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Integration of cytokinin and gibberellin signalling by *Arabidopsis* transcription factors GIS, ZFP8 and GIS2 in the regulation of epidermal cell fate

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The effective integration of hormone signals is essential to normal plant growth and development. Gibberellins (GA) and cytokinins act antagonistically in leaf formation and meristem maintenance and GA counteract some of the effects of cytokinins on epidermal differentiation. However, both can stimulate the initiation of defensive epidermal structures called trichomes. To understand how their relative influence on epidermal cell fate is modulated, we investigated the molecular mechanisms through which they regulate trichome initiation in *Arabidopsis*. The control by cytokinins of trichome production requires two genes expressed in late inflorescence organs, *ZFP8* and *GIS2*, which encode C2H2 transcription factors related to GLABROUS INFLORESCENCE STEMS (GIS). Cytokinin-inducible *GIS2* plays a prominent role in the cytokinin response, in which it acts downstream of SPINDLY and upstream of GLABROUS1. In addition, *GIS2* and *ZFP8* mediate, like *GIS*, the regulation of trichome initiation by gibberellins. By contrast, *GIS* does not play a significant role in the cytokinin response. Collectively, *GIS*, *ZFP8* and *GIS2*, which encode proteins that are largely equivalent in function, play partially redundant and essential roles in inflorescence trichome initiation and in its regulation by GA and cytokinins. These roles are consistent with their pattern of expression and with the regional influence of GA and cytokinins on epidermal differentiation. Our findings show that functional specialization within a transcription factor gene family can facilitate the integration of different developmental cues in the regulation of plant cell differentiation.

KEY WORDS: Cytokinins, Epidermis, Gibberellins, Transcription factor, Trichomes, *Arabidopsis*

INTRODUCTION

Phytohormones play distinct but overlapping roles in the regulation of growth and development in plants. Both the spatiotemporal pattern of hormone signal generation and the proper integration of these signals by downstream regulators are therefore essential in the production of appropriate responses. Recent data indicate that plants employ at least two strategies for the treatment of competing hormone signals. As one strategy, they use a centralized system that involves upstream integrators of hormone signalling; members of the DELLA family of transcription factors have been shown to play such a role in the control of plant growth by gibberellins (GA), auxins, ethylene and abscisic acid (Achard et al., 2006; Achard et al., 2003; Fu and Harberd, 2003; Peng et al., 1997; Silverstone et al., 1998). As another strategy, which is suggested by gene expression studies, they deploy more specialized regulators that may act further downstream and control distinct gene networks (Nemhauser et al., 2006). Little information is available so far on the nature of such regulators.

It is known that epidermal differentiation is regulated by hormone signals in plants. In particular, hormone levels affect the density of trichomes, which are large defensive epidermal structures found on aerial organs in many plant species (Chien and Sussex, 1996; Gan et al., 2006; Greenboim-Wainberg et al., 2005; Kazama et al., 2004; Perazza et al., 1998; Telfer et al., 1997; Traw and Bergelson, 2003). As trichome production is tightly regulated and easily monitored, it is a robust system for studying how epidermal differentiation is controlled and the role played by phytohormones in this process. Trichome initiation in *Arabidopsis* requires gibberellin signalling, and GA applications have been shown to cause an increase in trichome density on leaves and stems (Chien and Sussex, 1996; Gan et al., 2006; Perazza et al., 1998; Telfer et al., 1997). In inflorescence organs, gibberellins act in part through the transcription factor GLABROUS INFLORESCENCE STEMS (GIS), a positive regulator of trichome initiation that acts upstream of GLABROUS1 (GL1) (Gan et al., 2006).

Recent data indicate that cytokinins can also stimulate trichome initiation, as cytokinin applications to flowering *Arabidopsis* plants cause trichome proliferation on flowers. This effect is counteracted by mutations in *SPINDLY*, which positively regulates cytokinin signalling (Greenboim-Wainberg et al., 2005). The stimulation of trichome initiation by both GA and cytokinins contrasts with other, conflicting effects of the two hormone signalling pathways. For example, gibberellin applications inhibit the effect of cytokinin treatments and block cytokinin signalling (Ezura and Harberd, 1995; Greenboim-Wainberg et al., 2005). Reciprocally, increases in cytokinin levels cause changes in gene expression that antagonise GA signalling (Brenner et al., 2005). These opposing effects have been found to be particularly important in the maintenance of the shoot meristem (Jasinski et al., 2005; Yanai et al., 2005).

In the present study, we have investigated molecular mechanisms through which gibberellin and cytokinin signalling modulate epidermal differentiation during inflorescence development. We report that the integration of cytokinin and gibberellin signalling requires the collective action of GIS and two novel related transcription factors, ZFP8 and GIS2. We show that GIS, ZFP8 and GIS2 are functionally interchangeable activators of trichome production and that the corresponding genes have specialized to play distinct roles in GA and cytokinin responses during development.

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MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia Col-0 was used for most experiments in this study and the Landsberg *erecta* ecotype was grown as a control if needed. The *gl1-1*, *ga1-3* and *spy-3* mutants were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). For all phenotypic analyses, the plants were grown in cycles of 16 hours of light (95 μmol cm^{-2} $second^{-1}$, 21°C) and 8 hours dark (18°C). Inflorescence organs were harvested from plants with a main stem that had reached approximately 17 cm in length. Trichome initiation on branches and the third cauline leaf was monitored by counting all trichomes on the first internode or the adaxial side of the leaf blade, respectively. Trichome production on the main stem was evaluated by counting trichomes on 2 cm of stem length, 1.5 cm from the base of the stem. Total trichome number on the first internode of the main stem was extrapolated from these figures using average internode lengths for a given genotype/treatment combination. Trichome density on the first and second cauline leaves was measured in a 0.6 cm^{-2} area of the mid-section of each leaf. Total leaf trichome production was then extrapolated using average leaf area measurements. Trichome production on sepals was evaluated by counting trichomes on 10-15 flowers per plant. Unless specified otherwise, a minimum of 20 plants was used for trichome analysis for each treatment × genotype combination.

Isolation of novel mutants and construction of multiple mutants

A transgenic line carrying a transposon in the exon of *GIS2* (SM_3_32778) was obtained from NASC (catalogue number N119489). Homozygous *gis2* mutants were selected using Basta (40 μm) and by PCR using gene-specific primers (5′-AATCATCAAGGCAAATCCGCA-3′ and 5′-TGCTACCACCACCGACATACG-3′), alone or in combination with a transposon-specific primer (Spm1: 5′-CTTATTTCAGTAAGAGTGTGGGGTTTTGG-3′) (Tissier et al., 1999). *Zfp8* was identified from the SAIL collection (SALK_045674) and also obtained from NASC (catalogue number N545674). Homozygous *zfp8* mutants were selected on kanamycin (50 μg/ml) and by PCR using gene-specific primers (5′-TTTGAAAAAGGAGGATTGATGG-3′ and 5′-TCGGAGTTATCACCGACGAGC-3′) or with a gene-specific/T-DNA-specific primer combination (T-DNA primer: 5′-GCGTGGACCGCTTGCTGCAACT-3′). *gis gis2* and *gis2 spy-3* double mutants were selected from F2 populations by double selection on basta and sulfadiazine (5.2 mg/l) or basta and paclobutrazole respectively. *gis*, *gis2* and *gis gis2* lines in which *ZFP8* is silenced were obtained by transforming a *ZFP8* RNAi construct into these mutants.

Hormone treatments

GA3 (Sigma) and BA (6-Benzylamino-Purine, Sigma) were used in all experiments that involved exogenous GA and cytokinin treatments respectively. Control and mutant plants were grown on soil until the first three to four leaves had emerged and sprayed twice a week with GA3, BA or mock solutions until the plants were ready for analysis. For measuring the effect of GA applications on gene expression, a minimum of eight mutant and control plants were grown on soil until young inflorescence shoots had reached a size of 2-3 cm. The plants were then sprayed with either 100 μM GA3, 100 μM BA or a mock solution and the shoots were harvested 4 hours (GA) or 2 hours (BA) after treatment for RNA extraction.

Molecular biology

RNA extraction, real-time and semi-quantitative RT-PCR

Plant RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Pooled tissue samples from at least eight soil-grown plants were used for RNA extractions. The following gene-specific primer sequences were used for real-time PCR analysis: *GIS*, 5′-TTCATGAACGTCGAATCCTTCTC-3′ and 5′-ACGAATGGGTTTAGGGTTCTTATCT-3′; *ZFP8*, 5′-AAGCCGCCATTATTCGTCTCT-3′ and 5′-CTGCGGATAAGTTGTCGGAGTT-3′; *GIS2*, 5′-ACCGCCAACAAAACCACATT-3′ and 5′-CGCGTCGTTGATTTGAACAG-3′; *ARR5*, 5′-TTGCGTCCCGAGATGTTAGAT-3′ and 5′-TGAGTAACCGCTCGATGAACTTC-3′; *UBQ10*, 5′-GGTTCGTACCTTTGTCCAAGCA-3′ and 5′-CCTTCGTTAAACCAAGCTCAGTATC-3′; *GL1*, 5′-CGACTCTCCACCGTCATTGTT-3′ and 5′-TTCTCGTAGATATTTTCTTGTTGATGATG-3′. Q-PCR primer design and reaction conditions were as previously described (Gan et al., 2006). *UBQ10* transcripts were used as an internal control for normalizing expression of the other genes (Gan et al., 2005).

Semi-quantitative PCR analysis of *GIS2* gene expression in *gis2* mutants was performed with the same primers that were used to clone the full-length coding sequence of the gene (see below). cDNA template amounts were first adjusted according to *UBQ10* product intensities and *GIS2* amplification was performed over 41 cycles.

Cloning

For the production of all overexpression and RNAi constructs, sequences were first inserted into the Gateway entry vector pENTR-1A (Invitrogen), before recombination into the appropriate destination vector using the Gateway LR reaction (Invitrogen). All destination vectors were obtained from VIB (Flanders Interuniversity Institute). pH2GW7 (carrying a hygromycin-resistance gene) was used as the destination vector for *35S:ZFP8* and *35S:GIS2* constructs; pK7GWIWG2(II) (carrying NPTII) and pB7GWIWG2(II) (Basta resistance), respectively, for *ZFP8*-RNAi and *GIS2*-RNAi constructs; gene-specific fragments were first PCR-amplified from inflorescence cDNA (overexpression and RNAi constructs) or genomic DNA (promoter fusion constructs) using primers containing *Sal*I and *Not*I restriction sites. The following primers were used: *ZFP8* overexpression: 5′-TTCCATTGTCGACTCTCCCTGATCTCTCTCTTCC-3′ and 5′-TTGCATTGCGGCCGCTTCACCGATCAGCGAGTCT-3′; *GIS2* overexpression: 5′-TCAACTGTCGACAGCCATCCAGAGTCATAACCA-3′ and 5′-AAGATAGCGGCCGCGAATGGAACTAGAGGCGTAGA-3′; *ZFP8*-RNAi construct: 5′-ACTTGTCGACCACCACATCTACGGCTTCCT-3′ and 5′-ATTGCGGCCGCTTGCACATTGGGTTTCATCA-3′; *GIS2*-RNAi construct: 5′-ATTCGTCGACTTCAACCTCCATTCAAACG-3′ and 5′-AAGCGGCCGCGAATGGAACTAGAGGCGTAGA-3′.

For the production of *pGIS* promoter fusion constructs, a 1.6 kb *GIS* promoter fragment was amplified using the primers 5′-ATCTTGGAGCTCGGGGGAATGAGTCAAGAGTTC-3′ and 5′-ATCTTGTCTAGAGAGAGATAAAAAGACTGGGCG-3′ and substituted for the 35S promoter in pH2GW7 after restriction with *Sac*I and *Spe*I. The coding sequences of *GIS*, *ZFP8* and *GIS2* were then recombined into the modified vector from the same entry vector that was used for the production of overexpression constructs. A modified strategy was taken for *pGIS2* constructs: a 1.5 kb *GIS2* promoter fragment was amplified using primers 5′-TCCTGAGAGCTCTCTCAAGTTGGCTTCGTGTG-3′ and 5′-TTAGTAGCGCTAGCGGTGGTTATGACTCTGGATGG-3′, restricted with *Sac*I, then ligated into the vector fragment of pH2GW7 that was first restricted with *Spe*I, blunt-ended, then cut with *Sac*I. The coding sequences of *GIS*, *ZFP8* and *GIS2* were then recombined as above.

All binary vector constructs were introduced into *Agrobacterium* strain GV3101 by electroporation. *Agrobacterium*-mediated transformation of all *Arabidopsis* genotypes was performed using the floral dip method (Clough and Bent, 1998).

In situ hybridization

Non-radioactive in situ hybridization was performed as previously described (Gan et al., 2006). For synthesis of the *ZFP8* and *GIS2* RNA probes, gene-specific fragments were amplified using the same primers as for generating the RNAi constructs (see above).

RESULTS

Shifting influences of gibberellin and cytokinin signalling on epidermal differentiation during inflorescence development in *Arabidopsis*

Cytokinins can promote the proliferation of trichomes on *Arabidopsis* flowers, an effect that is offset by gibberellin applications and mutations in SPINDLY (Greenboim-Wainberg et al., 2005). We observed that applications of 6-benzylaminopurine (BA) also stimulate trichome production on cauline leaves and stems

and that this effect increases in intensity with successive branches or paraclades (Fig. 1A). As evidence that cytokinin is not only limiting, but also required for the production of trichomes, we found that trichome initiation is markedly decreased on flowers and on upper inflorescence stems of *Arabidopsis* plants in which the gene encoding cytokinin breakdown enzyme CKX2 is overexpressed (Werner et al., 2003). Similarly, we found that the flowers of loss-of-function SPY mutant *spy-3* are glabrous (Table 1).

The antagonistic effect of GA on flower trichome initiation is in contrast with their known stimulation of trichome production on leaves (Gan et al., 2006; Perazza et al., 1998; Telfer et al., 1997). We therefore examined in detail the effects on epidermal differentiation of applying a wide range of GA3 concentrations to wild-type plants and GA-deficient *ga1-3* mutants. GA applications restored trichome initiation on normally glabrous *ga1-3* flowers, although GA concentrations exceeding 10 μM caused a decrease, rather than a further increase, in trichome production – an effect also seen with wild-type plants (Fig. 2A,B). At GA concentrations exceeding 100 μM, we also observed a reduction in trichome production on cauline leaves and branches in *ga1-3* (Fig. 2A). These results were consistent with the inflorescence trichome phenotype of *spy-3*, in which GA signalling is increased and cytokinin signalling inhibited (Table 1) (Greenboim-Wainberg et al., 2005).

The above observations indicated that gibberellins and cytokinins play changing roles in epidermal differentiation as the inflorescence develops. GA signalling is required throughout development, while the requirement for cytokinins is limited to upper inflorescence organs. Consistently with the antagonistic roles of GA and cytokinins, the growing requirement for cytokinin signalling during inflorescence development is accompanied with an increasingly inhibitory effect of GA.

GIS homologues ZFP8 and GIS2 are required for trichome initiation late in inflorescence development and, in contrast to GIS, are necessary for the cytokinin response

The transcription factor GIS is required for trichome production on inflorescence organs and modulates the regulation by GA of trichome initiation (Gan et al., 2006). On the basis of the observed interplay between gibberellins and cytokinins on inflorescence trichome initiation, we asked whether GIS also mediates the cytokinin response. To this end, we tested the effects of cytokinin applications on trichome production in the *gis* loss-of-function mutant. As the overall response of cauline leaves and flowers to cytokinin treatments was similar in *gis* and control plants (Fig. 1A), we concluded that *GIS* is not required in this process.

We previously identified two genes, At2g41940 (*ZFP8*) and At5g06650, that encode proteins closely similar in sequence to GIS (Gan et al., 2006; Tague and Goodman, 1995). Moreover, their relatedness to GIS suggested that they might play a redundant role in the control of trichome production. We therefore investigated whether *ZFP8* and At5g06650, which we termed *GIS2*, play a role in this process and investigated their possible implication in the cytokinin response. We first searched public collections for mutants containing insertions in either of the two genes and identified two lines meeting this criterion. One line carried a transposon 166 bp upstream of the start codon in *GIS2* and the other a T-DNA in the promoter region of *ZFP8*, 139 bp upstream of the coding region. We found using RT-PCR that the expression of either of these genes was strongly downregulated or abolished in these lines (Fig. 1B). To further assess possible effects of loss of function, we also produced transgenic plants in which *ZFP8* and *GIS2* were silenced by RNAi, and selected lines

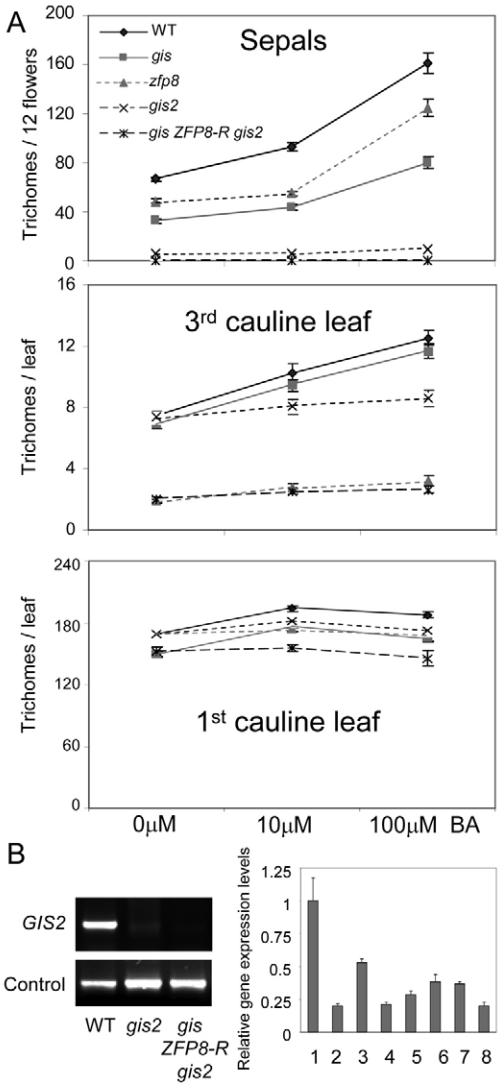

Fig. 1. Induction of trichome initiation by cytokinins in wild-type *Arabidopsis* plants and GIS clade mutants. (**A**) Trichome initiation on sepals (top panel) and first (bottom panel) and third (middle panel) cauline leaves of wild-type, *gis*, *zfp8*, *gis2* and *gis gis2 ZFP8-RNAi* line 2 (termed *gis ZFP8-R gis2*) plants that were treated with increasing concentrations of 6-benzylaminopurine. Values represent averages and standard error for 20 plants. (**B**) Expression of *ZFP8* and *GIS2* in loss-of-function mutants and RNAi lines; left panel: RT-PCR analysis of *GIS2* expression in *gis2* and *gis ZFP8-R gis2* mutants; right panel: real-time PCR analysis of *ZFP8* expression in wild-type (1), *zfp8* (2), *gis ZFP8-R1* (3), *gis ZFP8-R2* (4), *gis2 ZFP8-R1* (5), *gis2 ZFP8-R2* (6), *gis ZFP8-R1 gis2* (7) and *gis ZFP8-R2 gis2* (8). Values are ratios to wild type of normalized transcript levels. Sepal trichome values represent the total number of trichomes for 12 flowers. WT, wild type.

in which their expression was significantly downregulated (Fig. 1B; see Table S1 in the supplementary material). We then examined the trichome phenotype of T-DNA and RNAi lines.

We observed that ZFP8 loss of function led to a significant reduction in trichome density on upper cauline leaves and branches (Fig. 3C; Table 2). By contrast, lines in which *GIS2* was knocked out showed a very strong decrease in trichome production on flowers (Fig. 3B) but only a small decrease on branches and cauline leaves, which was only noticeable on the third paraclade (Table 2). The phenotype

Table 1. Influence of cytokinin signalling on trichome initiation in inflorescence organs

Genotype	Average number of trichomes	
	Sepals	Second branch, first internode
Wild type	36.2 (1.5)	52.6 (4.2)
35S:CKX2	18.8 (1.1)	23.4 (3.9)
spy-3	0.0 (0.0)	75.6 (6.1)

Trichome production of cytokinin-deficient line *35S:CKX2* and *spindly3*; only organs showing a significant difference between the genotypes are shown. *35S:CKX2* overexpressors only produced two cauline leaves under our growing conditions. Sepal trichome values represent the number of trichomes for a total of ten flowers. Branch trichome values are the total number of trichomes for the chosen internode. Values represent averages and standard error (in parentheses) for 20 plants.

of the different lines was otherwise similar to that of controls. In particular, we did not observe significant differences in trichome production on vegetative organs (see Table S2 in the supplementary material). As a confirmation that the mutant phenotypes were caused by the insertions, we found that silencing either of the genes by RNAi had a similar impact on trichome initiation (see Table S1 in the supplementary material). We were also able to complement the *zfp8* and *gis2* mutants by expressing the coding regions of the corresponding genes under either native or constitutive promoters (see Figs S1, S2 in the supplementary material).

To determine whether *ZFP8* and *GIS2* play a role in the induction by cytokinins of trichome initiation, we treated *zfp8* and *gis2* with increasing concentrations of BA and compared their response to that of control plants. We found that, while the responses of *gis2* and wild-type plants were similar in the first cauline leaf, the induction of trichome production was nearly abolished on third cauline leaves and on sepals in *gis2* (Fig. 1A) and significantly less pronounced on second cauline leaves (data not shown). ZFP8 loss of function affected the cytokinin response in the third cauline leaf, but the response was not significantly different from wild type in other parts of the inflorescence (Fig. 1A). These observations indicated that, in contrast to GIS, GIS2 and, to a lesser degree, ZFP8 are required for the induction of trichome initiation by cytokinins on inflorescence organs.

GIS, *ZFP8* and *GIS2* encode functionally equivalent proteins and their effects on trichome initiation are additive

To assess the level of functional redundancy between *GIS*, *ZFP8* and *GIS2*, we first examined the effects of overexpressing *ZFP8* and *GIS2* in a wild-type background. We had found that overexpressing *GIS* leads to high levels of trichome initiation on all inflorescence organs and ectopic trichome formation on floral organs (Gan et al., 2006). As a first indication that the activities of GIS, ZFP8 and GIS2 are similar, we found that *35S:ZFP8* and *35S:GIS2* plants closely resembled *GIS* overexpressors. In particular, trichome production on inflorescence stems and leaves was increased and ectopic trichomes were visible on flowers, in particular carpels (Fig. 3D). This phenotype was visible in more than half of the 30 *35S:ZFP8* and *35S:GIS2* transgenic lines that we examined. As in *35S:GIS* plants, overexpression of either of these genes also delayed flowering and caused the appearance of aerial rosettes (data not shown) (Gan et al., 2006).

As a second step, we tested whether the coding regions of *GIS*, *ZFP8* and *GIS2* had the ability to complement any of the *gis*, *zfp8* or *gis2* mutants. We first used the 35S promoter to overexpress the three coding regions in the different mutant backgrounds and obtained a minimum of 30 transformants with each the

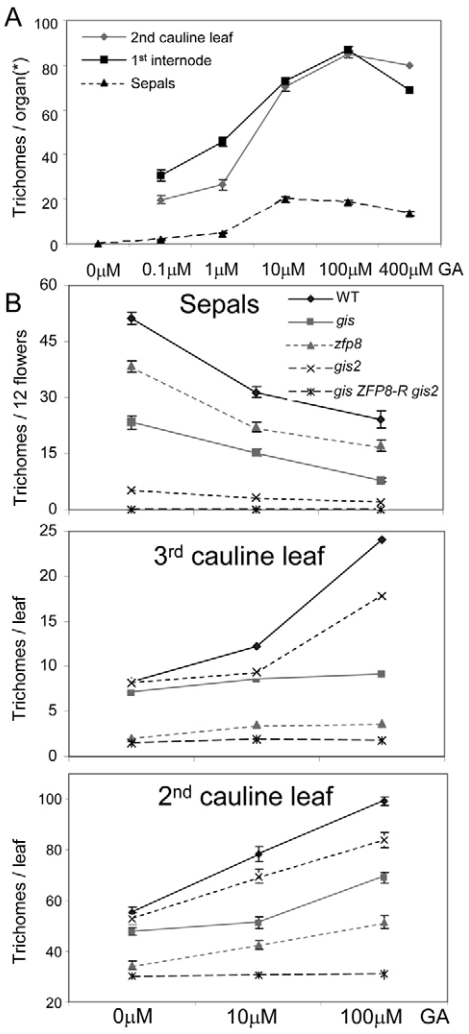

Fig. 2. Inflorescence trichome initiation in response to gibberellins in wild-type *Arabidopsis* plants and GIS clade mutants. (**A**) Trichome initiation on inflorescence organs of GA-deficient *ga1-3* mutants treated with increasing concentrations of GA3. (*) Sepal trichome values represent the total number of trichomes for 12 flowers. (**B**) Differential response of GIS clade loss-of-function mutants to GA applications at increasing concentrations. Values represent averages and standard errors for 20 plants. WT, wild type.

overexpression constructs. We found that, in at least 40% of the transgenic lines, overexpression of either of the three genes was sufficient to restore trichome production on cauline leaves, stems or flowers to normal levels or higher in all of the mutants (examples shown on Fig. 3E and Fig. S2 in the supplementary material). As the effects of ectopic expression could have masked minor differences in transcription factor activities, we repeated our complementation experiment of the *gis* and *gis2* mutants using promoter fragments of *GIS* (*pGIS*) or *GIS2* (*pGIS2*), obtaining a minimum of 40 transgenic lines for each of the combinations. We first confirmed that *pGIS* and *pGIS2* had comparable levels of activity to native regulatory sequences by complementing the *gis* and *gis2* mutants with *pGIS:GIS* (Fig. 3F) and *pGIS2:GIS2*, respectively (see Fig. S1 in the supplementary material). Complementation was observed in six *pGIS:GIS gis* and seven *pGIS2:GIS2 gis2* lines, respectively. We then transformed the *gis* mutant with *pGIS:ZFP8* and *pGIS:GIS2*

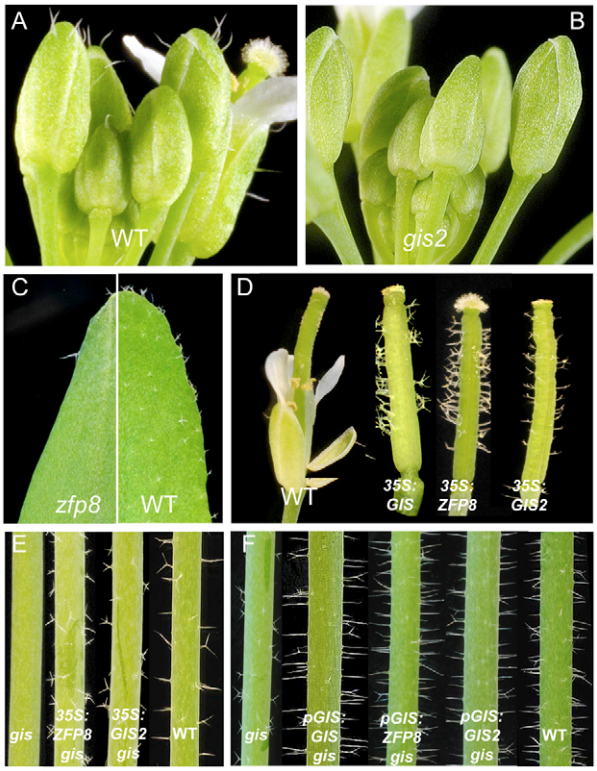

Fig. 3. GIS, ZFP8 and GIS2 have equivalent activities. (**A**,**B**) Trichome initiation on sepals of wild-type (A) and *gis2 Arabidopsis* flowers (B). (**C**) Trichome production on *zfp8* (left) and wild-type cauline leaves (right). (**D**) Production of ectopic trichomes on carpels of wild-type *35S:GIS*, *35S:ZFP8* and *35S:GIS2* plants. (**E**) Effects of *ZFP8* and *GIS2* overexpression on trichome initiation in wild type and in the *gis* mutant background (second branches). (**F**) Trichome branching and density on the main stems of *gis* (left), *pGIS:GIS gis*, *pGIS:ZFP8 gis* or *pGIS:GIS2 gis* mutants compared with wild-type stems (right). WT, wild type.

constructs and the *gis2* mutant using *pGIS2:GIS* and *pGIS2:ZFP8* constructs, generating a minimum of ten transgenic lines with each construct. Consistently with the results of constitutive overexpression, at least four independent lines had a trichome phenotype that was indistinguishable from wild type. Interestingly, while *35S:ZFP8* and *35S:GIS2* constructs could not restore the morphology of *gis2* stem trichomes, which produce supernumerary branches (Fig. 3E) (Gan et al., 2006), *pGIS:ZFP8 gis* and *pGIS:GIS2 gis* plants (like *pGIS:GIS gis* plants) produced normal trichomes (Fig. 3F). Possibly, the timing and localization of *pGIS*-driven expression were more appropriate for restoring a normal trichome developmental programme than 35S-driven expression. We also found that expression of *pGIS2:GIS* and *pGIS2:ZFP8* constructs in the *gis2* mutant restored trichome production on flowers (see Fig. S1 in the supplementary material). The results of these cross-complementation experiments provided strong evidence that GIS, ZFP8 and GIS2 have largely equivalent activities, at least in the control of trichome production.

The phenotypes of double and triple mutants were also consistent with a partially redundant role for the three genes. We constructed the double and triple mutant genotypes either by combining T-DNA insertions (for *GIS* and *GIS2*) or by transforming the *gis*, *gis2* and *gis gis2* mutants with an RNAi construct in order to silence *ZFP8*. We used this strategy because lines in which *ZFP8* is silenced were found to have a similar phenotype to the *zfp8* loss-of-function mutant and levels of *ZFP8* expression that were equivalent to that of the T-DNA insertion line (Fig. 1B; see Table S1 in the supplementary material). We could also verify that *ZFP8* expression was significantly downregulated in selected *gis*, *gis2* and *gis gis2 ZFP8*-RNAi lines (Fig. 1), which we termed *gis ZFP8-R*, *gis2 ZFP8-R* and *gis ZFP8-R gis2*, respectively (Table 1; Figs 1, 2). We found, by comparing the trichome phenotypes of the different mutants, that the effects of GIS, ZFP8 or GIS2 loss of function were largely additive. In particular, *gis ZFP8-R gis2* plants produced inflorescence organs that were either glabrous or with strong reductions in trichome density (Table 2). This additive effect was also seen in the cytokinin response, as the triple mutant was completely insensitive to cytokinin treatments (Fig. 1A). The double and triple mutants were otherwise similar to wild type in growth and development (see Table S2 in the supplementary material). These observations indicated that, collectively, *GIS*, *ZFP8* and/or *GIS2* are absolutely required for trichome initiation and cytokinin-induced trichome production in most of the inflorescence.

Table 2. Influence of GIS, ZFP8 and GIS2 on inflorescence trichome initiation

Genotype	Sepals	Second caul. leaf	Third caul. leaf	Second br.	Third br.	Stem (first internode)
Wild type	58.5 (0.9)	54.3 (2.6)	9.0 (0.7)	42.5 (2.6)	10.5 (1.7)	115.8 (1.2)
RNAi control	60.1 (1.0)	55.1 (2.0)	9.7 (0.7)	43.6 (1.9)	12.7 (2.1)	119.1 (1.8)
gis	32.6 (0.7)	45.6 (2.6)	7.7 (0.6)	0.5 (0.3)	0.0 (0.0)	79.7 (1.7)
zfp8	41.3 (0.9)	23.9 (2.0)	3.2 (0.4)	38.7 (3.5)	5.6 (1.2)	101.6 (2.4)
gis2	2.2 (0.3)	59.5 (1.6)	7.6 (0.4)	41.7 (2.1)	6.1 (0.9)	110.1 (1.8)
gis gis2	0.0 (0.0)	41.7 (2.0)	7.2 (0.4)	0.1 (0.1)	0.0 (0.0)	80.6 (1.5)
gis ZFP8-R1	29.2 (1.5)	32.0 (3.0)	3.0 (0.5)	0.0 (0.0)	0.0 (0.0)	76.7 (2.4)
gis ZFP8-R2	27.0 (1.3)	27.9 (2.2)	2.6 (0.4)	0.0 (0.0)	0.0 (0.0)	75.5 (2.9)
gis2 ZFP8-R1	2.3 (0.2)	28.8 (2.6)	2.6 (0.3)	28.0 (3.4)	3.8 (0.9)	98.4 (2.1)
gis2 ZFP8-R2	2.7 (0.3)	31.2 (2.0)	3.8 (0.5)	35.5 (3.1)	5.4 (1.1)	101.3 (2.0)
gis ZFP8-R1 gis2	0.0 (0.0)	25.7 (1.8)	2.1 (0.4)	0.0 (0.0)	0.0 (0.0)	75.2 (2.1)
gis ZFP8-R2 gis2	0.0 (0.0)	28.3 (2.2)	3.4 (0.4)	0.0 (0.0)	0.0 (0.0)	74.0 (2.7)

Trichome production on inflorescence organs of mutants and RNAi lines that are deficient in GIS, ZFP8 and/or GIS2 function. All trichomes were counted on the first internode of branches or main stems. In the case of main stems, the values were extrapolated from average internode lengths using measurements made on a 1.5 cm-long segment 2 cm from the base. Leaf trichome values were extrapolated using average leaf areas from counts obtained within a 0.6 cm^2 area. Values represent averages and standard error (in parentheses) for 20 plants. RNAi control, transgenic plants that were transformed with the insert-less RNAi vector; *ZFP8-R*, line in which the *ZFP8* gene was silenced by RNAi; caul. leaf, cauline leaf; br., branch.

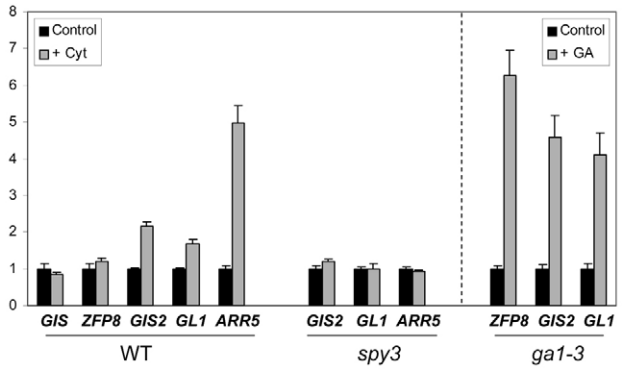

Fig. 4. Expression of *ZFP8, GIS2* and *GL1* in response to GA and cytokinin treatments. Left: expression of *GIS*, *ZFP8*, *GIS2*, *GL1* and primary response regulator *ARR5* in inflorescence organs of *Arabidopsis* plants 2 hours after 6-benzylaminopurine applications (100 μM) to wild-type plants. Middle: expression of *GIS*, *GL1* and *ARR5* in response to 6-BA applications (100 μM) in the *spy-3* mutant background. Right: induction by GA of *ZFP8*, *GIS2* and *GL1* expression in the inflorescence of *ga1-3* mutants 4 hours after treatment. Black bars: mock treatments; grey bars: hormone treatments. Transcript levels were measured by real-time PCR and the values represent ratios of normalized levels to controls.

GIS2 and *GL1* are cytokinin-inducible, but not *GIS* or *ZFP8*

To start defining how cytokinins might modulate ZFP8 and GIS2 action and ultimately trichome initiation, we measured the expression of *ZFP8*, *GIS2* and *GL1* in wild-type inflorescence organs in response to increases in cytokinin signalling. For this analysis, developing inflorescence organs were harvested 2 hours after BA treatment. The strong increase in the expression of primary response gene *ARR5* indicated that hormone applications led to a significant increase in cytokinin signalling. They also caused a significant elevation in *GIS2* and *GL1* transcript levels. This response was abolished in the *spy-3* mutant, an indication that the transcriptional effect was specifically due to variations in cytokinin signalling and required SPY (Fig. 4). By contrast, this treatment had little effect on *ZFP8* expression and, consistently with the cytokinin response of *gis* mutant plants, no effect on *GIS* transcript levels. These observations indicated that, in line with the contrasting sensitivities of *gis*, *zfp8* and *gis2* to cytokinin applications, *GIS*, *ZFP8* and *GIS2* are also differentially responsive to increases in cytokinin signalling. They also suggested that the induction of trichome initiation by cytokinins proceeds in part through the transcriptional activation of *GIS2* and *GL1*.

ZFP8 and *GIS2* participate in the GA response and their expression is GA-inducible

Our results indicated that *GIS*, *ZFP8* and *GIS2* encode partially redundant transcription factors that are divergent in their response to cytokinins. To determine whether the genes also play distinct roles in gibberellin responses, we first examined the effect of GA treatments on the *zfp8*, *gis2* and *gis ZFP8-R gis2* mutant phenotypes. We found that both *zfp8* and *gis2* mutants were locally less responsive to GA applications than wild-type plants. Consistently with the trichome phenotype of *zfp8* mutant plants, *zfp8* showed less or no increase in trichome production after GA treatment on the third cauline leaves (Fig. 2B). Similarly, the flowers and third cauline leaves of *gis2* mutants were less sensitive to GA. Strikingly, *gis ZFP8-R gis2* plants were completely unresponsive to GA as they were to cytokinins (Fig. 2B). The above experiment established that *GIS*, *ZFP8* and *GIS2* are collectively required for the response of inflorescence organs to GA.

To determine whether increasing transcript levels of these redundant genes can be sufficient for saturating the response, we examined the effect of GA applications on *35S:GIS2* overexpressors. We found that GA applications had no effect on trichome production on lower cauline leaves and branches, where they normally stimulate initiation, or on flowers, where they are normally inhibitory (see Fig. S4 in the supplementary material). These observations suggested that the control of trichome initiation by gibberellins in *Arabidopsis* inflorescence organs requires the transcriptional regulation of *GIS*, *ZFP8* and/or *GIS2*.

On the basis these results, we examined whether exogenous GA treatments affect *ZFP8* and *GIS2* gene expression. We found that GA applications to *ga1-3* mutants strongly induced the expression of *ZFP8* and *GIS2*, as we had previously observed with *GIS* and *GL1* (Fig. 4) (Gan et al., 2006). This result indicated that, although they differ from *GIS* in their response to cytokinins, *ZFP8* and *GIS2* are similarly inducible by GA.

GIS2 and ZFP8 act downstream of SPY and upstream of GL1

To further investigate the genetic control of trichome initiation by cytokinins, we first examined genetic interactions between *GIS2* and *SPY*. As GIS2 loss of function strongly inhibits trichome initiation on sepals and *spy-3* flowers are glabrous (Fig. 5A), we first overexpressed *GIS2* in the *spy-3* background. We found that trichome initiation was restored on flowers of *35S:GIS2 spy-3* plants (Fig. 5B), a result that was also obtained when *ZFP8* was overexpressed in *spy-3* (see Fig. S3 in the supplementary material). We also produced *gis2 spy-3* double mutants and found that their trichome production was most similar to *gis2* on branches and cauline leaves but that they produced glabrous flowers (Table 3).

The similar activities of GIS and GIS2 and the effect of cytokinin applications on *GL1* expression suggested that the two regulators might influence trichome initiation by acting on the same downstream pathways. To test this hypothesis, we investigated genetic interactions between *GIS2* and trichome initiation regulatory genes *GL1* and *GLABRA3* (*GL3*) (Payne et al., 2000). We found that both *35S:GIS2 gl1* and *35S:GIS2 gl3* lines were glabrous, like *gl1* and *gl3* (Fig. 5C). Conversely, as we had seen in the case of *gis* mutants, overexpressing in *gis2* the maize *R* gene, which is functionally equivalent to *GL3*, restored trichome initiation on flowers and increased trichome production on branches (Fig. 5D). Similarly, *R* overexpression increased trichome density on cauline leaves in *zfp8* (see Fig. S3 in the supplementary material), but *ZFP8* overexpression did not induce trichome production on *gl1* or *gl3* plants (data not shown). We also found that *GL1* expression was increased in inflorescence organs of *35S:GIS2* plants but decreased in *gis2* mutants, in particular late in development (Fig. 5E).

The results of these experiments suggested that GIS2 is a positive regulator of *GL1* expression and that both ZFP8 and GIS2 act downstream of SPY but upstream of the trichome initiation complex.

ZFP8 and *GIS2* are differentially expressed in inflorescence organs

To determine whether the expression pattern of the genes in the plant are consistent with their roles in trichome initiation and hormone signalling, we examined the distribution of *ZFP8* and *GIS2* transcripts in different tissues using quantitative RT-PCR and in the inflorescence by in situ hybridization. *ZFP8* and *GIS2* were found

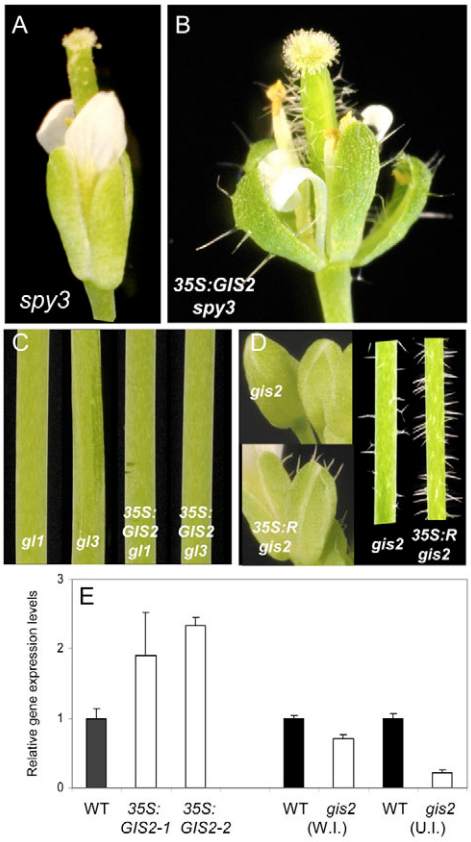

Fig. 5. Genetic interactions between *GIS2*, *SPY* and trichome initiation regulators in *Arabidopsis*. (**A**,**B**) Trichome initiation on flowers of *spy-3* (A) and *35S:GIS2 spy-3* mutants (B). (**C**) Trichome initiation on stems of *gl1*, *gl3*, *35S:GIS2 gl1* and *35S:GIS2 gl3* plants. (**D**) Effect of *R* overexpression on trichome initiation on *gis2* flowers (left) and third branches (right). (**E**) *GL1* expression in inflorescence organs of two *35S:GIS2* overexpressor lines (left) and in the *gis2* mutant (right). Transcript levels were measured by real-time PCR and the values represent ratios of normalized levels to controls. U.I., upper developing inflorescence; W.I., whole developing inflorescence; WT, wild type.

to be expressed at early stages of inflorescence development but at different levels in different inflorescence organs. Specifically, *ZFP8* was most expressed in cauline leaves (Fig. 6B,D,E) and *GIS2* in the primary and secondary meristems and in developing flowers (Fig. 6A,C,E). We also found that *GIS2* was expressed at increasing levels in successive cauline leaves (Fig. 6E). These patterns of expression were consistent with the phenotype of *gis2*, which is most pronounced in later cauline leaves and flowers, and with the cauline leaf phenotype of *zfp8*. They were also consistent with the patterns of hormone response during inflorescence development and the relative influences of ZFP8 and GIS2 on this process.

In summary, we found that, while the activity of the proteins are largely equivalent, the regulatory mechanisms controlling the expression of *GIS*, *ZFP8* and *GIS2* during inflorescence development have diverged (Gan et al., 2006).

DISCUSSION

In this study we have investigated the molecular mechanisms integrating gibberellin and cytokinin signalling in the control of trichome initiation in *Arabidopsis*. We found that the influence of the two hormone classes on trichome production is modulated by the combined actions of GIS and two related transcription factors, ZFP8 and GIS2. While all three transcription factors are required in the regulation of trichome initiation by gibberellins throughout inflorescence development, only ZFP8 and GIS2 modulate cytokinin signalling. Cytokinin-inducible *GIS2*, which plays a predominant role in the cytokinin response, encodes a transcriptional activator of *GL1* that acts downstream of SPINDLY. The products of *GIS*, *ZFP8* and *GIS2* are largely equivalent in function, but the genes have specialized and are differentially regulated during inflorescence development, in a way that is consistent with their roles in trichome initiation and the influence of gibberellins and cytokinins on this process.

Table 3. Genetic interactions between *GIS2* and *SPY*

Genotype	Sepals	Second caul. leaf	Third caul. leaf	Second br.
gis2	6.5 (0.5)	44.9 (2.4)	9.0 (0.5)	35.9 (2.5)
spy-3	0.0 (0.0)	57.5 (2.4)	14.4 (1.1)	75.6 (6.1)
gis2 spy-3	0.0 (0.0)	46.8 (3.2)	10.1 (0.5)	37.4 (3.7)

Trichome production of different inflorescence organs in *gis2*, *spy-3* and *gis2 spy-3* mutants. See Table 2 legend for detail on measurements. Values represent averages and standard error (in parentheses) for 20 plants. caul. leaf, cauline leaf; br.: branch (first internode).

Integration of hormone signalling by transcription factors

Our findings show that related transcription factors play different roles in regulating the influence of different hormone signals on epidermal differentiation in *Arabidopsis*. The roles of GIS, ZFP8 and GIS2 appear to be mainly in the modulation of specific epidermal responses to cytokinins and gibberellins, as the triple mutant does not show a general hormone response phenotype. In this respect, the GIS clade differs from regulators such as the DELLA transcription factors, which play a general role in modulating growth and differentiation in response to a variety of hormone signals (Achard et al., 2006; Achard et al., 2003; Fu and Harberd, 2003). In a further contrast with GIS, ZFP8 and GIS2, the DELLA proteins are post-transcriptionally regulated (Dill et al., 2004; Sasaki et al., 2003; Silverstone et al., 2001), and the relative influence of the different members of the clade may depend more on their abundance in a particular tissue than on their specific responsiveness to hormone signals (Sun and Gubler, 2004). In mainly modulating hormone responses, the GIS clade proteins also differ from the KNOX-type transcription factors that regulate gibberellin and cytokinin signalling in the meristem, as the latter act upstream, rather than downstream, of the hormone biosynthesis pathways (Jasinski et al., 2005; Yanai et al., 2005). The same holds true of FUSCA 3, which has been proposed to regulate gibberellin and abscisic acid biosynthesis in the embryo (Gazzarrini et al., 2004). The role of the GIS clade therefore highlights the central role played by transcription factors not only upstream but also downstream of hormone production.

Functional specialization of *GIS*, *ZFP8* and *GIS2*

The *GIS*, *ZFP8* and *GIS2* genes encode functionally equivalent proteins but have diverged in their response to phytohormones and in their role during inflorescence development. The functional specialization of genes encoding paralogous transcription factors appears to be an important mechanism through which plants regulate similar differentiation programs at different stages in development. For example, MYB transcription factor WEREWOLF 1 (WER) is

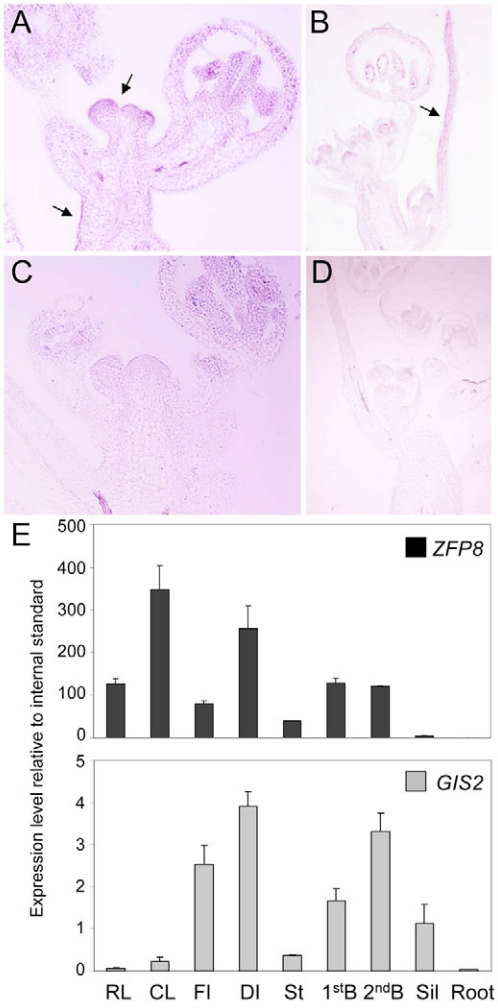

Fig. 6. Distribution of *ZFP8* and *GIS2* transcripts in different *Arabidopsis* plant tissues. (**A-D**) In situ hybridization of *GIS2* (A,C) and *ZFP8* (B,D) probes to developing inflorescence organs of wild-type plants. *GIS2* is strongly expressed in inflorescence meristems, floral meristems and the epidermis (arrows, A). *ZFP8* is most highly expressed in developing cauline leaves (B). (C,D) Sections that were hybridized using sense RNA. (**E**) Real-time PCR analysis of *ZFP8* (top) and *GIS2* (bottom) expression in various plant tissues. Values are normalized to the expression of the *UBQ10* gene. 1stB, first branch; 2ndB, second branch; CL, cauline leaf; DI, developing Inflorescence; Fl, flower; RL, rosette leaf; Sil, silique; St, main stem (base).

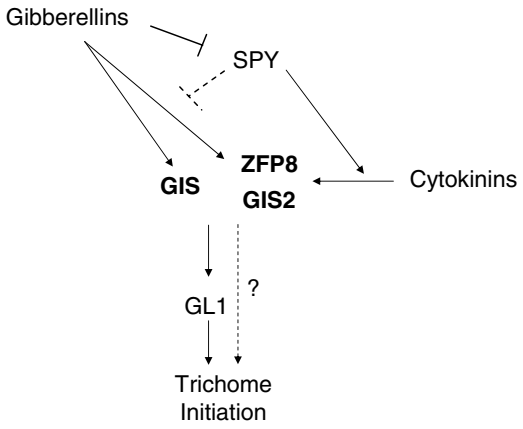

Fig. 7. Model of GIS, ZFP8 and GIS2 action in *Arabidopsis* inflorescence organs in response to GA and cytokinins. Broken lines: the interaction between GIS and SPY was previously examined. The antagonistic role of SPY in the regulation by ZFP8 and GIS2 of GA signalling is only suggested, as is the possibility that the regulation of trichome initiation by cytokinins is not solely dependent on GL1.

functionally equivalent to GL1; however, *WER* is expressed in the root, where it regulates epidermal hair development, whereas *GL1* regulates trichome development in the shoot (Lee and Schiefelbein, 2001). Similarly, GL1 and MYB23 are functionally interchangeable, but *MYB23* and *GL1* have distinct although overlapping expression domains during leaf development (Kirik et al., 2005).

In the case of the GIS clade, the divergence of their transcriptional control may be an adaptive mechanism that is important for modulating hormone responses during inflorescence development. This mechanism is fairly unique, as it allows the plant to respond similarly to seemingly conflicting hormone signals (Fig. 7). As the developmental patterns of *GIS*, *ZFP8* and *GIS2* expression have also diverged, their influence on trichome initiation could be seen as a mere reflection of a local balance in gibberellin and cytokinin levels within inflorescence organs. This scenario seems unlikely, however, as transcripts for the different genes are distributed across largely overlapping locations (Gan et al., 2006) and both the roles and inducibility of the genes in response to cytokinins are clearly different. Rather, our data suggest that *GIS*, *ZFP8* and *GIS2* have diverged in their responses to both developmental and hormonal signals and that these signals are not strictly interdependent.

ZFP8, GIS2 and the regulation of phase change

The phenotype of *GIS* overexpressors and loss-of-function mutants suggested that GIS influences trichome initiation as part of a more general role in the regulation of phase change (Gan et al., 2006). The heterochronic phenotypes of *35S:ZFP8* and *35S:GIS2* lines could indicate that ZFP8 and GIS2 also play a role in phase change, although these phenotypes may just reflect the fact that the three transcription factors have similar activities. Because of the localized nature of the *zfp8* and *gis2* phenotypes, it seems unlikely that ZFP8 and GIS2 play a significant role in phase change. As *gis ZFP8-R gis2* mutants go through normal phase transition, such a role, if it exits, is not just masked by genetic redundancy between GIS, ZFP8 and GIS2. Elucidating how increases in GIS activity influence phase change therefore awaits further investigation.

In conclusion, our results illustrate the importance of functional specialization within a plant transcription factor family in the control of cellular differentiation and in response to various hormonal and developmental cues. A better understanding of how developmental and hormonal signals are integrated in plants is likely to emerge as our knowledge of transcription factor function becomes more complete. Progress in this field will also benefit from system biology approaches that integrate the analysis of gene networks with the precise mapping of hormone influence within the plant.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/11/2073/DC1

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