Journal of Integrative Plant Biology 2008, **50** (7): 906–917

SAD2 in *Arabidopsis* Functions in Trichome Initiation through Mediating GL3 Function and Regulating *GL1*, *TTG1* and *GL2* Expression

Ying Gao[1,2,3], Ximing Gong[1,2], Wanhong Cao[2], Jinfeng Zhao[2], Liqin Fu[2], Xuechen Wang[1], Karen S. Schumaker[4] and Yan Guo[2*]

([1]*State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University*, Beijing 100094, China;
[2]*National Institute of Biological Sciences*, Beijing 102206, China;
[3]*Institute of Crop Sciences, Chinese Academy of Agricultural Sciences*, Beijing 100081, China;
[4]*Department of Plant Sciences, University of Arizona*, Tucson, AZ 85721, USA)

Abstract

Most genes identified that control *Arabidopsis* trichome initiation and formation are transcription factors or regulatory components in transcriptional networks and include GLABROUS1 (GL1), GLABRA2 (GL2), GLABRA3 (GL3) and TRANSPARENT TESTA GLABRA1 (TTG1). Herein, we report that an importin *β*-like protein, SENSITIVE TO ABA AND DROUGHT2 (SAD2), is required for trichome initiation. Mutations in SAD2 disrupted trichome initiation resulting in reduced trichome number, but had no effect on trichome development or root hair number and development. Expression levels of *GL1*, *MYB23*, *GL2* and *TTG1* were reduced in shoots of *sad2* mutants while expression levels of *GL3* and ENHANCER OF GLABRA3 (*EGL3*) were enhanced. Overexpression of *GL3* increased trichome numbers in wild type but not in *sad2* mutants, indicating that the function of the GL3 protein is altered in the *sad2* mutants. In contrast, overexpression of *GFP-GL1* decreased trichome number in both wild type and *sad2*. Double mutant analysis of *gl1 sad2* and *gl3 sad2* indicated that SAD2 functions genetically, at least in part, in the same pathway with these two genes. Co-immunoprecipitation indicated that the *sad2* mutation does not disrupt formation of the TTG1-GL3-GL1 complex. Analysis of GFP fusions of GL1, GL2, GL3 and TTG1 suggested that these proteins are most likely not direct cargo of SAD2. Our data suggest that SAD2 is involved in trichome initiation by regulating these nuclear genes.

Key words: *Arabidopsis*; GL3; nuclear transport; SAD2; trichome.

Gao Y, Gong X, Cao W, Zhao J, Fu L, Wang X, Schumaker KS, Guo Y (2008). SAD2 in *Arabidopsis* functions in trichome initiation through mediating GL3 function and regulating *GL1*, *TTG1* and *GL2* expression. *J. Integr. Plant Biol.* **50**(7), 906–917.

Available online at www.jipb.net

The control of cell determination is critical for many aspects of plant development including the formation of leaf hairs or trichomes. Trichomes are large, single-celled epidermal hairs that are thought to be important for protecting the plant against herbivores and loss of water through transpiration (Serna and Martin 2006). Trichomes have been used extensively to study how a specialized cell type can develop from a uniform cell layer. Mutations have been identified that disrupt trichome initiation, development, spacing, density and morphology. From these studies, six patterning genes in *Arabidopsis* have been linked to trichome initiation and development: *GLABRA1* (*GL1*), *AtMYB23*, *GLABRA2* (*GL2*), *GLABRA3* (*GL3*), *ENHANCER OF GLABRA3* (*EGL3*) and *TRANSPARENT TESTA GLABRA1* (*TTG1*) (Larkin et al. 1993; Payne et al. 2000; Kirik et al. 2001; Ohashi et al. 2002). Many of these genes are also involved in the development of root hairs (Schiefelbein 2003).

GL1 and *AtMYB23* encode R2R3-MYB-type transcription factors. Mutations in GL1 result in the absence of trichomes on the leaf surface, but do not affect root hair development (Larkin et al. 1994a, 1999). AtMYB23 functionally overlaps with GL1

Received 7 Apr. 2008 Accepted 9 Apr. 2008

Supported by the Hi-Tech Research and Development Program of China (2003AA210100) to Y. Guo and the Department of Energy/Energy Biosciences (DE-FG02-04ER15616/G) to K.S. Schumaker.

*Author for correspondence.

Tel: +86 10 8072 3279;

Fax: +86 10 8072 6671;

E-mail: <guoyan@nibs.ac.cn>.

doi: 10.1111/j.1744-7909.2008.00695.x

during regulation of trichome initiation at leaf margins. Loss of AtMYB23 function also leads to a defect in trichome morphogenesis (Kirik et al. 2001, 2005). *GL2* encodes a homeodomain transcription factor and is required for trichome morphogenesis. Mutations in GL2 cause abnormal trichome development in which most of the trichomes do not expand and have a single branch (Rerie et al. 1994; Masucci et al. 1996; Fyvie et al. 2000; Ohashi et al. 2002). *GL3* and *EGL3* encode basic helix-loop-helix (bHLH) type transcription factors. Mutations in GL3 have a moderate effect on trichome initiation and a strong effect on trichome branching, endoreduplication and cell size (Payne et al. 2000; Szymanski et al. 2000; Zhang et al. 2003). EGL3 functionally overlaps with GL3. Mutations in EGL3 reduce trichome number and branching and *gl3 egl3* double mutants show a glabrous leaf phenotype (Zhang et al. 2003). *TTG1* encodes a protein with WD-40 repeats. Mutations in TTG1 also result in a glabrous leaf phenotype and a transparent seed coat (Larkin et al. 1994a, 1999; Walker et al. 1999). Ectopic expression of a maize *GL3* homolog (*R* gene) can rescue most of the *ttg1* phenotypes, indicating that GL3 functions downstream of TTG1 (Payne et al. 2000). Studies have also shown that both TTG1 and GL1 interact with GL3 in yeast and that a threshold level of a GL1-GL3-TTG1 complex is likely required for cell fate determination and trichome formation (Payne et al. 2000; Schwab et al. 2000; Szymanski et al. 2000; Schellmann et al. 2002). The predicted consequence of reaching this threshold is generation of an autoregulatory loop that results in the upregulation of this complex of genes in certain cell types.

While most of the regulators involved in trichome initiation and development identified are transcription factors or regulatory components of transcriptional networks that function in the nucleus, little is known about how these proteins are translocated into the nucleus and whether they function in complexes in planta. In this study, we report that an importin β-like protein, SENSITIVE TO ABA AND DROUGHT2 (SAD2), is involved in trichome initiation by controlling *GL1*, *GL2*, *GL3* and *TTG1* transcription. Our data suggest that SAD2 may affect an upstream regulator of GL3 and disrupt complex function.

Results

sad2 mutant phenotype

Previously we reported that a knockout of the *SAD2* gene, which encodes an importin β-like protein, produced a mutant in *Arabidopsis* that is hypersensitive to ABA during seed germination (Verslues et al. 2006). In this study, we show that mutations in *SAD2* virtually abolished leaf trichome initiation in the *Arabidopsis* C24 genetic background, with only an occasional trichome produced on *sad2-1* leaves (Figures 1,2 and Table 1). We cloned a 10.5 kb genomic DNA fragment from BAC clone T9H9 including 2 kb of the promoter and 1 kb of the untranslated 3′-UTR region of *SAD2* into the pCAMBIA1200 vector (Verslues et al. 2006) and used this construct to transform the *sad2-1* mutant. The mutant trichome defect was rescued by the transgene, indicating that loss of SAD2 function was responsible for the lack of trichomes in the *sad2* mutant (Figure 1B and Table 2).

Two additional *sad2* T-DNA insertion mutants in the Columbia-0 (Col-0) genetic background (SALK_133577, *sad2-2*; and SAIL_313_B03, *sad2-3*) were obtained from the Arabidopsis Biological Resource Center (ABRC). Homozygous lines were identified by polymerase chain reaction (PCR) using T-DNA left border and *SAD2* gene-specific primers (data not shown). The T-DNA insertions are located in the 16th intron in *sad2-2* and the ninth exon in *sad2-3*. Reverse transcription (RT)-PCR results showed that *sad2-3* is a knockout mutant and *sad2-2* is a knockdown mutant (Figure 1C). Both *sad2-2* and *sad2-3* produced fewer trichomes on the leaf surface than wild type (Figure 1 and Table 1). Trichome numbers in the first four true leaves of *sad2-2* and *sad2-3* were reduced to less than 73% of the numbers in wild type (Table 1). Leaves of Col-0 plants produce approximately eight times more trichomes than leaves of C24 (Table 1), indicating that trichome numbers vary in different genetic backgrounds. Mutations in *SAD2* in both genetic backgrounds had no effect on trichome development, branching, patterning or root hair number (Figure 1 and Tables 1,3).

Scanning electron microscopy showed the absence of trichome initiation in the *sad2-1* mutant. However, in the Col-0 background, even though the number of trichomes was reduced in *sad2-3*, whenever a trichome was initiated, it developed into a fully expanded trichome as large as those found in wild type, with a covering of papillae and a base of supporting cells (Figure 2). These data demonstrate that the *SAD2* gene affects trichome initiation but not development.

Expression of genes involved in trichome initiation in *sad2* mutants

To determine if SAD2 functions in a pathway with genes previously reported to regulate trichome initiation and development, we monitored the mRNA levels for these gene products in the *sad2* mutants. Total RNA was extracted from shoots of 9 d-old wild-type and *sad2* mutant seedlings and the corresponding cDNA was used for quantitative real-time PCR analysis. In shoot tissue, expression of *GL1*, *AtMYB23*, *GL2* and *TTG1* was reduced in the *sad2* mutants compared to their levels in wild type (Figure 3); however, this reduction was never lower than 50% of the expression found in wild type. In comparison, expression of *GL3* and *EGL3* was not reduced in shoots of the *sad2* mutants but increased when compared to levels in wild type. Expression of *GL3* increased more than four times in the C24 background and more than double in the Col-0 background. Expression of

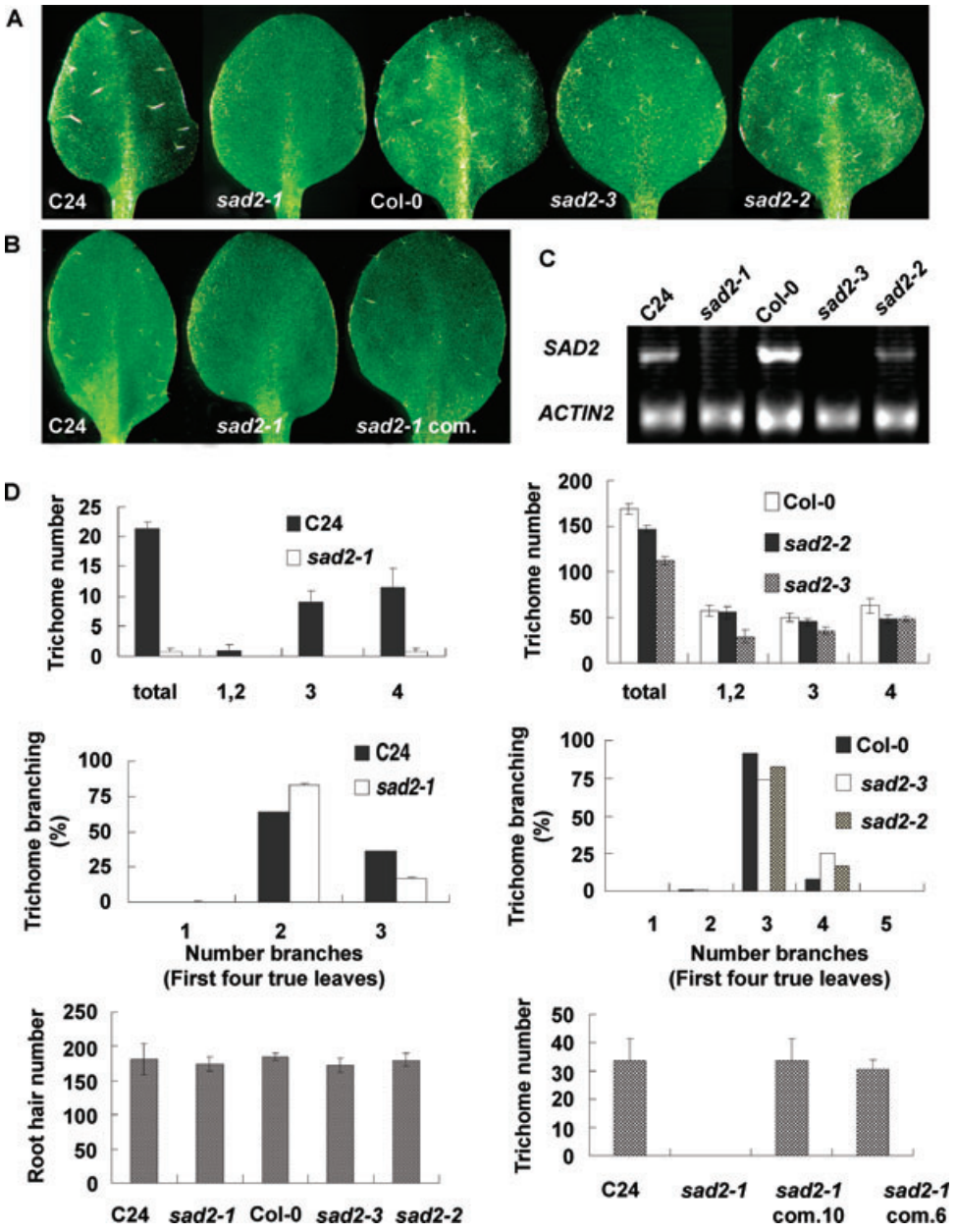

Figure 1. Trichome and root hair phenotypes of the *sad2* mutants.

(A) The third true leaf of C24 and *sad2-1*; the first or second true leaves of Columbia-0 (Col-0), *sad2-2* and *sad2-3*.

(B) The fifth true leaf of wild-type C24, the *sad2-1* mutant and the *sad2-1* complementation line.

(C) Expression of *SAD2* in wild type and the *sad2* mutants. Total RNA was extracted from 12 d-old seedlings of wild type and the *sad2* mutants. The resulting cDNAs were used for reverse transcription polymerase chain reaction analysis with *SAD2* primers located upstream of the T-DNA insertions.

(D) Trichome and root hair statistics for wild type, the *sad2* mutants and mutant complementation lines are listed in Tables 1, 2 and 3. Upper panels, trichome number in all leaves (total) or in the first and second (1,2), third (3) or fourth (4) true leaves in C24 and *sad2-1* (left panel) or Col-0, *sad2-2* and *sad2-3* (right panel). Middle panels, trichome branching as a percent of total number of trichomes in the first four true leaves of C24 and *sad2-1* (left panel) or Col-0, *sad2-3* and *sad2-2* (right panel). Lower panels, number of root hairs in 4 d-old seedlings from wild type and the *sad2* mutants (left panel). Number of trichomes in the first six true leaves from wild type, *sad2-1* and the *sad2-1* complemented lines (right panel). Error bars in **(D)** represent standard deviation, $n \geq 10$.

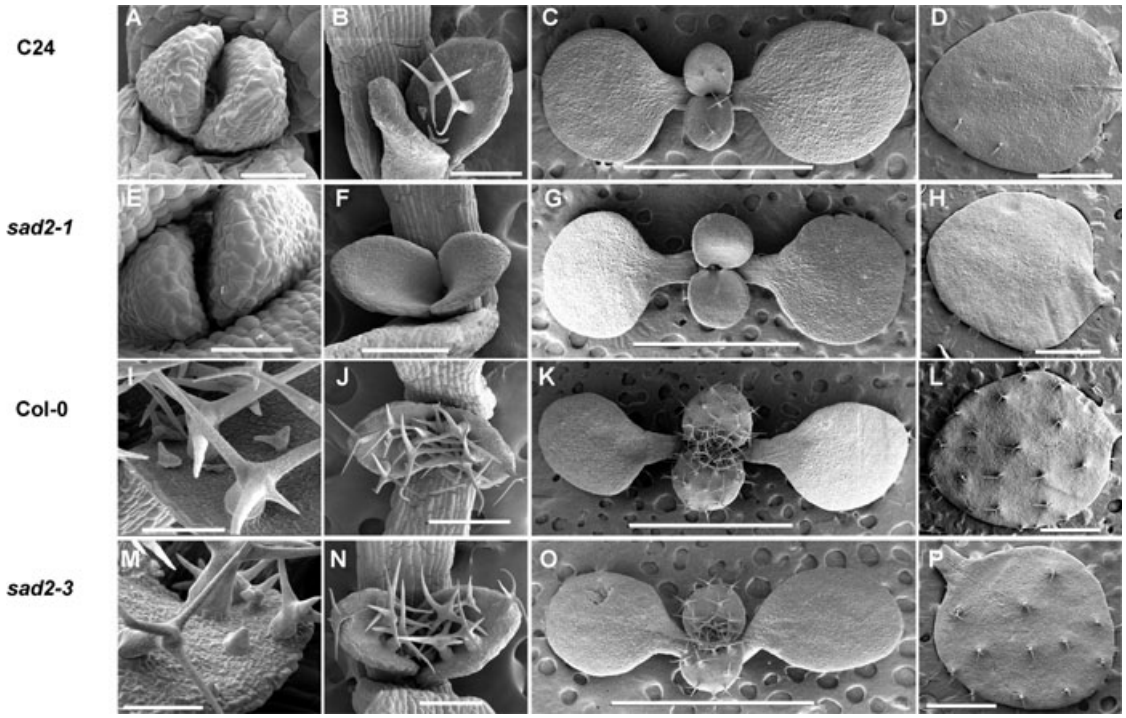

Figure 2. Scanning electron micrographs of trichome development in the first pair of true leaves of wild type and the *sad2* mutants.

(A,E,I,M) Trichome emergence.
(B,F,J,N) Early trichome development.
(C,G,K,O) Fully expanded trichomes.
(D,H,L,P) Mature trichomes.
Col-0, Columbia-0.
Bars on graph indicate **(A,E)** 50 μm, **(I,M)** 100 μm, **(F,J)** 300 μm, **(B,N)** 200 μm, **(C,G,K,O)** 2 mm and **(D,H,L,P)** 1 mm.

EGL3 also increased slightly in the C24 background; however, in Col-0, *EGL3* expression was either slightly higher or similar to levels measured in wild type (double in *sad2-2* and no increase in *sad2-3*). The results of this analysis indicated that expression of all genes in shoot tissue was higher in Col-0 than in C24, explaining why numbers of trichomes are more than eight times higher in leaves of Col-0 than in leaves of C24.

Subcellular localization of GL1, GL2, GL3 and TTG1

SAD2 encodes an importin β-like protein involved in nuclear protein transport. Because *sad2* is defective primarily in trichome initiation, we reasoned that GL1 and EGL3, also involved in trichome initiation, might be candidates for SAD2 targets. To look for putative targets, we fused GFP/YFP/CFP reporters to GL1, EGL3 and also to AtMYB23, GL2, GL3 and TTG1 at their N-termini under the control of a 35S promoter to investigate whether the nuclear localization of these proteins was altered in the *sad2* mutants.

As shown in Supplementary Figure S1, GFP-GL1, GFP-GL2 and CFP-GL3 signals were observed only in the nucleus in roots in both wild type and *sad2* mutant transgenic lines. In trichomes of transgenic lines, GFP-GL1 was observed only in the nucleus in both backgrounds (Supplementary Figure S1A). In root tissue, GFP-GL2 was detected only in the nucleus; whereas two GFP-GL2 localization patterns were found in trichomes in Col-0 and *sad2-3*. One pattern was restricted to the nucleus and the other was in both the nucleus and the cytoplasm (Supplementary Figure S1B). CFP-GL3 was detected in the nucleus in the root and in both the nucleus and the cytoplasm in trichomes from both Col-0 and *sad2-3* (Supplementary Figure S1C). Our data suggest that none of proteins tested was a direct target of SAD2; however, we cannot rule out the possibility that the nuclear localization of these proteins may be altered at a very early stage of trichome initiation or in specialized cell types in the *sad2* mutants.

Overexpression of *GL1* and *GL3*

Expression of *GL3* is enhanced, but trichome numbers are reduced in the *sad2* mutants. It has been shown that overexpression of *GL3* in wild type increases leaf trichome number

Table 1. Leaf trichome phenotypes in the *sad2* mutants

Genotype	Leaf number	No. of trichome branches					Total no. of trichomes
		1	2	3	4	5	
C24 wild type, $n=10$	1,2	0	2.4 ± 0.89	0.4 ± 0.89	0	0	2.8 ± 1.09
	3	0	5.2 ± 1.79	3.8 ± 2.29	0	0	9.0 ± 3.39
	4	0	5.2±1.64	3.0±1.87	0	0	8.2±1.3
	Total	0	12.8 ± 2.28	7.2 ± 4.31	0	0	20.0 ± 4.63
sad2-1, $n=10$	1,2	0	0.2 ± 0.6	0	0	0	0.2 ± 0.4
	3	0	0.3 ± 0.5	0.1 ± 0.3	0	0	0.4 ± 0.3
	4	0	0.1 ± 0.3	0	0	0	0.1 ± 0.2
	Total	0	0.6 ± 0.7	0.1 ± 0.32	0	0	0.7 ± 0.67
Col-0 wild type, $n=30$	1,2	0	1.0 ± 1.0	43.2 ± 6.14	2.8 ± 1.93	0	47.0 ± 7.04
	3	0	0	58.6 ± 16.89	5.6 ± 2.30	0	64.2 ± 18.25
	4	0	0	73.0 ± 14.63	7.0 ± 3.87	0	80.0 ± 17.22
	Total	0	1.0 ± 1.0	174.8 ± 31.50	15.4 ± 4.67	0	191.2 ± 34.87
sad2-3, $n=30$	1,2	0	0.2 ± 0.45	23.6 ± 2.07	7.8 ± 3.03	0	31.6 ± 2.07
	3	0	0	32.4 ± 8.82	11.4 ± 2.41	0	43.8 ± 10.08
	4	0	0.4 ± 0.89	36.2 ± 5.97	12.2 ± 5.12	0.2 ± 0.45	49.0 ± 9.95
	Total	0	0.6 ± 0.89	92.2 ± 12.30	31.4 ± 5.50	0.2 ± 0.45	124.4 ± 15.47
sad2-2, $n=30$	1,2	0	0.29 ± 0.76	28.29 ± 2.36	4.43 ± 2.37	0	33.0 ± 2.45
	3	0	0	42.71 ± 7.63	9.86 ± 3.67	0.14 ± 0.38	52.71 ± 11.12
	4	0	0.14 ± 0.38	44.43 ± 13.21	8.71 ± 2.75	0.29 ± 0.76	53.57 ± 14.63
	Total	0	0.43 ± 0.79	115.43 ± 17.19	23.0 ± 4.40	0.43 ± 0.79	139.29 ± 19.25

n, number of plants counted. The number 1 in the "No. of trichome branches" column refers to an unbranched trichome. Data represent means ± standard deviation. Col-0, Columbia-0.

Table 2. Leaf trichome phenotype of *sad2-1* after complementation

Genotype	Total no. of trichomes
C24	33.6 ± 7.86
sad2-1	0
sad2-1 com.6	30.6 ± 3.58
sad2-1 com.10	33.6 ± 7.83

The total number of trichomes was counted for the first six true leaves of ten plants. Data represent means ± standard deviation.

Table 3. Root hair numbers in wild type and *sad2* mutants

Genotype	Total no. of root hairs
C24	181.3 ± 23.06
sad2-1	174.5 ± 10.34
Col-0	184.2 ± 5.40
sad2-3	172.4 ± 10.71
sad2-2	180.4 ± 10.11

The total number of root hairs was counted for the primary root of 10, 4-d-old plants. Data represent means ± standard deviation. Col-0, Columbia-0.

(Payne et al. 2000). To determine if GL3 functions in the *sad2* mutant, *GL3* cDNA under the control of a 35S promoter was ectopically expressed in both wild type and the *sad2* mutants. Overexpression of *GL3* was confirmed in the transgenic plants using RT-PCR (data not shown). Consistent with the published data, overexpression of *GL3* in C24 and Col-0 wild type increased trichome numbers in transgenic plants (Figure 4A,B). However, overexpression of *GL3* in the *sad2* mutants did not result in an increase in the number of trichomes (Figure 4A,B). These data suggest that the GL3 protein does not fully function in the *sad2* mutants. Overexpression of *GL3* suppressed part of the *ttg1* mutant phenotype (Payne et al. 2000); however, this was not the case in the *sad2* mutants, demonstrating that reduction of trichome number in *sad2* is possibly independent of TTG1 function and supports the conclusion that TTG1 is not a target of SAD2.

It has been shown that overexpression of *GL1* and *GL2* leads to a reduction in trichome number in wild type (Oppenheimer et al. 1991; Larkin et al. 1994b; Ohashi et al. 2002). In our *35S:GFP-GL1* and *35S:GFP-GL2* wild-type Col-0 transgenic plants, trichome number was reduced (Figure 4C and data not shown). Similar results were also obtained with expression of this plasmid in the *sad2-3* mutant (Figure 4D and data not shown), indicating that the function of the GL1 and GL2 protein was not affected in the *sad2* mutant.

Double mutant analysis

To determine if there is a genetic interaction between *SAD2* and *GL1* or *GL3*, *sad2-2* mutants was crossed into *gl1* or *gl3* mutants to obtain *gl1 sad2-2* and *gl3 sad2-2* double mutants. Information about the mutants used in this study is given in

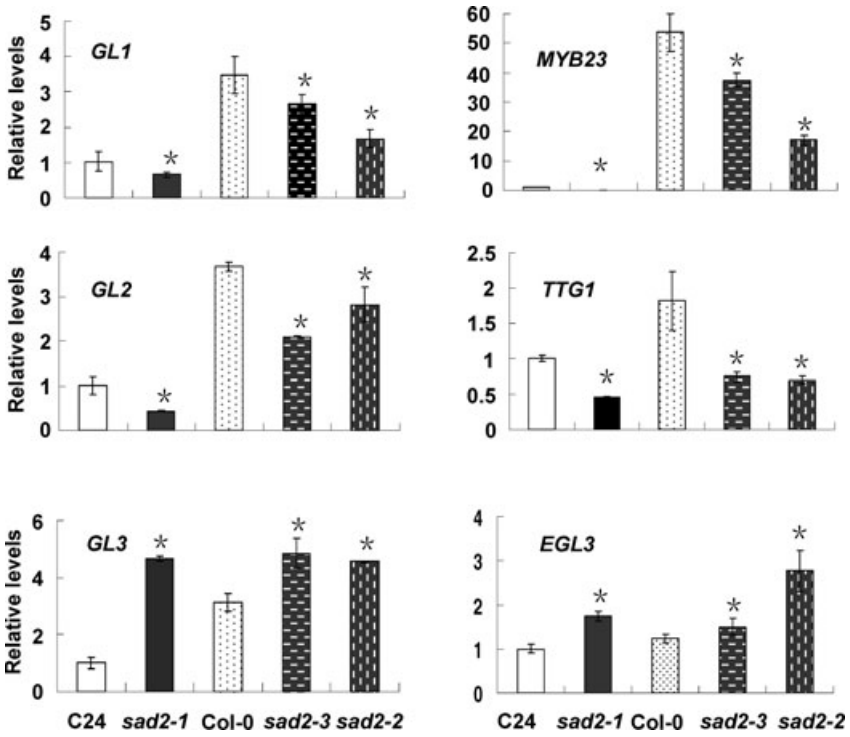

Figure 3. Expression of *GL1*, *GL2*, *GL3, TTG1, MYB23* and *EGL3* in wild-type and *sad2* mutant shoots.

Total RNA was extracted from 9 d-old seedlings of wild type and the *sad2* mutants. The resulting cDNAs were used for real time polymerase chain reaction analysis. Col-0, Columbia-0.

Error bars represent standard deviation, $n = 3$. Asterisks indicate significant difference at $P < 0.05$ (Student's *t*-test).

Tables 1, 4 and 5 and the phenotypes of the double mutants are summarized in Table 4. Mutations in *SAD2* enhanced the *gl1* mutant glabrous phenotype. Occasionally, *gl1* formed trichomes at the leaf margins. However, in *gl1 sad2-2* double mutants, no trichomes were observed in the first four true leaves (Figure 5A,B; Table 4). To generate the *gl3 sad2* double mutant, we obtained a *GL3* T-DNA insertion line (SALK_118201) from the ABRC. We verified that this line (*gl3kd*) was homozygous for the mutation and that the *GL3* transcript was knocked down (data not shown). Comparable with other *gl3* mutants, this *gl3kd* mutant also manifested trichome initiation and development defects. Trichome number in *gl3kd* was reduced to 87% of the wild-type level and most of the trichomes had two branches. The *gl3kd sad2-2* double mutant formed fewer trichomes than either *gl3kd* or *sad2-2* (Figure 5C,D; Table 4), suggesting that SAD2 carries other activities not found in GL3.

Mutations in SAD2 do not lead to disassociation of the TTG1-GL3-GL1 complex

The mutation in SAD2 did not affect the nuclear transport of TTG1, GL1 and GL2, but did reduce their transcriptional levels. It is thought that TTG1, GL3 and GL1 form a protein complex that further triggers leaf trichome initiation. To test for the presence of this complex in planta, and to determine if complex formation was affected by the *sad2* mutation, six myc-tags in a tandem repeat were fused to the N-terminus of GL3 and three flag-tags in a tandem repeat were fused to the N-termini of TTG1 and GL1, each under the control of a 35S promoter. *35SP:myc-GL3* and *35SP:flag-GL1* were co-transfected into wild-type and *sad2-1* leaf protoplasts. Proteins were extracted from these protoplasts and myc-GL3 was immunoprecipitated with anti-myc conjugated agarose. The pull-down products were then analyzed on immunoblots with an anti-flag antibody. The results demonstrate that GL3 could pull down GL1, indicating that they form a complex (Figure 6A). However, this interaction was not disrupted in the *sad2* mutant (Figure 6A). To determine if TTG1, GL3 and GL1 form a complex, *35SP:myc-GL3*, *35SP:flag-GL1* and *35SP:flag-TTG1* were co-transfected into leaf protoplasts from both wild type and *sad2-1*. Anti-myc conjugated agarose was used to pull down the complexes and the pull-down products were then analyzed on immunoblots with anti-flag antibody; the results are shown in Figure 6B. Bands representing both flag-TTG1 and flag-GL1 were detected in the pull-down products and mutations in *sad2* did not alter this complex formation.

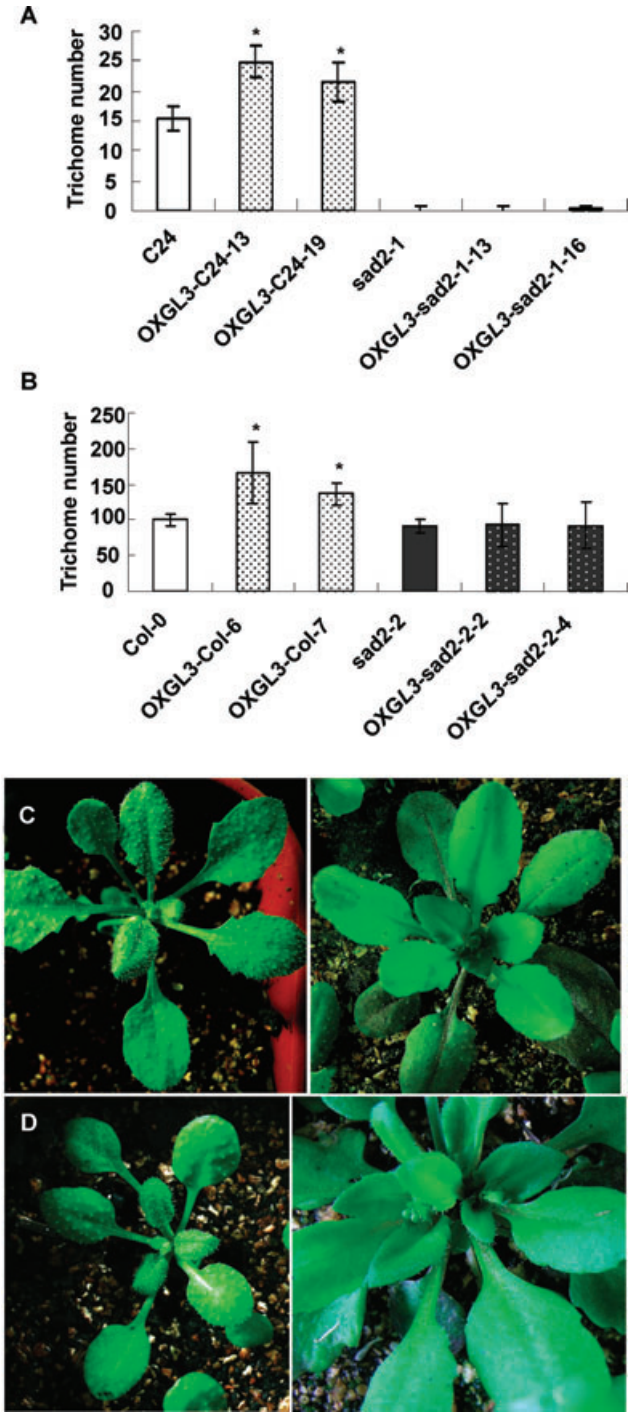

Figure 4. *GL3* and *GL1* overexpression in wild type and *sad2* mutants.

(A) *GL3* overexpression in the C24 background.

(B) *GL3* overexpression in the Columbia-0 (Col-0) background.

(C) Overexpression of *GFP-GL1* in Col-0. Left panel, Col-0; right panel, a *35S:GFP-GL1* transgenic line in Col-0.

(D) Overexpression of *GFP-GL1* in *sad2-3*. Left panel, *sad2-3*; right panel, a *35S:GFP-GL1* transgenic line in *sad2-3*.

Discussion

We have isolated three *SAD2* T-DNA mutants: *sad2-1*, *sad2-2* and *sad2-3*. *sad2-1* lacks trichomes in the C24 genetic background and *sad2-2* and *sad2-3* have reduced trichome numbers in the Col-0 genetic background. Mutations in *SAD2* affect the expression of *GL1*, *GL2*, *GL3* and *TTG1*. Both *GL1* and *TTG1*, which are required for trichome initiation, were downregulated in the *sad2* mutants, indicating that the reduction in trichome numbers in *sad2* is at least partially due to the decreased levels of these genes. This conclusion is also supported by the decreased expression level of *GL2* in *sad2*, as expression of *GL2* relies exclusively on the function of GL1 and TTG1 (Larkin et al. 2003).

The expression levels of *GL1*, *GL3*, *TTG1* and *GL2* in the C24 background were significantly lower than levels of these genes in the Col-0 background. This is consistent with the finding that trichome numbers in C24 are lower than in Col-0 and supports the hypothesis that a threshold level of GL1-GL3-TTG1 activating complex is important for the formation of trichomes (Marks and Esch 2003). In C24, the TTG1-GL3-GL1 complex may not function efficiently enough to allow transcripts of these genes to reach levels high enough to promote trichome initiation.

SAD2 did not affect the nuclear localization of GL1, TTG1 or GL3 in leaf trichomes or roots, suggesting that these proteins are probably not direct cargo of SAD2. It has been shown that loss-of-function mutations of HASTY, an importin-like protein, affect many developmental processes in *Arabidopsis*, including abaxial trichome initiation (Bollman et al. 2003). Mutations in SAD2 resulted only in defects in trichome number and not in trichome branching, shape and size. In addition, in the *sad2* mutants, there was no apparent effect on non-trichome pathways including those regulating root hair formation or seed coat pigment development, also supporting the conclusion that these proteins are not SAD2 cargo. In the C24 background, *sad2-1* showed a glabrous phenotype suggesting that there is no functional overlap within the importin β family in *Arabidopsis*. There are three importin β-like proteins in *Arabidopsis*; these proteins share significant sequence similarity. Double mutants generated by crossing *sad2-3* to a knockout mutant from each of the SAD2 homologous genes did not further decrease trichome number in the Col-0 background (data not shown), supporting the conclusion that the function of SAD2 in controlling trichome initiation is unique in *Arabidopsis*. Expression of *GL3* was upregulated in the *sad2* mutants, and ectopic overexpression of *GL3* increased wild-type trichome numbers but did not have a similar effect in the *sad2* mutants, suggesting that reduction

←

Trichome statistics are given for the first four true leaves. Asterisks indicate significant difference at $P < 0.05$ (Student's *t*-test).

Table 4. Leaf trichome numbers in wild type and mutants

Genotype	Background	Total no. of trichomes
gl1	Col-0	0.7 ± 0.82
sad2-1	C24	0
sad2-2	Col-0	129.2 ± 18.54
gl1 sad2-2		0
gl3kd	Col-0	107.4 ± 3.44
sad2-1	C24	0
sad2-2	Col-0	146.8 ± 3.31
gl3kd sad2-2		80.4 ± 5.12

The total number of trichomes was counted for the first four true leaves for 10 plants for the *gl1 sad2-2* and *gl3kd sad2-2* double mutants and their corresponding single mutants. Data represent means $\pm$ standard deviation. Col-0, Columbia-0.

of trichome number in *sad2* is partially due to the GL3 functional defect. Our results suggest that SAD2 may include an activity that positively regulates GL3 function, as a negative feedback control of *GL3* transcript by its own protein that may exist in *Arabidopsis* (Morohashi et al. 2007). It is also possible that SAD2 even functions downstream of GL3; however, the expression of *SAD2* was not affected by mutations in GL3, GL1, GL2 and TTG1. We are also not able to exclude that the effect of overexpression of *GL3* in *sad2* on trichome number might be duo to the limiting expression of *GL1*.

Recently, we reported that SAD2 is also involved in ultraviolet (UV)-B responses in *Arabidopsis* by mediating AtMYB4 nuclear trafficking (Zhao et al. 2007). SAD2 co-immunoprecipitated with AtMYB4 but not its homolog, GL1. Knockout *atmyb4* mutants were not ABA sensitive and did not have a trichome-deficient phenotype (Zhao et al. 2007; data not shown). These results suggest that SAD2 functions in various responses by regulating multiple nuclear proteins. Mutations in SAD2 altered the expression of *GL1*, *GL3* and *TTG1* and impaired the function of GL3, indicating that SAD2 functions in trichome initiation through regulation of this pathway and that one of the SAD2 targets regulates GL3 activity. Although this reduction in SAD2 did not affect TTG1-GL3-GL1 complex formation, it likely altered complex self-activation. As a result, the TTG1-GL3-GL1 complex was not able to reach the level required to positively regulate transcription of the complex components and trigger trichome initiation. The *gl3kd sad2-2* double mutant had fewer trichomes compared to its corresponding single mutants, suggesting that, in addition to GL3, SAD2 also regulates another factor(s) that mediates trichome initiation.

Materials and Methods

Plant materials

The following *Arabidopsis thaliana* strains were used in this study: C24 and Col-0 wild type; *sad2-1* (Verslues et al. 2006) in the C24 background; *sad2-2* and *sad2-3* in the Col-0 background; the *sad2-1* complementation lines, *sad2-1*com.6 and *sad2-1*com.10; *gl1* in Col-0; and *gl3kd* in Col-0 and double mutants *gl1 sad2-2* and *gl3kd sad2-2*. Genotype identification of homozygous *sad2-2*, *sad2-3* and *gl3kd* single mutants and of the double mutants used in this study is described below.

Mutant identification

The T-DNA lines, SAIL_313_B03 (*sad2-3*), SALK_133577 (*sad2-2*) and SALK_118201 (*gl3kd*) were obtained from the ABRC and homozygous lines of *sad2-2*, *sad2-3* and *gl3kd* were identified by PCR genotype screening with gene-specific primers (*sad2-3*: forward, 5′-GTTGCTTCTGCCTCGGC-3′; reverse, 5′-CTCCAAAACGCAGCCGCCTG-3′; *sad2-2*: forward, 5′-AGTTGACTCCATCACGGTAGC-3′; reverse, 5′-ATACACCAAGCGTCAAACCTG-3′; *gl3kd*: forward, 5′-CCG AAAGAATCACTGGAGGAGC-3′; reverse, 5′-TCGAGGA TTGAACCGAATGAGA-3′), and the T-DNA left border primers, LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) and LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′).

Leaf trichome and root hair measurements

Mutant and wild-type seeds were surface sterilized in 20% NaClO and 0.1% Triton X-100 and plated on Murashige and Skoog (MS) medium with 2.5% sucrose and 0.3% agar. After stratification at 4 °C for 3 d, plates with seeds were transferred to 22 °C under continuous light for germination and growth. The total number of primary root hairs was counted when seedlings were 4 d old. Leaf trichome numbers and number of trichome branches were counted in soil-grown seedlings when the first four leaves were fully expanded. Root hair and trichome observations were made with a Leica MZFL III dissecting microscope.

Scanning electron microscopy

Arabidopsis seeds were sprinkled on soil and grown in a chamber for 16 h in the light at 22 °C and 8 h in the dark at 18 °C. At specific stages of development, plants were fixed over-night in 2% glutaraldehyde and 0.1 mol/L cacodylate, followed by an alcohol dehydration series (once in 35%, 50%, 65%, 75%, 85% and 95% ethanol and twice in 100% ethanol, for 2 h per step) as described by Payne (Payne et al. 2000).

RT-PCR and real-time quantitative RT-PCR

Total RNA was extracted with TRI reagent from shoots and roots of 9 d-old seedlings grown on 0.6% MS medium. Ten micrograms of total RNA was treated with RNase-free DNase I to remove DNA and 2 μg of the RNA was then used for RT

Table 5. Leaf trichome branch numbers for *gl3kd* mutant and wild type

Genotype	No. of trichome branches					Total no. of trichomes
	1	2	3	4	5	
Col-0	0.1 ± 0.18	1.6 ± 0.73	127 ± 8.47	13.2 ± 3.77	0.2 ± 0.25	142.1 ± 19.50
gl3kd	10.5 ± 2.83	41.1 ± 5.46	72.1 ± 20.04	0	0	123.7 ± 17.09

The total number of trichome branches was counted for the first four true leaves of 10 plants. Data represent means ± standard deviation. Col-0, Columbia-0.

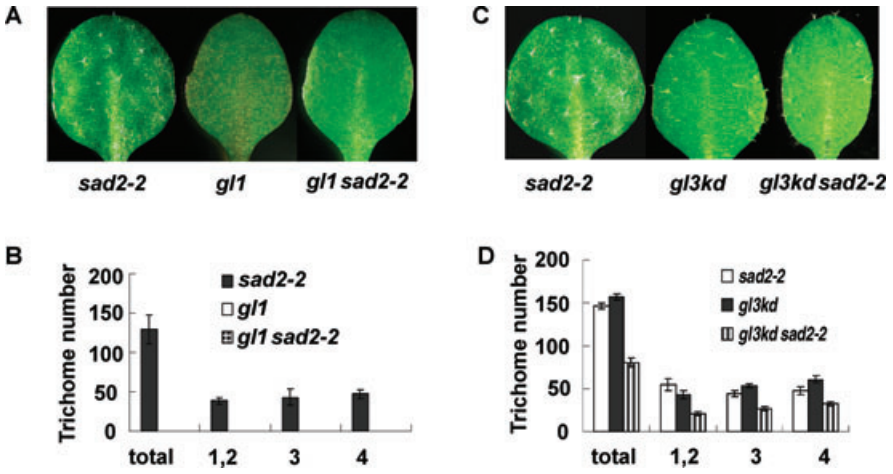

Figure 5. Trichome phenotypes of *gl1 sad2* and *gl3kd sad2* double mutants.

(A) First or second true leaf of the *gl1 sad2-2* double mutant and its corresponding single mutants.

(B) Trichome statistics for the first four leaves (total) or for the first and second (1,2), third (3) or fourth (4) true leaves for the *gl1 sad2-2* double mutant and its corresponding single mutants shown in **(A)**.

(C) The first or second true leaf of the *gl3kd sad2-2* double mutant and its corresponding single mutants.

(D) Trichome statistics for the first four leaves (total) or the first and second (1,2), third (3) or fourth (4) true leaves for the *gl3kd sad2-2* double mutant and its corresponding single mutants shown in **(C)**.

with M-MLV Reverse Transcriptase according to the manufacturer's instructions. First strand cDNA was used for RT-PCR and real-time quantitative PCR analysis. Real-time PCR was performed using the ABI 7500 Fast Real-Time PCR System and the SYBR Premix Ex Taq kit. The real-time PCR results obtained from the different cDNA samples were standardized using the gene expression levels of *ACTIN2*. Dissociation curves were analyzed for all samples to ensure the amplification of only one product. The following primer sequences were used: *SAD2* (forward: 5′-CACCCAAGTTCCAACCTTACTC-3′; reverse: 5′-TACTTATGGCACGCAAACAACC-3′), *GL1* (forward: 5′-CAATGGAACCGCATCGTCAG-3′; reverse: 5′-TGATGAACAATGACGGTGGA-3′), *MYB23* (forward: 5′-ACAGATGGTCGTTGATAGCG-3′; reverse: 5′-GTCAGTTGGTGTTGCGTGGA-3′), *GL2* (forward: 5′-CTCAGTGGCAATCCAGACAG-3′; reverse: 5′-ACTCTACTCCATCAGGTATG-3′), *GL3* (forward: 5′-CGCAGGAGAAAGAACATCAG-3′; reverse: 5′-CGAGGATTGAACCGAATGAG-3′), *EGL3* (forward: 5′-TCGGTTATGCTGGTCTAACG-3′; reverse: 5′-CCATGCAACCCTTTGAAGTG-3′), *TTG1* (forward: 5′-TATTCGTTCGCCGACTATGCC-3′; reverse: 5′-ATTGGGTCCAGCAACAGTAGG-3′) and *ACTIN2* (forward: 5′-GTCGTACAACCGGTATTGTG-3′; reverse: 5′-GAGCTGGTCTTTGAGGTTTC-3′).

Overexpression of *GL3* and *GL1* in wild type and the *sad2* mutants

GL1 and *GL3* cDNAs were amplified by RT-PCR, sequenced and then cloned into pCAMBIA1205 between the *Bam*HI and *Eco*RI sites under the control of a 35S promoter. The resulting plasmids were introduced into *Arabidopsis* plants through *Agrobacterium tumefaciens* strain GV3101 using the floral dip method. Positive transgenic lines were identified by PCR. Complementation of the *sad2* phenotype was described previously (Verslues et al. 2006). Young seedlings of 12 independent T_3 transgenic lines growing in MS medium with appropriate antibiotics were transferred to soil. When the first four true leaves were fully expanded, the total number of trichomes on these four

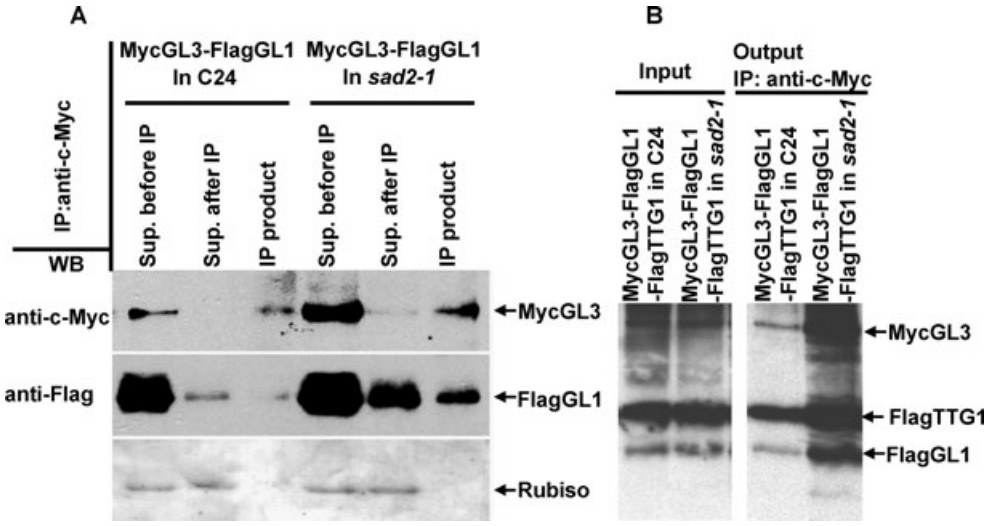

Figure 6. GL3-GL1 and TTG1-GL3-GL1 complex formation.

(A) Supernatant (Sup.) before and after immunoprecipitation (IP) with anti-c-myc conjugated agarose. IP products were analyzed by immunoblot (WB) analysis. One percent of the IP products (lanes 3 and 6) were used for immunoblot analysis. Top panel, result of the anti-c-myc hybridization; middle panel, result of the anti-flag hybridization; lower panel, presence of RUBISCO with Ponceau S staining. FlagGL1 was co-immunoprecipitated with mycGL3 in both C24 and *sad2-1*. When only *mycGL3* was expressed in C24, no signal was detected with anti-flag antibody (data not shown).
(B) *MycGL3*, *FlagGL1* and *FlagTTG1* were co-expressed in protoplasts from C24 and the *sad2-1* mutant. Total protein was analyzed using anti-c-myc and anti-flag antibodies (Input). After immunoprecipitation with anti-c-myc conjugated agarose, the components in the pellet were detected via immunoblot analysis (Output). Both flagGL1 and flagTTG1 co-immunoprecipitated with mycGL3 in both C24 and *sad2-1*.

leaves was counted. Primers used for cloning *GL1* and *GL3* and for identification of positive transgenic lines were as follows: *GL1* (forward: 5′-CGGGATCCATGAGAATAAGGAGAAGAG-3′; reverse: 5′-GGAATTCCTAAAGGCAGTACTCAACATC-3′) and *GL3* (forward: 5′-CGGGATCCATGGCTACCGGACAAAACA-3′; reverse: 5′-GGAATTCTCAACAGATCCATGCAAC-3′).

Double mutant isolation and trichome development

To obtain *gl1 sad2-2* and *gl3kd sad2-2* double mutants, two single mutants were crossed and the resulting F_2 progeny were used to identify homozygous double mutant lines. To confirm homozygous T-DNA insertions, T-DNA left border primers and gene-specific primers were used. To confirm homozygous point mutations for *gl1*, genomic DNA was sequenced. The trichomes of the first four or six true leaves of the double mutants and their corresponding single mutants were counted. The primers used for identification of *sad2-2* and *gl3kd* were given above.

CFP/GFP/YFP-fusion plasmid construction and image analysis

Plasmid *pCAMBIA1205:GFP-GL1* was generated by extracting *GL1* cDNA from the plasmid *pCAMBIA1205:GL1* as a 5′*Bam*HI and 3′*Eco*RI fragment and cloning it into the corresponding sites of *pCAMBIA1205:GFP*. *pCAMBIA1205:GFP-GL2* and *pCAMBIA1205:CFP-GL3* were generated as described for *pCAMBIA1205:GFP-GL1*. To generate plasmid *pCAMBIA1205:GFP-TTG1*, *TTG*1 was amplified by PCR from Col-0 cDNA, sequenced and then cloned into the *pCAMBIA1205:GFP* between the *Eco*RI and *Kpn*I sites. Plasmids *pCAMBIA1205:YFP-MYB23* and *pCAMBIA1205:YFP-EGL3* were generated by cloning *MYB23* and *EGL3* cDNAs into *Bam*HI and *Eco*RI sites of *pCAMBIA1205:YFP.* The binary constructs were introduced into *Arabidopsis* plants through *A. tumefaciens* strain GV3101 using the floral dip method. Green fluorescent protein (GFP) and cyan fluorescent protein (CFP) fluorescence was detected in elongating roots growing on MS medium and mature leaf trichomes of T_2 transgenic lines using a Carl Zeiss LSM 510 META confocal microscope and LSM Image Brower software. *TTG1, MYB23* and *EGL3* cDNAs were amplified with the following primers: *TTG1 (*forward: 5′-CGGAATTCATGGATAATTCAGCTCCAG-3′; reverse: 5′-GG GGTACCTCAAACTCTAAGGAGCTGCA-3′), *MYB23* (forward: 5′-CGGGATCCATGAGAATGACAAGAGATGG-3′; reverse: 5′-GGAATTCTCAAAGGCAATACCCATTAG-3′) and *EGL3* (forward: 5′-CGGGATCCATGGCAACCGGAGAAAACAG-3′; reverse: 5′-GGAATTCTTAACATATCCATGCAAC-3′).

Co-immunoprecipitation

To generate plasmids for expressing 6myc-GL3, *GL3* cDNA was extracted from *35S:GL3* with *Eco*RI and *Bam*HI and cloned into the *pRT105:6myc* vector at the same sites. *GL1* and *TTG1* cDNAs were extracted from *35S:GL1* and *35S:GFP-TTG1* vectors, respectively, and cloned into the *pRT105:3flag* vector. *Myc-GL3*/*flag-GL1* and *myc-GL3*/*flag-TTG1*/*myc-GL1* were co-transformed into protoplasts as indicated in the text. The protoplasts were incubated at room temperature for 12 h and harvested by centrifugation. Total proteins were extracted in 300 μL extraction buffer containing 10 mmol/L Tris (pH 7.5), 0.5% NP-40, 2 mmol/L ethylenediaminetetraacetic acid, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride and 1% protease inhibitor cocktail (Sigma P9599). Ten microliters of settled anti-c-myc agarose conjugate (Sigma A7470) were used for immunoprecipitation. After the antibody and antigen were incubated at 4 °C for 3 h, the agarose beads were washed in 1 mL of extraction buffer five times. The IP products were detected using an immunoblot. Both anti-c-myc (Sigma M-4439) and anti-flag (Sigma F-3165) were used at dilutions of 1:5 000.

Acknowledgements

We thank Mr Cheng Zhan and Ms Jun Zhang for excellent technical assistance and the ABRC (Ohio State University) for the T-DNA insertion lines and plasmids.

References

Bollman KM, Aukerman MJ, Park MY, Hunter C, Berardini TZ, Poethig RS (2003). HASTY, the *Arabidopsis* ortholog of exportin 5/MSN5, regulates phase change and morphogenesis. *Development* **130**, 1493–1504.

Fyvie MJ, Murray JA, Kilby NJ (2000). Mosaic analysis of GL2 gene expression and cell layer autonomy during the specification of *Arabidopsis* leaf trichomes. *Genesis* **28**, 68–74.

Kirik V, Lee MM, Wester K, Herrmann U, Zheng Z, Oppenheimer D et al. (2005). Functional diversification of *MYB23* and *GL1* genes in trichome morphogenesis and initiation. *Development* **132**, 1477–1485.

Kirik V, Schnittger A, Radchuk V, Adler K, Hulskamp M, Baumlein H (2001). Ectopic expression of the *Arabidopsis AtMYB23* gene induces differentiation of trichome cells. *Dev. Biol.* **235**, 366–377.

Larkin JC, Brown ML, Schiefelbein J (2003). How do cells know what they want to be when they grow up? Lessons from epidermal patterning in *Arabidopsis*. *Annu. Rev. Plant Biol.* **54**, 403–430.

Larkin JC, Oppenheimer DG, Lloyd AM, Paparozzi ET, Marks MD (1994a). Roles of the *GLABROUS1* and *TRANSPARENT TESTA GLABRA* genes in *Arabidopsis* trichome development. *Plant Cell* **6**, 1065–1076.

Larkin JC, Oppenheimer DG, Marks MD (1994b). The *GL1* gene and the trichome developmental pathway in *Arabidopsis thaliana*. *Results Probl. Cell Differ.* **20**, 259–275.

Larkin JC, Oppenheimer DG, Pollock S, Marks MD (1993). *Arabidopsis GLABROUS1* gene requires downstream sequences for function. *Plant Cell* **5**, 1739–1748.

Larkin JC, Walker JD, Bolognesi-Winfield AC, Gray JC, Walker AR (1999). Allele-specific interactions between ttg and gl1 during trichome development in *Arabidopsis thaliana*. *Genetics* **151**, 1591–1604.

Marks MD, Esch JJ (2003). Initiating inhibition. Control of epidermal cell patterning in plants. *EMBO Rep.* **4**, 24–25.

Masucci JD, Rerie WG, Foreman DR, Zhang M, Galway ME, Marks MD et al. (1996). The homeobox gene *GLABRA2* is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*. *Development* **122**, 1253–1260.

Morohashi K, Zhao M, Yang M, Read B, Lloyd A, Lamb R et al. (2007). Participation of the *Arabidopsis* bHLH factor *GL3* in trichome initiation regulatory events. *Plant Physiol.* **145**, 736–746.

Ohashi Y, Oka A, Ruberti I, Morelli G, Aoyama T (2002). Entopically additive expression of *GLABRA2* alters the frequency and spacing of trichome initiation. *Plant J.* **29**, 359–369.

Oppenheimer DG, Herman PL, Sivakumaran S, Esch J, Marks MD (1991). A myb gene required for leaf trichome differentiation in *Arabidopsis* is expressed in stipules. *Cell* **67**, 483–493.

Payne CT, Zhang F, Lloyd AM (2000). *GL3* encodes a bHLH protein that regulates trichome development in *Arabidopsis* through interaction with GL1 and TTG1. *Genetics* **156**, 1349–1362.

Rerie WG, Feldmann KA, Marks MD (1994). The *GLABRA2* gene encodes a homeo domain protein required for normal trichome development in Arabidopsis. *Genes Dev.* **8**, 1388–1399.

Schellmann S, Schnittger A, Kirik V, Wada T, Okada K, Beermann A et al. (2002). TRIPTYCHON and CAPRICE mediate lateral inhibition during trichome and root hair patterning in *Arabidopsis*. *EMBO J.* **21**, 5036–5046.

Schiefelbein J (2003). Cell-fate specification in the epidermis: a common patterning mechanism in the root and shoot. *Curr. Opin. Plant Biol.* **6**, 74–78.

Schwab B, Folkers U, Ilgenfritz H, Hulskamp M (2000). Trichome morphogenesis in *Arabidopsis*. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **355**, 879–883.

Serna L, Martin C (2006). Trichomes: different regulatory networks lead to convergent structures. *Trends Plant Sci.* **11**, 274–280.

Szymanski DB, Lloyd AM, Marks MD (2000). Progress in the molecular genetic analysis of trichome initiation and morphogenesis in *Arabidopsis*. *Trends Plant Sci.* **5**, 214–219.

Verslues PE, Guo Y, Dong CH, Ma W, Zhu JK (2006). Mutation of SAD2, an importin beta-domain protein in *Arabidopsis*, alters abscisic acid sensitivity. *Plant J.* **47**, 776–787.

Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL et al. (1999). The TRANSPARENT TESTA

GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *Plant Cell* **11**, 1337–1350.

Zhang F, Gonzalez A, Zhao MZ, Payne CT, Lloyd A (2003). A network of redundant bHLH proteins functions in all TTG1-dependent pathways of *Arabidopsis*. *Development* **130**, 4859–4869.

Zhao JF, Zhang WH, Zhao Y, Gong XM, Guo L, Zhu GL et al. (2007). SAD2, an Importin β-Like protein, is required for UV-B response in *Arabidopsis* by mediating MYB4 nuclear trafficking. *Plant Cell* **19**, 3805–3818.

Supplementary Material

The following supplementary material is available for this article:

Supplementary Figure S1. GFP-GL1, GFP-GL2 and CFP-GL3 localization in roots and trichomes of transgenic wild-type and *sad2* mutant lines.
(A) GFP-GL1, bar = 100 μm;
(B) GFP-GL2, bar = 100 μm;
(C) CFP-GL3, bar = 100 μm.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1744-7909.2008.00695.x

(This link will take you to the article abstract).

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

(Handling editor: Chun-Ming Liu)