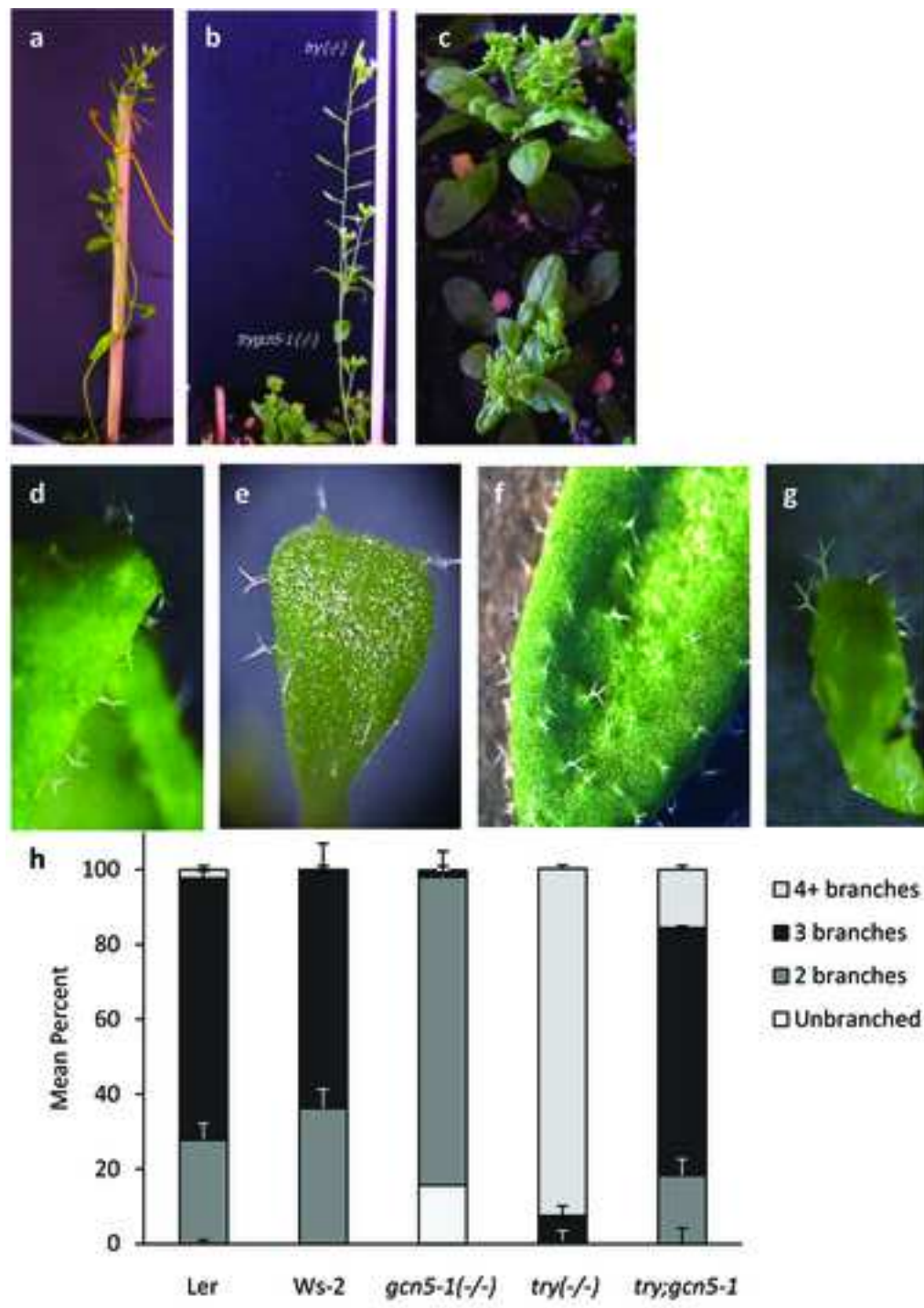


Figure 8

Target	<i>ZWI</i>	<i>KAK</i>	<i>TRY</i>	<i>TRY</i>	<i>GCN5</i>	<i>GCN5</i>	<i>GCN5</i>
Background	<i>gcn5-6</i>	<i>gcn5-6</i>	<i>gcn5-1</i>	<i>gcn5-6</i>	<i>zwi</i>	<i>kak</i>	<i>try</i>
(n)	4	3	5	4	5	3	5

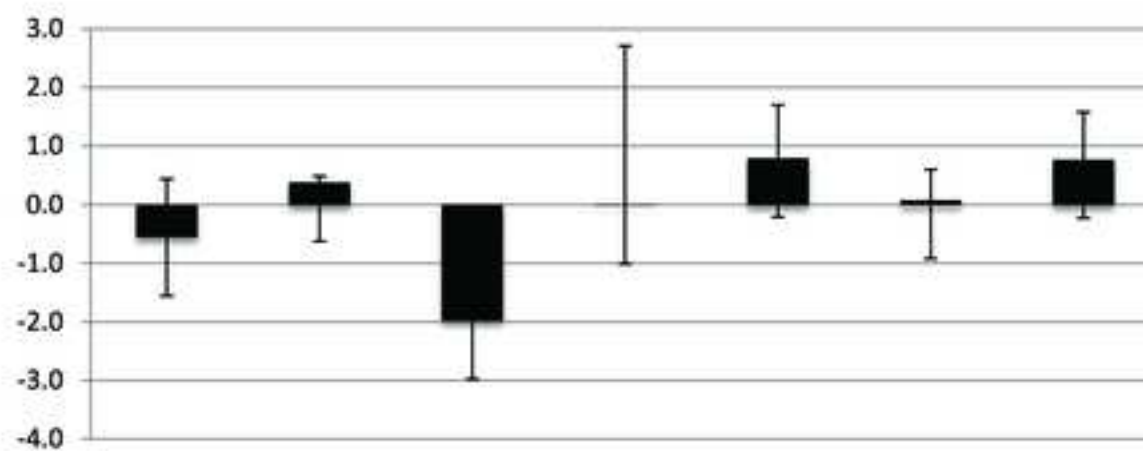


Figure 9

**Table 1.** Summary of mutant allelic backgrounds. References for trichome phenotypes of other *zwi* alleles are given in italics.

<b>Mutant allele</b>	<b>Ecotype background</b>	<b>Type of mutation</b>	<b>Trichome phenotype</b>	<b>References</b>
<i>gcn5-1</i>	Ws-2	T-DNA insertion in last exon, disrupts bromodomain	Reduced branching (present study)	Vlachonasios et al., 2003
<i>gcn5-5/hag1-5</i>	Col-0	T-DNA insertion in intron 10	Trend towards decreased branching (present study)	Long et al., 2006
<i>gcn5-6/hag1-6</i>	Col-0	T-DNA insertion at end of exon 1	Increased branching (present study)	Long et al., 2006
<i>ada2b-1</i>	Ws-2	T-DNA insertion in fifth intron, results in shortened, non-functional transcript	Reduced branching (present study)	Vlachonasios et al., 2003
<i>zwiA</i>	Col-0	T-DNA insertion in third exon	Reduced branching and shortened stalk (present study)	Alonso et al., 2003; Buschmann et al., 2015; <i>Hulskamp et al., 1994</i> ; <i>Oppenheimer et al., 1997</i>
<i>try-EM1</i>	Ler	EMS lesion	Increased branching and trichome clusters	<i>Hulskamp et al., 1994</i>
<i>kak-1</i>	Ler	EMS lesion	Increased branching	<i>Hulskamp et al., 1994</i> ; <i>Perazza et al., 1999</i>





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