Understanding the function of the Arabidopsis *GLABRA 2* gene in Trichome patterning, morphogenesis and differentiation

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To

Amma, Appa and Prashanth.

“Reality is merely an illusion, albeit a very persistent one.”

Albert Einstein

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Zusammenfassung

Die Blatthaare von *Arabidopsis thaliana* (Trichome) sind die mit am besten geeigneten Zelltypen zur funktionellen Untersuchung von Musterbildungsprozessen und Zelldifferenzierungsvorgängen. In dieser Arbeit wurde die Funktion des *GLABRA2* Gens in diesen beiden Prozessen näher untersucht. Die Befunde können wie folgt zusammengefasst werden:

- es konnte mit Hilfe von Trichom-Markerlinien gezeigt werden, dass in *glabra2* Mutanten während der Blattinitiation mehr Epidermiszellen als in Wildtyp ein Trichomschicksal wählen. Während der Blattentwicklung scheinen viele dieser Zellen wieder zu de-differenzieren, da in älteren Blättern deutlich weniger Trichome als in Wildtyp gefunden werden.

- *GLABRA2* hat eine Funktion in der Trichom-Differenzierung und Morphogenese. Viele Trichome beginnen sich wie in Wildtyp zu entwickeln, stoppen dann jedoch ihre Entwicklung und nehmen dann ein Epidermiszellschicksal an. Trichome, die sich weiter entwickeln, zeigen oft eine reduzierte Verzweigungsanzahl.

- *GLABRA2* und das Musterbildungsgen *TRIPTYCHON* regulieren sich gegenseitig in einer positiven Rückkopplungsschleife.

- Mit Hilfe von Trichom-Markerlinien konnte gezeigt werden, dass einzelne Trichome aus einer Gruppe von kompetenten Zellen ausgewählt werden und das dabei die Entscheidung, ob die Zelle weiter mitotische Zyklen durchläuft oder mit Endoreduplikation beginnt, wichtig ist.

- Experimente bei denen *TRIPTYCHON* und *CAPRICE*, zwei Musterbildungsgene, die an der lateralen Inhibition beteiligt sind, als GFP-Fusionen transient exprimiert wurden, zeigen, dass beide Proteine in benachbarte Zellen wandern können.

- Es wurde eine neue Methode entwickelt (MEPI) die es ermöglicht, die Expression mehrerer Gene simultan *in vivo* zu untersuchen. Zusätzlich zu GFP-Varianten mit verschiedenen Farbspektren werden bei dieser Methode GFP-Fusionen verwendet, die spezifische Kompartimente der Zelle markieren.

Summary of the Thesis

Arabidopsis trichomes (leaf hairs) are one of the best studied plant model cell types with respect to understanding the molecular mechanisms underlying the process of cell patterning and differentiation. Many mutants which exhibit altered trichome patterning and differentiation have been analyzed and insights into the molecular nature of interactions among the genes involved have been obtained. The main focus of this thesis has been to understand the role of the *GLABRA 2* (*GL2*) gene in trichome cell patterning, morphogenesis and differentiation. The main findings can be summarized as follows:

i. In the absence of GL2 function more protodermal cells than wild-type get specified as trichome precursors but most of them exit the trichome differentiation pathway at different stages of their development resulting in lesser number of trichomes on mature *gl2* mutant leaves. The role of GL2 in patterning was established by analyzing various gl2 double mutants as well as by ubiquitous expression of GL2 which showed that it is required both for trichome specification and differentiation. The development and fate of trichomes on *gl2* leaves was carefully analyzed and it is concluded that in the absence of GL2 function trichomes lose their identity and likely adopt the default epidermal differentiation pathway of pavement cells. GL2 was also found to regulate trichome branching positively. A positive feedback loop between TRIPTYCHON (TRY) and GL2 was discovered and is hypothesized to be important in the final steps of trichome pattern resolution. In a nutshell, GL2 was found to be involved in trichome patterning, branching and differentiation. All the results have been incorporated in a model discussed at the end of chapter 2.

ii. Analysis of an early trichome marker in young wild-type leaves showed that trichome patterning is a de novo process, meaning only a few cells get selected to become trichomes from a pool of apparently equivalent cells. Based on the above analysis it is hypothesized that in addition to the expression of specific transcription factors the final resolution of the trichome pattern is likely an outcome of competition between endoreplication and mitosis modes of cell cycle.

iii. Using transient assays it was found that TRY and CPC gene products exhibit ability for intercellular movement, an essential property for them to act as inhibitors during patterning.

iv. A novel method to simultaneously analyze multiple gene expression patterns in vivo (MEPI) has been proposed which is based on targeting many fluorescent reporter genes

(GFP variants) to distinct intercellular structures / organelles in the same specimen. A proof-of-concept has been demonstrated by simultaneously analyzing three different reporter genes in Arabidopsis epidermal cells using a transient assay.

1 Introduction

Arabidopsis trichomes as a model system to understand cell patterning and differentiation

Plants, like other multicellular eukaryotes, develop from a single celled zygote that ultimately gives rise to the many specialized cell types of the adult organism. Cell patterning is when cells are guided to their appropriate differentiated fate at the correct time and place in the developing organism. Understanding the mechanisms underlying cell patterning, cell fate specification and cell differentiation has long been the goal of developmental biology. The plant Arabidopsis thaliana has been successfully used as a model system to address such questions in plant biology.

1.1 The Plant epidermis

The plant epidermis is the outermost cell layer of a plant. It contains many specialized cell types which function primarily in protecting the plant from various external threats. The presence of a layer of waxy cuticle and different cell types serve in defending the plant against various pathogens and herbivores, dehydration, UV damage and other factors. The different specialized cell types found on the epidermis include the trichomes, stomatal guard cells, root hair cells, various secretory glands and nectaries and epidermal pavement cells among others.

The plant epidermis is an excellent tissue for studying cell patterning. The epidermis of the root, hypocotyls and the leaf consist of only a few cell types and is easily accessible for observation. The root and the hypocotyl consist of only two cell types: the root hair and the non-root hair cells in the root epidermis and the stomatal cells and the non stomatal cells on the hypocotyl epidermis. The cells are arranged in files of alternating types in both the tissues and are thought to arise due to a position dependent mechanism. However, the epidermis on the adaxial surface of the leaf consists of three different cell types. The trichomes, which are large branched hair like cells, stomatal guard cells, which help in gas exchange of the plant with its surrounding atmosphere and the epidermal pavement cells which are mostly jigsaw puzzle shaped cells covering the entire leaf.

1.2 Trichomes: An excellent model cell type to study cell patterning, cell fate specification, differentiation and cell cycle

Trichomes, also called as plant ‘leaf hairs’, are present on the aerial surfaces of most plants, ranging from ferns to angiosperms. Trichomes come in various shapes and forms, from single celled to multicellular, and include both glandular secretory hairs

and nonglandular hairs. They have been thought to function in providing the plants with resistance to insect herbivores, reducing water loss by excess transpiration, increasing freeze tolerance and protecting plants from UV light. One of the most thoroughly studied plant cell differentiation pathways is the development of Arabidopsis trichomes. Trichomes on Arabidopsis are large single polyploidy cells that protrude from the epidermis of aerial organs that include rosette leaves, cauline leaves, sepals and stems. They are surrounded by a ring of 8-10 specialized accessory cells, also called as socket cells (Fig.1; page 16) visible at very low magnifications and are accessible for manipulations. Under laboratory conditions they are completely dispensable to the plants and thus numerous mutants affected in various steps of trichome fate specification, morphogenesis and differentiation are available for genetic analysis which makes them an unparalleled model cell type for scientific investigation in the above areas.

1.3 Development of trichomes and their spatial distribution pattern

Trichome development in wild-type (wt) Arabidopsis begins near the distal tips of leaves when they are approximately 100 μm long, and proceeds basipetally (Larkin et al., 1996). Trichomes are found adjacent to one another much less frequently than would be expected by chance, suggesting that an active mechanism exists to govern trichome spacing (Hulskamp *et al*., 1994).

Trichomes are the first differentiated cell type formed on the developing leaf epidermis. Trichome progenitor cells are specified in young leaf primordia in a field of morphologically similar, undifferentiated dividing epidermal cells. The first steps of trichome differentiation start with an enlargement of the nucleus and an increase in cell size. The growing trichome cell extends out of the leaf surface, elongates and eventually initiates two branching events. Secondary branching occurs in a plane perpendicular to the primary branch plane and is followed by elaboration of the secondary cell wall thickening which eventually results in a mature trichome decorated with cell surface papillae. The single nucleus of a wild-type trichome continues to replicate its genomic DNA during differentiation, reaching an average nuclear DNA levels of 20C – 32C (Hulskamp *et al*., 1994; Melaragno *et al*., 1993), a process known as endoreplication or endoreduplication. Endoreplication is a common variant of cell cycle where mitosis and cytokinesis are suppressed, but cycles of DNA replication

continue. Trichome patterning and differentiation continue in the growth zone at the base of the leaf during further leaf expansion.

1.4 Genetic dissection of the various steps of trichome development

A powerful tool to address various questions regarding the regulatory mechanisms underlying the different steps of trichome development is genetic analysis using various mutants defective in each of those steps. More than 36 genes have been identified so far in several mutagenesis screens that affect different aspects of trichome development. The genes can be grouped into categories which affect a particular step of trichome development (Hulskamp *et al*., 1999). The trichome initiation step includes the genes *GL1*, *TTG1*, *GLl3*, *EGL3*, *TRY*, *CPC*, *ETC1* and *ETC2* which when mutated show either increased or decreased trichome initiations compared to wt. The other categories include local outgrowth (*GL2*), extension growth (*CRK, DIS1*, *DIS2*, *GRL*, *KLK*, *SPI* and *WRM*), endoreplication and primary branching (*GL3*, *STI*), secondary branching (*AN*) and maturation step (*UDT*, *TBR*, *CHA*, *CDO* and *RTS*).

1.5 Molecular dissection of Trichome patterning

The first step of trichome development is the selection of a single epidermal cell as a trichome initial, the step of trichome specification. Several mutants affecting this step have been identified and molecularly characterized (Hulskamp *et al*., 1994; Larkin *et al*., 2003). They are broadly classified as positive and negative regulators of trichome initiation. The mutation in the positive factors like *GLABRA1* (*GL1*), *TRANSPARENT TESTA GLABRA1* (TTG1) and *GLABRA3* (*GL3*) leads to either a reduction or complete absence of trichomes on leaves. Recently a close homolog of *GL3*, *THE ENHANCER OF GLABRA3* (*EGL3*), has been identified which may function redundantly with *GL3* during trichome patterning (Bernhardt *et al*., 2003). Whereas *gl1* and *ttg1* mutants display completely glabrous leaves, *gl3* mutants show a reduction in trichome number. But the *gl3 egl3* double mutant is completely glabrous supporting the redundancy idea. GL1 encodes an R2-R3 MYB transcription factor with two repeats of MYB DNA-binding domain. Mutation in *gl1* only specifically affects trichome initiations with the leaves being completely glabrous. TTG1 on the other hand encodes for a WD-40 protein which is thought to mediate protein-protein interactions and a mutation in this gene has pleiotropic effect showing reduced anthocyanin pigmentation, absence of seed

coat mucilage, and an increase in the number of root hairs (Walker *et al*., 1999) along with fully glabrous leaves. The third important gene in this process is *GL3*. *gl3* mutants, along with their reduced trichome number phenotype, also show smaller and less branched trichomes. Their nuclear DNA content is also reduced. GL3 encodes a basic helix-loop-helix (bHLH) protein closely related to the maize R gene (Payne *et al*., 2000). Recently one report has suggested that *GLABRA2* (*GL2*) could also be involved in the step of trichome initiation by showing that an additional copy of the *GL2* gene expressed under its own promoter increased the number of trichome initiations as well as trichome clusters / nests (Ohashi *et al*., 2002). GL2 encodes a homeo domain transcription factor of the HD-Zip IV class (Rerie *et al*., 1994).

The *TRIPTYCHON* (*TRY*) and *CAPRICE* (*CPC*) genes encode for single repeat MYB protein with no apparent transcriptional activation domain. Mutations in *try* lead to formation of adjacent trichomes at a much higher frequency than in wt. It has been shown to act non-cell autonomously in inhibiting cells neighboring trichomes from acquiring trichome fate. *cpc* mutants on the other hand show an increased number of trichome initiations. That both TRY and CPC act redundantly in lateral inhibition was suggested by the phenotype of the double mutant (*try cpc*) where huge nests of trichomes containing sometimes upto 30 were observed (Wada *et al*., 2002; Schellmann *et al*., 2002). Recently two close homologs of TRY and CPC have been identified. *ENHANCER of TRIPTYCHON and CAPRICE 1* (*ETC1*) and *ETC2* also seem to act redundantly along with *TRY* and *CPC* during trichome patterning (Viktor Kirik, 2004. personal comm.).

Protein interaction studies using the yeast two hybrid method has shown that GL3 interacts with itself, GL1 and TTG1 by its different domains. Similarly EGL3 has also been shown to interact with GL1 and TTG1 suggesting that a homo or hetero-dimer of GL3/EGL3 along with GL1 and TTG1 bound to them acts as a complex during trichome patterning. TRY has been shown to compete with GL1 to bind to the same site on GL3 and thus form an alternative complex consisting of TRY, GL3/EGL3 and TTG1.

1.6 Current model of the mechanism of trichome patterning

The position of trichomes on leaves does not seem to correlate with the position of underlying cell types nor is a cell lineage dependent stereotyped cell division

mechanism involved in the generation of the their distribution pattern (Larkin *et al*., 1996; Schnittger *et al.,* 1999). It was therefore postulated to be generated *de novo*. According to this concept, trichome precursors are selected from initially equivalent cells by a competition mechanism and that incipient trichome cells inhibit their neighboring cells from acquiring the trichome fate. A mathematical model proposed by Gierer and Meinhardt (Meinhardt and Gierer 2000; Gierer and Meinhardt 1972) has been used to explain the generation of such a de novo pattern starting from a pool of initially equivalent cells. In brief, the model proposes that an 'Activator' positively regulates the production of an 'Inhibitor' which in turn represses the production of the activator (Fig.1a). The activator also leads to more production of itself because of a positive feedback loop. The inhibitor is proposed to have a higher diffusion rate than the activator. With these properties embedded in the system, one can start with a scenario where the concentration of both the activator and the inhibitor are more or less the same across a field of cells. Due to stochastic fluctuations the activator concentration may slightly increase over that of the inhibitor in one of the cells. This small change is sufficient to be amplified by the positive feedback loop of the activator combined with the ability of the inhibitor to diffuse faster to neighboring cells, finally resulting in this cell having a much higher concentration of the activator than any of its neighbors, resulting in its specification as a trichome (Fig.1b). Thus, a spacing pattern evolves from an initially equipotential field of cells over time.

Currently it is speculated that trichome patterning is in principle based on this model. The positive patterning genes GL1, TTG1 and GL3/EGL3 represent the 'active complex' which functions to activate the immediate downstream target gene *GL2* leading to trichome fate specification(Larkin *et al.,* 2003). They are assumed to locally activate their own expression and that of TRY and CPC. The inhibitors counteract their activity by a competition mechanism by forming the 'inactive complex' as described above and thus inhibiting trichome specification. Cell-cell interactions are likely to be mediated by the movement of TRY and CPC through the plasmodesmata (Fig.1c). This is supported by the finding that in the root system CPC can move from the cells in which it is expressed into neighbouring cells (Wada *et al.,* 2002). However it remains to be seen whether these two proteins move between cells in the context of trichome patterning. Many other aspects of the meinhardt model also need to be tested, though

the protein interaction studies have already given some insights into how these genes possibly interact during trichome patterning.

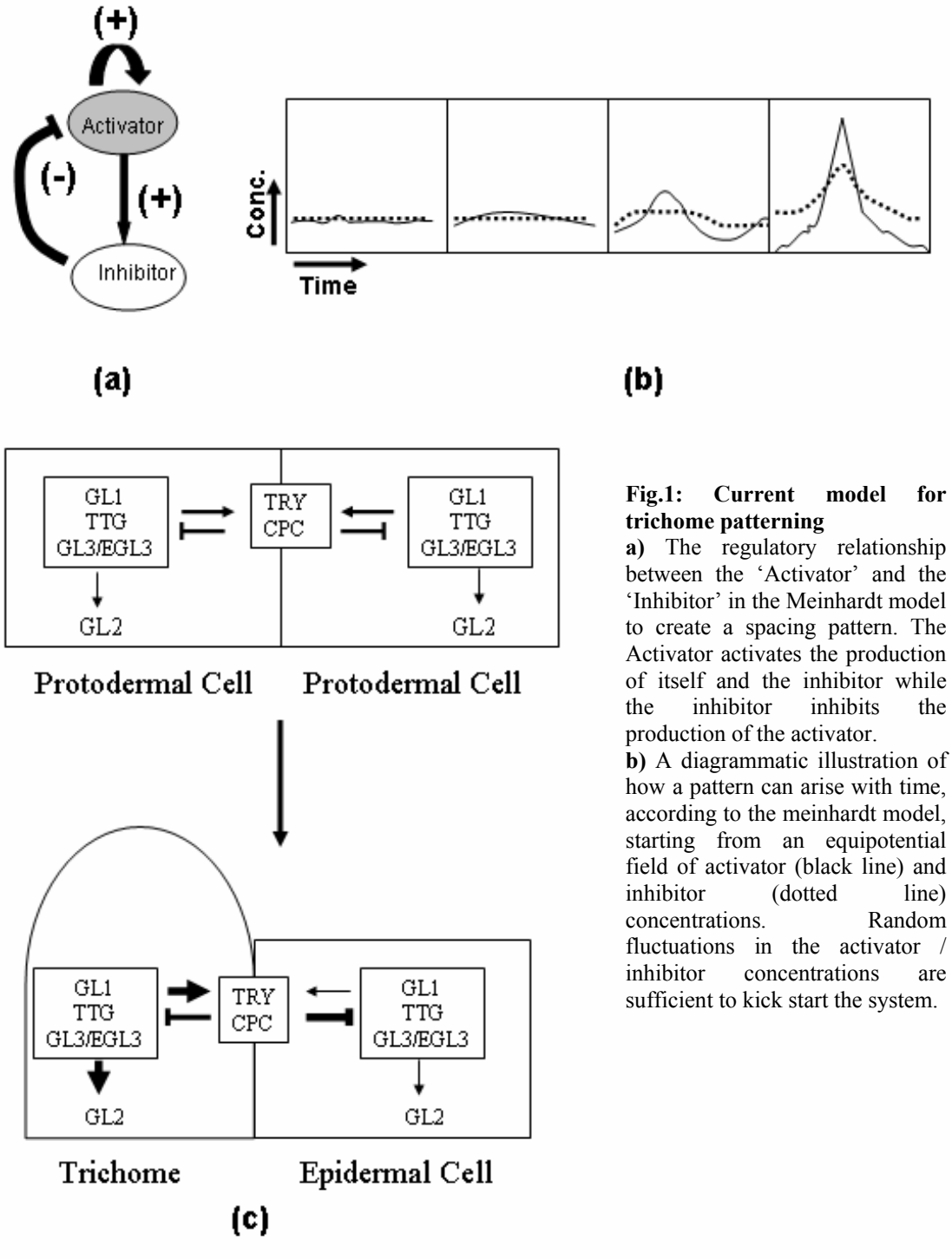


Fig.1: Current model for trichome patterning
a) The regulatory relationship between the 'Activator' and the 'Inhibitor' in the Meinhardt model to create a spacing pattern. The Activator activates the production of itself and the inhibitor while the inhibitor inhibits the production of the activator.
b) A diagrammatic illustration of how a pattern can arise with time, according to the meinhardt model, starting from an equipotential field of activator (black line) and inhibitor (dotted line) concentrations. Random fluctuations in the activator / inhibitor concentrations are sufficient to kick start the system.

c) The meinhardt model as applied to trichome patterning: Initially, all epidermal cells are equivalent (protodermal cells) expressing the activators GL1, GL3/EGL3 and TTG1 and begin to communicate with each other via TRY/CPC that are believed to move from cell to cell (top diagram). A bias in the balance of the activators concentration is postulated to increase the activity of the activators in one cell. The increased levels of the activator leads to trichome cell fate determination and causes increased levels of the inhibitor which in turn laterally suppress the neighbouring cells (bottom diagram). (Fig.1: modified from Srinivas BP and Hulskamp M, 2004)

2 The Arabidopsis *GLABRA 2* gene functions in trichome cell patterning, morphogenesis and differentiation

2.1 Summary

This chapter presents and discusses results of a detailed analysis on two aspects of trichome development: a) The role of the GLABRA 2 gene in trichome patterning and b) The development and fate of trichomes on *gl2* mutant leaves. A model incorporating all the results is presented at the end of the chapter. The main findings were:

a. Double mutant analysis of *gl2* with *cpc*, *try* and *gl3* revealed that GL2 positively regulates trichome initiation. GL2 was found to positively regulate *TRY* expression. In the absence of GL2 function more cells seem to enter the trichome pathway on *gl2* leaves, likely due to ineffective lateral inhibition, but cannot proceed further in the pathway to initiate the morphogenetic program. Ubiquitous expression of GL2 in wild-type leaves strongly inhibits trichome initiation but has a mild effect in *try cpc* double mutant leaves suggesting that its inhibitory function could be mediated by TRY / CPC or their homologs. An unexpected positive feedback loop between *TRY* and *GL2* was discovered which may have interesting consequences during trichome patterning.

b. Cells which have entered the trichome differentiation pathway lose their way during development in the absence of GL2 function. They start developing as wt trichomes and express different trichome specific markers but they either abort or exit the differentiation program at different points and appear to enter the default epidermal differentiation pathway of pavement cells. They eventually end up showing many features of pavement cells and the results suggest that they also re-enter mitosis implying de-differentiation. GL2 was also found to positively regulate trichome branching.

2.2 Introduction

gl2 mutants show defects in the differentiation of various epidermal cell types in Arabidopsis(Rerie *et al.,* 1994; Masucci *et al.,* 1996). Wild-type (wt) Arabidopsis roots and hypocotyls have alternate files of root hair / non-root hair and stomatal / non-stomatal cells respectively. In gl2 mutants it has been documented that the non-root hair cells (also called atrichoblasts) develop as root hair cells and some of the non-stomatal cells in the hypocotyl differentiate as stomatal cells. Whereas wt seeds are covered with a coat of mucilage, it is absent around gl2 seeds. GL2 expression pattern in arabidospsis has been well studied. Its expression starts early during embryogenesis where its position dependent expression pattern is established and maintained throughout the remainder of embryogenesis (Costa and Dolan, 2003; Lin and Schiefelbein, 2001). In roots and hypocotyls GL2 is preferentially expressed in non root hair and non-stomatal cell files respectively in a position dependent manner. The cells expressing GL2 directly lie over one underlying cortical cell suggesting that a position dependent mechanism controls GL2 expression (Hung *et al.,* 1998). It has also been shown that CPC plays an important role in this mechanism by repressing GL2 expression in hair cell / stomatal cell files (Lee and Schiefelbein, 2002).

Wild-type (wt) leaves contain trichomes which grow out the leaf surface and show a typical 3 branched morphology. Loss-of-function *gl2* mutants produce trichomes that expand aberrantly along the plane of leaf surface (Rerie *et al.,* 1994) or in weaker alleles with reduced branching thereby resembling the combined phenotype of several other differentiation mutants. This phenotype suggests that *GL2* acts downstream of the patterning genes after trichome cell selection to activate those genes specifically required for trichome specific differentiation. The *GLABRA2 (GL2)* gene encodes a putative transcription factor of the Homeo-domain leucine zipper (HD-Zip-IV) family. It has been hypothesized that *GL2* is downstream target of the patterning genes as its expression is dependent on *GL1*, *TTG1* and *GL3* (Szymanski and Marks, 1998). In leaves *GL2* is found to be expressed in trichomes at all stages of its development including its earliest stage of specification (when it is morphological similar to the neighboring epidermal cells). Studies on Gl2 expression in young leaves showed that it is expressed both in trichomes as well as to a lower level in the cells surrounding it. But

as the trichome develops GL2 expression increases steadily in it and concomitantly ceases to be expressed in other epidermal cells.

The *gl2* trichome phenotype combined with promoter regulation studies was used to conclude that GL2 is the essential downstream target of the patterning genes and it initiates the differentiation process by activating other genes. The idea received support by the finding that in roots GL2 directly binds and regulates the activity of the phospholipase Dζ gene, which is possibly involved in signal transduction, thereby acting as an intermediary between the cell patterning and morphogenesis steps (Ohashi *et al.,* 2003). Only one report so far suggested that GL2 quantitatively regulates trichome initiation and spacing using two experiments to support the claim. An additional copy of the GL2 gene under its own promoter was expressed in wt background (entopic expression), which lead to increased trichome initiations as well as trichome clusters. Also, gl2 heterozygotes were reported to have reduced number of trichomes on leaves, 8.1 ± 2.1 as against in wt leaves which had 6.2 ± 1.7. This difference in trichome numbers between gl2 heterozygotes and wt, however, does not appear to be very significant (Ohashi *et al.,* 2002).

Hence it was aimed in this study to investigate whether GL2 is indeed involved in trichome patterning. If so, how does it participate in the process? With which other trichome patterning genes does it interact and how? Also, a thorough analysis of the mutant itself was carried out to understand its role in trichome differentiation, the process which was initially identified to be the main defect in the mutant.

2.3 Results

Understanding the function of GL2 in trichome patterning

2.3.1 Increased trichome cell specifications on *gl2* leaves

The first pair of *gl2* leaves look superficially glabrous but later leaves show unbranched spike trichomes towards the leaf margin. A closer look however shows that trichomes are present on the leaf blade but fail to expand out of the leaf surface like wt (Fig.1). Reports so far in literature indicate that *gl2* leaves do not show any apparent trichome patterning (initiation) defect but show only trichome morphogenesis /differentiation defects resulting in abnormally expanded trichome cells. However, lately a report by Ohashi et al, suggested that GL2 quantitatively regulates trichome initiation in a positive way ( ). As it is not easy to detect and score the mutant trichomes using normal light microscopy Ohashi et al used *gl2* heterozygotes and wt plant lines expressing an additional copy of the *GL2* gene (driven by its own promoter; called entopic expression) to study the effect of GL2 on trichome patterning and concluded that GL2 positively regulates trichome initiations.

As *gl2* mutants are defective in trichome differentiation it appeared to be a good idea to score for the number of trichome initiations using a trichome molecular marker, *GL2::GFP-ER*, rather than relying on trichome morphology. In wt plants this marker is expressed in trichomes at all developmental stages irrespective of cell morphology and size (including in trichomes even before they have enlarged or expanded out of the leaf surface) and has thus been considered as an early marker for trichome cell fate (Szymanski and Marks, 1998). It has also been shown that GFP accumulation in plants carrying this construct accurately reflects the transcription pattern of the *GL2* gene (Lin and Schiefelbein, 2001). In young leaf primordia the marker is strongly expressed in very early trichome initials and to a lesser extent in the neighboring cells. During further development the intensity of the marker progressively increases in the trichome cell and concomitantly ceases in neighboring cells (Szymanski DB and Marks MD, 1998).

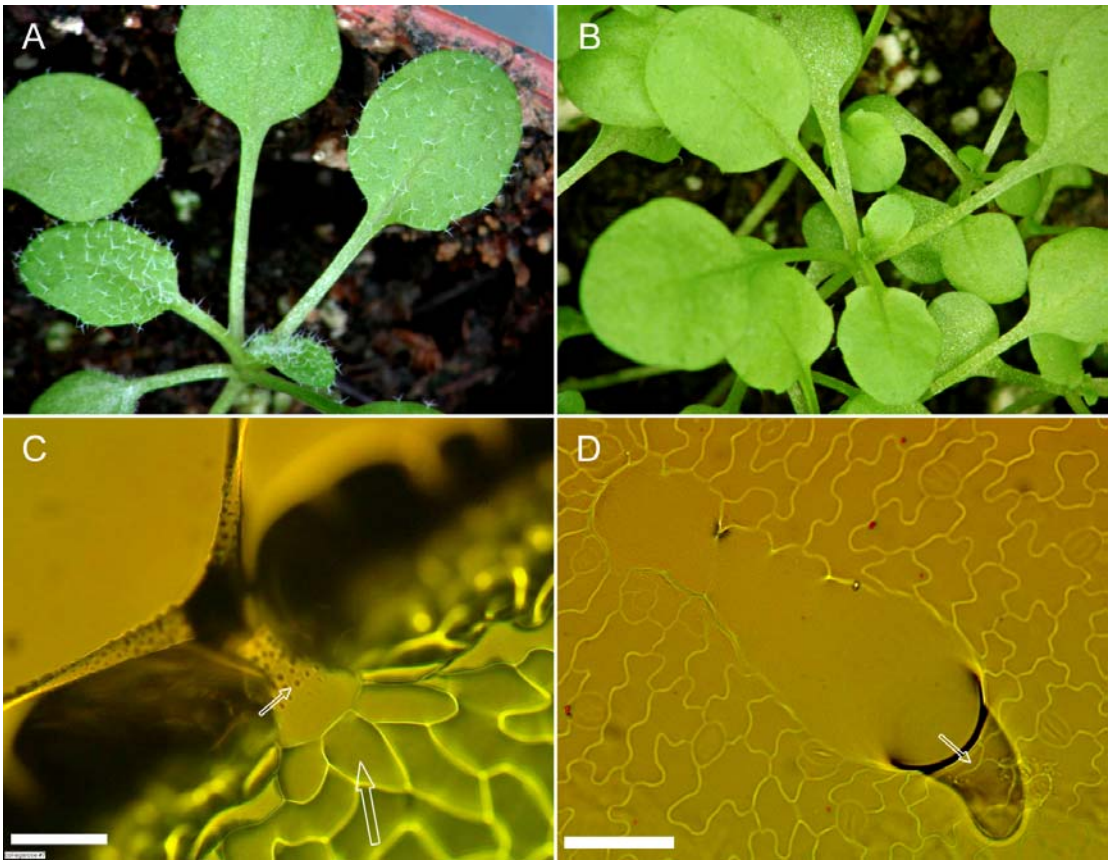


Figure 1: ***gl2* plants look superficially glabrous**.
View of a wt (A) and *gl2* mutant (B) plant. WT leaves show many trichomes distributed on their surface while gl2 leaves appear superficially glabrous. However, leaf epidermal imprints on agarose show that laterally expanded trichomes are present on *gl2* (D) with a small peak projecting out (arrow). Wild-type trichomes (C) grow out the leaf surface, normally have three extended branches, and at maturity show surface papillae (thin arrow). They are also surrounded by a ring of socket cells (thick arrow). Scale bar: C and D = 50 μm.

Both WT and *gl2* mutant plants carrying this reporter construct were analyzed and the number of trichome specifications on the first pair of young leaves (of an average length of 400 μm) was counted. In this analysis all cells that evidently exhibited a highly increased GFP fluorescence level relative to their neighboring cells were considered as trichomes (Fig.2). On *gl2* leaves trichome number was increased by about 30% compared to wt (Fig.2, Table.1). This result contradicted the expectations from published reports.

Therefore, to see the extent to which these cells, specified as trichomes at the marker level, proceed in the trichome developmental pathway, epidermal surface imprints of older leaves (first pair), of an average length of 1.5 mm, were made using agarose (see materials and methods section) and observed under a microscope. Young leaf primordia of the stage used in the above marker analysis experiment could not be used because of the limitations of this imprint technique. The number of morphologically identifiable trichomes was counted. Surprisingly, the number of trichomes was less in *gl2* when compared to wt-col leaves (Table.1). In summary the above two results suggest that in *gl2* mutants many cells get specified and enter the trichome pathway but only some among them proceed further in their development initiating cell morphogenesis steps. This implies that *GL2* positively functions to initiate trichome development but does it negatively regulate the first step of trichome cell selection?

2.3.2 Ubiquitous expression of GL2 inhibits trichome initiations

The mutant analysis described above suggests that *GL2* may act as a negative regulator during early trichome patterning, though its 'positive' function is needed after specification to push the cells entering the trichome pathway to completely undergo morphogenesis and differentiate as trichomes. One possibility to test this would be to ubiquitously express the *GL2* gene which should result in a glabrous phenotype similar as observed with *TRY* or *CPC*, both of which are inhibitors of trichome initiation. This has been attempted previously. Ubiquitous expression of *GL2* cDNA under the control of the *CaMV 35S* promoter has been reported to be toxic to plants leading to scarcely viable phenotypes (Ohashi *et al.,* 2002). In a wild-type background it was also observed that surviving plants show a *gl2*-like phenotype and it was concluded that ectopic GL2 expression interferes with endogenous GL2 function thereby effecting normal trichome cell morphogenesis. In order to avoid this toxic affect of ectopic GL2 expression during

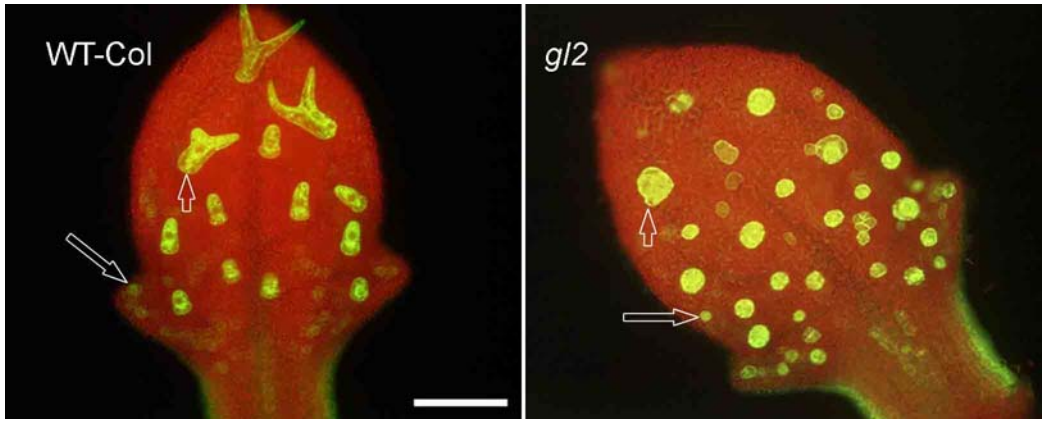


Figure 2: Number of trichome specifications / initiations in wt and *gl2*
First pair of leaves of WT-col (left) and *gl2* plants expressing an early trichome molecular marker (*GL2:GFP-ER*) were analyzed to count and compare the number of trichome cell specifications. Big arrows point to the earliest cell which has been specified as a trichome and small arrows point to developing larger trichomes. Scale bar = 100 µm

	Molecular marker criteria	**Morphological criteria**
WT-Col	22.28 ± 3.4 (n=38)	28 ± 4.6 (n=12)
gl2	28.94 ± 3.0 (n=36)	19.33 ± 3.8 (n=15)

Table 1: Number of trichome specifications / initiations counted using two different methods (trichome molecular marker and morphological criteria) on first pair of *gl2* and wt leaves. Young leaves of about 400 µm were used to count GFP marked trichome cells, whereas larger leaves of 1.5 mm were used for the other method (due to limitations of the imprint technique) and hence should not be directly compared.

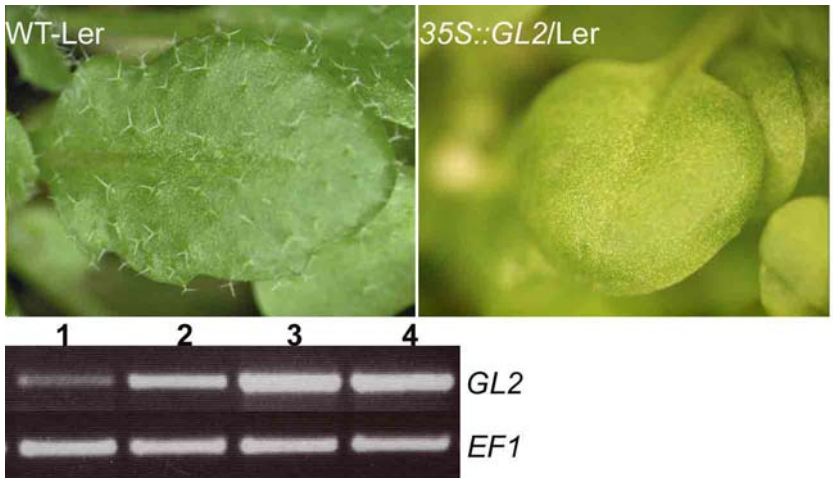


Figure 3: Ectopic over-expression of GL2 using the constitutively expressed *CaMV 35S* promoter in wild type (*Ler*) plants leads to inhibition of trichome initiation (above). Control wt plants after heat shock no trichome inhibition phenotype.
Bottom: RT-PCR analysis of the *GL2* gene. Wt-*ler* (1), *35S:GL2/ler* (2), *try cpc* (3) and *35S:GL2/try cpc* (4) (see text for explanation). *Elongation factor 1 (EF1)* is used as the internal control transcript. Note the increase in *GL2* transcript level in lane 2 when compared to lane 1.

seed germination a modified version of a published recombinase mediated transcriptional induction system (Hoff *et al.,* 2001) was used. The system is designed such that a heat shock induces a recombination event that generates an active *35S::GL2* arrangement on the chromosome (see materials and methods). After heat shock, trichome initiation was compared between wild-type plants and plants carrying the construct system. Wild-type plants showed no patterning defects. Heat shock treated plants containing the construct system showed extreme variability in the phenotype of different siblings of the same line or even between different leaves of the same plant. Although this variability prevents a statistical analysis, a qualitative description revealed surprising results. Six independent lines were analyzed; 3 lines showed inhibition in trichome initiation after heat shock treatments, with reduced number of trichomes per leaf compared to wt. Lines # 1 and 2 had strong trichome inhibition phenotype with line # 2 showing the most severe phenotype. Most plants were completely glabrous (Fig.3). Leaf epidermal imprints on agarose of these glabrous plants were analyzed to see if *gl2* like laterally expanded trichomes are found. The leaves were completely glabrous and had no resemblance to *gl2* phenotype where mutant laterally expanded trichomes can still be found. The inducible system showed leakiness, as reported in the original publication, such that also uninduced plants showed trichome inhibition phenotypes to varying degrees (data not shown). Genomic DNA PCR analysis of the lines which showed the trichome inhibition phenotype (both induced as well as uninduced) confirmed that the recombination event had occurred in these lines as expected. In addition, RT-PCR analysis of the lines showing the glabrous phenotype indicated that the level of *GL2* transcript was increased as compared to corresponding WT control plants (Fig.3). These results indicated that ubiquitous GL2 expression inhibits trichome initiation. The seeds of the two lines showing strong inhibition phenotype (line # 1,2) did not germinate in the next generation confirming published reports that ectopic over-expression of GL2 during embryogenesis is lethal.

2.3.3 Genetic interaction of *GL2* with *TRY*

a) *GL2* positively regulates *TRY* expression:

The finding that GL2 appears to suppress trichome initiations during trichome patterning raises the question of how GL2 function is linked to that of those factors (TRY and CPC) already known to have this function. Hence, it was sought to find if

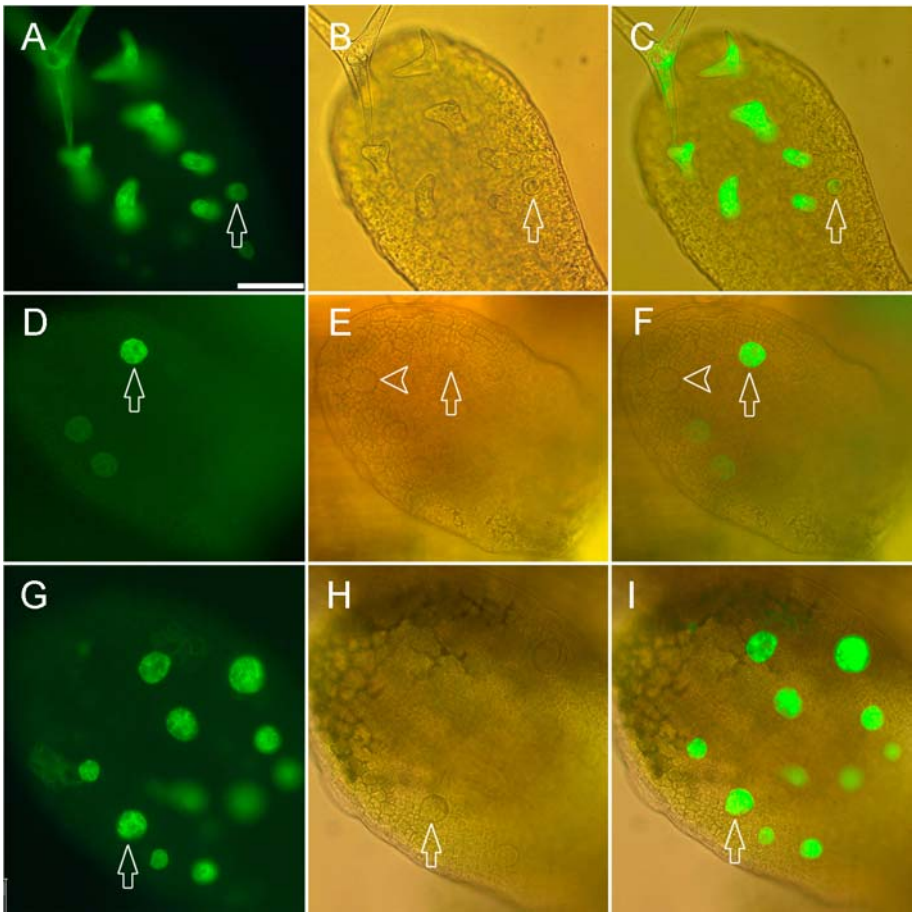


Figure 4: GL2 positively regulates *TRY* expression.
TRY gene expression pattern in very young leaves as revealed by the *TRY:GFP-ER* construct. A,D,G) Fluorescence of the GFP marker. B,E,H) DIC-light micrograph from the same leaves as A,D,G. C,F,I) Overlay of (A,B), (D,E) and (G,H) respectively. (A-C) *TRY* expression in wild type. *TRY* expression is seen as early as incipient trichomes can be recognized by morphological criteria (arrow). (C-E) *TRY* expression in *gl2* mutant leaf expressing *TRY* only in some (arrow) but not in other (arrow head) trichome initials. (c) *AtMYB23* expression in a *gl2* mutant leaf. Note this gene is expressed in all trichome initials. Scale bar: 50 µm.

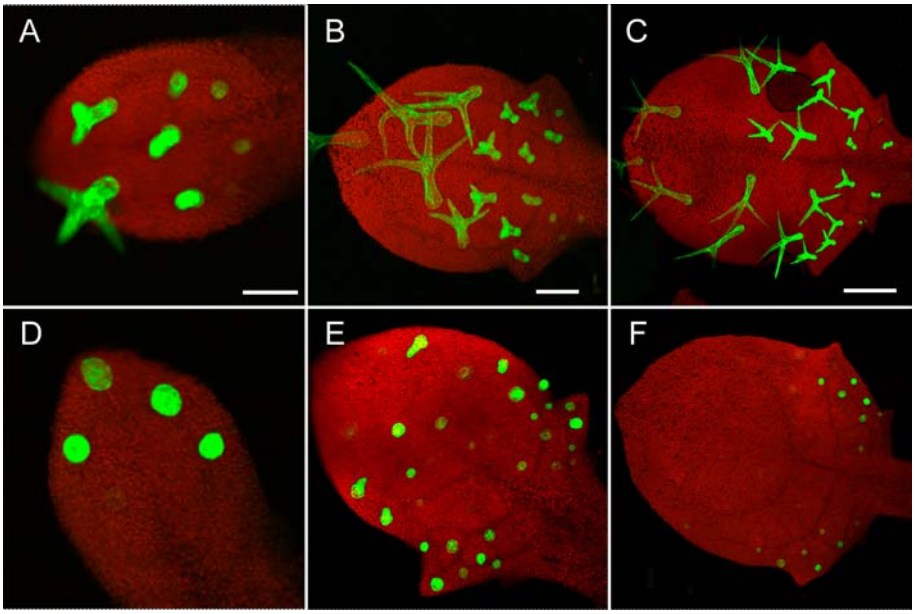


Figure 5: Temporal expression changes of *TRY* in *gl2* mutants and wild type
TRY gene expression pattern in very young leaves as revealed by the *TRY:GFP-ER* construct. The expression is compared at three different stages of leaf development. A-C) Wild type leaves. D-F) *gl2* mutant leaves. A,D) *TRY* expression in the youngest leaf stage. B,E) *TRY* expression in an intermediate leaf stage. C,F) *TRY* expression in a mature leaf. Note that mature trichome cells at the apex of a fully expanded wild type leaf still express the *TRY* gene while at the same stage there is no detectable *TRY* expression in *gl2* leaves. Scale bars: A,D = 40µm; B,E = 80µm; C,F = 200µm

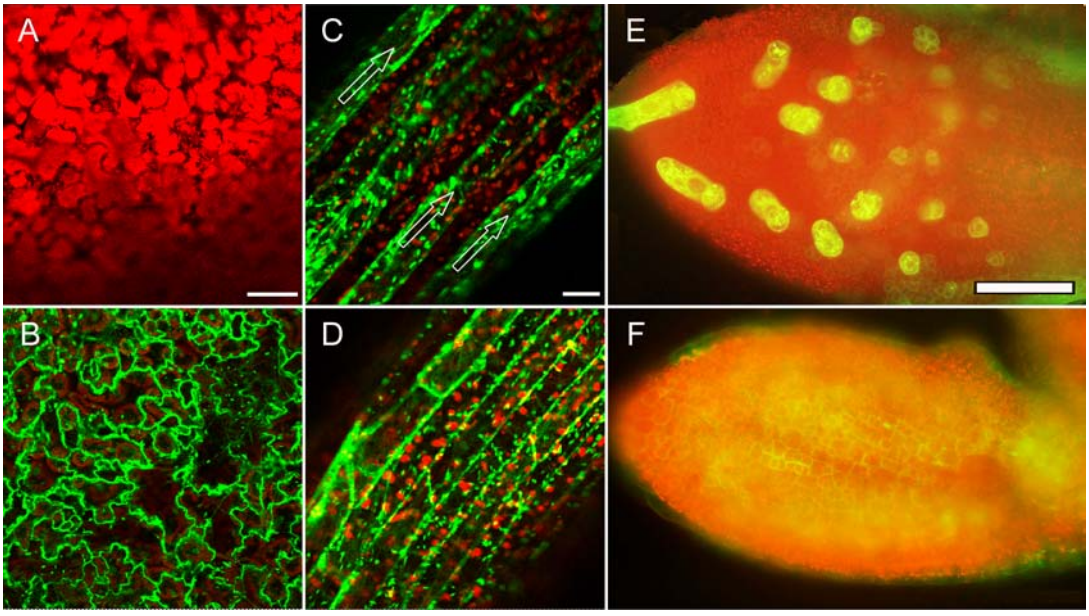


Figure 6: *TRY* is a positive regulator of *GL2*.
GL2:GFP-ER reporter construct expression pattern in wild-type and *35S:TRY* plants. A,C,E: wt plants. B,D,E: *35S:TRY* plants. A) Wild type cotyledon. No expression of *GL2* is seen. B) *35S:TRY* cotyledon. Strong expression of *GL2* is found in all epidermal cells. C) Epidermal cells of the wild-type hypocotyl. Cells of alternating files express *GL2* (arrows). D) Epidermal cells of the *35S:TRY* hypocotyl. Note, all cell files express *GL2*. E) wt young leaf. *GL2* expression limited to trichomes and some epidermal cells. F) *35S:TRY* young leaf primordium. Trichomes are absent but most epidermal cells express *GL2*. Scale bars: A,B = 80 µm; C,D = 40 µm; E,F = 50 µm

any regulatory interaction exists between *GL2* and *TRY*. The *TRY* gene has been shown to act as a negative regulator of trichome development (Schnittger *et al.,* 1999) and is thought to be important in mediating lateral inhibition during trichome patterning (Schellmann *et al.,* 2002). The latter is suggested by the finding that *try* mutants exhibitnests of 2 or 3 trichomes instead of single separate trichomes as in wild type. *TRY* expression was studied in *gl2* mutant plants at different stages of leaf development using the *TRY::GFP* marker line. It has been shown before, that the promoter used in this construct corresponds to the expression pattern observed in *in situ* hybridizations and is sufficient for rescuing the *try* mutant phenotype (Schellmann *et al.,* 2002). In very young wild-type leaves *TRY* is expressed consistently at high levels in all morphologically distinct and recognizable trichome initials (Fig.4 A,B,C). By contrast, in *gl2* mutants *TRY* expression is very variable. Frequently young trichome cells with a typical wt morphology and which have clearly expanded out of the leaf surface show much reduced expression levels or do not express any detectable *TRY* at all (Fig. 4 D,E,F). Two scenarios can explain these findings. Due to the differentiation defects in some *gl2* trichomes the expression of all trichome specific genes could be generally reduced or alternatively, the observed regulation of *TRY* reflects a specific regulation of *TRY* by *GL2*. To distinguish between these two possibilities, we analyzed the expression of another trichome specific gene, AtMYB23 (Kirik *et al.,* 2001). AtMYB23 is expressed in all morphologically distinct trichome initials both in WT as well as in *gl2* leaves (Fig.4G,H,I).

The variability of *TRY* expression intensity suggests that the temporal expression of *TRY* might be generally effected. We therefore compared its temporal expression pattern in *gl2* and wild type leaves. A comparison of different leaf stages revealed that *TRY* expression is almost absent in more mature leaves of *gl2* when wild type plants still show high expression levels (Fig.5). This indicates that GL2 is required not only to initiate *TRY* expression in all trichome initials but also to maintain it.

b) *GL2* is ectopically activated by ubiquitous expression of TRY but not CPC

To test if *GL2* expression is regulated by *TRY*, the *GL2::GFP-ER* marker was analyzed in a *35S::TRY* background. At least 10 independent transgenic lines which were analyzed showed the same result. We focused on the analysis of cells in which *GL2* is normally not expressed such as the epidermis of the cotyledons (Fig.6A) and the cell

files of the hypocotyls not overlying a cleft of the underlying cells (Fig.6C) ( ). All epidermal cells of the cotyledons (Fig.6B) and all hypocotyl cell files (Fig.6D) exhibited high levels of *GL2* in a *35S::TRY* background. In wt, *GL2* expression is mostly restricted to trichomes and some epidermal cells at the basal portion of the leaf (Fig.6E). In *35S::TRY* plants *GL2* was found to be ectopically expressed in most epidermal leaf cells of young leaf primordia (Fig. 6F), though its expression was relatively very weak as leaves developed, with the exception of cells at the margin and towards the apex where high levels of *GL2* expression was still seen (data not shown). Thus, ectopic expression of TRY causes ectopic expression of *GL2* indicating that TRY positively regulates GL2 expression. The findings suggest the existence of a positive feedback loop of TRY and GL2. It remains, however, to be determined whether this regulatory relationship is relevant in the context of trichome patterning and if it is dependent on developmental stages. An interesting exception was observed in the roots where only cells in the inner most tissue and not in the epidermis or cortex showed ectopic *GL2* expression (data not shown).

As TRY and CPC are highly homologous it has been postulated that they may act partially redundantly during epidermal cell patterning in Arabidopsis. It has been previously shown that *35S::CPC* inhibits *GL2* expression in roots (Lee and Schiefelbein, 2002). *GL2* expression was checked in a *35S::CPC* line (which has been previously published and was a kind gift from Takuji Wada, Japan) (Wada *et al.,* 2002). No expression of *GL2* was detected in the hypocotyls/roots/leaves or cotyledons of these plants (data not shown) confirming that CPC represses GL2 expression and suggesting a functional difference between TRY and CPC with respect to the regulation of *GL2*.

2.3.4 Lateral inhibition during trichome patterning appears to be compromised in *gl2*

It has been proposed that lateral inhibition is an integral component of the mechanism resulting in the trichome spacing pattern. TRY and CPC are implicated to function in this pathway by inhibiting the cells around a trichome from acquiring a trichome fate. The finding that *GL2* could be involved in early trichome patterning combined with the fact that *TRY* expression is controlled by *GL2* lead us to study patterning during early stages of trichome development as recognized with the trichome molecular marker,

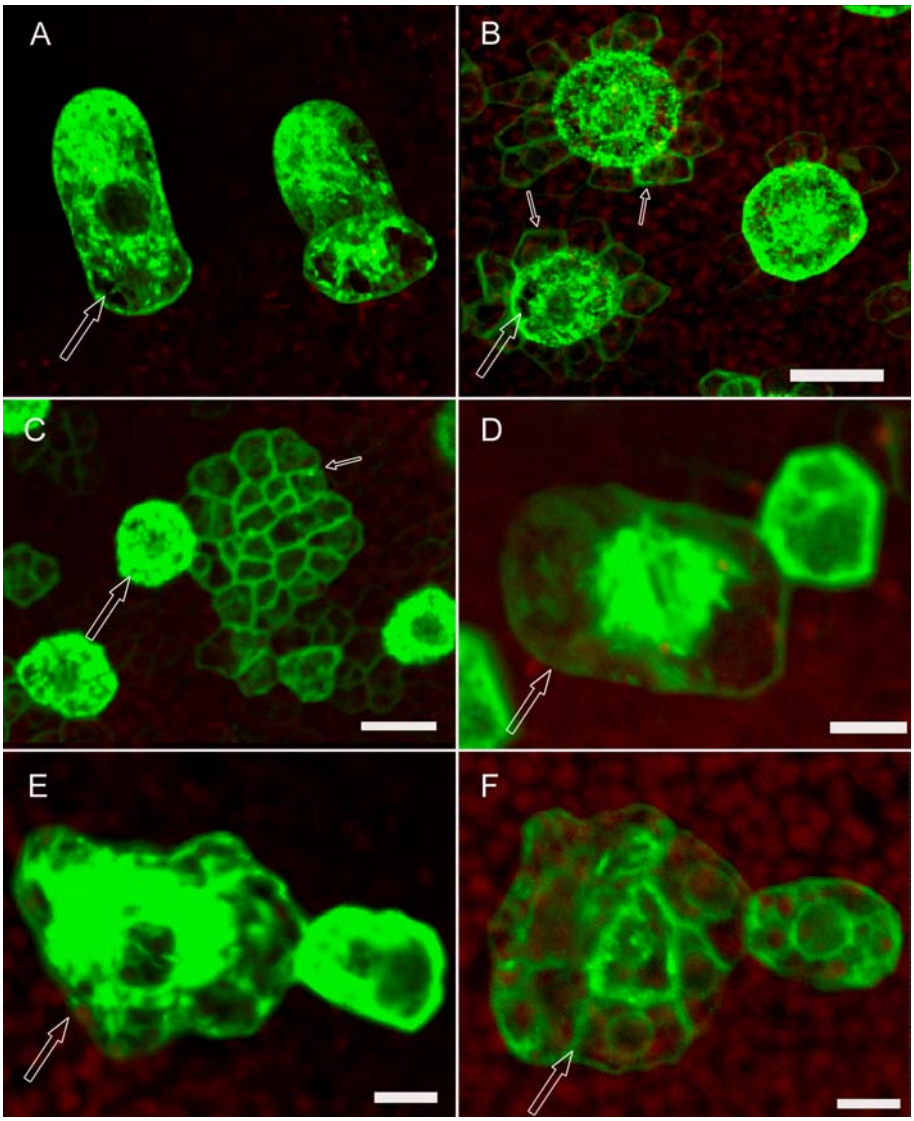


Figure 7: Ineffective lateral inhibition on *gl2* leaves
Expression of an early trichome marker (*pGL2:GFP-ER*) in wt and *gl2* leaves. Epidermal cells surrounding wt (A) trichomes (arrow) express the trichome marker at a very low level or do not express at all suggesting effective lateral inhibition from the trichome. However, in *gl2* (B) epidermal cells (thin arrows) surrounding some trichomes (thick arrow) still express the trichome marker relatively at higher levels implying compromise in lateral inhibition (The microscopic settings used were the same for wt and *gl2* leaves). Also, occasionally large groups of cells expressing the marker are found in the immediate neighborhood of trichome in *gl2* (C). Towards the base of the *gl2* leaves trichome clusters resembling *try* mutants are seen (D, E). However unlike try clusters, the *gl2* clusters always have one large dominant trichome (thick arrow) and 1-3 smaller cells surrounding it which start to express the trichome marker as strong as the dominant cell. At exactly the same positions on the leaves similar clusters are found in which the dominant cell is composed of many individual cells (F). Note that D,E and F are 3D reconstructions of many confocal sections (and same magnification) showing the top view of the dominant cell which is clearly bulged out of the leaf surface. In C the dominant cell is clearly one large cell. But in F it is composed of many individual cells with clear cell walls and nuclei. Scale bars: A,B = 20 µm; C = 16 µm; D,E,F = 8 µm.

GL2::GFP-ER. At stages in which epidermal cells around a wild-type trichome initial (Fig.7A) show either very little no marker expression anymore, in *gl2* mutants epidermal cells immediately surrounding some trichomes of similar stage were found expressing the marker at relatively high levels (Fig. 7B). Also, in the immediate neighborhood of some trichomes large groups of cells very strongly expressing the marker were found (Fig.7C).This suggests that weaker lateral inhibition in gl2 leaves could lead to such a phenotype. This ineffective inhibition of cells from getting specified as trichomes may consequently lead to more number of trichome specifications in *gl2* when compared to wt leaves. This is reminiscent of the *cpc* mutant phenotype where the number of trichome initiations is increased compared to wt.

The observation that expression of *TRY* in all trichome initials depends on *GL2* led us to speculate that small clusters of trichomes (2 - 3) as seen on *try* mutants could also be seen on young *gl2* leaves. Indeed, trichome clusters reminiscent of those observed in *try* mutants were observed in *gl2* mutants with an average frequency of around 5 - 10%. Typically, one to two adjacent cells next to a large trichome start to express *GL2* at high levels when compared to the other epidermal cells in the neighborhood where no marker expression is found anymore (Fig.7 D, E). Though this phenotype is reminiscent of the *try* phenotype, the difference lies in the fact that whereas in the *try* clusters the trichomes are equally large and developed, the *try*-like cluster seen on *gl2* has one dominant large trichome cell surrounded by one – two much smaller cells. At about the same frequency and position on the leaves of similar age try-like clusters of the same size as mentioned before were found but the difference was that the dominant cell was not one single cell any longer but composed of many individual cells (Fig.7F) with clear cell walls and large nuclei (By DAPI staining it was found that they are large nuclei and not vacuoles). 3D reconstructions of many individual confocal sections of all these try-like trichome clusters showed that they are bulged out of the leaf surface considerably. Fig.7D and Fig.7F are two representative pictures of these two classes of try-like clusters. Whereas the dominant cell in Fig.7D is clearly one large cell, the one in Fig.7F is not. The above observations pose the obvious question of whether the trichomes which are seriously compromised in lateral inhibition result in try-like clusters (Fig.7D) and possibly divide later (Fig.7F). The size of the dominant cell, their frequency of occurrence and position on the leaves in both the cases all are suggestive (but not proofs) that it may be the case. It also needs to be investigated in more detail

to see if the large group of cells found in the neighborhood of trichomes as seen in Fig.7C are a later consequence of the event seen in Fig.7F or completely unrelated to it. Epidermal imprints of *gl2* leaves were analyzed carefully to detect any morphologically identifiable *try*-like clusters seen by the marker study. None were detected. This again points to the fact that in the absence of GL2 function, new trichome specifications seen at the marker level do not proceed further in the pathway by initiating morphogenesis processes. Is the ineffective lateral inhibition on *gl2* leaves an indirect effect related to the differentiation status of trichomes? Or is GL2 directly involved in the process of lateral inhibition during trichome patterning in wt?

2.3.5 TRY and / or CPC may possibly mediate the inhibitory function of *GL2* during patterning

The finding that *GL2* positively regulates *TRY* expression in trichomes and *gl2* mutants show defective lateral inhibition raises the possibility that *TRY* and/or *CPC* may be important to mediate the inhibitory effect of *GL2*. In order to test this, heat shock inducible *GL2* expression in the *try cpc* double mutant background was used. The *try cpc* double mutant shows large clusters of trichomes containing between 2-30 trichomes in each cluster (Fig.8G) (Schellmann *et al.,* 2002). If *TRY* and/or *CPC* would mediate the inhibitory effect of *GL2* one would expect that the *try cpc* double mutant would be insensitive to ubiquitous *GL2* expression.

The *try cpc* double mutant plants were transformed with the same heat inducible GL2 construct as earlier (see "ubiquitous *GL2* expression inhibits trichome initiation in wt leaves" paragraph) and 6 resulting transgenic lines were analyzed. After heat shock four lines showed new phenotypes. In all four lines ectopic trichomes on cotyledons and hypocotyls was increased compared to *try cpc* control plants after heat shock (Fig.8 C, F). Line # 6 showed a strong effect on reduction of trichome cluster size and trichome numbers on leaves when compared to *try cpc* control after heat shock. Most leaves on line #6 plants had single isolated trichomes in the middle of the leaf and small clusters of 2-4 trichomes on the edges (Fig.8 H). Lines # 3 and 5 produced very high numbers of ectopic trichomes on cotyledons and hypocotyls (more than line # 6) but did not show any discernible difference in their leaf trichome phenotypes (Fig.8 I). Line # 4 had an intermediate leaf trichome phenotype with respect to trichome number and cluster size reduction. Two observations are interesting. No completely glabrous leaves

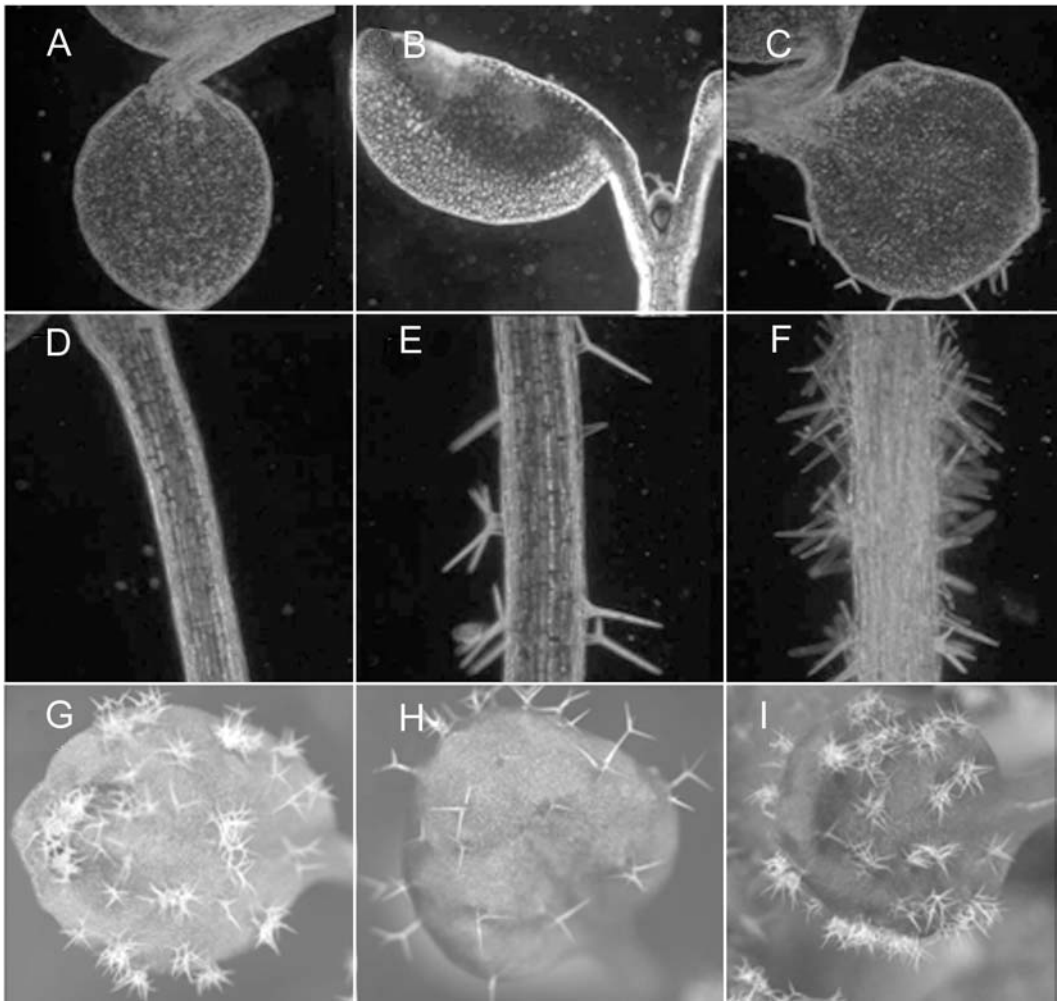


Figure 8: Ubiquitous expression of GL2 in *try cpc* double mutant.
A,B,C) Cotyledons of wt-*ler*, *try cpc* and *35S:GL2/try cpc* plants. Note the occurrence of many ectopic trichomes on C. Trichomes are not seen on wild type hypocotyls (D) but try cpc plants do produce some trichomes on their hypocotyls (E). But *35S:GL2* increases the number of trichome initiations on *try cpc* hypocotyls enormously (F). Two lines both showing ectopic trichomes on cotyledons and hypocotyl but having different effects on leaves are shown in H (line 6) and I (line 5). Line 6 shows a drastic reduction in cluster size and trichome number (H) when compared to control *try cpc* plants (G). But Line 5 (I) has a similar trichome phenotype as that of control.

were found. Does it have to do with the expression level of GL2 being lower in these lines than in *35S::GL2*/wt which showed glabrous leaves? RT-PCR analysis was done to compare the expression levels of *GL2* in these lines. The level of *GL2* expression appears to be similar in *try cpc* plants and the corresponding GL2 over-expressing plants (see RT-PCR analysis in Fig.3) possibly because the already high expression levels of GL2 in the *try cpc* background masks a further increase in expression level. However, the levels are much higher than in both the wt and *35S::GL2*/wt transgenic line (line #2 - which showed glabrous leaves) suggesting that even high levels of GL2 cannot inhibit trichome initiation strongly in the absence of TRY / CPC function. The other interesting observation is the strong reduction of cluster size and trichome numbers in the *35S::GL2*/*try cpc* line # 6. It is possible that the other two TRY homologs in Arabidopsis (ETC1 and ETC2) may mediate GL2 inhibitory function to some extent in the absence of TRY / CPC. But the ectopic trichome production on Line # 3 and 5 without any discernable effect on leaves suggests that TRY / CPC could mainly mediate GL2 inhibition effect seen in *35S::GL2*/wt leaves (Fig. 3) though a direct effect of GL2, independent of TRY / CPC, on trichome inhibition cannot be ruled out.

2.3.6 Analysis of *gl2* double mutants

To understand the function of *gl2* better in the context of trichome patterning double mutants of *gl2* with other genes which are known to be involved in patterning were analyzed. Specifically three double mutants, *gl2 gl3*, *gl2 cpc* and *gl2 try* were analyzed. The *gl3* and *cpc* mutants exhibit completely opposite phenotypes with respect to trichome patterning. The *gl3* mutants have reduced number of trichomes compared to wt (Payne *et al.,* 2000). But *cpc* mutants have higher number of trichomes than wt (Schellmann *et al.,* 2002). The *try* mutants exhibit a mixture of trichome branching and pattern phenotype. They have large over-branched trichomes which are found in clusters at a frequency of around 5-10%. The number of trichomes on *try* leaves is reduced to a small extent when compared to wt (Schellmann *et al.,* 2002). Different *gl2* alleles available and the double mutants were analyzed for trichome numbers using leaf epidermal imprints (Table 2).

The *gl3 gl2* double mutant leaves have been reported to completely lack trichomes and look glabrous (Hulskamp *et al*., 1994). It was also reported using a

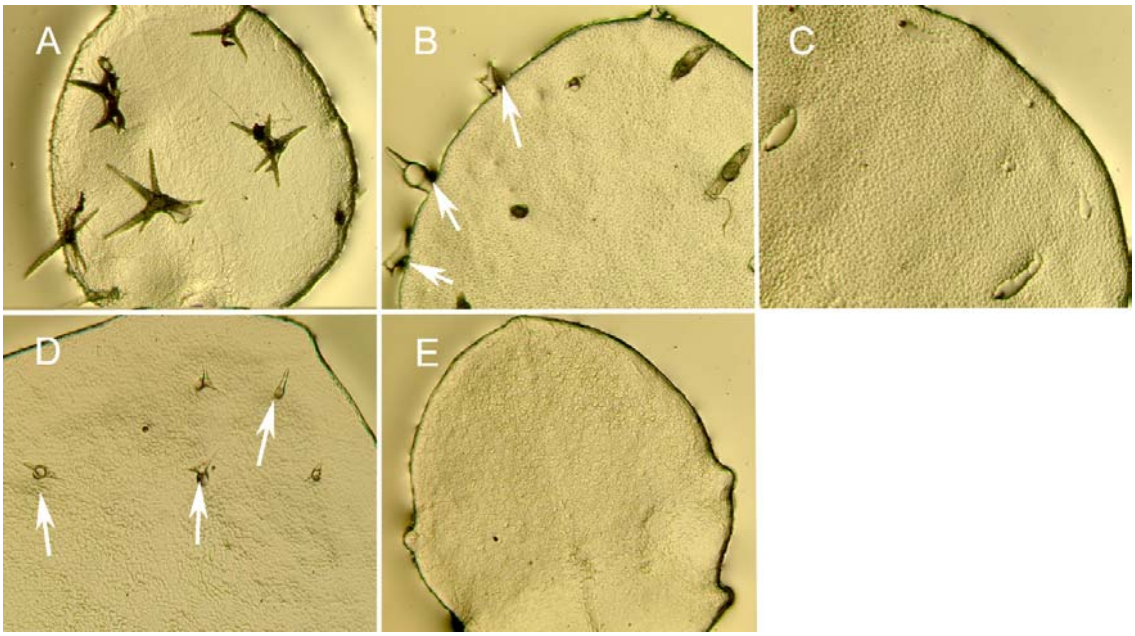


Figure 9: Analysis of trichome initiation in *gl2* double mutants
Epidermal leaf imprints of the various mutants were analyzed. *gl2* leaves consist of large laterally expanded trichomes (C), whereas the trichomes on *try gl2* double mutant leaves (B) grow out of the leaf surface and branch in a irregular way (arrows). Over-branched trichomes on *try* leaves can be seen in (A). Trichomes are seen on *gl3* leaves (arrows - D) though their number and size are smaller than wt. However the *gl3 gl2* double mutant (E) lacks trichomes completely.

Mutant lines	Number of trichomes
gl2-Bos (Ler)	7.5 ± 1.2 (n = 11)
gl2-5 (Col)	22.1 ± 2.1 (n = 15)
gl2-362 (Col)	21.8 ± 2 (n = 16)
WT (Ler)	13.3 ± 1.3 (n = 12)
WT (Col)	40.4 ± 5.3 (n = 12)
try	9.3 ± 1.2 (n = 11); 7.6%*
try gl2	12.3 ± 3.2 (n = 27); 3.6%*
gl3	5.7 ± 1.6 (n = 12)
gl3 gl2	0

Table 2: Number of trichome initiations in various mutants
The first pair of leaves of various alleles of *gl2* and *gl2* double mutants along with the corresponding wt ecotype controls was used to make agarose epidermal imprints and the number of trichome initiations was counted. Standard deviations are indicated as ± values with n indicating the number of leaves counted. The ecotype backgrounds are indicated in brackets next to the allele names.
* % of cells present in clusters (immediately adjacent to each other)

GL2::GUS transgene in the *gl2 gl3* double mutant background that a spotted pattern of GUS activity somewhat similar to wt leaves still exists, indicating presence of some patterning process (Ohashi *et al*., 2002). To see if there are still any laterally expanded trichomes, similar to the ones on *gl2* leaves, present leaf epidermal imprints on agarose were prepared and observed microscopically at high resolution. The leaf epidermis did not have any trichomes (Fig.9 E) indicating that both GL3 and GL2 may participate in trichome initiation, in a redundant way to some extent, and thus in the absence of both together no trichome initiations can occur.

The above inference of requirement of GL2 for trichome patterning as a positive regulator was supported further with the analysis of the *try gl2* double mutant. The *try gl2* mutant has been shown earlier to rescue the trichome morphology phenotype (Hulskamp *et al*., 1994) of *gl2*; meaning that the double mutant no longer consists of laterally expanding trichomes, typical of *gl2*, but they grow out of the leaf surface and show branching, though in some irregular ways. But the number of trichome initiations and clustering frequency has not been studied. Hence, the *try gl2* mutant was analyzed in more detail in the context of trichome patterning. It was found that the average number of trichome initiations was reduced (12.3) (see Table 2 for comparison) and about 3.6% of the trichomes developed in clusters. Thus it is clear that along with the number of trichome initiations, the cluster frequency is also reduced in *try gl2* compared to *try* (7.6%).

Note: The ecotype background of this double mutant is mixed (*gl2*-Col + *try*-Ler) and hence the number of trichome initiations cannot be directly compared between *gl2*, *try*

and *try.gl2*. However looking at Table shows that the number of trichome initiations are reduced by almost half in the double mutant (12.3) compared to *gl2* alone (22.1), and is increased only modestly compared to *try* (9.3). This effect is more than what can be accounted for by the mixing of both the ecotypes (Ler = 13.3 & Col. = 40.4) and suggests that removal of GL2 function during trichome patterning leads to reduced number of trichome initiations (Table 2).

2.3.7 Sub-cellular localization of GL2 protein in Arabidopsis epidermal cells

GL2 encodes a homeo-domain protein of the HD-ZIP IV class and is expected to localize to the nucleus as it is a putative transcription factor. In roots *GL2* is expressed in the atrichoblast cell files and has been shown that it functions to suppress root-hair cell (outgrowth) determination (Masucci *et al*., 1996) and thus promote non-root hair cell (atrichoblast) determination. On the contrary, in leaves *GL2* function is needed to promote trichome (outgrowth) formation. Thus, it appears to suppress local outgrowth (root-hair) in the roots while it promotes an outgrowth (trichome) on leaves. Could the intracellular localization of GL2 (as in partition between cytoplasm and nucleus) in the root and leaf tissues be different which may result in these two apparently contrasting effects? Immuno-localization studies using polyclonal antibody against an epitope in the C terminus of the GL2 protein showed that GL2 protein localizes to the nucleus inside trichome cells and is distributed both in the cytoplasm and nucleus in sub-epidermal cells (Szymanski and Marks, 1998). However, the staining in this study were not very convincing enough. GL2 localization in other epidermal cells of root and hypocotyl was not studied at all. To see whether there are differences in GL2 intracellular localization in different epidermal cells GL2 was fused to EYFP at its N-terminus and introduced into *gl2* mutant plants under its own promoter. The *GL2::EYFP:GL2* construct completely rescued the trichome differentiation phenotype of *gl2* leaves (Fig.10). In all the epidermal cells checked by fluorescence microscopy GL2 was localized exclusively in the nucleus (Fig.10 D, E and F). In roots, files of atrichoblast cells were found having the EYFP-GL2 protein in the nucleus (Fig.10 E). Similarly in the non-stomatal cells of hypocotyls, epidermal cells of the petiole as well as undifferentiated cells on the adaxial surface of young leaf primordia and trichomes GL2 was exclusively localized to the nucleus. Though GL2 was expected to be found

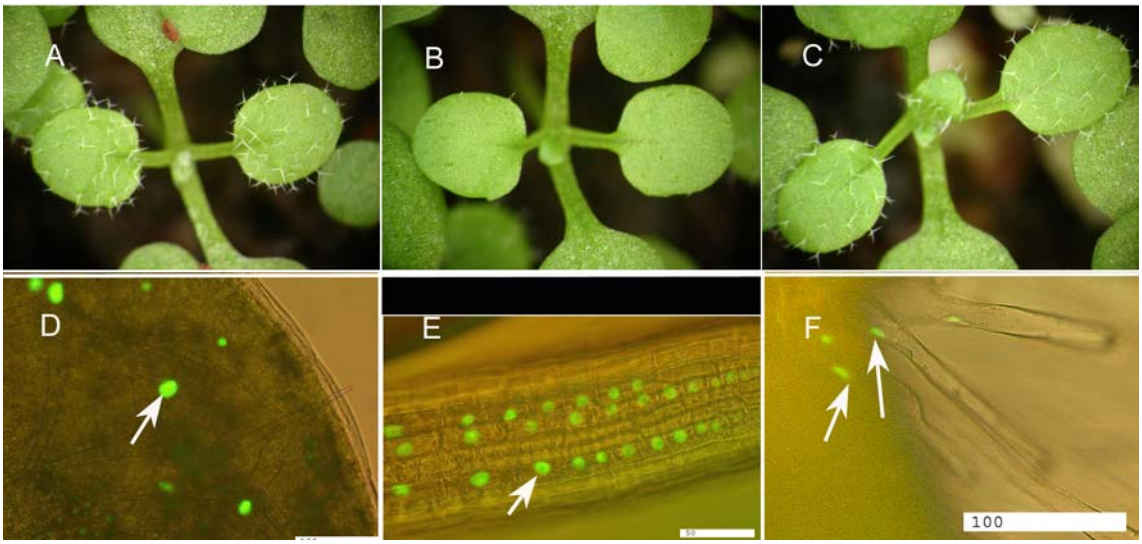


Figure 10: Rescue of *gl2* with *pGL2::EYFP:GL2* construct and sub-cellular localization of EYFP-GL2 protein
The *pGL2::EYFP-GL2* construct rescues the *gl2* mutant phenotype completely (C). Compare it with *gl2* (B) and Col-wt (A). The rescued plants were analyzed to see the localization of the GL2 protein. As can be seen, the EYFP-GL2 protein localizes to the nucleus (arrows) in trichome cells (D), in atrichoblast cells (E) and root hair cells near the root-hypocotyl junction (F). Scale bars: D, F = 100 µm; E = 50 µm.

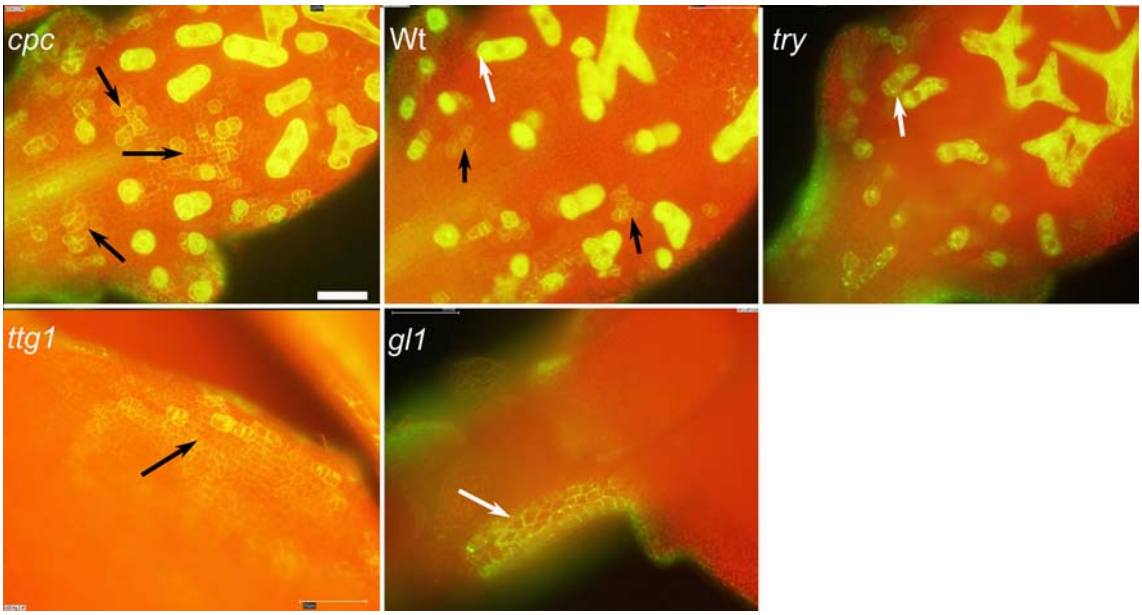


Figure 11:Regulation of *GL2* expression
The expression pattern of the *pGL2::GFP-ER* construct in various mutant backgrounds was analyzed by fluorescence microscopy. In *gl1* background *GL2* expression is seen only in cells of the petiole (arrow), towards the margin and not on the leaf blade whereas in *ttg1* mutants weak *GL2* expression is seen in epidermal cells on the leaf blade, which can be considered as the basal level of expression. *GL2* expression is up regulated in *cpc* (black arrows) where many groups of cells are expressing basal levels of *GL2* compared to wt. In *try* the expression is mostly limited to trichomes and basal expression is slightly lower than in wt. Scale bar: 50 µm

only in non-root hair cells frequently root hairs, especially more so in the root-hypocotyl junction also had GL2 localized in the nucleus (Fig.10F). One report (Wada *et al*., 2002) however has shown by careful in situ studies to localize GL2 expression pattern that GL2 is weakly expressed even in trichoblast cells initially. So, this does not come as a big surprise.

2.3.8 Regulation of the *GL2* promoter

The regulation of *GL2* expression in *gl1*, *ttg1* and *gl3* backgrounds using the *GL2::GUS* reporter has been previously studied and it was concluded that *GL1*, *TTG1* and *GL3* positively regulate *GL2* expression (Szymanski and Marks, 1998; Hung *et al*., 1998; Lin and Schiefelbein, 2001). But the expression of the *GL2* promoter in leaves of *cpc* and *try* mutants has not been studied thus far in the context of trichome patterning. Using *GL2::GFP-ER* as the reporter it was sought to analyze *GL2* expression in the different mutant backgrounds at a much higher resolution. *GL2* expression in wt leaves is found in trichomes at all stages of their development. In young leaves it is also found in some small groups of cells in the patterning zone (lower end of the leaf) where new trichome specifications occur (Fig.11) and these small groups of cells expressing *GL2* weakly but distinctly could be considered as trichome competence groups (TCGs). Fig.11 shows that the expression of *GL2::GFP-ER* in *gl1* is completely absent on the leaf blade but still present in cells of the petiole. However, weak *GL2* expression was seen on cells in the leaf blade of *ttg1* mutants indicating that TTG is not absolutely essential for *GL2* expression, though it may increase the strength of expression in combination with other factors. Interestingly, though weak *GL2* expression can be seen in *ttg1* leaves no competence groups are seen indicating that both GL1 and *TTG1* are essential for the patterning mechanism to get started. *cpc* mutant leaves show an increased activity of *GL2*, as reflected by more and / or larger TCGs, suggesting that *CPC* inhibits *GL2* during trichome patterning. This result is in agreement with roots where inhibition of *GL2* by *CPC* has been shown ( ). Though *CPC* and *TRY* are close homologs and are believed to act as inhibitors in a redundant manner during trichome patterning, the expression of the *GL2* reporter activity in *try* mutants is not upregulated like in *cpc*. Contrastingly, there appears to be fewer TCGs, which correlates with the fact that the number of trichome initiations on *try* leaves is reduced (9.3) compared to wt (13.3) whereas *cpc* mutants have increased number of trichome initiations (44.6)

(see Table.2 for comparisons with respective mutant and wt ecotypes). This suggests that *TRY* positively regulates the basal *GL2* expression to a small extent in wt leaves.

2.3.9 Effects of ectopic expression of *GL2* on trichome patterning in different mutant backgrounds

All the studies so far have suggested that *GL2* is the essential downstream target of the other patterning genes. It was also shown in roots that GL2 directly binds and regulates the expression of the phospholipase D gene thereby connecting cell patterning with morphogenesis. To check if GL2 expression is sufficient to initiate trichomes in the glabrous leaves of *gl1* and *ttg1* mutants GL2 was ectopically expressed under different promoters (*GL1, TRY* and *CaMV35S*) in these mutants. The *GL1* promoter is known to be active in both *gl1* and ttg mutant backgrounds. The *TRY* promoter however is not active in *gl1* but weakly active in *ttg1* background (Swen Schellman, personal comm.). Transgenic plants were created by introducing *pGL1:GL2*, *pTRY:GL2* and *p35S:GL2* (The *35S::GL2* construct was the same heat shock inducible one as used in the experiments described before) in both ttg*1* and *gl1* backgrounds. None of the transgenic lines rescued the mutant phenotypes indicating that expression of GL2 is not a sufficient criterion to initiate trichomes in the absence of GL1 and TTG1 function.

To see if the effect on trichome patterning when the regulation of *GL2* is changed by expressing it under two different trichome promoters, *gl2* mutants were transformed with the *pGL1::GL2* and *pTRY::GL2* constructs and the resulting transgenic lines were analyzed with respect to number of trichome initiations. Both the lines rescued *gl2* trichome phenotype to wt trichomes. But the number of trichome initiations was reduced (14.8 and 13.2) compared to wt (17.6). As a control *pGL2:GL2* was also transformed into *gl2* mutants and it too rescued the trichome phenotype but the number of initiations was still lower (15.2) than wt (17.6).

Understanding the function of GL2 in trichome growth and differentiation

2.3.10 Differentiation defects in gl2 trichomes

As mentioned earlier, trichomes on mature *gl2* leaves show a failure to grow out of leaf surface and instead are expanded laterally along the leaf surface (Fig.1). The obvious trichome phenotype lead earlier reports to infer that defective cell morphogenesis, in the absence of GL2 function, results in such abnormal trichomes. To better understand how these trichomes develop *gl2* plants carrying a trichome marker (*pGL2::GFP-ER*) were analyzed. The development of a single trichome initial was followed over time. Wild type (wt) trichomes initially swell after specification, then elongate perpendicular to the leaf surface, initiate branching and after further expansion undergo the process of cell wall maturation (Hulskamp *et al*., 1994). Fig.12 shows the development of a single trichome initial which was followed up to 43 hrs using confocal microscopy (Beyond 43 hrs the cells could not be tracked because of severe bleaching by the laser and problems with plant growth). Pictures were taken approximately at an interval of 12 hrs each. The *gl2* trichome starts its development initially like WT by projecting out of the leaf surface (Fig.12A, considered as 0hrs) but later starts to expand along the leaf surface rather than growing outwards. It does not initiate any branching event but continues to expand laterally. It eventually adopts a jigsaw puzzle shape very typical of the epidermal pavement cells (Fig.12C). This implies that though *gl2* trichomes start their initial development like wt (by expanding out of the leaf surface and expressing the trichome markers) they eventually end up looking like epidermal pavement cells with respect to their shape. Does this mean that they lose their way during development, forget their identity and adopt the default epidermal differentiation pathway? If so, do they show any other features typical of pavement cells?

A more careful examination of trichomes on mature *gl2* leaves was performed to answer the above questions. Epidermal leaf imprints were prepared using agarose and observed microscopically at high resolution. All trichomes were laterally expanded along the leaf surface either radially or in an elongated way. The trichomes could be broadly classified into two classes: a) those mostly closer to the leaf margin were elongated and were slightly jigsaw puzzle shaped (Fig.13B) and b) the rest of the trichomes either expanded radially (Fig.13D) or elongated but were highly puzzle

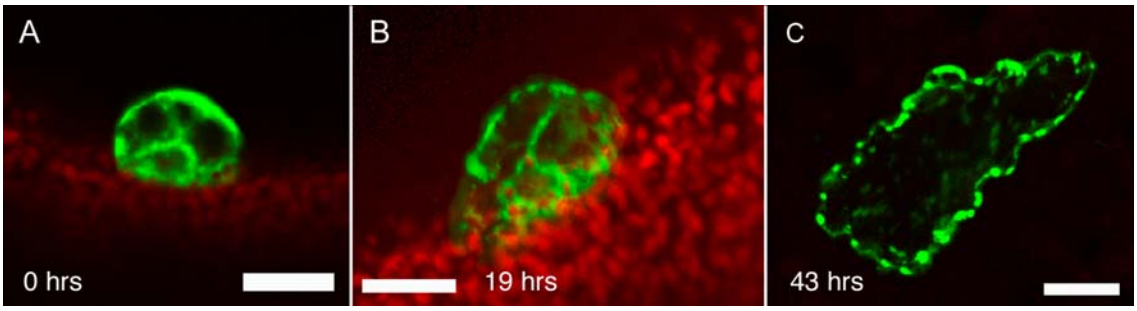


Figure 12: Development of a *gl2* trichome.
gl2 plants expressing a trichome marker (*pGL2::GFP-ER*) were used to follow the development of trichomes. A single *gl2* trichome initial (A) was followed as it begins to enlarge (0 hrs) and grow out of the leaf surface like a wt trichome initial (wt not shown here). In about 20 hrs it already starts to expand laterally along the leaf surface (B) and by 45hrs it has completely expanded laterally and has some puzzle like cell shape similar to pavement cells. Scale bars: 20 µm.

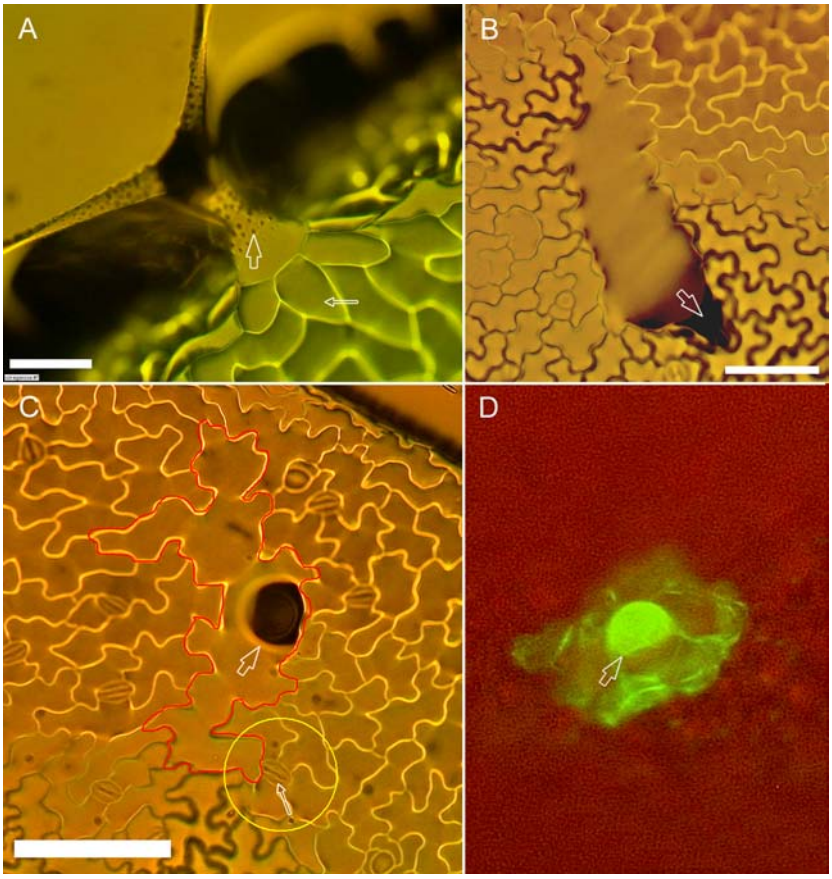


Figure 13: Leaf epidermal imprints and a fluorescent micrograph showing the features of wt and *gl2* trichomes.
Wild-type trichomes (A) grow out of the leaf surface, branch, show cell surface papillae (thick arrow) and are surrounded by specialized socket cells (thin arrow).However, *gl2* trichomes (B-D) expand along the leaf surface and are either elongated (B,C) or radially expanded (D). They have a small peak either at the end of the cell (B – arrow) or in the middle (C, D – thick arrow). The trichome in C is highly jigsaw puzzle shaped resembling the pavement cells adjacent to it. The picture in D shows a 'jellyfish-like-collapsing' *gl2* trichome which has a peak in the middle and is expanding radially. It is expressing the trichome marker *GL2::GFP-ER*. Note that socket cells are absent around *gl2* trichomes and instead stomatal guard cells are found in immediate contact frequently (C – thin arrow within circle). Scale bars: A,B and C = 50 µm; D = 100 µm.

shaped (Fig.13C), resembling epidermal pavement cells but for their huge size and the presence of a small peak, not as distinct as the ones on the trichomes at the edges.

Wild-type trichomes are always surrounded completely by a group of specialized cell type called the socket cells / accessory cells and are never found immediately adjacent to stomatal guard cells (Fig.13A). In *gl2* however, surprisingly stomata were frequently found in contact with the trichome cells (Fig.13C). Also, whereas wt mature trichomes exhibit surface papillae reflecting changes in the cell wall structure as the trichome matures the *gl2* trichomes are smooth and completely lack any surface papillae (Fig.12 B, C). The above observations, viz., pavement cell shape, absence of specialized socket cells around them, their large size comparable to wt trichomes and yet lacking the surface papillae and more importantly presence of stomata immediately adjacent to them, all are clear markers of defective differentiation of the trichomes on *gl2*. All published reports so far have concluded that trichomes on *gl2* mutants are defective in polar expansion and differentiation (Rerie *et al*., 1994; Larkin *et al*., 2003). But however the analysis presented here allows one to distinguish whether it is just defective morphogenesis (resulting abnormal trichome shapes) or whether cells which start with the trichome differentiation program lose their way in between, exit the pathway and end up differentiating as pavement cells. There are many other trichome mutants like *DISTORTED 1*, *SPIRRIG*, *DISTORTED 2*, *CROOKED* and others, which also show defective morphogenesis with respect to cell expansion, size and branching. But nevertheless they are known to be similar to wt trichomes in all other respects discussed above (accessory cells, no contact with stomata, surface papillae, no puzzle shape). This implies that mature *gl2* trichomes are not just defective but may well have lost their trichome identity and behave as pavement cells. Though all the observations presented so far suggest that some *gl2* trichomes may end up differentiating as pavement cells (implying trans-differentiation) it needs to be checked yet if they lose trichome specific marker expression and gain pavement cell specific marker expression. To answer the latter question *gl2* plants have been crossed to a marker line which is negative for trichomes and positive for pavement cells. This line of experiments needs to be pursued further in future. However when the expression of a trichome marker was analyzed in mature *gl2* leaves some of the older trichomes had ceased to express the marker while trichomes of the same stage in wt leaves still continued to express

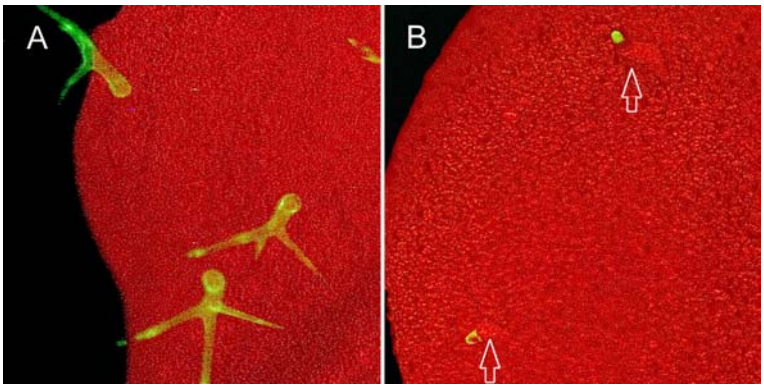


Figure 14: Trichomes on *gl2* lose their 'trichome identity' during their development.
Some trichomes on mature *gl2* (B) leaves (arrows) cease to express the trichome specific marker, *GL2:GFP-ER*, whereas trichomes on wt leaves (A) of the same stage still continue to express it suggesting the loss of their trichome identity. Note that some residual GFP is still seen in the two *gl2* trichomes shown in the picture, which have expanded laterally.

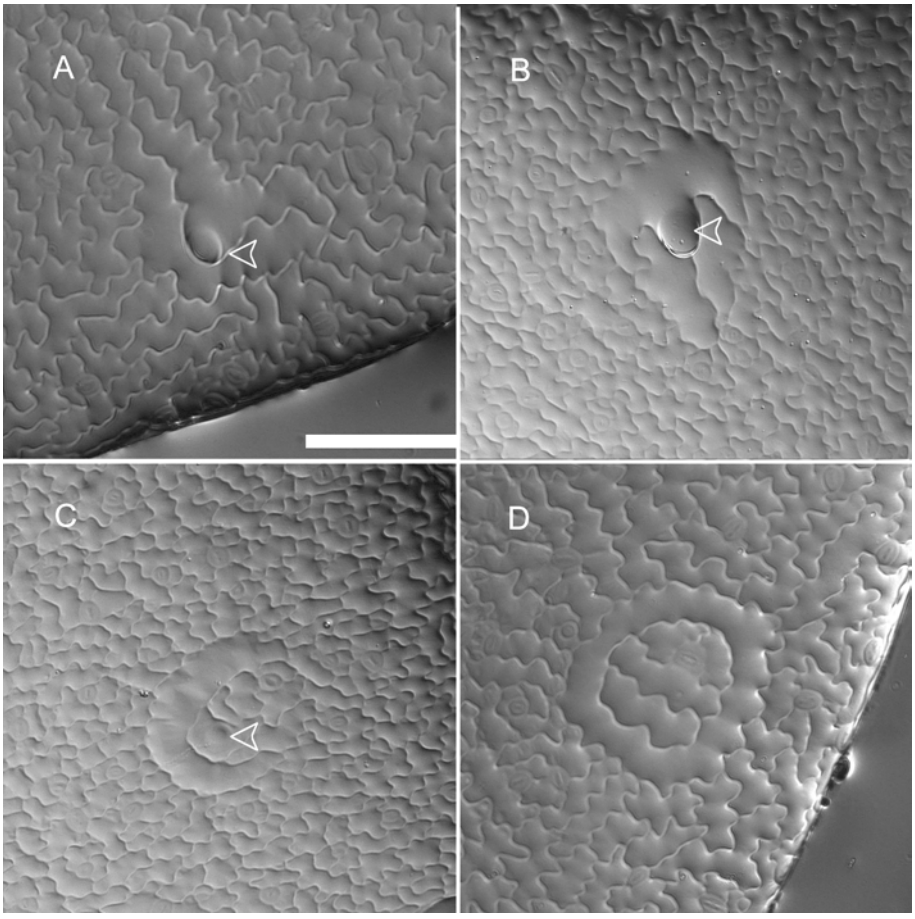


Figure 15: **Some trichomes on *gl2* appear to be divided**.
Epidermal leaf imprints of mature *gl2* leaves were observed at high magnification using DIC light microscopy. Frequently trichomes which appeared to be divided were seen. Most of them (A, B, C) still had a small peak (arrow head) which is more obvious is younger leaves. Their cell division patterns are very varied and atypical. Note the very strange cell division pattern in C and D. In D one large cell completely encircles many cells. Scale bar: 100 µm.

(Fig.14). This supports the conclusion that *gl2* trichomes exit the trichome differentiation pathway during their development.

Note: The down regulation of the *TRY* marker is more specific in the *gl2* background though some trichomes also switch off other trichome markers in mature leaves. When a comparison of *TRY* with *GL2* and *Myb23* marker expression was done, it was clear that when only very few trichomes are expressing *TRY* both *GL2* and *Myb23* are expressed in most trichomes present on the gl2 leaves. See Fig.16 for this comparison, though the figure is intended for a different purpose. Leaf number 1 / 2 was used for *GL2* and *Myb23* marker analysis while leaf 3 was used for *TRY* analysis because it's expression was too weak in leaf 1 / 2 (see Fig.5F which shows *TRY* expression in leaf 1/2).

2.3.11 *gl2* mutant trichomes appear to be divided

A careful examination of mature leaf epidermal imprints of *gl2* showed that quite often trichomes appeared to be divided around 1 - 4 times (Fig.15) with complete cell walls resulting in a cluster of cells of varying sizes. Each of these individual cells was about 2 – 6 times larger than the neighboring pavement cells surrounding them. No such cell clusters were seen on wt or other patterning gene mutant leaves analyzed so far. They were easily spotted because of the presence of the typical *gl2* peak in one of these cells, their obviously large size relative to normal epidermal pavement cells and their relative position between other trichomes showing the typical spacing pattern. DAPI staining of the nuclei of these cells showed that all the cells of such a divided cluster contained nuclei (data not shown) indicating complete cell division. Also, three trichome specific markers (*TRY*, *GL2* and *AtMyb23*) are expressed by these clusters (Fig.16) showing they all are trichomes. The origin of cells in such clusters is debatable. Whether they represent the division of one single trichome or the expansion of some cells surrounding a single trichome needs to be clarified. Analysis by making clonal sectors passing through such clusters would resolve this question beyond doubt. In the absence of such an analysis, other indirect ways and reasoning as summarized below are very suggestive that trichomes on gl2 exit the trichome differentiation pathway (as seen in the preceding paragraphs) and divide.

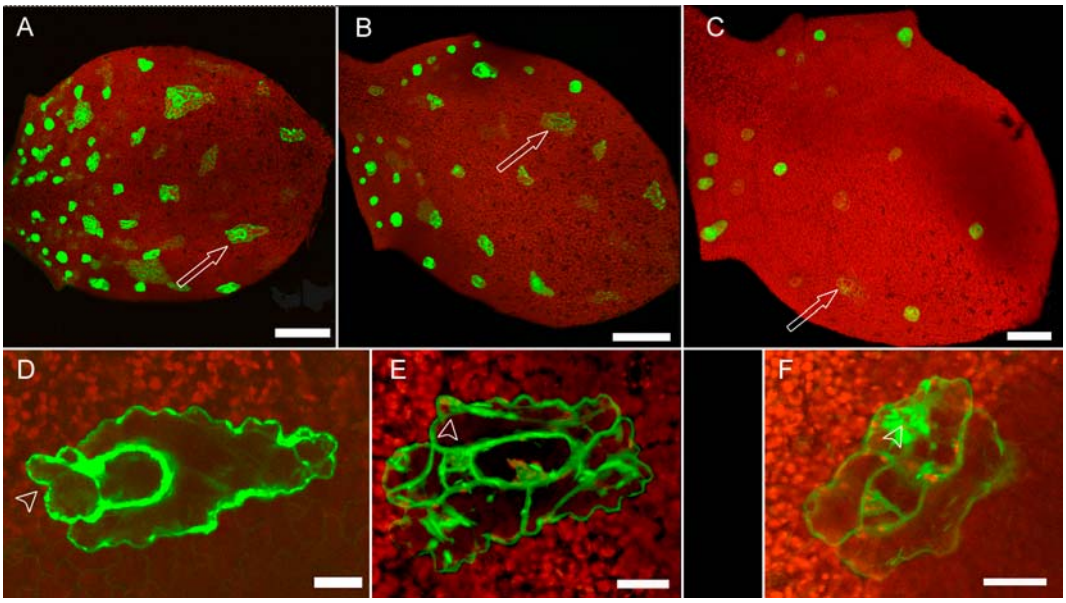


Figure 16: **Expression analysis of three different trichome specific markers in *gl2* leaves.**
Three dimensional reconstruction of confocal microscope pictures showing *gl2* leaves expressing the trichome specific markers *pGL2::GFP-ER* (A,D), *pMyb23::GFP-ER* (B,E) and *pTRY::GFP-ER* (C,F). Some *gl2* trichomes appear to be divided (arrows). D, E & F are high magnification pictures of the trichomes indicated by arrows on A, B & C respectively. The arrow heads in D, E & F indicate the typical small peak of *gl2* trichomes. First pair of leaves in A & B. Leaf number 4 is shown in C (because *TRY* expression in older first pair of leaves is very weak). Scale bars: D, E, F = 20 µm; A,B = 160 µm; C = 80 µm.

a) Expression of TRY marker:

Analysis of the *GL2::GFP-ER* marker in young *gl2* leaves shows that it is expressed strongly in trichome initials as well as in large group of smaller cells in the neighborhood (Fig. 17A). The expression of the same marker is restricted to a few large cells in a mature leaf, when no patterning is going on (Fig. 17B). So, one cannot be sure of whether the few large cells expressing the marker in the older leaf are the result of division of a single trichome cell or due to ineffective lateral inhibition. To overcome this problem the trichome specific marker *TRY::GFP-ER* was analyzed. It is always exclusively expressed in trichomes in young leaves (Fig. 17C) (both in wt and *gl2*; see also Fig.5 and Fig.4). But in older leaves, where patterning has already stopped, the marker was found expressed in a cluster of few large cells (Fig. 17D). This suggests that some trichomes have divided as the leaf develops. This result agrees well with the earlier findings that *gl2* trichomes appear to lose their way during development, exit the pathway and possibly differentiate as pavement cells.

b) Cell size criteria:

Epidermal leaf imprints of mature *gl2* leaves were used to compare cell sizes of large trichomes, individual cells of trichome clusters, and combined area of the cells in a cluster. The area of typical trichomes on *gl2* was found to be 11.3 units (s.d.± 3.3; n = 10). The area of the individual cells in the trichome cluster was about 4 (s.d. ± 1.7; n = 26) but the combined area of the cells in the cluster was 10.7 (s.d. ± 2.4; n = 11), which is close to that found for large undivided trichomes. This is again suggestive of the above made conclusion.

Note: see materials and methods section for details on how the measurements were done, units,etc.

c) Time course analysis of trichome initiation on gl2 leaves:

If as suggested in the previous paragraphs some trichome on *gl2* do divide and become pavement cells then one would expect the final number of trichomes on mature *gl2* leaves to be lesser than in a young leaf. To test this, *gl2* leaves of three different developmental stages were analyzed. Both wt and *gl2* leaves were classified into three categories based on their length (less than 1.5 mm, 1.5 mm -3 mm & greater than 3 mm) and the number of morphologically distinct trichomes was counted using leaf epidermal imprints. As shown in Graph 1 the number of trichomes on wt increased steadily as the leaf developed (28, 33.6 and 40.4) while the number of trichomes on *gl2*

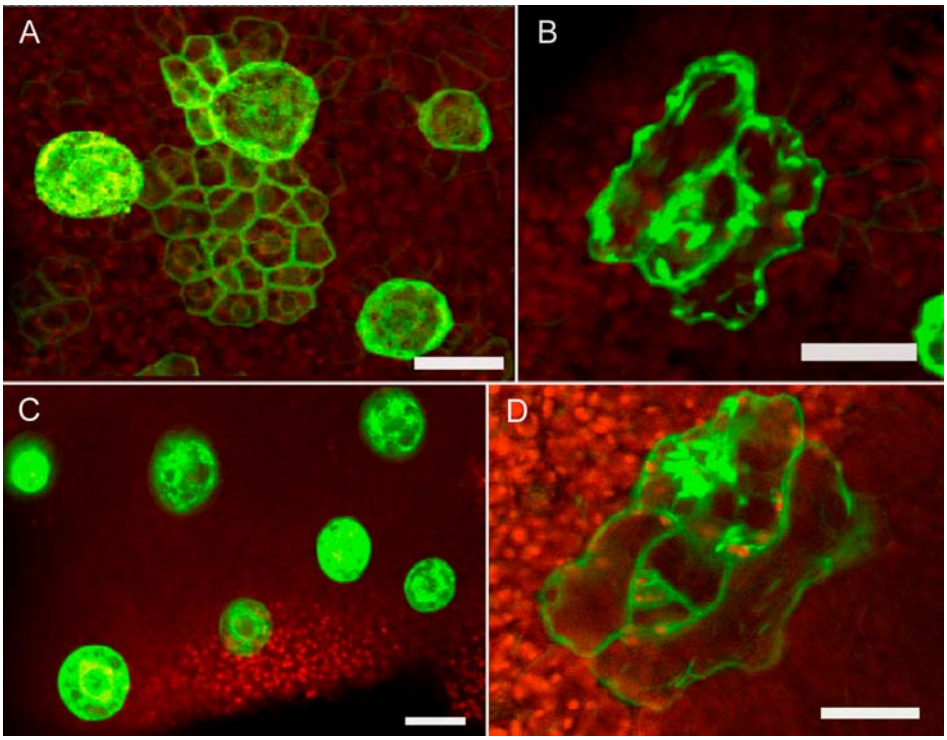
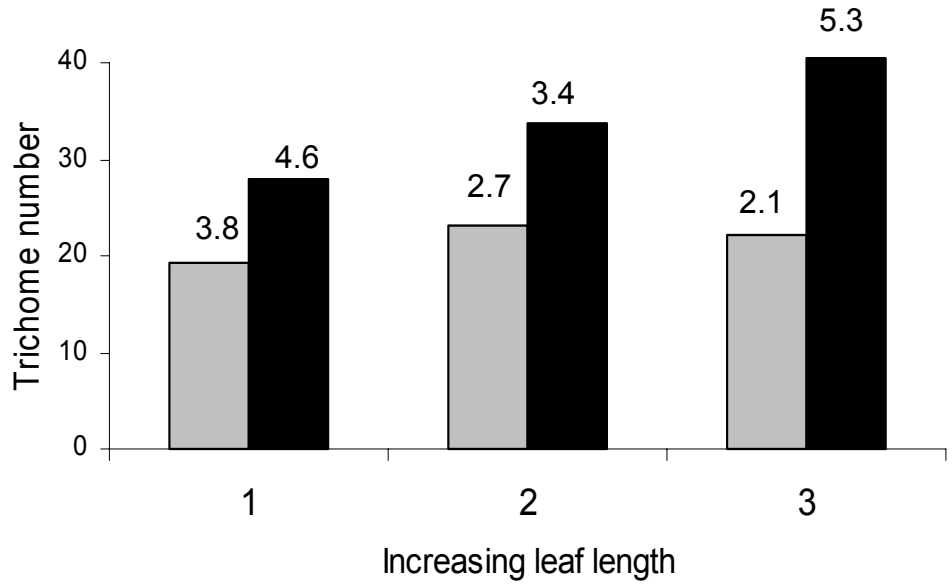


Figure 17: Expression analysis of two trichome markers in *gl2* leaves.
The *GL2:GFP-ER* marker is expressed strongly in trichomes and also in large groups of cells in the neighborhood of some trichomes in young leaves (A). In older leaves its expression is found restricted to a few large cells which occur as a cluster (B). The *TRY:GFP-ER* marker on the other hand is exclusively expressed only in trichomes in leaf primordia and young leaves (C). In older leaves (D) a cluster of few cells are found expressing the marker suggesting that some trichomes divide during their development. Scale bars: 20 µm.



Graph 1: **Time course analysis of trichome development on wt-Col and *gl2* leaves**
First pair of leaves on wt and *gl2* leaves were classified into three developmental stages (<1.5 mm, 1.5 – 3mm & >3mm) based on their length and the number of trichomes on them were counted. The bar graph shows that the number of trichomes on wt leaves (black bars) keeps increasing as a function of leaf length (left to right) whereas the trichome number on *gl2* leaves (grey bars) increases initially and then decreases slightly. Standard deviations from the mean are indicated above each bar.

leaves show an initial increase but decrease to a very small extent later (19.3, 23.2 and 22.1). One could speculate that this stage reflects equilibrium between trichome initiation and division?

All results presented so far about *gl2* trichomes namely, suggest that GL2 is required for complete and proper trichome cell differentiation. In the absence of its function, cells which enter the trichome differentiation pathway frequently exit the pathway at various stages, and enter the default epidermal differentiation pathway.

2.3.12 GL2 positively regulates trichome branching

The *gl2* mutant analysis presented so far shows that GL2 function is required for proper trichome morphogenesis and differentiation. One important aspect of trichome development is the process of branching. Many genes like *GL3*, *TRY*, *AtMYB23*, *AN*, *STI*, and others regulate trichome branching and in many mutants there exists a positive co-relation between trichome branch number and endoreplication level. Trichomes on *gl2* show a gross defect with respect to expansion out of the leaf surface and hence one cannot know whether GL2 is involved in the regulation of trichome branching or not just by looking at the mutant phenotype. Hence another approach was taken. To check whether GL2 is involved in the regulation of trichome branching and if it depends on its expression levels, *GL2* was expressed under two different trichome gene promoters which are known to differ in their timing and level of expression. The *TRY* promoter is active in all trichome initials at high levels in wt and continues to be active for a long time even in mature trichomes (Schellmann *et al*., 2002). However, the *GL1* promoter is active in early trichome initials but becomes inactive as early as branching is initiated and is not expressed at all in maturing trichomes (Larkin *et al*., 1993). Both the promoter fragments used in this study have been published to be sufficient to rescue the respective mutant phenotypes when the corresponding cDNAs are expressed by them. The idea was to use the *gl2* mutant where no GL2 activity is present and express GL2 in these plants under these two trichome promoters to see if branching process is regulated by GL2 differently in the two scenarios. A control experiment to demonstrate the difference in the activity of these promoters was done by analyzing the expression pattern of *TRY::GFP-ER* and *GL1::GFP-ER* constructs in wt background. The *TRY* promoter expression was stronger and active for a longer period than the *GL1* promoter (Fig.18. Also, note the absence of GFP marked trichomes near leaf tips of plants

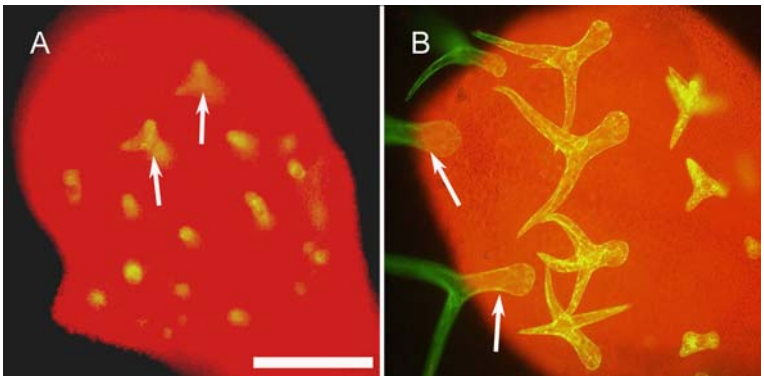


Figure 18: **Differential expression pattern of *GL1* and *TRY* promoters in trichomes.**
Wild type leaves expressing either *pGL1:GFP-ER* (A) or *pTRY:GFP-ER* (B) constructs were analyzed for a qualitative measure of expression pattern. As published previously, the *GL1* promoter is weak (arrow) after the first branching is initiated and no three branched trichome is seen expressing the marker. However, the *TRY* promoter is expressed strongly even in the oldest trichomes on leaf tips (arrow). Note the absence of GFP marked trichomes at leaf tip in A (though trichomes are present they cannot be seen in this picture because of the red chlorophyll fluorescence). Scale bar: 100 µm

	% number of trichomes with the following branch number				
	4	**3**	**2**	**1**	**Stubs**
Col-wt	11.9	87.9	0.2	0	0
pGL1::GL2 # 10	21	74.8	2.6	0.7	0.7
pGL1::GL2 # 11	38.9	54.5	2.2	0.4	4
pTRY::GL2 # 12	3.7	94.4	1.9	0	0
pTRY::GL2 # 8	6.4	92.9	0.7	0	0

Table2. Trichome branch number regulation by GL2.
The number of trichomes having branches from 1- 4 in different transgenic lines, expressing GL2 under different promoters, is given as % values. Values from two independent transgenic lines in each case are presented. Typical *gl2* mutant trichomes are represented as 'stubs'. The % number of trichomes having different number of branches was calculated by counting 200 – 600 trichomes for each line (see text).

expressing *GL1::GFP-ER* unlike those expressing *TRY::GFP-ER*). *gl2* plants were then transformed with either of the two constructs and the resulting transgenic lines were analyzed with respect to trichome branch number formation. Both the lines rescued the *gl2* trichome phenotype and hardly the typical stretched *gl2* trichomes were found in these lines. The frequency of occurrence of trichomes with various branch numbers was counted and tabulated. Values of two lines of *GL1::GL2/gl2* that were analyzed is presented. 3 independent lines of *TRY::GL2/gl2* were analyzed. Values of 2 lines (the other line had intermediate value) are presented as representative. It is very clear from Table.3 that in *TRY::GL2* lines the number of 4 branched trichomes increased significantly (21%, n = 266 and 38.9%, n = 275) when compared to wt (11.9%, n = 663). But in pGL1::GL2 lines the number of 4 branched trichomes decreased (3.7%, n = 268 and 6.4%, n = 296) when compared to wt and most trichomes were three branched (92.9 and 94.4% as compared to 87.9% in wt). This result clearly demonstrates that GL2 positively regulates trichome branching.

2.4 Discussion

2.4.1 GL2 is required for complete trichome morphogenesis and differentiation

Though the *gl2* mutant was first identified to be involved in trichome morphogenesis, based on its phenotype, in 1982 a detailed phenotypic characterization of the mutant with respect to trichome development had not been done. The *GL2* gene was found to be activated by *GL1, TTG1* and *GL3* and hence assumed that it represents the downstream most target of patterning genes, which further activates other target genes required for trichome morphogenesis. A fairly detailed analysis of the mutant presented in this study shows that *gl2* presents a unique opportunity to study cell patterning, cell morphogenesis and differentiation by being involved in all these steps of cellular development. Cells enter the trichome differentiation pathway in *gl2* mutants as they would in wt and start their initial steps of cell expansion and expression of trichome specific genes (*TRY*, *AtMyb23* and *GL2* have been examined in this study). But in the absence of GL2 function they appear to exit from the differentiation program they have started and enter the default epidermal pathway, eventually showing many characteristics of pavement cells. The highly puzzled cell shape, contact with stomatal guard cells, absence of trichome specific cell wall marker – the papillae but having smooth cell surface and the loss of expression of trichome specific genes all point to the above conclusion. The presence of highly puzzle shaped and not so puzzle shaped (and intermediate phenotypes) trichomes on mature *gl2* leaves is by itself suggestive of being snapshots of trichomes differentiating to different degrees as pavement cells. One elegant experiment to prove the above conclusion would be to see if a trichome negative but epidermal cell positive marker would be expressed in some of these 'epidermalized' trichomes in *gl2*. The required crossings of *gl2* plants with the marker lines have been done but need to be pursued in the near future.

Some trichomes appeared to be divided on *gl2* leaves when observed by morphological criteria. These trichome clusters also express the three trichome markers that were analyzed. The strongest support for the idea that *gl2* trichomes may indeed divide comes from the analysis of the *TRY* gene marker. *TRY::GFP-ER* expression in very young leaf primordia and young leaves was found to be always exclusively restricted to trichome initials. When older leaves were analyzed occasionally trichome

clusters containing a few large cells were found expressing the marker. Some trichomes on *gl2* expand radially and look like a jelly fish on young leaves (Fig. 13D). It would not be far fetched to speculate that trichome cell division patterns seen on mature *gl2* leaves as seen in Fig.15 C and D are a consequence of such radially expanded cells dividing. It should be noted in this juncture that continuous cell divisions, as the leaf develops, is also a characteristic feature of pavement cells (they are the last to differentiate on the epidermis, with trichomes being the first cell type to do so). Thus, it would not be surprising to find 'trichomes' to divide after they have lost their trichome identity and entered the pavement cell differentiation pathway. Another independent support to this conclusion comes from the observation that misexpression of cyclin D inhibitors, *ICK1 / KRP1* reduces endoreplication in trichomes and also produces large divided trichome like clusters indicating that blocking endoreplication can lead to entry into mitosis mode of cell cycle (Arp Schnittger, personal comm.)

Several genes are known to be regulators of trichome branching and morphogenesis. The genes functioning as components of the actin / microtubule cytoskeletal machinery (*DIS 1*, *DIS 2*, *CRK* and others) show defects in the axis of general cell expansion, and branch initiation / expansion when mutated. Another group of genes (*STI*, *GL3*, *TRY*, *AN* and others) only effect trichome branch number. To see if GL2 is also involved in regulating trichome branching it was expressed under two different promoters which differ in their strength and timing of expression. The experiment was carried out in gl2 mutant background where no GL2 activity is present. It was found that when *GL2* was expressed under the *GL1* promoter the number of 4 branched trichomes was dramatically reduced (~6.4%) and most of the trichomes were three branched (92.9%) when compared to wt plants (4 branched = 11.9%; 3 branched = 87.9%) . Whereas expression of *GL2* under the *TRY* promoter which is expressed for a longer time in trichomes and is stronger than *GL1*, lead to a strong increase in the number of 4 branched trichomes (38.9%). All the trichomes however, irrespective of branch numbers, were found to have cell surface papillae, a marker for trichome maturity. This experiment very nicely demonstrates that GL2 regulates trichome branching positively. How does GL2 regulate trichome branching? Does it interact with genes which are already known regulators? The answer could well be Yes. The double mutant analysis showed that *GL2* interacts with both *TRY* and *GL3*, both of which are involved in this process. More importantly the positive feedback loop of TRY and GL2

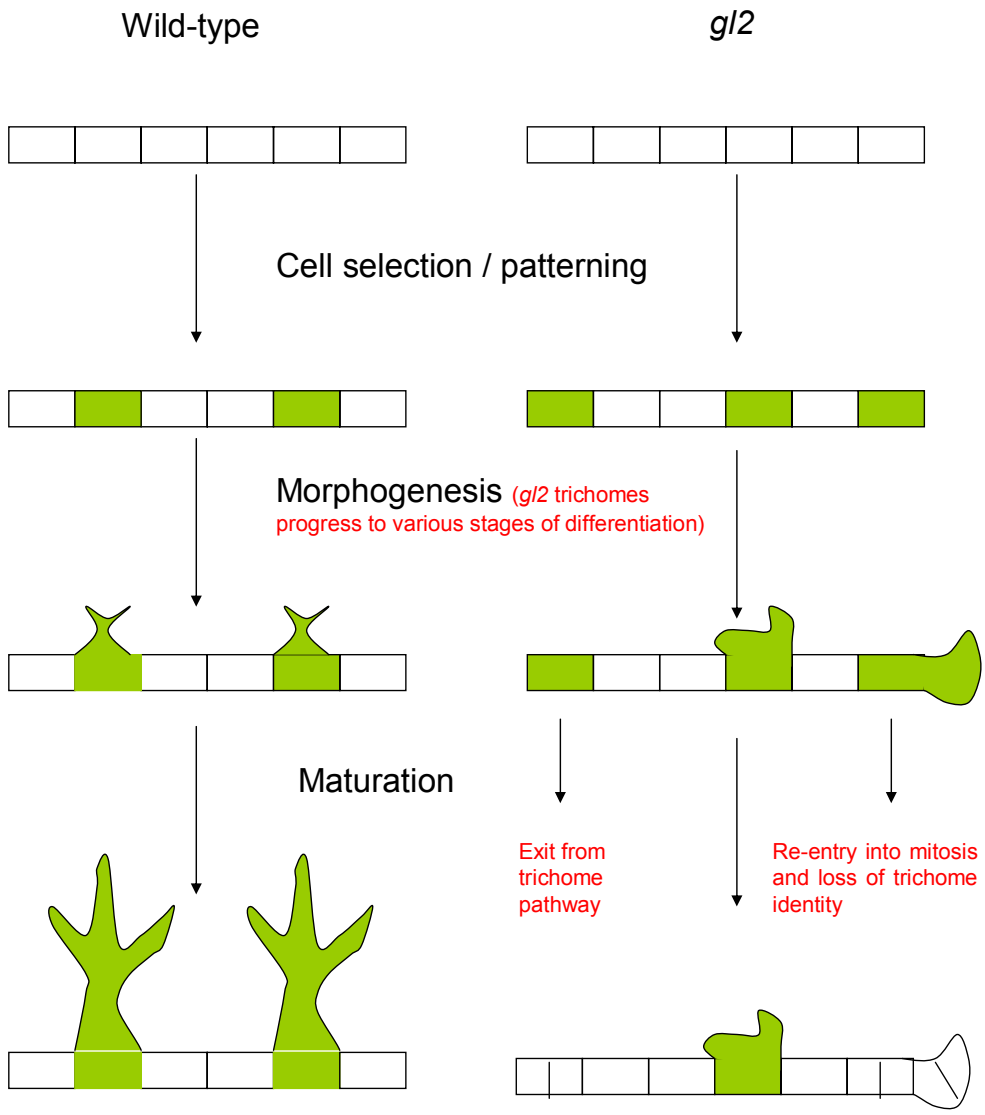


Figure 19: **A cartoon model depicting the development and fate of trichomes on *gl2* mutant leaves.** The first step in trichome development is the selection of a few cells from the epidermal cell pool to enter the trichome differentiation pathway (shaded box). In *gl2* mutant leaves more cells enter the trichome pathway than wt, likely due to ineffective inhibition. In the second step, all cells which have entered the trichome pathway start the morphogenesis process in wt, whereas in *gl2* only some of them continue with this second step while others exit the pathway. Those which do start morphogenesis show defective expansion. Mature trichomes arise on wt after completion of the morphogenetic program. In *gl2*, some trichomes which had initiated morphogenesis tend to exit the pathway by re-entering mitosis and losing their fate. This finally results in lesser number of trichomes on mature gl2 leaves when compared to wt.

found in this study may have a crucial role in this step. One observation which supports this hypothesis is the temporal expression pattern of *TRY* and *GL2*. Both are expressed in trichomes at high levels for a long time even in mature trichomes, long after genes like *GL1*, and *AtMyb23* cease their expression which suggests their continuous activation by each other. The above discussed ideas about the fate of trichomes on gl2 mutant leaves are summarized in a cartoon model in Fig.19.

2.4.2 Does nuclear endoreplication level influence the decision of 'to be' or 'not to be' a trichome?

Trichomes on *try* mutants have very high DNA content, reaching between 64 -128 C, when compared to wt trichomes which have on an average 32C DNA content. This suggests that TRY inhibits endoreplication. Trichomes on *gl2* appear to have lower DNA content than wt (Arp Schnittger, personal comm.) which suggests that GL2 promotes endoreplication in trichomes, probably by inhibiting their entry into mitosis. The rescue of trichome differentiation and morphology phenotypes in the *try gl2* double mutant thus indicates that TRY and GL2 may act in opposing ways with respect to the endoreplication cycle of trichomes and this in turn has an effect on trichome morphogenesis and differentiation. The divided trichome clusters found on *gl2* were completely absent in the *try gl2* double mutant. Thus there is a very good positive co-relation between endoreplication levels and the fate (or differentiation status) of trichomes. The rescue in *try gl2* double mutant could be attributed to an increased endoreplication level in trichomes which encourages them to stay on course to differentiate as trichomes rather than exit the pathway. This however needs to be checked in the future by doing DNA content analysis in *gl2*, *try* and *try gl2* mutants. Is endoreduplication a critical step which determines proper cell differentiation? If so, can one increase endoreplication cycles in *gl2* and thus rescue the trichome phenotype? This causal relationship needs to be addressed and is still an open question. It may well turn out that endoreplication is just one aspect for proper trichome differentiation. There may be other parallel processes which are regulated by GL2 which guide the cell through the differentiation steps. It is however possible that by increasing endoreplication levels in *gl2* trichomes by expressing some appropriate cell cycle genes (*E2F, DP*, etc.), one might be able to stop the *gl2* trichomes from re-entering mitosis but they would still be not completely differentiated, resembling the other aborted trichomes on *gl2* which do not divide. This reasoning is based on *gl3* mutant which has

low DNA content (16C) than wt trichomes (32C) but only shows defect in trichome branching (and initiations). The complete absence of trichome initiations in the *gl2 gl3* double mutant underscores the importance of endoreplication in trichome initiation. As mentioned before, some patterning activity is still on in the *gl2 gl3* mutant but no visible trichome initiations take place.

2.4.3 How does GL2 function during trichome patterning?

The finding that the number of trichome specifications, at the marker level, in *gl2* is more compared to wt suggests that trichome specification is not inhibited effectively in *gl2*. Further, occurrence of *try*-like trichome clusters and cells surrounding trichomes expressing an early trichome marker also indicate that lateral inhibition is compromised in *gl2* leaves. These phenotypes are reminiscent of *cpc* (more initiations) and *try* (trichome clusters) mutants. So, it is reasonable to speculate that GL2 positively regulates these inhibitors, *TRY* and *CPC*, which in turn mediate lateral inhibition during trichome patterning in wt leaves. This speculation is supported by the findings that GL2 is specifically required to initiate and maintain *TRY* expression in leaves. But the time course analysis of trichome initiation on *gl2* leaves showed that at any given point of time during leaf development the number of trichomes that develop in *gl2* is always lower than in wt, though more cells enter the trichome pathway, as observed by the marker. This indicates that GL2 function is required to further promote the cells which enter the trichome pathway to get committed to the trichome fate by initiating the process of morphogenesis and complete differentiation. In its absence, the inhibitors (TRY / CPC) are down regulated and more cells may enter the trichome pathway but do not succeed in initiating the morphogenesis process, which leads to their exit from the pathway. *CPC* expression is not found to be regulated by GL2 in roots but *GL2* expression has been shown to be negatively regulated by CPC. But the predictions from results in this study suggest that in leaves GL2 may positively regulate not only *TRY* but also *CPC* expression. The other explanation for the decreased levels of inhibitors leading to an increase in trichome cell specifications in *gl2* background is that GL2 may not be directly regulating the expression of *CPC* but due to trichome differentiation defects CPC levels may generally decrease.

Ubiquitous GL2 expression abolished trichome initiation in wt leaves which came as a surprise because GL2 promotes trichome initiation, as seen by the double

mutant analysis (*gl2 gl3*, *gl2 cpc* and *gl2 try*). According to the meinhardt model for trichome patterning an activator activates an inhibitor which in turn diffuses quickly to neighboring cells and inhibits the activator production in those cells. Accordingly, GL2 which is an activator (needed to promote trichome initiation) activates TRY (and possibly its homologs like CPC, ETC1 and ETC2) which inhibits trichome initiation. Thus when GL2 is ubiquitously expressed all the cells also express TRY (and other inhibitors) and thus it would resemble *35S::TRY* / *35S::CPC* phenotype, where trichome initiation is abolished. This interpretation is supported by the fact that *35S::GL2* in *try cpc* background did not dramatically inhibit trichome initiation in all the lines tested. The same lines however showed a very strong induction of ectopic trichomes on cotyledons and hypocotyls, a phenotype similar to what is found when GL1 or AtMyb23 are ubiquitously expressed in the *try cpc* background. A notable difference is the fact that GL1 and AtMyb23 expression also leads to ectopic trichome formation in the sub-epidermal layer as well as increases the number of trichome initiations on the leaf dramatically. Why does GL2 expression not lead to such an effect on leaf? On the contrary one of the lines showed a strong reduction in trichome number and clustering. This shows a difference between the other activators of trichome initiation like GL1, AtMyb23 and GL2. Though GL1, TTG and GL3 are found to act upstream of GL2 by positively regulating its expression, a mere expression of GL2 is not sufficient to produce the effects of *35S::GL1* expression for instance. Ectopic expression of GL2 (under *35S / GL1 / TRY* promoter) can neither rescue *gl1* or *ttg1* mutants, showing that GL2 expression is not sufficient to initiate trichomes in the absence of GL1 / TTG1.

Why does ubiquitous expression of GL2 using *35S* promoter lead to inhibition of trichome initiation but an additional copy of the gene (*pGL2::GL2*) in wt lead to increase in trichome initiations? The answer may lie in the fact that transcriptional regulation of the positive and the negative factors is important in creating the trichome pattern. According to the meinhardt model as discussed in the introduction, initially both positive (activator, say GL2) and negative factors (inhibitors, TRY / CPC) are equally present in all epidermal cells. Small changes in their concentration due to random fluctuations can be amplified given the fact that inhibitors can diffuse faster than activators to neighboring cells and inhibiting the activator production there while the activator levels in the less inhibited cells keeps increasing due to a positive feedback

loop. When GL2 expression is unregulated, as is the case while using a constitutive promoter like *CaMV 35S*, it is expressed in all epidermal cells, which leads to the production of TRY / CPC in all these cells. According to the model, in such a case there would be no chance to amplify small changes in activator/inhibitor concentrations and hence no trichome initiations, as all cells are equally inhibiting all other cells. But if GL2 levels are increased by expressing an additional copy of GL2 under its own promoter, it can be imagined that the patterning system would still work but the imbalance would slightly be shifted towards the activator peaks being produced at a higher frequency in a field of cells thus leading to higher number of trichome initiations.

It is not quite apparent what role the positive feedback loop seen between GL2 and TRY may play during trichome patterning. However it has to be noted that it is the first instance that a positive feedback loop identified in the context of trichome patterning, though it is one of the prime requirements of the meinhardt model. Knowing that gene redundancies are an integral component of most eukaryotic transcriptional networks and this interaction between *TRY* and *GL2* is just one component of the larger network involving other genes, it would be misleading to discount the importance of this feedback loop during trichome patterning. It has been hypothesized that robustness of biological networks to individual parameter variations would be a commonly occurring theme and properties conferring robustness would be conserved across species (Meir *et al*., 2002). In this light, one could speculate that some of the genes like *GL1* and *TTG* which are absolutely required for trichome initiation whereas interactions like the positive feedback loop between GL2 and TRY could have pattern resolving properties. This idea is discussed again in the next chapter where the final steps of resolving the trichome pattern in wt have been studied and the TRY / GL2 interaction finds more support. The GL2 gene is the founding member of a family of proteins called the '*GL2*-like homeodomain proteins'. So, it may well be that close homologs of GL2 belonging to this class may also function redundantly in this process along with the homologs of TRY. Fig.20 is the illustration of a modified model being proposed for trichome patterning with the inclusion of GL2 in the patterning step.

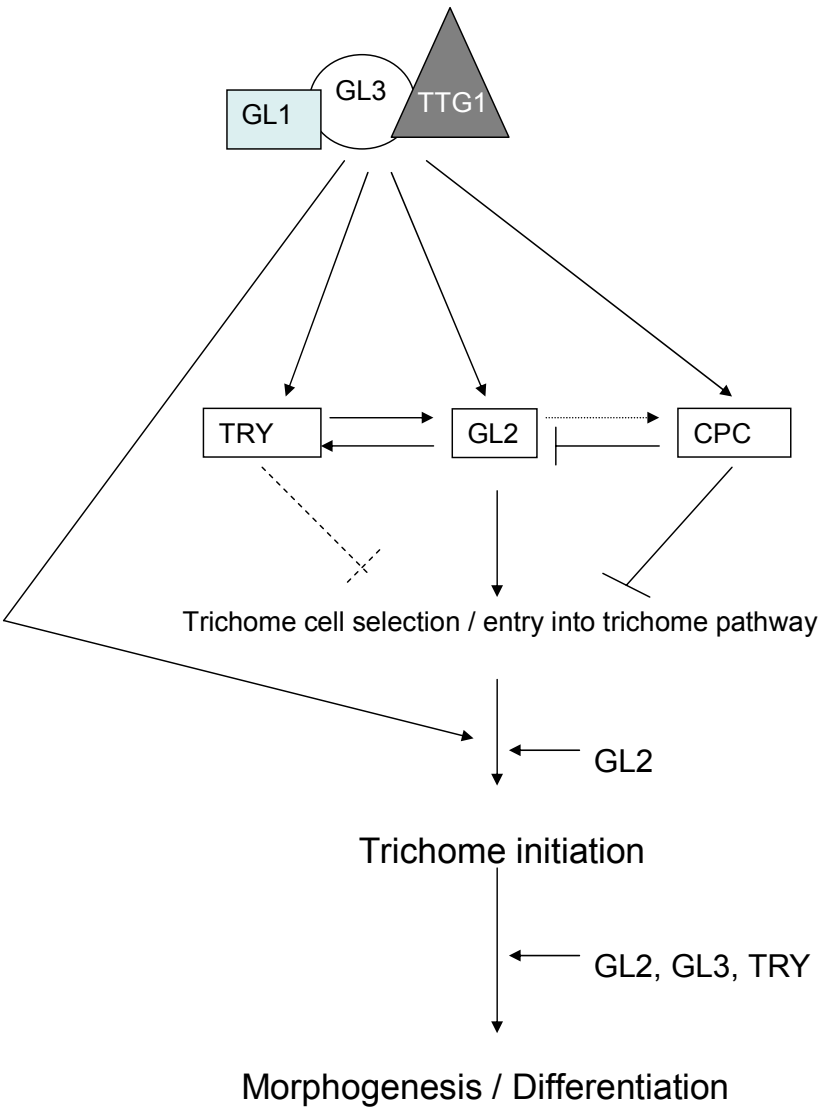


Figure 20: A genetic model for trichome patterning and differentiation
The transcriptional complex mainly made of GL1, GL3 and TTG1 activate TRY, CPC and GL2. Interactions between TRY, CPC and GL2 result in selecting cells to enter the trichome pathway and also create the spacing pattern. The next step is the initiation of the morphogenetic program (outgrowth of trichomes) for which both the function of GL2 and GL1 / TTG1 are required ectopic GL2 expression is not sufficient to rescue the glabrous phenotype of either gl1 / ttg1 mutants. The morphogenesis and differentiation step which also involves branching regulation is regulated by GL2, GL3, TRY and other downstream genes. Solid lines show relationships which have been studied so far. The dotted lines are speculations.
Note: Only genes well studied in the context of patterning are included in the model.

3

Understanding the mechanism of trichome patterning

3.1 Summary

To understand the process of trichome patterning the expression of an early trichome marker, *GL2::GFP-ER*, was analyzed at cellular resolution. Single cells getting selected from a group of apparently equivalent cells (representing a competence group) were found demonstrating de novo patterning during leaf development. The genetic interactions between *TRY*, *CPC* and *GL2* have been hypothesized to be sufficient for final resolution of the trichome pattern, which may be an outcome of competition in the trichome precursor between endoreplication and mitosis. Two predictions of the Meinhardt model have been tested and it was found that both *TRY* and *CPC* gene products show the ability for intercellular movement and when the concentration of either of them is increased to the levels of activators by expressing them with the *GL2* (activator) promoter, it results in glabrous leaves.

3.2 Results

3.2.1 Sub-cellular localization and ability for intercellular movement of TRY, CPC, GL2 and GL3

According to the Meinhardt model the inhibitors are expected to diffuse faster to neighboring cells than the activators and thus all the current models proposed to explain epidermal patterning either in root (root hair / non – root hair) or leaves (trichome) assume that neighboring cells communicate through the inhibitors TRY / CPC which can move between cells and result in the final pattern (Schellmann *et al.,* 2002; Hulskamp and Schnittger 1999). In roots it has been shown, by comparing the expression of the *pCPC::GFP* reporter with that of *pCPC::GFP-CPC* fusion protein construct that the CPC protein moves from the atrichoblast cells, in which it is produced, to the trichoblast cells (Wada *et al.,* 2002). Studies done so far have also concluded that TRY and CPC, though close homologs at the sequence level, functionally differ in their action during patterning.

It was attempted to check for the ability for intercellular movement of two inhibitors (TRY and CPC) and two activators (GL2 and GL3). The coding sequence of all the four genes was fused to EYFP in a construct with the constitutive *CaMV 35S* promoter and

terminator. The resulting four constructs *35S::EYFP:TRY*, *35S::EYFP:CPC*, *35S::EYFP:GL2* and *35S::EYFP:GL3* were used in a transient expression assay in Arabidopsis. Each of the constructs were co-bombarded with a *35S::ECFP-mTalin* vector into Arabidopsis cotyledon epidermal cells by microprojectile bombardment method (see materials and methods). The *ECFP:mTalin* protein binds to F-actin filaments and is cell autonomous. Therefore it acts as a good neutral control to detect the cells which initially get transfected by this method. After approximately 20 – 24 hrs plants were checked by fluorescence microscopy to detect the fusion proteins. Both the TRY and CPC proteins were found localized to the nucleus of the cell which got transfected (as visualized by the blue actin marker and yellow nucleus) as well as in neighboring cells (where no actin filaments were labeled but only yellow nucleus) demonstrating that the gene products had moved from the originally transfected cell to its neighbors (Fig.21). This clearly demonstrates the ability of transcription factors for inter-cellular movement. However, both EYFP-GL2 and EYFP-GL3 proteins were localized only in the cell which got transfected and were not detected in neighboring cells showing that they do not have the ability for intercellular movement (Fig.21). Both TRY and CPC were found localized both in the nucleus and cytoplasm of cells whereas GL3 and GL2 were strictly localized in the nucleus.

3.2.2 Mechanism of Trichome cell selection

It is assumed that cells on the leaf epidermis are initially equivalent and a patterning mechanism leads to the selection of some as trichomes. As there does not seem to be a position dependent or cell lineage dependent mechanism involved in creating the trichome pattern, it is suggestive that it could occur by a *de novo* patterning mechanism starting from a pool of equivalent cells. But it has not been shown whether that indeed is the case. Expression analysis of the *GL2:GUS* reporter (which is an early marker for trichomes) showed that the marker is very strongly expressed in trichomes but to a weaker extent in cells surrounding it (Szymanski *et al.,* 1998). But that was already a late event when the trichome can be morphologically identified to be different from its neighbors and hence whether cells were equivalent before one of them got selected to be a trichome remains an open question. Such a question can be addressed by a more careful analysis of a GFP reporter which is cell autonomous. Hence the

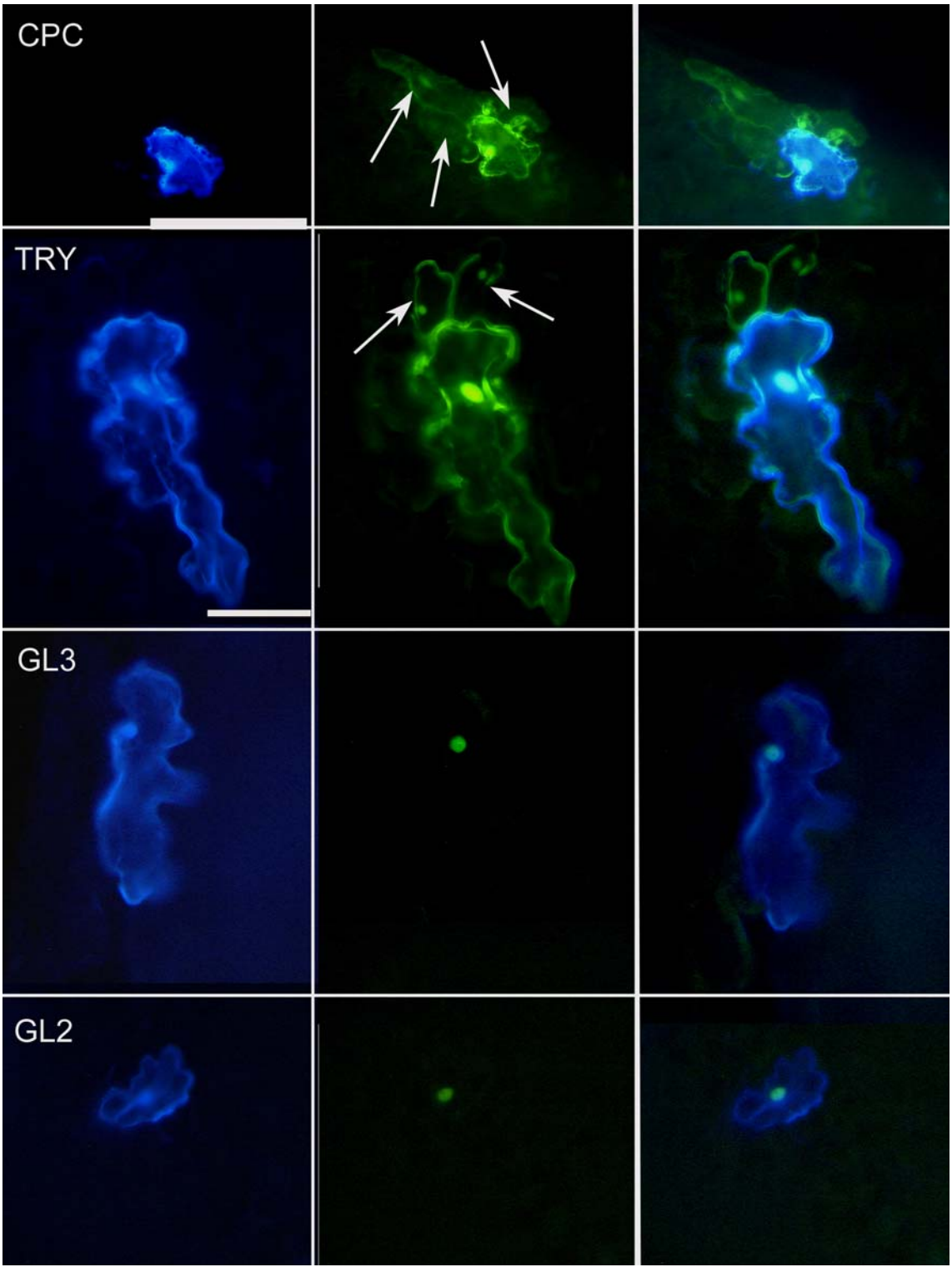


Figure 21: Intercellular mobility of genes involved in trichome patterning.
In frame translational fusions of TRY, CPC, GL2 and GL3 were created by fusing with EYFP and the resulting constructs (*35S::EYFP:TRY, 35S::EYFP:CPC, 35S::EYFP:GL2 and 35S::EYFP:GL3)* were co-bombarded with 35S::ECFP-mTalin construct into Arabidopsis cotyledon cells by the microprojectile bombardment method. Transfected cells expressing the respective genes were analyzed after 16 – 24 hrs post bombardment. The left panel shows pictures taken using the CFP specific filter, middle panel with YFP-specific filter and the right most panel shows an overlay of both the CFP and YFP images. The cells transfected by the bombardment method express CFP-mTalin which binds to F-actin filaments. The same cells also expressed all the EYFP protein fusions tested which were localized to the nucleus. However, both EYFP:TRY and EYFP:CPC could be found also in cells (arrows) neighboring the transfected cell whereas GL2 and GL3 were not. This clearly shows that both TRY and CPC, but not GL2 and GL3, move from the cells where they are expressed to the neighboring cells. Note that both TRY and CPC are localized both in the nucleus as well as in the cytoplasm of cells whereas GL2 and GL3 are strictly localized only in the nucleus. Scale bars: top most panels = 100 µm, all other pictures = 50 µm.

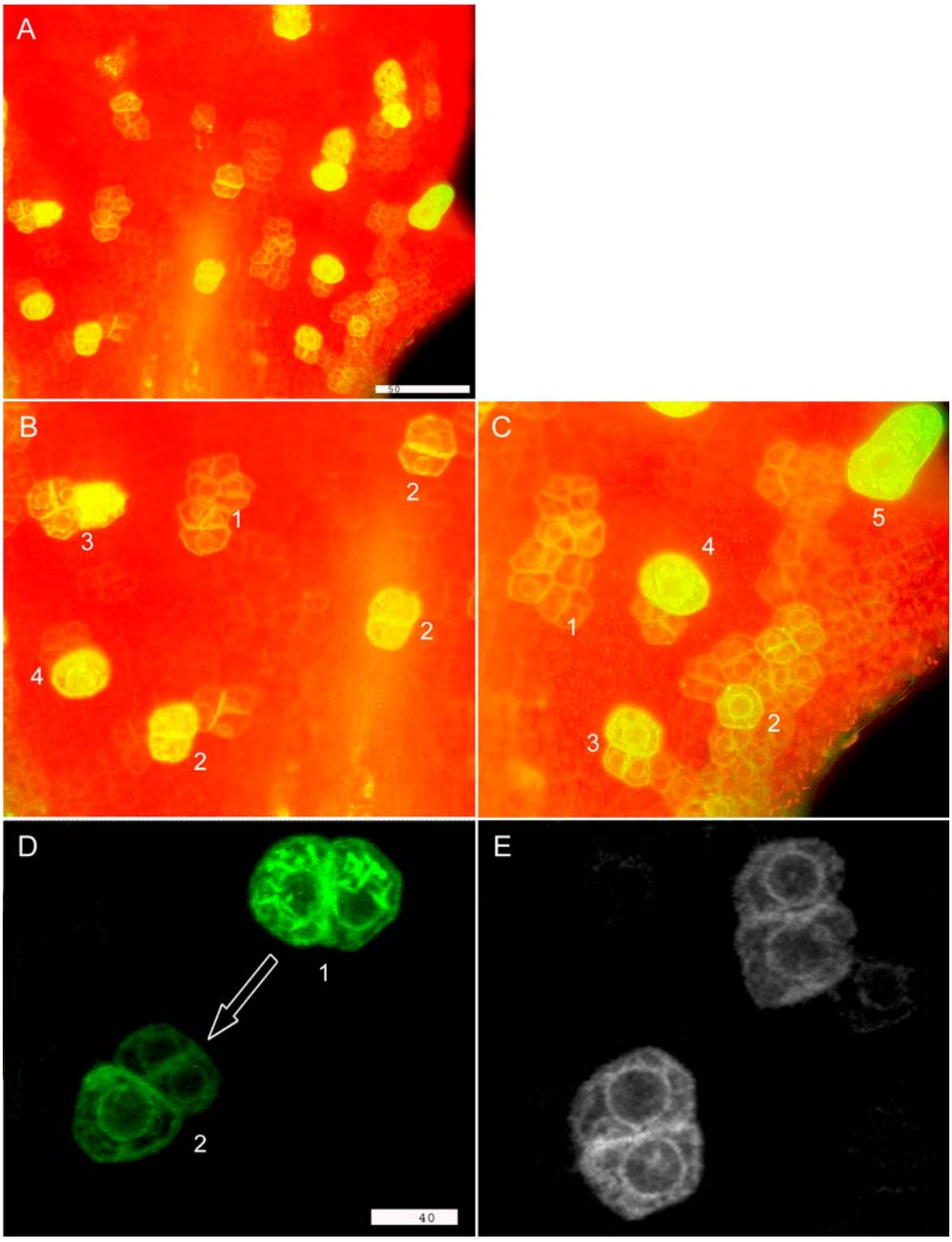


Figure 22: Analysis of the *GL2:GFP-ER* marker to understand trichome patterning
Wild-type (Ler) plants expressing the *GL2::GFP-ER* construct were analyzed (2nd pair of leaves) by fluorescence microscopy. The basal portion of young leaves showed highly dynamic patterning activity (A) with many groups of cells (competence groups) expressing the marker at apparently same levels. One of the cells in most of these groups was expressing the marker at very high levels indicating its commitment to the trichome pathway. Selection of one single cell from a group of equivalent cells (with respect to the expression of this marker) can be clearly seen in C 2. Frequently two equally large cells (doublets) expressing the marker at the same levels appeared to compete with each other to become trichomes (B 2, D 1 and E). Triplets of cells where one of the cells in the doublet had divided also occurred frequently (C 3 and D 2) suggesting that mechanistically winning the competition to become a trichome lies in one cell (winner) inducing the other (loser) to enter mitosis and divide. From this point one can expect that the two smaller cells in the triplet more effectively inhibit each other than they can inhibit their dominant neighbor, while the dominant cell continues to further inhibit them. This spiraling imbalance in inhibition continues and results in further division of the smaller cells as can be seen in B 3, C 4 and C 5 by which time the 'winner' has already initiated the trichome morphogenetic program (C 5).

GL2:GFP-ER was used as the early trichome marker and young leaf primordia were analyzed to gain insights into how trichome cell selection takes place and if indeed cells are equivalent to start with.

The basal portion of young wt leaf primordia showed very dynamic patterning activity with many cells being selected to enter the trichome pathway (Fig.22A). Small groups of cells expressing the marker at very similar levels could be found which seemed to be still competing with each other to get selected as trichomes (Fig.22 B.1, C.1). A single cell which gets selected from such competing groups could also be seen as shown in Fig.22 C.2. This is the first documentation of cells being equivalent, with respect to their size and the expression of an early trichome marker, from which single cells get selected as trichome initials. Thus intensity of the marker expression is one criterion, besides increase in cell size, for the commitment of a cell to trichome fate before it shows any morphological feature of a trichome by growing out of the leaf surface and expanding (Fig.22 C.2).

What was more interesting to observe was the presence of two cells, which were equally large and expressing the marker at equal levels, seemingly competing with each other to become trichomes. These doublets (Fig.22 B.2, 1.D) were very frequently seen on wt leaves. But as trichome clusters very rarely exist (< 0.1%) on wt leaves it means that one cell from this doublet wins the race to become a trichome while the other is effectively inhibited. What is the mechanism by which one cell inhibits the other in such doublets? An obvious clue to this question came from the finding of triplets of cells (Fig.22 C.3) where one of the cells in the doublet symmetrically divides with a concomitant reduction in marker intensity compared to the undivided cell (Fig.22 D). This shows that one of the cells wins the competition to proceed further in the trichome pathway by probably going into a endoreplication mode (as is apparent by its increasing size) while the cell which loses enters mitosis and divides till all its daughter cells are completely inhibited and express no trichome marker any more (Fig.22 B.3, B.4, C.4, C.5). Figure 22 B and 1C illustrate the possible sequence of events that may eventually lead to the selection of single isolated trichomes on leaves.

3.2.3 Expression of TRY or CPC by *GL2* promoter leads to trichome inhibition

To test what happens when the regulation of *TRY* and *CPC* are changed by expressing them with the promoter of a positive factor (GL2), transgenic plants in wt background were created which expressed either *GL2::TRY* or *GL2::CPC*. More than 10 lines in both the cases were analyzed and a majority of them showed completely glabrous first pair of leaves (Fig.23; pg. 63). In the others there were a few trichomes left on the leaves indicting strong inhibition of trichome initiation. Expression of *GL2::GL1* did not appear to have any effect on frequency of trichome initiation, whereas *GL2::GL3* resulted in increased trichome initiations similar to the effect seen on *35S:GL3* plants (Payne, 2000).

3.3 Discussion:

3.3.1 How does the trichome spacing pattern arise?

Analysis of the GL2::GFP-ER expression pattern in young wt leaves has shown for the first time that single cells get selected from a competence group to become trichomes. It needs to be noted however that selection to enter the trichome pathway and commitment to become trichomes are two different steps, as noted in chapter 2 with the analysis of GL2 function in this regard. It is possible that one of the crucial steps in selection and commitment to the trichome pathway is an outcome of the competition between the endoreplication and mitosis modes of cell cycle. How do the doublets arise in the first place? It is very likely that even after a cell gets selected from a group of cells (Fig.22 C.2) by some mechanism of lateral inhibition the cell may still be encountering the competition within to either enter the endoreplication or the mitosis mode. If it enters mitosis and divides once, it gives rise to the doublet of cells which are both equally competent to become trichomes (Fig.22 D.1, E). Irrespective of how the doublet arose it is more revealing to understand the mechanism of how, starting from this point, one of the cells wins the race to become a trichome while the other adopts the default epidermal fate. This step may be considered as the final pattern resolving step during trichome selection. One can speculate that mechanistically the way one cell inhibits the other in this step may be by itself continuing in the endoreplication mode but induce the other cell to enter mitosis and divide. This would then result in a triplet

(Fig.22 C.3, D.2). The two newly formed daughter cells in the triplet are now far behind in the competition to become trichomes when compared to their large dominant neighbor and hence they only end up inhibiting each other while their 'strength' to inhibit the larger cells is not sufficient enough, and at the same time its all the more easier for the larger cell to inhibit the two smaller cells. The net result of this now biased race between these three cells is that the two smaller cells again enter mitosis and divide to give rise to a quartet (Fig.22 B.3) and eventually a group of small cells dividing with less and less of the marker (reflecting their low chances of getting selected as trichomes) while their neighbor has already progressed very far in the trichome pathway, through additional rounds of endoreplication and having initiated morphogenesis (Fig.22 C.5).

3.3.1 The interactions between TRY, CPC and GL2 may be sufficient to explain the final resolution of the trichome pattern

Two genes, *TRY* and *CPC*, have been implicated in lateral inhibition during trichome patterning. While *cpc* mutants show an increased number of trichome initiations, *try* mutants show an increased frequency of trichome clusters where trichomes develop adjacent to each other. This indicates that their mode of action during lateral inhibition is different. Also, *try* mutant trichomes show increased levels of DNA content (64C – 128C) compared to wt (average 32C) suggesting that TRY suppresses endoreplication in trichomes (Schnittger *et al.,* 1998; Szymanski and Marks, 1998) and there by may facilitate its entry into mitosis. Is there any mechanistic link between the role of TRY in cell cycle and its ability to prevent adjacent trichome formation by lateral inhibition? From the results presented above it appears that one frequent (not always) situation encountered during the final resolution of pattern in trichome cell selection is the competition between two equally large cells. Is it possible that TRY mediates lateral inhibition at such situations that finally results in the selection of one single cell as trichome? The *try* mutant trichome phenotype of two adjacent trichomes occurring frequently is suggestive of its role in this context. If TRY is indeed involved then what is the mechanism by which it mediates inhibition? The model proposed in Fig.24 explains how the patterning system may work. One can imagine the situation to be as proposed in the meinhardt model. The two cells in the doublet are equivalent to start with and are competing with each other to become a trichome. Due to stochastic

reasons one of the cells produces slightly more TRY and CPC than the other. When TRY / CPC diffuse quickly to neighboring cells (look at next section for proof) TRY inhibits endoreplication while CPC represses *GL2* (a positive factor required for trichome initiation) and TRY expression in that cell. In effect the cell enters mitosis and its ability to enter the trichome pathway reduces. Thus one cell gets selected to become a trichome while the other enters the default epidermal pathway. The regulatory relationship shown in the model (Fig.24) can be simplified to a two component system with the properties described by the meinhardt model. As shown in the previous chapter, GL2 and TRY can be considered as the 'activator' component with the self activation loop (positive feedback) and CPC as the 'inhibitor' component which inhibits the activator (GL2 and TRY) production. With TRY and CPC able to communicate with neighboring cells by intercellular movement (while GL2 does not) the final resolution of the pattern can occur with only one cell being successfully selected to become a trichome. The positive feedback loop between TRY and GL2, as shown in chapter 1, could be very important in this final step to resolve the pattern. The model proposed assumes that GL2 also positively regulates CPC. But in roots it has been shown that the expression of CPC is not regulated by GL2 (Lee and Schiefelbein, 2002). Does it mean that in the root and leaf tissues the mechanism is different? It needs to be clarified further by more experiments. The observation that an additional copy of the GL2 gene in wt (GL2::GL2) leads to increased trichome initiations and trichome clustering (twins) fits the above model well. When frequently two cells are locked in a competition to become trichomes in the final step, increased levels of the positive factor, GL2, may over-ride the mechanism leading to both these cells acquiring trichome fate which may explain the increased frequency of trichome twin clusters.

When the inhibitors TRY / CPC are expressed by the *GL2* promoter their concentration in cells would be as high as the activators and hence would not meet one of the requirements of the meinhardt model where the levels of inhibitors, though present in trichomes, is lower than that of activators. The absence of this difference in relative levels of activator / inhibitor concentrations could explain why there is complete inhibition of trichomes in plants carrying *GL2::TRY* / *GL2::CPC*.

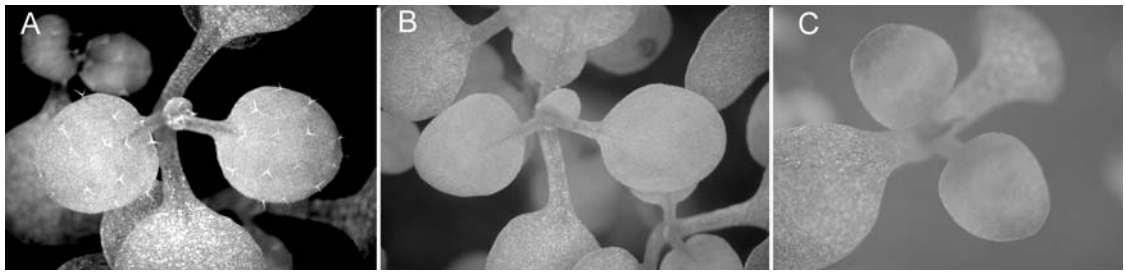


Figure 23: Expression of either TRY or CPC by the GL2 promoter leads to glabrous leaves
Wild type (Ler) plants have regularly distributed trichomes on leaves (A). But plants expressing the GL2::TRY (B) or the GL2::CPC (C) construct have glabrous leaves showing a complete inhibition of trichome initiation when their levels (inhibitors) are increased to possibly the same levels as the activators (by using a activator promoter).

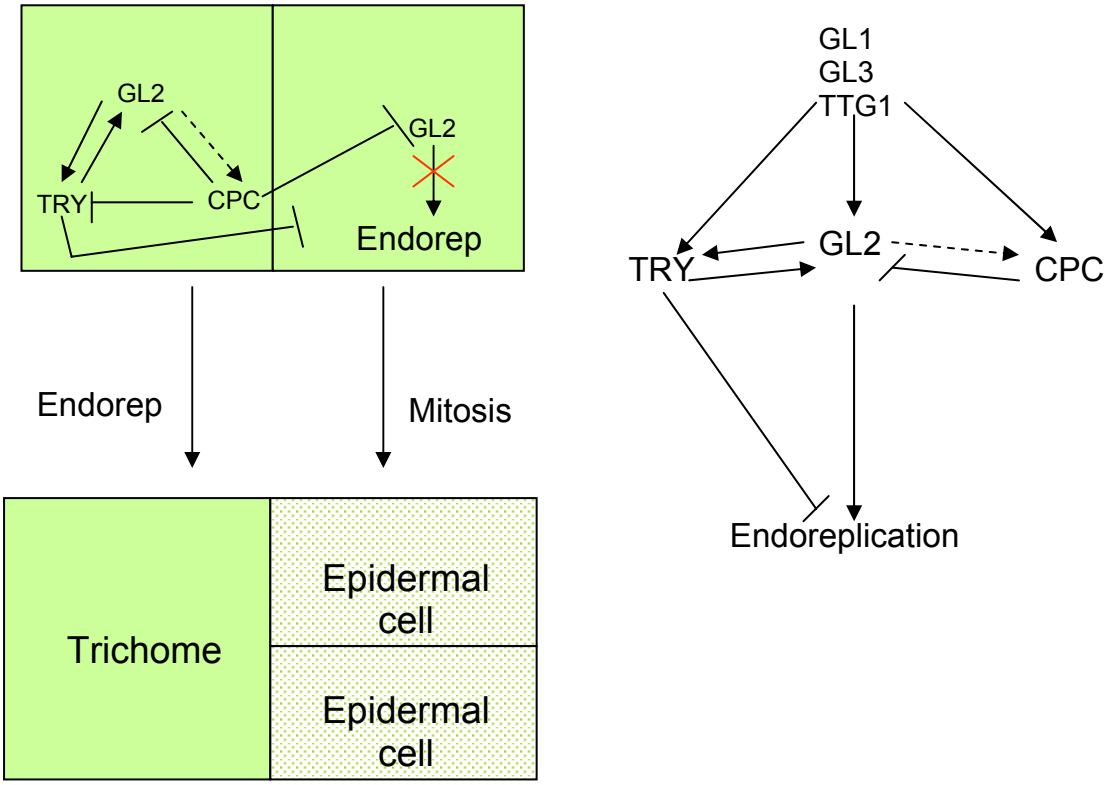


Figure 24: Cartoon model explaining how the final trichome pattern is resolved.
Shown on the above left is the doublet trichome precursor which is frequently seen on wt leaves. It is hypothesized that interactions between TRY, GL2 and CPC as indicated in the diagram are sufficient to select only one cell to become a trichome. At this stage, mechanistically 'Inhibition' can be thought of as competition between endoreplication and mitosis modes of cell cycle. GL2 promotes endoreplication and thus commitment to trichome fate. GL2 also activates the production of both TRY and CPC. Whereas CPC represses GL2 expression, TRY suppresses entry into endoreplication mode. The interactions between TRY, GL2 and CPC can be reduced to the Meinhardt model with TRY and GL2 representing the activator with an auto activation loop and CPC being the inhibitor. Starting with two cells in the doublet being equally likely to become trichomes, competition between them, as mediated by TRY and CPC movement between the cells, results in one cell inhibiting the other more which mechanistically results in the 'winner' entering the endoreplication mode while inducing the 'loser' into the mitosis mode. Both the inhibitors differ in their mode of action with TRY directly inhibiting entry into endoreplication while CPC represses GL2 production and thus indirectly inhibits entry into endoreplication. Failure to enter endoreplication mode results in mitosis. The diagram on the right shows that all three components (TRY, CPC and GL2) are activated by the activator complex consisting of GL1, TTG1 and GL3.

4

A novel method to simultaneously analyze multiple gene expression patterns *in vivo* (MEPI)

4.1 Summary

The use of reporter genes for the study of gene expression patterns in vivo has become routine. Among the various reporter genes used the green fluorescent protein (GFP) has become the tool of choice because it can be used to continuously monitor changes in gene expression patterns in live cells by fluorescence microscopy. Currently many GFP variants exist with different spectral properties and increased fluorescence levels. However, because of the overlaps in the emission spectra of these not all of them can be simultaneously used as reporters in the same specimen. To obviate this problem of spectral overlap which limits the number of reporter genes that can be used at a time, a novel approach which combines color and sub-cellular localization to differentiate between the different reporters is proposed. Accordingly, instead of GFP (or its variant) localizing to the entire cell it can be targeted to different distinct sub-cellular structures / organelles, which can be easily visualized and distinguished from each other, to create many reporters using the same color (fluorescent protein). Thus, by using this method, which has been named **MEPI** (for **m**ultiple gene **e**xpression **p**atterns ***i****n vivo*) potentially many genes can be analyzed simultaneously in a single specimen to understand their relative spatial and temporal expression patterns. For example: by using two spectral variants of GFP, namely CFP and YFP, and localizing them to 2 different sub-cellular structures (F-actin and peroxisomes) four different reporter genes can be created and used together at the same time. A proof-of-concept has been demonstrated using a transient method where three different promoter::reporter constructs have been visualized in the same plant. Future perspectives and potential applications of the method as a tool in developmental biology are discussed.

4.2 Introduction

Development is a cumulative effect of dynamic changes in gene expression in different cells within an organism. All developmental processes involve the action of many genes interacting as part of regulatory networks and hence it becomes imperative to know: a) The different genes that are expressed in defined cell types / tissues b) The sub-cellular localization of the gene products and c) The spatial and temporal pattern of gene expression in the given developmental context. Several methods currently exist to understand global changes in gene expression patterns in a defined cell type/s,

developmental state or after some kind of environmental / external stimuli are applied to a cell / organism. Microarray analysis of gene expression patterns has become one of the most popular methods to analyze global gene expression patterns. Simpler methods exist which allow examination of gene expression through measurement of either RNA or protein distribution within fixed tissue. Gene expression can be measured either directly by using probes and antibodies or indirectly by detecting the product of a fusion between the gene of interest and a reporter gene. Reporter genes have been used as convenient markers to visualize gene expression and protein localization in vivo in a wide spectrum of prokaryotes and eukaryotes. However, the detection of the commonly used reporters such as β-glucuronidase (GUS) (Jefferson *et al.,* 1987), β-galactosidase (LacZ) (Teeri *et al.,* 1989), chloramphenicol acetyltransferase (CAT) (Seed and Sheen 1988) and luciferase (LUC) (Gallie *et al.,* 1989) requires exogenous substrates / co-factors or antibodies. Their applications are sometimes limited by problems of substrate uptake, leaky product, cell fixation and cell permealization, especially in multicellular organisms. For purposes of monitoring gene expression dynamics continuously in real time in developing tissues in an organism the above mentioned methods cannot be used. The green-fluorescent protein (GFP) from jellyfish Aequorea *Victoria* has thus become a vital and convenient new tool as a reporter gene in various heterologous systems because the expression of GFP is independent of cell type and location and just blue or UV light and oxygen are required for GFP emission but not any other exogenous substrates (Chalfie *et al.,* 1994; Heim *et al.,* 1994). Unlike enzyme markers, GFP can be visualized at high resolution in living cells using conventional fluorescence or confocal microscopy. The images are not prone to fixation or staining artifacts, and can be of exceptional clarity (Haseloff 1999; Haseloff and Amos 1995). Moreover, the activities of living cells, such as cytoplasmic streaming, are clearly evident during microscopy. It is possible to monitor dynamic events by time-lapse confocal microscopy, and this combination of a vital fluorescent reporter with high-resolution optical techniques is ideal for use in studying cell biological or developmental processes.

GFP has been, in the last few years, extensively used as a transcriptional reporter, fusion tag, and biosensor. In plants GFP has been used very routinely by fusing it to the 5' promoter regions of genes and creating transgenic plants carrying such a reporter construct to enable visualization of the specific gene expression pattern at cellular resolution. Although in recent years many GFP variants with new colors,

improved fluorescence properties and expression levels have been generated by mutagenesis experiments, it has not been possible to simultaneously use more than two reporters in the same sample because of overlaps in the emission spectra of these variants. The only combination of GFP variants that have their excitation and emission spectra well separated are the Cyan Fluorescent protein (CFP) and Yellow Fluorescent Protein (YFP) and hence are suitable for such a dual labeling experiment. Another red shifted GFP variant, the Red Fluorescent protein (RFP), is of not much use in plants as the emission spectra of chlorophyll, which is present in all aerial parts of plants, overlaps and masks the RFP signal.

GFP has also been used to target / decorate almost all cell organelles and structures inside living cells by fusing GFP to a signal peptide or protein which can target it to the specific organelle or structure. This method has found several applications in cell biological studies at very high resolution. Examples of organelles and structures that have been studied by targeting GFP or GFP variants to them are: golgi apparatus, peroxisomes, chloroplasts, nucleus, endoplasmic reticulum, mitochondria, actin filaments, microtubules, cell wall, vacuoles and many others.

Instead of using just color to differentiate between different reporters one can target the given fluorescent protein (FP) to some distinct intracellular organelle or structure like nucleus, or peroxisomes or F-actin filaments which very clearly differ in their size, shape and motility inside a cell and thus create many different reporters using the same color. Existing methods based only on color differentiation allow the use of CFP and YFP as two reporters simultaneously. Now, if each of these is targeted to two different sub-cellular locations like peroxisomes and F-actin filaments then in effect we have created 4 different reporters. This new approach which combines color with sub-cellular localization of FPs to distinguish between different reporters has been named **MEPI** (for **m**ultiple gene **e**xpression **p**atterns **i**n vivo). Using sub-cellular targeting of FPs with a good fluorescence microscopy technique (at cellular resolution) will allow the visualization of multiple reporter gene expression patterns in living specimens. Another important advantage of targeting the FP to a specific structure or organelle inside a cell is that it makes the protein cell autonomous and would be restricted strictly to the cell in which it was expressed. In plants it has been shown that when GFP is expressed in a cell it passively diffuses to neighboring cells through the plasmodesmatal connections and this problem could be successfully overcome by targeting GFP to

endoplasmic reticulum (Haseloff *et al.,* 1997). Especially in plants, when it is intended to examine gene expression patterns at the cellular resolution, it would be best not to use untargeted GFP for the above mentioned reasons.

As an example consider analyzing 3 different genes *TRY*, *CPC* and *GL1*, involved in trichome patterning (Fig.25). Instead of fusing the respective gene promoters to untargeted YFP we do the following:

a) Fuse *TRY* promoter to YFP with a peroxisome targeting singal.
b) Fuse *CPC* promoter to YFP with a nuclear localization signal and
c) Fuse *GL1* promoter to a chimeric YFP-mTalin gene which targets the YFP to F-actin filaments.

Transgenic plants carrying all these three promoter:reporter (PR) gene constructs can now be analyzed using a fluorescence microscope. We observe cells and see which sub-cellular structure is fluorescent. Some cells may contain only yellow peroxisomes (which shows that only *TRY* gene is expressed there) or yellow nucleus (*CPC* gene is expressed) and some may contain yellow peroxisomes, yellow nucleus as well as yellow actin filaments, showing that these cells express all the three genes (*TRY, CPC* and *GL1*). Thus by just identifying the sub-cellular structures having fluorescence a relative gene expression map could be created for many genes involved in a particular pathway. Using the same principle if two colors are combined with 3 different structures then one can potentially analyze 6 different genes simultaneously.

4.3 Results

4.3.1 Creating individual promoter: reporter (PR) constructs

As a proof of concept, a small scale project was initiated to simultaneously monitor the expression of three genes. The promoters selected to be used in the study were *p35S* (0.85 Kb), *pTRY* (1.4Kb), *pCPC* (1.2 Kb), *pGL2* (2.1 Kb) and *pTTG1* (2.2 Kb). The genes selected to be used as reporters were YFP-mTalin (localizes to F-actin filaments), CFP-mTalin and YFP-Peroxi (localizes to peroxisomes). YFP and CFP were amplified by PCR with 5' XhoI and 3' NaeI sites using the EYFP and ECFP constructs bought from Clontech®. The PCR products were digested and used in a regular cloning procedure to replace the GFP part in *35S::GFP-mTalin* construct (obtained from Jaideep Mathur), thus creating the YFP and *CFP-mTalin* reporter genes. *YFP-Peroxi*

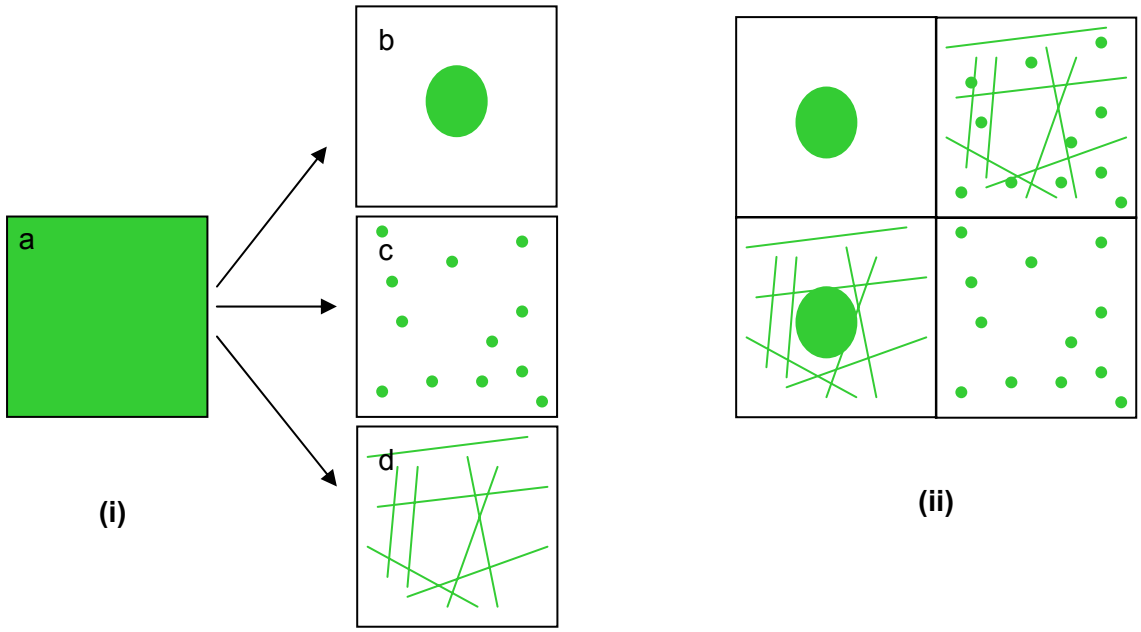


Figure 25 : A cartoon illustration of how MEPI works.

- If untargeted GFP (or its variant) is used as a reporter it localizes to the entire cell where the gene is expressed (a). Instead, one can target the same fluorescent protein (FP) to various distinct organelles or structures inside a cell like nucleus (b), peroxisomes (c) or the F-actin filaments (c) which clearly differ in their shapes and motility properties, and thus create 3 different reporter genes using the same FP (color).
- When a field of cells are expressing different combinations of genes (involved in some particular cell patterning process) MEPI can be used to visualize the relative gene expression patterns at cellular resolution. Three different gene:reporters (represented by green Nucleus, green peroxisomes and green actin filaments) can be simultaneously used in a single specimen and their combinatorial expression pattern detected which provides a more holistic perspective of their action.

(Clontech®) was subcloned into an amplifying vector containing *CaMV 35S* promoter and terminator sequence. The different promoter fragments were also amplified by PCR with suitable restriction sites flanking them. In the next step the promoters were sub-cloned into the reporter constructs resulting in different individual PR constructs.

4.3.2 Testing the PR constructs by transient expression in Arabidopsis

To test whether expression of three different reporter genes targeted to F-actin filaments (YFP and CFP) and peroxisomes (YFP) can be clearly distinguished in living cells the PR constructs were expressed both separately and in all combinations in onion cells by a transient method. Figure 26 (upper panel) shows an example where *p35S::YFP-mTalin*, *pGL2::CFP-mTalin* and *pTRY::YFP-peroxi* DNA constructs were introduced into onion cells by microprojectile bombardment and visualized after ~16 hrs by fluorescence microscopy. Cells expressing the individual constructs were easily visible with bright fluorescence from the respective sub-cellular location. Two different filter sets to visualize YFP and CFP were used. F-actin filaments were visible as either long cables or as a reticulate meshwork of fine threads. Peroxisomes were visible as brightly colored small dots with spherical or oval morphology and an approximate length of 1.5 µm, as has been reported previously (Mathur *et al.,* 2002). The constructs were then co-bombarded in different combinations of two's and all three together to see if they can still be easily distinguished in a single cell. As can be seen in figure 26 even when cells were expressing all three constructs together the reporter genes could easily be detected and distinguished. While using the YFP filter both yellow peroxisomes and yellow F-actin filaments could be detected and cyan actin was visualized when the CFP filter was used. Further, the same transient expression method was used to express the constructs in Arabidopsis epidermal cells (Fig 26 lower panel) and the results were the same, with the expression of all the three reporters in a single cell also being distinguished very easily. The peroxisomes were distinct by their small ovoid shape as well as their mobility. The pictures presented in figure 26 were taken at a single plane by normal fluorescence microscopy and hence only a few peroxisomes are visible. This demonstration shows that the method can be used to analyze at least 3 different genes simultaneously in Arabidopsis and can be further extended by selecting proper reporter gene combinations of color and structure (localization) to analyze more than 3 genes

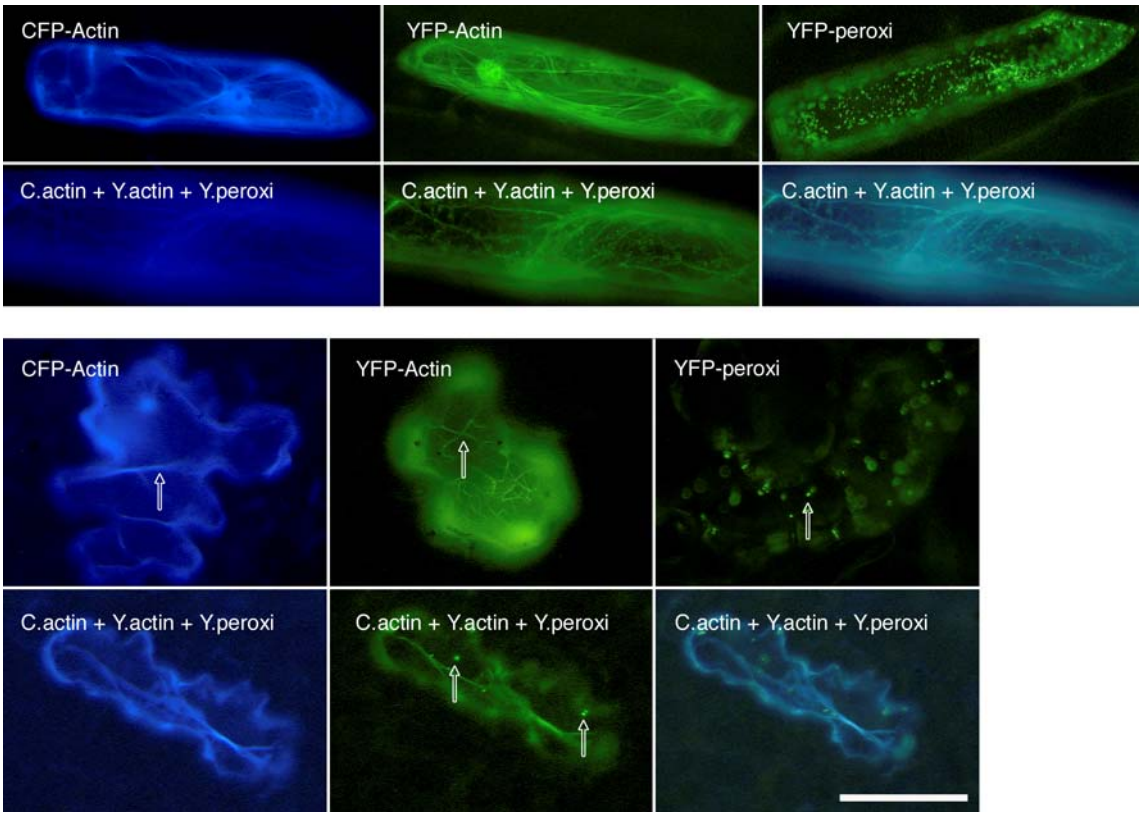


Figure 26: Expression analysis of different reporter constructs in Onion and Arabidopsis epidermal cells.
DNA constructs of three reporter genes (*pGL2::CFP-mTalin, p35S::YFP-mTalin* and *pTRY::YFP-peroxi*) were introduced into Onion (upper panel) and Arabidopsis (lower panel) epidermal cells by microprojectile bombardment method and their transient expression was analyzed by fluorescence microscopy after ~20 hrs.

Upper panel: Onion epidermal cells expressing the three reporter gene constructs separately (top three). The *pGL2::CFP-mTalin* and *p35S::YFP-mTalin* constructs label the F-actin filaments which are clearly visible as long strands crisscrossing the cell while *pTRY::YFP-peroxi* construct labels peroxisomes which are visible as small motile ovoid bodies. A CFP specific filter was used to visualize CFP-actin while YFP-actin and YFP-peroxisomes were visualized using a YFP specific filter. The 2nd row of pictures shows the expression of all the three constructs in a single cell. The CFP filter image is shown on the left (CFP-actin), YFP filter image in the middle and an overlay of the two channels on the right. Note that the expression of all the three constructs can be clearly detected and distinguished in this single cell (2nd row right most). (The nucleus is also labeled in cells expressing the YFP/CFP-mTalin construct due to the actin cage that is formed on its outer surface).

Lower Panel: Arabidopsis epidermal cells expressing the individual constructs (3rd row) show actin filaments as distinct strands (arrow - CFP-actin and YFP-actin) and peroxisomes (YFP-peroxi) as small motile bodies (arrow).A single epidermal cell (4th row) expressing all the three reporters simultaneously. Peroxisomes (arrow) and actin filaments can be seen in the middle cell.

simultaneously in vivo. It has to be noted that the 35S::YFP/CFP:mTalin constructs label actin effectively but also label the nucleus (as actin forms a cage around the nucleus) and hence cannot be used in combination with a reporter targeted to the nucleus.

4.3.3 Creating a suitable binary vector to carry many PR genes for transformation

The PR fusion genes were created such that they could easily be excised out as either NotI or AscI or SbfI (all 8 cutters) fragments. The pGreenII-Bar (Basta resistance) and p-GreenII-Kan (Kanamycin resistance) were used as the binary vectors to clone the PR genes. The pGreenII vector was first modified by removing the existing multiple cloning site between SacI and KpnI. To this region a polylinker was added which contained the 3 unique 8 cutter restriction sites in the following order SacI, **NotI, AscI, SbfI**, KpnI. This modified pGreen vector can now be used to clone the individual PR fragments sequentially in the unique 8 cutter sites and then to create transgenic plants for the analysis of the expression pattern of at least 3 genes.

4.4 Discussion

4.4.1 The challenge in creating transgenic plants carrying many PR genes

The transient experiment using the three different PR constructs demonstrates that MEPI could be potentially used to analyze the expression patterns of at least three or more genes simultaneously *in planta*. The main challenge in achieving that objective would be to create transgenic plants carrying the many different promoter:reporter (PR) fusion genes. One could consider cloning at least 3 PR fusions into one binary vector and use it to create transgenic plants by agrobacterium mediated plant transformation protocol. In this case each of the individual PR genes must be sequentially cloned into a suitable binary vector, preferably the pGreen vector as its starting size (3.3 Kb) is much smaller compared to other available binary vectors. The maximum number of genes that can be transferred into plants by agrobacterium mediated transformation would

depend on the final size of the T-DNA in the binary vector. If each PR fusion on an average is about 3.5 – 4 Kb then, at the most 3 genes can be transferred with reasonable efficiency. Longer T-DNAs do not seem to be able to be faithfully transferred by the agrobacterium transfer method. One way to overcome this problem would be to do sequential transformation of plants with the different PR gene constructs or to cross two transgenic plants which already harbor two or more PR genes. This obviously would consume more time than a one step transformation method. However, this could be the best way of achieving the goal.

4.4.2 The challenge of monitoring dynamic changes in gene expression patterns

The use of confocal laser scanning microscopy would best suit a continuous monitoring of changes in gene expression patterns at very high resolution. But the Leica TCS SP2 confocal system is not well equipped to illuminate and detect CFP at reasonable expression levels. However, the same levels of CFP expression can be very well detected in a conventional fluorescence microscope, though the problem of background fluorescence blurring is an undesirably problem encountered. But given the fact that very good specific filters to detect CFP, YFP and a dual filter set to detect CFP and YFP together are available to be used with conventional fluorescence microscopes it should be a better bet than confocal microscopy.

4.4.3 Future perspectives and potential applications of the method

MEPI can potentially be used to address different kinds of questions like the hierarchy of action of a set of genes involved in any developmental process, or the differential regulation of gene expression with time among a network of genes. Some of the questions relevant to epidermal cell patterning in Arabidopsis which were encountered during the course of research work presented in this thesis and could be addressed using MEPI are:

a) The molecular nature of non-TRY expressing trichome initials on gl2 leaves

As discussed in a previous chapter the *gl2* mutant leaves produce trichome initials some of which express *TRY* and some which do not. Among those which do express TRY there is variability in the levels of *TRY* expression

as monitored by the *pTRY::GFP* marker. g*l2* mutant leaves also show compromise in lateral inhibition where some of the trichome initials appear to be not so effective in inhibiting their neighbors from expressing a trichome marker. Also, some try like clusters are found on *gl2* leaves, the dominant cell of which in many cases is divided. In this context it will be interesting to simultaneously monitor the expression pattern of *TRY*, *GL2* and *CPC*. Do the cells which show try like clusters express *CPC* but not *TRY*? What about the trichomes which apparently enter mitosis and divide. Do they lack *TRY* expression? It is obvious that MEPI would best suit to answer such questions.

b) The molecular differences between trichomes in the try gl2 double mutant

Though the *try gl2* leaves produce trichomes which grow out of the leaf surface and branch, there are very clear differences between trichomes with respect to their maturation and the number of branches they produce. As TRY and GL2 have been shown to be involved in branching of trichomes (in opposite ways) it will be interesting to use MEPI and find out if there are differences in the expression levels of these two genes in the different trichomes on *try gl2* leaves, which can be co-related with the differences in their morphology and maturation.

c) Epidermal cell patterning in roots and leaves

It has been speculated that the epidermal cell pattern in the root (producing rows of root hair and non-root hair cells) and the spacing pattern of trichomes on leaves is created by a competition mechanism based on the principles of the meinhardt model and a common set of transcription factors function in both systems. According to the model initially both the positive and negative regulators are expressed at the same levels in a field of cells and then their levels of expression changes due to a positive feedback loop of the activator and the movement of the inhibitor to the neighboring cells. Using MEPI one can test if such changes in gene expression patterns among the known positive and negative regulators do happen when trichome / root hair patterning occurs.

Materials and Methods:

Materials:

Plant mutant alleles and transgenic plants used in this study:

The gl2 mutant allele mainly used in this study is *gl2 5ap-2* (referred to as *gl2-5*). The other mutants used are: *gl1-1*, *gl2-Bos*, *gl2-4aa* (referred to as *gl2-4*), *gl3-1*, *ttg1*, *try82*, cpc. The *gl2* double mutants used in this study existed in the lab collection and had been created using the *gl2 4aa* allele. The *gl2-5* and *gl2-4* alleles show the strongest gl2 trichome phenotype. Unless otherwise mentioned in this study gl2 refers to gl2-5 allele. The *GL2::GUS* / 35S-CPC line was obtained from Takuji Wada (Japan) and was used to analyze GUS expression by the standard method.

Promoter::GFP-ER plants:

The *GL2::GFP-ER* construct was transformed into *gl2-5*, *cpc*, *try cpc*, *gl1-1*, *ttg-1* and *35S-TRY* mutant backgrounds and plants were analyzed either in the T_2 or later generations. The *GL2::GFP-ER* / *gl2* plant was crossed to wt-col (female) and wt plants expressing the marker were analyzed in T3 or later generation after continuously selecting for GFP lines. Similarly *TRY::GFP-ER*, *GL1::GFP-ER* and *AtMYB23::GFP-ER* constructs were transformed into both gl2-5ap and wt (Ler) backgrounds and transgenic plants expressing the markers were analyzed in T2 or later generations. Transgenic plants were also confirmed by genomic DNA PCR analysis where appropriate.

DNA constructs:

The *mgfp5-ER* gene (Haseloff J et al., 1997) was fused downstream of the published promoter fragments of *GL2* (~2.1Kb), *TRY* (~1.4Kb)and *AtMYB23* (~2 Kb) ( ) to create the respective promoter:GFP-ER reporter gene constructs. To create the *GL1::GFP-ER* both the 5' and 3' enhancers of *GL1* (Larkin J. C et al., 1993) were fused to the GFP-ER gene. For the recombination based induction system of GL2, the *GL2* cDNA was subcloned into *pCROX-19* vector (http://biobase.dk/~mundy) at the EcoRI/KpnI site, which results in the CaMV 35S::stuffer DNA::GL2 configuration. After heat shock mediated recombination it is converted into *CaMV 35S::GL2*.

***CaMV 35S::EYFP-protein* fusion constructs used in transient assays:**

GL1, GL2, GL3, TTG1, TRY and CPC ORFs were PCR amplified with appropriate primers and cloned into an amplification vector containing *CaMV 35S* promoter, EYFP (Enhanced Yellow Fluorescent Protein) / ECFP and a terminator so that an in-frame translational fusion between each protein and ECFP / EYFP was achieved. (Note: EYFP and ECFP have been referred to just as YFP and CFP respectively throughout this thesis and similarly *CaMV 35S* promoter is often referred to as just *35S*). The YFP:Protein fusion constructs were used in transient assays, both in onions and Arabidopsis, to check for the localization and inter-cellular mobility.

The GL2::EYFP:GL2 fusion construct:

To check for GL2 protein localization in different epidermal cell types *GL2* cDNA was fused to the C terminus of EYFP and cloned into a binary vector (pCAMBIA-1300) containing the *GL2* 5' promoter. *gl2* mutant plants were transformed with the above construct and the lines showing rescue phenotype were analyzed further.

MEPI binary vector (Chapter 4):

The pGreen II binary vectors (Kanamycin and Basta resistance) were a kind gift from Dr.Rüdiger Simon. More information about the vector can be obtained from http://www.pgreen.ac.uk/. It was modified by cloning a unique site containing 8 cutter sites.

***35S::YFP/CFP-Talin* and *35S::YFP-Peroxi* constructs:**

The published *35S::GFP-mTalin* construct (Kost et al., 1998) was used to replace GFP with YFP and CFP to create the variants. Later different promoter fragments were used to replace the *35S* promoter to create individual promoter:reporter constructs.

Methods:

Plant growing conditions and creating transgenic plants:

All plants used for fluorescence microscopic analyses were grown on MS agar plates (MS salts, 3% sucrose, 1% Phytagar; 23°C, 16 hours daylight / 8 hours dark). For

counting trichome initiations and for leaf epidermal imprints soil grown plants were used.

All the analysis with respect to trichome initiations were carried out on the first pair of true leaves, unless other wise mentioned. Transgenic plants were produced by transforming plants with agrobacterium (GV3101), carrying the appropriate DNA constructs, by floral dip method (Clough and Bent, 1998). T_1 plants were screened on MS medium (Murashige and Skoog, 1962) selection plates containing Hygromycin B (25 µg/ml) or Kanamycin (50 µg/ml).

Recombinase mediated transcriptional induction (35S::GL2) by heat shock:

Ectopic misexpression of GL2 using *CaMV 35S* promoter has been shown to be lethal to plants as it affects the proper function of GL2 during embryogenesis. Hence a recombinase mediated transcriptional induction method ( ) was used for ectopic GL2 expression. *GL2* cDNA was cloned in between the EcoRI / Kpn I sites of the pCROX19 vector which was a kind gift from Dr. John Mundy (University of Copenhagen, Denmark) resulting in SR4 construct. The *GL2* cDNA is separated from the *35S* promoter by a stuffer fragment flanked by two lox sites. The stuffer fragment itself codes for the expression of CRE with NLS driven by a *CaMV 35S* promoter. After heat shock the CRE enzyme is expected to excise the stuffer fragment and thus bring the GL2 cDNA immediately downstream of the 35S promoter to be constitutively expressed.

T_2 transgenic plants containing the *SR4* construct grown at 18 °C on MS plates / soil and after complete germination (as the cotyledons begin to open up) 2 consecutive heat shock treatments of ~12 hrs at 37 °C interrupted by a ~16- 24 hrs recovery period at 18 °C were given. The plants continued to grow after heat shock at 18 °C and were analyzed after 2-7 days. Control plants were also subjected to the same heat shock procedure.

Microscopy:

Light and fluorescence microscopy was carried out on a LEICA-DMRE microscope equipped with a high resolution KY-F70 3-CCD JVC camera and frame grabbing software (DISKUS, Technisches Büro, Königswinter). A spectrophotometric confocal laser scanning microscope (Leica TCS-SP2) was used in some experiments to visualize

cells labeled with GFP / YFP / CFP. For comparison of GFP expression patterns between WT and mutant / transgenic lines the same microscopic settings (laser strength, PMT and pinhole) were used, after initially standardizing for wt plant levels.

Transient gene expression in cells using particle bombardment method:

50 µl aliquots of gold particles (BioRad, Hercules, CA) of either 0.6 or 1 µm size were prepared after washing in 100% ethanol thrice with vortexing in every step. The final aliquots of gold suspended in ethanol were stored at -20° C. Samples for bombardment were prepared by first removing ethanol, washing with H_20 thrice, and resuspending the gold particles in 50 µl H_20. 5 µl of the appropriate DNA construct (1 µg/µl) was added along with 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine. The contents were vortexed vigorously for about 3 minutes, centrifuged at 10 K rpm for 1 minute and the aqueous phase discarded. The particles were then washed with 250 µl of 100% ethanol, vortex for about 25 seconds, centrifuged at 10K rpm for 1 minute and the liquid discarded. Finally the particles now coated with DNA were suspended in 50 µl of 100% ethanol and used for bombardment.

Particles were delivered into Onion (*Allium cepa*) epidermal cells using the Biolistic PDS-1000/He system (Bio-Rad) with 1,100 pounds $inch^{-2}$ rupture discs under a vacuum of 25 inches of Hg. After bombardment, tissue was maintained on moist filter paper in parafilm-sealed plastic Petri dishes. Fluorescence microscopy was carried out on the epidermal peel, removed using a pair of forceps and mounted in tap water, 24 to 72 h after bombardment. For assays in Arabidopsis, seeds were sown in the center of plates containing MS medium and used when the seedlings were 7-9 days old (first leaf primordia just visible). 900 pounds $inch^{2}$ rupture discs were used. For co-bombardment experiments the constructs were mixed in a 1:1 ratio and then coated on the particles as described above. CFP-mTalin construct which labels F-actin filaments was often used in co-bombardment experiments. Plants in such experiments were first screened to locate *35S::CFP* expressing cells (using a CFP specific filter) and later switched to the YFP specific filter to visualize the YFP fusion proteins.

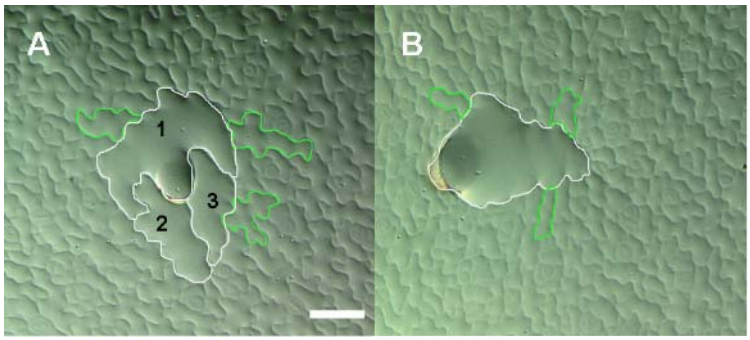
Preparation and analysis of leaf epidermal imprints

Leaf epidermal imprints were prepared by the agarose method as described before (Mathur and Koncz, 1997). 2.5% agarose solution was prepared by dissolving agarose

in water in a microwave. A layer of the hot agarose liquid was poured on a regular microscope glass slide using a Pasteur pipette and quickly leaves were cut from the plants and laid on the agarose. The agarose was allowed to gradually solidify for about 2 - 4 minutes, the leaves were carefully removed and the slides observed under a light microscope using the DIC optics. For better quality of pictures the slides were observed after a few hours by which time the agarose layer was thin due to evaporation of its water content.

Measurement of Relative Cell Size (RCS) of epidermal cells using epidermal imprints:

Relative cell size (RCS) was used as an index to measure the size of trichome cells on *gl2*. Leaf epidermal imprints of gl2 were prepared as described above and the pictures were analyzed using the computer program DISKUS ( ). The cell borders were marked and the area of the cell determined. The RCS value was calculated as the ratio of the area of the trichome cell to the average area of 3 largest pavement cells immediately surrounding it as shown in the figure below. An apparently divided trichome cluster (A) and an undivided trichome (B) on the same mature *gl2* leaf. Cells with green border are pavement cells while the trichome has a white border.



Molecular Biology experiments:

Routine molecular biology work involving DNA cloning, PCRs, RT-PCR, genomic DNA isolation from plants, DNA sequencing and others were performed using the standard protocols in the Sambrook and Maniatis manual (Sambrook J, et al., 1989).

References:

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Appendix 1

Abbreviations used:

AtMyb23	Arabidopsis thaliana MYB23
bp	Base pair(s)
C	DNA content of haploid genome
pCaMV 35S	Cauliflower mosaic virus 35S promoter
cDNA	Complimentary de-oxy ribonucleic acid
CFP	Cyan fluorescent protein
Col	Columbia
CPC	CAPRICE
DAPI	4-6-Diamidino-2-phenylindol
dH_2O	deionized water
DNA	De-oxy ribonucleic acid
ECFP	Enhanced cyan fluorescent protein
EGL3	ENHANCER OF GLABRA 3
ETC1	ENHANCER OF TRIPTYCHON AND CAPRICE 1
ETC2	ENHANCER OF TRIPTYCHON AND CAPRICE 2
EYFP	Enhanced yellow fluorescent protein
FP	Fluorescent protein
GFP	Green fluorescent protein
GL1	GLABRA 1
GL2	GLABRA 2
GL3	GLABRA 3
GUS	Glucuronidase
Kb	Kilo bases
kD	Kilo Dalton
Ler	Landsberg *erecta*
MAP4	Microtubule associated protein 4
mRNA	Messenger ribonucleic acid

mTalin	mouse TALIN
NLS	Nuclear localization signal
p	Promoter
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
STI	STICHEL
T-DNA	Transfer-DNA
TRY	TRIPTYCHON
TTG1	TRANSPARENT TESTA GLABRA 1
WT / wt	wild-type
YFP	Yellow fluorescent protein

Erklärung

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