Analysis of trichome pattern formation in *Arabidopsis thaliana*:
The role of KAKTUS in protein degradation

Inaugural-Dissertation

zur

Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

vorgelegt von

**Elena Galiana Jaime**

aus Valencia

2007
Berichterstatter: Prof. Dr. Martin Hülskamp
Prof. Dr. Ulf-Ingo Flügge

Prüfungsvorsitzender: Prof. Dr. Wolfgang Werr

Acknowledgements

This PhD thesis would not exist without the support of many individuals that have helped me all those years to make my dream of becoming a doctor come true.

My deepest gratitude goes to Professor Dr. HÜLSKAMP for his stimulating advice and constructive criticism during the years of research. I am thankful for the opportunity given to me to develop my own ideas and to work independently.

I extend my gratitude to Professor Dr. FLÜGGE, Professor Dr. WERR and Dr. LUDEWIG who have found time to evaluate this thesis.

I gratefully acknowledge the advice of Dr SCHELLMANN. His knowledge added much to my understanding of the importance of depletion to the trichome pattern formation.

This study could be performed thanks to the assistance of the technicians of the department: Birgit KERNEBECK, Britta MÜLLER, Irene KLINKHAMMER, Bastian WELTER and Uschi CLAẞEN. The personal encouragement and actual help I received from them has made it possible to complete my studies during my time in the university of Cologne.

I am deeply appreciative to many of my colleagues in the university for their understanding and indispensable cooperation. I have learned very much from the long discussions with the members of the Patterning Group: Dr. BOUYER, Dr. PESCH, Rachappa BALKUNDE, Burcu DARTAN, Simona DIGIUNI and Katja WESTER as well as from the members of the Escort Complex Group: Dr. SCHELLMANN, Dr. SPITZER, Aneta SABOVLJEVIK, Mojgan SHAHRIARI and Channa KESHAVAIAH.

I am indebted to Dr. SAEDLER, Dr. PESCH, Dr. SCHELLMANN and Dr. SANTAELLA TENORIO for the critical reading of the whole text and for the valuable contribution to the clearness of the manuscript.

I would like to express my indebtedness to Professor Dr. HERZOG who suggested and stimulated my first steps in the area of trichome development, several years ago, at the university of Grenoble. I realize the great importance of the support of Dr. PERAZZA, Dr. BONNEVILLE and Dr. VACHON to educate me on what critical scientific reasoning actually means.

In the first three years of my investigation I received financial support from the Graduate School for Molecular Analysis of Developmental Processes for which I express my gratitude.

Several friends have made my life very pleasant during the last four years. Many from them could call themselves experts in the development of trichomes even though they never studied
biology, just because they have been curious and patient to listen to me talking about my project. Very often, they made me see aspects from the question that I hadn’t considered. Beyond any doubt, they deserve to see their names associated to this work. I am also very grateful to those that were there for me any time I needed them. I am talking about Nele DICKMANN, Santiago GÁLVEZ SETTIER, Deborrah GHIRMAI, Delphine GIRAUD, Pari HEIDENREICH, Nermin KOCALAN, Verónica MAURINO, Claudia RAMBKE, Rainer SAEDLER, Mariana SAIGO, Marcella SANTAELLA TENORIO, Enrique TORRES PRIETO and Christina WEINL.

And, last but not least, I was lucky to be working in the best office one can find in the whole universe, thanks to Moola MUTONDO, Silke UHRIG, Valerie MACH, Cho-Chun HUANG and Cordula JÖRGENS, and to the “guests”: Simona DIGIUNI, Philipp THOMAS and Claudia RAMBKE.
This work is dedicated to five persons who are proud of who I am, and have always supported my choices:

Elvira, my mother
Ana, my sister
Elvira, my grandmother
Carmen and Asunción, my grandaunts
# Table of contents

Table of contents ........................................................................................................................................ I
Abbreviation list ........................................................................................................................................ IV
Figure index ................................................................................................................................................ V
Abstract ...................................................................................................................................................... VI
Zusammenfassung ....................................................................................................................................... VII

## A Trichome Morphogenesis

A 1 Introduction ......................................................................................................................................... 2
  1.1 General description of the trichome morphogenesis ...................................................................... 2
  1.2 The hormone gibberellin is implicated in trichome branching ..................................................... 3
  1.3 Aim of this work ............................................................................................................................... 5

A 2 Results ................................................................................................................................................. 7
  2.1 Genetic mapping of POLYCHOME (PYM) ..................................................................................... 7
    2.1.1 Generation of the mapping population .................................................................................. 7
    2.1.2 Creation of markers around PYM ......................................................................................... 7
    2.1.3 PYM is located between T11A7 and F7D19 ......................................................................... 7
  2.2 Phenotypic characterization of POLYCHOME (PYM) ................................................................ 8
    2.2.1 Hypocotyl development and stem height ............................................................................ 9
    2.2.2 Trichome development ......................................................................................................... 11
    2.2.3 Flowering ............................................................................................................................ 12

A 3 Discussion .......................................................................................................................................... 14
  3.1 Genetic mapping of the POLYCHOME gene .............................................................................. 14
  3.2 Morphological characterization of the polychome mutant and analysis of its dependency on the gibberellins .................................................................................................................................. 15

## B Trichome Pattern formation

B 1 Introduction ......................................................................................................................................... 19
  1.1 Trichome initiation in Arabidopsis thaliana .................................................................................... 19
  1.2 A model to explain two-dimensional pattern formation ................................................................. 19
  1.3 Is protein degradation relevant for trichome pattern formation? ................................................ 20
  1.4 Elements of the trichome pattern system and their molecular nature ....................................... 21
  1.5 Outlook and aim of the work ......................................................................................................... 23

B 2 Results ................................................................................................................................................. 25
  2.1 At4g38600 is the KAKTUS gene ...................................................................................................... 25
  2.2 The kaktus plants carry a mutation in a patterning ........................................................................ 25
  2.3 The 35S::YFP::KAKTUS gene is not able to rescue the aberrant trichome patterning phenotype on the kaktus mutant plants .................................................................................................................................. 26
  2.4 KAKTUS interacts in yeast and in Arabidopsis thaliana with proteins implicated in trichome patterning .................................................................................................................................. 28
2.5 KAKTUS and GLABROUS1 interact genetically in the trichome initiation pathway. ................................................................. 31
2.6 The degradation of GL1 is mediated by the 26S proteasome ............... 32
2.7 KAKTUS is implicated in the degradation of GL1........................................ 33

B 3 Discussion .......................................................................................... 35
3.1 KAKTUS plays a role in the trichome patterning .................................... 35
3.2 KAKTUS is a HECT E3 ligase very likely implicated in the degradation of regulators of trichome initiation...................................................... 36
3.3 GL1 is degraded via the 26S proteasome in a KAK-dependent manner ...... 37
3.4 The KAKTUS-dependent degradation of GL1 is relevant for trichome pattern formation................................................................... 38
3.5 Towards a new model to explain trichome pattern formation: the key role of depletion................................................................. 39
3.6 Outlook................................................................................................... 41

C 1 Material.................................................................................................. 43
1.1 Chemicals, antibiotics .............................................................................. 43
1.2 Enzymes, primers and kits ......................................................................... 43
1.3 Cloning vectors .......................................................................................... 43
1.4 Microbiotical strains.................................................................................... 43
1.5 Plant lines .................................................................................................. 44

C 2 Methods .................................................................................................. 44
2.1 Plant work.................................................................................................. 44
  2.1.1 Plant growth conditions .......................................................................... 44
  2.1.2 Crossing of plants ................................................................................... 44
  2.1.3 Plant transformation ................................................................................ 44
  2.1.4 Seed surface sterilisation and subsequent plant treatment .................... 45
  2.1.5 Selection of transformants ...................................................................... 45
  2.1.6 Gibberellin treatment ................................................................................ 45
  2.1.7 Uniconazol-P treatment .......................................................................... 45
  2.1.8 MG132 (Z-Leu-Leu-Leu-H) treatment ...................................................... 46

  2.2 Microscopy and cytological methods .......................................................... 46
    2.2.1 Microscopy ............................................................................................ 46
    2.2.2 Yeast two-hybrid assay ......................................................................... 46
    2.2.3 BiMolecular fluorescent complementation ............................................. 46
    2.2.4 Kinetics of protein degradation ............................................................... 47

  2.3 Molecular-biological methods: ................................................................. 48
    2.3.1 RNA isolation ....................................................................................... 48
    2.3.2 Reverse transcription ............................................................................. 48
    2.3.3 Semiquantitative RT-PCR ..................................................................... 48
    2.3.4 Genomic DNA preparation .................................................................. 49
    2.3.5 Plasmid DNA preparation from bacteria ................................................. 49
    2.3.6 DNA-manipulation ............................................................................... 50
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.7</td>
<td>Cloning of the KAK cDNA</td>
<td>50</td>
</tr>
<tr>
<td>2.3.8</td>
<td>Primers used for the mapping of PYM</td>
<td>51</td>
</tr>
<tr>
<td>2.4</td>
<td>Biological-chemical methods</td>
<td>51</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Protein extraction</td>
<td>51</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Western blotting</td>
<td>51</td>
</tr>
</tbody>
</table>

References | 53
Erklärung | 61
Lebenslauf | 62
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>°</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>μ</td>
<td>micro</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AD</td>
<td>Activation domain</td>
</tr>
<tr>
<td>AtKLI5</td>
<td><em>Arabidopsis thaliana</em> KAKTUS LIKE 5</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>BD</td>
<td>Binding domain</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular fluorescent complementation</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CaMV</td>
<td>35S promoter from <em>Cauliflower Mosaic virus</em></td>
</tr>
<tr>
<td>C</td>
<td>DNA-content of a haploid genome</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CDS</td>
<td>coding sequence</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>cm</td>
<td>centimeters</td>
</tr>
<tr>
<td>Col</td>
<td>Columbia</td>
</tr>
<tr>
<td>COP</td>
<td>Constitutive morphogenesis</td>
</tr>
<tr>
<td>CPC</td>
<td>CAPRICE</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dCAPs</td>
<td>derived cleaved amplified polymorphic sequence</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitinating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>Enzyme 3</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia [Lat.] for example</td>
</tr>
<tr>
<td>EGL3</td>
<td>ENHANCER OF GLABRA3</td>
</tr>
<tr>
<td>et al.</td>
<td>et alterni [Lat.] and others</td>
</tr>
<tr>
<td>ETC 1/2</td>
<td>ENHANCER OF TRY CPC1/2</td>
</tr>
<tr>
<td>Fig</td>
<td>Figure</td>
</tr>
<tr>
<td>GA</td>
<td>gibberelic acid</td>
</tr>
<tr>
<td>GAI</td>
<td>GA INSENSITIVE</td>
</tr>
<tr>
<td>GAL</td>
<td>galactosidase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GL1/3</td>
<td>GLABRA1/3</td>
</tr>
<tr>
<td>HECT</td>
<td>homologous to E6-associated protein carboxyl terminus</td>
</tr>
<tr>
<td>k</td>
<td>kilo</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilo bp</td>
</tr>
<tr>
<td>kDA</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KAK</td>
<td>KAKTUS</td>
</tr>
<tr>
<td>Ler</td>
<td>Landsberg erecta</td>
</tr>
<tr>
<td>LUC</td>
<td><em>Photinus-luciferin 4-monooxygenase</em> LUCIFERASE</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Shoog</td>
</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>p</td>
<td>promoter</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PYM</td>
<td>POLYCHOME</td>
</tr>
<tr>
<td>RACE-PCR</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RGA</td>
<td>REPRESSOR OF GA1</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription PCR</td>
</tr>
<tr>
<td>SSLP</td>
<td>simple sequence length polymorphism</td>
</tr>
<tr>
<td>SPY</td>
<td>SPINDLY</td>
</tr>
<tr>
<td>TTG1/2</td>
<td>TRANSPARENT TESTA GLABRA1/2</td>
</tr>
<tr>
<td>TRY</td>
<td>TRIPHYCHON</td>
</tr>
<tr>
<td>UPL1/7</td>
<td>Ubiquitin protein like 1/7</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
</tr>
<tr>
<td>YFPc/n</td>
<td>C/N terminal sub-fragment on the YFP gene</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>

All gene and mutant names are written in italics.
WT genes are written in capital letters.
Proteins are written in non-italic letters.
Figure index

Fig. 1: Steps in trichome development
Fig. 2: Phenotype of the kak-like mutants potentially implicated in the gibberellin pathway
Fig. 3: The transduction of the gibberellic acid message
Fig. 4: Mapping of POLYCHOME
Fig. 5: Phenotypical characterization of the pym hypocotyl
Fig. 6: Number of trichomes per mm$^2$ in the second pair of leaves
Fig. 7: Kinetics of flowering of Ler wild-type and pym mutants
Fig. 8: Pattern formation by autocatalysis and long-range inhibition
Fig. 9: Activator-inhibitor model applied to the trichome patterning system
Fig. 10: Involvement of KAKTUS in the establishment of trichome patterning
Fig. 11: Interaction of KAK with the proteins implicated in the trichome patterning in yeast two-hybrid assays
Fig. 12: Interaction profile of patterning proteins with KAKTUS in bimolecular fluorescence complementation assays
Fig. 13: Study of the dependence of GL1 degradation on the 26S proteasome
Fig. 14: Kinetics of the degradation of GLABROUS1
Fig. 15: Model to explain the role of the KAKTUS-dependent degradation of GL1 in the stabilization of the trichome pattern
Abstract

In this PhD thesis I have studied two different aspects of the cell differentiation: the mechanisms underlying pattern formation and the hormonal regulation of cell morphogenesis. I concentrated on the development of trichomes, the leaf hairs of *Arabidopsis thaliana*, as a model system. This cell type is very well suited for the analyses of those processes because it presents a simple two-dimensional spacing pattern on the rosette leaves and develops a predictable and characteristic shape.

The gibberellic acid is implicated in the regulation of cell morphogenesis. In the trichome, this regulation takes place via a *SPINDLY*-dependent pathway. I have investigated the role of *POLYCHOME* in this pathway. I have attempted to map it, and I could provide evidence for its implication in the transduction of the GA signal leading to the development of trichome branches in *Arabidopsis thaliana*.

In the second part of my thesis I examined the part played by protein degradation in the processes that control the formation of a regular trichome spacing pattern on the leaf surface. My thesis revealed the implication of *KAKTUS*, which encodes a protein homologous to HECT E3 ligases, in the establishment of a wild-typical patterning. I showed that GL1 is degraded in a KAK-dependent manner through the 26S proteasome. Finally, I could demonstrate the physiological relevance of this regulated depletion for trichome initiation. These observations are summarised into a model that suggests that the function of KAK in the early events during trichome pattern formation is to stabilize the incipient trichome pattern.
Zusammenfassung

In dieser Doktorarbeit habe ich zwei verschiedene Aspekte der Zelldifferenzierung untersucht: den Mechanismus der Musterbildung und die hormonelle Regulation der Zellmorphogenese. Ich habe mich auf die Entwicklung von Trichomen, den Blatthaaren von Arabidopsis thaliana, als Modellsystem konzentriert. Dieser Zelltyp ist für die Analyse dieser Prozesse sehr geeignet, da er ein simples zweidimensionales Abstandsmuster präsentiert und eine vorhersehbare und charakteristische Form entwickelt.


Im zweiten Teil meiner Arbeit habe ich untersucht welche Rolle der Proteinabbau im Prozess der Bildung eines geordneten Trichommusters auf der Blattoberfläche spielt. Meine Arbeit zeigte, dass KAKTUS an der Bildung des wildtypischen Musters beteiligt ist. KAKTUS kodiert ein Protein, das der HECT E3 Ligase homolog ist. Ich konnte zeigen, dass GL1 KAK-abhängig durch das 26S Proteasom abgebaut wird. Schließlich konnte ich die physiologische Relevanz dieser regulierten Abnahme für die Trichominitiation demonstrieren. Diese Beobachtungen werden in einem Modell zusammengefasst, das darauf hinweist, dass die Funktion von KAK in der frühen Trichommusterbildung darin liegt das beginnende Trichommuster zu stabilisieren.
Trichome Morphogenesis
A 1 Introduction

The nature presents an amazing diversity of shapes, ranging from isomorphic bacteria to spermatozoids. The fascinating question of how a cell establishes, regulates and maintains its shape has motivated a wide field of research. Plants represent a good model for the study of those questions, because it is relatively easy to observe some of their cells while they are performing their morphogenesis. In Arabidopsis thaliana, a plant model system, the development of leaf trichomes (Hülskamp et al., 1994; Folkers et al., 1997; Mathur and Hülskamp, 2002), root hairs (Carol and Dolan 2002), pollen tubes (Hepler et al., 2001) and stomata cells (Nadeau and Sack 2003) have been studied in great detail.

The trichomes (also called leaf hairs) of Arabidopsis thaliana are particularly well suited to study cell morphogenesis. Firstly, they are single cells that emerge from the epidermal layer and are therefore easily accessible for observation and experimentation. Secondly, they present a predictable, characteristic and complex stellate shape. Thirdly, it is possible to define genetic distinct steps in trichome development (Hülskamp et al., 1994). Fourthly, trichomes are dispensable for survival of the plant under laboratory conditions.

Figure 1: Steps in trichome development. Scanning electron micrographs of developing wild-type trichomes. (A) Incipient unbranched trichome. (B) Trichome with primary branch point. Note orientation of the branches with respect to the basal-distal leaf axis. (C) Trichome with primary and secondary branch. (D) Mature trichome. (Figure modified from Schwab et al., 2000).

1.1 General description of the trichome morphogenesis

Hülskamp et al. (1994) have described the steps involved in the development of a trichome (Fig1). Trichomes are the first cells that start to differentiate on leaf protoepidermis. Such differentiating cells undergo three rounds of endoreduplication (DNA synthesis without
cytokinesis) that bring the DNA content from 2C to 16C. Thus, the first morphological evidence of a cell entering this pathway is the increase of the nuclear volume. Then, the incipient trichome expands out of the leaf plane and initiates a first branching event. As a consequence, at this stage a plant hair is composed of a stem and two branches. Subsequently, a fourth round of endoreduplication occurs, which brings the DNA content to the one of a mature trichome (32C). The next step of the development of a trichome is the formation of a second branching point on the branch pointing to the distal end of the leaf. Finally, on the last maturation step of the plant hair, incrustations appear on the surface of the cell. The nucleus of a mature trichome has a triangular shape, and is characteristically located under the second branch point.

1.2 The hormone gibberellin is implicated in trichome branching

To date, two studies have focused on the relationship between trichome branching and the gibberellins. The link has been first established by the analysis of glabrous1.2, a weak allele of the MYB transcription factor GLABROUS1 (GL1), which develops two-branched trichomes. The promoter of this gene is positively regulated by the gibberellins, indicating that this hormone may regulate trichome branching by up-regulating GL1 (Herman et al., 1989, Esch et al., 1994, Chien and Sussex, 1996; Perazza et al., 1998). More recently, some genes that lead when mutated to an increased DNA content and to the formation of up to eight branches (Fig. 2) have been implicated in the transduction of the gibberellic message. These genes are KAKTUS, SPINDLY and POLYCHOME (Perazza et al., 1999). KAKTUS and SPINDLY are positively implicated in the sensing of the gibberellic acid message: the spy mutant is able to germinate in the absence of the hormone (Jakobsen et al., 1993), whereas an application of gibberellins the kak mutant leads to an abnormal elongation of the hypocotyl (Downes et al., 2003). POLYCHOME seems to act downstream from SPY: the double mutant pym spy displays the same number of trichome branches than the parental lines alone (Perazza et al., 1999). Therefore, PYM may be also involved in the sensing of the GA message. Since the double mutant kak-2 spy-5 presents no additivity of the overbranching phenotype, it has been suggested that SPINDLY and KAKTUS function on a linear way. Genetic analyses suggest that PYM acts to repress branch formation through a pathway independent of KAK: the double mutant kak-2 pym is highly overbranched compared to the parental lines. (Perazza et al., 1999).
Introduction

Figure 2: Phenotype of the *kak*-like mutants potentially implicated in the gibberellin pathway. Scanning electron micrographs of mature trichomes (A to D) and DNA fluorescence distributions of trichome nuclei (E to H) (A) Three-branched wild-type trichome (*Ler*). (B) *kak*-2 mutant trichome (C) *spy*-5 mutant trichome (D) *pym* mutant trichome. DNA fluorescence distributions in wild-type *Ler* trichome nuclei (E), in *kak*-2 mutant trichome nuclei (F), in *spy*-5 mutant trichome nuclei (G) and in *pym* mutant trichome nuclei (H). (Figures modified from Perazza *et al.*, 1999 and Downes *et al.*, 2003).

GA signalling operates as a de-repressible system moderated by DELLA-domain proteins, which are transcriptional regulators that repress GA responses (Figure 3). Five DELLA protein genes have been identified in Arabidopsis: *GA-insensitive (GAI)*, *Repressor of ga1.3 (RGA)*, *RGA-like1 (RGL1)*, *RGL2* and *RGL3*, with *RGA* and *GAI* being the major repressors during vegetative growth and floral induction (Richards *et al.*, 2001, Olszewski *et al.*, 2002). The regulatory steps used by the plant to regulate trichome branching through the gibberellins are unclear. Nonetheless, the cloning of *SPY* and *KAK* has provided with strong clue about it. *SPY* encodes a O-linked N-acetylglucosamine transferase that transfers O-Glc-Nac residues to target proteins (Jakobsen *et al.*, 1996). Based on this, *SPY* has been hypothesized to modify DELLA proteins in response to the GA (Thomas *et al.*, 2004). One of the target proteins of *SPY* might be a DELLA protein implicated in the repression of trichome branch formation, and therefore the gibberellins might activate trichome branching by positively regulating *SPY*, which in turn modifies the DELLA proteins and eventually leads to the activation of trichome branching. *KAK* encodes for a HECT domain protein and thus may be implicated in the ubiquitin degradatory pathway (Downes *et al.*, 2003, El Refi *et al.*, 2003). Since in response to GA DELLA proteins are rapidly degraded via the ubiquitin proteasome pathway (Sun and Gubbler, 2004), it is reasonable to hypothesize that the role of *KAK* in trichome branch formation is to degrade activators of this trichome developmental stage in response to the GAs. It appears that *PYM* is the only gene described to be implicated in the regulation of trichome branching through the GAs that has not been cloned yet. This impeaches to draw a complete picture about this regulatory process.
1.3 Aim of this work

The gibberellic acid is implicated in the regulation of cell morphogenesis. In the trichome, this regulation takes place by the means of a SPINDLY-dependent pathway. To date, no investigation has been carried out to improve our understanding on the mechanisms by which this regulatory cascade leads to the development of branches in the trichome cell. This is partially due to the fact that not all the proteins implicated in the transduction of this hormonal message have been cloned and studied.

![Diagram of gibberellin transduction](image)

**Figure 3: The transduction of the gibberellic acid message.** The gibberellins regulate developmental processes through the action of SPY. This acetylglucosamine transferase is supposed to transfer O-Glc-Nac residues to the five DELLA proteins existing in *Arabidopsis thaliana* (represented in orange boxes) in response to the hormone. The DELLA proteins repress the processes indicated in the box, among others. PYM and KAK are implicated in the regulation of this pathway downstream of SPY. Nonetheless, their exact function has not been elucidated yet.

Genetic analysis have shown that *PYM* plays a role in the hormonal regulation of trichome branching downstream of *SPY*. This point has not been confirmed by further experimental data. Also, the molecular nature of this gene has not been described. In order to increase the knowledge on the hormonal regulation of cell morphogenesis, it is important to confirm the
implication of *POLYCHOME* in the gibberellin pathway and to determine its molecular function.

To better understand the role of the gibberellic acid in the context of cell morphogenesis in *Arabidopsis thaliana*, I focused my thesis work on the study of *POLYCHOME*, a gene implicated in the trichome branching. I proceeded to a genetic mapping of *PYM*, as well as to a detailed morphological characterization of the *pym* mutant and to an observation of the relationship between PYM and the gibberellins.
A 2  Results

2.1  Genetic mapping of POLYCHOME (PYM)
To understand the exact molecular function of the PYM protein, I initiated a genetic mapping of PYM, exploiting positional cloning.

2.1.1  Generation of the mapping population
To generate the mapping population, a Ler pym plant was crossed to a Col-0 wild-type plant and the offspring was screened in the F2 for individuals presenting a pym trichome phenotype. The mapping was performed with 850 plants generated by two independent crosses.

2.1.2  Creation of markers around PYM
Previous studies have shown that PYM is located at the bottom of chromosome II. pym revealed a linkage to the markers Ubique and nga168 (Perazza et al., 1998). Therefore, I analyzed the mapping population with molecular markers located either in the proximity or between those two. However, the available molecular markers (nga168 and Ubique) for the region of interest on Chromosome II were not sufficient to map the gene. So, I designed new markers based on either simple sequence length polymorphism (SSLP) or derived cleaved amplified polymorphic sequence (dCAPS) techniques. The corresponding data about existing polymorphisms between Col-0 and Ler were obtained from the ‘Monsanto Arabidopsis thaliana polymorphism and Ler sequence collection’.

2.1.3  PYM is located between T11A7 and F7D19
The chromosomal walking was performed in two steps. Firstly, to get an insight into the location of PYM on the second chromosome and to confirm the published localization of this gene, I analyzed 49 plants with 6 SSLP markers located within 37 Bacterial Artificial Chromosomes (BACs). Their analysis showed that no chromatide had recombined between the PYM gene and F18O19-EGJ. Hence, PYM is located between F11A7-EGJ and F4I18-EGJ (Figure 3a). Secondly, to map PYM more precisely, I analyzed the 850 plants comprising the entire mapping population with F11A7-EGJ, F4I18-EGJ and with 6 markers located within
those two (19 BACs). Three plants and one plant had recombined between *pym* and T11A7-EGJ and between this gene and F7D19-EGJ, respectively. It was not possible to detect any further recombination in the area between those markers (Figure 3b). To conclude, these data show that the genetic mapping allowed to map *PYM* between T11A7-EGJ and F7D19-EGJ, but was not sufficient to localize it precisely.

**Figure 4:** Mapping of *POLYCHOME*. (a) *POLYCHOME* is located on the chromosome II between T11A7-EGJ and F4I18-EGJ. (b) *POLYCHOME* is located on the chromosome II between T11A7-EGJ and F7D19-EGJ. The horizontal lines represent fragments of chromosome II. Each vertical line represents one bacterial artificial chromosome (BAC). The markers used for the mapping are shown on the corresponding BAC. The ratios indicate the number of recombinant events that took place between *PYM* and the given marker for the tested population.

### 2.2 Phenotypic characterization of *POLYCHOME* (*PYM*)

Genetic analyses indicate that *PYM* and *SPY* are part of the same pathway. Since *SPY* is implicated in the transduction of the gibberellin signal (Jakobsen *et al.*, 1993), it has been suggested that *PYM* function may be linked to this hormone (Perazza *et al.*, 1999). To test whether this *PYM* is part of the GA pathway, I observed the behaviour of the corresponding mutant plant during some of the developmental processes controlled by the hormone (hypocotyl and stem elongation, endoreduplication of the hypocotyl epidermal cells, trichome
density and flowering). Subsequently, I analysed the growth of the pym hypocotyl as well as its ability to switch to the reproductive face in the presence of exogenous gibberellic acid. Finally, I quantified the effect of a drug known to block the biosynthesis of the GAs, the Uniconazol-P, to the trichome branching.

### 2.2.1 Hypocotyl development and stem height

In *Arabidopsis*, the hypocotyl appears as the result of a series of apical-basal and radial divisions taking place during the embryonic development followed after germination by a gibberellin-dependent elongation of the cells as well as by endoreduplication (Mayer *et al.*, 1991, Gendreau *et al.*, 1999, Berger *et al.*, 1998a). The requirement of the gibberellic acid for the hypocotyl elongation can be best seen in the GA deficient mutant *ga1.3*, which is dwarf consistently with the positive role of the gibberellic acid in the elongation of the hypocotyl. The stem elongation is also under the control of the gibberellins. This organ has a reduced size in the original GA-defective mutants as well as in plants overexpressing genes that encode GA-catabolizing enzymes, the GA2-oxydases (Schomberg *et al.*, 2003) and is abnormally long in the *spy* mutant plants (Jakobsen *et al.*, 1996).

After 8 days on MS media 3% under long day conditions, the size of the pym’s hypocotyl is reduced of 59.6% compared to the corresponding Ler wild-type (Fig. 5a; *pym*: 2.25 (+/- 0.55) mm; *Ler*: 4.21 (+/- 0.74) mm; n= 91). After 25 days growing on soil under green house conditions, the pym stem is almost half as short as the corresponding control (Fig.5b; *Ler* wild-type: 14.7 (+/- 1.2) cm; *pym*: 7.8 (+/- 1.3) cm. n=47).

The shortage of the pym’s hypocotyl can be due to a reduction in the number of cells comprising this organ or to a decrease in the cell size. To discriminate between these two hypotheses, I compared the number of cells of both *pym* and wild-type embryonic hypocotyls, as well as the size of the cells of this organ and of the wild-type one 8 days after germination. For this assay, the plants were grown on MS media 3% under long day conditions. On the one hand, one hypocotyl cell row of *pym* is made of 25.7 (+/- 3.86) epidermal cells on average, while the wild-type one comprises 23.5 (+/-3.92) cells (Fig. 5c; n=50). On the other hand, the length of 8-days old wild-type cells fall into four classes: less than 100 μm, 100 to 200 μm, 200 to 300 μm and more than 300 μm (Fig. 5d). The pym mutant presents about 300% more cells smaller than 100 μm than the wild-type (*pym*: 49.73%; *Ler*: 16.36%). Also, the number of cells ranging from 100 to 200 μm and from 200 to 300 μm is reduced in the mutant of 33% and 52.8% respectively (100 to 200 μm: *pym*: 43.72%, *Ler*: 65.45%; 200 to 300 μm: *pym*: 6.01%, *Ler*: 12.73%). Finally, 5.45% of the wild-type cells are bigger than 300 μm. No *pym*
Results

A cell belongs to this category. To summarize, the pym hypocotyl comprises the same number of cells than the wild-type one, and the mutant cells in this organ are on average smaller than the wild-type ones: the pym mutant hypocotyl phenotype is due to an elongation defect.

To determine whether this defect is due to a deficiency on hormone gibberellin or to an inability of the mutant to sense the GA message, I have measured the length of this organ in a Ler wild-type and in a pym backgrounds in the presence of increasing concentrations of gibberelic acid (Fig.5e. n>40). The length of the Ler wild-type hypocotyl is the same before and after the application of concentrations of GA ranging from 0,01 μM to 10 μM (0,0 μM: 4,35 +/-0,69 mm; 0,01 μM: 4,53 (+/- 0,67) mm; 0,1 μM: 4,54 (+/- 0,79) mm; 0,5 μM: 5,21(+/- 0,73) mm; 3,5 μM: 4,63 (+/-1,01) mm; 5 μM: 5,51 (+/-0,99) mm; 10 μM: 5,61 (+/- 0,70) mm). The presence of exogenous hormone at a concentration of at least 0,1 μM results in the mutant in a hypocotyl elongation comparable to the one observed for the corresponding wild-type (0 μM: 1,83 (+/- 0,47) mm; 0,01 μM: 2,71(+/- 0,49) mm; 0,1 μM: 3,54 (+/- 0,68) mm; 0,5 μM: 4,72(+/- 1,37) mm; 3,5 μM: 5,2 (+/-1,47) mm; 5 μM: 5,26 (+/-1,47) mm; 10 μM: 4,89 (+/- 0,91) mm). Therefore, an exogenous application of gibberellins rescues the aberrant hypocotyl mutant phenotype.

To characterize the endoreduplication profile of the pym hypocotyl cells, I have determined the ploidy pattern of both pym and wild-type hypocotyls (Fig 5f; n=39). The DNA content of Ler wild-type hypocotyl cells grown for 8 days on MS 3% media can be distributed in 4 classes: 2C (ploidy level corresponding to unreduplicated DNA), 4C (one round of endoreduplication), 8C (two rounds of endoreduplication) and 16C (three rounds of endoreduplication). Respectively, 43,53%, 28,21%, 20,51% and 7,69% of the cells belong to these classes. 10,53%, 21,05%, 28,95% and 26,32% of the mutant nuclei contain 2C, 4C, 8C and 16C respectively. 13,16% of the mutant cells have undergone a fourth round of endoreduplication (32C). Therefore, the pym mutant is affected on the control of the endoreduplication.
Figure 5: Phenotypical characterization of the pym hypocotyl. (a) Hypocotyl length of 8 days old plants. n=91. (b) Flowering Ler wild-type (left) and pym (right) plants grown for 25 days on soil under green house conditions. (c) Hypocotyl cell number of embryonic plants. n=50. (d) Distribution of hypocotyl cell size of 14 days old plants n>150. (e) Dependency of the hypocotyl size of 8 days old plants on GA4+7. n>40. (f) Distribution of hypocotyl cell DNA content of 14 days old plants. n=39. All the plants except the ones depicted on the figure 3b were grown on MS3% at 22°C. Vertical bars represent the standard deviation.

2.2.2 Trichome development

A mutation in the POLYCHOME gene leads to trichome overbranching (Perazza et al., 1999). To clarify the relationship between this trichome phenotype of pym and the gibberellins, I have quantified the leaf hairs branches in the presence of different concentrations of Uniconazol-P, an inhibitor of the GA synthesis.

A deficiency in gibberellins caused by an exogenous application of uniconazol-P does not significantly affect the number of pym three-branched trichomes (0 mM: 32,4 (+/- 4,52) %; 10^-6 mM: 37,41 (+/-5,66) %; n=30). The same concentration of uniconazol-P is sufficient to
trigger a significant decrease in the percentage of three-branched trichomes of the Ler wild-type plants (0 mM: 59.52 (+/- 2.62) %; 10^{-6} mM: 23.5 (+/-3.39) %; n=25).

In addition to playing a role in trichome branching, the gibberellic acid is implicated in trichome initiation (Herman et al., 1989, Chien and Sussex, 1996, Perazza et al., 1998, Payne et al., 2000). To find out whether PYM is implicated in this aspect of the trichome development, I quantified the trichomes in pym compared to the wild-type.

The amount of trichomes per mm² is significantly reduced in the second pair of leaves of the pym mutant compared to the wild-type (Fig.6; Ler wild-type: 0.35 (+/-0.04) trichomes/ mm²; pym: 0.23 (+/-0.04) trichomes/ mm²). This difference does not exist in the third pair of leaves (Ler wild-type: 0.44 (+/-0.05) trichomes/ mm²; pym: 0.44 (+/-0.03) trichomes/ mm²).

![Figure 6: Number of trichomes per mm² in the second and third pair of leaves. n=20. The bars represent the standard deviation.](image)

2.2.3 Flowering

The gibberellic acid is known to play various roles in reproductive development. For example, GA application accelerates flowering, particularly in short days (Landridge 1957, Bagnall 1992). Consistently, mutations that block GA biosynthesis or responsiveness (e.g. sleepy1) cause delayed flowering, whereas mutants with increased GA signalling (e.g. rga, gai and spy) flower early (Olszewski et al., 2002).

I have compared the kinetics of flowering of pym and Ler wild-type (Fig. 7a, 7b; n=27). The plants were monitored for 25 days and were growing on soil under long day conditions.
Figure 7: (a) Kinetics of flowering of Ler wild-type and pym plants. The results are shown in percentage of flowering plants in relation to the total population. n=27. (b) The histograms represent the number of rosette leaves of the plants shown in Fig 6a when the vegetative meristem switched to a reproductive meristem. The vertical bars indicate the standard deviation.

It appears that after 18 days, 71.4% of the wild-type plants are able to flower, and after 19 days, it is possible to observe a floral bulge in 100% of the population (Fig. 7a). In contrast, after 20 days, 25% of the pym plants have flowered. This percentage increased to 41.6% and to 58.3% after 21 and 22 days, respectively. On the 23rd day, 83.3% of the plants flowered. No further flowering was observed between the 23rd and the 25th day. On average, Ler produces 5.29 (+/- 0.47) leaves before flowering. The pym mutant produces 6.71 (+/- 0.49) leaves (Fig. 7b). Taken together, those results indicate that pym presents a delay in flowering. The retard in flowering observed in the pym mutants can be completely rescued by the application of exogenous gibberellins. 100% of both Ler wild-type and pym plants are able to flower after 19 days of growth under GA treatment. When grown without exogenous GAs, only 86.95% Ler wild-type plants and 38.8% pym mutants flowered.
The shaping of the leaf trichomes of *Arabidopsis thaliana* is under the positive regulation of the gibberellic acid (Chien and Sussex 1996, Perazza *et al*., 1998). Three genes have been implicated in the hormonal regulation of the trichome cell morphogenesis: *SPY*, *KAK* and *PYM*. Although the cloning *SPY* and *KAK* are helping to get a better picture of the mechanisms by which this regulation takes place, this regulatory pathway has been poorly investigated and much is still unknown about its way of action. To perform substantial progress in our understanding of the GA-dependent regulation of cell morphogenesis, it is essential to characterize other members of the pathway.

At the time of the beginning of my theses work, *POLYCHOME* had been proposed to encode for a protein involved in the GA transduction cascade in the trichome, downstream from *SPINDLY* (Perazza *et al*., 1999). To acquire further knowledge on the regulation of cell morphogenesis by the hormone gibberellin, I concentrated my work on the investigation of the *POLYCHOME* gene. I attempted to map it and I proceeded to a morphological characterization of the mutant, as well as to the observation of the dependency of several pym’s developmental stages on the gibberellins by the application of exogenous gibberellic acid or by the blockage of the hormone biosynthetic pathway.

### 3.1 Genetic mapping of the *POLYCHOME* gene

At the beginning of my work, it was known that the *PYM* gene is located in the south of chromosome II in the genome of *Arabidopsis thaliana*. It co-segregates with the markers nga168 and Ubique, indicating that *PYM* and those two markers are in close proximity (Perazza *et al*., 1999).

To locate *PYM*, I further fine-mapped the portion between and around nga168 and Ubique. The *PYM* gene always co-segregated with the markers located within T11A7-EGJ and F7D19-EGJ. Therefore, my work allowed to locate *PYM* in the region of 423.140 nucleotides existing between those two markers, but was not sufficient to map *PYM* precisely.

To overcome this result, one could imagine sequencing all the genes located in this region, to apply a candidate approach or to increase the mapping population. The economical cost of the sequencing is too high to justify the choice of the first possibility. A candidate approach
consists on emitting an educated guess about the molecular nature of the gene of interest, and to screen the area to which the gene has been mapped down looking for candidates to be the one in concern. The only knowledge about \textit{PYM} is that it may play a role downstream of \textit{SPY}. Therefore, it is not possible to attribute a potential molecular nature to this gene. All what can be done is increasing the mapping population and continuing with the chromosomal walking.

Nonetheless, the identification of the \textit{pym} mutants in the \textit{pym Ler x wild-type Col F2} population presented specific problems related to the variability of the occurrence of trichome overbranching on the rosette leaves of \textit{Arabidopsis}. Indeed, the percentage of 3-branched trichomes on a rosette leaf is not constant; it depends instead on unknown internal and external factors. In addition, the ecotype Columbia presents somewhat more overbranched trichomes than Landsberg \textit{erecta} (Larkin et al., 1996). Therefore, the offspring of the crossing \textit{Ler pym x Col wild-type} comprised wild-type and heterozygous plants with a higher overbranching than the \textit{Ler} wild-type as well as \textit{pym} mutant plants with a moderate percentage of 4- to 6-branched trichomes. Given the difficulties encountered to obtain a reliable mapping population comprising only \textit{pym} mutant plants, it was decided not to map \textit{PYM} further.

There are about 120 genes in the region where \textit{PYM} is located. From those, 15 are transcription factors, 8 are protein kinases that have not been involved in any specific cascade, 5 play a role in the degradatory pathway, 4 are implicated in protein-protein interactions and 3 are part of the cytoskeleton. All the other genes have no attributed function. None of them have been implicated in the biosynthesis of the GAs or on the transduction of the gibberellin acid message.

3.2 Morphological characterization of the \textit{polychome} mutant and analysis of its dependency on the gibberellins

The relevance of the PYM protein in the trichome morphogenesis was revealed by the characterization of the corresponding mutant phenotype: the \textit{pym} mutant trichomes are overbranched and present higher DNA contents than the wild-type. Thus, PYM is essential for the inhibition of the endoreduplication and the branching of the trichomes (Perazza et al., 1999). In addition, my work has shown that the \textit{pym} mutant plants present pleiotropic alterations: both the hypocotyl and the stem of the mutant are shorter than the ones of the wild-type, the trichome density is reduced, the hypocotyl cells are defective in the regulation
Discussion A

of the endoreduplication and the pym mutant plants flowers late. How to explain those aberrations?

Genetic analyses indicate that PYM functions downstream of SPY in the GA signalling pathway. Since both mutants present the same trichome phenotype, pym may be affected in the same manner than spy: the mutant might transduct constitutively the gibberellin signal. To test this hypothesis, I observed the effect of a blockage of the GA biosynthesis in the trichome morphogenesis. If the transduction of the GA signal is constitutively activated in the pym mutant, the mutant trichomes must be able to branch in the absence of the hormone. In the assay performed for this thesis work, a blockage of the gibberellin biosynthesis did not reduce the branch number in the mutant trichomes, but did in the Ler wild-type ones. This data confirms the function of PYM as a negative regulator of trichome morphogenesis via the GA signalling cascade.

Interestingly, the pym mutant does not present the phenotypic alterations that are characteristic for a constitutive transduction of the gibberellin signal. These mutants typically elongate more, develop more trichomes and flower earlier than the wild-type, which is contrary to the phenotypes observed in pym plants. Moreover, the application of exogenous GA to the pym seedlings resulted in a complete rescue of the hypocotyl length and restored the ability of the mutant to flower, suggesting that the aberrations observed in the mutant are due to a deficiency in GAs. This apparent contradiction can be explained in two ways. It is possible that the phenotypes observed in the pym mutant are the result of mutations in two different genes: in this scenario, a mutation in the PYM gene will affect a protein negatively implicated in the transduction of the gibberellin message specifically in the trichome, and will lead to overbranching. A second side mutation would lead to short stem and hypocotyl as well as to a delay in flowering due to a defect on a gene of the GA biosynthetic pathway. To verify this hypothesis, it is important to perform a genetic analysis of the offspring from a pym x Ler wild-type crossing: the different phenotypes should segregate in an F2 population if they are due to mutations in different genes. In another scenario, all the phenotypes are caused by a single mutation affecting the transduction cascade in the trichomes. This exiting hypothesis is based on the discovery that mutations activating constitutively the transduction of the gibberellin signal lead to decreased levels of bioactive GAs (Xu et al., 1995, Hedden and Kamiya, 1997, Cowling et al., 1998, Silverstone et al., 1998). The role of PYM as a negative regulator of signal transduction seems to be limited in the plant to the control of trichome morphogenesis and to the regulation of endoreduplication in both the trichome and the hypocotyl since all the other aberrant phenotypes that were described during this work
Discussion A

typically correspond to a deficiency on gibberellins. The fact that different proteins in different organs mediate the transduction of the GA signal has already been described, even though the mechanisms implicated in this process have not been understood yet. For instance, \textit{DWARF1} of Potato seems to play a specific role in the ability of the second leaf to sense the gibberellin hormone (Ashikari \textit{et al.}, 1999; Fujisawa \textit{et al.}, 1999).

To summarize, \textit{PYM} is negatively implicated in the transduction of the gibberellin signal leading to the development of trichome branches in \textit{Arabidopsis thaliana} and very likely also to the control of endoreduplication in the trichome and in the hypocotyl cells. The aberrant phenotypes observed in the \textit{pym} plants might be due to a second side mutation in the genome of the \textit{pym} plants but could as well be the result of a deficiency in the GA levels provoked by the up-regulation of the gibberellin signal transduction.
Trichome

Pattern formation
B 1  Introduction

1.1  Trichome initiation in *Arabidopsis thaliana*

The study of the mechanisms underlying pattern formation is a fascinating area of research on developmental biology. How are cells recruited from initially equivalent cells to differentiate and how are they arranged in a well-ordered manner? The development of trichomes, the leaf hairs of *Arabidopsis thaliana*, is an excellent model system to study pattern formation because they present a simple two-dimensional spacing pattern and are initiated at regular distances to each other (Hülskamp et al., 1994, Marks 1997, Hülskamp *et al.*, 1999, Szymanski *et al.*, 2000, Larkin *et al.*, 2003). The resulting pattern must be tightly controlled because the distance between the developing trichomes is at least three to four cells and trichomes adjacent to one another (clusters) are much less frequent as would be expected by a random distribution (Hülskamp *et al.*, 1994, Larkin *et al.*, 1996). Cell lineage is not involved in the decision of becoming a plant hair since trichomes do not derive from systematic cell division patterns (Larkin *et al.*, 1996, Schnittger *et al.*, 1999). Finally, trichomes do not seem to emerge as a response to positional cues, since they are not found associated to any pre-pattern (Hülskamp *et al.*, 1994). It is likely that the trichome pattern is established on the epidermal layer of very young leaves as a result of cell-cell interactions taking place between the trichome precursor and its neighbouring cells (Larkin *et al.*, 1996, Schnittger *et al.*, 1998, Schnittger *et al.*, 1999).

1.2  A model to explain two-dimensional pattern formation

Meinhardt and Gierer have proposed a model to explain biological pattern formation based on the reaction diffusion mechanism studied by Turing in the 50s. Turing demonstrated that two interacting chemicals could generate a spatial concentration pattern if one of the substances diffuses faster than the other (Turing 1952). In the model of Meinhardt and Gierer, thereafter called activator-inhibitor model, the two interacting chemicals described by Turing are an activator and an inhibitor (Meinhardt and Gierer 1974, Koch and Meinhardt 1994, Meinhardt and Gierer 2000). A stable pattern is established by local self-enhancement of a short-ranging activator and an inhibition of this autocatalysis by a long-ranging inhibitor, production of which depends upon the activator (Figure 8a). The activator is engaged in a self-activation
loop, e.g. a slight increase of the activator’s concentration leads to a further increase of this activator. However, this is not sufficient to create a regular pattern because every small fluctuation would lead to an exponential increase on the activator’s concentration. The fast diffusion properties of the inhibitor in this system prevents that the activation takes place in the surrounding tissue and at the same time does not disturb the incipient local increase of the activator. The reaction scheme is shown in figure 8b. A local minute increase of the activating substance (green line) above the concentration range will grow further due to the self-enhancing process while the concomitantly produced inhibition (red line) down-regulates the activation of the surrounding field. A final, stable situation is reached when the local self-enhancement is at equilibrium with the surrounding cloud of inhibition.

Figure 8: Pattern formation by autocatalysis and long-range inhibition. (a) The simplest reaction scheme: an activator (blue) catalyses its own production and that of its highly diffusing antagonist, the inhibitor (red) (b) Computer simulation of pattern formation in a chain of cells. Random fluctuations in the ability of the cells to perform the reaction (blue squares) are sufficient to initiate pattern formation. In a field larger than the diffusion range of the inhibitor (red), several activator (green) maxima emerge. Under this condition, the spacing is somewhat irregular and minimum distance is maintained. The initial, an intermediate and the finally stable distributions are shown. (Adapted from Meinhardt and Gierer, 2000)

1.3 Is protein degradation relevant for trichome pattern formation?

The activator-inhibitor model incorporates the existence of a depletion mechanism regulating the concentration on activators and inhibitors (Gierer and Meinhardt 1972, Meinhardt and Gierer 2000). The ability to control the removal of these regulatory elements from the system appears to represent a big advantage for fine-tuning the process, as it may allow rapidly creating and stabilizing incipient concentration maxima. To date, the existence of such a mechanism in the context of trichome pattern formation has not been described. Interestingly, KAKTUS, a gene implicated in trichome morphogenesis, encodes a HECT E3 domain protein and thus may target proteins to degradation via the 26S proteasome (Perazza et al., 1999, El Refi et al., 2003, Downes et al., 2003). Several of the proteins involved in trichome
morphogenesis also play a role in trichome pattern formation (Hülskamp et al., 1994, Larkin et al., 1994, Larkin et al., 1999, Schnittger et al., 1999, Payne et al., 2000, Johnson et al., 2002, Schellmann et al., 2002). Thus, it is reasonable to hypothesize that KAK could play a role in the establishment of trichome formation by promoting the degradation of elements involved in the trichome pattern system.

A protein destined to be degraded by the 26S proteasome enters the pathway via an ATP-dependent conjugation cascade, involving the sequential action of an universal E1 ubiquitin-activating enzyme, one of several E2 ubiquitin-conjugating enzymes and one member of the E3 ubiquitin-ligase family. In the final step, an E3 ligase recruits both the target and an ubiquitinated E2 intermediate and then stimulates ligation of the C-terminal carboxyl group of the ubiquitin to free amino groups in the target (Hershko and Ciechanover 1998, Pickart 2001). A polyubiquitin chain is synthesized by successively adding activated ubiquitin moieties to internal lysine residues on the previously conjugated ubiquitin molecule. In a final step, the 26S proteasome recognizes the chain and degrades the polyubiquitinated substrate.

1.4 Elements of the trichome pattern system and their molecular nature

Several genetic screens have allowed the identification of two classes of mutants that show defects in trichome initiation and pattern formation. One class of mutants present few or no trichomes on the leaf surface, and thus the genes affected are positively implicated in trichome initiation. GLABROUS1 (GL1), AtMYB23 (MYB23), GLABROUS2 (GL2), GLABROUS3 (GL3), ENHANCER-OF-GLABROUS3 (EGL3), TRANSPARENT-TESTA-GLABRA1 (TTG1) and TRANSPARENT-TESTA-GLABRA2 (TTG2) belong to this class. In another class of mutants, more or clustered trichomes develop on the leaf: the genes affected encode for inhibitors of trichome formation. The second class of mutants comprises alterations in CAPRICE (CPC), TRYPTICHON (TRY), ENHANCER-OF-TRYPTICHON-AND-CAPRICE1 (ETC1) and ENHANCER-OF-TRYPTICHON-AND-CAPRICE2 (ETC2) (Koornneef 1981 Oppenheimer et al., 1991, Hülskamp et al., 1994, Wada et al., 1997, Johnson et al., 2002, Schellmann et al., 2002, Zhang et al., 2003, Kirik et al., 2004a, Kirik et al., 2004b). GL1 and its homolog MYB23 encode for an R2R3-type MYB transcription factor. GLABRA2 has been sequenced and shown to have sequence similarity to homeodomain proteins and therefore is likely to coordinate the expression of target genes (Rerie et al., 1994). GLABRA3 and EGL3 are members of the basic helix-loop-helix (bHLH) transcription factor family (Oppenheimer et al., 1991, Payne et al., 2000, Zhang et al., 2003, Kirik et al.,
The TTG1 protein contains seven WD-40 domains that are thought to mediate protein-protein interactions (Walker et al., 1999, Larkin et al., 2003). The TTG2 gene encodes a WORKY domain protein and plays a role as a transcription factor (Johnson et al., 2002). CPC, TRY, ETC1 and ETC2 are highly homologous to each other. They encode for proteins that possess a R3 MYB repeat and do not have a recognisable activation domain (Schellmann et al., 2002, Wada et al., 2002, Kirik et al., 2004b).

Figure 9: Activator-inhibitor model applied to the trichome patterning system. GL1, GL3 and TTG1 form an activating complex in the leaf protoepiderm and will autocatalyze their own activity and activate the transcription of the activator GL2 and of the inhibitors TRY, CPC, ETC1 and ETC2. GL2 will positively regulate the transcription of downstream genes implicated in the trichome initiation. The repressors move to the neighbouring cells, form an inhibitory complex with GL3 and TTG1 and repress the transcription of GL2. The interaction between TRY and GL3 has been formally described and is the only one depicted here, but the high similarity existing among the inhibitors makes it likely that they all act in a similar manner.

The current model to explain trichome pattern formation is based on the activator-inhibitor model. It postulates that initially, all the cells in the protoepiderm are competent to develop as trichomes. These cells produce low levels of GL1, GL3, EGL3, TTG1 and possibly TTG2 (Larkin et al., 1993, Johnson et al., 2002, Zhang et al., 2003, Baudry et al., 2004). Together, those proteins comprise a transcriptional activator complex that induces transcription of the downstream genes GL2, TRY, CPC and possibly ETC1 and ETC2 as well (Paynes et al., 2000, Szymanski et al., 1998, Schellmann et al., 2002, Wada et al., 2002, Zhang et al., 2003).

MYB23 is also active in promoting trichome initiation, but it is only expressed in the developing trichomes (Kirik et al., 2005). The GL2 protein promotes trichome cell fate while the other proteins are very likely transported into the neighbouring cells, where they inhibit the expression of GL1, GL2 and perhaps GL3 and TTG1 by forming a inhibitory complex.
with GL3 and TTG1 (Paynes et al., 2000, Szymanski et al., 1998). At first, the cells are locked in mutual inhibition. This state is metastable, and owing to random variations in gene expression levels, some cells will have higher levels of the transcriptional activators. These cells will ultimately become committed to the trichome fate (due to the action of GL2) and succeed in inhibiting the neighbours from doing the same (because of the inhibition of GL2 transcription via the inhibitory complex). This mechanism is represented on the figure 9.

Experimental evidences corroborate that the activator-inhibitor model can explain the establishment of a trichome pattern. The overexpression of GL1 does result in enhanced trichome production and strongly increased cluster frequency in the try mutant only, suggesting that TRY prevents cluster formation by inhibiting GL1 as would be expected by the model (Schnittger et al., 1998, Szymanski et al., 1998a, Schnittger et al., 1999). The enhanced cluster formation was also found for the GL3 homologous gene R from maize in try mutant background and is additionally enhanced if both activators, R and GL1, are ectopically expressed in the absence of TRY (Schnittger et al., 1999). Therefore TRY is supposed to mediate lateral inhibition by suppressing GL1 and GL3 function (Schnittger et al., 1999). The activator-inhibitor model predicts that the negative regulator follows the expression pattern of the activator. In fact the activators and the inhibitors show the same dynamic expression pattern with initial ubiquitous distribution throughout the patterning zone of the leaf and the following restriction to the trichomes (Larkin et al., 1993, Schellmann et al., 2002, Zhang et al., 2003). There is some evidence that the activity of TRY depends on the dosage of the positive regulator TTG1 (Schnittger et al., 1999, Larkin et al., 1999).

1.5 Outlook and aim of the work

The activator-inhibitor model explains how a biological pattern can be established de novo from identical primordia cells. This model has been used as a frame to investigate how Arabidopsis thaliana regulates the distribution of trichomes on rosette leaves. As a result from those investigations, many regulators of trichome pattern formation have been identified and the interactions between activators and inhibitors are starting to be understood. Nonetheless, one intriguing aspect of the model, namely the regulation of the concentration on activators and inhibitors by depletion has not been investigated. The finding that KAK, a gene implicated in trichome morphogenesis, encodes an E3 ligase permits to hypothesize that the degradation of patterning proteins might have a physiological relevance for the establishment of a trichome patterning.
My thesis first aimed to provide evidences for an implication of KAKTUS in the establishment of trichome patterning. Second, I questioned whether KAKTUS is responsible for the degradation of at least one of the patterning proteins. Third, I proposed that GL1 is the target of the KAK-dependent degradation and I studied whether the depletion on GL1 takes place via the 26S proteasome. Finally, I provided with proves of a genetic interaction between KAK and GL1 making it likely that the role of KAKTUS as E3 ligase in the degradation of GL1 has a physiological relevance for the trichome initiation. The results are summarized in a new model to explain the very early steps of trichome pattern formation.
B 2 Results

2.1 *At4g38600* is the *KAKTUS* gene

The overbranching phenotype observed on the kaktus trichomes correlates to mutations in the annotated gene *At4g38600*. It was demonstrated by RACE-PCR that this gene encodes a homolog of HECT E3 ligases thereafter assumed to be the transcript of the *KAKTUS* gene (El Refi et al., 2003, Downes et al., 2003). To prove the relevance of the proposed gene in trichome morphogenesis, I analysed the relative amounts of three-branched trichomes on the third rosette leaf of 20 T1 35S:*YFP:KAK kaktus* plants compared to *Ler* wild-type plants and to a knockout mutant of *KAKTUS*, *kaktus2*. In the wild-typical background, 70% of the trichomes are three-branched structures. In contrast, up to 80% of *kaktus2* trichomes are overbranched. From the 20 transgenic lines observed, 9 presented a wild-typical trichome branching phenotype (they develop more than 70% of wild-type trichomes), 6 develop between 20% and 70% of three-branched trichomes and 5 lines displayed only trichomes with four branches or more. Therefore, it is possible to conclude that *At4g38600* is the *KAKTUS* gene and that it plays a role in the inhibition of trichome branching.

2.2 The *kaktus* plants carry a mutation in a patterning

In an attempt to assess whether *KAKTUS* is implicated in the establishment of a trichome patterning in *Arabidopsis thaliana*, I compared the density and the distribution of trichomes on the third rosette leaf of both the wild-type and *kaktus2* plants. The trichome density on the third mutant leaf is in average double than the wild-type one (*Ler* wild-type: 29,0 (+/- 12,2) trichomes /cm²; *kaktus*: 58,4 (+/-16,0) trichomes /cm²; n=50;). Since *KAKTUS* is expressed in the cotyledons and in the hypocotyl (El Refi et al., 2003), I quantified the number of trichomes on those organs in *kak2* compared to the wild-type. Neither the *Ler* wild-type nor the *kaktus* mutant develops trichomes on the cotyledons. But, although *Ler* wild-type displays a glabrous hypocotyl, the *kaktus* mutant initiates on average 4,0 (+/- 1,5) trichome-like structures on this organ (Figures 10a and 10b, and Table 1). Those structures resemble trichomes in that they are single cells developing from the epidermal layer. Nonetheless, they appear to be always unbranched and somewhat bigger than trichomes.
2.3 The 35S:YFP:KAKTUS gene is not able to rescue the aberrant trichome patterning phenotype on the kaktus mutant plants

To find out whether a mutation in the KAKTUS gene is responsible for the increased trichome density observed in the kaktus mutant plants, I analysed the distribution of trichomes on the 20 35S:YFP:KAK kaktus T1 plants described above as well as the density and the distribution of plant hairs on 50 T2 plants arising from 3 of those 20 T1 lines. To make sure that the phenotypes observed are a consequence of the expression of YFP:KAK, I verified the presence of the YFP:KAK fusion protein in those plants.

Figure 10: Involvement of KAKTUS in the establishment of trichome patterning. (a) Glabrous hypocotyl of a Ler wild-type plant. (b) Trichome-like structures on the kaktus mutant hypocotyl. (c) Trichome patterning on the rosette leaves from 35S:YFP:KAKTUS kaktus plants. (d) Nuclear localization of YFP: KAKTUS in a trichome of a rosette leaf (e) Glabrous cotyledon of a Ler wild-type plant. (f) Trichomes on a 35S:GL1:HA kaktus cotyledon.

From the 20 T1 lines observed, 11 develop trichomes only on the edges of the lamina and display the fusion protein in the nucleus of the Arabidopsis thaliana cells (Figures 10c and 10d). Nine T1 plants present a similar phenotype than the kaktus background leaves with respect to the trichome patterning: among them, 4 contain YFP:KAK in the nucleus and 5 do not express the fusion protein in detectable levels. There is no correlation between the rescue of the trichome branching phenotype described above and the alteration of the trichome pattern presented here: 6 out of the 9 35S:YFP:KAK kaktus plants that develop more than 70% of wild-typical trichomes present trichomes exclusively at the edges of the lamina.
Conversely, only 3 \(35S:YFP:KAK\) \(kaktus\) plants from the 11 that present an aberrant trichome distribution are wild-typical in respect to the trichome morphogenesis. This first analysis of the ability of \(35S:YFP:KAK\) to rescue the aberrant trichome density on the \(kaktus\) plants indicates that this gene is not correcting the \(kaktus\) phenotype but alters the trichome distribution on the lamina.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Trichome number (lamina)</th>
<th>Trichome number (hypocotyl)</th>
<th>Trichome number (cotyledons)</th>
<th>Lamina surface (cm(^2))</th>
<th>Trichomes per cm(^2)</th>
<th>Trichome distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ler) wild-type</td>
<td>17.7 (+/- 3.9)</td>
<td>0</td>
<td>0</td>
<td>0.6 (+/- 0.2)</td>
<td>29.0 (+/- 12.2)</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>(kaktus)</td>
<td>28.0 (+/- 6.0)</td>
<td>4.0 (+/- 1.5)</td>
<td>0</td>
<td>0.5 (+/- 0.1)</td>
<td>58.4 (+/- 16.0)</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>(35S:YFP:KAK) in (kaktus)</td>
<td>45.0 (+/- 17.6)</td>
<td>0</td>
<td>0</td>
<td>0.8 (+/- 0.2)</td>
<td>57.2 (+/- 21.6)</td>
<td>On the edges of the lamina</td>
</tr>
<tr>
<td>(35S:GL1:HA) in (kaktus)</td>
<td>19.8 (+/- 2.7)</td>
<td>0</td>
<td>0</td>
<td>1.8 (+/- 0.3)</td>
<td>11.5 (+/- 2.6)</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>(35S:GL1:HA) in (kaktus)</td>
<td>30.1 (+/- 2.4)</td>
<td>15.2 (+/- 8.8)</td>
<td>5.8 (+/- 1.6)</td>
<td>1.8 (+/- 0.3)</td>
<td>17.1 (+/- 4.1)</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>(gl1(65C))</td>
<td>15.1 (+/- 4.1)</td>
<td>0</td>
<td>0</td>
<td>0.9 (+/- 0.3)</td>
<td>18.0 (+/- 7.1)</td>
<td>On the edges of the lamina</td>
</tr>
<tr>
<td>(gl1(65C)) x (kaktus)</td>
<td>34.1 (+/- 4.5)</td>
<td>0</td>
<td>0</td>
<td>0.9 (+/- 0.3)</td>
<td>40.0 (+/- 12.5)</td>
<td>Homogeneous</td>
</tr>
</tbody>
</table>

Table 1: Trichome number, density and distribution of several mutants and over expressing lines. The density is indicated in trichomes per cm\(^2\) on the lamina. The seventh column refers to the trichome distribution on the lamina. This analysis only takes into consideration the lamina of the third rosette leaf.

To analyse in detail how the over expression of the \(35S:YFP:KAK\) gene interferes with the establishment of a wild-typical trichome pattern, I selected randomly 3 lines among the ones presenting an altered trichome distribution and I analysed both the trichome localization and the density on the third rosette leaf as well as the presence of trichomes on the hypocotyl of 50 T2 plants per line. All the plants included in this study presented the YFP:KAK fusion protein in the nucleus of the cells. A t-test showed that the three lines displayed a comparable trichome pattern in all the organs \((p > 0.05\%)\). The results obtained for one representative line are summarized on the Table 1. The trichomes are distributed homogenously on the lamina surface of the \(Ler\) wild-type plants as well as of the \(kaktus\) mutant plants. As expected, the offspring of one \(35S:YFP:KAK\) \(kaktus\) plant that initiates trichomes exclusively at the edges of the lamina behaves like the parental plant. Hence, the most prominent effect of an over expression of the \(35S:YFP:KAK\) gene is that the transgene has a negative effect on the
initiation of trichomes on the middle of the lamina. In addition, the Ler wild-type plants develop 29.0 (+/- 12.2) trichomes/ cm² on the lamina (n=50) while both the kaktus mutants and the 35S:YFP:KAK kaktus transgenic plants present approximately twice as many trichomes/ cm² than the wild-type (kaktus: 58.4 (+/-16.0) trichomes/ cm²; 35S:YFP:KAK kaktus: 57.2 (+/- 21.6) trichomes/ cm²; n=50). Nonetheless, the actual trichome density on the lamina is different between the three populations, because the leaf hairs of the transgenic plants are all located at the edges. Thus, the trichome density on the edges of the 35S:YFP:KAK kaktus lamina is higher than the one in the kaktus plants. Therefore, the over expression of YFP:KAK in kaktus results in an increased trichome density compared to both the Ler wild-type and the kaktus mutant plants. To conclude, the over expression of YFP:KAK abolishes the trichome initiation in the middle of the leaf and increases the trichome density of the kaktus plants at the edges of the lamina. By contrast, the expression of 35S:YFP:KAK rescues the aberrant trichome initiation on the kaktus mutant hypocotyl: the kaktus mutants develop on average 4.0 (+/-1.5) trichome-like structures on the hypocotyl while the transgenic plants present a glabrous hypocotyl.

2.4 KAKTUS interacts in yeast and in Arabidopsis thaliana with proteins implicated in trichome patterning

Targeting of a protein via the ubiquitin system must involve specific binding of the protein to the appropriate HECT E3 ligase (Gonen et al., 1991, Scheffner et al., 1993, Varshavski et al., 2000). Therefore, a direct interaction between an E3 ligase and a protein provides a hint about a potential role of the ligase in the degradation of this polypeptide. To reveal targets of a potential KAK-dependent degradation, I analysed whether KAK and the proteins implicated in trichome patterning are able of physical interaction in yeast and in Arabidopsis thaliana.

To perform the yeast two-hybrid assay, I fused the KAKTUS cDNA either to the GAL4 DNA binding domain (BD) or to the GAL4-activation domain (AD) in vectors allowing the expression of the fusion proteins in yeast. In yeast, the KAKTUS:GAL4 DNA BD fusion protein interacts with GLABROUS1:GAL4 AD. If GLABROUS1 is missing the last 27 amino acids, which contain the DNA transactivation domain of the protein, it does not interact with neither the KAK:GAL4 AD nor the KAK:GAL4 DNA-BD proteins. Similarly, KAKTUS is able to direct interaction with the full-length version of GL3, but not with the truncated version missing the last 29 amino acids (containing the transactivation domain of
the protein). In addition to interacting with the GL1 and GL3 proteins, the KAKTUS protein fused to the GAL4 DNA-BD interacts in yeast with GL2:GAL4 AD. The KAK:GAL4 AD protein interacts in yeast with GL2, TTG1, TTG2, CPC and TRY fused to the GAL4 DNA-binding domain. MYB23 is not able of direct interaction with KAKTUS in yeast (figure 11). None of the fusion proteins alone enabled the yeast cells to grow on media lacking histidine.

Figure 11: Interactions of KAK with the proteins implicated in the trichome patterning in yeast two hybrid assays. Growth on the histidine-deficient medium indicates an interaction of the fusion protein with KAK-AD (a) and with KAK-BD (b) AD: GAL4 transcriptional activation domain, BD: GAL4 binding domain. ΔGL1 and ΔGL3: truncated versions of the GLABROUS1 and the GLABROUS3 proteins, where the last 27 and 29 amino acids, respectively, are missing.

To check whether the interactions observed in a heterologous system also take place in the plant, I fused KAKTUS to the N- and C-terminal sub-fragments of the YFP gene in a vector allowing the transient expression of the fusion proteins in plants and I used these constructs to perform a bimolecular fluorescence complementation assay (BiFC) in protoplast cells of Arabidopsis thaliana. The BiFC (also known as "split YFP") assay is based on the observation that the N- and C-terminal sub-fragments of the YFP (YFPn and YFPC respectively) do not spontaneously reconstitute a functional fluorophore. However, if fused to interacting proteins, the two non-functional halves of the fluorophore are brought into tight contact, refold together and generate de novo fluorescence. Thus, by BiFC, the interaction status of two proteins can be easily monitored via fluorescence emission upon excitation with a suitable wavelength (Bhat et al., 2006). I tested the ability of GL1, GL2, GL3, TTG1, TTG2, TRY, CPC and MYB23 fused either with YFPC or YFPn to interact with the KAKTUS fusion proteins. This method allowed the verification of an interaction in the nucleus of Arabidopsis thaliana between KAKTUS and all the proteins tested except MYB23 (Figure 12).
Figure 12: Interaction profile of patterning proteins with KAKTUS in bimolecular fluorescence complementation assays (BiFC). BiFC assay testing the interaction of GL1:YFPc with KAK:YFPn (a to c), GL2:YFPn with KAK:YFPc (d to f), GL3:YFPc with YFPn:KAK (g to i), TTG1:YFPc with KAK:YFPn (j to l), TTG2:YFPc with YFPn:KAK (m to o), TRY:YFPn with KAK:YFPc (p to r) and CPC:YFPn with YFPc:KAK (p to r). The figures on the left column represent the protoplast under white light. The figures on the middle column display an emission of light in the nucleus of the corresponding protoplast due to the reconstituted YFP protein. The figures on the right column show the light emitted by the nuclear GL3:CFP. YFPc: C-terminal fragment of the YFP protein. YFPn: N-terminal fragment of the YFP protein.
2.5 **KAKTUS and GLABROUS1 interact genetically in the trichome initiation pathway.**

To better understand the role of depletion in the formation of a pattern, I decided to focus my work in the relationship between *KAK* and *GL1*. To find out whether these two genes interact to establish the trichome patterning, I analysed the density and the distribution of trichomes on $35S:GL1:HA$ *kaktus* plants compared to the ones on $35S:GL1:HA$ *Ler* plants and on the corresponding backgrounds. The overexpression of *GL1* does not alter the number of trichomes on the rosette leaves of the *kaktus* plants compared to the wild-type ($35S:GL1:HA$ *Ler* wild-type: $11.5$ (+/-$2.6$) trichomes/cm$^2$; $35S:GL1:HA$ *kaktus*: $17.1$ (+/-$4.1$) trichomes/cm$^2$; $n=50$). Interestingly, the $35S:GL1:HA$ x *kaktus* cotyledons develop $5.8$ (+/-$1.6$) leaf hairs. By contrast, the cotyledons of all the other genotypes tested are glabrous (Figure 10e and 10f). In addition, the number of trichome-like structures on the hypocotyl is increased in a $35S:GL1:HA$ *kaktus* compared to the *kaktus* mutant alone but not in a $35S:GL1:HA$ *Ler* background (kaktus: $4.0$ (+/-$1.5$) trichomes on the hypocotyl; $35S:GL1:HA$ x *kaktus*: $15.2$ (+/-$8.8$) trichomes on the hypocotyl; $n=50$). These findings point towards a genetic interaction between *KAK* and *GL1* in the context of trichome initiation. All the results described above are summarized on the Table 1.

To better understand the genetic relationship between *KAK* and *GL1*, I analysed the effect of an absence of *KAK* transcript on the trichome patterning of plants expressing a mutated version of the GL1 protein, gl1(65C). This weak mutant allele carries a point mutation in the 383$^{rd}$ nucleotide, which turns the 128$^{th}$ amino acid from a tryptophane into an isoleucine and abolishes completely the trichome initiation in the middle of the lamina. In this analysis, I identified homozygous *kak* gl1(65C) double mutants among a F2 population. Then, I quantified the amount of trichomes on the third rosette leaf of these backgrounds and I performed a qualitative analysis of the plant hair distribution on the lamina and on the hypocotyl. The trichome density on the third rosette leaf of gl1(65C) kak and is similar to that on the *Ler* wild-type and on the gl1(65C) plants (*Ler* wild-type: $29$ (+/-$12.2$) trichomes /cm$^2$; gl1(65C): $18.0$ (+/-$7.1$) trichomes /cm$^2$; gl1(65C) kak: $40.0$ (+/-$12.5$) trichomes /cm$^2$; $n=50$). Nonetheless, the trichome distribution of the double mutant is comparable to the wild-typical one, but not to the distribution observed on the gl1(65C) plants: the leaf hairs are homogeneously distributed on the lamina of both the gl1(65C) kak and the *Ler* wild-type plants while they are exclusively localized on the edges of the gl1(65C) mutants leaf. In addition, my investigation revealed that in contrast to the *kaktus* plants, the gl1(65C) x *kak* plants do not develop trichome-like structures on the hypocotyls. Taken together, these
Results B

genetic data suggest that *GL1* and *KAK* are implicated in the same pathway to regulate trichome initiation.

2.6 The degradation of GL1 is mediated by the 26S proteasome

The 26S proteasome is the only complex able to recognize, bind and degrade polyubiquitinated proteins and therefore a severe malfunction of it leads to a complete blockage of the proteolysis of target proteins, and thus to an accumulation of them in the cell. To test whether the degradation of GL1 takes place via the 26S proteasome, I induced a complete blockage of the complex via the application of MG132 to 35S:*GL1*:HA plants (Bogyo et al., 1997). Subsequently, I revealed by western blotting the amounts of HA-tagged protein in the plant before and after the blockage.

![Western Blot](image)

**Figure 13: Study of the dependence of GL1 degradation on the 26S proteasome.** (a) Detection of the GL1:HA protein before and after treatment with a proteasome inhibitor (MG132). The GL1:HA protein was detected on the western blot using an anti-HA antibody. (b) Semi-quantitative RT-PCR to compare the RNA levels of GL1:HA before and after treatment with MG132. As control for the RNAs extraction efficiency, the different RNAs were used as templates to amplify the *Elongation Factor* gene (*EF*).

The amount of GL1:HA protein in five 10 days-old plants is under the limit of detection of the western blot technique and therefore the fusion protein cannot be visualized on a blot. After the treatment with MG132, the concentration of protein in the same amount of material is sufficient to allow its visualisation. The GL1:HA fusion protein is predicted to be 30,5 kDa in size. On the blot, nonetheless, the band corresponds to a protein of over 35 kDa (Figure
13a). The transcription levels of *GL1:HA* before and after blockage of the 26S proteasome are constant, as demonstrated by RT-PCR analysis (Figure 13b).

2.7 **KAKTUS is implicated in the degradation of GL1**

To assess whether the degradation of GL1 is dependent on KAK, I analyzed the consequences of the absence of **KAKTUS** for the efficiency of GL1 degradation. Therefore, I monitored the kinetics of the degradation of the GL1 protein in a *kaktus* knockout background compared to the wild-type situation during a so-called luciferase assay. The luciferase (*Photinus-luciferin 4-monooxygenase*, LUC) is an enzyme that catalyzes a light-producing reaction: the oxidation of the luciferine. In a luciferase assay, the plants are treated with cycloheximide (to block translational elongation) and with luciferine (to provide a substrate for the enzymatic activity of the luciferase). Subsequently, a light sensitive apparatus detects the photon emission and quantifies the intensity of the fluorescence emitted during the oxidation of the luciferine. This intensity is correlated to the amount of luciferase in the organism. By this mean, it is possible to follow in vivo the degradation of the luciferase, as well as the degradation of any protein fused to it, by monitoring the decrease in the intensity of the fluorescence emitted by an organism (Greer III and Szalay 2001). To take advantage of this property, **GLABROUS1** was fused to the *Photinus-luciferin 4-monooxygenase* in a binary vector and the construct was transformed into Col-0 wild-type plants. I generated two *35S:GL1:LUC kaktus* lines by crossing two independent *35S:GL1:LUC* plants to *kaktus* mutants. The offspring from those lines was used to perform independent luciferase assays (Figure 14a and 14b). The depletion on a fusion protein is the result of the synergistic effect of the degradation of each sub-fragment. To quantify the relative importance of the degradation of the luciferase in the decrease on the amounts of GL1:LUC, I followed the kinetics of the degradation of the LUC protein by Col-0 wild-type plants. This assay was repeated three times with similar results (p>0.05%). The average values obtained from the measurements are represented on the Figure 14 (blue curve).
Figure 14: Kinetics of the degradation of GLABROUS1. The abscissa axis represents the time in minutes. The curves represent the decay in the luminescence emitted through luciferine oxidation in the 35S:LUC Col-0 plants (in blue), in the 35S:GL1:LUC kaktus plants (in red) and in the 35S:GL1:LUC Col-0 plants (in green). The assay was performed independently with two lines: line 1 (a) and line 2 (b). The intensity of the luminescence at t₀ is reported to 100% and can be assimilated to the total pool of LUC or of GL1:LUC present in the plant at the beginning of the assay.

The degradation of the LUC by the 35S:LUC Col-0 transgenic plants takes place at a speed that is almost constant: the average rate is 13% of protein degraded per hour for the time of the assay. After 225 minutes, the 35S:LUC Col-0 plants are depleted from 50,1% of the enzyme. The Col-0 plants degrade the GL1:LUC fusion protein at a higher rate than the luciferase enzyme alone (green curve). The plants from the two lines tested are able to degrade almost 80% of the GL1:LUC fusion protein in 225 minutes (77% and 76,5% respectively). The degradation rate is 30% of protein degraded per hour during the first 100 minutes, and then 13% of protein degraded per hour until the end of the assay. The 35S:GL1:LUC kaktus knockout mutants degrade the fusion protein at a lower rate than the transgenic wild-type plants (red curve). These plants degrade the fusion protein at a rate of 24% of protein degraded per hour for the first 100 minutes and at a rate of 12% until the end of the assay. Therefore, during the assay, the GL1:LUC kaktus mutant plants degrade 60,5% (line 1) and 64,2% (line 2) of the protein. Taking into consideration the proportion of the GL1:LUC protein that is degraded in the cells due to the depletion on LUC, it appears that the wild-type cells degrade approximately 26,5% of GL1 protein in 225 minutes (line 1: 26,4%; line 2: 26,4%) whereas the kaktus mutants degrade between 10,4% and 14,1% of protein in the same time (lines 1 and 2 respectively).
B 3 Discussion

The process of trichome formation on the leaves of Arabidopsis thaliana is used as a model system to study the establishment of a biological pattern. Currently, it is assumed that this pattern is created via the so-called activator-inhibitor model (Meinhardt and Gierer 1974, Larkin et al., 1997, Hülskamp and Schnittger, 1998). This view implicates that the biological pattern appears as a result of local self-enhancement of an activator and an inhibition of this autocatalysis by an inhibitor. The model describes that the depletion on those proteins is necessary to regulate their concentration. To date, this aspect of the model has not been studied. Interestingly, the recent cloning of KAKTUS allows hypothesizing that a KAK-dependent depletion may be relevant for trichome initiation: KAK, a protein implicated in the repression of trichome branching, is highly homologous to HECT E3 ligases that targets proteins to degradation via the 26S proteasome pathway (Perazza et al., 1999, El Refi et al., 2003, Downes et al., 2003). To determine how regulated depletion plays a role in the establishment of the trichome pattern, I revealed a role of KAKTUS in leaf hair initiation. In addition, I provided evidence for the implication of the 26S proteasome in the degradation of a positive regulator of trichome formation, GL1. I could demonstrate as well that KAK is necessary for this degradation. Finally, I showed that the KAK-dependent degradation of GL1 has a physiological relevance for the establishment of a wild-typical trichome pattern and I propose a model to explain the implication of depletion in the early steps of trichome formation.

3.1 KAKTUS plays a role in the trichome patterning

The analysis of trichome number and distribution in the kaktus mutant plants reveals alterations of the trichome pattern. First, the trichome density is higher on the kaktus third rosette leaf than on the Ler wild-type one. Second, the kaktus mutant develops trichome-like structures on the hypocotyl while this organ is always glabrous in the wild-type. Thus, the kaktus plant carries a mutation in a patterning gene.

To demonstrate the implication of the KAKTUS gene in the trichome initiation process and to exclude that the aberrant patterning phenotypes are the result of a second site mutation in the
genome of the \textit{kaktus} plants, it is important to analyze the effect of the presence of \textit{KAKTUS} on the trichome patterning of the \textit{kaktus} mutant plants. It appears that the ectopic overexpression in \textit{kaktus} mutant background of the \textit{KAKTUS} cDNA does not lead to a wild-typical initiation of trichomes on the rosette leaves but results instead in an altered distribution of leaf hairs: the trichomes develop exclusively at the edges of the lamina. This observation suggests that \textit{KAKTUS} is implicated in the trichome patterning process as a repressor of trichome initiation but is not sufficient to conclude that it is responsible for the alterations observed in the \textit{kaktus} mutant plants. Interestingly, the presence of 35S:\textit{KAKTUS} is sufficient to rescue the initiation defect observed on the \textit{kaktus} hypocotyl: this organ is glabrous on the overexpressing lines as well as on the wild-type plants. Taken together, the fact that \textit{KAKTUS} is able to repress trichome formation on the rosette leaves and to correct an initiation defect on the \textit{kaktus} hypocotyl strongly suggests that this gene is responsible for the control of the patterning processes altered in the \textit{kaktus} mutant. Nonetheless, an analysis of the trichome initiation pattern should be performed on \textit{kaktus} plants expressing the \textit{KAK} cDNA under its endogenous promoter. In addition, a genetic study should be carried out to verify that mutations on the \textit{kaktus} gene co-segregate with the patterning defects. In this work, \textit{KAKTUS} will be assigned to the repressor of trichome initiation that causes, when mutated, the patterning defects observed in the \textit{kaktus} mutant plants.

3.2 **KAKTUS is a HECT E3 ligase very likely implicated in the degradation of regulators of trichome initiation**

Sequence analysis of \textit{KAKTUS} provides strong cues about its molecular function. The presence of a 400-amino acid HECT domain at the C-terminal end of the protein that includes the catalytic cysteine residue required for the ubiquitin binding activity of HECT proteins strongly suggests that \textit{KAKTUS} belongs to the family of HECT E3 ligases. This family comprises seven members in \textit{Arabidopsis thaliana}. Their implication in protein degradation has not been investigated previously. The HECT E3s are unique among the E3s in that they directly participate in the ubiquitin transfer, using a conserved cysteine in the HECT domain to which the activated ubiquitin is transferred from E2, and so they have a catalytic role (Hershko and Ciechanover 1998, Pickart 2001, El Refi et al., 2003, Downes et al., 2003). Those proteins are implicated in the ubiquitin-mediated degradation of polypeptides via the 26S proteasomal pathway. As \textit{KAK} is involved in the establishment of a trichome pattern, it is
possible to speculate that it is implicated in the degradation of the proteins playing a role in the specification of the trichome fate.

Within the ubiquitin system, substrates must be recognized by the appropriate E3 as a prerequisite to their ubiquitination. The ability of E3 ligases to specifically bind to their substrates has been successfully used to identify the targets of the polyubiquitination. For instance, direct interaction of PARKIN (the ubiquitin ligase responsible for the pathogenesis of autosomal recessive juvenile parkinsonism, one of the most common forms of Parkinson disease) with CDCrel-1 led to the discovery of the role of PARKIN in the degradation of this GTPase, implicated in the inhibition of exocytose through its interaction with syntaxin (Zhang et al., 2000). In Arabidopsis, the RING E3 ligase CONSTITUTIVE MORPHOGENESIS 1 (COP1) interacts with the transcription factor HYPOCOTYL5 (HY5) to promote its degradation (Ang et al., 1998). One approach to identify the potential substrates of KAKTUS was therefore to identify interactors in yeast and in Arabidopsis thaliana. Among the proteins positively implicated in the establishment of the trichome fate, GL1, GL2, GL3, TTG1 and TTG2 are able of direct interaction with KAK in yeast and in plant. The two repressors of trichome formation tested in these assays, TRY and CPC, also bind to KAK. Therefore, KAKTUS might function to promote the degradation of these members of the trichome patterning system.

It is worthwhile to notice that the existence of a physical interaction between MYB23 and KAKTUS could not be demonstrated suggesting that MYB23 is not degraded in a KAK-dependent manner. Since MYB23 is specifically relevant for the initiation of trichomes at the edges of the lamina (Kirik et al., 2005), it may be that 35S:YFP::KAK kaktus plants develop trichomes exclusively in this area because KAKTUS is not implicated in the degradation of MYB23 and therefore this transcription factor promotes trichome initiation on the leaf edges.

### 3.3 GL1 is degraded via the 26S proteasome in a KAK-dependent manner

In this work, I have focused on the study of the KAK-dependent degradation of GL1. This decision was motivated by to facts. First, since the kaktus mutant presents an overabundance of trichomes, it is reasonable to assume that what is relevant for the establishment of a wild-typical trichome pattern is the KAK-dependent degradation of positive regulators of trichome initiation. Second, as GL1 is the only activator implicated exclusively in the formation of the activation complex (Fig. 9), it is very likely that the maintenance of differences on the GL1
levels between neighbouring protoepidermal cells is the key mechanism that stabilizes an incipient trichome pattern.

The absence of KAK in the plant results in a reduced efficiency of GL1 degradation. *Arabidopsis thaliana* cells are depleted of about 30% of the intracellular GL1 protein in 225 minutes, and that KAKTUS is required for the degradation of two thirds of this amount. This result confirms the implication of KAK in the degradation of GL1. Interestingly, the concentration on GL1 tends to stabilization, suggesting that a pool of protein remains in the cell independently of the KAK-dependent degradation.

To study the implication of the 26S proteasome in the degradation of GL1, I checked whether this protein accumulates in the absence of functional proteasomal activity. Previous investigations have used the same reasoning to elucidate the role of this complex in the degradation of proteins. In rice, for instance, inhibition of the 26S proteasome activity causes the accumulation of high molecular mass ubiquitin conjugates resulting in reduced pollen growth while in kiwifruit, an application of MG132, a peptide aldehyde that inhibits the 26S proteasome activity in a potent manner (Bogyo *et al.*, 1997), leads to the accumulation of polyubiquitinated SLR, a repressor of GA signalling. In both cases, it was shown that under physiological conditions, the proteins are degraded via the 26S proteasome complex and that the high molecular ubiquitin conjugates cumulate as a result of the blockage on their degradation (Speranza *et al.*, 2001, Sasaki *et al.*, 2003). This work has shown that GL1:HA cumulates in the plant as a results of an application of MG132. Thus, the accumulation of the fusion protein in the absence of functional proteasomal activity strongly suggests that GL1 is degraded via the 26S proteasome. Interestingly, the GL1:HA detected during this investigation has a size that corresponds to the one predicted for the fusion protein fused to one ubiquitin moiety. By contrast, all the investigations published to date confirm that the blockage of the proteasome results in a accumulation of polyubiquitinated substrate. One possibility is that GL1 may be the target of deubiquitination enzymes (DUBs). These enzymes are capable of specifically removing covalently bound Ubs and thus help preventing the turnover of specific targets by the 26S proteasome (Wilkinson 2000).

### 3.4 The KAKTUS-dependent degradation of GL1 is relevant for trichome pattern formation.

Although it appears that KAKTUS plays a role in trichome formation and that the degradation of GL1 depends on this homolog to the HECT E3 ligases, the study of the mechanisms of
Discussion B

3.5 Towards a new model to explain trichome pattern formation: the key role of depletion.

A major finding of my work is that the establishment of a wild-typical trichome pattern in *Arabidopsis thaliana* requires the KAK-dependent depletion of the key activator GL1, and that this process involves the 26S proteasome pathway. In addition, the study of the kinetics of GL1 degradation suggests that the depletion on this protein is negatively regulated under physiological conditions, since the concentration of GL1:LUC in the plant tends to stabilize after 225 minutes. How does the regulation of the GL1 degradation take place in the leaf protoepidermal cells, and how is this leading to a wild-typical trichome pattern? To propose a model explaining it, it is important to take into consideration that GL1 forms an activator complex with GL3 and TTG1 in the trichome precursor cells and binds to the promoter of *GL2* to induce its transcription which eventually leads to the initiation of trichomes (Fig. 9). Since it has been shown that certain transcription factors have to dissociate from the cis
regulatory sequence in order to be recognized by E3 ligases (Hatoum et al., 1998), it is tempting to speculate that the regulation of the KAK-dependent degradation of GL1 takes place by this means. The model illustrated in the figure 15 proposes a view that takes advantage of this mechanism to explain how the differential regulation of the GL1 degradation stabilizes the incipient trichome pattern. In this view, the protein that is attached to the GL2 promoter and forms the activator complex cannot be recognize by KAK and thus is not degraded. In contrast, in the cells where the inhibitor complex (comprising TRY, GL3 and TTG1) is bound to the GL2 promoter, KAK promotes the degradation GL1. In this situation, the role of KAK would be to stabilize the fate of the non-trichome cells by decreasing their concentration on transcriptional activator while it does not affect the initiation of incipient trichomes. In addition, since KAK is also able of physical interaction with the inhibitors of trichome formation, it could also be targeting them to degradation when they would not be binding to the GL2 promoter as part of the inhibitory complex.

To summarize, this new model postulates that KAK comes into play to stabilize the newly created trichome pattern. To do so, it allows the emergence of striking differences in the levels of regulators of trichome formation between the incipient trichome and the surrounding population of cells.
Figure 15: Model to explain the role of the KAKTUS-dependent degradation of GL1 in the stabilization of a trichome pattern. The GLABROUS1 protein that is not bound to the DNA is ubiquitinated in a KAKTUS dependent manner and degraded by the 26S proteasome. By contrast, DNA binding of this transcription factor in an activation complex inhibits the degradation. Similarly, the inhibitors of trichome initiation are degraded only in the cells that contain the activation complex. Therefore, the incipient trichomes as well as the surrounding cloud of inhibition are stabilized on their fate.

3.6 Outlook

The activator-inhibitor model postulates that a biological pattern can be established de novo by local self-enhancement of an activator and long-range lateral inhibition. My work is not contradictory with this view. Nonetheless, it demonstrates that Arabidopsis thaliana utilizes the 26S proteasome pathway to regulate the degradation of at least one activator of trichome formation, and that this is important to create a wild-typical trichome pattern. Rather than opposing to the established model of trichome pattern formation, this thesis suggests that the regulation of depletion plays a role in the stabilization of the incipient pattern. Interestingly, the control of the degradation of transcription factors seems to be generally important for mechanisms that need to be fine-tuned. For instance, the regulation of the expression of BANYULS, an anthocyanidin reductase committed to the biosynthesis of proanthocyanidins, seems to involve the degradation of TRANSPARENT TESTA8 (TT8), a transcription factor.
homologous to GL3. In this system, the degradation of TT8 is inhibited through binding to TTG1. The rapid turnover of TT8 in tissues lacking TTG1 might be important to restrict more efficiently the expression of BAN in the pigmented cell layer (Debeaujon et al., 2003, Baudry et al., 2004). Consistently with this view, the rapid depletion on GL1 in incipient non-trichome cells may restrict the number of trichomes in the leaves of *Arabidopsis thaliana*. In addition, it may be that the KAK-regulated depletion on regulators of trichome patterning has an effect on the establishment of the pattern by promoting the positive feedback loop that enhances concentration of GL1 in the incipient trichome.

To conclude, the relevance of the KAK-dependent degradation of patterning proteins could have two different effects. First, it could contribute to the autoactivation of *GL1* transcription. Second, it could stabilize the incipient trichome pattern.
C.1 Material

1.1 Chemicals, antibiotics
All used chemicals and antibiotics in analytical quality have been used from Sigma (Deisenhofen), Carl Roth GmbH (Karlsruhe) and Appligene (Heidelberg).

1.2 Enzymes, primers and kits
Restriction enzymes were used from MBI-fermentas (St.Leon-Rot, Germany) and New England Biolabs (Frankfurt/Main, Germany). Modifying enzymes were used from MBI-fermentas (St.Leon-Rot, Germany), Invitrogen (Karlsruhe, Germany), Roche (Mannheim, Germany), usb (Cleveland, USA), Qbiogene (Heidelberg, Germany), TaKaRa (Otsu, Japan). Primers were generated by ARK Scientific and Invitrogen (Karlsruhe). Kits were supplied from peqlab (Erlangen, Germany), Roche (Mannheim, Germany) and QIAGEN (Hilden, Germany).

1.3 Cloning vectors
The following vectors were used in this work:
- pBluescript KS (pBS) (Stratagene) for standard clonings and PCR-product clonings;
- pGEM-T easy (Promega) for PCR-product clonings;
- pDONR201 vector (Invitrogen) was used as a donor in gateway based clonings;
- pACT and pAS plasmids (Clontech) for the yeast-two hybrid assays;
- pColP1 (created by Jean-Marc Bonneville) containing the 1125 bp at the N-terminal end of the KAKTUS cDNA fragment in a pBluescript KS vector backbone;
- pF2aR2b (created by Jean-Marc Bonneville) containing 1630 bp of the KAKTUS cDNA fragment (including the nucleotides 1079 to 2709) in a pBluescript KS vector backbone;
- pEarlyGate 104 (N-YFP) (Early et al., 2006) as a binary gateway target vector containing a CaMV 35S promoter cassette, the YFP protein and conferring resistance to BASTA;

1.4 Microbiotical strains
For standard clonings the Escherichia coli strains DH5α and XL1blue were used. For gateway cloning of destination vectors the DB3.1 strains were used which are resistant to the ccdB
gene. For plant transformation *Agrobacterium tumefaciens* strains GV3101 were used. The gateway cloning required the usage of a modified strain of GV3101, pMP90RK. For yeast-two hybrid assays, the strain AH 109 was used.

1.5 Plant lines

In this study Landsberg *erecta* (Ler) and *Colombia* (Col) were used. The mutant alleles of *glabrous1*, *polychome* and *kaktus* are in Ler background (Hülskamp *et al.*, 1994, Perazza *et al.*, 1998).

C 2 Methods

2.1 Plant work

2.1.1 Plant growth conditions

Seeds were sown on humid freshly prepared *Arabidopsis* culture soil, covered with a plastic lid and stored for three to seven days at 4°C. Plants were grown at constant 16h light and 8h dark condition at constant temperatures at either 18°C or 23°C and the lid was removed after three to four days.

2.1.2 Crossing of plants

Using fine-tweezers the anthers of flowers at a stage when the petals grew out of the sepals were removed. All remaining older and younger flowers were removed and the prepared flower was fixed on a wooden stick. After one to three days the stigma of the carpels were pollinated.

2.1.3 Plant transformation

Plants were transformed according to the “floral dip” method (Clough & Bent 1998). To gain strong plants, these were allowed to grow at 18°C and till the first flowers appeared at stalks of approximately 10 cm in length. Four days before plant transformation a 5 ml pre-culture in
YEB medium of the agrobacterial clone was incubated for two days at 29°C and 1 ml of this pre-culture was used to inoculate the final 200 ml culture. This culture was incubated again for two days at 29°C and afterwards precipitated at 5800 rpm for 12 minutes. The pellet was resuspended in a 5% Sucrose solution containing 0.05% Silwett L-77. Plants were dipped for approximately 20 seconds and afterwards covered with a lid. The lid was removed after two days and after that plants were treated as usual.

2.1.4 Seed surface sterilisation and subsequent plant treatment
Before placing seeds on MSAR-agar-plates (1% Murashige-Skoog salts, 3% sucrose, 0.7% agar-agar, pH5.7, when required: with kanamycin (50µg/ml) or hygromycin (25µg/ml) they were incubated for five minutes in 95% Ethanol (Rotisol) and afterwards incubated for 15 minutes in a 3% NaClO3 solution containing 0.1% triton X-100. Afterwards seeds were washed two times with 0.01% Triton-X100.

2.1.5 Selection of transformants
The seeds of transgenic plants carrying in their T-DNA a kanamycin resistance were selected on MSAR-Agar plates with 50µg/ml kanamycin or 25 µg/ml hygromycin, respectively. Transgenic plants containing the BASTA resistance were grown on soil for 10 to 15 days. The seedlings were sprayed with a 0.001% BASTA solution, the spraying was repeated after three to seven days.

2.1.6 Gibberellin treatment
Plants were grown on MSAR media at 22°C until the cotyledons were opened. Then they were transferred to MSAR media containing gibberellic acid (C₁₉H₂₂O₆, Carl Roth GmbH, Karlsruhe) at concentration ranging from 0,01 µM to 10 µM. Hypocotyl elongation measurements were taken after 10 days grown on vertical plates. Alternatively, the plants were grown on MSAR media containing gibberellins until the flowering bud was visible.

2.1.7 Uniconazol-P treatment
Plants were grown on MSAR media at 22°C until their first pair of leaves was visible. Then they were transferred to MSAR media containing the GA-biosynthesis inhibitor Uniconazol-P (Dr. Ehrenstorfer GmbH, Augsburg) at a concentrations of 10⁻⁶ M as described by Perazza et al., 1998. The measurements were done on the second pair of leaves.
2.1.8 MG132 (Z-Leu-Leu-Leu-H) treatment
Plants were grown on MSAR media at 22°C until their first pair of leaves was visible. Then they were transferred to 3 mL of liquid MSAR media with 1 mM of MG132 (Peptides International, Inc., Louisville, USA) solved in DMSO or with 150 uL of DMSO. After two days, the proteins were extracted and analysed.

2.2 Microscopy and cytological methods

2.2.1 Microscopy
Light and epifluorescence microscopy was performed using a LEICA-DMRE microscope using DIC optics (LEICA). Images were taken using a high resolution KY-F70 3-CCD JVC camera and a frame grabbing DISKUS software (DISKUS, Technisches Büro, Königswinter).

2.2.2 Yeast two-hybrid assay
Fusions with the GAL4 activation domain and the GAL4 DNA-binding domain were performed in the pACT and pAS plasmids (Clontech): Full-length KAKTUS and GLABROUSI cDNAs were fused to the GAL4 activation domain in the pACT plasmid. Full-length KAKTUS cDNA and a truncated version of GL1 missing 27 amino acids at the C terminus were fused to the GAL4 DNA-binding domain in the pAS plasmid. All used constructs and empty vectors did not show any self activation in yeasts. The yeasts were transformed using a standard AcLi/ssDNA/PEG method and plated on SD media lacking leucine and tryptophane. After 4 days, the yeasts were transferred to a SD media lacking leucine, tryptophane and histidine with 5mM 3-amino-1,2,4-triazole (Sigma, Deisenhofen). Two proteins are said to interact when a population of yeast transformed with them fused either to the GAL4 activation domain or the GAL4 DNA-binding domain has acquired the ability to grow on histidine lacking media.

2.2.3 BiMolecular fluorescent complementation
KAKTUS was fused with the N-terminal and C-terminal fragments of the YFP protein in the pCL112, pCL113, pSYC and pSYN. GLABROUSI cDNAs was fused with the N-terminal and C-terminal fragments of the YFP protein in the pSYC and pSYN. The Arabidopsis thaliana protoplasts were transformed as described below with two fusion proteins: one containing the N-terminal fragments of the YFP protein and another one containing the C-terminal fragments.
of the YFP protein. Finally, the protoplasts were observed after 10 to 12 hours with epifluorescence microscopy. Two proteins are said to interact when the protoplasts expressing the fusion proteins present an emission of light due to the reconstituted YFP protein. To create the protoplasts, 50 mL of cell suspension culture (MS powder: 4,3 g, sucrose: 30 g, vitamin B5: 4 mL, NAA: 0,5 mg, kinetin 0,1 mg, pH 5,5) was spun down at 1500 rpm for 5 minutes. 50 mL of enzyme solution (1% cellulase, 0,2% mazerozym in MS-0,34M Glucose manitol medium (MS powder 4,3 g, glucose 30,5 g, mannitol: 30,5 g, pH 5,5) was added to the pellet in two steps of 25 mL each. The suspension was transferred to two large petridishes (diameter: 145 mm, height: 20 mm) and shaken in the dark for 4 hours at 50 rpm. Subsequently, the protoplasts were made ready for transformation transferred to two Falcon tubes and were spun down at 800 rpm. The pellets were resuspended in 25 mL MS-0,34M Glucose manitol medium and spun down like described previously. The pellet was resuspended in 5 mL of MS-0,28M sucrose medium (MS powder: 4,3g.L⁻¹, 0,28M sucrose, pH 5,5) and centrifuged at 800 rpm for 5 minutes. The supernatant containing the protoplasts was then ready for transformation. Each transformation required to mix 50 μL of protoplasts and 15 μg of DNA with 150 μL PEG solution (25% PEG 6000, 0,45 M mannitol, 0,1M Ca(NO₃)₂, pH 9) in a 2 mL Eppendorf tube. The mix was incubated 20 minutes in the dark and the PEG was washed away by the means of two subsequent additions of 500 μL of 0,275 M Ca(NO₃)₂. The transformed protoplasts were centrifuged at 800 rpm for 7 minutes and transferred to 500 μL MS 0,34 M glucose mannitol solution. The Eppendorf tube was left laying for 10 to 12 hours in the dark before observation of the protoplasts.

### 2.2.4 Kinetics of protein degradation

Transgenic plants expressing 35S:GL1:LUC or 35S:LUC were grown on soil under greenhouse conditions until they had developed 6 leaves. Then, the plants were cleaned from soil and put on 100 μM cyclohexidine 30 minutes before the assay. Finally, the plants were sprayed with a 5 mM luciferine solution and the emission of light from the whole plant was monitored with a new light detection cooled charged coupled device (CCCD). The results were analysed with the Excel software from Microsoft.
2.3 Molecular-biological methods:

2.3.1 RNA isolation

Young rosette plants with four to five leaves were used. Plants were homogenised with a mortar and pistil under constant addition of liquid nitrogen and the powder transferred into a 2-ml-tube. 250 µl extraction buffer (1M Tris/HCl pH 7.4, 1% SDS, 5mM EDTA) was added and directly mixed with 500 µl PCI (Phenole-chloroform/isoamyl alcohol, 24+1) and immediately mixed. After centrifugation at 13 krpm the aqueous (=upper) phase carefully transferred into a new 1.5-ml-tube and again immediately mixed with 500 µl PI and thoroughly vortexed. After centrifugation (5 min. 13 krpm) the upper phase was transferred into a new 1.5-ml-tube and mixed with Chloroform (Cl) and again centrifuged (5 min. 13 krpm). This step was repeated for one time. The resulting aqueous phase was transferred into a new 2-ml-tube and 50 µl sodium acetate and 1500 µl 100% EtOH added, carefully mixed and incubated for at least one hour at – 70°C. In this step the RNA (and DNA) will precipitate and can be pelleted by centrifugation (10 min, 13 krpm, 4°C). After careful removing the supernatant the pellet was dried and resolved afterwards in 200 µl DEPC-treated H2O, mixed with 200 µl 4M LiCl and incubated over night at 4°C. During this step the RNA will be specifically precipitated. After centrifugation (15 min. 13 krpm, 4°C) the pellet is washed with 1 ml 2 M LiCl solution and again centrifuged (10 min. 13 krpm, 4°C). The supernatant was carefully removed and the pellet rinsed with 70% EtOH. After centrifugation the pellet was dried and resolved in 50 µl H2O.

2.3.2 Reverse transcription

Prior to reverse transcription residues of genomic DNA were removed using the DNA-free kit from Ambion (Ambion AMS biotechnology) was used according to the manufacturer’s manual. Reverse transcription of isolated RNA was performed using the SUPERSCRIPT II RNase H+ Reverse Transcriptase kit from GibcoBRL (Life Technologies/GibcoBRL, Cleveland USA) according to the manufacturer’s manual. After reverse transcription residual RNA was removed by adding 5 units of RNAse H (MBIfermentas) and incubated for 30 minutes at 37°C.

2.3.3 Semiquantitative RT-PCR

The expression levels of GL1:HA overexpressing plants were estimated by semiquantitative RT-PCR analysis. RT-PCR was carried out with Titan One tube RT-PCR mix (Roche
Diagnostics, Indianapolis, USA). The primer pair ACGACTCTCCACCGTCATTGTTCATC and TAAAAGATGCGCCCATCAACCACCTT was designed to only amplify the 35S:GL1:HA transcript. The elongation factor1 transcript was used as an internal control for the efficiency of mRNA extraction (EF1: ATGCCCCAGGACATCGTGATTTCAT and EF2: TTGGCGGACCCCTTAGCTGGATCA). The PCR reaction was stopped after 40 cycles.

2.3.4 Genomic DNA preparation
For PCR analysis CTAB-preparation of genomic DNA was performed (Rogers & Bendich 1988). For southern blotting, the following protocol was used. One gram of plant material (around 10 six week old rosettes) was grinded with mortar and pistil and continuous addition of liquid nitrogen. To the homogenised powder 4ml of extraction buffer (2% (w/v) CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris/HCl pH 8.0, 0.2% \( \beta \)-mercaptoethanol) was added and incubated at 65°C for 30 minutes. After addition of 4ml Chloroforme/Isoamylalcohol (24:1) and careful shaking, the probes were centrifuged for 15 minutes at 4000 rpm. The aqueous phase was transferred into a new tube and mixed with 3 ml isopropanol and centrifuged for 15 min. at 4000rpm. The upper was again transferred into a new tube and mixed with 3 ml Isopropanol and the precipitate gained by centrifugation for 20 min. at 4000 rpm. The supernatant was removed and the pellet rinsed with 70% EtOH with 10 mM ammonium acetate. After centrifugation and rejection of the supernatant the pellet was dried and afterwards resolved in 250 \( \mu \)l H2O and transferred into a 1.5 ml tube. After addition of 5 \( \mu \)l RNAse (10 mg/ml) for 30 min at RT, the solution was mixed again with 150 \( \mu \)l Phenol/Chloroform. The mixture was centrifuged (16000rpm, 5 min.) and the upper phase transferred into a new 1.5-ml-tube and mixed with Chloroform. After centrifugation (2 min. 16 krpm) the upper phase was transferred into a new 1.5-ml-tube and mixed with 1/10 vol. Na-acetate and 1 vol. Ethanol and incubated at 4°C for at least 15 min. After centrifugation the pellet was rinsed with 70% EtOH and again centrifuged. The supernatant was removed and the pellet was dried and resolved in Tris/HCl pH 8.0 over night at 4°C.

2.3.5 Plasmid DNA preparation from bacteria
Plasmid DNA from E.coli was prepared according to the manufacturer’s protocol using a column pEQ-LAB Plasmid Miniprep Kit (PEQLAB Biotechnology GmbH, Erlangen) to obtain plasmid concentrations of up to 150 ng.\( \mu \)L\(^{-1}\) or using HiSpeed Plasmid Purification Kit (Sigma) to obtain concentrations of 400 ng.\( \mu \)L\(^{-1}\). Plasmid DNA from Agrobacteria was isolated using Qiagen plasmid miniprep kit.
2.3.6 DNA-manipulation

DNA manipulation and cloning were carried out according to Sambrock and Russel (2001) or Ausubel et al. (1994), using standard procedures. All polymerase-chain reaction (PCR)-amplified fragments were sequenced prior to further investigation. Sequencing was carried out on an ABI 310 Prism (Perkin-Elmer Applied Biosystems, Foster City, CA) sequencing equipment according to the manufacturer’s instructions. Sequencing reactions were performed using Big-Dye kit 1.1 (Perkin Elmer Applied Biosystems, Foster City, CA). PCR-Primers and constructs were designed using the VectorNTI-suite 7.1 software (InforMax, Paisley PA4 9RF United Kingdom).

2.3.7 Cloning of the KAK cDNA

The KAKTUS cDNA was cloned with attached gateway sequences into pBS SK+. The first 2708bp, without gateway sequences, were amplified in two fragments of 1125 bp and 1629 bp, and brought into pGEM-T easy vector. They were called pColP1 and pF2aR2b, respectively. Those two molecules were donated by Dr. Jean-Marc Bonneville. The first 1125 bp were introduced into pF2aR2b using Not I and Nsp V. The first 628 bp of the gene as well as the last 3128 bp divided in tow fragments were amplified by PCR using the primer combinations 5’GWF-CC-ATGAAAACATCGGAGCCGCAAGCGGGCGGAG 3’ with 5’TTTCCCTCTTCTCTCTTTCA3’, 5’AAATTTGCCCAAAACTTCGCCAGG 3’ with 5’GGCATGCTCTTAGTCACATGGTACG3’ and 5’CACACCGTCAAAATCCAC3’ with 5’GWR-C-TTATGAGAGGTCGAACGATCCTTGCC3’ respectively. Each fragment was sequenced and ligated to reconstitute the entire KAKTUS cDNA by the means of classical molecular methods. The strategy used to reconstitute the KAKTUS cDNA in pBS SK+ is summarised in the table below. The primers used for the amplification as well as the restriction enzymes required for the cloning steps are specified.

<table>
<thead>
<tr>
<th>POSITION IN THE SEQUENCE (in bp)</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
<th>RESTRICTION ENZYMES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 2708</td>
<td>ATGAAAACATCGGAGCCGCAAGCGGGCGGAG</td>
<td>-</td>
<td>Apal and Bam HI</td>
</tr>
<tr>
<td>1 to 628</td>
<td>ATGGAAACTCGGAGCCGCAAGCGGGCGGAG</td>
<td>G TTTCCCTCTTCTCTTTCA</td>
<td>Apal and Bst XI</td>
</tr>
<tr>
<td>2539 to 4259</td>
<td>AATTTGCCCAAAACTTCGCCAGG</td>
<td>GGCATGCTCTAGTCACATGGTACG</td>
<td>PpuMI and SacI</td>
</tr>
<tr>
<td>3867 to 5667</td>
<td>CACACCGTCAAAATCCAC</td>
<td>TTATGAGAGGTCGAACGATCCTTGCC</td>
<td>Apal and Aphi</td>
</tr>
</tbody>
</table>
### 2.3.8 Primers used for the mapping of PYM

<table>
<thead>
<tr>
<th>BAC</th>
<th>Marker Name</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Tm</th>
<th>Amplion Length</th>
<th>Restriction Enzyme</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1F6</td>
<td>nga 168</td>
<td>nga 168-1</td>
<td>TCGTCTACTGCACTGCCG</td>
<td>53°C</td>
<td>Col: 151</td>
<td>16 bp/-16bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>nga 168-2</td>
<td>GAGGCCAGATGTGATAGGCAG</td>
<td>52°C</td>
<td>Lcr: 135</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2O85</td>
<td>T2O85-EGJ</td>
<td>T2O85-EGG35</td>
<td>TGGAGGTGTCAAACCGTGACAAGCA</td>
<td>59°C</td>
<td>Col: 152</td>
<td>14bp/-14bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2O85-EGG36</td>
<td>AGCCAGACGAAARCACACAACAC</td>
<td>58°C</td>
<td>Lcr: 158</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T11A7</td>
<td>T11A7-EGJ</td>
<td>T11A7-EGG37</td>
<td>CSGAATGGGAAATATGATAGGCG</td>
<td>58°C</td>
<td>Col: 152</td>
<td>16bp/-16bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T11A7-EGG38</td>
<td>TCTGTAATAGTATTTGCTCCG</td>
<td>57°C</td>
<td>Lcr: 136</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T6020</td>
<td>T6020-EGJ</td>
<td>T6020-EGG185</td>
<td>GATTCGCCACCTCATATTTG</td>
<td>58°C</td>
<td>Col: 1235</td>
<td>Au I</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T6020-EGG186</td>
<td>CAAAACAATAATGACACCCGT</td>
<td>57°C</td>
<td>Lcr: 384+851</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T24P15-EGG194</td>
<td>TCAGCTCCTTCTGACAGGT</td>
<td>58°C</td>
<td>Lcr: 1336</td>
<td>Mnl I</td>
<td></td>
</tr>
<tr>
<td>MHK10</td>
<td>MHK10-EGJ</td>
<td>MHK10-EGG159</td>
<td>CTTGACTGCTGATTTGGTTAAC</td>
<td>59°C</td>
<td>Col: 517+935</td>
<td>BstX I</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MHK10-EGG160</td>
<td>TGCCGAAACTATCTGACCG</td>
<td>58°C</td>
<td>Lcr: 1463</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F14N22</td>
<td>F14N22-EGJ</td>
<td>F14N22-EGG157</td>
<td>AGCCAGGAGG7A7TGACAGAAA</td>
<td>58°C</td>
<td>Col: 1492</td>
<td>BerG I</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F14N22-EGG158</td>
<td>TTGCAGCCTAATGATCGT</td>
<td>57°C</td>
<td>Lcr: 453+1039</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F7D19</td>
<td>F7D19-EGJ</td>
<td>F7D19-EGG201</td>
<td>AGGTACCAAACCGGCCAATA</td>
<td>59°C</td>
<td>Col: 200</td>
<td>8bp/-8bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F7D19-EGG202</td>
<td>AGATTACGGCAAAGTACTGCTT</td>
<td>58°C</td>
<td>Lcr: 208</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F14B2-EGG212</td>
<td>GCAGCTGCTGAGTACATCC</td>
<td>54°C</td>
<td>Lcr: 224+93</td>
<td>Mfe I</td>
<td></td>
</tr>
<tr>
<td>F18O19</td>
<td>F18O19-EGJ</td>
<td>F18O19-EGG39</td>
<td>TCGTTGATTGTTTACAGGGA</td>
<td>58°C</td>
<td>Col: 270</td>
<td>56bp/-56bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F18O19-EGG40</td>
<td>CTTTCTACTTCTCCTCCCTCA</td>
<td>57°C</td>
<td>Lcr: 214</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F411</td>
<td>F411-EGJ</td>
<td>F411-EGG53</td>
<td>GGGATCAAGTGGAACTACCA</td>
<td>58°C</td>
<td>Col: 209</td>
<td>58bp/-58bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F411-EGG54</td>
<td>GCCTGTTGATTGTTGACAGT</td>
<td>57°C</td>
<td>Lcr: 151</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F418</td>
<td>F418-EGJ</td>
<td>F418-EGG41</td>
<td>TGGATTAGATGCGAACCACAG</td>
<td>58°C</td>
<td>Col: 320</td>
<td>43bp/-43bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F418-EGG42</td>
<td>TGGTTTATAGCTGACCTGAAC</td>
<td>57°C</td>
<td>Lcr: 277</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F11C10</td>
<td>F11C10-EGJ</td>
<td>F11C10-EGG43</td>
<td>CGAAGTTCTTATGCGAAGAAT</td>
<td>57°C</td>
<td>Col: 238</td>
<td>42bp/-42bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F11C10-EGG44</td>
<td>TTACCACCTTATGGCTACCC</td>
<td>56°C</td>
<td>Lcr: 196</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 2.4 Biological-chemical methods:

#### 2.4.1 Protein extraction

The plants were grounded in 20 μL of extraction buffer (50 mM Tris pH 6.8, 2% SDS, 36% Urea, 30% Glycerin) and 1 μL of mercaptoethanol. Then, the suspension was incubated for 10 minutes at 95 °C and centrifuged at 12000 rpm for 30 seconds. The supernatant was transferred to a fresh Eppendorf tube and kept frozen until usage. The protein concentration was established using the Bio-Rad Protein Assay (Bio-Rad, München) according to the manufacturer’s instructions.

#### 2.4.2 Western blotting

The extracted protein was loaded on a gel (separation gel: 7.5% acrylamide (Rotigel30), 5.625mL of 1M Tris pH 8.8 and 150 μL of SDS 10% in 15 mL H2O) and ran for 60 minutes under 20 mA. The blot was carried on under semi-dry conditions to a Roti-PVDF membrane (Carl Roth GmbH, Karsruhe) according to the manufacturer’s instructions.

The membrane was blocked for 1 hour in 5% milk dissolved in phosphate buffered saline-tween (PBST: 8 g of NaCl 0.2 g of KCl, 1.44 g of Na2HPO4, 0.24 g of KH2PO4, 2 ml of tween-20 in 1L of H2O; pH 7.2). The first antibody (Rat anti HA antibody, Roche) 1:2000 was applied over night at 4°C in 5% milk in PBST. The blot was washed three times in PBT.
for 10 minutes. The second antibody (goat anti rat HRP, Jackson Immuno Research) was applied for 90 minutes at room temperature in 5% milk in PBST. The blot was washed three times in PBST for 10 minutes. Finally, the detection was performed with the kit ECL Western Blotting Analysis system (Amersham Biosciences, UK) following the instructions given by the manufacturers.
References


Koorneef M (1981): The complex syndrome of ttg mutants. Arabid Inf Serv 18, 45-51

Landridge J. (1957): Effect of day-length and gibberellic acid on the flowering of Arabidopsis. Nature 180, 36-37


Larkin JC, Oppenheimer DG, Lloyd AM, Paparozzi ET, Marks MD (1994): Roles of the GLABROUS1 and TRANSPARENT TESTA GLABRA Genes in Arabidopsis Trichome Development Plant Cell. 6(8), 1065-1076


References


Erklärung


Elena Galiana Jaime
Lebenslauf

Elena Galiana Jaime

egaliana78@hotmail.com
Kornelimünsterstrasse, 36
50933 Köln

Geburtsdatum: 15. 10. 1978
Geburtsort Valencia, Spanien
Staatsangehörigkeit: Spanisch


2003: Beginn der Doktorarbeit mit dem Titel: Analysis of Trichome Pattern Formation in Arabidopsis thaliana: The role of KAKTUS in protein degradation

2001 - 2002: Grad des Diplom Biologen (Diplome d’Etudes Approfondis Biologie Cellulaire et moléculaire (Université Joseph Fourier - Grenoble, Frankreich)

1999 - 2000: Maîtrise en Biologie Cellulaire et Physiologie (Université Joseph Fourier-Grenoble, Frankreich); Praktikum über Trichome Initialisierung (CERMO, Université Joseph Fourier - Grenoble, Frankreich)

1998 - 1999: Licence en Biologie Cellulaire et Physiologie (Université Joseph Fourier - Grenoble, Frankreich)

1996 - 1998: Vordiplom in Biologie (Diplome d’Etudes Universitaires Générales Université Joseph Fourier-Grenoble, Frankreich) Praktikum über Trockenheitstress in Mais (Centre de Biologie Alpine, Université Joseph Fourier - Grenoble, Frankreich)

1995-1996: Allgemeine Hochschulreife (Bacchalaréat und Selectividad; Lycée Français de Valencia - Valencia, Spanien)

SS 1995: Sommer-Intensivkurs der Deutche Grundstufe III (Goethe Institut - Europäischen Jugendgägestehaus Todtmoos, Deutchland)