A contradictory *GLABRA3* allele helps define gene interactions controlling trichome development in *Arabidopsis*

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Summary

Previously characterized *Arabidopsis gl3* mutants have trichomes that are smaller, less branched and undergo fewer rounds of endoreplication than wild-type trichomes. A new *gl3* mutant, called *gl3-sst*, has oddly shaped trichomes that over expand during early development, undergo more endoreduplication and that have a striking nuclear morphology. The mutant nuclei consist of many interconnected lobes; however, only a single set of polytene-like chromosomes reside in the mutant nuclei. The predicted gl3-sst polypeptide has a Leu to Phe substitution (codon 78) within a region responsible for protein-protein interaction. Yeast interaction assays comparing GL3 with gl3-sst proteins show that the mutant protein interaction with GL1 and TTG1 is decreased by 75% and 50%, respectively, but there is no difference in its interaction with TRY.

Furthermore, TRY has the ability to prevent the GL1 GL3 interaction and the GL1 gl3-sst interaction is even more sensitive to TRY. Analysis of plants expressing functional GFP-tagged versions of GL1, GL3 and TRY show that the proteins are localized in trichome nuclei. These results have been used to model trichome initiation in terms of protein interactions and threshold levels of activator complex.

Supplemental data available online

Key words: Trichome, bHLH, MYB, GL1, TTG1, GL3, CPC, TRY, WER, Transcription factor, pBridge, Lac OP/I-GFP, Endoreduplication, Endoreplication, Elemental analysis, GFP, Nuclear localization, Interphase chromosomes, Cell fate, Differentiation, Cell cycle, Cytoskeleton, Root hair

Introduction

Arabidopsis leaf trichomes are unicellular, and are composed of a stalk and two to four branches. Trichome development can easily be observed on young leaves where the mature trichomes are found at the tip and in various stages of development toward the base (Hülskamp et al., 1994; Szymanski et al., 1998). Trichome development can be divided into six stages (Szymanski et al., 1999). At stage one an epidermal cell ceases to divide and undergoes radial expansion. During subsequent stages developing trichomes expand out of the leaf surface and produce two to four branches. They then cease to expand and form a thick cell wall containing surface papillae during the sixth and final phase. There is a pattern to trichome development in that trichomes rarely develop next to one another and this pattern appears to be controlled by cell-cell communication (Larkin et al., 1996). The development of leaf trichomes in *Arabidopsis* is being used to address questions concerning cell fate specification, pattern formation and cellular differentiation (Hülskamp et al., 1998; Marks et al., 1991; Szymanski et al., 2000).

Genetic analyses have identified three genes that are involved in promoting trichome initiation: *GLABROUS1* (*GL1*), a R2R3-Myb-type transcription factor; *TRANSPARENT TESTA GLABRA1* (*TTG1*), a protein with WD-40 repeats; and *GLABRA3* (*GL3*), a bHLH type transcription factor (Oppenheimer et al., 1991; Payne et al., 2000; Walker et al.,

1999). Severe loss-of-function mutations in either *GL1* or *TTG1* result in greatly reduced trichome initiation. Mutations in *GL3* moderately reduce trichome initiation and result in smaller trichomes that have fewer branches (Koornneef et al., 1982; Payne et al., 2000). The *Arabidopsis* genome contains another gene closely related to *GL3*, *ENHANCER OF GLABRA3* (*EGL3*). Plants containing mutations in both *GL3* and *EGL3* display the greatly reduced trichome initiation phenotype that is exhibited in *gl1* and *ttg1* mutants (Zhang et al., 2003). Mutations in *EGL3* alone only slightly alter trichome initiation and development. Unlike *gl1* mutants, which only affect trichome development, *ttg1* and *gl3 egl3* mutants display additional defects that include the production of extra root hairs and the loss of both pigmentation and seed coat mucilage production (Galway et al., 1994; Koornneef, 1981; Zhang et al., 2003).

The expression patterns of *GL1* and *GL3* have been characterized. Both genes are diffusely expressed at low levels in fields of epidermal cells on young leaves, and then expressed at higher levels in early stage trichomes (Larkin et al., 1993; Zhang et al., 2003). Additional studies have shown that the GL1 and GL3 proteins probably act in a complex with TTG1. It is thought that a threshold level of the GL1 GL3 TTG1 activator complex is needed to irreversibly push a cell into the trichome pathway (Payne et al., 2000; Schellmann et al., 2002; Szymanski et al., 2000). The predicted consequence of reaching

the threshold is the generation of an autoregulatory loop that results in the up-regulation of the complex genes, as well as genes needed for the initial stages of trichome differentiation, including *TRYPTICHON (TRY)*. *TRY*, which encodes a single-repeat R3 Myb, limits trichome initiation and prevents neighboring cells from becoming trichomes (Hülskamp et al., 1994; Schellmann et al., 2002). In yeast assays, TRY physically interacts with GL3 and may function to reduce the ability of the GL1 GL3 TTG1 activation complex to activate gene expression in developing trichomes and those cells that neighbor trichomes (Zhang et al., 2003). To mediate this latter role, it has been proposed that once translated in developing trichomes, TRY protein diffuses through the connecting plasmodesmata to neighboring cells (Schellmann et al., 2002).

A model similar to the trichome initiation model has been proposed for root hair (H-cell) development where the WER, TTG1 and GL3/EGL3 proteins form an initiation complex to promote the N-cell fate (non hair) (Schiefelbein, 2003; Zhang et al., 2003). The *CAPRICE* (*CPC*) gene, which encodes a single-repeat R3 MYB homologous to *TRY* and is expressed in N-cells, acts as a negative regulator of the N-cell fate (Lee and Schiefelbein, 2002; Wada et al., 1997). CPC protein has been shown to be able to travel to H-cells and to down-regulate the expression of N-cell genes like *WER* in H-cells (Lee and Schiefelbein, 2002; Wada et al., 2002).

This paper presents the characterization of a new allele of *GL3*. In contrast to previously described *gl3* mutants, the trichomes of the new mutant display greatly increased levels of endoreduplication and over expand early during development. This novel contradictory *gl3* phenotype has provided a new insight into trichome development.

Materials and methods

Plant strains and growth condition

The *gl1* mutant used in this study was isolated from the Weigel Activation tagged T-DNA lines (Weigel et al., 2000). The *try* and *egl3* mutants were identified using the SIGnAL website (http://signal.salk.edu) and were derived from SALK lines 29760 and 77439, respectively. *gl3-1* was obtained from Dr Koornneef in a L*er* background and was backcrossed three times into a Col background before being used in these analyses. Plants were grown on soil (LG3 and LP5, Sungrow Horticulture, Bellevue, WA) at 22°C and constant illumination of 80 µE/m^2/second.

Mapping and isolation of *sst*

Bulk segregation analysis of an F$_2$ population derived from a cross between Col *sst* and L*er* was used to determine the general chromosomal location of *sst* (Lukowitz et al., 2000). To more finely map the chromosomal location of *sst*, DNA samples from 302 *sst* F$_2$ plants were tested with PhyC, CiW9 and CiW10 SSLP marker primer pairs (Research Genetics). Data from these reactions placed the *sst* locus between PhyC and CiW9 on chromosome 5. Additional SSLP markers were created utilizing information from the insertion/deletion (InDel) polymorphism collection generated by Cereon Genomics (now Monsanto Co.) (Jander et al., 2002). InDels 454480 and 457742 were ultimately used to limit the *sst* locus to either TAC clone K1O13 or P1 clone Myc6. Because the Myc6 clone contained the candidate *GL3* gene, *sst* was tested for allelism to *GL3*.

F$_2$ genotyping of *gl3-1, gl3-sst* and SALK TDNA line 77439

The *gl3-1* mutation causes a C to T transition at codon 378, which results in the loss of an *Aci*I site. Using forward 5′-TCAGTAC-

GGAGCCTTTTCCAACAGC-3′ and reverse 5′-CTTTAACATTCCT-TGTGATGGTGCC-3′ primers, a 509 bp genomic fragment was amplified using the Extract-N-Amp PCR system (Sigma). Digesting the samples with *Aci*I generated fragments of 174 bp and 73 bp for Col compared with a 247 bp fragment for *gl3-1*.

The *gl3-sst* mutation causes a C to T transition at codon 78 leading to the elimination of a *Dde*I site. Using forward 5′-GCGAAT-TCGCCATGGCTACCGGACAAAACAGAAC-3′ and reverse 5′-GCGGAAGCTCGTCTGGTGAC-3′ primers, a 661 bp fragment was generated by PCR. Digesting the samples with *Dde*I generated fragments of 460 bp and 89 bp for Col compared with a 549 bp fragment for *gl3-sst*.

SALK line 77439 contains a T-DNA insert in the third exon of *At1g63650*. To distinguish between the insertional mutant and wild loci two sets of primers were used. One set of primers (forward 5′-CCCCGGAGGAGCGTTATCCAATGGA-3′ and reverse 5′-CCCT-TAAGTGACGATAAATACACTCCG-3′) was used to detect the wild-type gene and another set of primers (forward 5′-GCGTGGAC-CGCTTGCTGCAACT-3′ and reverse 5′-CCCTTAAGTGACGATA-AATACACTCCG-3′) was used to detect the T-DNA insertion.

Scanning electron microscopy and x-ray elemental analysis

The plant specimens were frozen in liquid nitrogen and processed with an Emitech K1150 cryo-preparation system where the specimens were sputter coated with 20 nm gold and imaged using a cold stage with a Hitachi S3500N scanning electron microscope. For X-ray elemental analysis, the samples were sputter coated with 20 nm of nickel and analyzed with the EDAX, Inc. Falcon System.

Vector construction for the Lac Operator/Repressor-GFP reporter system

The p928 LacOP construct, which contains 256 repeats of the Lac Operator sequence, was generated by moving a 12.5 kb fragment containing the repeated sequence from pSV2-dhfr 8.32 (Robinett et al., 1996) into the T-DNA vector p928 (gift from Dr Mitra, University of Nebraska).

The coding sequence of a modified LacI repressor protein and a C-terminal SV40 nuclear localization sequence used for the pEGAD LacI construct originated from a 1276 bp *Eco*RI-*Dra*I fragment from p3′SSdimer-Cl-EGFP (Robinett et al., 1996). This fragment was cloned into the *Eco*RI and blunt-ended *Hin*dIII sites of the 35S::GFP-containing T-DNA vector pEGAD (Cutler et al., 2000) to create plasmid pEGAD LacI.

Construction, transformation and imaging of GFP-fusions in *Arabidopsis*

The promoter regions from *MYB5* (forward 5′-GAGCTCTGCTG-GAGAAATTCATCCCAA-3′ and reverse 5′-ACCGGTCTCCGCC-GTCTTCAACAAAGC-3′), *ATML1* (forward 5′-GAGCTCGATCC-ATAGTTTCTAAAATGTGC-3′ and reverse 5′-ACCGGTGGATTC-AGGGAGTTTCTTTAACC-3′), *TRY* (forward 5′-AAGCTTGAGCT-CGTTAGTTTAATGGGTCGA-3′ and reverse 5′-GGATCCACCGG-TGATGAAATTTTGAGTTTGG-3′) were amplified from Columbia genomic DNA using JumpStart Taq Redimix (Sigma) and cloned into pCR2.1 using the Topo-TA cloning system (Invitrogen). Each promoter was then excised as a *Sac*I-*Age*I fragment and was swapped with the 35S promoter of pEGAD (Cutler et al., 2000). *pEMYB5::GFP-cGL3* was generated by excising the coding region of *GL3* from pD2CD-7 (a gift from Dr Lloyd, University of Texas) as an *Eco*RI-*Bam*HI fragment and cloning into the corresponding sites of pE*GAD MYB5 Pro. pEATML1::GFP-cGL1* was generated by excising the coding region of *GL1* from *pGL1-A* (a gift from Dr Lloyd) with *Eco*RI and *Bgl*II and cloning it into the corresponding sites of *pEGAD ATML Pro. pETRY::GFP-cTRY* and *pEMYB5::GFP-cTRY* were generated by excising the coding region of *TRY* from *pCR-TRY-BD* as an *Eco*RI/*Bam*HI fragment and cloning it into the

appropriate sites of *pEGAD TRY Pro* and pE*GAD MYB5 Pro*, respectively

The binary constructs were introduced into *Agrobacterium tumefaciens* C58C1 by electroporation. The *Arabidopsis* Columbia strains were transformed with *Agrobacterium* by dipping and selected on soil with Basta (Clough and Bent, 1998). GFP flourescence was detected in mature trichomes using a Nikon Eclipse E800 microscope with a Cool Cam color CCD camera (Cool Camera Co., Decatur, GA) and Imago Pro Plus version 3.0 software (Media Cybermetics, Silver Spring, MD).

DAPI staining

Mature leaf tissue from Columbia wild-type, *gl3-1* and *gl3-sst* was stained with DAPI (4′6-diamidino-2-phenylindole; Sigma) as previously described (Szymanski and Marks, 1998). The staining was visualized with a Nikon Diaphot-200 inverted microscope with DIC optics and a Kodak MDS290 photo capture system.

Construction, transformation, and analysis of yeast constructs

p*GL3-AD* was constructed by cloning an *Eco*RI/*Bam*HI fragment containing the *GL3* coding region from pD2DC-7 [(Payne et al., 2000) a gift from Dr Lloyd] into the *Eco*RI and *Bgl*II sites of pGAD424 (Clontech). p*sst-AD* was generated by swapping a 702 bp *Bam*HI/*Bgl*II fragment from pCR-*sst-N* with the corresponding fragment from p*GL3-AD*. The *GL3* coding region from the *gl3-sst* mutant (pCR-*sst-N*) was generated by PCR amplifying a 1.08 kp fragment from *gl3-sst* cDNA using the primers, forward 5′-GC-GAATTCGCCATGGCTACCGGACAAAACAGAAC-3′ and reverse 5′-CGGATCAAGAACGTTGTCGATGTG-3′. The fragment was cloned into pCR2.1 (Invitrogen) and the presence of only the *gl3-sst* specific C to T change at positon 235 was confirmed by DNA sequencing. The coding regions for *GL1*, *TTG1* and *TRY* used to make GAL4 binding domain fusions originated from p*GL1-B*, pWS10 and pCR-*TRY-BD*. Both p*GL1-B* and pWS10 were gifts from Dr Lloyd (Payne et al., 2000), whereas pCR-*TRY-BD* was a PCR-generated *TRY* coding region from Columbia cDNA (forward 5′-GAATTCG-CCATGGATAACACTGACCGTCGT-3′ and reverse 5′-GGATCCCT-AGGAAGGATAGATAGAAAAGCG-3′) that was cloned into pCR2.1 (Invitrogen) and verified by DNA sequencing. The p*GL1-BD*, p*TTG1-BD* and p*TRY-BD* were generated by cloning a 0.62 kb *Eco*RI/*Pst*I fragment, 1 kb *Eco*RI/*Sal*I fragment and 0.32 kb *Eco*RI/*Bam*HI fragment, respectively, into the corresponding sites of pBridge (Clontech).

The construct used for the GL1-TRY competition assay, p*GL1-BD/TRY-free,* was generated by cloning a 0.32 kb *Not*I/*Bam*HI fragment from pCR-*TRY*-FR into the *Not*I and *Bgl*II sites of p*GL1-BD*. pCR-*TRY-FR* was generated by cloning a PCR-generated *TRY* coding region from Columbia cDNA (forward 5′-GCGGCCGCAGC-CATGGATAACACTGACCG-3′ and reverse 5′-GGATCCCTAGGA-AGGATAGATAGAAAAGCG-3′) into pCR2.1 (Invitrogen).

The appropriate pGAD424- and pBridge-based constructs were transformed sequentially into the yeast strain Y190 using electroporation. The cells were selected on plates containing SD synthetic medium (2% glucose, 1× yeast nitrogen base) lacking first only Leu, then Leu and Trp. Liquid cultures of SD synthetic medium lacking Leu and Trp were used to measure β-galactosidase (β-gal) activity (Ausubel et al., 1995). The cells were grown to an OD_{600}=0.7-1.0, pelleted by centrifugation and suspended in z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0). The cells were permeabilized by adding a final concentration of 0.005% SDS and 3.5% chloroform (v/v). ONPG (o-nitrophenyl-D-galactopyranoside; Sigma) was added as a substrate. After incubation at 30°C, the reaction was stopped with sodium carbonate and measured for activity with an OD_{420}. Units of β-gal activity was determine by the equation U=1000×[OD_{420}]/time (in seconds) × volume (in ml) × [OD_{600}]. For each comparison, multiple

independent yeast isolates (*n*=4-8) were tested multiple times (*n*=3-6).

Results

The *sst* mutation represents a new allele of the *GL3* locus

A new trichome mutant with a highly pleiotropic trichome phenotype was found in an EMS-treated Col population (Fig. 1B). Trichomes on wild-type leaves typically consist of a stalk and three to four branches (Fig. 1A,D). The trichomes on the leaves of the mutant varied from nearly normal (Fig. 1E) to lacking a normal stalk and exhibiting multiple abnormal branches (Fig. 1F-I), to lacking branches (Fig. 1J). For this reason, the mutant was tentatively named *shapeshifter (sst)*. The *sst* trichomes also varied in their degree of maturation. During the final phase of development, wild-type trichomes develop papillae on the outer surface of the cell wall (Fig. 1M). While the normal appearing *sst* trichomes developed a few papillae (Fig. 1O), most mutant trichomes lacked papillae (Fig. 1N). In addition to the morphological defects in trichome
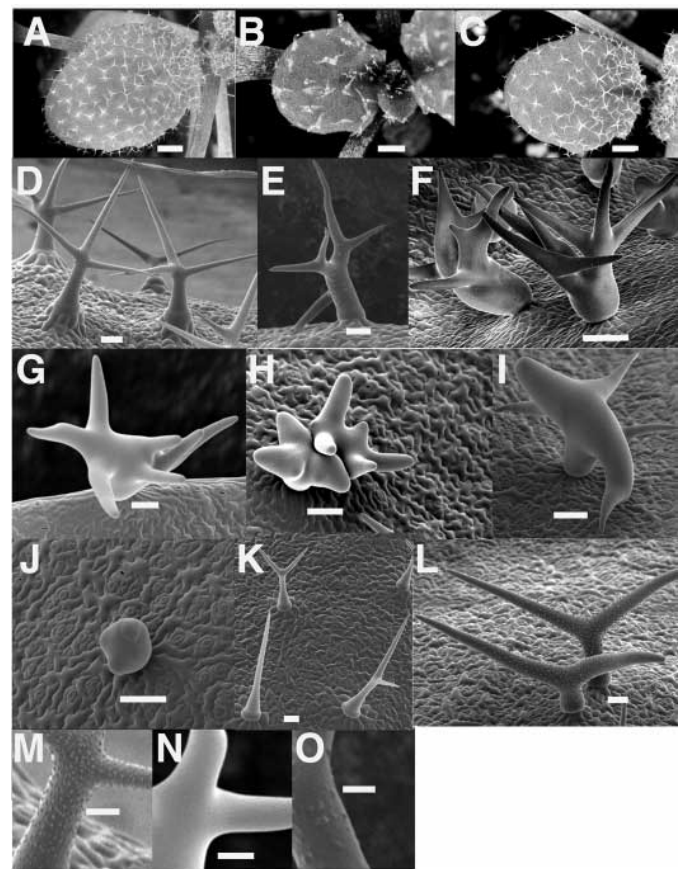
Fig. 1. Comparison of *shapeshifter* (*sst*), Col wild-type and *gl3-1* leaf trichomes. (A-C) Stereomicroscopic images of young leaves of Col, homozygous *sst* and heterozygous *sst* plants, respectively. Scale bars: 0.5 mm. (D-O) Scanning electon micrographs of mature trichomes. (D) Col. (E-J) Homozygous *sst* leaves. Note the size of D compared with G and H; scale bars in D-L 50 µm. (K) *gl3-1* and (L) *sst* × *gl3-1* F₁ leaf trichomes. (M-O) Closeup of Col trichome, papillaeless *sst* trichome, *sst* trichome with papillae, respectively. (M-O) Scale bars: 25 µm.

development, *sst* leaves exhibited fewer trichomes that were abnormally clustered (Table 1).

The F$_1$ plants from crosses between *sst* and Col shared two trichome abnormalities with homozygous *sst* mutants. First, there were fewer trichomes per leaf and second, the F$_1$ trichomes consistently had more branches than wild-type trichomes (Table 1 and Fig. 1C). Neither of the alterations in the F$_1$ phenotype was as extreme as seen in the homozygous mutant. The segregation analysis of the F$_2$ population indicates that the severe *sst* phenotype was recessive and probably caused by a mutation at a single locus (53 *sst*; 155 wild type + extra branches; fits 1:3, χ^2=0.025, P>0.8). These findings were further supported by the analysis of selected F$_3$ populations. It was found that all selected F$_2$ plants with wild-type trichomes only produced wild-type F$_3$ plants, *sst* F$_2$ plants only produced *sst* F$_3$s, whereas the F$_2$ plants with trichomes containing extra branches gave rise to segregating F$_3$ populations (data not shown).

The *sst* mutation was mapped to a chromosomal region containing the candidate gene *GL3*. To determine if *sst* represented a new allele of *GL3*, *sst* was crossed with the *gl3-1* mutant. The F$_1$ plants had a distinct phenotype as shown in Fig. 1L. The trichomes on these plants tended to be less branched than wild-type trichomes, but the branches were much larger in diameter than *gl3-1* mutant trichomes (Fig. 1K). These results suggested that *sst* is an allele of *GL3*. To obtain molecular evidence that *sst* was a new *GL3* allele, the *GL3* locus in the *sst* mutant was isolated by PCR and sequenced. The *GL3* locus of *sst* contained a single base change of C to T in codon 78, which converts Leu to Phe. As a final indicator that *sst* was a new allele of *GL3*, a green fluorescent protein (GFP)-tagged version of the wild-type *GL3* coding region was moved into *sst* plants (see Materials and methods). This gene fusion was placed under the control of the *Arabidopsis MYB5* gene promoter, which has previously been shown to drive gene expression in trichomes (Li et al., 1996). This construct rescued the phenotype resulting from both *sst* and *gl3-1* mutations (Fig. 2). These results demonstrated that *sst* represented a new *GL3* allele, which hereafter will be called *gl3-sst*.

gl3-sst mutant trichomes display defects in early cell wall expansion, cell wall maturation and endoreduplication

The *gl3-sst* trichomes exhibit a dramatic increase in cell expansion during early stages of trichome development (Fig.
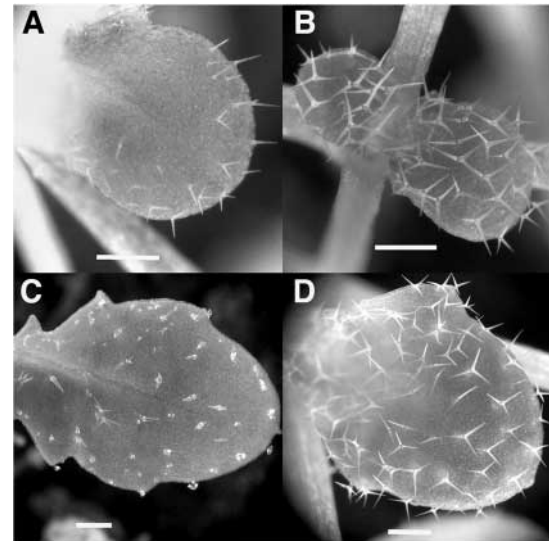
Fig. 2. Molecular complementation of *gl3-1* and *gl3-sst* mutants. Young leaves of (A) *gl3-1* and (C) *gl3-sst* mutants. (B,D) Leaves of (B) *gl3-1* and (D) *gl3-sst* plants transformed with pE*MYB5*::*GFP-*c*GL3* construct, Scale bar: (A,B) 0.25 mm; (C,D) 0.5 mm.

3A-F; note differences in bar scales between A and D, B and E, C and F). This resulted in most stage two *gl3-sst* trichomes being much larger than wild-type trichomes (Fig. 3E). The increased surface area of early stage mutant trichomes appeared to support a larger number of branch initiation sites (Fig. 3F). The degree to which the branches expanded varied greatly (compare Fig. 1F with 1H). While trichome expansion eventually ceased, very few *gl3-sst* trichomes developed papillae. In contrast to the excessive expansion seen for *gl3-sst* trichomes, *gl3-1* trichomes expanded less during early stages (Fig. 3G,H).

The elemental composition of Col and *gl3-sst* trichome cell walls was compared using SEM EDAX, which can determine the major elemental composition of selected surfaces (see Materials and methods). The papillae on Col trichomes contained carbon, oxygen, magnesium, calcium and phosphorus, whereas the smooth wall regions between the papillae were lacking phosphorus (see Supplemental data Fig. S1 at http://dev.biologists.org/supplemental/). For the walls of *gl3-sst* trichomes that contained papillae, it was found that both the papillae and intervening regions lacked phosphorus and

Table 1. Analysis of leaf trichome and trichome branch number

	Number of trichomes on first leaf*	Number of trichomes on third leaf*	Percent of trichomes in clusters[†]	Number of branches per trichomes*
Col	34.8±2.3	83.8±6.0	0.3%	61% three branches 39% four branches
Shapeshifter	15.5±3.3	39.7±8.9	14.2%	Not determined[‡]
Heterozygote	26.6±5.9	55.5±8.5	0.5%	32% three branches 41% four branches 25% five branches 1% six branches

*Average±s.d.
[†](number of trichomes neighboring one another/total number of trichomes) × 100 (%).
[‡]Many branches were too small to be accurately counted.
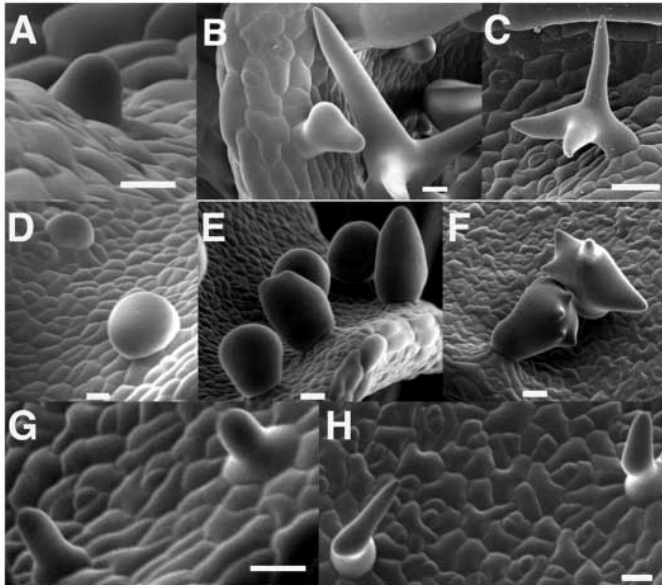
Fig. 3. SEM analysis of developing trichomes on Col, *gl3-sst* and *gl3-1* leaves. (A,D,G) Stage 2 trichomes on Col, *gl3-sst* and *gl3-1* leaves, respectively. (B,E,H) Stage 3-4 trichomes on Col, *gl3-sst*, and *gl3-1* leaves, respectively. (C,F) Stage 5 trichomes on Col and *gl3-sst* leaves, respectively. Scale bar: (A,B,D-H) 10 μm; (C) 25 μm.

Fig. 4. Comparison of Col, *gl3-sst* and *gl3-1* nuclei.
(A-C) Fluorescence images of DAPI-stained Col, *gl3-sst*, and *gl3-1* leaf trichomes. (D-F) Maximum projections of confocal images of trichome nuclei expressing the nuclear envelope-localized N7 GFP fusion gene: (D) Col, (E,F) two different *gl3-sst* trichome nuclei. Scale bar: (A-C) 50 μm; (D-F) 25 μm.

magnesium. The *gl3-sst* trichomes that lacked papillae also lacked calcium, as well as phosphorus and magnesium. These cell walls were similar in composition to the mature guard and pavement cells surrounding the trichomes on wild-type leaves, which only showed peaks for carbon and oxygen.

The DNA content of mature Col trichomes ranges from 16C to 64C (Hülskamp et al., 1994; Melaragno et al., 1993; Szymanski and Marks, 1998). The analysis of images of DAPI-stained *gl3-sst* trichome nuclei indicated that the *gl3-sst* trichomes contain much more DNA than wild-type or *gl3-1* trichomes (compare Fig. 4A, B and C, captured at the same magnification and exposure settings). Interestingly, the DAPI-stained *gl3-sst* mutant nuclei appeared to be composed of numerous lobes collapsed upon one another. To observe the nuclei in living plants, Col and *gl3-sst* plants expressing the nuclear-localized N7 GFP fusion were used for confocal analysis (Cutler et al., 2000) (see Materials and methods). This analysis showed that wild-type N7 nuclei were single spheres (Fig. 4D), whereas the *gl3-sst* N7 nuclei were composed of a series of interconnected lobes (Fig. 4E,F).

The *gl3-sst* trichome nuclear defect was further characterized to determine if each nuclear lobe in *gl3-sst* trichomes contains a distinct set of chromosomes. This question was addressed using the two component Lac Operator/Repressor-GFP (Lac OP/I-GFP) reporter system that has been used to visualize interphase chromatin (Robinett et al., 1996) (see Materials and methods). Fig. 5 shows representative confocal images of Col and *gl3-sst* nuclei from plants that contained integrated Lac Operator sequences and that expressed the Lac I-GFP fusion protein. To compare cells that undergo different degrees of endoreduplication, the trichome nuclei (Fig. 5A,B) were compared to the nuclei of guard cells and epidermal pavement cells (Fig. 5C,D). All nuclei exhibited a low level of green fluorescence, which
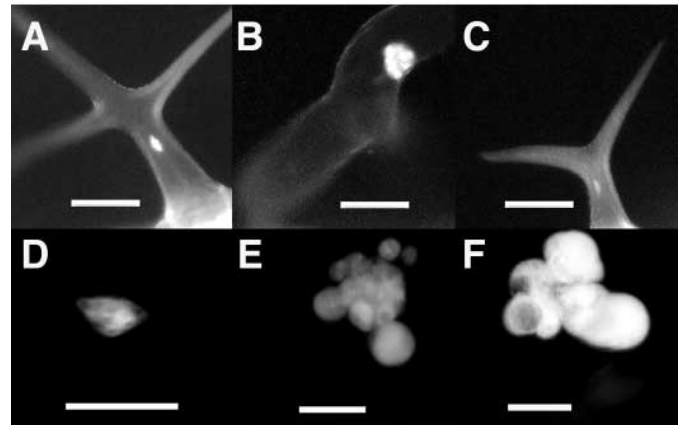
allowed the outline of the nuclei to be visualized. The guard cells have been shown to contain 2C DNA (Melaragno et al., 1993) and a single faint spot, corresponding to the binding of Lac I-GFP to the integrated Operator, was seen in most of the guard cell nuclei of Col and *gl3-sst* leaves (Fig. 5C,D). Epidermal pavement cells typically have a DNA content ranging from 4C to 16C and, as shown in Fig. 5C,D, these cells typically contained brighter spots. Col trichomes contain an average of 32C and, as shown in Fig. 5A, the integrated Lac operator was most often visualized in interphase chromatin as a single intense spot (*n*=3 independent lines), although occasionally closely clustered bright spots were seen (not shown). Shown in Fig. 5B is an image of a *gl3-sst* trichome nucleus (*n*=5 independent lines) with a relatively large focus of fluorescence. Two lines (one *gl3-sst* and one Col) had trichome nuclei with two intense spots and these lines probably have two Lac OP inserts. Lines expressing Lac I-GFP, but lacking the integrated Lac OP repeat did not have spots. These lines were first generation transformants for the Lac OP repeat, so the copy number of Lac OP units has not been assessed. This analysis also showed that the morphology of non-trichome epidermal cell nuclei in *gl3-sst* was not affected by the mutation (compare Fig. 5C,D).

The *gl3-sst* allele is functional

It was previously noted that *EGL3* is functionally redundant to *GL3* and it has been shown that *gl3-1 egl3* plants lack trichomes (Payne et al., 2000; Zhang et al., 2003). To determine if *EGL3* is needed for the *gl3-sst* trichome development, an *egl3* mutant was crossed to *gl3-sst*. The *egl3* mutant used for this analysis was the previously uncharacterized SALK line 77439, which contained a T-DNA insertion in the third exon of *EGL3*. Thus, to determine if the T-DNA insertion mutant line behaved in the same matter as previously described *egl3* mutants, the T-DNA mutant was also crossed to *gl3-1*. As previously described for *egl3* and *gl3-1* interactions, it was found that plants homozygous for the insertion and *gl3-1* were glabrous. In the F$_2$ population of plants derived from the cross

between the insertional mutant and *gl3-sst*, 24 plants had a wild-type phenotype and another 10 closely resembled *gl3-sst*. Progeny from five of the F₂ *gl3-sst*-like lines were analyzed. One of the five F₃ populations (*n*=25 plants) consisted solely of plants with a reduced number of *gl3-sst*-like trichomes. These plants were found to be homozygous for both *gl3-sst* and the T-DNA insertion (see Materials and methods for genotyping). In addition to the reduced number of leaf trichomes, it was found that the double mutants lacked stem trichomes (see Supplemental data Fig. S2 at http://dev.biologists.org/supplemental/).

## The *gl3-sst* mutation alters GL3 interaction with GL1, TTG1 and TRY

The mutation responsible for *gl3-sst* allele is in a region that encodes a protein domain which mediates interaction with GL1 and TRY proteins (Payne et al., 2000; Zhang et al., 2003). To determine if the mutation affected protein-protein interactions, the yeast two-hybrid system was used to compare the interactions of gl3-sst and GL3 proteins with GL1, TTG1 and TRY. To study the interactions, the coding regions of the *GL3* and *gl3-sst* were fused to the activation domain (AD) of GAL4, whereas *GL1*, *TRY* and *TTG1* were fused to the binding domain (BD) of GAL4. Yeast containing either empty pGAD424 (AD) or pBridge (BD) vectors in conjunction with any of the corresponding trichome protein fusions did not

exhibit significant β-gal activity (data not shown), whereas yeast containing either sst-AD or GL3-AD and GL1-BD, TRY-BD or TTG1-BD exhibited β-gal activity (Fig. 6A-C). However, there was a significant difference in activity level between the strains containing either sst-AD or GL3-AD. Yeast isolates containing GL1-BD and GL3-AD exhibited approximately fourfold higher levels of β-gal activity than isolates containing GL1-BD and sst-AD (Fig. 6A). Yeast containing TTG1-BD and GL3-AD exhibited approximately a twofold higher level of β-gal activity than isolates containing TTG1-BD and sst-AD (Fig. 6B). Finally, it was found that TRY-BD interacted equally well with either GL3-AD or sst-AD (Fig. 6C).

In a previous model, it has been posited that TRY and GL1
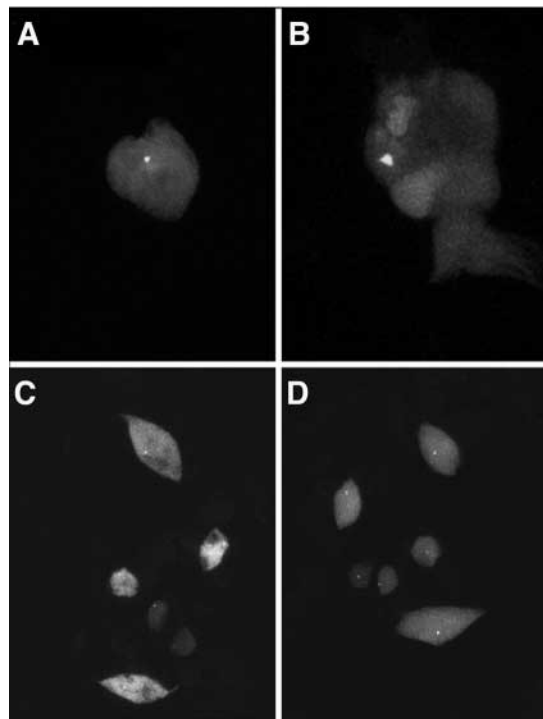
Fig. 5. Visualization of interphase chromatin in Col and *gl3-sst* epidermal cell nuclei. Confocal microscopy of Col and *gl3-sst* plants containing a chromosomally integrated Lac operator array and a constitutively active *35S::GFP-Lac I* transgene. (A,B) Trichome nuclei from Col and *gl3-sst* plants, respectively. (C,D) Guard and neighbor cell nuclei on leaf epidermis of Col and *gl3-sst* plants, respectively. The pairs of images shown in A and B, and in C and D are maximum projections of confocal images captured at the same magnifications and laser intensities.
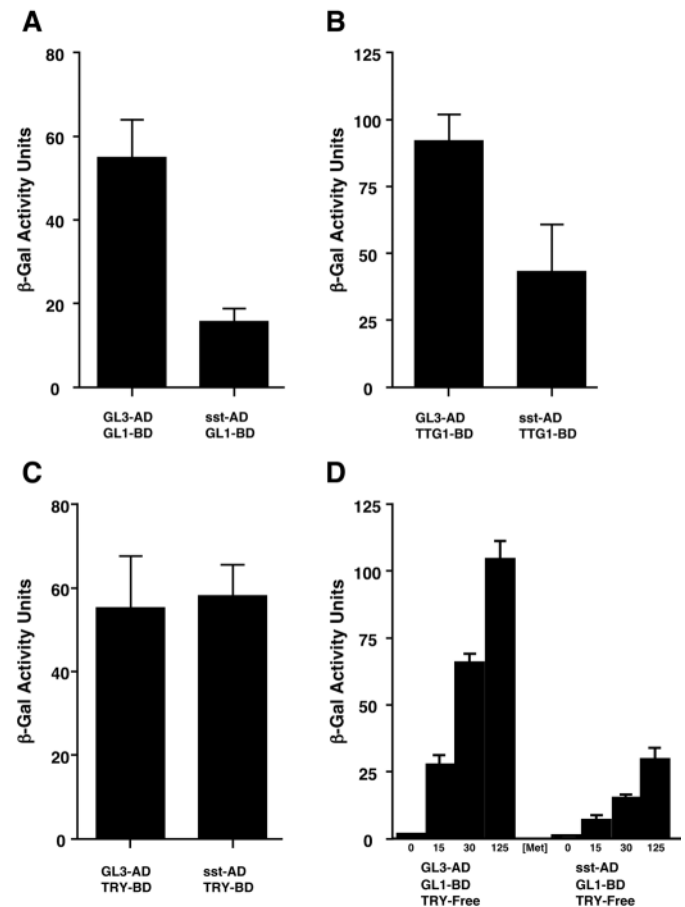

Fig. 6. Protein interactions with GL3 versus gl3-sst. Comparison of protein interactions using a yeast two-hybrid assay between GL3 and gl3-sst with (A) GL1, (B) TTG1, (C) TRY. D shows the ability of TRY to compete for the GL1 binding sites of GL3. Using the pBridge vector (Clontech), a third protein (TRY) under the control of a methionine-repressible promoter was expressed in a yeast interaction assay comparing the interactions of GL3 or gl3-sst with GL1. The assay was performed at varying methionine concentrations (0 μM, 15 μM, 30 μM, and 125 μM). The GL3 and gl3-sst proteins were compared as GAL4 activation domain (AD) fusions whereas GL1, TTG1 and TRY were expressed as GAL4 binding domain fusions (BD) except in D where TRY was expressed as a free protein (no AD or BD domain). The samples were normalized by using OD₅₅₀ readings. The strength of the interaction was determined by using β-galactosidase activity as measured using an ONPG assay.

compete for the same binding site on the GL3 protein to form alternate types of complexes (Marks and Esch, 2003). To test this model and to determine if the mutation in *gl3-sst* alters the proposed competitive interaction between TRY and GL1, the pBridge system (Clontech) was used to create a competitive yeast interaction assay. In addition to expressing a GAL4 binding site fusion protein, pBridge can also express a second protein (free), which is under the control of a methionine repressible promoter and does not contain either a BD or AD domain. To determine if TRY can compete with GL1 for a binding site on GL3, *TRY* was cloned into the free site of the GL1-BD construct to create the plasmid GL1-BD/TRY-free. In the absence of methionine, where TRY-free expression would be at maximum levels, it was found that yeast containing GL3-AD or sst-AD and GL1-BD/TRY-free exhibited very little β-gal activity (Fig. 6D). As the concentration of methionine was increased, which should depress TRY-free expression, the levels of β-gal activity increased (Fig. 6D). The differences in the levels of β-gal activity were not due to the presence or absence of methionine, as yeast containing either GL3-AD or sst-AD and GL1-BD were able to express β-gal to the same level in the presence or absence of 1 mM methionine (data not shown). These results are consistent with the model, which predicts that free TRY protein should be able to inhibit the interaction between GL3-AD and GL1-BD. Furthermore, with the reduction in GL1 GL3 interactions in the *gl3-sst* plants, it would take less TRY protein to reduce the level of the activation complex.

To corroborate the yeast interaction data, the cellular location of the GL3, GL1 and TRY proteins was ascertained. This analysis made use of the *GFP-GL3* fusion construct that was used to rescue the *gl3-1* and *gl3-sst* mutants. In addition, GFP fusions with GL1 (GFP-GL1) and TRY (GFP-TRY) were generated (see Materials and methods). The L1 layer-specific promoter from *ATML1,* which is upregulated in developing trichomes, was used to drive the expression of GFP-GL1 and the *TRY* promoter was used to express the GFP-TRY fusion (Schellmann et al., 2002; Sessions et al., 1999). Both of these constructs were able to rescue their respective mutants (see supplemental Fig. S3 at http://dev.biologists.org/supplemental/). Strong *gl1* mutants typically lack all adaxial leaf surface trichomes. The expression of the *GFP-GL1* construct in the *gl1* background restored the development of wild-type-like trichomes. *try* mutants typically have trichomes that are extra branched, and 10-15% of *try* trichomes develop next to one another. The *GFP-TRY* construct was able to rescue both of these phenotypes. As shown in Fig. 7A-I, all of the fusion proteins can be clearly detected in the trichome nuclei. The filter set used to collect the images limits the fluorescence emission to the green range. At the higher gain and exposure setting on the CCD camera, the primarily red chlorophyll autofluorescence was still detected in the green range. This background autofluoresecence was detected in the mesophyll cells of all samples at the higher gain and exposure settings. In addition, a low background of green autofluorescence was emitted from control Col wild-type trichome cell walls at the higher settings. Importantly, Col wild-type trichome nuclei lacked detectable fluorescence (Fig. 7J-L). These results place TRY, GL3 and GL1 proteins in the trichome nuclei, where they could interact as observed in the yeast system.

Enhanced *TRY* expression can rescue the *gl3-sst* clustering phenotype

To determine if increased *TRY* expression could alter the *gl3-sst* phenotype, mutant plants were transformed with a *GFP-TRY* construct under the control of the *MYB5* promoter, which drives expression in trichomes (see Materials and methods). This construct has the ability to rescue the phenotype of *try* mutant plants (data not shown). The phenotype of *gl3-sst* plants containing the *TRY* gene was modified. Firstly, it reduced the number of leaf trichomes on these plants. On the third leaf, the Col plants had 71±9 trichomes, the *gl3-sst* mutant had 44±5 trichomes, and the TRY-expressing *gl3-sst* line had only 26±5 trichomes. Secondly, extra TRY expression greatly reduced the prevalence of clustered trichomes. The percentage of trichome clustering observed on the third leaf was 14.5% for the *gl3-sst* mutant as compared with 1.5% for the TRY-expressing *gl3-sst* line. No trichome clusters were detected in the Col plants.
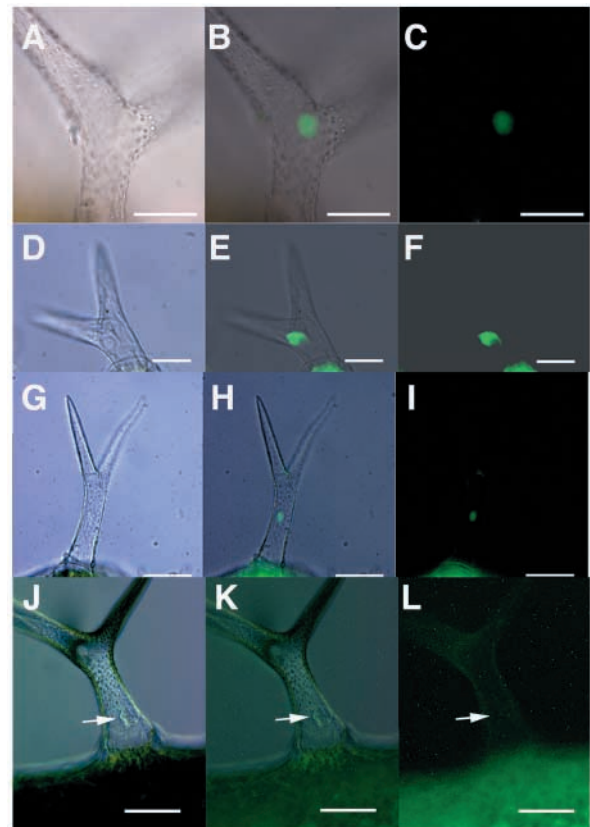
Fig. 7. Nuclear localization of GL1, GL3 and TRY proteins. (A,D,G,J) Light microscopic images of stage 5 (D) and stage 6 mature (A,G,J) trichomes; (C,F,I,L) the same specimens imaged with fluorescence microscopy to visualize GFP; (B,E,H,K) a merged images of the first and third columns. (A-C) Leaf trichome on *gl3-1* plant expressing the *pEMYB5::GFP-cGL3* construct. (D-F) Leaf trichome on *gl1* plant expressing *pEATML1::GFP-cGL1* construct. (G-I) Leaf trichome on Col plant expressing *pETRY::GFP-cTRY*. (J-L) Leaf trichome on untransformed control Col leaf (arrow indicates the nucleus). Scale bar: (A-F) 25 μm; (G-L) 50 μm.

Discussion

gl3-sst is a novel allele of the *GL3* locus

A new recessive trichome mutant with a novel phenotype has been described. The trichomes on the new mutant were highly variable in shape and for this reason, the mutant was named *shapeshifter* (*sst*). Genetic mapping of *sst* placed it in the same chromosomal region as the *GL3* gene. Three lines of evidence were used to show that *sst* represents a new *GL3* allele. First, the analysis of F_1 and F_2 populations derived from a cross between *sst* and *gl3-1* plants failed to produce any wild-type plants. Second, sequencing of the *sst GL3* locus revealed a base substitution. Third, a wild-type *GL3* transgene was able to rescue the *sst* phenotype.

The *gl3-sst* mutant is phenotypically distinct from the other loss-of-function *gl3* mutants. The trichomes on previous *gl3* mutants typically had more slender stalks and fewer branches than wild-type trichomes (Koornneef et al., 1982; Payne et al., 2000). In contrast, the *gl3-sst* trichomes have a swollen stalk and vary widely in branch number. Another difference is in the degree to which the nuclear DNA of *gl3-1* and *gl3-sst* trichomes undergoes endoreduplication. Wild-type Col trichome nuclei typically contain a range between 16C and 64C with an average of 32C, while *gl3-1* trichomes average 16C (Hülskamp et al., 1994; Melaragno et al., 1993; Szymanski and Marks, 1998). While the exact levels of nuclear DNA were not determined, it was clear from the intensity of DAPI staining and the increased nuclear size that *gl3-sst* trichomes exhibited higher than wild-type levels of endoreduplication.

The *EGL3* gene, located on chromosome one, is closely related to *GL3* (Payne et al., 2000; Zhang et al., 2003). To determine if this gene has a role in the *gl3-sst* phenotype, the genetic interactions between an *EGL3* T-DNA mutant and both *gl3-1* and *gl3-sst* were studied. As previously reported, it was found that the trichomes that develop on *gl3-1* mutants required *EGL3* function (Zhang et al., 2003). Furthermore, it was found that *gl3-sst* plants produced trichomes in the absence of a functional *EGL3* gene. This showed that the *gl3-sst* allele is functional.

Some *gl3-sst* trichome abnormalities may be due to cytoskeletal alterations

Many of the mutant *gl3-sst* trichome defects could be due to alterations in the microtubule cytoskeleton. For example, the swollen appearance of early stage *gl3-sst* trichomes is similar to trichomes on wild-type plants treated with microtubule destabilizing agents such as oryzalin (Mathur and Chua, 2000; Szymanski et al., 1999). Likewise, the abnormally lobed structure of *gl3-sst* trichomes nuclei is similar to nuclei in plants treated with antimicrotubule drugs. In this case, the lobate nuclear structure is thought to result from spindle microtubule disassembly, which results in a disruption of karyokinesis.

gl3-sst trichomes exhibit cell wall defects

The majority of the cell walls of the mature *gl3-sst* leaf trichomes had a 'glassy' appearance when viewed by stereoscopic microscopy. SEM analysis revealed that the walls lacked the papillae that are normally associated with wild-type trichomes. This suggested that most *gl3-sst* trichomes failed to undergo a final maturation step. To further analyze these differences, the elemental composition of the trichome cell wall was ascertained. Compared to wild-type trichomes, the *gl3-sst* trichomes showed two forms of cell wall immaturity. In *gl3-sst* trichomes with a papillate surface, magnesium, which was found throughout the cell wall of wild-type trichome, and phosphorus, which was localized within the wild-type trichome papillae, were both absent. In papillae-less *gl3-sst* trichomes, calcium, in addition to magnesium and phosphorus, was missing from the cell wall, which suggested that the deposition of these elements play an important role in trichome cell wall maturation.

Role of cell cycle control in the *gl3-sst* phenotype

Extra endoreduplication occurred in *gl3-sst* trichome nuclei. In both Col wild-type and *gl3-sst* trichomes, the Lac OP/I-GFP reporter experiments suggested that the chromatids of endoreduplicated chromosomes are tightly associated with one another. Comparable results have also been reported using a similar version of the Lac OP/I-GFP system to study the nature of chromosomes in pavements cells (Kato and Lam, 2001; Kato and Lam, 2003). However, Kato and Lam (Kato and Lam, 2003) reported the presence of multiple Lac I-GFP binding sites in pavement cells, which is contradictory to the idea that the sister chromatids are associated with one another as in polytene chromosomes. In the present study, only single binding sites were observed in most nuclei, whether from trichome, pavement, or guard cells. Overall our results suggest that the chromatids of endoreduplicated chromosomes are highly associated with one another (or are polytene). Furthermore, the results provided evidence that the highly lobed *gl3-sst* nuclei contain single sets of chromosomes that have not undergone karyokinesis.

The mutation in *gl3-sst* highlights the importance of protein-protein interaction in trichome development

It was previously shown that GL1 and TTG1 can interact with GL3 in yeast two-hybrid assays (Payne et al., 2000). Thus, the interactions between either GL1 or TTG1 with either GL3 or gl3-sst were compared. The location of the mutation in gl3-sst in the 78th codon predicted that gl3-sst might display an aberrant interaction with GL1, and indeed, this result was seen. The mutation resulted in a 75% reduction in the interaction between gl3-sst and GL1, based upon reproducible quantitative beta-gal assays. In addition, it was found that the gl3-sst interaction with TTG1 was reduced by 50%. Since GL1, GL3 and TTG1 proteins have been proposed to form an activating complex that regulates genes needed for trichome development, it is likely that the *gl3-sst* phenotype is due to a reduction in this complex.

A model based on the dynamic interactions between GL1, GL3 and TTG1 activating complex has previously been presented (Szymanski et al., 2000). The model proposed that the loss of trichomes on plants that overexpress the CPC gene were due to the ability of CPC to inhibit the physical interaction between GL3 and GL1. Because it has subsequently been shown that *TRY* encodes a CPC-like protein, the model was modified to propose that TRY functions to inhibit the physical interaction between GL3 and GL1 (Marks and Esch, 2003). In this paper, evidence is presented that supports this model. Competitive yeast interaction assays demonstrated that TRY can interact with GL3 and that this interaction can prevent the GL3-GL1

interaction. Interestingly, the mutation in *gl3-sst* does not influence the interaction between gl3-sst and TRY.

TRY has been found to be expressed in young trichomes and it has been predicted that TRY protein can migrate from initiating trichomes into neighboring cells (Schellmann et al., 2002). Given the interaction data described, it is likely TRY would be able to disrupt any residual GL1 GL3 complexes in neighboring cells and that may be the mechanism by which TRY acts to inhibit the initiation of neighboring trichomes.

Further support for TRY movement comes from the analysis of *CPC*. It has recently been shown that CPC protein is produced in the non root hair cells and can move into the adjacent files of cells that become root hairs (Wada et al., 2002). In the root, CPC is probably inhibiting the formation of a complex between WER and GL3/EGL3. Direct movement of TRY could not be detected in this study, because the level of fluorescence from the GFP-TRY fusion was below the background autofluorescence emitted by chlorophyll in the mesophyll. Given the expression pattern of *TRY* and the recent *CPC* findings, the movement of TRY into cells neighboring developing trichomes would not be unexpected.

Threshold model for trichome initiation

It has been posited that a cell enters the trichome pathway only after a critical threshold concentration of GL1 GL3 TTG1 complex has accumulated (Szymanski et al., 2000). Once the threshold is reached, it is predicted that an auto regulatory loop is generated that results in the up-regulation of the complex genes. Over time, as the level of the activator increases, it is predicted that different genes needed for trichome differentiation would be induced. In order for trichome initiation to proceed, it is predicted that genes needed to positively influence trichome development would be expressed first and then genes needed either to modulate or inhibit trichome development would be expressed later. Genes activated early during trichome initiation, when the activator complex would be relatively low, would be induced because they have a higher affinity for the complex than genes that are activated later.

In terms of this threshold model it is interesting to note that different promoters can be used to functionally drive the activator genes. For example, it may seem surprising that the *MYB5::cGL3* construct could rescue the *gl3-1* mutant trichome phenotype. However, the *MYB5* promoter is up-regulated in developing trichomes and *gl3-1* mutants do initiate trichomes, albeit undersized ones (Li et al., 1996). Thus, GL3 protein would be expressed in the trichomes of *gl3-1* plants containing the *MYB5::cGL3* construct. Given that native *MYB5* expression increases as trichomes develop, it is probable that the levels of GL3 protein would increase. This increase would allow the sequential activation of positive and negative regulators of trichome development as predicted by the threshold model. The ability of the *ATML1::cGL1* construct to rescue the *gl1* mutant appears to be further complicated by the fact that *gl1* mutants do not initiate trichomes. The *ATML1* promoter is expressed at a low level in all the epidermal cells of the developing leaf and in situ analyses have shown that *ATML1* expression is enhanced in young developing trichomes (Sessions et al., 1999). In terms of the threshold model it is possible that the *ATML1* promoter provides a threshold level of GL1 protein in all the epidermal cells. However, only the few cells that achieve the threshold

level of GL3 enter the trichome pathway. Once a cell enters the pathway the levels of *ATML1* expression would be enhanced and the level of GL1 protein would increase, again allowing the sequential activation of genes as predicted by the threshold model.

Trichome development in *gl3-sst* provides some additional evidence for the threshold model. This support comes from two aspects of the *gl3-sst* phenotype, which may seem paradoxical. First, there are fewer trichomes on the leaves of *gl3-sst* plants, which could indicate increased trichome inhibition. In contrast, there are more trichome clusters, which suggest that trichome inhibition is relaxed. Given the reduced interaction between gl3-sst and both GL1 and TTG1, the reduction in trichome number could be the result of fewer cells reaching a critical concentration of activation complex. The reduced interaction between gl3-sst and GL1 or TTG1 could also result in increased trichome clustering if the inhibition signal emitted from *gl3-sst* trichomes was reduced. This latter possibility is supported by the observation that increased *TRY* expression in *gl3-sst* can prevent trichome clustering. Other aspects of the *gl3-sst* phenotype such as excess expansion and extra endoreduplication could be due to the unregulated activation of genes needed to mediate these cellular phenomenon during early trichome development. However, levels of activator complex needed to stimulate the expression of genes that either modulate or inhibit these early processes may not be reached in *gl3-sst* trichomes. Layered on top of this relatively simple threshold model, there is probably an added level of complexity. The activator complex possibly does not consist of just GL1, GL3/EGL3 and TTG1, but contains additional components as well. It is more likely that gene regulation in developing trichomes is controlled by both the quantity of activator complex and its composition. Thus, some aspects of the *gl3-ss*t phenotype could result from aberrant ratios of complexes containing or lacking GL1, TTG1 or other components.

Perspectives

While the pleiotropic nature of the *gl3-sst* mutant trichomes may appear confusing, this very feature provides an excellent resource for studying all aspects of trichome development. These mutants are deficient in trichome initiation, trichome cell patterning, cell expansion, endoreduplication and maturation. Through future genetic screens for suppressors and enhancers of the various components of the phenotype and through detailed expression profiling of the mutant, the understanding of many processes involved in cellular differentiation will be advanced.

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