GLABROUS1 Overexpression and TRIPTYCHON Alter the Cell Cycle and Trichome Cell Fate in Arabidopsis

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Cellular competence, initiation cues, and inhibition signals control the distribution of trichomes on the Arabidopsis leaf. The GLABROUS1 (GL1) gene has a dual role in that it is required for trichome initiation, but GL1 overexpression reduces trichome number. We have found that a mutation in the TRIPTYCHON (TRY) gene partially suppresses the GL1 overexpression phenotype but not in a way that indicates that TRY directly controls an epidermal inhibition pathway. Surprisingly, cauliflower mosaic virus 35S::GL1 try plants contain a subclass of trichomes derived from the subepidermal layer. Altered cell cycle control was also detected in 35S::GL1 and try plants. A mutation in TRY led to increased epidermal and mesophyll cell number, a reduction in endoreduplication in the epidermis, and an increase in endoreduplication in trichomes. GL1 overexpression also reduced endoreduplication levels in both the epidermis and trichomes; however, in the presence of try, it synergistically enhanced trichome endoreduplication. Interactions with the COTYLEDON TRICHOME1 (COT1) gene indicate that GL1 and TRY control trichome development and may be involved in cell cycle control during leaf development.

INTRODUCTION

Arabidopsis trichome development has been used to address fundamental biological questions concerning the regulation of cell fate and pattern formation (Marks, 1997). Arabidopsis trichomes are unicellular structures derived from protodermal cells. They are found on the adaxial surface of early rosette leaves, both adaxial and abaxial surfaces of later rosette leaves, inflorescent stems, cauline leaves, and sepals. They normally are not found on hypocotyls, cotyledons, petals, stamens, or carpels. The developmental timing and spatial arrangement of trichome initiation events are highly regulated (Hülskamp et al., 1994; Larkin et al., 1994, 1996). Rosette leaf trichome initiation does not occur until the leaf primordium reaches a length of \( \approx 100 \, \mu m \). Scanning electron microscopy (SEM) of developing leaves, a histological examination of developing Arabidopsis leaves, and the use of a dominant inducible transgene that regulates trichome initiation all indicate that the positions of trichome initiation coincide with mitotically active regions of the leaf (Pyke et al., 1991; Lloyd et al., 1994; Marks, 1994). The experiments detailed in this study begin to address how trichome initiation is coordinated with the cell cycle and leaf development.

The temporal changes in leaf mitotic activity and postmitotic DNA synthesis have been described in Arabidopsis (Galbraith et al., 1991; Pyke et al., 1991). It was found that DNA synthesis continues in most cells after cell division has ceased. Typically, Arabidopsis leaf cells complete between one and three rounds of endoreduplication, cycling through successive S phases without cytokinesis or the generation of multiple nuclei. At 21 days after germination, \( \approx 50\% \) of nuclei derived from the first leaf pair are either 8C or 16C (Galbraith et al., 1991). The developmental consequences of the observed changes in DNA content are unclear, but the positive correlation between increases in DNA content and cell size has been noted (Galbraith et al., 1991; Melaragno et al., 1993). Changes in cell cycling accompany trichome initiation, and endoreduplication cycles punctuate trichome morphogenesis (Hülskamp et al., 1994). Once a cell enters the trichome pathway, cytokinesis is blocked, and the nucleus undergoes variable rounds of endoreduplication. The DNA content of fully expanded trichome nuclei ranges from 4C to >64C (Melaragno et al., 1993). Unlike epidermal pavement cells, DNA content and cell volume are not correlated in wild-type trichomes (Melaragno et al., 1993).

The balance of initiation and inhibition cues is hypothesized to regulate entry into the trichome pathway. Genetic models in which interacting positive and negative pathways regulate initiation have been proposed (Hülskamp et al., 1994; Larkin et al., 1994, 1997), and the dual role of initiation genes in an inhibition pathway is consistent with this idea. GLABROUS1 (GL1) and TRANSPARENT TESTA GLABRA (TTG) are essential genes for normal trichome initiation, and...
there is evidence that both genes also inhibit other epidermal cells from entering the pathway (Larkin et al., 1994). GL1 encodes a myb-like transcription factor, and TTG encodes a protein that contains WD40 repeats (J. Gray and M. Walker, personal communication), which mediate protein–protein interactions. Wild-type plants containing a GL1 gene under the transcriptional control of the cauliflower mosaic virus 35S RNA promoter produce fewer leaf trichomes (Larkin et al., 1994; Szymanski et al., 1998b). This reduction in trichome number is not due to cosuppression because 35S::GL1 plants overexpress both the GL1 mRNA and gene product (Larkin et al., 1994; D.B. Szymanski, unpublished results).

Previous models have suggested that the 35S::GL1 phenotype, defined here as GL1oe, could be due to a squelching mechanism, but the data are more consistent with GL1 overexpression activating a tissue-level inhibition program that operates in the leaf epidermis (Larkin et al., 1994; Szymanski et al., 1998b). There must be a temporal or developmental component to 35S::GL1-mediated inhibition, because the early phase of trichome initiation is not affected even though high levels of GL1 message accumulate in young leaves (Larkin et al., 1993; Szymanski et al., 1998b). Although the overall effect of GL1 overexpression in the leaf is trichome inhibition, GL1 overexpression also leads to limited trichome initiation on the cotyledon (Larkin et al., 1994). This latter result highlights the capacity of GL1 expression to induce trichomes.

In addition to GL1 and TTG, several other genes are known to influence trichome initiation. The REDUCED TRICHOME NUMBER (RTN) gene is required for trichome initiation after the leaf reaches the 400- to 500-μm stage of leaf development (Larkin et al., 1996). The COTYLEDON TRICHOME1 (COT1) gene limits trichome initiation on the cotyledon and leaf in the presence of 35S::GL1, but the cot1 mutation alone produces no detectable phenotype (Szymanski et al., 1998b). The TRIPTYCHON (TRY) and GL3 genes also affect trichome initiation, trichome endoreduplication, and morphogenesis (Koomneef et al., 1982; Hülskamp et al., 1994; Folkers et al., 1997). TRY has been proposed to play a role in the putative inhibition pathway, because mutations in TRY result in a few clustered leaf trichomes (Hülskamp et al., 1994). TRY mutants also have increased DNA content in trichome nuclei (Hülskamp et al., 1994). Mutations in GL3 cause decreased branching and endoreduplication in trichomes (Hülskamp et al., 1994; D.B. Szymanski, unpublished results).

To determine whether TRY plays a role in the GL1oe phenotype, we crossed the try mutation into plants overexpressing GL1. We found that try partially suppressed GL1oe in the leaf; however, the mode of suppression did not suggest that TRY was directly involved in an epidermal inhibition pathway. Surprisingly, we found that 35S::GL1 try plants contain a subclass of trichomes derived from the subepidermal layer, a phenotype defined here as Try–G. The competence of subepidermal cells to enter the trichome pathway is unexpected, given that such cells are not observed in wild-type plants that overexpress GL1. As indicated below, this could be related to the role of TRY in cell cycle control. The number of epidermal cells that become trichomes is similar in 35S::GL1 and 35S::GL1 try plants. However, when try is combined with the cot1 mutation and the 35S::GL1 transgene, the induction of epidermal trichomes is greatly increased. This suggests that COT1 and TRY have an overlapping function to limit trichome initiation in the leaf epidermis.

GL1 overexpression and the try mutation differentially affected cell cycle parameters. Cell number and endoreduplication measurements in mutant and wild-type backgrounds demonstrated that try plants have increased cell numbers in the epidermis and mesophyll. Unlike the elevated endoreduplication levels in try trichomes, try pavement cell endoreduplication levels were reduced. In 35S::GL1 leaves, cell numbers were similar to those of the wild type, but endoreduplication levels were skewed toward lower values in both the epidermis and trichomes. The lower level of endoreduplication in 35S::GL1 trichomes required wild-type TRY, because 35S::GL1 try trichome nuclei had greatly elevated DNA content. These observations indicate that GL1 and TRY are involved in complex interactions that affect the cell cycle during leaf development and underscore the importance of cell cycle control in cell differentiation. These results widen the scope of the analysis of genes such as GL1 and TRY to include the balance of cellular proliferation and differentiation during leaf development.

RESULTS

try Suppression of GL1oe

To determine whether the TRY gene mediates the GL1oe phenotype, we crossed plants overexpressing GL1 with plants homozygous for try. In the Columbia (Col) wild-type background, the mean number of trichomes on the adaxial surface of the first leaf was 28 (Table 1). The number of trichomes on the first leaf of try plants was comparable to those in the wild type, and the clustering observed in try plants accounted for the possible small increase in trichome number (Table 1). The plot of trichome number against leaf length in try was indistinguishable from that of the wild type (data not shown). As previously reported, GL1 overexpression led to a reduction in leaf trichome number (Table 1). As would be predicted for a gene involved in negative regulation of trichome initiation, the try mutation partially suppressed the reduced trichome phenotype of 35S::GL1 plants (Table 1). However, as shown below, the nature of the suppressed phenotype was entirely unexpected.

Trichome development in wild-type, try, GL1oe, and Try–G plants was examined using SEM (Figure 1). For this analysis, a leaf stage was chosen at which trichome initiation was still detectable. In wild-type leaves, the trichomes were evenly distributed and contained three to four branches (Figure 1A).
Table 1. Cotyledon and Leaf Trichome Phenotype and Gene Dosage Sensitivity of 35S::GL1 and try

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cotyledon Trichomes</th>
<th>Leaf Trichomes</th>
<th>Trichomes in a Cluster (%)</th>
<th>Subepidermal Large Cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>n (^a)</td>
<td></td>
<td></td>
<td>Nonerupting (^b)</td>
</tr>
<tr>
<td>Col</td>
<td>22</td>
<td>0</td>
<td>28.0 ± 0.97</td>
<td>0</td>
</tr>
<tr>
<td>35S::GL1</td>
<td>22</td>
<td>0.41 ± 0.73</td>
<td>10.2 ± 1.1</td>
<td>2.6</td>
</tr>
<tr>
<td>TRY/try(^f)</td>
<td>23</td>
<td>0</td>
<td>29.9 ± 2.8</td>
<td>0.9</td>
</tr>
<tr>
<td>try/try</td>
<td>22</td>
<td>0</td>
<td>33.0 ± 3.4</td>
<td>8.5</td>
</tr>
<tr>
<td>35S::GL1/-f</td>
<td>23</td>
<td>2.2 ± 2.7</td>
<td>12.6 ± 3.3</td>
<td>7.7</td>
</tr>
<tr>
<td>try/try</td>
<td>35S::GL1</td>
<td>23</td>
<td>11 ± 3.7</td>
<td>16.4 ± 3.2</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

\(^a\) For each plant, measurements were obtained from one cotyledon or from one of the first two leaves from each of the genotypes analyzed.

\(^b\) Cotyledon trichomes were defined as any cell that expanded out of the plane of the epidermis and was \(\sim\)100 \(\mu\)m in length.

\(^c\) The total number of trichomes on the adaxial surface of fully expanded first or second leaves was measured.

\(^d\) The adaxial surface of leaves was scored by using light microscopy and SEM for the characteristic bumps derived from subepidermal large cells.

\(^e\) The adaxial surface of leaves was scored by using light microscopy and SEM for the irregular expansion of trichomelike cells that did not penetrate the epidermis (nonerupting) or that penetrated the epidermis (erupting). N and Y indicate the absence or presence of the above cell types, respectively.

\(^f\) Analyzed as an F1 plant. Dash indicates hemizygous.

The gross morphology of try leaves was similar to that of the wild type, except that the typical first leaf pair usually contained at least one pair of clustered trichomes (Table 1 and Figure 1B). In addition, try trichomes were larger than those of the wild type and contained four to five branches (Figure 1B). The distribution and frequency of trichomes in 35S::GL1 plants were modified (Figure 1C). Trichomes in GL1\(^{co}\) leaves were largely excluded from the leaf midblade, but trichome size and branch number resembled those of the wild type. Trichomes in 35S::GL1 plants homozygous for try were dramatically altered in their distribution and the manner in which they develop (Figures 1D and 1E). The emerging trichomes on these plants were spaced over the entire leaf surface and were no longer limited to the leaf periphery, as in 35S::GL1 plants. The most striking change was that many of the trichomes appeared to erupt from the subepidermis, whereas trichomes on wild-type plants arose from clearly distinct epidermal cells (Figure 1F). The outer diameter of the mounded epidermal cells in 35S::GL1 try was larger than wild-type trichomes and caused distortions in the epidermal layer. The pavement cells that border the putative erupting trichomes did not have the clear support cell structure that could be observed in the wild type (Figure 1G).

The transition from putative subepidermal large cell to erupting trichome was sensitive to TRY gene dosage, and only the nonerupting class of subepidermal large cells was observed in TRY/try 35S::GL1 plants (Table 1). The spacing of the trichomes in 35S::GL1 try leaves was altered compared with both try and the wild type; 24% of the trichomes appeared to emerge in small clusters (Table 1). Compared with try clusters, in which 91% \((n=22)\) of all clusters shared a common cell wall boundary, only 48% \((n=35)\) of 35S::GL1 try clusters shared cell wall continuity.

Erupting trichomes were examined in more detail in serial sections through Try\(^{-}\)-G leaves at different stages of development. Serial sections through Try\(^{-}\)-G leaves at approximately the 1-mm stage were viewed by using differential interference contrast (DIC) microscopy. In proximal to distal transverse serial sections, enlarged subepidermal cells were detected (Figures 2A through 2F). In all Try\(^{-}\)-G leaves, large subepidermal cells were found directly beneath the existing epidermal cell layer. The onset of the subepidermal Try\(^{-}\)-G phenotype occurred very early in leaf development, when subepidermal cells were densely cytoplasmic and had not obviously differentiated into photosynthetically competent mesophyll cells. The development and fate of chloroplasts in this cell type are not known, but subepidermal large cells and erupted trichomes in fully expanded leaves lack chloroplasts. In Try\(^{-}\)-G plants, some subepidermal large cells appeared to arrest in a highly vacuolated state and ceased to expand (Figure 2A), but in many cases they retained densely staining cytoplasm and continued to expand into the overlying epidermis. Subepidermal large cell expansion was polar, and clear differences in the apical and basal cell wall structure were detected. The basal cell wall was often clearly defined (Figures 2B through 2F); however, in restricted apical regions, the cell wall was difficult to identify (Figures 2C, 2E, and 2F). In limited apical regions, the expanding cell edge appeared to invade the overlying epidermal cell layer (Figures 2C and 2E). The DNA content and nucleus position of subepidermal large cells varied. The DNA content of Try\(^{-}\)-G subepidermal large cells in the first leaf pair was measured.
trichomes through a fully expanded Try leaf (Figures 3A through 3G), and the final morphology was roughly similar to that of the wild type. Stable GL2::GUS expression was detected in trichomes along the leaf perimeter and centered on regions of subepidermal cell expansion (Figure 4B). GL2::GUS activity in Try−G plants was also seen in epidermal bumps, and strong staining was observed in erupted trichomes (Figure 4C). Dark-field illumination of a semithin transverse section through a highly vacuolated nonerupted subepidermal large cell in TRY/try 35S::GL1 leaves also contained subepidermal large cells that did not penetrate the epidermal layer (Figures 1D and 1E). Neither the subepidermally derived trichomes nor greatly enlarged mesophyll cells have been seen in developing wild-type, 35S::GL1, or try leaves. The first Try−G leaf pair also contained trichomes of epidermal origin that were initiated during the 100- to 500-μm stage. Their development was indistinguishable from that of the wild type (data not shown).

Because the subepidermal large cells displayed many of the hallmarks of wild-type trichome morphogenesis, it was important to test the idea that this unique cell type used the molecular mechanisms of wild-type trichome initiation and morphogenesis. To address this issue, a transgene containing a GL2 promoter fragment fused to β-glucuronidase (GL2::GUS [dMR]) was crossed into the Try−G background (Szymanski et al., 1998a). In the wild type, GL2::GUS expression first occurs throughout the leaf primordium and then becomes limited to trichomes (Szymanski et al., 1998a). The staining pattern of developing leaves in try, TRY/try 35S::GL1, and try/try 35S::GL1 was qualitatively similar to that of the wild type. Stable GL2::GUS expression was detected in trichomes on fully expanded try leaves (Figure 4A). In TRY/try 35S::GL1 plants, stable GL2::GUS expression was detected in trichomes along the leaf perimeter and centered on regions of subepidermal cell expansion (Figure 4B). GL2::GUS activity in Try−G plants was also seen in epidermal bumps, and strong staining was observed in erupted trichomes (Figure 4C). Dark-field illumination of a semithin transverse section through a highly vacuolated nonerupted subepidermal large cell in TRY/try 35S::GL1 confirmed that significant GL2::GUS expression was associated with the subepidermal cell type (Figure 4D). High background GL2::GUS expression in all cell layers of young leaves precluded a similar analysis at earlier stages of leaf development.

GL1 Overexpression and the try Mutation Cause Ectopic Trichome Formation

Wild-type and try plants did not produce cotyledon trichomes. Approximately 10% of the plants that were homozygous for 35S::GL1 produced a few cotyledon trichomes, and cotyledon trichome production was enhanced in 35S::GL1 plants homozygous for try (Table 1). In this latter population, all plants produced cotyledon trichomes with an average of 11.0 trichomes per cotyledon (Table 1). Likewise, F1 plants hemizygous for the transgene and heterozygous for try displayed an enhanced cotyledon trichome number. In this population, plants had a mean cotyledon trichome number of 2.2 (Table 1), and 83% of the F1 plants had at least one cotyledon trichome. Further analysis demonstrated that the

Figure 1. SEM Images of the Adaxial Surface of Developing Wild-Type and Mutant Leaves at the Four-Leaf Stage.

(A) Adaxial surface of a developing Col leaf.
(B) Adaxial surface of a developing try leaf.
(C) Adaxial surface of a developing Gl1oe leaf.
(D) Adaxial surface of a developing Try−G leaf.
(E) High-magnification image of an erupting trichome found within the box in (D).
(F) High-magnification image of an early-stage wild-type trichome.
(G) High-magnification image of a wild-type mature trichome and adaxial epidermis.

bt, extra branched trichome; ct, clustered trichome; ert, erupting trichome; et, expanding trichome; gc, guard cell; mt, mature trichome; pc, pavement cell; sc, support cell; set, subepidermal trichome. Bars in (A) to (D) and (G) = 50 μm; bars in (E) and (F) = 10 μm.
dosage of 35S::GL1 had little impact on the phenotype, and
the major reduction in cotyledon number in the F\textsubscript{1} progeny
was due to heterozygosity at TRY (data not shown). Try\textsuperscript{−}G
plants also produced ectopic trichomes on the abaxial surface
of the first leaf pair, stamens, and carpels (data not shown). 35S::GL1 plants contained abaxial trichomes, but
stamen and carpel trichomes were unique to Try\textsuperscript{−}G plants.
Previous analyses indicated that <1\% of 35S::GL1 tri-
chomes develop support cell trichomes. Support cell tri-
chomes initiate from the specialized cells that surround an
existing trichome. This phenotype is greatly enhanced in
35S::GL1 cot1 plants (Szymanski et al., 1998b). Try\textsuperscript{−}G
plants show a slight increase in the production of this class
of ectopic trichomes compared with 35S::GL1 alone.

GL1 Overexpression, try, and Effects on Cell
Proliferation and Endoreduplication

A histological examination of sectioned leaves at several de-
velopmental stages indicated an apparent difference in epi-
dermal and mesophyll cell number between Col and try
(data not shown). In addition, DAPI staining of whole-
mounted trichomes revealed apparent differences in nu-
cleus size and fluorescence intensity between Col and Try\textsuperscript{−}G
plants. Cell number in the adaxial epidermis and underlying
mesophyll cell layer was measured, and the nuclear DNA
content of pavement cells and trichomes was examined to
test the idea that TRY and GL1 overexpression affects the
mitotic and endoreduplication cycles. Because the genes
that control trichome initiation exert their effects in a spa-
tially and temporally restricted manner in the leaf, it was im-
portant to develop accurate methods to analyze the cell
number and DNA content in intact leaf samples. To maintain
consistency between samples, we examined the cell num-
er and the extent of endoreduplication in leaf cells in the
first leaf pair of the wild type and mutants 21 days after ger-
mination. This stage was chosen because the leaf was fully
expanded and did not exhibit any evidence of senescence.
In addition, whole-leaf estimates of the proportions of 2C,
4C, 8C, and 16C nuclei have been measured at this stage of
development (Galbraith et al., 1991).

Pavement cell numbers of wild-type and mutant leaves
were measured from bright-field images of impressions ob-
tained from the epidermis (see Methods). For all genotypes,
three independent measurements of mean cell number per
unit area were made on different leaves. Each measurement
was expressed as the mean cell number of six randomly
chosen fields per leaf (0.083 mm\textsuperscript{2} per field), and the final es-
timate of the cell density was calculated as the mean of

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**Figure 2.** Serial Transverse Sections through Developing Subepidermal Trichomes in Young Try\textsuperscript{−}G Leaves.

(A) Subepidermal large cell in Try\textsuperscript{−}G.
(B) Low-magnification view of the Try\textsuperscript{−}G leaf highlighted in (C) to (F). Box indicates the magnified region shown in (C) to (F).
(C) to (F) Serial sections through two developing subepidermal trichomes sampled from the proximal to distal axis of the leaf. The left cell is la-
beled with a\textsubscript{1} and with open arrowheads and the right cell with a\textsubscript{2} and with filled arrowheads; a, apical region; b, basal region.
Bars in (A) through (F) = 50 \(\mu\text{m}\).
three independent measurements. The wild-type epidermis contained a mean cell density of $33 \pm 4$ cells per field (Table 2). The measured epidermal cell densities in 35S::GL1 and Try\textsuperscript{−}G plants were not significantly different from the cell density of the wild type (Table 2). Mean epidermal cell density in try (40 ± 3) was slightly elevated compared with that of the wild type. For each genotype, mesophyll cell density in the layer directly underlying the adaxial epidermis was measured 21 days after germination (Table 2). Cell wall boundaries of this layer of mesophyll cells were easily visualized in intact leaves that had been fixed and cleared (see Methods). Col and GL1\textsuperscript{oe} leaves had mean mesophyll cell densities of 65 ± 11 and 68 ± 5, respectively. Mesophyll cell densities in try (81 ± 4) and Try\textsuperscript{−}G (100 ± 9) were higher compared with those of the wild type. The first leaf pair for each genotype was roughly spherical, and first-leaf measurements indicated that leaf dimensions in millimeters for Col (length $l$, 5.2 ± 0.2; width $w$, 5.25 ± 0.2; $n = 6$), try ($l$, 5.3 ± 0.4; $w$, 5.1 ± 0.3; $n = 6$), and GL1\textsuperscript{oe} ($l$, 5.5 ± 0.2; $w$, 5.1 ± 0.3; $n = 6$) were not significantly different. However, leaf size in Try\textsuperscript{−}G was reduced compared with that of the wild type ($l$, 4.2 ± 0.2; $w$, 3.7 ± 0.5; $n = 6$). If the ~40% reduction in leaf area in Try\textsuperscript{−}G is taken into account, Try\textsuperscript{−}G leaves would have fewer epidermal cells and approximately equal numbers of mesophyll cells compared with wild-type epidermal and mesophyll cell numbers.

To measure the relative DNA content of epidermal cells, we adopted an in situ digital imaging approach based on the DNA binding activity and fluorescence properties of DAPI. Unlike flow cytometry or measurement of epidermal peels,
this technique preserved the positional information of measured cells with respect to the whole leaf and did not include any tissue disruption that could skew the cell types that were examined. Collection and analysis of digital images at several focal planes accommodated the spatial heterogeneity in nucleus shape and location within an epidermal cell. In addition, the definition of guard cell nuclei DNA content of \(\sim 2C\) provided an important internal standard for each observation field and allowed fields within a leaf to be pooled into a single data set (Melaragno et al., 1993). The mean relative fluorescence intensity and area of guard cell nuclei were not significantly different in all genotypes examined and served as useful standards for comparisons between genotypes. The standard deviation of stomatal measurements within a given field was routinely \(<10\%\) of the mean.

For previous flow cytometric studies of the systemic changes in endoreduplication over time and measurement of trichome DNA content, researchers used Arabidopsis plants grown on plates (Galbraith et al., 1991; Melaragno et al., 1993). Therefore, the proportions of polyploid pavement cell nuclei were measured on plates. To obtain preliminary information on the relative impact of growth conditions and genotype on endoreduplication, we also analyzed soil-grown plants. The frequency distributions of pavement cell nuclei DNA content of wild-type and mutant plants grown on plates are shown in Figures 5A to 5D. The ratio of relative fluorescence units (RFU) of a given nucleus over the mean stomatal nucleus RFU for a given field was converted to log base 2. This converted the skewed distribution of ratios to a simple symmetric scale (e.g., \(\log_2 (16C\ \text{nucleus}/2C\ \text{guard cell}) = 3\) and \(\log_2 (32C\ \text{nucleus}/2C\ \text{guard cell}) = 4\)) upon which cutoffs for putative DNA categories could be assigned to all data sets (Figure 5).

In the wild type, a major population of putative 2C nuclei fell \(<40\%\) below the value of 0 that would be expected if guard cell nuclei were from G1 phase-arrested 2C cells, but subsequent peaks corresponding to the predicted DNA content of 4C, 8C, and 16C were in phase with the measured 2C peaks (Figure 5A). The \(<1C\) DNA content measured for a subpopulation of cells in each genotype could reflect an incorrect assumption regarding guard cell DNA content or differences in structure of guard cell and pavement nuclei. The latter factor most likely accounts for part of the discrepancy in the predicted 2C DNA content. Guard cell nuclei in an open stoma were circular, condensed, and strongly fluorescent; however, many putative 2C pavement cell nuclei were diffuse, and portions of the nucleus fell below the detection limit (Figures 5A to 5D; lowest DNA content category). In either case, all epidermal nuclei in a given observation field were counted for an accurate measurement of proportions within the measured leaf, and low DNA content nuclei were included in the 2C category. Putative 4C, 8C, and 16C nuclei measurements predominantly fell within discrete categories and were less problematic to quantify.
The effects of treatment (plate versus soil) and genotype (Col, 35S::GL1, try, and 35S::GL1 try) on the proportions of 2C, 4C, 8C, and 16C nuclei were quantified (see Methods). The magnitude of the effect of genotype and treatment on endoreduplication was analyzed using a multifactorial analysis of variance (split plot analysis). The interaction between genotype and endoreduplication was clearly the main effect, and the null hypothesis of equal 2C, 4C, 8C, and 16C proportions between genotypes was rejected (degrees of freedom of variance (split plot analysis). The interaction between endoreduplication was analyzed using a multifactorial analysis.

The magnitude of the effect of genotype and treatment on endoreduplication was quantified (see Methods).

The frequency distribution was divided into logical categories based on expected C values. The frequency of nuclei having between 48C and 64C DNA content compared with 25% in the wild type (Table 3). In try and Try−G, the distribution of trichome nuclei was skewed toward a higher DNA content (Figures 5G and 5H, respectively). In both try and Try−G, ~47% of the trichome nuclei contained between 48C and 64C amounts of DNA compared with 25% in the wild type. In addition, 29% of 35S::GL1 try trichome nuclei had at least 96C DNA content compared with zero in all other genotypes (Table 3). The trichome DNA content of each genotype was also measured in soil-grown plants, and the same effects of genotype on endoreduplication levels were observed.

### Interaction between COT1 and 35S::GL1 try

An interesting feature of the Try−G phenotype is that the number of leaf epidermal cells that become trichomes does not differ greatly from the number on 35S::GL1 leaves. We have previously identified the COT1 gene as another regulator of trichome number on 35S::GL1 plants. 35S::GL1 cot1 plants show a modest increase in leaf epidermis-derived trichomes, and similar to 35S::GL1 try, they have an increased cotyledon trichome number (Szymanski et al., 1998b).

To determine whether COT1 interacts with TRY in the leaf, we crossed 35S::GL1 cot1 and 35S::GL1 try plants. In the F2 population, approximately one-sixteenth of the plants displayed a new phenotype. It has been difficult to isolate a 35S::GL1 cot1 homozygous line, presumably because putative triple mutants have reduced seedling viability and seed set. However, it was possible to generate a line homozygous for 35S::GL1 and heterozygous for cot1 and try. Putative triple mutants in this F2 population produced far more cotyledon trichomes than did either parent (Szymanski et al., 1998b), and the leaf shape and surface area were greatly altered compared with the wild type at the same stage (Figure 7A). Higher magnification SEM images showed that the morphology of a large fraction of the epidermal cells was greatly altered in the putative triple mutant (Figure 7B).

### Table 2. Cell Number and Proportions of 2C, 4C, 8C, and 16C Pavement Cell Nuclei in Col, GL1try, and Try−G Leaves

<table>
<thead>
<tr>
<th>Plant</th>
<th>No. of Pavement Cells</th>
<th>No. of Mesophyll Cells</th>
<th>2C</th>
<th>4C</th>
<th>8C</th>
<th>16C</th>
<th>32C</th>
<th>n^c</th>
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<tr>
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<td>33 ± 4</td>
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<td>0.18</td>
<td>0.38</td>
<td>0.23</td>
<td>0.03</td>
<td>252</td>
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<tr>
<td>GL1</td>
<td>33 ± 4</td>
<td>68 ± 5</td>
<td>0.41</td>
<td>0.35</td>
<td>0.19</td>
<td>0.03</td>
<td>0</td>
<td>285</td>
</tr>
<tr>
<td>try</td>
<td>40 ± 3</td>
<td>81 ± 4</td>
<td>0.42</td>
<td>0.30</td>
<td>0.19</td>
<td>0.09</td>
<td>0</td>
<td>355</td>
</tr>
<tr>
<td>Try−G</td>
<td>37 ± 2</td>
<td>100 ± 9</td>
<td>0.52</td>
<td>0.33</td>
<td>0.12</td>
<td>0.01</td>
<td>0</td>
<td>363</td>
</tr>
</tbody>
</table>

^cEpidermal pavement and mesophyll cell numbers were measured directly from bright-field images that were collected digitally. The reported numbers are the number of cells from an image field of 0.083 mm^2 ± SD.

^dEndoreduplication categories are based on the use of a guard cell nucleus with a 2C DNA content as a standard. Division categories are defined as shown in Figure 5.

^eTotal number of pavement cell nuclei measured.
Many of the epidermal cells resemble the abortive trichomes that are found on gl2 leaves (Oppenheimer et al., 1991). Trichomes on gl2 generally contain one or more small aerially expanding spikes, whereas the remainder of the cell expands along the epidermal surface. SEM of additional putative 35S::GL1 cot1 try leaves detected subepidermal large cells and erupting trichomes (data not shown).

**DISCUSSION**

**GL1<sup>OE</sup>, TRY, and Cell Cycle Control**

The differential response of the epidermis and the mesophyll to alterations in GL1 expression and TRY gene function emphasizes the role of tissue level control of proliferation, competency, and differentiation during multicellular development. A mutation in TRY caused an increase in cell number and trichome DNA content. This result is consistent with a role for TRY in limiting both mitotic and endoreduplication cycles and mirrors its genetically separable effects on trichome clustering and morphology (Hülskamp et al., 1994). A model of such a shared activity in wild-type epidermis and trichome development is diagrammed in Figure 8. The model assumes a loss-of-function mutation in TRY, but the mutation could be gain of function, and similar models based on positive regulation could be made.

TRY is shown as the only negative regulator of cell cycle activity in Figure 8, but other factors are likely to be involved. A mutation in TRY has only moderate effects on mitotic cycling and does not display logarithmic expansion of cell number that would be expected with runaway cell division. The phenotypic effects of try become more prominent in the sensitized GL1<sup>OE</sup> background, especially in the double mutant combination with cot1. Cell cycle studies with both yeast and animals have shown that there are multiple and redundant points of cell cycle control. The loss of only one regulatory factor often leads to either undetectable or modest changes in cell cycle parameters that are compatible with cell viability (Andrews and Measday, 1998; Brugarolas et al., 1998).

This model does not fully explain the reduced endoreduplication in mature try epidermal cells. Even if one assumes that the additional cell divisions in try give rise to cells that have a lower DNA content relative to surrounding cells that had entered the endoreduplication cycle at an earlier time, the magnitude of the effect is not sufficient to explain the ~15% decrease in try pavement cell nuclei in the 2C, 4C, 8C, and 16C categories when compared with the wild type. These results suggest that cell size and DNA content are partially uncoupled in the try epidermis when compared with the wild type. Mitotic and endoreduplication cycles may be coordinately regulated in the developing wild-type leaf, and local regions of prolonged cell division in try could disrupt the normal timing of organ level events that drive pavement cell endoreduplication. Either the signal to initiate endoreduplication or the ability to respond to that signal could be delayed or attenuated when TRY function is altered.

GL1 overexpression reduces endoreduplication levels in the epidermis and in trichomes but does not dramatically affect pavement or mesophyll cell number in the wild-type background (Table 2). Therefore, it is likely that GL1 overexpression primarily affects cells that have entered the endoreduplication cycle. Trichome-specific GL1 regulation of TRY could influence endoreduplication kinetics.

It is not known whether the differences in endoreduplication levels between 35S::GL1 and wild-type plants are due to altered timing or expression levels of GL1. Temporal differences in GL1 expression between 35S::GL1 and wild-type plants certainly exist because 35S promoter–dependent GL1 expression is stable in trichomes of mature leaves (Larkin et al., 1996), whereas wild-type GL1 transcription is only transiently detected in developing trichomes and leaf primordia (Larkin et al., 1993, 1996). Prolonged or elevated 35S promoter–dependent GL1 expression could increase TRY activity and decrease the endoreduplication level of 35S::GL1 trichomes (Figure 8). The mode of endoreduplication inhibition in Gl1<sup>OE</sup> pavement cells is not clear. The proportions of 2C, 4C, 8C, and 16C nuclei in Gl1<sup>OE</sup>, try, and Try<sup>−</sup>G pavement cells are similar, and the effects of GL1 and TRY in this cell type cannot be separated.

The spatial and temporal control of trichome initiation is sensitive to GL1 expression and TTG gene dosage (Larkin et al., 1994; Szymanski et al., 1998b), and it involves some form of cell-to-cell communication (Larkin et al., 1996). It is possible that reduced endoreduplication in Gl1<sup>OE</sup> plants reflects cell-to-cell communication and the effects of a mis-regulated inhibitory pathway that normally restricts endoreduplication in fields of epidermal cells. Alternatively, ectopic GL1 expression could abnormally alter the activity of a factor such as TRY that is required for normal endoreduplication rates in a cell-autonomous manner and consequently downregulate pavement cell endoreduplication levels.

The reduction in cellular DNA content in Gl1<sup>OE</sup> epidermal and trichome cells did not significantly alter cell size. The clear uncoupling of DNA content from cell size in 35S::GL1 contrasts with the positive correlation between pavement cell size and DNA content in the wild type (Melaragno et al., 1993). However, DNA content and cell size were also uncoupled in transgenic tobacco plants expressing the dominant negative cell cycle mutant of Arabidopsis cdc2a (Hemerly et al., 1995). Therefore, endoreduplication levels do not necessarily control cell size.

The detection of a synergistic effect of 35S::GL1 and try on trichome endoreduplication suggests the presence of two GL1-dependent activities: (1) TRY gene function is required, either directly or indirectly, for GL1 overexpression-dependent endoreduplication inhibition in trichomes; and (2) a GL1-dependent endoreduplication activation pathway must also exist in trichomes. If there were not a compensatory
Figure 5. Endoreduplication Levels in Col, Gl1oe, try, and Try-G Adaxial Epidermis Pavement Cells and Trichomes from Leaves 21 Days after Germination.

Histograms relate the frequency of nuclei to DNA content per nucleus. DNA content is expressed as the log base 2 of the ratio of RFU of a given nucleus divided by the mean stomatal nucleus RFU.

(A) to (D) Frequency distribution of the pavement cell endoreduplication levels.

(E) to (H) Frequency distribution of trichome endoreduplication levels.

(A) and (E) Col; (B) and (F) Gl1oe; (C) and (G) try; and (D) and (H) Try-G. Dashed lines indicate category divisions for predicted DNA content per nucleus. The estimated DNA content for each category is indicated along the top of each histogram. Filled arrowheads indicate the predicted DNA content of a guard cell nucleus. The broken lines in (A) through (D) indicate that the x- and/or y-axis scales are discontinuous.
activation pathway, and if 35S::GL1 operated solely through TRY-dependent suppression, 35S::GL1 try trichome nuclei would have DNA contents similar to try alone (Figure 8). The GL3 gene is a good candidate as a regulator of the endoreduplication-promoting pathway; recessive mutations in GL3 reduce endoreduplication levels in trichomes, and GL3 is epistatic to TRY (Hülskamp et al., 1994). It is possible that the timing or level of GL1 expression in trichomes affects the balance of positive and negative regulation of the endoreduplication cycle.

Subepidermal Trichomes: A Developmental Oddity

The combination of overexpression of GL1 and homozygosity for the try mutation leads to several novel phenotypes, the most prominent being the entry of subepidermal cells into the trichome developmental pathway. The actual origin of subepidermal large cells and erupting trichomes is difficult to determine. Rare periclinal divisions in the protoderm that send epidermal cells into the subepidermis occur in the wild type and have also been detected in Try^{-}G leaves (data not shown). It is possible that these cells ultimately give rise to subepidermal large cells and erupting trichomes. Regardless of their origin, plant cell identity during leaf development is largely determined by position (Demchenko, 1960; Stewart and Burk, 1970; Stewart and Demchenko, 1975), and the erupting trichomes are clearly subepidermal in position when they display attributes of trichome initiation. GL1 overexpression and an alteration in TRY activity appear to override normal positional control of trichome cell fate.

The growth dynamics and changes in nucleus position during the transformation of a subepidermal large cell into a developing trichome are illustrated in Figure 9. In the early phase of subepidermal transformation, the cell expands isodiametrically and undergoes variable numbers of endoreduplication cycles. The cell acquires apical/basal polarity, and diffuse cell expansion is observed in regions of the apical surface (Figure 9A). After the cell apex penetrates the epidermis, the distribution of cell expansion changes, and subsequent expansion occurs primarily in the aerial portions of the expanding stalk and branches. During the aerial phase of trichome growth, the nucleus migrates into the expanding stalk (Figure 9B). The nucleus position and cell wall shape of a recently erupted trichome are shown in Figure 9C. Unlike the support cells of wild-type trichomes, the epidermal cells adjacent to the erupting trichomes in Try^{-}G leaves had the appearance of pavement cells.

Several aspects of subepidermal cell trichome initiation are not understood. First, why do subepidermal cells enter the trichome pathway? In Arabidopsis, competency to enter the trichome pathway is limited to epidermal cells. Perhaps the loss of TRY function extends the temporal and spatial boundaries of competency for trichome formation, and misexpressed GL1 simply unmasksthat competency. If the function of TRY is to regulate the cell cycle, then this competency could be related to the relaxation of a cell cycle checkpoint.

Second, why does GL1 overexpression not inhibit subepidermal trichome initiation? With respect to the trichome phenotype, loss of try function in Try^{-}G leaf primarily affects subepidermal initiation, and these cells enter the pathway despite high 35S promoter activity in the leaf mesophyll (Larkin et al., 1994). Presumably GL1-dependent inhibition is not due to high levels of GL1 within a given cell but may reflect cell-to-cell communication and an inhibition pathway (Szymanski et al., 1998b). Subepidermal initiation events may reflect tissue-dependent differences in response to GL1 inhibition signals.

Third, what controls the tissue and cellular polarity of subepidermal trichomes? Only cells that contact the epidermis enter the pathway (Figure 2). This positional control exists despite the apparent effects of TRY on cell number in all layers of the mesophyll, and 35S-dependent expression of GL1 is detected throughout the developing mesophyll (Larkin et al., 1994). It is possible that some positional information, mediated by cell-to-cell contact or a diffusible morphogen(s), restricts ectopic mesophyll initiation to a single subepidermal layer. Subepidermal trichomes also display cellular polarity. To date, all subepidermal trichomes that enter a polar expansion pathway preferentially expand toward the epidermal surface. However, subepidermal cells in other plant species undergo complex morphogenetic changes. For example, the Osmanthus leaf sclereids are derived from palisade cells that underlie the epidermal layer. These cells expand into branched structures with thickened cell walls that resemble Arabidopsis leaf trichomes, but in this case, the committed cell expands inward (Griffith, 1968; Oppenheimer et al., 1992).

Last, why is homozygosity of try required for the committed subepidermal cell to penetrate the epidermal surface? In GL1^{oe} leaves heterozygous for try, some subepidermal cells are greatly expanded relative to the surrounding cells, and these cells specifically express the GL2 marker for mature trichomes. This abortive morphogenesis phenotype
could be related to TRY dosage sensitivity in the wild-type background. Plants heterozygous for try have trichomes with increased size and branch number (Folkers et al., 1997), but only homozygous try plants display significantly enhanced clustering (Table 1). Dosage sensitivity is commonly observed with mutations that affect the cell cycle (reviewed in Nasmyth, 1993), and TRY dosage sensitivity could reflect quantitative aspects of TRY function in cell cycle control.

**Try**<sup>-G</sup> and Ectopic Trichome Initiation

In strict genetic terms, try both suppresses the Gl1<sup>oe</sup> leaf phenotype and enhances the production of ectopic trichomes in Gl1<sup>oe</sup> plants. For example, cotyledon trichomes are never found on wild-type plants and are only found on 10% of 35S::GL1 plants, but cotyledon trichomes are observed on all Try<sup>-G</sup> plants. Ectopic trichomes were also observed on the stamens and carpels of Try<sup>-G</sup> plants. In other species, such as Brassica hirta, these organs normally contain trichomes (Lamb, 1980). It is possible that TRY expression limits the distribution of trichomes in Arabidopsis. Similar to what occurred in the mesophyll, TRY expression in Arabidopsis cotyledons and floral organs may render these tissues insensitive to the inductive GL1 trichome signal.

COT1 is another gene that enhances ectopic trichome production and suppresses the reduced trichome leaf phenotype of Gl1<sup>oe</sup> (Szymanski et al., 1998b). As in the 35S::GL1 try cotyledon, all 35S::GL1 cot1 plants produce cotyledon trichomes. We have previously reported that the cotyledon phenotype is synergistically enhanced in the try background (Szymanski et al., 1998b). This suggests that TRY and COT1 act through distinct mechanisms to limit cotyledon trichome initiation. 35S::GL1 cot1 plants also have a leaf trichome phenotype. On these plants, many of the support cells that surround trichomes and a small fraction of adaxial pavement cells near the leaf margin enter the trichome pathway. These trichomes initiate late, are misformed, and appear to arrest at various stages of development. In this report, we found that a defect in TRY function in the 35S::GL1 cot1 background leads to greatly enhanced trichome initiation in the adaxial epidermis. Enhanced trichome initiation on 35S::GL1 cot1 try leaves also occurs late in leaf development, and the mutant trichomes are abortive. These results suggest that TRY and COT1 have overlapping functions to limit trichome initiation in the epidermis of 35S::GL1 leaves. Because not all cells in these plants become trichomes, the presence of additional factors that limit entry into the trichome cell fate is likely.

The interactions between try, cot1, and GL1 overexpression mimic the interactions seen between cell cycle regulators in other organisms. In yeast, for example, functional redundancy and multiple mechanisms to regulate cell cycle progression are common themes. Transcription factors, inhibitors, proteases, as well as cyclins and cyclin-dependent kinases interact in complex multibranched pathways to regulate the cell cycle. In plants, many of these components have been identified (recently reviewed in Francis et al., 1998). The molecular genetics of trichome initiation may

### Table 3. Proportions of Different Classes of Endoreduplicated Trichome Nuclei in Col, Gl1<sup>oe</sup>, try, and Try<sup>-G</sup> Leaves

<table>
<thead>
<tr>
<th>Plant</th>
<th>8C&lt;sup&gt;a&lt;/sup&gt;</th>
<th>12 to 16C</th>
<th>24 to 32C</th>
<th>48 to 64C</th>
<th>&gt;96C</th>
<th>n&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>0.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.20</td>
<td>0.38</td>
<td>0.25</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Gl1&lt;sup&gt;oe&lt;/sup&gt;</td>
<td>0.28</td>
<td>0.31</td>
<td>0.34</td>
<td>0.06</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>try</td>
<td>0</td>
<td>0.14</td>
<td>0.39</td>
<td>0.47</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Try&lt;sup&gt;-G&lt;/sup&gt;</td>
<td>0</td>
<td>0.07</td>
<td>0.16</td>
<td>0.48</td>
<td>0.29</td>
<td>38</td>
</tr>
</tbody>
</table>

<sup>a</sup>Endoreduplication categories are based on the use of a guard cell nucleus with a 2C DNA content as a standard. Division categories are defined as shown in Figure 5.

<sup>b</sup>Total number of nuclei measured within assignable categories.

<sup>c</sup>Proportion calculations did not include ambiguous values within a midpoint category between upper and lower intervals of consecutive categories.

![Figure 7. SEM Images of the Adaxial Surface of the First Leaf of a Putative 35S::GL1 cot1 try Leaf 21 Days after Germination.](image)

(A) Putative 35S::GL1 cot1 try viewed at low magnification. (B) Higher magnification of the boxed region in (A). et, erupting trichome; mt, mature trichome. Bars in (A) and (B) = 50 μm.
Interaction between try and GL1 Overexpression

Conclusion

In this study, we describe a relationship among GL1 overexpression, TRY gene function, and cell cycle regulation during leaf and trichome development and provide a basis for understanding the dual function of genes that regulate the cell cycle and differentiation. Our results also suggest that mitotic and endoreduplication cycles are coordinately regulated and contain both shared (TRY) and unique (GL1) components. The nontrichome try phenotype raises questions concerning its role in trichome development. This mutant was originally isolated based on increased trichome clustering, branch number, and trichome DNA content, but it appears that TRY plays a more general role in leaf development. The actual function of TRY during trichome initiation and morphogenesis will remain unclear until the gene is cloned and additional genes in the pathway are identified. The observed cell cycle effects of GL1 overexpression and mutation in TRY highlight the importance of the relationship between control of the cell cycle and differentiation, but many questions regarding the mechanism of GL1 and TRY cell cycle control remain unanswered. The challenge of future experiments is to define the pathways and cell cycle components that GL1 and TRY regulate.

Schnittger et al. (1998) have recently reported that ectopic GL1 expression in a try background leads to the formation of subepidermally derived trichomes and an enhancement of ectopic trichome formation in other regions of the shoot. These authors analyzed the effects of TTG dosage and ectopic expression of the maize R gene on this phenotype; they showed that TTG dosage did not affect subepidermal trichome formation and that try mutants ectopically expressing R did not produce subepidermal trichomes.

Trichome endoreduplication levels were also measured by Schnittger et al. (1998). The endoreduplication levels they reported for most of the genotypes are comparable to those provided by try and GL1 overexpression.
reported here, but the trend toward elevated endoreduplication in 35S::GL1 trichomes that they observed is not consistent with our results. Differences in methodology and data analysis could explain this discrepancy. For example, Schnittger et al. (1998) used a photometer to collect analog fluorescence intensity data, whereas we adopted optical sectioning and digital imaging for fluorescence quantitation. We feel that this latter technique better accommodates the heterogeneity in nucleus shape, orientation, and background fluorescence.

Our model of the effects of GL1 and TRY is based upon the endoreduplication levels in the trichome, which suggest to us that GL1 is a positive regulator of TRY. By contrast, in the model presented by Schnittger et al. (1998), TRY is depicted as an inhibitor of GL1 activity during trichome formation. Clearly, determining the true nature of the interactions between these proteins must await the cloning and characterization of TRY.

METHODS

Plant Strains and Growth Conditions

For all phenotypic characterizations, Arabidopsis thaliana seeds were grown on a soil mixture consisting of a vermiculite base overlaid with ~5 cm of Peters soil mix (Scotts Company, Marysville, OH) and fertilized with 0.5 × Hoagland solution. Growth chambers were maintained at 22°C with constant illumination at 80 μE m⁻² sec⁻¹. Seeds germinated on plates were surface-sterilized and propagated on minimal Murashige and Skoog salts (Life Technologies, Gaithersburg, MD), 1% sucrose, and 0.8% Bacto Agar (Life Technologies). Imbibed seeds were cold treated at 4°C for 8 days before incubation at 22°C. At 21 days after germination, seedlings were fixed for 30 min at room temperature in a 3:1 mixture of 95% ethanol-glacial acetic acid and 1 mM MgCl₂, MgCl₂ (1 mM) was maintained throughout sample preparation and analysis. Samples were cleared in 95% ethanol overnight and then rehydrated in a series into staining buffer (10 mM Tris-HCl, pH 7.0, and 1 mM MgCl₂). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) at 0.1 μg mL⁻¹ for 10 sec at room temperature. Samples were rinsed with staining buffer and then washed at 4°C overnight with gentle shaking. Stained samples were stable for at least 3 weeks.

Intact leaves from the first leaf pair were mounted in staining buffer in a chambered slide for fluorescence microscopy. To minimize spatial heterogeneity in epidermal DNA content within each leaf and to limit analysis to the most mature regions of the leaf, we focused sampling on the leaf perimeter 2 to 5 mm from the leaf margin. Three plate-grown leaves were sampled in at least six positions to measure ~200 nuclei per genotype. All intact and accessible trichomes on a given leaf were also analyzed.

Preparations were viewed using a photomicroscope (Eclipse E800; Nikon) equipped with bright-field, DIC, dark-field, and fluorescence optics, including a 100-W mercury lamp epifluorescence illumination with standard UV light (excitation filter 330 to 380; barrier 420 nm). The samples were viewed using a 10×, 0.30 n.a. plan fluor; or 40×, 0.75 n.a. plan fluor; or 60×, 1.40 n.a. plan fluor objective. Digital images were collected using a CoolCam liquid-cooled, three-chip color CCD camera (Cool Camera Co., Decatur, GA) and captured to a 486DX2 personal computer using Image Pro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD). Digital images were stored on an Iomega ZIP drive (Iomega Corp., Roy, VT).

The fluorescence images used for quantitation were the maximum projection of between two and five focal planes for epidermis nuclei analysis and one or two focal planes for trichomes. Composite images were converted to gray-scale eight-bit images, and background was corrected using Image Pro Plus, version 3.0. DAPI fluorescence was quantitated using National Institutes of Health (NIH) Bethesda, MD) Image ppc v1.61 (NIH ftp site: ftp://codon.nih.gov/pub/NIH Image/). To correct for spatial heterogeneity in DAPI fluorescence in different regions of the leaf, all epidermal observation fields contained at least three guard cell nuclei that were used as an internal standard and as an indicator of relative DNA content of epidermal pavement and trichome cell nuclei.

The calculated relative fluorescence intensity values were analyzed using Microsoft Excel (Microsoft Corp.). The calculated guard cell relative fluorescence intensity was used as an indicator of image quality. The magnitude of the standard deviation of the guard cell quantitation was used as an indicator of image quality, and a standard deviation of <25% of the mean within a field was used as a criterion for acceptance of the data. Relative fluorescence values for pavement and trichome nuclei were expressed as a ratio over the mean guard cell fluorescence within a given observation field, and the measurements taken from several fields within the same leaf were pooled. Because trichome nuclei and guard cell nuclei were not nearly parfocal, trichome DNA measurements for a given leaf were calculated using the mean guard cell intensity calculated from at
least eight guard cell nuclei randomly sampled over the leaf of interest. The log base 2 of relative fluorescence intensity ratios was plotted as frequency distribution histograms. To calculate the proportions of nuclei in discrete ploidy categories, we applied specific cutoff values to the frequency distributions of each genotype.

Cell Counting

Measurements of epidermal cell numbers were obtained from impressions of fully expanded leaves 21 days after germination. A thin layer of Wet-n-Wild nail polish protector (Pavion Ltd., Nyack-on-Hudson, NY) was applied to the adaxial leaf surface and allowed to dry. Removal of the dried nail polish impression yielded near complete replication of the leaf surface. Peels were mounted in 80% glycerol and viewed at 400×. Measurements from three different leaves. Mesophyll cell number was the mean of three independent measurements from three different leaves. Mesophyll cell number was measured using the same scheme, except that a monolayer of mesophyll cells was visualized in whole mounted leaves that had been fixed, cleared, and rehydrated as described above.

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REFERENCES


GLABROUS1 Overexpression and TRIPTYCHON Alter the Cell Cycle and Trichome Cell Fate in Arabidopsis

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