Mosaic Analysis of GL2 Gene Expression and Cell Layer Autonomy During the Specification of Arabidopsis Leaf Trichomes

Mark J. Fyvie, James A.H. Murray, and Nigel J. Kilby*

Institute of Biotechnology, University of Cambridge, Cambridge, United Kingdom

Received 3 August 2000; Accepted 6 September 2000

Summary: Homozygous glabra2 (gl2) mutant Arabidopsis thaliana Landsberg erecta plants with only a few rudimentary single spiked trichomes on the leaf margin were transformed with a genomic clone of GL2, resulting in partial restoration of the normal leaf trichome phenotype. The introduced GL2 transgene was configured as part of an FLP recombinase-responsive gene switch, which permitted visibly marked gl2 mutant clonal sectors to be generated by FLP recombinase-mediated deletion of the GL2 transgene with concomitant activation of a previously silent β-glucuronidase (GUS) marker gene. GUS marked sectors extending through all three leaf cell layers (L1, L2, and L3) displayed the anticipated gl2 mutant phenotype, whereas immediately adjacent unmarked tissue, and unmarked tissues overlaying GUS sectors restricted to the L2 and/or L3 cell layers, retained the GL2 restored phenotype. These data support the view that the GL2 gene product acts in a region-autonomous manner within a single cell layer and indicate that GL2 gene expression in the L1 layer is sufficient for GL2-directed outgrowth of trichomes. genesis 28: 68–74, 2000. © 2000 Wiley-Liss, Inc.

Key words: genetic mosaics; Arabidopsis trichomes; FLP recombinase

INTRODUCTION

The mosaic analysis of gene function provides an unparalleled opportunity to examine the cell autonomy of a gene product, to dissect signal transduction pathways, and, if the timing of mosaicism can be precisely controlled, to address the temporal importance of gene expression. In plants, the traditional method of choice for generating genetic mosaics has been to mutagenise seed that is heterozygous for a recessive gene of interest. However, this approach is inherently limited because the induction of mosaicism is restricted to the seed stage of development and a ubiquitous phenotype-independent cell marker that can be used unambiguously to correlate phenotype with genotype is lacking. In Drosophila, equivalent technical restrictions have been mitigated by the use of the yeast FLP recombinase to generate mosaic flies, either by inducing illegitimate mitotic recombination (Xu and Rubin, 1993) or by a process of recombinase-mediated gene switching, termed the “FLP-out” approach (Struhl and Basler, 1993; Basler and Struhl, 1994). The basic strategy used in the “FLP-out” approach has been to flank a cloned gene of interest by two FLP recombinase target (FRT) sites arranged in an excision-competent (i.e., direct) orientation relative to one another. The FRT flanked gene cassette can then, e.g., be cloned between the coding region of a visible marker gene and its promoter to prevent expression of the marker gene by physical intervention of transcription. In the presence of active FLP recombinase, base-pair perfect recombination between the two directly oriented FRT sites is predicted to occur (Vetter et al., 1983), resulting in excision of the cloned gene with concomitant activation of the marker gene. If the excision-competent gene cassette is transformed into the corresponding mutant (thus complementing the mutant phenotype) and FLP recombinase is transiently expressed within the same genetic background, the cloned gene will be excised in a proportion of cells; if an excision reaction occurs in a cell that subsequently undergoes rounds of mitotic division, a visibly marked mutant cell lineage will be generated within an otherwise phenotypically wild-type, unmarked background.

Here we describe the use of the “FLP-out” approach to generate visibly marked Arabidopsis plants that are genetically mosaic for glabra2 (gl2) to investigate the autonomy and cell layer specificity of GL2 gene expression in leaf trichome cell development. Plants mutant for GL2 possess a few rudimentary (unbranched) single spiked trichomes on the leaf margin and are typically devoid of trichomes across the entire leaf blade. This phenotype is consistent with a role for GL2 in cell expansion processes that specify the local outgrowth of trichomes (Hülskamp et al., 1994; Rerie et al., 1994).
an earlier effort to determine the spatial importance of GL2 gene expression in trichome development, Hülskamp et al. (1994) generated Arabidopsis plants that were mosaic for GL2 by chemical mutagenesis of seeds heterozygous for gl2. They observed patches of mutant trichomes on leaves within an otherwise wild-type background and inferred that GL2 must therefore act locally. However, as these authors pointed out, there was no independent cell marker available in their experiments that could be used to correlate genotype with observed phenotype, leaving the issue of whether GL2 acts locally, unresolved.

GL2 has been cloned from Arabidopsis by two groups who used plants with different genetic backgrounds as their source material; Di Christina et al. (1996) cloned GL2 from the Columbia ecotype, and Rerie et al. (1994) cloned GL2 from the Wassilewskija ecotype. The Columbia ecotype GL2 clone (designated Athb-10) differs from the Wassilewskija ecotype GL2 clone in that it encodes a predicted protein product that is 87 amino acids longer than the predicted GL2 protein product published by Rerie et al. (1994). Transformation of the Wassilewskija ecotype GL2 clone into gl2 mutant plants complements the gl2 mutant phenotype (Rerie et al., 1994) providing convincing proof that the GL2 clone represents the GL2 locus. Upstream promoter elements (two putative myb binding sites) included in the GL2 clone(s) used by Rerie et al. (1994) are, however, absent on the Athb-10/ GL2 clone described by Di Christina et al. (1996) and appear to be required for full complementation of the gl2 phenotype (Rerie et al., 1994). It is noted though that a clear distinction can be made between the gl2 aborted trichome phenotype and that of the partially restored phenotype (predominantly double branched trichomes distributed across the entire leaf blade) of a gl2 mutant plant transformed with the Columbia GL2 (Athb-10) clone (this study).

To generate Athb-10/GL2 genetic mosaics in Arabidopsis, we transformed gl2 mutant plants with the Athb-10/GL2 transgene configured as part of an FLP recombinase-responsive GL2 excision cassette, designed to allow FLP recombinase-mediated excision of the GL2 transgene with concomitant activation of a previously silent visible marker gene, β-glucuronidase (GUS). In our scheme, single insert-containing plant lines homozygous for the FLP-excisable Athb-10/GL2 gene cassette are crossed to gl2 mutant plants homozygous for a single insert heat-shock-responsive source of FLP recombinase. Progeny from the cross, hemizygous for each insert, are briefly heat-shocked to activate transient FLP recombinase expression. FLP recombinase-mediated excision of the Athb-10/GL2 transgene in a cell(s) that subsequently undergo mitosis is predicted to generate a GUS marked clonal gl2 mutant cell lineage. By comparison of trichome development in gl2 mutant sectors clonal in all three leaf cell layers (L1, L2 and L3) with trichome development in immediately adjacent Athb-10/GL2 restored unmarked leaf sectors, we confirm that the Athb-10/GL2 gene product acts locally. Furthermore, given that gl2 mosaic sectors clonal in the L2 and/or L3 alone had no discernable effect on the local outgrowth of Athb-10/GL2 restored trichomes from overlying epidermal cells in the L1, we suggest that Athb-10/GL2 gene expression may only be required in the L1 cell layer for Athb-10/GL2-directed local outgrowth of trichomes.

RESULTS

Phenotypic and Molecular Characterisation of Athb-10/GL2 Transformants

Homozygous gl2 mutant Arabidopsis thaliana Landsberg erecta plants lacking branched trichomes on the leaf surface and possessing all but a few rudimentary single spiked trichomes on the leaf margin (Fig. 2B) were either transformed with the FLP excisable Athb-10/GL2 gene cassette, pMJF19 (Fig. 1A), or an excision control construct, pMJF20. NPTII = neomycin phosphotransferase II coding region with nopaline synthase (nos) promoter and nos 3’ termination sequence; 35S = 35S CaMV promoter; FRT = FLP recognition target; HYG = hygromycin phosphotransferase coding region; ocs = octopine synthase 3’ termination sequence; Athb-10/GL2 = Athb-10/ GL2 coding region with 1kb of upstream native promoter and 3’ termination sequence; GUS = β-glucuronidase coding region with nopaline synthase (nos) 3’ termination sequence; Br = right T-DNA border; Bl = left T-DNA border.

FIG. 1 T-DNA arrangement of (a) the FLP excisable Athb10/GL2 gene cassette, pMJF19 and (b) the excision control construct, pMJF20. NPTII = neomycin phosphotransferase II coding region with nopaline synthase (nos) promoter and nos 3’ termination sequence; 35S = 35S CaMV promoter; FRT = FLP recognition target; HYG = hygromycin phosphotransferase coding region; ocs = octopine synthase 3’ termination sequence; Athb-10/GL2 = Athb-10/ GL2 coding region with 1kb of upstream native promoter and 3’ termination sequence; GUS = β-glucuronidase coding region with nopaline synthase (nos) 3’ termination sequence; Br = right T-DNA border; Bl = left T-DNA border.
tion of branching was at normal positions along the trichome stalk. In contrast to the Athb-10/GL2 restored phenotype of Athb-10/GL2 transformed gl2 mutant plants, gl2 mutant plants transformed with the excision control construct retained their gl2 mutant status (Fig. 2C). GUS staining of seedlings from a representative stably transformed excision control line showed that the recombined product remaining after FLP-mediated excision was competent for GUS expression in planta (Fig. 2D); in the absence of FLP recombinase, lines 19g-3 and 19g-79 were GUS negative (data not shown). Chi-squared analysis of segregation of kanamycin resistance in hemizygous lines 19g-3 ($\chi^2 = 0.29$; not significant at the 5% level) and 19g-79 ($\chi^2 = 0.11$; not significant at the 5% level) indicated that each of these lines contained one T-DNA insert; this was confirmed by Southern blot analysis (data not shown).

Mosaic Analysis of Athb-10/GL2 Cell Layer Autonomy and Gene Expression

GUS marked gl2 mutant sectors were generated in Athb-10/GL2 restored Arabidopsis plants by heat-shock treatment of germinating seed (as described; Kilby et al., 2000) derived from crosses between homozygous, single insert-containing FLP-excisable Athb-10/GL2 restored lines 19g-3 and 19g-79, and a gl2 mutant line homozygous for a single insert, heat-shock activatable source of FLP recombinase (see methods). GUS marked half leaf blade sectors in which GUS marking extended from the mid-rib to the leaf margin were identified (Fig. 3B); sectors extending from the mid rib to the leaf margin were assumed to be clonal in all three leaf cell layers (L1, L2, and L3) as previously demonstrated by sectioning of GUS marked half leaf blade sectors (Kilby et al., 2000). The leaf trichome phenotype associated with GUS marked L1/L2/L3 sectors (Fig. 3A) was identical to that of a gl2 mutant plant, i.e., trichomes were unbranched and were restricted to the leaf margin. In contrast, leaf trichomes that developed from the epidermal cell layer in immediately adjacent unmarked half leaf blade sectors (Fig. 3C) displayed the Athb-10/GL2 restored phenotype (cf. Figs. 2E and 2F with Fig. 3C); trichomes in unmarked half leaf blade sectors were distributed normally across the leaf epidermis. No difference was observed between the trichome phenotype of GUS marked and unmarked sectors produced when either line 19g-3 or line 19g-79 was used as the FLP-excisable Athb-10/GL2 restored parent.
In addition to observing trichome development in GL2 marked L1/L2/L3 sectors, we also studied trichome development in unmarked L1 epidermal cells overlaying GL2 marked sectors that were clonal in the L2 and/or L3 leaf cell layers. GL2 marked leaf sectors were assumed to be clonal in the L2 and/or L3 leaf cell layers when GL2 marking did not extend to the leaf margin (Furner and Pumfrey, 1992; Irish and Sussex, 1992). More than fifty GL2 marked L2/L3 sectors were generated; a typical example of an L2/L3 sector is shown in Fig. 4A where GL2 marking is restricted to the interior of the leaf blade and does not extend to the leaf margin. In all GL2 marked L2/L3 sectors examined, the phenotype of trichomes that developed from overlying epidermal cells (Fig. 4B) was identical to that of leaf trichomes in Athb-10/GL2 restored gl2 plants (cf. Figs. 2E and 2F with Fig. 4B). As predicted, trichomes in the L1 cell layer overlying L2/L3 sectors did not stain for GUS activity (Fig. 4B).

**DISCUSSION**

*Arabidopsis* leaf trichomes are formed from leaf epidermal cells by a process of directed morphogenesis. Mature leaf trichomes have a characteristic architecture, typically a single-stalked structure that, depending on ecotype, branches two, three, or four times at its apex (Marks and Esch, 1994) and is surrounded at its basal end by a “nest” of approximately eight accessory cells. The development and the regularity of spacing of trichomes on the *Arabidopsis* leaf is well documented (Hülskamp et al., 1994; Marks and Esch, 1994; Hülskamp and Schnittger, 1998; Szymanski et al., 1998, Schnittger et al., 1999) and involves more than 20 genes, many of which have multiple alleles (Hülskamp et al., 1994). The mutant phenotype of various of these genes reveals that trichomes develop by a step-wise series of progressions that begins with trichome initiation and endoreplication immediately followed by local outgrowth of corresponding epidermal cells and concludes with incrustation, just prior to trichome maturation (Hülskamp et al., 1994). Leaf trichome development can therefore be described in terms of phenotype as revealed by analysis of mutants and by the expression of the corresponding early and late acting genes. One of the earliest acting genes known to influence the development of *Arabidopsis* leaf trichomes is GL2. As already noted, the gl2 null mutant phenotype suggests that the GL2 protein is involved in directing the local outgrowth of trichomes (Hülskamp et al., 1994; Rerie et al., 1994). Given that trichomes are formed by specification of epidermal cells, it is intuitive to suggest that the GL2 gene product is required in the L1 cell layer for GL2 directed outgrowth of trichomes. Indeed, antibodies raised against GL2 protein detect GL2 protein in the L1 leaf cell layer, but notably the GL2 protein is also present in the L2 and L3 leaf cell layers (Symanski et al., 1998). In planta GL2 promoter-GUS fusion studies (Symanski et al., 1998) also indicate that although the spectrum of GL2 promoter activity may change during the time course of trichome development, the GL2 promoter is active in all three leaf cell layers. What these observations do not resolve, however, is the issue of whether there is a strict requirement for GL2 gene expression in any particular leaf cell layer(s) for the local outgrowth of trichomes; to address this question it is necessary to establish if the GL2 gene product acts in a region-autonomous manner.

To investigate GL2 autonomy and cell layer specificity, we have used a site-specific FLP recombinase-based method, analogous to the “FLP-out” approach pioneered in *Drosophila* (Struhl and Basler, 1993; Basler and Struhl, 1994), to generate mosaic *Arabidopsis* plants that contain GUS-marked gl2 mutant cell lineages. It is noted that the precedent for this approach *in planta* was originally established using Cre recombinase in tobacco (Oddell et al., 1990; Bayley et al., 1992) and in *Arabidopsis* using FLP recombinase (Kelby et al., 1995, 2000). Recently, the first use of a recombinase for the mosaic analysis of gene function in plants has been reported, wherein Cre recombinase was used to generate *Arabidopsis* plants mosaic for AGAMOUS (*AG*) (Sieburth et al., 1998); in contrast to our approach of positive cell marking, the *AG* mosaic analysis conducted by Sieburth and coworkers in *AG* transgene-complemented *ag* mutant plants involved deactivation of the *AG* transgene with concomitant loss of a GUS marker gene.

To address the question of GL2 region autonomy in leaf trichome development we generated GUS-marked gl2 mutant cell lineages (in Athb-10/GL2 restored gl2 plants) that were clonal in all three leaf cell layers (Fig. 3A); the trichome phenotype associated with GUS marked gl2 mutant cell lineages was identical to that of the gl2 null mutant (Fig. 2B). In contrast, the phenotype of trichomes in immediately adjacent unmarked leaf tissues (Fig. 3C) was identical to that of leaf trichomes in Athb-10/GL2 restored gl2 plants (cf. Fig. 3C with Figs. 2E and 2F). Importantly, the boundary between gl2 mutant and Athb-10/GL2 restored trichome sectors corresponded exactly with GUS marking, indicating that the Athb-10/GL2 gene product acts in a region-autonomous manner.

The influence of cell layer specificity of Athb-10/GL2 gene expression on Athb-10/GL2-directed local outgrowth of trichomes from cells in the L1 leaf cell layer, was assessed by generating GUS marked gl2 mutant sectors that were clonal in the L2 and/or L3 leaf cell layers alone. The phenotype of trichomes that developed from epidermal cells in the L1 cell layer overlying L2/L3 GUS marked sectors (Fig. 4B) was identical to that of leaf trichomes in Athb-10/GL2 restored gl2 plants (cf. Fig. 4B with Figs. 2E and 2F) but in contrast to cells in the underlying L2 and L3 cell layers, trichomes did not stain for GUS activity. Given that the Athb-10/GL2 gene product is region autonomous, and that the Athb-10/GL2 transgene is absent in the L2 and/or L3 leaf cell layers in L2/L3 sectors as indicated by GUS activity, these data provide strong evidence that Athb-10/GL2 gene expression is only required in the L1 leaf cell layer to direct the local outgrowth of trichomes. This suggests that al-
though the GL2 protein is present in all three leaf cell layers (Symanski et al., 1998) the essential requirement for localised trichome outgrowth is limited to the L1 cell layer. Because the construct that we used did not fully restore wild-type trichomes, presumably due to the limited promoter sequence included, we cannot exclude the possibility that GL2 expression in the L2 or L3 might be needed to attain a fully normal trichome morphology. We predict that the strategy described will be useful for the mosaic analysis of the other pathway components acting in trichome development. Once the region autonomy and cell layer specificity for each gene product has been determined, then the temporal importance of gene expression can be assessed either by controlled induc tion of mosaicism at defined stages in development or by adapting the strategy described here to incorporate the “real-time” cell marker, green fluorescent protein.

METHODS

Cloning

The FLP activatable Athb-10/GL2 gene excision cassette pMJJ19 (Fig. 1A) was constructed by cloning the Arabidopsis Columbia ecotype Athb-10/GL2 gene (kindly provided by Giorgio Morelli as an 8.3 Kb SalI/BglIII subclone with an additional 1 kb SalI/SalI subclone corresponding to the GL2 promoter region) between two directly oriented FRT sites configured as part of a FLP activatable β-glucuronidase gene switch, resident on the T-DNA of the binary vector pNJK14 (Kilby et al., 1995). The Athb-10/GL2 excision control construct pMJJ20 (Fig. 1B) was cloned by passage of pMJJ19 in the FLP expressing Escherichia coli strain BL-FLP (Snaith et al., 1996). Full details of the constructions can be obtained from the corresponding author.

Plant Transformation

Homozygous gl2-1 mutant Arabidopsis thaliana Landsberg erecta plants were transformed with pMJJ19 and pMJJ20 (previously mobilised into Agrobacterium tumefaciens LBA4404 by triparental mating) as described (Kilby et al., 1995). Transformants were selected using 50 ppm kanamycin sulphate.

Generation of a Heat-Shock-Inducible, FLP-Expressing gl2 Mutant Line

A heat-shock-inducible, FLP-expressing gl2 mutant line was generated by crossing homozygous gl2-1 plants with the heat-shock-inducible, FLP recombinase-expressing line HFC* (Kilby et al., 2000); HFC* is homozygous for a single insert, heat-shock-inducible source of FLP recombinase in an Arabidopsis thaliana Landsberg erecta background. Progeny from the cross were allowed to self, and F2 plants that were resistant to kanamycin and that also displayed the gl2 mutant phenotype were identified. F3 seed from these gl2 mutant transformants was screened for kanamycin resistance and lines that were 100% resistant to kanamycin were thereby identified as being homozygous for the HFC* insert.

Generation of GUS Marked gl2 Mosaic Sectors, GUS Staining and Photography

Crosses between gl2 mutant plants that were homozygous for a single insert, heat-shock-inducible source of FLP recombinase (HFC*) and Athb-10/GL2 restored gl2 lines (lines 19g-3 and 19g-79; see results) homozygous for the FLP activatable Athb-10/GL2 gene cassette, pMJJ19 (Fig. 1A) were made. Seed from crosses was collected, stratified (4°C for 5 days), and then acclimated to room temperature prior to heat-shock at 42°C for 4 h, as described (Kilby et al., 2000). Heat-shocked seed maintained in vitro was incubated at 20°C for 10 days prior to GUS staining and photography, as described (Kilby et al., 2000).

ACKNOWLEDGMENTS

The authors are grateful to Liam Dolan for providing seed of gl2-1 and to Giorgio Morelli for providing Athb-10/GL2.

LITERATURE CITED


