# BRANCHLESS TRICHOMES links cell shape and cell cycle control in Arabidopsis trichomes 

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#### Abstract

SUMMARY Endoreplication, also called endoreduplication, is a modified cell cycle in which DNA is repeatedly replicated without subsequent cell division. Endoreplication is often associated with increased cell size and specialized cell shapes, but the mechanism coordinating DNA content with shape and size remains obscure. Here we identify the product of the BRANCHLESS TRICHOMES (BLT) gene, a protein of hitherto unknown function that has been conserved throughout angiosperm evolution, as a link in coordinating cell shape and nuclear DNA content in endoreplicated Arabidopsis trichomes. Loss-of-function mutations in BLT were found to enhance the multicellular trichome phenotype of mutants in the SIAMESE (SIM) gene, which encodes a repressor of endoreplication. Epistasis and overexpression experiments revealed that BLT encodes a key regulator of trichome branching. Additional experiments showed that BLT interacts both genetically and physically with STICHEL, another key regulator of trichome branching. Although b/t mutants have normal trichome DNA content, overexpression of BLT results in an additional round of endoreplication, and b/t mutants uncouple DNA content from morphogenesis in mutants with increased trichome branching, further emphasizing its role in linking cell shape and endoreplication.


KEY WORDS: Arabidopsis, Cell cycle, Endoreduplication, Endoreplication, Trichome

## INTRODUCTION

The acquisition of plant form requires the development of multiple cell types from meristematic cells to form the tissues and organs that make up the mature plant. These diverse cell types must not only be arranged in the correct spatial pattern but also take the correct shape. Indeed, much of plant growth involves the directionally controlled expansion of cells, as opposed to cell proliferation. The cytoskeleton plays an important role in determining plant cell shape by influencing the pattern in which cell wall materials are deposited in expanding cells (Smith and Oppenheimer, 2005; Szymanski, 2009). Cell expansion is achieved by the cell wall yielding to turgor pressure from within, the resultant breakage and reformation of bonds between cell wall components and the controlled deposition of new wall materials (Szymanski and Cosgrove, 2009). The determinants of the polarity of cell expansion remain unclear, although recent work has implicated interactions between Rho family GTPases and cytoskeletal components (Yang, 2008; Szymanski, 2009).

Cell differentiation is also often accompanied by alterations in the cell cycle. These modifications range from simple arrest of the cell cycle in the differentiating cell to patterns of stereotyped

[^0]cell divisions, as are seen in stomatal and root development (Verkest et al., 2005b; Abrash and Bergmann, 2009). One type of altered cell cycle that is commonly seen in large or metabolically active differentiated cells is endoreplication, also known as endoreduplication (Vlieghe et al., 2007). This modified cell cycle involves repeated replication of nuclear DNA without subsequent cell division. The DNA content of endoreplicated cells is typically correlated with cell size (Melaragno et al., 1993; Hülskamp et al., 1994). Although we are beginning to understand the alterations in the plant cell cycle that result in endoreplication (Vlieghe et al., 2007), essentially nothing is known of the mechanisms that coordinate DNA content and cell size in an endoreplicated cell.

Arabidopsis trichomes (shoot epidermal hairs) are large specialized single cells that project from the epidermis of leaves, petioles, sepals and stems. Trichomes have a unique branched cellular architecture; on leaves, they typically have three or four branches (Schellmann and Hülskamp, 2005). Arabidopsis trichomes are well established as a system for studying cell fate determination, cell morphogenesis and endoreplication (Larkin et al., 2007; Pesch and Hülskamp, 2009; Szymanski, 2009). The initiation of trichome development on Arabidopsis leaves is promoted by a transcription factor complex consisting of the bHLH transcription factors GL3 and EGL3, the Myb transcription factors GL1 and MYB23 and the WD-repeat protein TTG. Selection of trichome precursor cells depends on cell-to-cell movement of both the positive regulator TTG and the negative transcriptional regulator TRY and related proteins (Bouyer et al., 2008). One of the earliest events in trichome development is the initiation of endoreplication, and the developing trichome typically undergoes three to four rounds of DNA replication without division during its development (Hülskamp et al., 1994). The developing trichome cell then expands from the plane of the epidermis and typically undergoes two to three branching events, resulting in a total of three to four branches in the mature trichome (Szymanski, 2009).

The final phase of expansion is driven by enlargement of the trichome vacuole, and development concludes with maturation of the thick cellulose cell wall.

Mutations in a number of genes are known to affect endoreplication levels in trichomes (for a review, see Larkin et al., 2007). These include cell cycle components that affect the G1/S transition and DNA replication such as $R B R$ (Desvoyes et al., 2006) and CDC6 (Castellano et al., 2004), and transcriptional regulators of trichome cell fate, such as GL3 and TRY (Hülskamp et al., 1994). In general, mutations in genes that increase or decrease the number of rounds of endoreplication increase or decrease, respectively, trichome cell size and the number of branches. Because both of these classes of genes encode proteins involved in nuclear processes, they presumably influence cell size and branch initiation indirectly. However, essentially nothing is known of the mechanism that coordinates endoreplication with cell size and shape.

Mutations affecting trichome branching and cell shape that do not affect endoreplication are also known (Schellmann and Hülskamp, 2005), and these presumably include factors that function primarily in branch initiation and expansion. The mechanism for polarizing branch initiation sites is not understood, but recent work has focused on two proteins. Recessive loss-offunction mutations in STICHEL (STI), which encodes a protein with sequence similarity to eubacterial DNA polymerase III $\gamma$ subunits (Ilgenfritz et al., 2003), and BRANCHLESS TRICHOMES $(B L T)$, which encodes a protein with no similarity to proteins of known function, both result in trichomes that are completely unbranched. Recently, both of these proteins were localized to newly initiated trichome branch points (Marks et al., 2009), indicating that STI and BLT are excellent candidates for proteins that determine branch sites. Mutations in STI appear to have no effect on endoreplication (Ilgenfritz et al., 2003), and the effect of $B L T$ on endoreplication has not been investigated previously.

The recessive siamese (sim) mutation results in multicellular trichomes in place of the unicellular trichomes of wild-type plants (Walker et al., 2000). The SIM gene encodes a cyclin-dependent kinase inhibitor that is a regulator of endoreplication onset during trichome cell differentiation (Churchman et al., 2006). Here, we report the isolation of a blt mutation based on its genetic enhancement of the sim multicellular trichome phenotype, and show that $B L T$, a major regulator of trichome branch initiation, also regulates endoreplication levels. In addition to its effects on the cell cycle, we show that BLT protein interacts directly with STI in the regulation of branching. This work suggests that BLT, a key regulator of trichome cell shape, also plays a role in integrating endoreplication levels with cell shape.

## MATERIALS AND METHODS

## Plant material and growth conditions

Plants were grown as previously described (Larkin et al., 1999). The loss-of-function blt insertion line SAIL_632_G06.V1 (Columbia ecotype) was obtained from the Arabidopsis Biological Resource Center. The origin of the sim-1 allele used for mutagenesis has been described previously (Churchman et al., 2006). The try-JC allele has also been described previously (Larkin et al., 1999; Schellmann et al., 2002); in the work of Schellman et al. (Schellman et al., 2002), which describes the molecular defect, it is mislabeled as the try-5C allele. The nok-122 and kak-2 alleles (Hülskamp et al., 1994; Folkers et al., 1997) and the GL2pro:GL3 line (Kirik et al., 2001) have been described previously. The $r f i$ allele (Perazza et al., 1999) was obtained from Jean-Marc Bonneville. The gl3-sst allele (Esch et al., 2003) was obtained from M. David Marks. The ethyl methane sulfonate (EMS)-induced sti-AS allele was obtained from Alan Sessions
(University of California, Berkeley). All alleles were either generated in $\mathrm{Col}-0$, or were backcrossed three times to $\mathrm{Col}-0$ before selfing to obtain homozygotes. Putative double mutants were confirmed by complementation, by crossing to wild-type plants and recovering the two parental mutant phenotypes in a segregating F2, or by sequencing.

## Isolation of the blt-3 allele

As described previously (Kasili et al., 2010), homozygous seeds of sim-1 were mutagenized with EMS and collected as 37 pools of $300 \mathrm{M}_{1}$ plants (a total of $11,100 \mathrm{M}_{1}$ plants) and $\sim 2000 \mathrm{M}_{2}$ plants per pool were screened for sim phenotypic modifiers. Plants exhibiting increased multicellularity and clustering of trichomes were selfed. These putative modifiers were then backcrossed three times to the sim-1 mutant, and plants having the enhanced sim phenotype were selected for further work. The presumed double mutants were crossed to Col-0 wild type and the F1 selfed to isolate the modifier as a single mutant. Plants with reduced trichome branching but no multicellular trichomes or trichome clusters were observed in this segregating population. This phenotype segregated as a recessive monogenic trait, and when the new mutant was crossed to either the sim- 1 or sim- 2 alleles it reconstructed the enhanced multicellularity of the original isolate, confirming that this mutant was the sim modifier of interest.

Genetic mapping and identification of BLT as the genetic modifier Using bulked segregant analysis (Lukowitz et al., 2000), the reduced trichome branching phenotype of the modifier was mapped to a region on chromosome I. Genomic DNA from 971 plants from an F2 population of a cross between the mutant (in Col-0) and Landsberg erecta (Ler) was genotyped by PCR with molecular markers generated using the Cereon database of the Columbia and Ler polymorphisms at TAIR (http://www.arabidopsis.org/browse/Cereon/index.jsp). Two restriction enzyme-cleavable PCR-amplifiable markers, F13O11-4 and F1N19-7, were used to localize the mutation within a region of $\sim 70 \mathrm{~kb}$ on two overlapping BAC clones, F1N19 and F13O11. Sequencing genes within this region revealed a G-to-A point mutation in the gene AT1G64690. This gene is known as BRANCHLESS TRICHOMES, and loss-of-function blt mutations reduce trichome branching (Marks et al., 2009). The EMS-induced allele described here was designated blt-3 in accordance with the naming of previously published alleles.

For molecular complementation, the genomic coding sequence of AT1G64690, together with 1447 bp of upstream sequence and 1228 bp of downstream sequence, was PCR amplified from the BAC clone FIN19 using primers ENS1CF ( $5^{\prime}$-CACCTGTGACAGACTTGCTCCTAC-3') and ENS1CR2 ( $5^{\prime}$-CAGCCACATAAGGACCCGAC- $3^{\prime}$ ). This fragment was cloned into the Gateway pENTR vector following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Error-free entry clones were confirmed by sequence analysis before attL recombination into the Gateway destination vector $\mathrm{pMDC100}$, a plant binary transformation vector, to create $\mathrm{pMDC100} / \mathrm{ENS} 1 \mathrm{C}-3.5$, which was then used for transformation of homozygous blt-3 plants.

## Phylogenetic analysis of BLT-related gene families

Predicted protein sequences for BLT, At1G50660, At5g22310, At3g11590 and At3g20350 were obtained from TAIR (http://www.arabidopsis.org) and used in NCBI BLAST searches with the TBLASTN option. The most similar predicted protein sequence to each of the five query sequences in the genomes of Arabidopsis lyrata (L.) O'Kane \& Al-Shehbaz, Vitis vinifera (L.), Ricinus communis (L.) and Populus trichocarpa (Torr. \& A.Gray) and Sorghum bicolor (L.) Moench was retained for analysis. Including the five query sequences, this resulted in 24 protein sequences, predicted from the following GenBank accession numbers: A. thaliana BLT, NM_105144; A. lyrata BLT, XM_002887821; R. communis BLT, XM_002518874; P. trichocarpa BLT, XM_002297906; V. vinifera BLT, XM_002272534; S. bicolor BLT, XM_002467436; At1G50660, NM_103948; At5g22310, NM_122136; At3g11590, NM_101041; At3g20350, NM_112926; A. lyrata-2, XM_002891541; A. lyrata-3, XM_002874015; A. lyrata-4, XM_002882692; R. communis-2, XM_002532506; R. communis-3, XM_002523807; R. communis-4, XM_002530473; P. trichocarpa-2, XM_002316730; P. trichocarpa-3, XM_002328072; P. trichocarpa-4, XM_002309373; V. vinifera-2

XM＿002280300；V．vinifera－3，XM＿002275870；S．bicolor－2， XM＿002448513；S．bicolor－3，XM＿002441284；and S．bicolor－4， XM＿002447617．

Alignment of these 24 predicted protein sequences was performed using Mega4（MEGA Software）using the ClustalW algorithm with default parameters（multiple alignment gap opening penalty＝10；gap extension penalty $=0.2$ ；Gonnet matrix，residue－specific penalties，on；hydrophilic penalties，on；gap separation distance $=4$ ；end gap separation，off；use negative matrix，off；delay divergent cut－off $\%=30$ ）．The phylogeny was built in Mega 4 using neighbor joining with 1000 bootstrap iterations．The resulting tree was arbitrarily rooted between the BLT subfamily and all other paralogs．For the alignment used to produce this tree，see Fig．S3 in the supplementary data，

## Generation of transgenic lines

The full－length $B L T$ coding region was PCR amplified from the BAC clone FIN19 using primers ENSIF（ $5^{\prime}$－CACCATGAAGGATATGAAGA－ －TGCAGAGC－3＇）and ENS1R1（ $5^{\prime}$－TCAAGGAGGAGGAGA－ GGAGAGA－3＇），which contains a stop codon，or ENS1R2（5＇－ AGGAGGAGGAGAGGAGAGAAGAG－3＇），which lacks a stop codon， and cloned into the Gateway vector pENTR following the manufacturer＇s protocol（Invitrogen）．Error－free entry clones were confirmed by DNA sequencing before attL recombination into the Gateway－compatible destination binary vector pLEELA－pGL2（Weinl et al．，2005）for overexpression in developing trichomes．The resulting plasmids were introduced into Agrobacterium tumefaciens by electroporation or by the freeze－thaw method（Weigel and Glazebrook，2002）and subsequently introduced into plants by the floral dip method（Clough and Bent，1998）． Transgenic plants were selected on MS medium containing the relevant antibiotics and then transferred to soil．

## Microscopy

The in situ DNA content of trichomes was measured as described（Walker et al．，2000），and the DNA values were normalized to reported wild－type epidermal cell nuclei values（Melaragno et al．，1993）．Statistical analysis was performed using SigmaStat（Systat Software，San Jose，CA，USA）． Flow cytometry was performed as described（Verkest et al．，2005a）．

Trichomes were examined using scanning electron microscopy（SEM） as previously described（Larkin et al．，1999）．Trichome branches were counted on all trichomes on the adaxial side of the first or second leaf from at least ten plants of each genotype．

## Yeast two－hybrid and bimolecular fluorescence complementation protein－protein interaction assays

Entry clones containing full－length cDNA of BLT or STI or a cDNA fragment of STI（encoding the N－terminal 454 amino acids）were transferred to the Gateway－compatible yeast two－hybrid vectors pAS－attR and pACT－attR by LR recombination following the manufacturer＇s protocol（Invitrogen）and used to transform yeast strain AH109．Assays for $\alpha$－galactosidase activity resulting from expression of the GAL4－responsive MEL1 gene present in the AH109 host strain were conducted as described in the Clontech Yeast Protocols Handbook（PR973283，July 2009）．Six transformed colonies were analyzed per genotype．Results are reported in milliunits（mU）of $\alpha$－galactosidase activity $\mathrm{ml}^{-1}$ cell ${ }^{-1}$ ；one unit of $\alpha$－ galactosidase activity is defined as the amount of enzyme that hydrolyzes $1 \mu$ mole p－nitrophenyl－$\alpha$－D－galactoside to p－nitrophenol and D－galactose in 1 minute at $30^{\circ} \mathrm{C}$ in acetate buffer（ pH 4.5 ）（Aho et al．，1997）．

For bimolecular fluorescence complementation（BiFC），entry clones of full－length $B L T$ and $S T I$ were fused with the C－terminal and N －terminal split fragments of YFP，respectively，in the BiFC pBatTL vectors（Uhrig et al．，2007）by Gateway recombination，and were introduced into onion cells by biolistic bombardment．

## RESULTS <br> Identification of blt as a genetic enhancer of the sim multicellular trichome phenotype

siamese－1（sim－1）is a loss－of－function mutation that results in multicellular trichomes that occur in clusters（Fig．1A，B；Table 1）． SIM encodes a CDK inhibitor that binds to CYCD－CDK


Fig．1．Identification of a genetic enhancer of sim multicellular trichomes．（A－C，E－H）Scanning electron micrographs（SEMs）of（A） wild－type（WT，Col－0），（B）sim－1，（C）blt－3 sim－1 double－mutant，（E）blt－ 3，（F）blt－1，（G）sti－AS and（H）sti－AS sim double－mutant Arabidopsis trichomes．Arrows indicate representative cell junctions．（D）Transverse section of a blt－3 sim－1 double－mutant trichome stained with Toluidine Blue．Arrows indicate individual cells．Scale bars： $50 \mu \mathrm{~m}$ in $\mathrm{A}, \mathrm{D}, \mathrm{H}$ ； $100 \mu \mathrm{~m}$ in B，E－G．
complexes and regulates endoreplication onset in Arabidopsis （Churchman et al．，2006；Peres et al．，2007）．In a search for genetic modifiers of the sim mutant trichome phenotype，we identified a recessive mutation that greatly enhanced the multicellularity of the sim mutant trichome phenotype in double mutants（Fig．1C，D； Table 1）．When separated from sim，the single mutant of this

Table 1．b／t increases the multicellularity of sim mutant trichomes

| Genotype | No．of nuclei per trichome＊ | Total trichomes $^{\dagger}$ |
| :--- | :---: | :---: |
| Col－0 | $1.0 \pm 0.0$ | 50 |
| blt－3 | $1.0 \pm 0.0$ | 47 |
| sim－1 | $2.2 \pm 1.2$ | 64 |
| blt－3 sim－1 | $9.8 \pm 3.3$ | 40 |
| sti | $1.0 \pm 0.0$ | 50 |
| sti $\operatorname{sim}$ | $1.2 \pm 0.5$ | 50 |

[^1]

Fig．2．BLT overexpression increases branching．SEMs of trichomes of（A） Col－0 wild－type，（B）blt－3 and（C） GL2 pro：BLT in Col－0．Scale bars： $100 \mu \mathrm{~m}$ ．
modifier produces predominantly unbranched trichomes on leaves （Fig．1E）．Root growth，plant size，growth rate，floral structure and general appearance of these single mutants were identical to those of wild type，and the number of trichomes on single－mutant leaves was indistinguishable from that of wild－type leaves，indicating that the mutant phenotype is primarily or exclusively limited to post－ initiation trichome development．

The unbranched trichome mutation was mapped，and sequencing of candidate genes revealed a point mutation in the gene AT1G64690，which has been named BRANCHLESS TRICHOMES （BLT）（Marks et al．，2009）．The genetic modifier responsible for enhancing the sim phenotype was shown to be allelic to blt by the following criteria．First，the trichome mutant identified here produced unbranched trichomes that were phenotypically identical to those of the blt－1 reference allele（Fig．1F），and the two mutations failed to complement．The phenotype of our unbranched trichome allele was also completely rescued by a genomic DNA fragment containing BLT（see Fig．S1 in the supplementary material）．Finally，when the blt－1 reference allele was combined with either of two sim alleles，it produced an enhancement of the sim multicellular trichome phenotype identical to that seen with our initial blt allele（see Fig．S2 in the supplementary material）．These results indicate that the gene responsible for the genetic enhancement of cell division in sim mutants observed in our screen was $B L T$ ，a gene with no known role in the cell cycle．

In accordance with the naming of blt alleles in previous work （Marks et al．，2009），we refer to the point－mutant allele generated in our screen as blt－3．This allele contains a G－to－A mutation in AT1G64690 that changes the amino acid tryptophan（TGG）to a stop codon（TGA）at codon 64 ，eliminating more than $70 \%$ of the predicted protein．Thus，$b l t-3$ is likely to be a null allele．The $b l t-3$ allele was used for the studies described below unless otherwise indicated．
Loss－of－function mutations in another gene，STI，result in unbranched trichomes very similar in phenotype to those of the blt mutants（Fig．1G）（Ilgenfritz et al．，2003）．Double－mutant sti sim plants，however，did not exhibit an increase in the number of cells per trichome relative to $\operatorname{sim}$ trichomes（Fig．1H；Table 1）．Thus，the
dramatic enhancement of the sim multicellular trichome phenotype seen in sim blt double mutants（Fig．1C，D；Table 1）represents a unique link between trichome branching and the cell cycle．

## BLT represents an ancient plant protein lineage of unknown function

The 273 amino acid product of BLT predicted by conceptual translation does not closely resemble any protein of known function， although sequences similar to $B L T$ are found in most，if not all， angiosperm genomes（see Fig．S3 in the supplementary material）． Additionally，four other Arabidopsis genes（AT3G20350， AT1G50660，AT5G22310 and AT5G22310），all of unknown function，encode proteins that share the predicted amino acid sequence REERVQMKL（residues 161－169 of BLT），as well as other limited similarity with BLT．There is a clear and strongly supported monophyletic clade of $B L T$－related genes in all of the angiosperm species examined，indicating that the $B L T$ clade originated before the last common ancestor of monocots and dicots（see Fig．S4 in the supplementary material）．This suggests that $B L T$ encodes an ancient conserved function，which is represented in the Arabidopsis genome only by $B L T$ itself．Sequences with similarity to $B L T$ also occur in Physcomitrella patens（Hedw．）Bruch \＆Schimp，but the sequences are from partial cDNAs and it is not possible to determine whether they represent $B L T$ orthologs or paralogs．

## BLT is a key positive regulator of trichome branching

$B L T$ was initially identified among a number of genes in a transcriptomics study of trichome development（Marks et al．， 2009）．To further investigate the role of $B L T$ in trichome branching， transgenic lines expressing $B L T$ from the $G L 2$ promoter，$G L 2_{\text {pro，}}$ were created．In leaves，this strong promoter drives expression predominantly in developing trichomes．Numerous transgenic lines were obtained，and most independent $G L 2_{\text {pro }}: B L T$ lines showed increased trichome branching（Fig．2）；counts of trichome branch points from three representative lines are shown in Table 2．Thus， when expressed from a strong trichome promoter，BLT expression actively promotes trichome branching．

Table 2．Plants overexpressing BLT have increased branching

| Genotype | No．of branch points＊ |  |  |  |  |  |  | No．of trichomes ${ }^{\dagger}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | 0 | 1 | 2 | 3 | 4 | 5 | 6 |  |
| Col－0 | 0.0 | 0.0 | 73.9 | 26.1 | 0.0 | 0.0 | 0.0 | 119 |
| blt－3 | 87.3 | 12.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 106 |
| GL2 pro：BLT line 1 | 0.0 | 0.0 | 25.9 | 30.2 | 29.3 | 12.9 | 1.7 | 116 |
| GL2 pro：$^{\text {SLIT }}$ line 2 | 0.0 | 0.0 | 32.3 | 36.5 | 23.9 | 7.3 | 0.0 | 96 |
| GL2 pro：BLT line 3 | 0.0 | 1.1 | 30.1 | 43.0 | 22.6 | 3.2 | 0.0 | 93 |

[^2]

Fig. 3. b/t is epistatic to mutations that increase trichome branching. SEMs of (A) Col-0, (B) blt-3, (C) try-JC, ( $\mathbf{D}$ ) blt-3 try-JC, (E) kak-2, (F) blt-3 kak, (G) GL2 pro:GL3, (H) blt-3 GL2 pro:GL3, (I) gl3-sst and (J) blt-3 gl3-sst Arabidopsis trichomes. Scale bars: $100 \mu \mathrm{~m}$.

The necessity for $B L T$ function in trichome branching was also addressed by constructing double mutants between blt and triptychon (try), noeck (nok), kaktus (kak), rastafari (rfi), the GL3
gain-of-function allele glabra3-shapeshifter (gl3-sst) and the GL3 overexpression construct $G L 2_{p r o}: G L 3$. Each of these latter mutants and constructs produces substantial numbers of trichomes with more than four branch points (i.e. at least five branches) (Folkers et al., 1997; Perraza et al., 1999; Esch et al., 2003). In the double mutants, the blt mutation greatly reduced trichome branching relative to the second mutation on its own, and very few trichomes had more than one branch point (Fig. 3; Table 3). Both blt alleles that we examined produced at least a few trichomes with one branch point (Table 3). Thus, blt is essentially epistatic to try, nok, kak and rfi. In double mutants, blt mutation also restricted the branching potential resulting from the gl3-sst gain-of-function allele or the artificially engineered GL3 gain-of-function resulting from expression from the GL2 promoter (Fig. 3), although owing to the complex branching pattern and trichome clustering that occur in these GL3 gain-of-function situations it was difficult to quantitate the degree of trichome branching.
It should be noted that the sole existing $r f i$ allele, as used here, contains a C-to-T point mutation in the GL3 gene that converts codon 116 from GCC (Ala) to GTC (Val). This $r f i$ allele maps close to $g l 3$ on chromosome five. Thus, $r f i$ might be another $g l 3$ gain-offunction mutation.

## BLT couples cellular morphogenesis and endoreplication in trichomes

In many, but not all, cases, a positive correlation exists between the average number of trichome branches associated with a particular genotype and the degree of endoreplication of trichome nuclear DNA (Hülskamp et al., 1994; Larkin et al., 2007). One mutant that does not show such a correlation is sti; trichomes of sti mutant plants have little branching, but have a nuclear DNA content similar to that of wild type (Ilgenfritz et al., 2003). Similarly, we found that blt mutation had no effect on trichome nuclear DNA content (Fig. 4A) or on the nuclear DNA content of leaf cells (Fig. 4B). These results indicate that, like STI, BLT function is not required for endoreplication. However, three $G L 2_{p r o}: B L T$ overexpression lines all showed a significant increase in DNA content relative to wild-type or blt mutant trichomes (Fig. 4C). This result suggested that BLT function is necessary to couple trichome branching to the degree of endoreplication. To test this hypothesis, we examined the nuclear DNA content of blt gl3-sst and blt try double mutants in comparison to the respective single mutants, $g l 3$ -

Table 3. Loss of BLT function limits branching potential in double mutants

| Genotype | No. of branch points* |  |  |  |  |  |  |  | No. of trichomes ${ }^{\dagger}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |  |
| Col-0 | 0.0 | 0.0 | 93.4 | 6.6 | 0.0 | 0.0 | 0.0 | 0.0 | 244 |
| b/t-3 | 82.9 | 17.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 258 |
| try-JC | 0.0 | 0.0 | 1.3 | 11.5 | 36.5 | 36.2 | 12.2 | 2.3 | 304 |
| b/t-3 try-JC | 55.7 | 37.8 | 6.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 246 |
| kak-2 | 0.0 | 0.0 | 0.6 | 14.5 | 33.0 | 35.8 | 11.7 | 4.3 | 324 |
| b/t-3 kak-2 | 16.9 | 83.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 242 |
| nok | 0.0 | 1.5 | 26.1 | 50.7 | 20.1 | 1.5 | 0.0 | 0.0 | 134 |
| blt nok | 34.3 | 65.1 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 169 |
| rfi | 0.0 | 0.0 | 0.4 | 21.3 | 36.8 | 31.6 | 7.6 | 2.2 | 225 |
| blt-3 rfi | 56.4 | 43.3 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 307 |
| gl3-1 | 63.2 | 33.5 | 3.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 151 |
| blt-3 gl3-1 | 97.7 | 2.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 299 |

[^3]

Fig. 4. BLT overexpression increases nuclear DNA content. (A) In situ measurements of trichome nuclear DNA content of b/t-3 and sti-AS loss-of-function mutants. Relative fluorescence units (RFU) have been normalized to approximately correspond to haploid genome equivalents ( $C$ values). None of the pairwise comparisons among these three genotypes is significant $[P>0.05$, Kruskal-Wallis one-way ANOVA on ranks and an all pairwise multiple comparison test (Dunn's test)]. (B) Nuclear DNA content of 21-day-old wild-type (Col) and blt Arabidopsis leaves, as determined by flow cytometry. The percentage of nuclei in each ploidy class is indicated. Error bars indicate s.d. (C) In situ measurements of trichome nuclear DNA content of $G L 2_{\text {pro }}: B L T$ overexpression lines. All three $G L 2_{\text {pro }}: B L T$ lines differ significantly from wild type and blt ( $P<0.05$, Kruskal-Wallis one-way ANOVA on ranks and Dunn's test). Data in A and C are presented as box plots, in which the box encompasses the 25th through to the 75th percentile of the data, the line within the box is the median (50th percentile), and the error bars represent the 5th (lower bar) and the 95th (upper bar) percentiles.


Fig. 5. Loss of BLT function uncouples DNA content from morphogenesis in increased branching mutants. (A) Comparison of $g / 3$-sst and blt-3 g/3-sst DNA contents. Based on a Kruskal-Wallis one-way ANOVA on ranks and Dunn's test, all pairwise comparisons differ significantly $(P<0.05)$, except for $g / 3$-sst versus blt-3 gl3-sst and for wild type versus blt. (B) Comparison of try and blt-3 try. In a Kruskal-Wallis one-way ANOVA on ranks and Dunn's test, all pairwise comparisons differ significantly $(P<0.05)$, except for try-JC versus b/t try$J C$ and for wild type versus blt. Box plot data are presented as described in Fig. 4. RFU are normalized to approximately correspond to haploid genome equivalents (C values).
sst and try, both of which result in increased branching and nuclear DNA content on their own. Both double mutants exhibited an elevated DNA content that was not significantly different from that of the corresponding single mutant (Fig. 5A,B), even though blt substantially restricts branching in the double mutants (Fig. 3D,J; Table 3). This was particularly striking for the gl3-sst allele. Although it was difficult to accurately count the number of branches on trichomes of plants homozygous for this $g l 3$ gain-offunction allele owing to highly variable branch expansion and trichome shape, it was clear that $\mathrm{gl3}$-sst single mutants have an increased number of branch points per trichome, and trichomes having greater than four branch points were common (Fig. 3I) (Esch et al., 2003). In spite of an obvious and dramatic reduction of branching in blt gl3-sst trichomes (Fig. 3J), these trichomes had a median DNA content that was more than fourfold higher than that of wild type and indistinguishable from that of gl3-sst singlemutant trichomes (Fig. 4A).


Fig. 6. Partial intergenic non-complementation between blt and sti. SEMs of (A) blt-3, (B) sti-AS, (C) blt-3/+ sti-AS/+ F1 and (D) blt-3 stiAS homozygous double-mutant Arabidopsis trichomes. Scale bars: $100 \mu \mathrm{~m}$.

In a separate experiment, we found that, whereas individual sim nuclei had a mean DNA content of $12.4 \pm 7.3$ relative fluorescence units (RFU), individual blt sim nuclei had a mean DNA content of 3.1 $\pm 3.3$ RFU. The DNA content of blt sim nuclei was significantly different from that of both sim and blt $[P<0.05$, Kruskal-Wallis one-way ANOVA on ranks and an all pairwise multiple comparison test (Dunn's test)]. When these data were combined with the number of nuclei per trichome initiation site for each genotype (Table 1), the total nuclear DNA content per trichome initiation site was similar in the two genotypes (27.2 RFU for sim and 30.4 for blt sim). Thus, in the blt sim double-mutant combination, blt increases mitotic divisions above the degree of division seen in the sim single mutant, without affecting the number of cycles of DNA replication.

We also considered the hypothesis that, although blt mutations do not affect the level of endoreplication reached by mature trichomes, they might prolong a juvenile developmental stage permissive for division, in which case we might see an alteration in the timing of endoreplication in early stages of blt trichome development. It is difficult to obtain accurate in situ DNA content values from early trichome developmental stages owing to the high background and close spacing of cells. Therefore, as a proxy for
endoreplication in early trichome development stages, we used the widest cross-sectional area of stage one and stage two trichome nuclei, i.e. the nuclei of trichomes that are less than twice as tall as they are wide, prior to the initiation of branching in wild-type trichomes (Szymanski et al., 1998). The nuclei of surrounding protodermal cells, which were still dividing, were also examined. Because the nuclei of these cells appear to be essentially spherical, this cross-sectional area should be indicative of cell volume (Walker et al., 2000). No difference was found between wild-type and blt nuclei for early-stage developing trichomes or dividing protodermal cells (see Table S1 in the supplementary material), indicating that there is no obvious alteration in the timing of endoreplication in the early development of blt trichomes.

## BLT interacts both genetically and physically with STI, another positive regulator of trichome branching

Mutations in only two genes, blt and sti, result in trichomes that are unbranched but otherwise develop normally. As noted by Marks et al. (Marks et al., 2009), blt sti double mutants resemble the two parental mutants, a result that is consistent with the two gene products acting in the same pathway. However, in constructing this double mutant, we noticed that $25-30 \%$ of the trichomes on F1 plants heterozygous for both $b l t$ and sti had only a single branch point (i.e. were two-branched; Fig. 6; Table 4), a phenotype that is almost never seen in the Col- 0 wild-type background in which both mutations were derived, and is rare (less than 3\%) in F1 heterozygotes between either blt or sti and wild type. Thus, blt and sti exhibit substantial intergenic non-complementation.

Intergenic non-complementation is often observed in cases in which two protein products act as part of the same complex (Huffaker et al., 1987). We tested the hypothesis that the BLT and STI proteins interact using two methods. Yeast two-hybrid experiments demonstrated a clear interaction between the N terminal 472 amino acids of STI and full-length BLT (Fig. 7A-D; Table 5). Interaction between STI and BLT was confirmed by in planta bimolecular fluorescence complementation (BiFC) experiments in which full-length $S T I$ and $B L T$ coding regions were respectively fused to the N -terminal and C-terminal split domains of YFP and co-bombarded into onion cells, which resulted in YFP fluorescence indicative of close association of the two test proteins (Fig. 7E,F).

We also investigated the localization of BLT and STI fluorescent protein fusions in developing trichomes of $s t i$ and $b l t$ mutants, respectively, using transient expression of $Y F P$ fusions introduced into developing trichomes via biolistic bombardment of developing

Table 4. Genetic interaction between blt and sti for trichome branching phenotype

|  | No. of branch points* |  |  |  |  |  |
| :--- | :---: | :---: | :---: | :---: | :---: | :---: |
| Genotype | 0 | 1 | 2 | 3 | 4 | No. of trichomes ${ }^{\dagger}$ |
| Col-0 | 0.0 | 0.0 | 78.0 | 12.8 | 0.2 | 392 |
| blt-3 | 90.7 | 9.3 | 0.0 | 0.0 | 0.0 | 236 |
| sti-AS | 99.7 | 0.3 | 0.0 | 0.0 | 0.0 | 292 |
| Col-0 $\times$ blt-3 F1 (heterozygote) | 0.0 | 2.7 | 97.3 | 0.0 | 0.0 | 480 |
| Col-0 $\times$ sti-AS F1 (heterozygote) | 0.0 | 2.8 | 96.8 | 0.4 | 0.0 | 497 |
| blt-3 $\times$ sti-AS F1 (heterozygote) | 0.0 | 31.3 | 68.7 | 0.0 | 0.0 | 399 |
| blt-3 sti-AS | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 | 368 |

Based on a Kruskal-Wallis one-way ANOVA on ranks and an all pairwise multiple comparison test (Dunn's test), branch counts of b/t-3 $\times$ sti-AS F1 trichomes differed significantly ( $P<0.05$ ) from both Col- $0 \times b l t-3$ F1 trichomes and Col- $0 \times$ sti-AS F1 trichomes, whereas Col- $0 \times$ sti-AS F1 trichomes and Col $-0 \times$ sti-AS F1 trichomes did not differ significantly.
*Shown is the percentage of trichomes having the indicated number of branch points (one branch point indicates a trichome with two branches). Counts were made on ten first or second leaves per genotype.
${ }^{\dagger}$ Total number of trichomes on which branches were counted.


Fig. 7. Interaction between BLT and STI in yeast two-hybrid and BiFC assays. (A-D) Yeast two-hybrid interaction of BLT with a domain of STI that comprises the N-terminal 454 amino acids. Yeast strain AH109 cells co-expressing bait and prey plasmids were allowed to grow on -Trp/-Leu plates and then on -Trp/-Leu/-His+3AT to test for proteinprotein interaction. (A) pBD-STI $N$-term/pAD-BLT, (B) pBD-SNF1/pAD-SNF4 (positive control), (C) pBD-STI N-term/pAD-GUS (negative control) and (D) pBD-GUS/PAD-BLT (negative control). (E) Confocal image of interaction between BLT and STICHEL (STI) using BiFC. BLT and STI fused with the Cterminal and $N$-terminal split fragments of YFP, respectively, were cobombarded into onion cells. The YFP fluorescence (yellow) from the BiFC complex serves as an indicator of the interaction between BLT and STI proteins. When both fusion clones were co-bombarded, 20-30 YFPpositive cells were observed in each of four independent experiments. No YFP-positive cells were observed when either fusion was bombarded individually, or in bombardments with empty vector. (F) Phase-contrast image of the cell shown in E. Scale bars: $50 \mu \mathrm{~m}$.
leaves (Fig. 8). In all sti early-stage trichomes expressing YFP:BLT, YFP fluorescence was localized apically, similar to the YFP:BLT localization seen in wild type (Fig. 8A,B). Twelve of fifteen blt cells expressing STI:YFP also exhibited the apical localization observed for STI:YFP in wild type (Fig. 8C,D), whereas three cells exhibited
a more lateral localization of the fluorescent signal not seen in wildtype controls. No trichomes in either experiment showed detectable nuclear-localized fluorescence.

## DISCUSSION

In most genotypes, the degree of endoreplication is highly correlated with trichome branching (Hülskamp et al., 1994; Larkin et al., 2007), but the mechanism that maintains this correlation is unknown. The cell polarity mechanism involved in trichome branch initiation is likewise poorly understood. Here, we have found that the product of the $B L T$ gene, previously known as a regulator of trichome branch initiation (Marks et al., 2009), can also influence the cell cycle in developing trichomes. This suggests that BLT is a component of the mechanism that coordinates branch initiation and the endoreplication cell cycle in developing trichomes.

The $B L T$ gene is unique in the Arabidopsis genome, with the most closely related Arabidopsis genes clearly being paralogs (see Fig. S3 in the supplementary material). Both monocots and dicots have BLT homologs (see Fig. S3 in the supplementary material), indicating that the function of the gene product has been conserved throughout angiosperm evolution, although neither BLT nor its paralogs resemble any genes of known function. Marks et al. predicted that the BLT protein sequence has a high probability of adopting a coiled-coil structure throughout much of its length (Marks et al., 2009). Based on data collated at the Genevestigator website (Hruz et al., 2008), BLT is widely expressed in Arabidopsis, with the highest levels seen in testa, the root hair zone and petals. The transcriptional pathways that control mucilage formation in the testa and root epidermal fate share transcription factors with the trichome cell fate pathway. However, we detected no defects in mucilage formation or root hair development in blt mutants (our unpublished observations).

Overexpression of BLT resulted in the initiation of supernumerary branches (Fig. 2; Table 2). In double-mutant combinations with increased trichome branching mutants, a blt mutation severely restricted branching and was essentially epistatic to the increased branching mutant in each case (Fig. 3; Table 3). Thus, BLT function is essential for branching, and, when overexpressed in wild type, $B L T$ is sufficient to promote branch initiation, properties that are expected of a major regulator of branch initiation. These results significantly strengthen the evidence indicating that $B L T$ plays a key role in trichome branch initiation.
Perhaps most significantly, the work presented here indicates that BLT forms a protein complex with STI, another protein directly involved in branch initiation. Four lines of evidence support this conclusion. First, F1 heterozygotes resulting from crosses between blt and sti show a degree of intergenic noncomplementation for branching, producing significant numbers of two-branched trichomes (trichomes with a single branch point; Fig. 6 C ), whereas the wild type and either individual mutant

Table 5. Yeast two-hybrid screen for interaction of BLT with STICHEL

| Bait | Prey | mM 3AT |  |  |  | $\alpha$-galactosidase ${ }^{\dagger}$ | $n$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | 0 | 3 | 5 | 10 |  |  |
| STI-N term | BLT | + | + | + | + | $1.7 \pm 0.3$ | 6 |
| STI-N term | GFP* | + | - | - | - | $0.1 \pm 0.1$ | 6 |
| GFP* | BLT | - | - | - | - | $0 \pm 0$ | 6 |

A $t$-test indicated that the $\alpha$-galactosidase activity resulting from the STI-N term/BLT combination differed significantly from that of the STI-N term/GFP negative control combination ( $P<1 \times 10^{-6}$ ).
+, Positive interaction; -, no interaction. 3AT, 3-amino-1,2,4-triazole
*, Negative control.
${ }^{\dagger}$ Milliunits of $\alpha$-galactosidase activity $\mathrm{ml}^{-1}$ cell ${ }^{-1}$. $\alpha$-galactosidase activity results from expression of the GAL4-responsive MEL1 reporter present in the AH109 host strain. Six independent colonies of each genotype were assayed for $\alpha$-galactosidase activity.

YFP:BLT


Fig. 8. Localization of YFP:BLT in sti and of STI:YFP in blt by transient expression in biolistically transformed developing trichomes. $(\mathbf{A}-\mathbf{J})$ To reduce the total number of bombardments needed, cpc mutant plants, which have an increased number of trichomes on leaves, were used as the wild-type control. Preliminary experiments demonstrated that CPC and cpc plants exhibit an identical apical localization of YFP:BLT and STI:YFP. YFP fluorescence (A-E) and bright-field (F-J) images are shown for each of the developing trichomes. Scale bars: $5 \mu \mathrm{~m}$.
heterozygote produce predominately three-branched trichomes (trichomes with two branch points) and very few two-branched trichomes (Table 4). Such intergenic non-complementation is often observed between components of a protein complex; examples include yeast (Huffaker et al., 1988) and Drosophila (Hays et al., 1989) $\alpha$ - and $\beta$-tubulin, components of a yeast G-protein signaling cascade (Akada et al., 1996), and the trichome cell fate transcription complex in Arabidopsis (Larkin et al., 1999). Second, full-length BLT interacted strongly with the N-terminal domain of STI in a yeast two-hybrid assay (Fig. 7A; Table 5), although interaction between BLT and full-length STI was at best weak (our unpublished observations). Third, full-length BLT and full-length STI interact in BiFC assays in living onion epidermal cells (Fig. 7E). Finally, Marks et al. have shown that biologically functional fluorescent protein fusions of both BLT and STI co-localize in developing trichome branch tips (Marks et al., 2009).

Taken together with the gain-of-function and loss-of-function phenotypes of blt and sti, these results make a strong case for a protein complex that contains both BLT and STI functioning as a key polarity determinant in trichome branch initiation. As with BLT, little functional information for the STI protein can be inferred from its protein sequence. STI has sequence similarity to eubacterial DNA polymerase III $\gamma$-subunits (Ilgenfritz et al., 2003), but given that sti mutants have no effect on DNA replication and that it appears to be localized in the cytoplasm, at the branch tips, the significance of this similarity is unclear. Nonetheless, the identification of a protein complex involved in branch initiation opens up new avenues to an understanding of cell shape using the trichome model. The primary role of BLT would thus seem to be at the branch initiation sites in a complex with STI. Not only do the proteins interact, but the effects on trichome branch initiation of manipulating $B L T$ or $S T I$ function are identical: loss-of-function mutations in either gene virtually eliminate branching, whereas overexpression of either gene results in increased branching. By contrast, only manipulation of $B L T$ function affects endoreplication: BLT overexpression
increases trichome nuclear DNA content (Fig. 4C), whereas STI overexpression has no effect on DNA content (Ilgenfritz et al., 2003).

Our data suggest that, in addition to its role in promoting branch formation, BLT functions in a signaling mechanism that originates at the branch tips and maintains homeostasis between the number of branches and nuclear DNA content. Given that blt loss-of-function mutants do not exhibit reduced endoreplication, BLT is presumably not directly necessary for endoreplication, but functions to increase endoreplication to 'catch up' if branching increases faster than DNA content. Excess free BLT that is not localized to branch points does not appear to be the signaling molecule as nuclear BLT has not been detected, and YFP:BLT is not detectably mislocalized in developing sti mutant trichomes (Fig. 8). We also found that, although blt mutations increase cell division in combination with $\operatorname{sim}$ (Fig. 1C,D; Table 1), blt sim mutant trichome initiation sites have the same total DNA content for all nuclei as sim trichome initiation sites, indicating again that blt loss-of-function has no effect on the number of rounds of DNA replication. Thus, BLT must also have a minor second function in suppressing mitosis in trichomes that is revealed only when the primary mitosis-suppressing function is compromised by a $\operatorname{sim}$ mutation.

A candidate nuclear target of such a signaling pathway linking branching and endoreplication is Chromatin assembly factor-1 (CAF-1), which is involved in the replication-dependent deposition of histones H3 and H4 onto DNA (Groth et al., 2007). Mutations in CAF-1 subunits result in trichomes with increased branching but normal endoreplication (Exner et al., 2006). Mutations in sti are epistatic to mutations affecting two different CAF-1 subunits, and CAF-1 function is necessary for the extra endoreplication cycles that result from kak mutations (Exner et al., 2008). CAF-1 thus resembles BLT in having a primary role in trichome branching and a more cryptic role in endoreplication. Several cell fate transcription factors affect the degree of endoreplication when mutated or overexpressed (Larkin et al., 2007), and these constitute other possible nuclear targets of such a pathway.

The results presented here provide insight into the coordination of cell shape and DNA content in an endoreplicating cell, as well as advancing our understanding of a protein complex that is involved in specifying cell shape. It has previously been speculated that trichome branching mechanisms were derived from the cell division machinery (Schnittger and Hülskamp, 2002). Although our results do not directly support this idea, the work presented here opens new avenues for investigating the relationship between cell shape, cell differentiation and the cell cycle.

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## Competing interests statement

The authors declare no competing financial interests.

## Supplementary material

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Supplemental data file: CLUSTAL W (1.8) multiple sequence alignment of 24 BLT-related predicted protein sequences.

A-thaliana-BLT
A-lyrata-BLT
R-communis-BLT
S-bicolor-BLT
P-trichocarpa-BLT
V-vinifera-BLT
At1G50660
A-lyrata-2
At5g22310
A-lyrata-3
A-lyrata-4
At3g11590
P-trichocarpa-3
P-trichocarpa-4
At 3 g 20350
P-trichocarpa-2
R-communis-2
R-communis-3
R-communis-4
S-bicolor-2
S-bicolor-3
S-bicolor-4
V-vinifera-2
V-vinifera-3

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R-communis-3
R-communis-4
S-bicolor-2
S-bicolor-3
S-bicolor-4
V-vinifera-2
V-vinifera-3

MKVTGKPYSTATLPAFES-DKNLFSHGPDLRAIQRATTVTKRRARNPS-L MKVTGKPYSTATLPAFES-DKNLFSHGPDLRAIQRATTVTKRRARNPS-L MEQRKKG---------CKIRKRGGSSSSS--SSLARR-NRFKRAIFAGKR ME-RKRG---------CKIRKRGGSSSSS--SSLARR-NRFKRAIFAGKR MPRQNQS--VENLLLLGKIRKRGCSSPTSSTSSILREGYRFKRAIVVGKR MPRQNQS--VENLLLLGKIRKRGCSSPTSSTSSILREGYRFKRAIVVGKR MEAKSREQ------QQCKIRKRGNSSSSS--SSLVKK-YRFKRAILVGKR MPRQKGS-ATMEELLPGKIRKRVCSSSASSSSSIIQN-YRFKRAILVGKR MKFTGKSNLTATLPAT----------VPNIRDIHR------RRARKPS-F

MKITGRPHSTPTFPGNTITCSR-APQNPQPHPDLKPHHRLRKTARTPV--MESKSRE--------HCKIRKRGNSSSSS--SSIVQR-YRFKRAILVGKR MPRQNQSRASVEELLPGKIRKRGCSSSASSSSSIIQN-YRFKRAILVGNR MSSRNRSAAAASCAA------------PAAAPSLRTPRRLKRRPVKSP--MARPRRSSPVAMTRS------------PARAAHGRAAATP-RQASPSS--MPRWDSE---------------DGGAADEAAAAVAVRLANKIRKRRAVSSS MKITATTELPTTIPAILSSRARNASPDSDLISKRSSRKPPRRKARTPAGG MPRQNHG---IQGLIPGKIRKRGCSSSSS-TSSVLQK-YRFKRAILVGKR

---RRRRSRSRTRTRPDTPFLKWKIDNNNNNNNDKGVQVHRHDDDDDGDV GGSTTPVPTWKTST--KSPTL-SMPNAESTKCPPSQSG--------GKT GGSTTPVPTWRLMGTTRTPSS-ALRARDSPKYAASQNGGIVKGKLMLQQQ ----AGAPGGGRRSGPATPLLKWDVGGGGRGEGRKGAGAADEA-------
----RHPRRGRRLLRVQSPSLASSSARRAPAPAPAHAPPPATPP-------GGSDPAAGRRLRSRRPAVLLPRRRTSAGGDMSESSRSR--------------ARLKRDGAAGGRRSRPETPLLRWKFDDIEREKDANVLDVD-------EKI GGSSTPVPTWRLMN-SRSPAS-AMRAMESP---RSMGG---------GKA

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A-thaliana-BLT A-lyrata-BLT
R-communis-BLT
S-bicolor-BLT


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QVARSETTRRKDR-RKISRPVSVRKLAAGLWRLQVPDASSSGG-------
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VEEKLDSGGRKGRRRFSSRAVSARKLAAGLWRLQLP-ETVVSAGHG----KEASVSARKLAATLWEINKIPSPKN-----LELAGDTKELRSGEK----QAAPVSARKLAATLWELNEMPSPKMKEVVGSEERRLRREGKGRERAGAAR ------GDAARAR-ETKAREVSVRRLAAGVWRLRPPEAVAGGG----------LRWPGDAVAPRGAGAAAAAAVRRIAASLWRAHPPPREPG--------------HCRGGGGNRLADGARPSASARRLVDAFWQDMDSGLLQGDAAAR----APEHGRRSGRKVRKGRE-VTVSSRRLASGLWRLQLPGVDAAHGGRW----KQAPVSARKLAATLWEMNEMPSPRA-----DDEKRSKREVRGRER--VAR
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[^4]P-trichocarpa-BLT
V-vinifera-BLT
At1G50660
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R-communis-BLT
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P-trichocarpa-BLT
V-vinifera-BLT At1G50660
A-lyrata-2
-------MEEVKVM-----MMSS-LHHSMNESSPQDPITTTTCTSWKLYE -------MEKMMMM-----MIRS-PENPSNETISEEPITATTCPSWKLYE PSTIATTKNGFLCKLEP---SMPFPHSAMEGATKWDPVCLDTME--EVHQ PSTIATTKNGFLCKLEP---SMPFPHSAMEGATKWDPVCLDTMD--EVHQ ------------IGANS-----------VKTRFKNVSDGLTTSKELVKVL -------------IVTNS-----------LKTRLKNVSEGLTTSKELVKVL ARDPISNGSFMDIETRSRVETPTGSTVGVKTRLKDCSNALTTSKELLKII ARDPINSGSFMDIETRSRVETPTGSTVGVKTRLKDCSNALTTSKELLKII GLDSVGNSSLMEIESHPKGRSR---TIGIKTCLKDVSNGLTTSKELLKVL AFDSVSNASLMEIETRSQAQTPSGSTVGVKPRLKDVSNALTTSKELLKII -------HSRFLCKHEP---SVPFPHCAMEGATKWDPICLDTRD--DVHQ -----------------------------MEGATKWNPVCLERSD--ELQQ PSSVSDMKNRFFCKVEP---SFQFSNTAMEGATKWDPVCLETID--EVRQ --DSVDNASFMEMETHTKGKNRRECNLGIRTRLKDVSNGLTASKELLKVL AFDAISNASLLEIETSSRAQTPSGSTVGAKTRLKDVSNALTTSKELLKII PISVLERKSGELHKVQLHATSAMLPVTTMEKATKWEPGDIKGMESHDAYL RTGTKPLGNAIIREVGPYSPS---PRIEMEVATKWDRPCLNTSGGADYDF SADMMSTGSAMEIGTFS--QDDVSRCPDKTVNLQDLQNSLIASKELVKVL PHSMHHHKSGFLCRLEP---SFQFSNSAMEGATKWNPVCSKTSD--EVRQ VMDSFSSASLMEIEIRSRGQTPSGSTVGVRTRLKDVSNALTTSKELLKII

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-KAFIWDLNFIKVFMES-ELGKAQDEIKELKAELDYERKARRRAELMIKK -KAFIWDLNFIKIFMES-ELGKAQDEIQELKAELDYERKARRRAELMNKR IAASFWDLTFLRPIMET-ELDFARAQIIELKAELEYERKARKKGETMNKR VDDQGYGYGYGYGAMSS-ELELCVARIRDLRAELEFERRMRRKAEALSEA IAASFWDLTFFRPIMDT-ELDFARAQILDLKAELEYERKARKKLETMSKR IAASFWDLTFFRPIMDS-ELDIARAQIIELKAELEFERKARKKVESMNKR LEQFLRKVSEERAAWRSREHEKVRAIIDDMKTDMNREKKTRQRLEIVNHK LEQFLRKVSEERAAWRSREHEKVRAIIDDMKTDMNREKKTRQRLEIVNHK

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A-lyrata-4
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R-communis-3
R-communis-4
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S-bicolor-3
S-bicolor-4
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At3g11590
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LAKDVEE--------------ERMAREAEEMQNKRLFKELSSEKSEMVRM LAKDVEE--------------ERMARVAEEMQNKRLFKELSSEKSEMVRM LAKELAE---------------ERRGREALERVCEQLAKEISFDKAEIDRM LAAELAD--------------ERRRGEAECR----------ALREEAERA LAKELAE--------------ERRGREALARVCEELAREISCDKEEIDHM LAKDLNE--------------ERRGREAMERVCEVLAKEISSDKAEISWM LVNELADSKLAVKRYMQDYEKERKARELIEEVCDELAKEIGEDKAEIEAL LVNELADSKLAVKRYMQDYEKERKARELIEEVCDELAKEIGEDKAEIEAL LGRELTEAKETERKMKEEMKREKRAKDVLEEVCDELTKGIGDDK------LGRELAEAKETERKMKEEMEREKRAKDVLEEVCDELAKGIGDDK-----LGKELAETKSALMKAVKEIENEKRARVMVEKVCDELARDISEDKAEVEEL LGKELAETKSALMKAVKEIENEKRARVMVEKVCDELARDISEDKAEVEEL LGKELADTKESLSKAMKELETEKRAKEILEQVCDELARGIGDDRAEVEEM LGKELAETKASLLKAVKELESEKRARVVMQKVCDELARDIGDDKAEVEEL LVNELADSKLAVKRYMHDYQQERKARELIEEVCDELAKEIEEDKAEIEAL LVNDLANAKVSAKRYMQDCEKERKARELIEEVCDELAKEIGEDKAEVEAL LVNELADAKVSAKRFMQDYEKERKARELIEEVCDELAKEIGQDKAEVEAF LGLELADTKASLSQALKELESEKRAKEILEQVCDELARGIGQDRAEVEEL LGKELAETKTSLMKAVKELENEKRTRVVMEQVCDELARDVDEDKAEVEEL LVNELKEAKMSAKQLLQEYDTERKARELTEEVCNELAREVEEDKAEIEAL LMDEFSELKSLAKRYLQDYEKERKARELMEEVCDELAKEIADDKAEVEAL LSIALANTEASLQAVTKELDRERKSKGRVEKICNELIRGIDEDKAEVQAL LVNELSDVKLSAKRFMODYEKERKDREVLEEVCDELAKEIGDDKAEAESL LGRELAETKTSLIKAVKELECEKRAREIMEQVCDELTQDVGEDKDEVEEI

KR-------DLEEERQMHRLAEVLREERVQMKLMDARLFLEEKLSELEEA KR-------DLEEERQMHRLAEVLREERVQMKLMDARLFLEEKLSELEEA KR-------EIEEERRMLRMAEVLREERVQMKLAEAKILFEEKLLELRGT LE-------GVEEERRMLRVAELWREERVQMKLADARAAVEEKLREIDDA KR-------EMEEERKMLRMAEVLREERVQMKLAEARMLFEEKLLELGGT KR-------EIEEERKMLRMAEVLREERVQMKLADAKFLLEEKLLELE-V KRESMSLREEVDDERRMLQMAEVWREERVQMKLIDAKVALEERYSQMNKL KRESMSLREEVDDERRMLQMAEVWREERVQMKLIDAKVALEERYSQMNKL --------KEMEKEREMMHIADVLREERVQMKLTEAKFEFEDKYAAVERL --------KEMEKEREMMHIADVLREERVQMKLTEAKFEFEDKCAAVERL KRESFKVKEEVEKEREMLQLADALREERVQMKLSEAKHQLEEKNATVDKL KRESFKVKEEVEKEREMLQLADALREERVQMKLSEAKHQLEEKNAAVDKL

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P-trichocarpa-2

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NRQGE---------------------------------------------------
NRQGE--------------------------------------------------QHSTEPDESSTLKMEQEQEQEKYKN------------------------------VAQLHAATTTAAAAAASVSDINK-------------------------------TTQTELHHNSASRMEQKYQEDKE--------------------------------TKQTQVERPSS-KMEHKNQEDNG-------------------------------VGDLESFLRSRD-IVTDVKEVREAELLRETAASVN-IQEIKEFTYVPANP VGDLESFLRSRD-IVTDVKEVREAELLRETAASVN-IQEIKEFTYEPANP KKELRRVLDGE-------EGKGSS-------EIRRILE---------------KVELRRVLDGE-------EGKGSS-------EIRRILE---------------RNQLQTYLKAKRCKE---KTREPPQTQLQNEETGDYLN--HHIGFGS-YN RNQLQTYLKAKRCKE---KTREPPQTQLHNEEAGDYLN--HHISFGS-YN RNELETYLREKVGEK---DGDGSP-NYDRIKELEAYLEEIQFGSCQQAER QNQLEAFLGTKRTKE---KGRSSS-NYMNDEEIAAYLSKNRFVSHQSEIN VGDMEAFLSSR--NTTGVKEVRVAELLRETAASVDNIQEIKEFTYEPAKP VADLEVFLRSRS-ATQGLKEMKEAESVIQAAASVH-IQDIKEFTYEPPNQ VADLETFLRSRT-ATPDLKEMREAESLVQAAASVD-FQDVKEFTYEPPNP RHELEAYLQEKVVSKENGDGNGSP-NYERIKEPEAYLNRIRDDSCQRIER RSQLEAFLGPKRTKE---KGRGS--YHMKDEEIAAYLNKTRSVSHQNEVN QQDVEAFIAACSTANADITAVEEAENIIQAIKSVR--AQDAEFRYEPPVA QANLESFLSFHRGSSVDKETVRDGERLREAISSMK--LHGKEFSYKPPPP RDELQAYLDTRKEQEPLNDPMQLSHASENGAPPADVVPD------------MADIAAFLRSKG-ANPDVKEMKEVESLCEAAAAVN-IQDVKEFTYVPPNP KNELEVFMRSKKAKE---KGRGSL-KHDNFSEIAAYLSRTHFGTLQNEEN

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-DDIYAVFEEMNIGE-AHDREMEKSVAYSPTSHGSKIHTVSPDANLMNKK
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-------IIDGSGSDDE--EESDLKSIELNMESGSKWG---------------
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IEDGE--VENG--NEEG-SGESDLHSIELNID--NKSYKWP----------
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R-communis-2
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S-bicolor-3
S-bicolor-4
V-vinifera-2
V-vinifera-3
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V-vinifera-BLT
At1G50660
A-lyrata-2
At5g22310
A-lyrata-3
A-lyrata-4
At3g11590
P-trichocarpa-3
P-trichocarpa-4
At3g20350
P-trichocarpa-2
R-communis-2
R-communis-3
R-communis-4
S-bicolor-2
S-bicolor-3
S-bicolor-4
V-vinifera-2
V-vinifera-3

A-thaliana-BLT
A-lyrata-BLT
R-communis-BLT
S-bicolor-BLT
P-trichocarpa-BLT
V-vinifera-BLT
At1G50660
A-lyrata-2
At5g22310
A-lyrata-3
A-lyrata-4
At3g11590
P-trichocarpa-3
P-trichocarpa-4
At3g20350
P-trichocarpa-2
R-communis-2
R-communis-3
R-communis-4
S-bicolor-2
---------------------RERNRMM---KPKILERACSSP-----------------------------RERNRMM---KPKILERACSSP--------FNRLFLGDTDKLCDENSCSDSRESTRAVFSGKSSYNDNLGSVPSMTI------------------------SSSCCSSPDGSKSTSQHGQQSPSRTQ---LNRLVL--SEKSCYDNSGADSKESTRVILSEMSSFNDKSRSISSMVI-------------------------ESARFIPGEKPTCNDSNAVVPSMAI-----GRHSDAYTHQNGDIEEDDSGWETVSHLEEQGSSYSPDGSIPSVNNKNH --GRHSDAFTHQNGDIEEDDSGWETVSHLEEQGSSYSPDGSIPSVNNKNH ------YVDSLKDRRR---FDGSGG-----DDDDDDPVEKRSVIVENG--------YVESGRDHRRESRFGGSGDEDDDDDDDDDDPVEKRSVIVENG--------YGEENRGRKST----PRKSLSLQKSISDCVDWVVQSEKLQK---------YGEENRGRKST----PRKSLSLQRSISDCVDWVVQSEKLQK---NSKWFSGGKDFKGRKSISDNIQWGNICLQRRNSNGIDGLGRDLISENQ--VRKAAIDEEDIKGRKSTSSKLPRRSTSLQRSISDGVEWDTRNERVPF-----GRHSNAFTDQNGEFEEDDSGWETVSHSEEHGSSYSPDESIPNISN--T GNHGHSEAFVHQNGDIEEDESGWETVSHLEDQGSSYSLDGSIPSVN---K GNHRHSDAFYDQNGDIEEDESGWETVSHLEDQGSSYSPEGSVPSVNN--K NPKRMSVDRDFRGRKSISENIQWGSICLQRRNSNGIDGPDSAFISKKQ--LRKAAIDEEEIKGRKSTSSKVPRRSTSLQRSISDGVDWNAQNERLSV---KAKVYSNKSPKDE-SVTEDGSSWETISHEDMQGSSGSPDGSEPSVSN--K PANKYS-TQPCAR-NEDDDDSGWETVSHVEEQGSSNSPDGSEPSVN---G RSASRHESISDRGMDGVNSWRLEQSFWDMDEELEGDWAEGCSNGMLN---DIRRHSNAFSEENGDIEEDESGWETVSHAEDQGSSYSPGGSDPSVH---K LRKVPIDEEETRVRKSTSGKMSRRSTSLQRSMSDGIDWGIQSENLQS---


NHRHSNASSGGTESLGKVWDDTMTPTTEISEVCSIPRRSSKKVSSIAKLW HHRDSNASSGGTESLGKVWDETMTPTTEISEVCSIPRRSSKKVSSIAKLW

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HHRNSNVSMNGTE-----YEKTLLR--EIKEVCSVPRRQSKKLPSMAKLW
NCRESNVSGSATEWEENACDETPIT--DISELCSMPRRQ-LKASSITRFW
NHRDSNVSGSGTEWEENACDETSIT--EITELCSVPIRQYKKVSSIAKLW

ICDGSISWKSRNGFEYMENEKLKDD--LADAYLTNMNQPKKKESAISKLW

S-bicolor-3
S-bicolor-4
V-vinifera-2
V-vinifera-3

A-thaliana-BLT
A-lyrata-BLT
R-communis-BLT
S-bicolor-BLT
P-trichocarpa-BLT
V-vinifera-BLT
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At5g22310
A-lyrata-3
A-lyrata-4
At3g11590
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P-trichocarpa-4
At3g20350
P-trichocarpa-2
R-communis-2
R-communis-3
R-communis-4
S-bicolor-2
S-bicolor-3
S-bicolor-4
V-vinifera-2
V-vinifera-3

A-thaliana-BLT
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V-vinifera-BLT At1G50660
A-lyrata-2
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R-communis-3
R-communis-4
S-bicolor-2
S-bicolor-3
S-bicolor-4
V-vinifera-2
V-vinifera-3

FCGGNGASASETDWEEDNCENCRSN--SGICSTTGEN--HRKGSSFARLW

FRQDSNASGSGTDWEENADEETPIT--EISEVRSVPMKQLKKGSSISRLW
-vinifera-3


RSTGASNGDRDSNYKVISMEGMNGGRVSNGRKSSAGMVSPDRVSSKGGFS RSSGASNGDRDSNYKVISMEGMNGGRVSNGRKSSAGMVSPDRVSSKGGFS
--------------------------------------ERDE-----------
-----------------------------------------ERDE----------
-----------------------------------------SGDGGLDWGRSI
----------------------------------------SGDGGDWGRSI
----------------------------------------ARPDMPDQERVA
--------------------------------------AGDG-IDWGRFS
SS----------------LEGMNG-RVSNARKSTVEMVSPETGSNKGGFN
R----------------SLDGLNS-RLSNGRKSNAGIMSPDKVSGESGIS RSG------GDNYKII-SVDGMNG-RLSNGRKSNGVIVSPDRGSGKGGLS
--------------------------------------EISNAFDRERLS
---------------------------------------TGDG-IDWEGFT
KSSR---PKNSEICKKDAVETVNA-RSSNVRLSVGTYSTVESGIQEIGLS RSS------NGDGRRKTGSDLLNG-RLSSSRMPNAALS-PDLKNSEVCQV

KSCP---NNGENYKIISVVEGMNG-RLSNGRISSAGIMSPDRGSGKGGLS -----------------------------------------SRDG-LDWGRFY
-----------ARRRCEN-PQIKRG-IN---PFPRVMRAIRSKSE----------------ARRSCEN-PQIKRG-IN---PFPRVMRAIRSKSE------------QR-RASPEPEN-PHIKRG-IKGFVEFPRVVRAIGSKSR------------PHRREPGGGEN-PHIRRG-IKGFVEFPKAVRLRGPREERV----------QRSRASPEPEN-PHIKRG-IKGFVEFPRVIRAIGSKNR-------------QR-RASPETEN-PHIKRG-IKGFVEFPRVVRAIGSRSR------PMMDLVGQWNSSPESANHPHVNRGGMKGCIEWPRGAQKSSLKSK------PMMDLVGQWNSSPESANHPHVNRGGMKGCIEWPRGAQKNSLKSK--------------------------SLKT--LRDYIVSNVRCIGPSS------------------------------SLKT--LRDYIVSNVRCVGPSS-----------EVEP----KGYLDETQAYKPNKASSKDHHLVSGSRLSNFRG-----------DVEP----KGYIDETQAYKPNKASSKDHHILSGSRLSNFRG----------ELGFNSQAQGYEDEIRKYRSVRT--LKDHILSGPKRSPIRNFASPTRLWG GLERQGQGKVYGDEMHGHQSVKG--LRDYLLSGSRLDSPRGYASPMRQAG -TLDLVGQWSSSPDSAN-ANLNRGGRKGCIEWPRGAHKNSLKTK-------PPDLVGQWSS-PDSVN-PHMTRG-MKGCIDWPRG-HKNSLKAK-------P-DLTGQWSS-PDSGN-PHITRG-MKGCIEWPRGAQKNSLKAK------ELDLHSQDE---DEISCYRSVKS--LRDHIVSGSRRQPIQNFASPT----ELERHLQGKGYVDDMHGYQSVKG--LRDYLLSGSRIDSTRGCASPTRQVG --PPSVEQWSS-PDSMN-IQFNRG-FKGCIEYPRTSQKHSLKAK--------SPSVGDWS--PDLLN-PRVVRA-MKGGVEWPQGTQKRTQKSK-------------------LDHDEERYLAIKNLREQMLAGSGFIMSQGRED--------PPDLAGQWSS-PDSGN-PHVNRG-MKGCIEWPRGAQKNSLKAK------ELEKEAQGKGYGDEMQRYKSVKG--LRDQILAGSRIGSARVFASPTRQWG
A-thaliana-BLT
A-lyrata-BLT
R-communis-BLT
S-bicolor-BLT
P-trichocarpa-BLT
V-vinifera-BLT
At1G50660
A-lyrata-2
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P-trichocarpa-4
At3g20350
P-trichocarpa-2
R-communis-2
R-communis-3
R-communis-4
S-bicolor-2
S-bicolor-3
S-bicolor-4
V-vinifera-2
V-vinifera-3


| A-thaliana-BLT | -- |
| :--- | :---: |
| A-lyrata-BLT | -- |
| R-communis-BLT | -- |
| S-bicolor-BLT | VV |
| P-trichocarpa-BLT | -- |
| V-vinifera-BLT | -- |
| At1G50660 | -- |
| A-lyrata-2 | -- |
| At5g22310 | -- |
| A-lyrata-3 | -- |
| A-lyrata-4 | -- |
| At3g11590 | -- |
| P-trichocarpa-3 | -- |
| P-trichocarpa-4 | -- |
| At3g20350 | -- |
| P-trichocarpa-2 | -- |
| R-communis-2 | -- |
| R-communis-3 | -- |
| R-communis-4 | -- |
| S-bicolor-2 | -- |
| S-bicolor-3 | S-bicolor-4 |
| V-vinifera-2 | -- |
| V-vinifera-3 | -- |



Table S1. Nuclear size of developing wild-type and blt mutant trichome and pavement cell nuclei

| Genotype | Cell type | Cross-sectional area $\left(\mu \mathrm{m}^{2}\right)^{*}$ | Estimated volume $\left(\mu \mathrm{m}^{3}\right)^{+}$ |
| :--- | :---: | :---: | :---: |
| Col-0 | Developing trichome | $45.8 \pm 10.3(12)$ | 233.0 |
| blt-3 | Developing trichome | $44.5 \pm 12.0(10)$ | 223.3 |
| Col-0 | Developing protoderm | $15.0 \pm 2.2(8)$ | 43.7 |
| blt-3 | Developing protoderm | $14.8 \pm 2.6(11)$ | 42.8 |
| Cross-sectional areas of DAPl-stained developing trichome and pavement cell nuclei were determined using images captured and |  |  |  |
| analyzed using Slidebook software (Intelligent Imaging Innovations, Denver, CO, USA). Based on a Kruskal-Wallis one-way ANOVA |  |  |  |
| on ranks and an all pairwise multiple comparison test (Dunn's test), all pairwise comparisons differ significantly ( $P<0.05)$, except Col- |  |  |  |
| 0 developing trichome nuclear size versus blt-3 nuclear size and Col-0 developing protoderm nuclear size versus blt-3 nuclear size. |  |  |  |
| *Mean $\pm s . d$. ( $n$ ). |  |  |  |
| +Volume estimated from cross-sectional area assuming that the nuclei are spherical. |  |  |  |


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[^1]:    The numbers of nuclei per trichome in sim－1 versus b／t－3 sim－1 are significantly different（ $P<0.001$ ，Mann－Whitney rank sum test）．
    ＊The number of nuclei per trichome was counted under a fluorescence microscope （trichomes were stained with DAPI）．Data are mean $\pm$ s．d．
    ${ }^{\dagger}$ The total number of trichomes for which nuclei were counted on first or second leaves．At least ten leaves were examined per genotype．

[^2]:    Based on a Kruskal－Wallis one－way ANOVA on ranks and an all pairwise multiple comparison test（Dunn＇s Test），branch counts of all GL2 pro：BLT lines differ significantly $(P<0.05)$ from both Col－0 and blt－3，and branch counts of Col－0 and blt－3 differ significantly from each other（ $P<0.05$ ）．
    ＊Shown in the percentage of total trichomes having the indicated number of branch points（one branch point indicates a trichome with two branches）．Counts were made on first or second leaves of each genotype．
    ${ }^{\text {T}}$ Total number of trichomes on which branches were counted．

[^3]:    For each comparison of a single trichome branching mutant with its double mutant with blt-3, as well as for the comparison of Col-0 with b/t-3, the median number of branch points per trichome differed significantly ( $P<0.001$ ) based on a Mann-Whitney rank sum test.
    *Shown is the percentage of trichomes having the indicated number of branch points (one branch point indicates a trichome with two branches). Counts were made on ten first or second leaves per genotype.
    ${ }^{\dagger}$ Total number of trichomes on which branches were counted.

[^4]:    -------MKDMKMOSSPETMMTRIPTPDPHSTGVREDAMDSVCKPWKLYE -------MKDMKMQSSSETMMTRIPTPDPHNTGVREDAMDTVCKPWKLYE -------MEEVKVM-----MMINGVENTRNESFPLEPITTTTCPTWKLYE ----------------------------MASVVQVEANHGAGSPPWKLYH

