Doctoral Thesis

Analysis and manipulation of gene expression patterns in leaf development

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Analysis and manipulation of gene expression patterns in leaf development

A dissertation submitted to the

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DOCTOR of NATURAL SCIENCES

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Cover picture: enhancer trap line 1493 displaying GFP expression in the leaf margin in *Arabidopsis thaliana*.
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I Summary

Previous data from our laboratory have shown that manipulation of the cell cycle and growth at the leaf margin resulted in changes in leaf shape (Pien et al., 2001a; Wyrzykowska et al., 2002). This indicates that events at the leaf margin might play a crucial role in leaf morphogenesis. The aim of part 1 of this thesis was to identify and characterise genes which are specifically expressed in the leaf margin of Arabidopsis thaliana. After screening an enhancer- and gene trap library and searching in the database for already identified enhancer traps, we found a recessive mutant, which we termed "hepatica" (hep). The mutation in HEP is putatively caused by an enhancer trap insertion in a gene expressed in the leaf margin. Coupled with this expression pattern, the line shows an altered pattern of margin cell differentiation and altered leaf phenotype. Therefore, HEP might be a candidate gene involved in regulating leaf shape via the regulation of leaf margin differentiation. Histological data suggest that the alteration in leaf shape is caused by a delayed cell division arrest front.

As soon as the HEP gene is identified, its function in leaf development could be determined by locally manipulating its expression at the leaf margin. It has been shown that such a microinduction approach is feasible in tobacco (Pien et al., 2001a). Therefore, the goal of part 2 of this study was to establish a comparable microinduction system in Arabidopsis. The microinduction system investigated here is based on an estradiol-inducible cre/lox system (CLX). Our data showed that, although the CLX system could be used as microinduction system in mature tissue, it failed to function in proliferating, young tissue in Arabidopsis.

Finally, the expression of genes encoding sucrose synthase (SuSy) and other enzymes involved in carbohydrate metabolism has been reported to be up-regulated at the site of incipient primordium formation in tomato (Pien et al., 2001b). The aim of part 3 of this work was to analyse SuSy expression in the maize shoot apical meristem (SAM) and to compare it with that found in tomato. Maize provides an extensive array of molecular and genetic tools to investigate the significance of altered SuSy expression in the SAM. Our results show that SuSy is not differentially expressed in the maize SAM. A careful analysis of SuSy expression in developing maize leaves revealed a different expression pattern compared to that previously reported and suggests a role for SuSy in determining sink/source relationships within the developing leaf.
Part of this work has been accepted for publication:

Emanuel Hänggi and Andrew Fleming

Sucrose synthase expression pattern in young maize leaves: implications for phloem transport.

Planta May 19, 2001 vol.214 pages 326 - 329
Zusammenfassung

II Zusammenfassung

Kürzlich wurde gezeigt, dass Wachstumsmanipulationen am Blattrand zu Veränderungen der Blattform führen (Pien et al., 2001a; Wyrzykowska et al., 2002). Diese Resultate demonstrieren, dass Entwicklungsvorgänge im Blattrand essentiell für die Blattmorphogenese sind. Das Ziel von Teil 1 dieser Doktorarbeit war die Identifizierung und Charakterisierung von Genen, welche spezifisch im Blattrand von Arabidopsis thaliana exprimiert werden.


Vor kurzer Zeit wurde demonstriert, dass Gene, die im Karbohydratmetabolismus involviert sind, am Ort der Blattinitierung in Sprossmeristem von Tomatenpflanzen erhöht exprimiert werden (Pien et al., 2001b). Unter diesen Genen befindet sich die Sacharose-Synthase. Um zu prüfen, ob die Sacharose-Synthase ein genereller Marker für die Blattinitierung ist, analysierten wir das Expressionsmuster der Sacharose-Synthase in Sprossmeristem von Maispflanzen. Unsere Daten zeigen, dass, im Unterschied zu Tomatenmeristem, die Sacharose-Synthase nicht an der Stelle der Blattinitierung akkumuliert wird. Allerdings, im Kontrast zu früheren Publikationen, weist die Sacharose-Synthase ein anderes Expressionsmuster in wachsenden Maisblättern auf. Wir behaupten, dass das
Expressionsmuster der Sacharose-Synthase in Maisblättern dessen Funktion in der Regulation der Senken- und Quellenverhältnisse wiederspiegelt.

Ein Teil dieser Arbeit wurde veröffentlicht:

Emanuel Hänggi and Andrew Fleming
Sucrose synthase expression pattern in young maize leaves: implications for phloem transport.
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III General Introduction

1. The organisation of the shoot apical meristem

In animals, the mature embryo usually generates a miniature version of the mature organism, whereas in plants the embryo gives rise to two structural cores, the shoot apical meristem and the root apical meristem. The later post-embryonic activity of these meristems leads to the formation of the whole, mature morphology of the plant.

The shoot apical meristem (SAM) provides the cells that form the aerial part of the plant. It consists of only ~60 tiny cells in the model plant *Arabidopsis thaliana*, but can designate a population of up to a thousand cells in other plant species (Fosket, 1994). Clonal analysis has revealed that the SAM consists of three cell layers in angiosperms (Fig 1). The two outermost cell layers, L1 and L2, consists of predominantly anticlinally dividing cells and are generally termed the tunica, whereas L3, which is also referred to as corpus, contains the core cells of the meristem that divide in random planes (Satina et al., 1940). Genetic chimera studies revealed that L1 derivatives generate the epidermis, whereas L2 and L3 progenies form the subepidermal and inner tissue in shoot and lateral organs. Although this might imply lineage dependent behaviour, differentiation in plants is based on positional cues, e.g., laser ablated L1 cells in the epidermis are replaced by underlying L2 (not L1) cells and these cells take on a fate appropriate for their new position (Marcotrigiano, 2001).

![Fig. 1 Organisation of the shoot apical meristem (SAM). a) Clonal analysis reveals 3 layers in SAM: epidermal layer (L1), subepidermal layer (L2) and the corpus (L3). (Adapted from Bowman and Eshed, 2000). According to histological and morphological studies the SAM can be divided in 3 zones: b) central zone (CZ), rib zone (RZ) and peripheral zone (PZ).](image)
On the other hand, the SAM can also be divided into three functional domains defined by cell division rates and morphological studies: the central domain, which is surrounded by the peripheral domain and, below these two zones, the rib domain. The central domain contains slowly dividing cells and harbours a small stem cell population from which almost all tissue above ground is derived. By contrast, the peripheral domain consists of relatively rapidly dividing cells and is involved in lateral organ formation, whereas the underlying rib zone provides the precursors of the stem tissue.

2. Molecular and genetic analysis of the shoot apical meristem

As mentioned above, the central domain harbours a small population of stem cells which provide precursor cells for all above ground tissue. Molecular and genetic analyses over the last few years have revealed that a self regulatory system works in the SAM to balance stem cell proliferation and organ initiation (Fig 2): CLAVATA1 (CLV1) and CLAVATA2 (CLV2) proteins are thought to work as a plasma membrane heterodimeric receptor (Brand et al., 2000). This putative receptor complex has been shown to interact with CLAVATA3 (CLV3), a small secreted protein that is thought to be the ligand. CLV3 is expressed in the stem cells, whereas CLV1 and CLV2 are expressed in the underlying cells within the central zone (Trotochaud et al., 2000). CLV1/2 signalling was shown to restrict the expression domain of WUSCHEL (WUS), a homeodomain protein, which is expressed within the CLV1/CLV2 expression domain. WUS function is essential in inducing and maintaining stem cell identity (Mayer et al., 1998). Loss of function of any of the CLAVATA genes increases the WUS expression domain and, therefore, the stem cell population within the meristem. This gives rise to a huge SAM, from which supernumery partially fused organs are initiated (Fletcher et al., 1999; Clark et al., 1993).

Interestingly, WUS can promote CLV3 expression and, therefore, signalling via the CLV1/CLV2 system. This led to the proposal of a model in which the WUS and CLV gene products interact to generate a self regulatory system for the maintenance of meristem size (Schoof et al., 2000). In this model, if there is a transient elevated need for stem cells, e.g., when organs are induced, CLAVATA signalling might be attenuated, which allows the WUS expression domain and, hence, the stem cell population to enlarge. However in return, the enhanced WUS expression would promote CLV3 expression and, therefore, elevate signalling via CLV1/CLV2, which would suppress WUS expression and, thus, suppress the signal for increased stem cell proliferation. This system would elegantly adjust the production to the consumption of cells within the meristem.
General Introduction

Fig. 2 Expression pattern of genes involved in meristem maintenance (pictures from Bowman and Eshed, 2000). a) CLAVATA3 is expressed in the two outermost cell layers (L1 and L2) of the central zone (red). CLAVATA1 transcripts are detected in the corpus (L3; purple), whereas WUSCHEL mRNA is restricted to a small group of cells within the L3 of the central zone (yellow). b) WUSCHEL mediates a positive signal which triggers CLAVATA3 expression. CLAVATA3 signals back and restricts the expression of WUSCHEL. In this way a regulatory feedback loop is created that preserves the number of stem cells in equilibrium.

Similar to WUS, other homeobox genes (STM, KNAT1) are also implicated in maintaining cells in an undifferentiated state (Lenhard et al., 2002). These genes are expressed all over the Arabidopsis SAM, but transcript levels decrease at the sites of incipient leaf primordia. KNAT1 ectopic expression in leaves interferes with differentiation, which is reflected by the presence of small densely cytoplasmic leaf cells and ectopic meristems forming in leaf lobes (Chuck et al., 1996). Moreover, plants are not able to preserve a functional SAM in loss of function STM mutants (Douglas et al., 2002). These data confirm that these homeobox genes are important for meristem maintenance, possibly by actively keeping cells in a less differentiated state or preventing differentiation towards leaf identity.

Other transcription factors seem to promote differentiation. Thus, ASI (asymmetric leaves1) is a myb transcription factor which downregulates KNAT1 in developing leaves and, therefore, promotes differentiation (Ori et al., 2000). By contrast, ASI is repressed by STM in the meristem to prevent differentiation. Evidence for this comes from ectopic ASI expression in meristems, which causes SAM abortion, and from stm/asi double mutants, where the stm phenotype is suppressed (Byrne et al., 2000; Semiarti et al., 2001).

In addition to genes encoding transcription factors, receptors and ligands, a number of other transcripts have been shown to accumulate in specific patterns within the SAM. For example, studies on the SAM of tomato indicated that a number of genes encoding enzymes implicated in aspects of carbohydrate metabolism were expressed at the site of incipient leaf formation (Pien et al., 2001b). One of them was sucrose synthase (SuSy) which catalyses the reversible
conversion of sucrose to fructose and UDP-glucose (Geigenberger and Stitt, 1993). SuSy has been implicated in a number of important physiological processes: These include sink/source relationships within the plant (Zrenner et al., 1995), response to anoxia (Ricard et al., 1998) and cell wall biosynthesis (Amor et al. 1995). In the latter SuSy is thought to be membrane-associated and might channel UDP-glucose to cellulose and callose synthases (Stone et al., 1992). Its role in the SAM, where it is asymmetrically expressed, has still to be unravelled. However, the absence of mutants in SuSy showing obvious meristem-associated phenotypes, and the potential complexity and flexibility of plant metabolism makes the elucidation of the functionality of SuSy (and other genes involved in carbohydrate metabolism) in the SAM an intriguing but challenging problem.

3. Molecular and genetic analysis of leaf development

Following leaf initiation, a primordium can undergo a wide variety of morphological processes. A key event in many leaves is the formation of a flattened lamina to generate organs with a distinct adaxial and abaxial polarity.

Snow and Snow performed pioneering work in examining the development of dorsoventrality in leaves (Snow and Snow, 1958). Microsurgical experiments where a cut was placed between the meristem and incipient primordium showed that the resulting leaves were radially symmetric and abaxialised. These results provided clues that a signal emanating from the meristem could be involved in promoting differentiation of the adaxial side of leaves, whereas abaxialisation occurs as default if this messenger is absent. Recent molecular genetic studies have provided an insight into the potential nature of this signal and into the mechanism by which adaxial and abaxial identity is determined. One of the targets of such a hypothetical adaxialising signal could be the PHABULOSA transcription factor, which is specifically expressed in the adaxial domain in developing leaves (Mc Connell et al., 2001). This transcription factor contains a START domain, which is implicated in sterol/lipid binding. Mutations at a specific site of this START domain result in a gain of function mutation, which is reflected by the ectopic presence of PHABULOSA in the abaxial sides of leaf primordia. This results in the formation of adaxialised leaves. Because of the sterol/lipid binding domain, one hypothesis was that brassinolides could be the signal emanating from the center of the meristem that, by interacting with PHABULOSA, could induce adaxial differentiation. However, brassinolide application on the abaxial side of leaf primordia did not cause adaxialised leaves (Barton, unpublished). This rather questions the implication of
brassinosteroids in the establishment of dorsoventrality in leaves (for an alternative hypothesis see following section).

Strikingly, mutation of ad/abaxial identity genes often leads to a lack in lamina outgrowth. For example, strong mutant alleles of the PHANTASTICA gene in Antirrhinum (involved in establishment of adaxial fate) result in radially symmetric abaxialised leaves (Waites et al., 1998). The leaves have a needle-like appearance and totally lack any outgrowth of the leaf blade. Observations on the PHANTASTICA mutant led to the proposal that lamina initiation requires the juxtaposition of tissues expressing adaxial and abaxial identity genes and that, in the absence of one or the other, radial leaves are generated. The recent identification and characterisation of adaxial identity genes (such as PHABULOSA) and abaxial identity genes (such as the YABBY and KANADI families) have supported this hypothesis and have led to a complex model of interacting factors which leads to the final formation of tissue of exclusively adaxial or abaxial identity (Siegfried et al., 1999; Kerstetter et al., 2001). These data indicate that communication between the ad- and abaxial domain of developing leaves is crucial in triggering leaf blade formation. However, the mechanism by which such interactions actually lead to controlled outgrowth to generate a leaf lamina is unknown.

4. The huge effects of microRNAs in leaf development

Recently, a significant new level of regulation of leaf development has been identified via the characterisation of small RNA pieces, called microRNAs (Carrington and Ambros, 2003). MicroRNAs originate from transcription of short non-coding RNA precursors that have the potential to form partial double-stranded RNA (dsRNA) through fold backs. The dsRNAs are targets of a specific RNAse which cuts the dsRNA precursors into small (between ~21-22 nucleotide) RNA pieces (microRNAs). The antisense microRNAs match incompletely or perfectly to sites on mRNAs and mark them for degradation or inhibition of translation. Therefore, microRNAs are a potential tool to regulate post-transcriptionally the expression of various genes. In plants, a few microRNAs have been characterised and two of them have implications on leaf development.

MicroRNA miR165/166 is fully complementary to a stretch in the START domain of the PHABULOSA transcription factor. As described above, PHABULOSA is expressed in the adaxial domain of the leaf and drives adaxial fate (Kidner and Martienssen, 2003). Mutations in the sequence which is complementary to miR165/166 result in ectopic expression of PHABULOSA in the abaxial domain and causes adaxialized leaves. It is thought that
mutations within the complementary site within START could interfere with annealing of microRNA and its processing in the abaxial side of the leaf. Therefore, PHABULOSA mRNA accumulates in the abaxial domain as well and causes adaxialisation. MiR165/166 seems, therefore, to be expressed exclusively in the abaxial side of the leaf to cause inactivation of PHABULOSA mRNA. It is noteworthy that loss of function mutations in ARGONAUTE-1 and PINHEAD/ZWILLE proteins lead to abaxialised leaves (Bohmert et al., 1998). These genes encode eukaryotic translation initiation factors whose homologues in C. elegans are involved in mRNA processing. These results indicate the potential major roles of microRNAs in the establishment of leaf polarity.

A more recent paper has shown that microRNAs might also be implicated in the timing and shape of the cell division arrest front in developing leaves. CINCINNATA loss of function mutants in Antirrhinum showed enlarged leaves which are crinkled at the leaf margin (Nath et al., 2003). The phenotype was due to changes in the cell division arrest front, which moves basipetally during normal leaf development. In the mutant, the arrest front was delayed and had a concave instead of a convex shape, which resulted in longer cell division activity in the margin compared to internal leaf zones. This was manifested in increased growth of the margin compared to middle regions of the leaf and, therefore, caused enlarged and crinkled leaves. CINCINNATA encodes a plant specific transcription factor belonging to the TCP family. TCP homologues in Arabidopsis were found which might have similar functions. Recently, a particular microRNA, jaw-3D, was discovered, which putatively induces cleavage of a number of TCP mRNAs (Palatnik et al., 2003). When this particular microRNA was constitutively expressed, it resulted in permanent downregulation of those genes and caused a leaf phenotype similar to the CINCINNATA mutant in Antirrhinum. This result again demonstrates the importance of microRNAs in leaf development and nicely indicates the relevance of an accurately timed and shaped arrest front. Differences in leaf morphogenesis might be caused in response to variability in timing and shape of this arrest front.

5. Signaling in leaf development

5.1 Auxin and leaf initiation

Mutants have been described that fail to initiate lateral organs on the inflorescence stem. Interestingly, most of them are associated with auxin signalling (e.g., monopteros) or transport (e.g., pin-formed1) (Berleth et al., 2001). Loss of function mutants of PIN-FORMED (PIN1), a putative auxin efflux carrier, are defective in polar auxin transport. This causes naked inflorescences which are impaired in primordia formation. Naphtylphthalamic
acid (NPA, an inhibitor of polar auxin transport) -treated tomato plants phenocopy the PIN1 mutant, but additionally fail to initiate leaf primordia. Interestingly, organ formation can be rescued by microapplication of auxin at the peripheral zone of PIN1 or NPA treated meristems (Reinhardt et al., 2000). The common view is that influx and efflux carriers are concentrating auxin at the location of incipient primordia (Stieger et al., 2002).

Whereas auxin peaks seem to initiate leaf development, cytokinin might antagonize auxin effects in the SAM. Recent evidence indicates that STM and KNAT1 could increase cytokinin levels (Rupp et al., 1999). Since both of these genes are expressed throughout the meristem except at the site of incipient primordia, cytokinins could play a role in keeping cells in an undifferentiated state in the meristem, whereas auxin drives cells into differentiation. Since expansin genes have been shown to contain auxin response elements in their promoter and are already expressed in the I1 position (where the next leaf will be initiated), they are very likely targets of auxin signalling (Caderas et al., 2000). Expansins have been implicated in driving cell expansion in plants (see next chapter). Other putative targets for an auxin signal at the site of leaf initiation could be AINTEGUMENTA (ANT) which regulates cell proliferation in organ primordia (Mizukami et al., 2000). This early primordium marker gene is expressed in a ring along the periphery of SAM in PIN1 mutants. However, after microapplication of auxin ANT is again locally expressed at the site of incipient primordia (Vernoux et al., 2000; Traas unpublished).

Interestingly, KNAT1 represses the expression of gibberellic acid 20 oxidase, a key enzyme in gibberellic acid (GA) synthesis, in the meristem (Sakamoto et al., 2001). Since KNAT1 is not expressed in developing leaves, gibberellic acid 20 oxidase transcript levels are high in leaf primordia, suggesting that elevated GA levels might play a role in the early stages of leaf development.

In summary, auxin peaks could prevent knotted- gene expression and hence, depress cytokinin levels in incipient organs, and could favour gibberellic acid synthesis and signalling. This is consistent with recent results that imply that auxin promoted root growth is mediated by gibberellic acid (Fu and Harberd, 2003). A similar scenario might work in the promotion of leaf growth.

5.2 From simple to complex leaves via gibberellic acid?

A major division can be made between leaves with a simple structure and those with a compound or complex structure in which a basic leaf shape (leaflet) is re-iterated within one
leaf. Recent findings indicate that the degree of complexity in leaf shape might be due to the level and pattern of “ectopic” KNAT expression (Hay et al., 2002). High ectopic KNAT1 expression in Arabidopsis leaves leads to shooty-like leaves with ectopic outgrowths on the leaf blade that resemble leaflets (Chuck et al., 1996). In other words a simple leaf became more complex. Exogenous gibberellic acid (GA) can restore the simple leaf phenotype in KNAT1 overexpressors (Hay et al., 2002). Moreover GA 20 oxidase (see previous section) expression is repressed by ectopic KNAT1 expression in the transgenic plants. Similar studies in the dissected leaves in tomato gave analogous results. Ectopic KNAT expression enhances the degree of dissection and this can be reversed by exogenous GA. Moreover, increased simple leaf characteristics are obtained in procera, a constitutive GA signalling mutant (Hay et al., 2002). This indicates that the degree of repression of GA levels by KNAT gene products might reflect the level of complexity in leaf shape. Since KNAT proteins are thought to trigger cytokinin synthesis, leaf complexity might rely on GA/cytokinin ratios.

6. What drives leaf morphogenesis: Cell division or expansion?
The data discussed above indicate that specific patterns of transcription factors define regional identity in the shoot apex and that signalling between these regions leads to the formation of appropriate leaf morphology and differentiation. What are the downstream effectors by which these patterns and signals are transduced into change of form?

Surprisingly, whether cell division or expansion mediates morphogenesis is still a matter of debate. It is puzzling that some unicellular algae can have a very complex structure achieved without cell division, implying that division is not required for morphogenesis. But, how is it in a multicellular organism? Pioneering experiments by Foard showed that wheat apices, which lack cell division due to gamma-irradiation, have the potential to initiate leaflike-outgrowths (Foard, 1971). For some, this is an argument that morphogenesis is mainly driven by cell growth in plants, whereas for others the formation of abnormal primordia implies the need of cell division to maintain leaf growth and correct patterning. Recent evidence shows that correct leaf morphogenesis probably is based on a minimal competence of both cell division and cell expansion.

In plants, cell growth is a biophysical process (Cosgrove, 2000). A turgor pressure acts outwards from the cell and is driven by the high osmotic potential of the vacuole, which allows water influx. This hydrostatic pressure is counteracted by the mechanical strength of the cell wall. At a given turgor pressure regulation of growth is thought to occur through an
increase in cell wall extensibility. Expansins are the best characterised proteins that have the potential to increase cell wall extensibility. They are secreted proteins that are supposed to allow slippage of wall polysaccharide fibers, permitting turgor-driven cell enlargement. Amazingly, the ectopic expression of expansin within the meristem was sufficient to initiate leaf morphogenesis, which resulted in correct mature leaves (Pien et al., 2001a). In addition, over- or underexpression of expansins in vascular tissue or local induction of expansin expression on the flanks of primordia changed leaf shape (Cho and Cosgrove, 2000; Pien et al., 2001a). Since in the latter case cells at the site of outgrowth are the same size as at the uninduced flank, this underlines that cell division merely acts to fill up the space provided by cell expansion. Additionally, it shows that cell expansion is enough to induce relative differences in growth rate and alter leaf shape.

Consistent with these results are some attempts to change leaf morphogenesis by manipulating cell division and cell division orientation, e.g., overexpression of a CyCD2 in tobacco resulted in an increase in cell division and growth rate, however did not result in morphological changes (Cockcroft et al., 2000). Moreover, the maize tangled-1 mutant has abnormally oriented cell divisions, yet develops a normal leaf with normal cell layers (Smith et al., 1996).

In contrast, there is evidence in favour of cell division driving the morphology of plants. Overexpression of CDK inhibitors (ICKs) resulted in serrated leaves with reduced blades, but bigger cells. The reduction of endoreduplication and the bigger cell sizes infer that cell divisions were reduced in these plants (Wang et al., 2000; de Veylder et al., 2001). Moreover, the role of cell division on plant morphology might be context dependent. For example, the local induction of cell division within tobacco meristems did indeed lead to an accumulation of smaller cells, however this did not result in any morphological changes. Interestingly, though, the same cell cycle manipulations at the margin of a leaf primordium resulted in a change of leaf shape, manifested by a strong indentation at the site of induction (Wyrzykowska et al., 2002). This indicates that at a particular stage and location (margin), cell division activity seems to be crucial for leaf morphogenesis. Indeed, histological data shows that cell division activity is high at the margin of young leaf primordia (P1-P2) (Donnelly et al., 1999).

How is cell expansion and cell proliferation co-ordinated in morphogenesis? One interesting phenomenon in regulating morphology is the uncoupling of cell growth and cell division. Since cells have tissue-specific sizes in an organism, there has to be a specific size / division relationship. However, there seems to be plasticity within that relationship. Global inhibition
of the cell cycle or increase in cell expansion led to plants with normal leaf morphology, but
greater cell size (Hemerly et al., 1995; Jones et al., 1998). It has been claimed that a supracellular
mechanism exists that balances cell division and expansion in a manner such that, if either of them
is handicapped, one compensates for the other (Fleming, 2002). The result would be an
appropriate sized and shaped organ with more but smaller cells, or with fewer but bigger cells.
However, the capacity of this growth control mechanism seems to be limited if the expansion
or cell division competency is too low.

7. The role of the leaf margin in morphogenesis?
Early interpretations of plant anatomy suggested that there was a group of meristematic cells
along the edge of a leaf (termed the marginal meristem) and that it was the activity of this
meristem which determined lamina size and shape. However, experiments using clonal
analysis by Poethig and Sussex (1985a) showed that the daughter cells derived from cells at
the margin did not make a significant contribution to the leaf mass. Following these
publications, interest in the margin decreased. However, a number of lines of evidence now
suggest that cells at the leaf margin might indeed play a key role in organ development. These
data are summarised below.

First, cells at the margin undergo a specific pattern of differentiation early in leaf formation.
This involves cessation of cell division and subsequent elongation perpendicular to the
direction of lamina expansion (Poethig and Sussex, 1985b) (Fig. 3).

Second, it is notable that the CINCINNATA-like genes whose misexpression leads to altered
leaf morphogenesis are expressed in a dynamic fashion along the leaf margin (Nath et al.,
2003).

Thirdly, local manipulation of leaf shape via altered expansin or cell-cycle gene expression
involved target tissue encompassing the leaf margin (Pien et al., 2001a; Wyrzykowska et al.,
2002).

Fourthly, there is indirect evidence that auxin fluxes are specialised in the region of the
margin. Transgenic plants that contain a DR5 promoter fused to a reporter gene are often used
to estimate auxin distribution within plants. DR5 is a synthetic promoter that contains
multiple auxin response elements (Himanen et al., 2002). Analysis of these transgenic plants
indicates that auxin is normally homogenously distributed within the leaf. However, after
treatment with NPA (a polar auxin transport inhibitor) auxin clearly accumulates at the
margin of the leaf. This indicates that auxin is funneled to or synthesized at the margin. Other
experiments focused on the developmental consequences of NPA treatment on leaves (Mattsson et al., 1999). After NPA treatment during leaf development, vascular strands formation was very intense at the margin compared to central areas of the leaf blade. Moreover, these thick vascular strands paralleled the leaf margin. Since auxin is known to trigger vascular formation, this is an indirect evidence that after polar auxin transport is blocked, auxin accumulates at the leaf margin. This resulted also in a leaf phenotype where the leaf blade is reduced and roundish, although this could also be an indirect effect from change in vascular patterns.

More precise direct auxin measurements have implicated that auxin is distributed in a gradient within the leaf (Ljung et al., 2001). Auxin levels are higher in the midrib at the leaf base and lower towards the leaf margins. Somewhat conflicting data are provided by overexpression of ABP (the auxin binding protein and putative auxin receptor) which did not result in any morphological effects in leaves (Jones et al., 1998). However, since auxin responses besides cell elongation were not changed in these plants, different auxin receptors might mediate leaf morphogenesis. In summary, auxin gradients might play an important role at the leaf margin co-ordinating differentiation and growth of ab-/adaxial fate. Moreover, its graded distribution within the leaf might have a morphogenic function, providing positional cues for developmental events.

Finally, on purely theoretical grounds the margin cells appear at the right place and time to play a role in defining leaf shape. In developmental systems, boundaries are crucial in pattern formation. Boundaries are the site where, via communication between two different cell types, new decisions are made in terms of growth and differentiation (Lawrence et al., 2000). As described earlier, lamina formation requires adjacent expression of adaxial and abaxial identity genes. The position of the margin cells falls on this junction. Since the whole ad- and abaxial domains of the leaf (including epidermis) are joined at the leaf margins, the margin might reflect an important boundary line which could coordinate growth and differentiation between the ad- and abaxial cell types.

Despite these correlative data, our understanding of leaf margin differentiation and its potential role in morphogenesis is very limited.
8. The use of inducible promoters to investigate gene function

Constitutive promoters have proved powerful and widely used tools to investigate gene function using transgenic technology. However, such constitutive promoters have limitations. For example, if altered expression of a gene at early stages of development is lethal, then potential functions of a gene at later stages of development are impossible to study. To circumvent this problem, a number of inducible promoter systems have been developed for plant systems (reviewed by Gatz, 1997; Zuo and Chua, 2000a). These systems generally involve supply of an inducer to all parts of a plant. A refinement of this approach is microinduction in which a chemical inducer is loaded onto a carrier (beads or lanolin) and then small portions of the carrier positioned onto appropriately engineered transgenic plants. This approach has proved useful in the investigation of the role of cell division and expansion during leaf morphogenesis (Pien et al., 2001a; Wyrzykowska et al., 2002). The original microinduction system was developed using a tetracycline-inducible promoter in tobacco (Pien et al., 2001a). However, this system has been reported not to function in Arabidopsis. Bearing in mind the genetic resources and tools available for Arabidopsis, the development of a microinduction system for this model plant would provide a novel and powerful method to investigate gene function.
9. The aim of this work

Part 1:
As described above, the leaf margin has special features and has been proposed to play a key role in leaf development.
The aim of the first part of this thesis was to find and characterise genes which are specifically expressed in the Arabidopsis leaf margin. A recessive mutant is characterised called “hepatica”. The mutation is putatively caused by an enhancer trap insertion in a gene expressed in the leaf margin. Leaves of hepatica plants show altered margin cell differentiation. In addition, the plants are dwarfed and have a changed leaf shape. The alteration in leaf shape is putatively caused by a delayed cell division arrest front.

Part 2
A long term aim of this project was to locally manipulate the expression of margin specific genes to investigate their function. Previous work had shown that such a microinduction approach was feasible in tobacco. The second aim of this thesis was to establish a comparable microinduction system in Arabidopsis. The investigation of an estradiol-inducible cre/lox-based system (reported here) indicates the possibilities and limitations of this approach in Arabidopsis.

Part 3
Previous work illustrated that genes encoding sucrose synthase (SuSy) and other enzymes involved in carbohydrate metabolism were upregulated at the site of incipient primordium formation in tomato (Pien et al., 2001b). The aim of part 3 was to analyse SuSy expression in maize meristems. SuSy has been extensively studied at the biochemical and genetic level in maize and, therefore, provides an array of molecular and genetic tools to investigate the significance of altered SuSy expression in the SAM. Our results show that SuSy is not differentially expressed in the maize SAM, in contrast to the situation in tomato. A careful analysis of SuSy expression in developing maize leaves revealed different expression pattern compared to that previously reported, and suggests a role for SuSy in determining sink/source relationships within the developing leaf.
IV PART 1

Characterisation and analysis of *hepatica* in *Arabidopsis thaliana*
1. Introduction

Recently, progress has been made elucidating the importance of the leaf margin in leaf development. Cells at the leaf margin have a number of special anatomical features. It consists of a so-called marginal blastozone consisting of long and thick epidermal cells which follow the leaf edge and which surround small internal cells which do not have clear ad- or abaxial characteristics (Hagemann and Geissberg, 1996). (Adaxial means upper side, whereas abaxial indicates the lower side of the leaf).

There are a number of clues that the leaf margin cells might be important in regulating the formation of the lamina. First, the margin marks the boundary line of the ad- and abaxial domain of the leaf. It has been shown that if either ad- or abaxial cell types are missing, the leaf primordia fail to initiate lamina outgrowth, which results in needle-like leaves (Waites et al., 1998; Mc Connell et al., 2002). Since boundaries in animal systems have been shown to be crucial in pattern formation, the leaf margin could function as a boundary which plays a role in coordinating growth and differentiation of ad- and abaxial fates. An amount of experimental data indicate that the leaf margin might act as an organizing center for lamina growth (Fleming, 2003).

First, genetic chimera assays in which clones of cells derived from a plant species with a particular leaf shape are present within the lamina of a plant with a different leaf shape, illustrated that it is mostly the genotype of the "host" tissue that phenotypically dominates. Strikingly though, if the "foreign" clone extends to the leaf margin zones of the "host", the leaf accepts rather the growth characteristics of the "foreign" clone (Marcotrigiano, 2001).

Secondly, transgenic plants that contain a DR5 promoter fused to a reporter gene can be used to reflect auxin distribution within the plants. DR5 is a synthetic promoter that contains multiple auxin response elements (Himanen et al., 2002). Analysis of such plants indicates that auxin is homogenously distributed within the leaf. However, after treatment with NPA (a polar auxin transport inhibitor) auxin clearly accumulates at the margin of the leaf. This suggests that auxin is funnelled to or synthesized at the leaf margin. In addition, the NPA-induced alteration in auxin accumulation at the leaf margin was linked with a change in leaf shape and vascular differentiation (Mattsson et al., 1994).

Thirdly, a hypothetical target of auxin at the margin could be PRESSED FLOWERS, a homeodomain transcription factor which is expressed exclusively in cells at the margin of lateral organs. Loss of function mutation of this gene causes a lack in lamina growth of sepals.
Although, the phenotype of leaves is not changed (probably due to gene redundancy), the change in sepal shape provides causal evidence that a gene expressed in the margin has the potential to regulate the morphogenesis of a lateral organ (Matsumoto et al., 2001). Fourthly, local promotion of cell divisions at the leaf margin of primordia leads to a reduction in lamina outgrowth at that position (Wyrzykowska et al., 2002) whereas increase in cell wall extensibility leads to an increase in lamina outgrowth (Pienn et al., 2001). These data identify tissue on the flanks of leaf primordia as being susceptible to altered parameters of cell division and growth with respect to change in leaf shape. These data are also consistent with the view that changes in cell division activity occur at early stages of leaf development and that these pattern of cell division are causally involved in controlling morphogenesis. For example, it has been shown that at the margin in young leaf primordia (P1-P2), cell division activity (as measured by cylinB promoter linked reporter gene activity) is higher than anywhere else in the primordium (Donnelly et al., 1999).

Observation of relatively high rates of cell division at the margins of leaf primordia is reminiscent of an old hypothesis, the marginal meristem hypothesis, which claimed that the progeny of a small source of initial cells at the lateral margins were relevant for the outgrowth of the leaf lamina (Foster, 1936). The work of Poethig and Sussex refuted this hypothesis. Using clonal analysis they could show that tissue derived from cells at the leaf margin did not make a significant higher contribution to leaf shape than elsewhere in the primordium (Poethig et al., 1985a). If the original marginal meristem hypothesis is incorrect, what is the function of the specific cellular events observed to occur at the leaf margin?

One possibility is that the margin could function as some sort of organizing center for lamina growth and differentiation. Cell cycle manipulation in this area could interfere with the boundary functions of the margin. Thus, the observed changes in leaf shape following manipulation of cell division pattern might be primarily due to a change of the function of the organizing center than to direct changes in local cell division rates. It is suspicious that cell cycle promotion at the margin of primordia resulted also in an inhibition of differentiation, i.e., reduction of vascular tissue and trichome formation, which indicates that patterning was affected (Wyrzykowska et al., 2002). Moreover, cells at the indentation were surprisingly bigger, which rather indicated a cell division delay than promotion.

The potential difficulty of simply relating altered patterns of cell division to final change in morphogenesis is shown by data from other systems. For example, changes in the organizing centers of the shoot (WUSCHEL-expressing domain) and root (quiescent center, (QC)) results in morphogenetic changes which are not primarily due to alteration in cell division rates.
Thus, altered WUSCHEL expression can lead to an increased stem cell population, which results in a change of meristem size. This alteration in meristem size is most probably causal in the altered shoot organisation observed in such situations (Schoof et al., 2000). In the root, it seems that the QC inhibits differentiation of surrounding initials cells, which allows them to proliferate. Laser ablation of the QC leads to various changes of cell division pattern in the root apex, but these are mainly indirect via altered patterns of signalling rather than directly due to altered proliferation of the QC. (van den Berg et al., 1997). These results highlight the potential problem of interpreting growth manipulations at the leaf margin. Are the reported change of shape and size of the lamina caused by directly manipulating growth patterns or by inhibition or promotion of the cell population of an organising center, which has the task of regulating these growth patterns in a non-autonomous way?

As a first step to understanding the potential role of the margin in leaf morphogenesis, we were interested in identifying genes which are specifically expressed in this tissue. The character of the proteins encoded by these genes, coupled with the genetic resources available for the manipulation of gene expression in plants, would provide a powerful set of tools to investigate margin cell differentiation and its relationship to leaf developent.

To identify margin-expressed genes, we took an enhancer/promoter trap strategy. This technology exploits the potential of random, high frequency insertion of foreign DNA into plant genomes by simple transformation procedures. The foreign DNA can be engineered to contain a reporter gene which allows easy and rapid visualisation of transformed cells. By appropriate engineering of promoter sequences, the reporter gene construct can be used to "trap" neighboring genes in the genome so as to both reflect the expression pattern of such "trapped" genes and to act as a mutagen for the these genes. In addition, molecular techniques have been developed which allow for the identification and cloning of "trapped" genes of interest. Such enhancer and promoter traps have proved a useful tool over the last few years in the identification of genes involved in a variety of functions (Springer et al., 2000).

Here, we report on the identification and characterisation of an enhancer trap line which shows reporter gene expression in the margin of developing leaves. Coupled with this expression pattern, the line shows an altered pattern of margin cell differentiation and an altered leaf phenotype. We have termed this mutant hepatica. We discuss the possibility that the HEPATICA gene product might be involved in regulating lamina growth via its action in the leaf margin.
2. Results

2.1 Screening for enhancer trap lines with activity in the leaf margin

1300 Arabidopsis plants of an enhancer trap library (provided by Ueli Grossniklaus, University of Zürich) were screened for genes active in the leaf margin. An enhancer trap is a reporter gene fused to a minimal promoter which is only able to drive gene expression when activated by neighbouring endogenous enhancer elements (Springer, 2000). Random insertion of enhancer traps throughout the genome leads to patterns of reporter gene expression, which reflect the expression pattern of flanking endogenous genes.

Only one enhancer trap line, namely 252, revealed GUS expression in the leaf margin, but showed no obvious phenotype (data not shown). TAIL-PCR was performed to find the location of the enhancer trap insertion (Liu et al., 1995). The enhancer trap turned out to be inserted between genes At3g22880 and At3g22890 which encode a recA-like protein (AtDMC1), and an ATP-sulfurylase (Couteau et al.; 1999), respectively. Both of these gene products were already characterised and were not reported to show expression at the leaf margin. This suggests that the enhancer trap activity in 252 is driven by a more distantly located promoter or more likely is gene-unspecific (i.e. reflects only some cis elements of a promoter or a cryptic promoter). Instead of looking for further possible candidate genes in line 252, we decided to analyse enhancer trap lines YJ158 (Fig. 4a) and 1493 (Fig. 6a), which both revealed reporter gene expression at the leaf margin. These lines were identified in other laboratories screening enhancer trap libraries and were made available to us for further analysis (gifts from J. Bowman (UC Davis, USA) and S. Poethig (University of Pennsylvania, USA), respectively).

GUS expression analysis of developing leaves in YJ158 indicated that reporter gene expression was first detected at the tip of young leaves (leaf length ~2mm). From the tip GUS expression expanded along the leaf margin basipetally as leaf growth proceeded. Histological analysis revealed that GUS expression was restricted to the typical long tube-like cells found along the margin of the leaf (Fig. 4b and c).

In this line, the enhancer trap was located between a MATE-(At5g17700) and GrpE-like (At5g17710) gene. MATE genes (multidrug and toxic compound extrusion) are supposed to be involved in the detoxification system as well as in various aspects of growth and development in plants (Eckardt, 2001). GrpE genes, on the other hand, are putative nucleotide exchange factors, which interact with heat shock proteins in the chloroplasts (Schroda et al.,
Fig. 4 GUS expression pattern in enhancer trap line YJ158. (a) GUS expression is first detected at the tip of young leaves (leaf length ~2mm). From the tip, GUS expression expands along the leaf margin basipetally as leaf growth proceeds. (b) and (c). Technovit cross sections of a leaf primordia and mature leaf of YJ158. (b) GUS expression in leaf primordia is restricted to 5-6 big tube-like cells, which go along the margin of the leaf. The number of those cells decreases during subsequent leaf growth. (c) In mature leaf only 2-3 typical margin cells are present. Bar in (a) = 5mm; Bars in (b) and (c) = 50μm.

Fig. 5 In situ hybridisation analysis of genes flanking the enhancer trap of YJ158. (a-f). Cross sections of leaf margins: (a and b) hybridised with an antisense or sense probe for RBCS as control, respectively. (c and d) hybridised with an antisense or sense probe for MATE, respectively. (e and f) hybridised with an antisense or sense probe for GrpE, respectively. MATE shows weak expression throughout the leaf with higher signals present in stomata (arrows), whereas GrpE is lowly expressed in internal tissue. Bars = 50μm.
Results

2001). In situ hybridisation was performed to test whether the expression pattern of one of these genes reflected the enhancer trap expression pattern of YJ158. (Fig. 5a-f).

Unfortunately, neither of the two indicated expression in the margin of the leaves, which could signify gene-unrelated activity of the enhancer trap: The MATE-like gene seems to be highly expressed in stomata and weakly throughout leaf tissue, whereas the GrpE-like gene is faintly expressed in photosynthetically active tissue in the leaf, confirming its possible role in chloroplasts.

We decided then to focus on the enhancer trap line 1493. This showed not only leaf margin expression, but also a phenotype. This is a nice feature since, potentially, not only the function but also the expression pattern of the gene is reflected in one line. According to its phenotype (described later) we named line 1493 “hepatica” and this name will be used in the following description of the mutant.

Hepatica was obtained from an enhancer trap library generated in the group of Scott Poethig (University of Pennsylvania) using a construct from the group of Jim Hasseloff (University of Cambridge, UK) (http://enhancertraps.bio.upenn.edu/).

2.2 Enhancer trap expression pattern in hep

Reporter gene expression pattern of hep in leaf margins is similar to the one described for YJ158. In leaves of hep GFP expression appears first at the tip of young leaves (rather earlier than in line YJ158; leaf length ~1mm; Fig. 6a). From the tip, GFP expression expands along the leaf margin basipetally as leaf growth proceeds. As the leaf matures, GFP expression fades out in a basipetal pattern (Fig. 6b).

GFP expression can also be detected in the margin of young sepals and some developing trichomes on the leaf blade (Fig. 6c, d). The enhancer trap is active in the epidermis of hypocotyls, as well as at the tip and base of maturing siliques (Fig. 6e).

In roots, GFP is present in the epidermis and faintly expressed in the columella (Fig. 6f).

2.3 Hep shows a recessive pleiotropic phenotype

The hep mutant displays a pleiotropic phenotype. Hep plants are dwarfed, their rosette leaves are Lotus-like in shape, crinkled and bent downwards (Fig. 7a-f). Moreover, the leaves are dark green and very tightly arranged, probably due to a lack of outgrowth laterally from the shoot axis. This gives the plant the appearance of a mini-lettuce (a bowl of tightly packed leaves). In addition hep leaves are more robust and fleshy than wild-type. The fleshy and
Fig. 6 GFP expression pattern in *hepatica*: (a) Leaf primordia show GFP expression at the tip. (b) Rosette leaves of 2 week old seedlings: GFP expression restricted to margin. (c) GFP is active in developing trichomes. (d) GFP is expressed in the margin of developing sepals. (e) GFP expression additionally appears at the tip and the base of growing siliques. (f) In roots, GFP is present in the epidermis and faintly expressed in the columella. Bars in (a, d, f) = 500 μm; Bar in (b) = 5 mm; Bar in (c) = 1 mm and bar in (e) = 200 μm.
Fig. 7 Hep displays a very pleiotropic phenotype: (a and c) 3 week old WT seedlings on MS plates. (b and d) 3 week old HEP mutant seedlings grown on MS plates; they are dwarfed, dark green and have densely packed leaves, which are bent downwards. (e) Mature WT rosette leaf. (f) HEP mutant rosette leaf, which is Lotus-like shaped and crinkled. They are more rigid and fleshy than WT leaves. (g and h) 5 week old WT and hep roots, respectively. HEP mutant roots turn green approximately 3 weeks after germination.

Bars in (a, b, c, d, h) = 1 cm. Bars in (e and f) = 1.25 cm and bar in (g) = 1.5 cm.
Results

A crinkled appearance of those leaves resemble thalli of mosses from the family hepaticae (i.e. *pellia endiviifolia*), hence the name “hepatica”.

The cauline leaves, which grow from a short but thick inflorescence stem, are not changed in shape, but decreased in size. In addition, the mutant has a bushy appearance due to a loss of shoot apical dominance (Fig. 8). Although heavily impaired in growth, the timing of development seems to be intact in *hep*: With the exception of a slight delay in flowering, no differences in timing of germination and leaf initiation were obvious. Flowers have smaller organs and reveal reduced fertility: Siliques, which are shorter, but thicker, harbour only a few seeds (about 10 seeds compared to 25-30 seeds in wild-type siliques).

Root growth seems to occur relatively normal in contrast to the crippled phenotype of the shoot. Surprisingly, though, roots start to green in two-to three-week old *hep* plants (Fig. 7h). Greening of roots is most intense at basal parts at the junction to the hypocotyl and fades out towards the main root tip. After 5 weeks, also lateral roots at the basal area of the main root start to turn green.

To investigate whether the observed phenotype was linked with the enhancer trap insertion, outcrosses of the *HEP* mutant to wild-type (WT) Arabidopsis (Col) were performed. Analysis of these progeny revealed a range of phenotype degree, but the phenotypic characteristics were consistent. Plants of moderate phenotypes were somewhat bigger and had less crinkled and curved rosette leaves than the strong phenotypes of *hep*, but the Lotus–like leaf shape was unchanged. Segregation analysis indicated that the phenotype of *hep* is a recessive trait. Moreover, the phenotype was always linked to strong GFP expression compared to faint GFP expression in putative heterozygotes (three quarters of F1 *hep* progenies express GFP). One quarter of F1 progenies show no GFP expression and no phenotype and are kanamycin sensitive, which implies that the enhancer trap insertion in *hep* causes the phenotype observed.

2.4 *Hep* is linked to a complex T-DNA insertion

TAIL and Genome Walker PCR (see Materials and Methods) were performed to identify the location of the enhancer trap. Both types of inverse PCRs are based on amplifying flanking regions from the right and left border of the T-DNA. After sequencing of a number of PCR products only T-DNA, but no genomic DNA sequence information was obtained. Moreover, the sequences of the PCR products indicate that besides T-DNA also Ti-plasmid fragments flanking the T-DNA have been inserted into the plant genome. Analysis of the PCR products, originating from the right or left border of the T-DNA, revealed overlapping regions.
Fig. 8 The *HEP* mutant shows loss of apical dominance (a and b) six week old WT and *hep* Arabidopsis plants, respectively. *Hep* has a bushy appearance due to a lack of dominant growth of the main inflorescence stem depicted in (b). White Scale bars = 1 cm.
The nature of the T-DNA insertion in hep is complex. Two kinds of inverse PCRs, TAIL- and Genome Walker PCRs (see Materials and Methods), were performed to identify the location of the enhancer trap. In TAIL- and Genome Walker PCRs, the right and left T-DNA border primer is used in combination with either a degenerated primer (which anneals ~ every kb in the Arabidopsis genome) or adaptor primer (after digestion of genomic DNA and adaptor ligation), respectively. The flanking regions obtained from the right and left border of the T-DNA are sequences present in the Ti-plasmid. Moreover, sequences from right and left border PCRs partially overlap in two cases, which indicates the presence of at least three T-DNAs, which according to the intervening sequences, are spaced by short stretches of Ti-plasmid (schematically shown in (a)). This indicates the insertion of a complex T-DNA or concatamer in hep. Putatively, the T-DNA concatamer or complex was formed by recombination events in the Agrobacterium before integration into the plant’s genome (as illustrated in (b)). Filled boxes indicate T-DNA; black lines or circles represent Ti-plasmid; cross indicates recombination events in Agrobacterium; R and L stand for right and left T-DNA border, respectively, and arrows in (a) reflect left and right border primer.

Fig. 10 Southern blot analysis indicates one T-DNA or T-DNA complex insertion in hep: Genomic DNA was extracted from HEP mutant and WT plants and digested with HindIII or SpeI. Equal amounts were used (10 μg) for gel electrophoresis. DNA was blotted on nitrocellulose membrane and hybridised with the left border region of the T-DNA. HindIII cuts once, whereas SpeI does not cut within the T-DNA or Ti-plasmid. Hybridisation of SpeI-digested hep genomic DNA results in only one band (~20 kb), which indicates that there is one T-DNA insertion. However, hybridisation of HindIII-digested hep genomic DNA reveals multiple bands, which confirms, that the insertion is a complex of more than one T-DNA; hybridisation of digested WT genomic DNA did not show any signals. L = DNA ladder.
According to these, right and left borders of the T-DNAs are separated from each other by a relatively short stretch of Ti-plasmid (Fig. 9). Since this is true for two T-DNA border regions, the insertion site might contain at least three T-DNA sequences plus additional partial Ti-plasmid regions. This indicates the insertion of a complex T-DNA or concatamer in the \textit{hep} mutant. It is possible that the T-DNA concatamer or complex was formed by recombination events in the Agrobacterium and was then inserted as one piece into the plant genome.

Southern blots were performed to detect the number of T-DNA insertions in \textit{hep} (Fig. 10). Genomic DNA from WT and the \textit{HEP} mutant was digested with SpeI (which does not cut within the Ti-plasmid) and HindIII (which cuts once within the T-DNA sequence). Fragments were separated by gel electrophoresis and transferred to a membrane. The blot was then hybridised with a radioactively labelled probe for the left border T-DNA sequence. The presence of one band in the SpeI digest of hep DNA indicates that the hep phenotype seems to be the result of one site of T-DNA insertion. The presence of one main band with a laddering of smaller fragments in the HindIII digest of the hep DNA is consistent with the proposed insertion of a concatamer or complex T-DNA insertion in this mutant.

2.5 Growth along the proximo-distal axis is affected in hep leaves

The leaves of plants showing a moderate hep phenotype are not as densely packed and are less crinkled and curved than leaves from plants showing a strong hep phenotype. Therefore these could be used for comparison of phyllotaxis and leaf geometry with WT plants.

Rosette leaves of six week old hep and WT plants were detached from the stem and aligned according to their age (Fig. 11). No obvious differences in leaf number or phyllotaxis were observed between the mutant and WT plants. The rosette leaves of hep plants showing a moderate phenotype varied from rather Lotus-like (first five leaves appearing at the shoot apex) to roundish leaves (following leaves), whereas the WT leaves display shapes from oval (first five leaves) to lanceolate (following leaves). Serration started at the sixth youngest leaf and increased in the successive leaves in both WT and hep plants. Cotelydons showed no major differences in size or shape in the mutant compared to WT.

Leaf length, width and area were measured for the eighth leaf of 30 mutant and WT plants (Fig. 12). This analysis indicated a statistically significant difference in dimensions between mutant and WT leaves. The eighth WT rosette leaf is approximately twice as long as the eighth leaf in the mutant (WT: 3mm, SD=±1.6mm; hep: 1.6mm, SD=±1.8mm) whereas in
Fig. 11 Rosette leaves of six week old hep and WT plants were detached from the stem and aligned according to their age. Hep moderate rosette leaf shapes vary from rather Lotus-like (first five leaves) to roundish leaves (following leaves), whereas the WT leaves display shapes from oval (first five leaves) to lanceolate (following leaves). Serration starts at the sixth leaf and is increasing in the successive leaves in both, WT and hep. Cotyledons (cot) show no major differences in size or shape in the mutant compared to WT. Asterisks mark the eighth leaf of the mutant and WT, respectively. On those leaf width, length and area were measured (see Fig 9). L1 indicates the first leaf. Bar = 1 cm.
Results

the WT leaf width is about the same as that of the mutant leaf (WT: 1.7 mm, SD= ±2.5mm; hep: 1.45mm, SD= ±2.1mm). The HEP mutant leaf area is approximately half that of the WT (WT: 3.1mm², SD= ±0.6mm²; hep: 1.6mm², SD= ±0.5mm²), and this change seems to be primarily due to a reduction in growth along the proximo-distal axis of the leaf.

2.6 Cell expansion in the proximal area of young hep leaves is inhibited

Technovit- and paraffin sections were taken at different stages of leaf development to investigate histological differences of the mutant compared to WT (Fig. 13-16). In this assay hep plants showing a strong phenotype were chosen.

At early stages of leaf development (P1-P4) no obvious distinction could be made between WT and HEP mutant leaves (Fig. 13 a-d). However, cross sections reveal that the arrangement of leaf primordia at the shoot apex in hep plants appears to be denser, probably due to a delay of internode growth (Fig. 13 a, b).

Sections of WT and mutant leaves (length 2-3mm, ~P5), revealed drastic differences in histology (Fig.14).

Expansion and differentiation occurs in a basipetal manner in Arabidopsis leaves, which means that, after an initial phase of expansion linked with cell division early in leaf development, the rate of cell division decreases at the distal tip of the leaf while the rate of growth is maintained, leading to cell expansion and differentiation. This process starts at the distal tip of the organ and extends towards the proximal base as leaf growth proceeds. Both WT and hep leaves display this basipetal behaviour, which is reflected by smaller and less differentiated cells in proximal parts compared to larger and more differentiated cells in distal parts of the lamina (Fig. 14a, b). However, in the hep mutant there is a tremendous contrast between large and differentiated cells in distal parts compared to tiny, densely cytoplasmic cells at proximal parts of the lamina (Fig. 14d, f). Such tiny cells, located at the proximal part of the leaf in the mutant, are normally only present at very early stages of leaf development (approximately stages P1-P3). Thus, there is a much steeper gradient in cell size within the hep mutant leaves along the proximal-distal axis compared with the WT situation. Moreover, the internal tissue of the proximal part of the mutant leaf reveals more cell layers (8–10 layers) compared to distal areas of HEP mutant leaves or to the proximal region of WT leaves (4-5 layers) at the same stage (Fig. 14e, f).

Technovit cross sections of slightly older leaves (~5mm) in proximal and distal regions confirm the accumulation of tiny, cytoplasmic cells at proximal regions (Fig. 15a-d), whereas
Fig. 12 Hep leaves have a reduced area due to a lack of growth along the proximo-distal axis. On the eighth leaf of 30 mutant and WT plants leaf length, width and area were measured. (a) The eighth WT rosette leaf (blue bars) is approximately twice as long as the eighth leaf in the mutant (transparent bars), whereas in width, the WT leaf is about the same as the mutant leaf. (b) Moreover, the WT leaf area is twice as big as in the mutant. This demonstrates that at least in moderate hep plants leaf area is primarily reduced due to a lack in growth along the proximo-distal axis. Columns represent the average of measurements done on 30 leaves.
Fig. 13 Early leaf development in hep is not altered. Paraffin cross and longitudinal sections were taken at early stages of leaf development to investigate histological differences of the mutant compared to WT. The sections were stained with methyleneblue. (a and b) Cross sections of WT and mutant primordia, respectively. (c and d) Longitudinal section of WT and hep meristem area, respectively. At early stages of leaf development no obvious distinction can be made between WT and the mutant. However, the arrangement of leaf primordia at the shoot apex in hep appears to be denser (compare a with b), probably due to a delay of internode growth. Scale bars = 100μm.
Fig. 14 Lack of expansion and differentiation in proximal parts of young hep leaves. Longitudinal technovit sections were taken from WT (a, c, e) and mutant (b, d, f) leaves (~2-3 mm in length) and stained with methylene blue. The orientation of sections and area where sections were taken is indicated by arrows in sketches. (a) Longitudinal section of a WT leaf exhibiting a basipetal gradient in cell size along the proximo-distal axis. c) Close up of cells at the distal region of a WT leaf indicating big and differentiated cells. e) Close up of cells at the proximal part of a WT leaf showing smaller cells. b) Hep mutants exhibit a very steep gradient of differentiation and expansion along the proximo-distal leaf axis. Cells at distal parts are big and differentiated (d), whereas cells at proximal regions are tiny and barely differentiated (f). Moreover, at proximal parts, there are more cell layers. Scale bars in (a and b) = 100 μm; Scale bars in (c, d, e and f) = 50 μm.
Fig. 15 Cells in proximal parts of *hep* leaves are small and barely differentiated. Technovit cross sections were taken from WT (a, c, e) and mutant (b, d, f) leaves (~5mm in length) and stained with methylene blue. The orientation of sections and locations where sections were taken are depicted by arrows in sketches above: WT cross sections were taken at the margin in proximal (a and c) and distal (e) areas of the leaves. (b and d) show cross section taken at the margin of proximal parts of *hep* mutant leaves, whereas f) exhibits a cross section taken at the margin of distal areas of *hep* leaves. Scale bars = 100 μm.
cross sections taken in more distal areas of *hep* leaves exhibit cells which are comparable in size to the analogous WT sections (Fig 15e, f).

These results imply that expansion and differentiation appears to be mainly absent or retarded in proximal parts of *HEP* mutant leaves. To investigate what impact this apparent defect in proximal cell elongation and differentiation had on mature leaves, histological analysis of adult *hep* and WT leaves was performed:

2.7 Basipetal gradient in cell size is maintained in mature *hep* leaves

Technovit cross sections of the lamina (at proximal and distal areas), midrib and margin (at equidistances between leaf tip and -base) of the fully matured eight youngest leaf of WT and *HEP* mutant seedlings were taken and the histology analysed (Fig 16).

Observation of sections of mutant mature leaf lamina indicated differences in cell size along the proximo-distal axis. Although cells in the distal regions of *hep* leaves were comparable to WT in terms of size and differentiation (Fig. 16a, b), cells in more proximal regions appeared smaller and more densely packed (Fig. 16c, d). Nevertheless, these cells were bigger than cells at a comparable position at earlier stages of *hep* leaf development (compare Fig. 15a to 16d). In addition, in some sections it was difficult to allocate the cells into spongy mesophyll-and palisade parenchyma cell types in the proximal part of the *hep* leaf (Fig. 16d), whereas in WT leaves at this stage of development and at this region the differentiation of these cell types was distinct (Fig. 16c). Therefore, the altered proximo-distal gradient in cell size and differentiation observed at early stages of development was maintained in mature *hep* leaves.

Cells at the margin and midrib of *hep* leaves also appeared smaller than the equivalent WT tissue (Fig. 16e-h). Additionally, at the margin the identity of the internal cells is unclear in the *HEP* mutant compared to WT tissue (Fig. 16g, h).

To make more precise conclusions about cell size, the number of cells per 50 μm² was counted in midrib, lamina and margin cross sections (Fig. 17). These sections were taken at regions equidistant from the leaf tip and base (i.e., in the middle portion of the leaf). In all areas the mutant was calculated to have more and, hence, smaller cells than WT leaves.

A more detailed examination of patterning and cell size of the epidermis was made using scanning electron microscopy.

2.8 Patterning and cell size in the epidermis is changed in *hep* leaves

Scanning electron micrographs were made from the adaxial (upper side) and abaxial (lower side) of adult mutant and WT leaves (Fig 18. a-d). In both cases the epidermis of the leaf blade
Fig. 16 In hep mature leaves the basipetal gradient in cell size is maintained. Technovit cross sections of the lamina (at proximal and distal areas), midrib and margin (at equidistances between leaf tip and base) of WT and mutant mature leaves were taken and stained with toluidine and methylene blue: Sections taken at distal (a) and proximal (c) areas of WT lamina tissue reveal no significant differences in cell size, whereas sections of the mutant mature leaf lamina still indicate differences in cell size along the proximo-distal axis: (b) Cells present in rather distal regions of the leaf are comparable to WT (a), whereas cells in rather proximal regions (d) appear smaller and more densely packed compared to WT (c). Cells at the midrib and margin of the mutant appear also smaller (f and h, respectively) than cells at the midrib and margin in analogous WT sections (e and g, respectively). Scale bars in (a, b, c, d, e and f) = 100 μm; scale bars in (g and h) = 50 μm.

Fig. 17 Cells in hep leaves are smaller. Cells per 50 μm² were counted in midrib, lamina and margin cross sections taken approximately at the middle between the leaf tip and the base. Blue columns represent WT; white columns represent hep.
in the mutant is changed compared to WT. The WT adaxial pavement cells are jigsaw shaped and relatively uniform in size (Fig. 18a), whereas in the mutant adaxial pavement cells are rather rectangular and appear smaller in size (Fig. 18b). The WT abaxial pavement cells are also jigsaw shaped, but less uniform in size than their adaxial counterparts (Fig. 18c). In the mutant, abaxial pavement cells are less sinuous and appear smaller (Fig. 18d). Intriguingly, also in the epidermis there is a gradient of increasing cell size towards the proximal regions detectable in the HEP mutant but not in WT leaves, suggesting again a defect along the proximo-distal axis of the leaf (Fig. 18g, h).

Another striking observation is the relatively high density of stomata on the leaf blade of the mutant (Fig. 18g, h). This might be due to simply the general smaller size of cells on the epidermis; however in some areas of the leaf blade stomata are clustered in a way that sometimes two stomata are adjacent to each other (Fig. 18b). This is in contrast to the consistent feature of WT stomata patterning in which stomata are separated from each other by at least one cell (Sachs, 1991; Nadeau et al., 2002). This indicates that besides a growth defect, patterning is impaired in the epidermis of hep leaves.

Epidermal cells at the WT leaf margin are very long tube-like cells (Fig. 18e). Astonishingly, in certain crinkled areas of the hep mutant, these tube-like cells are replaced by smaller rectangular cells, which are also present on the leaf blade (Fig. 18f). These long tube-like cells might provide mechanical strength to the WT leaf and crinkles might form in the mutant, because they are absent at certain spots (see discussion).

In addition to this alteration in margin cell phenotype, growth and branching of trichomes is impaired (Fig. 18g, h). Trichomes in Arabidopsis are polyploide cells, which can have from two up to multiple branches (Larkin et al., 1997). The mutant harbours trichomes which are more slender, smaller and predominantly contain only two branches. At the same time, the papillae on the surface of trichome appear bigger in the mutant than in the WT (Fig. 18j, k). Mutants have been described, which have a dysfunctional fucosyltransferase (FUT1) and exhibit also changes in size and form of papillae (Vanzin et al., 2002). FUT1 is thought to be required for the establishment of a proper cell wall. Problems in cell wall properties are also suggested by the SEM analysis. In the mutant, the surface of some pavement cells appears rough, whereas the surface of pavement cells in the WT is generally smooth (Fig. 18c, d). A more detailed analysis of cell wall structure with focus on the leaf margin was provided by transmission electron microscopy.
Fig. 18 Patterning and cell size is changed in the epidermis of HEP mutants. SEM pictures taken from the epidermis: (a) The WT adaxial pavement cells are jigsaw shaped and relatively uniform in size, whereas in the mutant adaxial pavement cells are rather rectangular and appear smaller in size (b). (c) The WT abaxial pavement cells are also jigsaw shaped, but less uniform in size than their adaxial counterparts. (d) In the mutant, abaxial pavement cells are less sinuous and are smaller. The surface of some pavement cells appears rough (white arrow), whereas the surface of pavement cells in the WT is generally smooth. There is a high density of stomata on the mutant leaf blade. (b) In some areas of the leaf blade stomata are clustered in a way that sometimes two stomata are adjacent to each other. (e) Epidermal cells at the leaf margin are very long tube-like cells (white arrow). (f) In the mutant in certain crinkled areas, those tube-like cells are replaced by smaller rectangular cells, which are also present on the leaf blade. (g) WT trichomes on leaf blade are two up to multiple branched. (h) Growth and branching of trichomes on the leaf blade of the mutant is impaired. Most of trichomes have only two branches, are slender and smaller than WT trichomes. Shown also in (h) basipetal gradient of cell size in the epidermis (red and white arrows point at big and small cells, respectively). (k) Papillae (knobs on surface of trichomes) are more pronounced, but less densely distributed in the mutant compared to WT trichomes (j). Scale bars in (a, b, c and d) = 20 µm; Scale bars in (e) = 100 µm. Scale bars in (f, g and h) = 50 µm. Scale bars in (j and k) = 25 µm
Fig. 19 Cell wall properties are altered in the leaf margin of *hep*. TEM pictures were taken of epidermal margin cells. (a) The outermost cell wall (white arrow) of margin cells is overlaid by the cuticle (black arrow). (b) The outermost cell wall of *hep* margin cells is thin and shows spike-like protrusions, which are overlaid by a massive cuticle. (c) TEM pictures from a leaf margin cells. The margin cell is flanked by extra cell wall material (black arrows) in the WT. (d) In the mutant this additional cell wall material is almost absent (black arrows). Scale bars in (c and d) = 10 μm.
Results

2.9 Cell walls are changed in the epidermis of hep

TEM (transmission electron micrographs) were taken of epidermal margin cells. Drastic differences were visible on the outermost cell wall of the leaf margin surface (Fig. 19): The outermost cell wall of the mutant is thin and shows spike-like protrusions (Fig. 19b, d), whereas the WT outermost cell wall is thicker and wavy (Fig. 19a, c). In addition, in the mutant epidermis the cuticle is massive and thicker than the WT cuticle (Fig. 19a, b). This indicates that in the HEP mutant the construction of the outermost cell wall of margin cells is impaired or changed.

Furthermore, TEM pictures at the margin indicate, that in WT leaves the big tube-like margin cells are flanked by extra cell wall material, which might stabilize the margin cell complex (Fig. 19c, d). However in the mutant this additional “seal” is almost absent. This missing cell wall material at the cell margin complex and the occasional absence of typical marginal cells at the leaf edges could both contribute to the change in leaf shape of the mutant (see discussion). Elsewhere in the region of the margin, the internal cell walls appear comparable in both mutant and WT.

2.10 HEP mutant phenotype is not primarily due to altered cytokinin levels or response.

The pleiotropic phenotype suggests that phytohormone synthesis, perception or response could be altered to a certain degree in the mutant. Because of the dark green color, loss of apical dominance and the dwarfed phenotype of the mutant, cytokinin could be a likely candidate. One of the better characterized responses of cytokinin in plants is its impact on retarding senescence (Mok and Mok, 2001). Therefore, we performed a detached leaf senescence assay: The fully expanded seventh leaves from mutant and WT plants were detached and floated on sterilized tap water in the dark for 1-6 days (Fig. 20 and 21). After each day, chlorophyll (Chl) contents were measured. The results revealed that HEP mutants do not show an obvious retardation in senescence: In both, mutant and WT leaves, rapid Chl loss started after 2 days of incubation and was highly reduced after 5 days. The hep leaves do, however, contain initially 1.5 fold higher Chl levels g⁻¹ fresh weight than WT leaves.

Although senescence occurs normally in HEP mutants, other cytokinin features might still be altered. Therefore RT-PCR and semi-quantitative RT-PCR of genes involved in cytokinin synthesis (IPT3), signalling (A-type ARRs) or response (CyCD3.3) were performed to reveal differences in expression levels between WT and mutant seedlings (Kakimoto, 2003; Hutchison and Kieber, 2002; Hwang and Sheen, 2001). The results indicated that all these genes were expressed and that A-type ARR and CyCD3.3 genes (whose expression is
Fig. 20 Leaves of the HEP mutant do not show retarded senescence. The fully expanded seventh leaf from mutant and WT plants were detached and floated on sterilized tap water in the dark for 5 days. (a and b) WT and HEP mutant leaves before incubation, respectively. (c and d) WT and HEP mutant leaves after 5 days incubation in the dark.

Fig. 21 Chlorophyll measurements based on detached leaf senescence assay. The fully expanded seventh leaf from mutant and WT plants were detached and floated on sterilized tap water in the dark for 1-6 days. After each day chlorophyll (Chl) contents were measured. Each data point represents the average of measurements from 3 leaves. In both, mutant (triangles) and WT (squares) leaves, rapid Chl loss started after 2 days of incubation and was highly reduced after 5 days. This, although hep leaves contain initially 1.5 fold higher Chl levels g-1 freshweight than WT leaves. The results confirm that HEP mutant do not show a retardation in senescence.
Fig. 22 In HEP mutants cytokinin signalling is not significantly altered. Semi-quantitative RT-PCRs of genes involved in cytokinin signalling (A-type ARRs: ARR4, 5 and 8) or -response (CyCD3.3) were performed to reveal differences in expression levels between WT and mutant seedlings. As a control semi-quantitative RT-PCR of a household gene AMT (arginin methyltransferase) was done in WT and hep. RNA was extracted from 1 week old WT and hep seedlings. Reverse transcription of 1 µg RNA from WT and hep was performed. For semi-quantitative RTPCR the resulting cDNA was used undiluted (first row) or diluted 1: 10; 1: 20 and 1:100 (second, third and fourth row, respectively). For various kb sizes of RT-PCR products see Materials and Methods.
enhanced by elevated cytokinin levels) were not differentially expressed in the mutant or WT seedlings (Fig. 22 and 23). This indicates that the phenotype of the mutant is unlikely to be due to a change in cytokinin signalling.

Finally, WT and mutant plants were grown on medium containing various concentrations (ranging from 1 to 100µM) of two different anticytokinins (gifts from Prof. E. Karanov, see Materials and Methods). These anticytokinins were originally shown to inhibit cytokinin responses in radish. However, unfortunately, they had no obvious effect on WT or hep plants. Therefore, it seems that these anticytokinins fail to act on Arabidopsis, at least under the conditions used in our assay (data not shown).

2.11 KNAT1 is not ectopically expressed in hep leaves

Transgenics which show increased cytokinin levels also display elevated expression of the KNAT1 gene (Rupp et al., 1999; see General Discussion). KNAT1 (class I knox genes) has been implicated in maintaining a functional shoot apical meristem (SAM), putatively by keeping cells in an undifferentiated state (Clark et al., 1996; Chuck et al., 1996). Moreover, ectopic expression of KNAT1 in young leaves results in highly lobed leaves, which occasionally form ectopic meristems. These transgenic leaves contain smaller and densely packed cells whose identity is not clear. The tight arrangement and the size of these cells resembles the internal histology of hep leaves (especially in the proximal areas). AS1 and AS2 encode myb transcription factors and have been shown to repress KNAT1 expression in young leaves and plants mutated at these loci show the same phenotype as KNAT1 overexpressors (Byrne et al., 2002; Iwakawa et al., 2002). Therefore, RT-PCR was performed to examine the presence or absence of AS1 and KNAT1 transcripts in developing HEP mutant leaves, respectively (Fig. 23). The genes were found to be normally expressed in mutant leaves, which means that the hep phenotype is not caused by ectopic KNAT1 expression. Since KNAT1 seems to be influenced by cytokinin, this also confirms the results obtained above: Cytokinin perception, signalling or response does not seem to be influenced in the hep mutant.

2.12 HEP does not seem to be linked to photomorphogenesis

The dark green appearance and loss of apical dominance of hep are also characteristics of some photomorphogenic mutants (i.e. det2) if grown in the light (Chory et al., 1991). These mutants were originally found because they exhibit characteristics of light grown plants in the dark, which includes thick hypocotyls, expanded cotyledons, primary leaf buds and constitu-
Fig. 23 *HEP* mutant phenotype is not caused by ectopic *KNAT1* expression. RT-PCRs were performed to examine the presence or absence of *AS1* and *KNAT1* transcripts in developing *HEP* mutant leaves, respectively. Additionally, the presence of transcripts of 2 genes involved in cytokinin synthesis (*IPT3*) and perception (*AHK4*) was tested in *hep* seedlings. All genes are adequately expressed in *hep*. As a control RT-PCR of an arginine methyltransferase (*AMT*) was performed. RNA was extracted from 1 week old seedlings and young leaves. Reverse transcription of 1 µg RNA from WT and *hep* seedlings or leaves were performed.

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Fig. 24 *HEP* is not primarily involved in the regulation of photomorphogenic responses. To examine if *hep* reveals characteristics of photomorphogenic mutants, WT and *hep* seedlings were grown in the dark. (a) After 5 days, expression of a photosynthetic marker (*RBCS*) was tested by RT-PCR. *RBCS* is not or hardly expressed in the dark, but strongly expressed in light-grown mutant and WT seedlings. However, *RBCS* is lowly expressed in roots of 3 week old *hep* seedlings grown in the light. (b) The presence of *DET1*, *2* and *3* (de-etiolated), *COP1* and *10* (constitutive photomorphogenesis) and *FUS3*, *4*, *5*, *6*, *7* and *11* (fusca) transcripts was tested by RT-PCR. In the case of *FUS*- and *DET*- and COP-RT-PCRs, RNA was isolated from 2 weeks old seedlings and reverse transcription was performed. In the case of *RBCS* RT-PCR, RNA was extracted from light and dark grown seedlings and root tissue of 3 week old *hep* and WT seedlings. Then reverse transcription was performed.
tive anthocyanin production. To examine, if hep reveals characteristics of photomorphogenic mutants, WT and hep seedlings were grown in the dark. After 5 days, expression of a photosynthetic marker (RBCS) and of various genes involved in the inhibition of photomorphogenesis were compared by RT-PCR (Fig. 24). Among them are the DET (dectiolated), COP (constitutive photomorphogenesis) and FUS (fusca) genes. Most of these genes are part of an ubiquitination and proteaseome mediated degradation machinery which destroys photomorphogenic activators in the dark (Ma et al., 2003).

All of those genes were expressed normally in WT and mutant seedlings. Furthermore, RBCS is hardly or not expressed in dark- but strongly expressed in light- grown mutant and WT seedlings. These results indicate that HEP is not involved in the regulation of photomorphogenic responses (Fig. 24b). However, dark-grown hep plants are shorter and thicker than dark-grown WT plants (data not shown). Moreover, as mentioned previously, the roots become green in three weeks old hep seedlings and express RBCS at that stage (Fig. 24a). Whereas the former phenotype might rather reflect a general growth problem of the mutant rather than a photomorphogenic behaviour, the latter phenotype is conflicting because it indicates a link between HEP and a photomorphogenic feature, namely, activation of ectopic chloroplast development. However, this phenomenon could also be indirectly linked to the HEP mutation (see discussion).

2.13 HEP mutants show retarded wilting

Drying HEP mutant plants for seed collection suggested that these plants remained turgid for a longer time than the WT. To investigate this observation further, 7-week old hep and WT plants grown on soil were kept for 7 days without water. After this time hep plants were still upright, whereas WT plants showed strong wilting (Fig. 25). Resistance to drought (wilting) is normally mediated by the phytohormone abscisic acid (ABA), which triggers closure of the stomatal pores. This process is vital for plants to conserve water by reducing transpirational water loss (Simonneau et al., 1998). The non-wilting phenotype could indicate that ABA functions are altered in the HEP mutant. However, the effect could also be of a more indirect nature: The thick cuticle (Fig. 19b), and the lack of typical intercellular spaces in internal tissue of hep leaves (Fig. 13d), could also take part in reducing transpirational water loss.
Fig. 25 Wilting is drastically delayed in hep compared to WT. a) 5 week old Arabidopsis WT plant and b) 5 week old hep plant before wilting assay. c) WT plant after 7 days without water. d) hep plant after 7 days without water. Scale bars = 1 cm.
3. Discussion

The pleiotropic phenotype of hep indicates that the HEP protein might have multiple functions or that it could be part of a developmental switch which influences the expression of various target genes.

The dwarfed phenotype of the shoot implies that HEP is required for growth and, coupled with its apparent restricted pattern of expression, that it seems to affect growth in a cell non-autonomous way. However, the activity of the enhancer trap in hep might also be genespecific (does not reflect the expression pattern of the gene which it mutates).

Histological data from the analysis of hep leaves suggests that the decrease in growth and the change in leaf shape might be due to inhibition of cell expansion. Reduction of cell expansion might be caused by various means, which are closer described in sections 1 to 3, focusing on leaf development. How differential growth of the margin might be related to overall growth of the leaf via biophysical forces and how margin cells might stabilize the planar leaf form is discussed in section 4.

In addition to the outcome on plant growth, HEP also seems to influence aspects of chloroplast development and this is discussed in section 5.

3.1 The role of HEP in cell expansion and differentiation

The smaller size and changed shape of cells in HEP mutant leaves could be due to a delay or change in the differentiation program. The accumulation of small undifferentiated cells in proximal parts of young hep leaves, where normally larger more differentiated cells are located, indicates that HEP might be involved in promoting differentiation (see Results Fig. 14 e and f). There are various genes characterised which interfere with development via keeping cells in a less differentiated state. For example, loss of function mutations of PASTICHINO (PAS) and TUMOROUS SHOOT DEVELOPMENT (TSD) genes induce calli on Arabidopsis plants and the encoded proteins have been implicated in inhibiting dedifferentiation (Harrar et al., 2003; Frank et al., 2002).

KNAT homeobox genes, on the other hand, are involved in keeping cells in a less differentiated state (Chuck et al., 1996). Transgenic plants overexpressing KNAT genes display densely packed small cells in leaves, which lack an obvious palisade parenchyma and spongy mesophyll. Since hep leaves do not appear tumorous, the accumulation of small and less differentiated cells rather resembles ectopic KNAT expression phenotypes. However, our
data show that KNAT1 is not ectopically expressed in hep leaves. Moreover, delayed senescence (which is usually a characteristic of ectopic KNAT expression in association with higher cytokinin levels) was not observed in hep leaves. So it is unlikely that the apparent delay in expansion and differentiation in hep leaves is caused by ectopic KNAT gene expression.

In addition, although cells in the proximal part of older hep leaves have histological differences compared to WT, the overall organisation of internal tissue seems to be intact. Hep leaves are radially polarised, i.e., they have cells which resemble palisade parenchyma- and spongy mesophyll cells but have reduced intercellular spaces (see Results Fig. 16).

Another possibility is that the observed alteration in the normal program of expansion/differentiation is an indirect affect of altered cell proliferation.

3.2 The role of HEP in cell division: the timing of the arrest front

Cells in wild-type leaves usually stay small during proliferation but increase in size following the arrest of division, when further growth is accommodated by cell expansion (Donnelly et al. 1999). In the first stages of leaf development, cells are rather small due to continued proliferation accompanied by growth. Cell expansion and cell division arrest starts at the tip of young leaves (~2mm in length) and moves basipetally as leaf growth proceeds. This leads to a wave of cell division arrest which travels along the proximal-distal axis of the leaf. At intermediate time points, this leads to a gradient of cell size in which cells at the distal end of the leaf are larger and more differentiated than cells in more proximal parts of the leaf.

Cells in young hep leaves also follow this pattern rule, but the gradient in cell size is very steep compared with the wild-type situation. Cells at the proximal part of the leaf remain extremely small and densely cytoplasmic, whereas large cells are present in the distal area of the same leaf (see Results Fig. 14a and b). This suggests that the cell cycle arrest front could be delayed in hep leaves compared with WT. Interestingly, the recently characterised CINCINNATA gene (CIN) in Antirrhinum is implicated in the timing of the arrest front and leads to an abnormal leaf shape (Nath et al., 2003). CIN, a TCP transcription factor, is generally expressed within the proliferation zone within the leaf, but is more highly expressed in the leaf margin compared to medial regions. Its expression declines basipetally during leaf growth, co-inciding with the gradual termination of cell division from the tip of the leaf towards the base. CIN is thought to somehow make cells (especially at the margin) more sensitive to a signal to exit from the cell cycle. It has been proposed that loss of function mutation of this gene leads to prolonged cell division activity (and growth) in the leaf margin,
resulting in a concave instead of convex arrest front. The resultant extra growth of the margin compared to medial regions of the leaf causes the observed enlarged crinkled leaves. Another related gene family in Arabidopsis (TCP-like) has also been implicated in regulating leaf shape via its differential pattern of expression in the leaf (Palatnik et al., 2003). Since HEP is expressed in the leaf margin and shows prolonged proliferation in the proximal region, it might also be involved in regulating the arrest front in a cell non-autonomous way. Surprisingly though, in contrast to cin, hep leaves are smaller. Considering the accumulation of small cells in proximal parts of young hep leaves, one might expect that after expansion hep leaves should be much wider than WT leaves in this area. However, histological data of older hep leaves reveals that the cells in the basal region do not fully expand. In contrast to CIN, HEP might play a more profound role in promoting cell cycle exit. Therefore, since the cells in hep keep proliferating, they might be less sensitive to expansion and differentiation signals. In this respect, HEP might antagonize AINTEGUMENTA, which is involved in regulating cell proliferation in lateral organs and which, when overexpressed, leads to prolongation of the proliferation phase of leaves (Mizukami et al., 2000).

3.3 The role of HEP in cytoskeletal and cell wall arrangement

A number of observations on the HEP mutant plants are consistent with an alteration in elements of the cytoskeleton and/or the cell wall. For example, the ton/fass mutants of Arabidopsis have a certain similarity to hep plants in that they are dwarfed and leaf shape sometimes is Lotus-shaped. In ton/fass mutants the microtubules are disorganized, which results in random cell division planes and irregular cell expansion (Traas et al., 1995). Three further observations give clues that cytoskeletal arrangement and, hence, cell division planes could be altered in the HEP mutant.

Firstly, in hep stomata distribution is abnormal, which is reflected by stomata clustering and the presence of adjacent stomata (see Results Fig. 18 d). This is in contrast to a consistent feature of normal stomata patterning in which stomata are separated from each other by at least one cell (Sachs, 1991; Nadeau et al., 2002a). Stomata patterning is based on a series of asymmetric divisions by which adjacent stomata or stomata precursor cells always divide away from each other to prevent clustering. In too many mouths (TMM) mutants, stomata clusters appear as a result of the plane of asymmetric divisions becoming randomised in the epidermis (Nadeau et al., 2002b).

Secondly, SEM images occasionally reveal a rough instead of smooth surface in the epidermis of hep leaves. This is confirmed by TEM pictures, which show that the cuticle forms spike-
like protrusions and that the cell wall appears thinner in *hep* compared to the outer epidermis of the WT (see Results Fig. 18 d and 19). The arrangement of the cell wall (cellulose microfibrils) depends on the orientation of cortical microtubules, and hence, the defects seen could be again due to a lack of proper cortical microtubule arrangements (Burk et al., 2002).

Thirdly, branching of trichomes in *hep* is reduced (see Results Fig. 18 e and f). Interestingly, *hep* is expressed in trichomes, indicating cell-autonomous action in that tissue. Loss of function mutation in the *ANGUSTIFOLIA (AN)* gene leads to reduced trichome branching due to a disarrangement of cortical microtubules. AN has been shown to interact with ZWICHEL, a kinesin motor protein, which is associated with the cytoskeleton (Folkers et al., 2002; Kim et al., 2002).

Although, these examples indicate a possible defect in the organization of the cytoskeleton in the *hep* epidermis, this could also be true for internal tissue, since internal cell shape and size are changed in *hep*. *HEP* could have an impact on the microtubule arrangement in the proximal parts in leaves and, therefore, could make those cells less competent to expand.

As outlined above, cytoskeletal organisation is linked to cell wall architecture. It is also possible that the *HEP* mutation leads to a direct change in biosynthesis or structure of a component of the cell wall. TEM analysis of the margin cells in the *HEP* mutant plants reveals a dramatic change in cell wall thickness and structure of the outer epidermal wall. The HEP protein thus might have a direct function in this striking aspect of the *hep* phenotype. However, this raises the question: if the alteration of *HEP* function is restricted to the margin, how could a change in margin cell function influence the entire growth characteristic of the leaf? This is discussed in the next section.

### 3.4 Biophysics at the leaf margin and its putative impact on leaf morphogenesis

Biophysical models have been described which propose that organogenesis is regulated by the balance of forces between different tissue layers. In the case of the SAM, it has been suggested that a tensile force in the epidermis (generated by anticlinally dividing cells in the tunica) balances a compression force in the inner tissue (generated by the randomly dividing cells in the underlying corpus) (Selker et al., 1992; Green et al., 1994). The tangential tension of the tunica is thought to restrict SAM growth by counteracting the compression force of the corpus. The cuticle (the outermost layer of the epidermal cell wall) builds a continuum and helps to strengthen and transmit the tangential tensile forces. Application of cell wall loosening agents (such as expansin proteins) weaken the tensile force of the outer epidermal cell wall, leading to an imbalance of forces between the inner and outer tissues, which results in bulging outward of the meristem.
Analogous to the situation in the meristem, local expression of expansin on the margin of primordia results in outgrowth of the leaf lamina (Pien et al., 2001a). Therefore, lamina outgrowth might be regulated by similar mechanical constraints. Intriguingly, the margin consists of anticlinally dividing robust, long and thick epidermal cells which are connected to neighbouring epidermal cells by extra cell wall material (see Results Fig. 18e and 19 c). This robust appearing margin complex might be expected to be highly resistant to any physical stress imposed on it. Thus, it might be involved in suppressing compression forces generated in the leaf blade by underlying margin tissue. What would be the outcome of a disruption to this biophysical equilibrium?

A simple prediction is that any weakening of the margin cell complex would lead to alteration of the growth characteristics of the whole leaf. Our observations on the HEP mutant support such a concept. In the HEP mutant plants the leaf margin cells are smaller, have thinner outer epidermal cell walls, and the structure of the cell wall itself is abnormal. Leaf growth is greatly disrupted in these plants. Relating the precise mechanism of this margin cell disruption to the final phenotype is more difficult, since it is possible that the resultant morphogenesis at least partially reflects the response of the plant to this disruption. Nevertheless, the crinkles, which are predominantly formed in absence of normal margin cells, provide hints that margin cells might stabilize the planar form of the leaf (see Results Fig. 10f).

**Fig. 26** Differential growth of the margin might be related to overall growth of the leaf via biophysical forces. **a)** A tensile force (generated by anticlinal divisions) in long and thick margin cells in the epidermis (blue cells) suppress compression forces created in underlying internal tissue (green cells). **b)** Disruption to this biophysical equilibrium by weakening of the margin cell complex (i.e. by presence of smaller margin cells or changed cell wall properties) could lead to alteration of growth characteristics of the whole leaf. (Vertical and horizontal arrows show orientation of compression and tensile forces, respectively.)
Discussion

Smaller epidermal cells at the margin provide more potential breaking points along the proximo-distal axis than long margin cells and this might be related to the tendency of the leaves to bend downwards (see Fig. 26).

3.5 The role of HEP in chloroplast development

In addition to altered morphogenesis, the HEP mutant plants display altered aspects of physiology. The dark green colour of the mutant leaves could simply reflect the smaller cell size in the mutant and the more compact nature of the plant. However, the observation that the roots also turn greenish (which does not occur in WT plants) indicates that HEP might be involved in repressing chloroplast development specifically in the light (since dark grown hep plants do not show obvious photomorphogenic traits; e.g., the chloroplast marker RBCS is not expressed). It is noticeable that the roots start greening at a rather late stage of development (3 weeks after germination) and at sites which are fully differentiated, i.e. at the junction to the hypocotyls. It might be that a factor was accumulating during root development and at a particular threshold triggers ectopic chloroplast development in HEP mutants. However, the green root phenotype could also be caused indirectly. The leaves in hep are epinastic and grow downwards from the shoot apex. After approximately 2 weeks, the growing leaves of hep touch the ground. Because of the robustness of the fleshy leaves, further growth of the leaves seems to lift the whole plant. Therefore proximal parts of the roots, which will green first, tend to get heaved into the air. Because of that the root might acquire certain shoot-like characteristics, which includes chloroplast development.
4. Materials and Methods

4.1. Primer list

primers used for RT-PCR (to generate probes for in situ hybridisation):

- ats1for: 5'-CGGATATCCGTTCGCCAGGCTGATGCC-3'
- ats1brev: 5'-GGACTAGTGCTTTGCTTTGCTAAGTCTCC-3'
- forceodper2: 5'-CGGATATCCGAGGATCGAGAGGTGTCAG-3'
- revper2: 5'-GGACTAGTCACGGCCACTAAACGGTTAGAG-3'
- grpe5: 5'-CGGATATCAGAAGGATCGAGAGGTGTCAG-3'
- grpe3: 5'-GGACTAGTATTTTCTATGGCCTTTAAGATGCC-3'

primers used for RT-PCR (to check differentiation status):

- as1for: 5'-GGACTATCTCAGAAGGAGGCTGATGCC-3'
- as1rev: 5'-GGACTAGTCAAGTTACACAAAGGATTTAGAG-3'
- as2for: 5'-GGACTATAGCAGGCTGATGCC-3'
- as2rev: 5'-GGACTAGTATAGCTATTAGTTTCTCATGACCAAG-3'
- knatfor: 5'-GGAACTTCCAGAAGGTAGGCTGATGCC-3'
- knatrev: 5'-GCTCTAGATTGTAACAAGAAGCAGAGGCTGATGCC-3'

primers used for semi-quantitative RT-PCR (cytokinin response):

- forarr5: 5'-GGACTAGTTATCTTCTCTCAGGAGTCCAGAGTCC-3'
- revarr5: 5'-GCTCTAGATTTTCTTCTTCTCTTTT-3'
- arr4for: 5'-AGGAATCTCGAGTATCTCCAGAGTCCAGAGTCC-3'
- arr4rev: 5'-AGCACCCTTCTCAAGGACATCC-3'
- arr8for: 5'-CATCGCCACAAATTCTTCTGATGCC-3'
- arr8rev: 5'-TAAACGCCGCTCTTTCTTTTCTCAGAGTCCAGAGTCC-3'
- ahk4for: 5'-ATTGAAGCTGGAAAGCTGGA-3'
- ahk4rev: 5'-CCGAATTTCCAAGGAGTCCAGAGTCC-3'
- cycd3.3for: 5'-TCTCCATTATCTCTCTCAGAGTCCAGAGTCC-3'
- cycd3.3rev: 5'-CAGCTTCATGATCGTACTCCAGAGTCCAGAGTCC-3'
- ipt3for: 5'-GTGGATGAGACTCGGAGTCCAGAGTCCAGAGTCC-3'
- ipt3rev: 5'-TCACGAGCTACTCCAGAGTCCAGAGTCC-3'
- amtfor (control): 5'-CGAACACTGCTGACTCCAGAGTCCAGAGTCC-3'
- amtrev (control): 5'-GCATCTCGCGATGCTCTGCTG-3'
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primers used for RT-PCR (photomorphogenesis):

- det1UP 5'-GTGCTGATCATC TTCAGCTTTCTTCT-3'
- det1RP 5'-TTGTTCAAGGGCATAGACTGTTATT-3'
- Det2UP 5'-TCCAAGCTCCTTACGGTAAACA-3'
- Det2RP 5'-TCCGGGTGAAATGAAATGAGAT-3'
- Det3UP 5'-GTGATTCTGCTTCCTCAAGGAAA-3'
- Det3RP 5'-TAAGTGGTGTACACCAACTGCAA-3'
- Cop1UP 5'-GTGGAGAGCGAGACAAACGGAAG-3'
- Cop1RP 5'-TCTTCCAGCAAACCGGACAATAC-3'
- Cop10UP 5'-TGTTGACACCTGCGGAG-3'
- Cop10RP 5'-TCAATGTGAAGCTGCACTTCAC-3'
- FUS3UP 5'-CTATATCCAGTACAGATGATGTCACCAC-3'
- FUS3RP 5'-AGATTCACTTACGGAGACCCAAAC-3'
- FUS4UP 5'-GGAGGACAGAGATTGATGCTCTTT-3'
- FUS4RP 5'-CTTCCTCCTCCTTCTGATCAA-3'
- FUS5UP 5'-ATGGATATACAGAGACGAAG-3'
- FUS5RP 5'-AACCTGGAACATTTGGTGAAG-3'
- FUS6UP 5'-CTCCTCCTTTAGCAACACATCT-3'
- FUS6RP 5'-GGATCCTTCAGGTAACGAGAG-3'
- FUS7UP 5'-GGGAGGCAGAGATTGATGCT-3'
- FUS7RP 5'-CTTCTCCGGTTTCTTGGAAT-3'
- FUS11UP 5'-TCTCAGGGATGCTGAACTCCG-3'
- FUS11RP 5'-CTTCAGGGAAATACAGGTAAC-3'

primers used for inverse PCRs:

- LB1 5'-TTGATTATACAGGATTTTGGGAG-3'
- RB1 5'-GTTTGGGAGAGTAGCGACACTC-3'
- LB3 5'-CCACCCAGTACATTAAAACGTC-3'
- RB3 5'-CTCTCAATGAATGAAATGAGAT-3'
- Ds3-1 5'-CGATTACGTATTATCCCGTGTCCG-3'
- Ds3-2 5'-CCGGTATATCCCGTGTCCG-3'
- Ds3-3 5'-GTACGCGGCTTTTCTGCTCC-3'
- AD2 5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3'
- Ds5-1 5'-CCGTTTACCCTTGTATATCCCGC-3'
- Ds5-2 5'-CTGTTCCTTGTACATTTACCC-3'
- Ds5-3 5'-GGTCAGGTACCGAATCTCCC-3'
4.2 Plant material and growth conditions

For growth on soil, Arabidopsis thaliana (ecotype Columbia) seeds were sown on 1/3 perlite and 2/3 soil, vernalised at 4°C for two days and then grown under long day conditions (16h light/8h dark) in a growth chamber (20°C, 80% humidity, 3500 Lux). For growth on medium, Arabidopsis seeds were first surface sterilised. The seeds were then incubated in 70% ethanol for 2 minutes. After that the seeds were incubated in bleach (2.5% hypochlorite) for 15 minutes. During these 15 minutes, the bleach was changed several times. Then they were washed 4 times with sterile milliQ H2O and stored in sterile tap H2O. Vernalised seeds were then sown on MS plates (with or without kanamycin at 50 µg/ml) and placed in a growth room (16h light/8h dark, 20°C).

4.3 Enhancer- and gene trap screening

The 1300 enhancer trap and 200 gene trap lines screened are based on the Cold Spring Harbour enhancer- and gene trap system (Sundaresan et al., 1995) (provided by Ueli Grossniklaus, University of Zürich). In short, in this system, the enhancer traps contain a GUS reporter gene which is fused to a 35S minimal promoter, whereas the gene traps contain a promoterless GUS reporter gene (Springer et al., 2000). Each construct is located on a Ds element (dissociation transposable element). In enhancer traps, GUS expression is activated by neighbouring endogenous elements. The gene traps, on the other hand, are only activated, if the Ds elements insert in a transcriptional unit. Random insertion of Ds elements throughout the genome leads to patterns of reporter gene expression, which reflects the expression pattern of neighbouring endogenous genes.

F3/F4 progenies of Arabidopsis enhancer- and gene trap lines were sterilized, six of each line was sown out on MS plates (2.2 g/L Murashige and Skoog basal medium (Sigma) (pH adjusted to 5.7 with KOH), 1% Agar (Fluka)). Then, 6 to 10 days old seedlings from each line were put into 2 ml of GUS medium in a 12 well plate. GUS medium consisted of 260 mM H2NaO4P, 2.6 mM HNa2O4P, 2mM C6FeK3N6 (III), 2mM C6FeK4N6, 10 mM EDTA, Triton X-100 and 0.5% X-Gluc (Biosynth AG). The plants were vacuumed for 2 x 30 sec in order to infiltrate the substrate. The plates were incubated for two days at 37°C. After that, the seedlings were visually screened for expression pattern in the leaf margin.
4.4 Semi-quantitative RT-PCR and inverse PCR

For semi-quantitative RT-PCR (described in Fleming et al., 1996), total RNA was extracted from 1 week old WT and hep seedlings by using Rneasy columns (Qiagen). 1 µg RNA was used for reverse transcription, cDNA substrate dilutions (0, 1/10, 1/20 and 1/100) were amplified within the linear range with primers for ARR4, ARR5, ARR8, CYCD3.3 and AMT (see primer list) which gave products at 400, 450, 420, 800 and 850bp, respectively. For inverse PCRs, TAIL- PCRs and Genome Walker PCRs were performed: DNA was isolated from either WT or hep plants using Nucleon Phyto Pure Plant DNA Extraction Kit (Pharmacia Biotech). For TAIL- PCR extracted genomic DNA was diluted 1000x . TAIL-PCR was performed as described in Liu et al. (1995). To amplify 5’ or 3’ border regions of Ds elements, Ds5.1 and nested primers (Ds5.2, Ds5.3) or Ds3.1 and nested primers (Ds3.2, Ds3.3) were used in combination with the degenerated primer AD2 (which anneals approximately every kb within the Arabidopsis genome). To identify 5’ or 3’ border regions of T-DNAs in hep, LB1 and RB1 primers were used in combination with AD2 (see primer list for sequences). Genome Walker PCRs were done according to manufacturer’s protocol (Universal GenomeWalker Kit, BD Biosciences, Clonetech Lab.). In short, 5 µg genomic DNA was digested with one of the supplied restriction enzymes (Dral, EcoRV, PvuII or Stul) to obtain blunt ends. 2.5 µg of digested DNA was used for adaptor ligation. PCRs were performed using adaptor primers in combination with either LB1 or RB1 primers to amplify the 3’or 5’ T-DNA border regions, respectively.

4.5 Histological analysis

Fixation of tissue was performed the same way as previously described for in situ hybridisation with minor alterations (Pien et al., 2001b): In short, 2 week old WT and hep Arabidopsis seedlings were taken, the roots and cotyledons removed by scalpel and the remaining parts (hypocotyls and leaves) fixed in 4% (w/v) formaldehyde in PBS (Sigma) (fixation medium). 4 x 5 minutes vacuum infiltration was performed (releasing vacuum every 90 sec.) before incubation in fresh fixation medium at 4°C overnight. Then, stepwise dehydration was performed at 4°C: 30%, 50%, 60%, 75% and 90% ethanol (Fluka) for 2 hours each step. After that, samples were incubated for at least 24 hours in 100% ethanol at 4°C (ethanol was exchanged twice during that time). For some tissue samples, solutions were stepwise replaced with Histoclear at room temperature (National Diagnostics, Chemie Brunschwig) before embedding in Paraplast Xtra Plus (Sigma). Other tissue samples were embedded in Technovit resin according to manufacturer’s instructions with the alteration that
longer incubation times with solvent A were used (10 hours instead of 1 hour) (Haska, Bern, Switzerland). Sections (8 μm) were mounted on Polysine slides (Menzel) (if embedded in Paraplast) or on conventional glass slides (Menzel) (in the case of using Technovit resin for embedding). All sections were stained with toluidine blue and/or methylene blue (Fluka).

4.6 Microscopy
Light microscopy and photomicrographs were taken in a Zeiss Axiophot microscope or a Zeiss Stemi SV 11-binocular. Transmission electron microscopy (TEM) was done on old leaf samples of HEP mutant and WT plants according to Sieber et al. (2000). Scanning electron micrographs (SEM) were performed on old leaves of WT and HEP mutant plants as described in Fleming et al. (1999).

4.7 Anticytokinin assay
WT and mutant plants were grown on MS plates containing increasing concentrations (0, 2, 50 and 100μM) of two different anticytokinins (N1-(methyl-4-pyridyl)-N2-phenylurea and 7-[N-ethoxycarbonyl)piperazinyl]-3-benzyl-3H-1,2,3-triazolo[4,5-d]-pyrimidine) (gifts from Prof. E. Karanov, Acad. M. Popov Institute, Sofia). The anticytokinins were initially dissolved in DMSO (Fluka) and then added to MS media to appropriate final concentrations.

4.8 Detached leaf senescence assay
2 x 21 seventh leaves from WT and HEP mutant plants, respectively, were detached with a scalpel. Chlorophyll content measurements were done immediately of 3 leaves from each WT and HEP mutant plants (day 0), the remaining 18 leaves of either WT or hep plants were equally distributed on 6 Petri dishes containing 30ml sterilized tap water, in as such as every Petri dish contained 3 leaves floating (with their abaxial side in contact with the water) on the water surface (day 1 to 6). The Petri dishes were then placed in the dark. After each day one Petri dish containing either WT or hep leaves was taken and chlorophyll content measurements were performed. Chlorophyll content determination was performed as described in environmental protection agency (EPA) protocol (www. ertresponse.com/sops/2030.pdf). 10 mg leaf tissue was ground in 250 μl extraction buffer (1 part 0.1 (N) ammonium hydroxide and 9 parts acetone (both Fluka)) in a Eppendorff with a sterile plastic pestle for 30 sec. The Eppendorffs were covered with aluminium foil and incubated for 30 min. on ice. After that additional grinding was performed (15 sec) and then, 500 μl 80% aceton (Fluka) was added. The extract was spun in a microcentrifuge at full speed.
for 1 min. at 4°C. Supernatants were taken and adjusted to 1 ml with 80% aceton. Absorbance was measured at 663nm (Chlorophyll a) and 645nm (Chlorophyll b) wavelengths. If necessary, the supernatant was diluted (especially from samples taken at day 1- and day 2 leaves) or more than 10 mg tissue were taken (samples from day 5- and day 6 leaves) to give absorbance readings between 0.2 and 0.8 at according wavelengths. For calculations of total mg chlorophyll contents per g fresh weight check formula from the EPA protocol (see above).

4.9 In situ hybridisation

In situ hybridisation was as previously described, with minor alterations, using digoxygenin labelled sense and antisense probes for Arabidopsis RBCS (At5g38430), MATE (At5g17700) and GrpE (At5g17710) (Pien et al., 2001b). In short, 6 to 12 days old Arabidopsis seedlings were taken, the roots and cotyledons removed by scalpel and the remaining parts (hypocotyls and leaves) fixed in 4% (w/v) formaldehyde in PBS (Sigma) (fixation medium): 4 x 5 minutes vacuum infiltration was performed (releasing vacuum every 90 sec.) before incubation in fresh fixation medium at 4°C overnight. Then, stepwise dehydration with ethanol was done at 4 °C and after that solutions were exchanged with Histoclear at room temperature (National Diagnostics, Chemie Brunschwig) before embedding in Paraplast Xtra Plus (Sigma) was performed. Sections (9μm) were mounted on Polysine slides (Menzel), digested with proteinase K for 15 min. at 37°C, treated with acetic anhydride, dried in ethanol, then hybridised with appropriate DIG- labelled probes overnight at 50°C. As hybridisation buffer microarray hybridisation buffer was used according to manufacturer’s instructions (Amersham Biosciences, UK). After washing with 0.2 x SSC at 55°C, the slides were treated with RnaseA for 30 min. at 37°C, washed again at 55°C with 0.2 x SSC, then processed for revealing the DIG antigen. This involved blocking with DIG-blocking reagent and BSA, followed by incubation with an anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics), washing with blocking reagent, then colour revealed by incubation in NBT and X-phosphate for periods of 12 to 48 hours. Reactions were stopped with milliqH₂O, slides air-dried, then mounted in Euparal (TAAB Laboratories) before viewing.

4.10 Cloning of probes for in situ hybridisation

RT-PCR as described above was performed with primers grpe5, forcodper2 or ats1bf or (containing an EcoRV site) and grpe3, revper2 or ats1brev (containing a SpeI site), respectively. RT-PCRs generated ~300 bp cDNA products including parts of the 3'
untranslated region. The RT-PCR products were digested with EcoRV/Spel and were purified with Stratagene Purification Kit (Stratagene). The pBluescript plasmids was digested with EcoRV/Spel. The digested vector (EcoRV/Spel) was dephosphorylated and purified (Stratagene Purification Kit). RT-PCR products were ligated into the pBluescript (EcoRV/Spel). Then the constructs were transformed into *E.coli* DHα with heat shocking at 37°C.

4.11 DNA gel blot analysis

Aliquots of 2.5 μg of genomic DNA isolated from either WT or line 1493 (*HEP* mutant) using Nucleon Phyto Pure Plant DNA Extraction Kit (Pharmacia Biotech) were digested with 160 U of SpeI and HindIII overnight at 37°C. The digests were run on a 0.8% agarose gel before transfer onto Hybond N+ nylon membrane by standard techniques (Maniatis). After fixing by a combination of baking and UV cross-linking, the membrane was hybridised with a ³²P- labelled probe equivalent to sequence 6808 - 6352 from the Ti-plasmid used for the enhancer trap lines generated by Scott Poethig (http://enhancertraps.bio.upenn.edu/). The membrane was hybridised overnight at 42°C in a buffer (50% formamide, 5 x SSC, 0.1 mg/ml denatured Salmon Sperms, 2x Denhardts, 50 mM KPO₄ buffer (pH7), 0.5% SDS). Then washed in 2x SSC, 0.2% SDS, 42°C for 10 min., after that, 2x SSC, 0.2% SDS, 60°C, 30 min. Signals were visualised using a phosphoimager.
V PART 2

Establishment of a microinduction system in
Arabidopsis thaliana
1. Introduction

Inducible promoter systems are an important tool to investigate the function of a particular target gene by ectopic expression or downregulation. Moreover, with the help of inducible promoter systems, manipulation of gene expression can be exerted at a particular developmental stage and for a desirable duration (Zuo and Chua, 2000a). Therefore, they provide a major advantage over constitutive expression systems where the continual expression of particular transgenes might result in, for example, lethality. In addition, inducible promoter systems can be used to mimic loss of function mutations by triggering the expression of a target gene in an antisense orientation or as via an RNAi strategy (Guo et al., 2003). Thanks to these approaches, embryo lethal effects can be circumvented and the role of such genes in later stages of plant development can be investigated.

Various inducible promoter systems have been described in plants (reviewed by Gatz, 1997; Zuo and Chua, 2000a) where the inducer was either added to the growth medium or sprayed over the whole plant. A microinduction technique has been developed in our group in which the chemical inducer is added as local source on plant tissue, allowing for highly localised induction of gene expression. The microinduction technique is based on a tetracycline-inducible promoter system (Gatz et al., 1992), in which tetracycline-loaded lanolin (acting as a local source) induces transcription of a cDNA cloned into a construct so as to be under control of an appropriate TetO promoter in transgenic tobacco plants. This approach was used to perform local manipulation of cell division and expansion at the margin of tobacco leaves and meristems (Pien et al., 2001a; Wyrzykowska et al., 2002). The establishment of a similar microinduction technique in Arabidopsis would provide a powerful tool to use the molecular and genetic resources now available for this model plant. Unfortunately, the tetracycline system does not work in Arabidopsis due to high background transcription of the transgene (Gatz, 1997). However, other inducible promoter systems have been reported to function in Arabidopsis. These include both chemical and heat shock inducible systems. Among these, the estradiol-based CLX system developed by Chua and co-worker offers a number of potential advantages. The CLX (Cre/loxP) system is a chemical regulated, site-specific DNA excision system (Zuo et al., 2001) which is itself based on the earlier described XVE system (Zuo et al., 2000b) (Fig. 27). The XVE construct consists of three transcriptional units. A strong constitutive promoter (G10-90) drives the expression of a trichimeric protein (XVE). The XVE protein consists of a fusion of a DNA-binding domain (LexA), a transcription
activation region (VP16) and the regulatory region of the human estrogen receptor (hER). The second transcriptional unit contains an hygromycin resistance gene (Hpt) fused to a nopaline synthase promoter (Pnos). The third transcriptional unit includes eight copies of the LexA operator sequence connected to a 35S minimal promoter (OlexA-46). This chimeric promoter drives any target gene of interest inserted downstream into appropriate cloning sites. Each of the three transcriptional units contains a different termination sequence to direct appropriate addition of poly (A) addition sequences (TE9, TNOS, T3a sequences, respectively).

After application of an estradiol inducer to transgenic plants containing the XVE construct, the XVE protein is activated and binds to the LexA operator sequences, leading to transcriptional activation of any target gene cloned downstream.

The CLX system also consists of three units. The first unit encodes the XVE protein. This unit is downstream of the G10-90 promoter. The second unit is a kanamycin resistance gene (KAN) fused to the nopaline synthase promoter. The third unit consists of the phage P1 Cre recombinase gene interrupted by an intron (cre-int) and regulated by the OlexA-46 promoter (see above). The intron insertion prevents background expression of cre in bacterial cells. The cDNA of any gene of interest can be inserted downstream of the cre recombinase gene. The Cre recombinase protein specifically recognizes loxP sites: One loxP site is inserted between the G10-90 promoter and the XVE unit, whereas the second is just upstream of the cDNA of the target gene (Zuo et al., 2001).

Since the Cre recombinase is under control of the XVE system, addition of the inducer estradiol to transgenic plants containing the CLX construct leads to expression of the Cre recombinase gene (Fig. 28). The Cre recombinase acts on the loxP sites and excises a DNA fragment containing the XVE, KAN, and the cre-int fusion gene, as well as associated promoters. This results in the subsequent splicing of the downstream target gene with the
upstream G10-90 constitutive promoter and, hence, to the constitutive expression of the target gene in those cells in which activation has taken place.

The CLX system was developed with the aim of generating a system by which unwanted selectable markers (i.e., antibiotic resistance genes) can be removed from transgenic material. However, if activation of the CLX system could be limited to a few cells within an organ, then (in theory) a clone of cells expressing the target gene would be produced in a background of transgene non-expressing cells. By comparison with the microinduction system developed in tobacco, the CLX system offers the potential of developing a similar system in Arabidopsis. The Arabidopsis system would lead to maintained local expression of any target gene. In combination with such approaches as RNAi and the wealth of defined genetic backgrounds in which the construct could be transformed, microinduction in Arabidopsis would provide a useful tool for the analysis of gene function. However, so far neither the CLX system (nor any others) have been used for microinduction experiments on Arabidopsis. In the following section I describe my attempts to establish a microinduction system in Arabidopsis based on the CLX system. These experiments were performed using the GUS reporter gene as a target for transcriptional induction, i.e., to characterise the system. The longer term view was to use the system to locally manipulate the expression of genes so as to test their potential role in leaf morphogenesis. Potential targets for such manipulation were genes involved in cell cycle regulation and cell expansion, as well as genes identified from
enhancer- and gene trap screening procedures showing activity in the leaf margin in Arabidopsis (described elsewhere in this thesis).
2. Results

2.1 Proliferating tissues of CLX-GUS transgenic plants show low reporter gene expression after induction

Initial experiments using the original CLX-GFP construct (a gift from Nam-Hai Chua, Rockefeller University, USA) indicated that the signal to noise ratio after whole seedling induction with estradiol was rather low (data not shown). Since the detection of GUS activity is very sensitive, we replaced the GFP coding sequence with that for GUS, generating the CLX-GUS construct (Lindsey et al., 1993). This construct was transformed into *A. tumefaciens* C58 before transformation into *Arabidopsis thaliana* (ecotype Columbia) via a floral dip method (Clough et al., 1998). 13 kanamycin-resistant T0 plantlets were recovered and a preliminary analysis for GUS activity was performed. Leaves from each plant were incubated over night in liquid MS media either containing or without estradiol (100 μM). The next day, histochemical assays were performed which revealed GUS expression after induction in all of the 13 plants. Zero to very low GUS activity was observed in non-induced transgenic seedlings.

A more detailed induction analysis was performed on the T1 progeny of these 13 plants. One to two week old T1 generation seedlings (grown on MS without kanamycin) from each line were incubated for 2 days in liquid MS media containing 2, 20 or 100 μM estradiol. These estradiol concentrations were selected based on published data (Zuo et al., 2001). As controls, WT and transgenic seedlings from line 19 were incubated with or without estradiol, respectively. Each of the T1 progeny from line 19 exhibited GUS expression upon induction with estradiol, suggesting the presence of multiple independent T-DNA insertions in this line. Although GUS expression in non-induced line 19 seedlings was very low, it was not zero. This is described in more detail below. Surprisingly, regardless of the estradiol concentration used, although all lines showed some level of GUS expression after induction with estradiol, none of the lines displayed GUS expression in root and shoot apical meristems or in young leaf primordia (Fig. 29b and d). In older leaves, GUS expression was rather restricted to distal parts where cell expansion already has occurred (Fig. 29b). Moreover, 4 out of 13 lines showed a patchy GUS expression pattern, namely line 5, 7, 12 and 20 (Fig. 29a), whereas the remaining lines generally exhibited homogenous expression except in young leaves and meristems (line 1, 3, 8, 11, 17, 19, 21, 23 and 29). Some of the transgenics of line 19 which were mock-inoculated showed GUS expression in leaf hydathodes (3 out of 20) (Fig. 29f).
test if these “escapes” were due to endogenous sterols activating the estradiol-sensitive CLX construct, transgenic seedlings from line 19 were incubated in liquid MS to which epibrassinolide was added to a final concentration of 50 nM. However, this did not significantly alter the number of “escapes” (4 out of 20).

The non-uniform induction of GUS expression in the 13 T1 lines investigated above could be due to a number of factors. A number of experiments (described in this and the following section) were performed to test these possibilities.

One possibility is that, although the seedlings were immersed in the inducing medium, the estradiol did not penetrate to all tissues. To improve the accessibility of the inducer to meristematic tissue, one to two week old T1 seedlings from four lines (line 11, 17, 19 and 21) were germinated on solid MS medium containing Kan and were transferred to liquid MS media containing 100 μM estradiol. After vacuum infiltration for 3 min. the seedlings were incubated in the inducer media for 5 days. For recovery, the seedlings were transferred to MS plates without estradiol for 2 days. If the CLX system functions, cells that have been induced should be switched permanently to express the target gene (i.e., subsequent growth under non-inducing conditions should not lead to a decrease in reporter gene activity). Although the intensity of the GUS staining was generally increased in these seedlings compared to experiments in which induction was performed for only 3 days, GUS expression in meristematic tissues and young leaf primordia was still very low or zero (Fig 29c). Essentially identical results were obtained when the experiment was performed with an even longer period of estradiol incubation (7 days instead of 5 days).

2.2 Microinduction of CLX-GUS transgenic plants leads to reporter gene expression in older but not younger leaves

Although the whole seedling induction of the 13 T1 lines gave negative data for young leaf primordia, we reasoned that it was still possible that simple immersion of the seedlings in the inducer gave only poor access to some tissues. Since the eventual aim of this part of the project was to develop a microinduction technique, we proceeded to investigate whether direct local application of the estradiol inducer on leaf primordia led to reporter gene expression. For these time consuming experiments, we focussed on four transgenic lines (T1 generation) which exhibited the strongest sensitivity to estradiol and no patchy GUS expression in our whole seedling induction experiments (line 3, 11, 19 and 29). Moreover, all T1 progenies of three of these lines (line 3, 11 and 19) exhibit GUS expression after induction which again is probably indicative of multiple independent T-DNA insertions. Lanolin
Results

containing 50 or 100 μM estradiol dissolved in DMSO was locally applied onto the margin of leaves at various developmental stages (from P5 onwards). As a control, transgenic seedlings were mock-inoculated with lanolin containing DMSO without the inducer. The locally induced transgenic seedlings were further grown on MS plates for 3 days, before histochemical assays were performed.

GUS activity was detectable in a localised region at the margin of many 15 out of 20 “older” leaves (>3mm in leaf length) following this manipulation, especially at distal parts where, putatively, cell proliferation is declining and cell expansion predominates (Fig. 30a-d). However, activation of the CLX system (as indicated by GUS activity) through microinduction at the margin of young leaves (<3mm in leaf length), was very infrequent (only 2 out of 22 local inductions were successful). The 2 positive assays resulted from local induction at rather distal areas of these young leaves (Fig. 30d). Repeated experiments in which either the inducer concentration (200μM) or incubation time before GUS assay was increased (from 3 to 5 days) did not improve the frequency at which local induction of GUS expression in young primordia was observed (data not shown).
Fig 29 Total induction of CLX-GUS transgenic Arabidopsis seedlings with estradiol. (a) Line 7 seedlings reveal a patchy GUS expression pattern after incubation with estradiol (100 μM) for 2 days. (b) Line 19 seedlings exhibit a homogenous GUS expression pattern, but GUS expression is lacking in youngest leaves (see arrowhead) after incubation with estradiol (100 μM) for 2 days. (c) Same as in (b) but infiltration was performed with the inducer media prior to incubation. The incubation period was extended to 5 days. GUS expression is enhanced, but still absent in youngest leaf tissues (arrowhead). (d) Close up of root apical region of line 7 seedlings reveal lack of GUS expression in meristematic tissue (arrowhead). (e) Mock-inoculated line 19 seedlings reveal no GUS expression or GUS expression predominantly in hydathodes in some ‘escapes’ (arrowheads). Scale bars in (a) = 6mm; in (b, c, e and f) =1mm; in (d) = 400 μm.
Fig 30 Local induction of CLX-GUS transgenic Arabidopsis seedlings. (a) Local application of estradiol (100μM) in margins of leaves at different developmental stages. Only local supply of estradiol in "older" leaves of line 19 (> 3mm in length) resulted in local GUS expression (black arrowheads). Red arrowhead indicates local application of estradiol in margin of a young leaf (< 3mm in length) which did not result in GUS expression. (b) Magnified picture of a local supply of estradiol in (a) where lanolin is still detectable (arrowhead). (c) Local supply of young primordia (arrows) did not cause local GUS expression. (d) Rare positive result of local supply of estradiol which triggered GUS expression in distal part of a young leaf (~ 3mm in length). Scale bars in (a) = 5mm; in (b) = 300 μm; in (c) = 1mm and in (d) = 3mm.
3. Discussion

3.1 Does the differential induction frequency of CLX-GUS in different tissues reflect a differential activity or sensitivity to recombination?

A surprising result of this investigation was the non-uniform response with respect to GUS activity of transgenic seedlings engineered to express the CLX construct after total immersion in the inducing agent. In particular, the tissues showing poor (or zero) induction were those tissues expected to be most active in terms of general metabolism, growth and cell division (i.e., root and shoot meristems and young leaves). A number of possible explanations can be put forward to explain this observation. These are discussed below.

One possibility is that proliferating tissue (such as the shoot apical meristem and young primordia) is less accessible to the inducer estradiol. The inducer might not be efficiently transported to these tissues, or might be rapidly metabolised. Although we performed a number of experiments to try to ensure that the estradiol fed to the seedlings gained access to these apical tissues, it is difficult to state categorically that the inducer level was not limiting. However, our observation that even parts of the root apex frequently showed very low GUS activity after induction, whereas other parts of the root showed a very strong staining patterns argues against inducer accessibility being a main problem. It is difficult to conceive how a small diffusible molecule such as estradiol might readily access one part of the root and yet have difficulty diffusing at all to a neighbouring region. An alternative explanation is that the CLX system itself is not equally efficient in all tissues. This interpretation suggests that excision or recombination events might be generally inhibited in proliferating cells.

Originally the CLX system was established for marker gene excision of transgenic crops (Zuo et al., 2001). Although most of the lines reported in this publication exhibited uniform somatic GFP expression upon supply of estradiol to MS plates, genetic and molecular analysis indicated that CLX activity seemed to be inhibited in germ cells or their progenitor L2 cells in the meristem. The potential downregulation of excision events in the meristem (especially in the L2 layer) would make sense, since double stranded breaks in those cells might have drastic consequences for further development and production of germ cells. In addition, there are recent papers that have shown that a surveillance system works in the shoot apical meristem, which works as a filter in trafficking only certain mRNA into the shoot apical meristem (Foster et al., 2002; Ruiz-Medrano et al., 1999). The filter might have evolved as a barrier for RNA viruses which might infect the stem cells in the meristem and which
otherwise might confer viruses to the progeny. For a similar reason, a surveillance system might exist in the meristem which makes sure that excision events which cause double stranded breaks (as occur in the CLX system or via DNA transposable elements) are inhibited in the meristem. In this respect, it is possible that the DNA repair mechanism is especially competent in proliferating tissue where it acts at cell cycle checkpoints.

3.2 Microinduction using the CLX system is not ideal for leaf developmental studies

Activation of the CLX system through microinduction was successful on „older“ leaves or in distal part of younger leaves, where predominantly cell expansion occurs. Microinduction of reporter gene expression in these leaves occurred precisely at the spot of lanolin application (Fig. 30b), which indicates that estradiol is not highly diffusible and acts within a short range from the application point. However, the CLX system was hardly activated upon local induction of transgenic leaves below 3mm in length and which are, therefore, predominantly in the proliferation phase. Nevertheless, previous studies indicated that the CLX system can be activated in young leaves. For example, Zuo and co-workers obtained uniform reporter gene expression in young leaves upon total induction of CLX-GFP transgenic Arabidopsis plants (Zuo et al., 2001). In these experiments, a relatively long induction period (2 weeks) was used, in contrast to the 3-5 day inductions reported here. However, in the context of microinduction, where a fast response to the inducer is required during organ differentiation, such a long incubation time is not favourable. After two weeks, a young leaf primordium in Arabidopsis will have grown to maturity, thus precluding any analysis of early stages of development. In addition, ~16% of mock inoculated transgenic CLX-GUS plants in our experiments (line 19) showed some background GUS expression, especially in the hydathodes of the leaves. The reason for this tissue-specific background activation of the CLX system is unclear. Putatively, oestrogen-like acting compounds (phyto-oestrogens) might be present in the tissue and activate the CLX system. For example, because of the putative presence of phyto-oestrogens in soybean, the XVE system (and hence the CLX system) shows high background activity in this species (Zuo et al., 2000a). Phyto-oestrogens are iso-flavones which are predominantly found in soya and other legumes. Sometimes these substances imitate the working of oestrogens (Valtuena et al., 2003).

Overall, the results reported here indicate that the CLX system might be not convenient for local induction experiments in Arabidopsis, at least not during early phases of organ development.
4. Materials and Methods

4.1 Plant material and growth conditions
For growth on soil, Arabidopsis thaliana (ecotype columbia) seeds were sown on 1/3 perlite and 2/3 soil, vernalised at 4°C for two days and then grown under long day conditions (16h light/8h dark) in a growth chamber (20°C, 80% humidity, 3500 Lux). For growth on medium, Arabidopsis seeds were first surface sterilised. The seeds were then incubated in 70% ethanol for 2 minutes. After that the seeds were incubated in bleach (2.5% hypochlorite) for 15 minutes. During these 15 minutes, the bleach was changed several times. Then they were washed 4 times with sterile milliQ H₂O and stored in sterile tap H₂O. Vernalised seeds were then sown on MS plates (with or without kanamycin at 50 µg/ml) and placed in a growth room (16h light/8h dark, 20°C).

4.2 Cloning of the CLX-GFP vector and transformation of Arabidopsis
CLX: GFP construct from Chua (pX6GFP(CLX)) was digested with Spel/Xhol to release the GFP insert. The empty vector (Spel/Xhol) was dephosphorylated and gel purified. Plasmid pJIT166 from Phil Mullineaux (JIC, Norwich UK) was digested with Sall/Xbal to release the GUS coding sequence. The insert was gel purified (Stratagene Gel Purification Kit) and ligated into the CLX (Spel/Xhol) vector to generate the CLX::GUS construct.

4.3 Transformation of Agrobacterium tumefaciens C58
Transformation of Agrobacterium tumefaciens C58 was done by electroporation. Single colonies of A. tumefaciens C58 (grown on LB medium with ampicillin and rifampicin, at 30°C) were used to inoculate 3 ml YEB- medium (0.5 w/v Bacto-Trypton, 0.5 w/v Bacto-Pepton, 0.1 w/v Yeast-extract (all Difco) and 2mM MgCl₂ (Fluka)) containing 100 mg/L rifampicin (Fluka), 100 mg/L ampicillin and grown at 30°C until OD₅₂₆ = 0.8. Then the cells were spun for ten min at 14000rpm at 4°C in 1.5 ml Eppendorff tubes. The cells were washed three times with 2 ml 10% (v/v) glycerol and then resuspended in 50µl 10% (v/v) glycerol. Then the cells were mixed in an electroporation cuvette (BioRad) with 50 ng of the CLX::GUS- plasmid. The electroporation was done at 2.5 kV, 400 Ohm, 25µF with the equipment from BioRad Laboratories. After the electroporation the cells were incubated in 1 ml YEB-medium at 30°C for 1 hour and then plated out on YEB-plates (YEB medium plus
1% Agar (Fluka)) with 100 mg/L rifampicin, 100 mg/l ampicillin and 100 mg/L streptomycin. The plates were then incubated at 30°C for 2 days.

4.4 Transformation of *Arabidopsis thaliana*

*Arabidopsis* were grown (as described above) until flower buds formed (bolt height 5-10 cm). Transformation was done by a floral dip method as described in Clough et al. (1998) with minor alterations. Briefly, a transformed colony of *A. tumefaciens* C58 containing the CLX::GUS vector was used to inoculate a preculture of 100 ml YEB- medium (see above) containing 100 mg/L rifampicin, 100 mg/L ampicillin and 100 mg/L streptomycin. This preculture was used to inoculated 500 ml of the same medium and was grown with shaking at 30°C until OD$_{526}$ = 1 (approximately 24h). The bacteria were spun down for 15 min. at 5000g and 4°C. An additional centrifugation was done of the supernatant to the same conditions. The pellets were resuspended in infiltration medium (MS medium (2.2g/L Murashige and Skoog basal medium (Sigma) adjusted to pH 5.7 with KOH), 5% sucrose, 4.4 μM Benzylaminopurine (Fluka), 0.03% v/v Silwet L-77 (Lehle Seeds)). 400 ml of the solution was poured in a 500ml beaker. The solution was under gentle agitation by the use of a magnetic stirrer. The floral buds were dipped for 30 sec. After that, the plants were placed in a plastic tray and covered with a tall clear-plastic dome to maintain humidity for 24h after treatment. Then, plants were put back into growth chambers. After 4 days the dipping process was repeated with fresh infiltration medium to the same conditions.

4.5 Total- and Microinduction method

Seeds from CLX::GUS transformed plants were sterilised and plated out on MS medium for 10 – 14 days. Then transgenic and WT (as controls) seedlings were transferred to 6 well plates containing liquid MS medium supplemented with β-estradiol (Sigma) to 2, 20 or 100 μM final concentrations. β-estradiol was first dissolved in DMSO (Fluka) before addition. Some seedlings were vacuum infiltrated for 3 minutes. Incubation in the induction medium ranged from 2 to 5 days. After that GUS stainings were performed, some were directly stained whereas others were first transferred to MS plates for 2 days (for recovery) before GUS stainings were done: Seedlings were put into 5 ml of GUS medium in a 6 well plate. GUS medium consisted of 260 mM H$_2$Na$_2$O$_4$P, 2.6 mM HNa$_2$O$_4$P, 2mM C$_6$FeK$_3$N$_6$ (III), 2mM C$_6$FeK$_4$N$_6$, 10 mM EDTA, Triton X-100 (all Fluka) and 0.5% X-Gluc (Biosynth AG). GUS incubation was performed for 24 hours at 37°C.
Localised induction of meristem and leaf primordium was performed using lanolin paste (3% paraffin in lanolin) (Pien et al. 2001a). The paste was melted for 1 hour at 50°C. β-estradiol was first dissolved in DMSO and was then added to the melted lanolin paste to a final concentration of 2, 10 20 50 or 100 μM. Very small portions of lanolin were applied onto the margin of Arabidopsis leaves at various stages using stretched plastic tips. For all inductions, controls were performed using the same mix without estradiol and the same application method. Locally induced seedlings were further grown for 3 to 5 days. GUS histochemical assays were performed for 24 hours at 37°C.
VI PART 3

Sucrose synthase expression pattern in young maize leaves: Implications for phloem transport
Introduction

Previously in our lab a differential display analysis was performed to identify genes that are asymmetrically expressed in the shoot apical meristem associated with leaf formation in tomato (Pien et al., 2001). Surprisingly, the analysis led to the identification of several genes encoding proteins involved in carbohydrate metabolism (ADPglucose pyrophosphorylase (AGPL1), SNF1-like kinase and sucrose synthase (SuSy, EC 2.4.1.13). This indicates that altered carbohydrate metabolism might play key roles in leaf formation. AGPL1 is a key enzyme in starch synthesis, SNF1-like kinase seems to play an important role in sugar signalling and SuSy has been implicated in a number of important physiological processes, which are described in more detail below. SuSy catalyses the readily reversible conversion of sucrose to fructose and UDP-glucose (Geigenberger and Stitt, 1993). This reaction has been implicated in sink/source relationships within the plant (Zrenner et al., 1995), response to anoxia (Ricard et al., 1998) and cell wall biosynthesis (Amor et al. 1995). In the latter SuSy is thought to be membrane associated and might channel UDP-glucose to cellulose and callose synthases (Stone and Clarke, 1992). Moreover, upon phosphorylation SuSy seems to dissociate from the membrane and is mainly present in a soluble form in the cytosol, where it might act to provide UDP-glucose for glycolysis (Winter et al., 1997).

With respect to sink/source relationships, one of the most consistently reported observations has been a specific localisation of SuSy expression to phloem cells in leaf tissue (Yang and Russell 1990; Martin et al., 1993; Nolte and Koch 1993; Fu and Park 1995). Taken in conjunction with data from experiments in which pyrophosphatase activity was manipulated in phloem cells (Lerchl et al., 1995), these observation have led to the hypothesis that SuSy plays a role in sucrose loading/unloading in the phloem. With the exception of the report by Nolte and Koch (1993), all these investigations have relied on an indirect assessment of SuSy gene expression (i.e., use of promoter-reporter gene constructs transformed into Arabidopsis, tobacco or potato). In this report, we show that, contrary to expectation, there is a specific exclusion of SuSy transcript and protein accumulation from sink phloem tissue of young maize leaves.

As mentioned above, our initial investigation of SuSy expression in young maize leaves was prompted by finding that SuSy (and other genes involved in carbohydrate metabolism) accumulate in the meristem of tomato at the site of incipient leaf formation. We wanted to test whether this pattern was also observed in the meristem of monocotyledonous plants. To do this we chose to examine maize, since there is an abundant literature on SuSy in maize.
including extensive information on molecular genetics and gene expression. In maize, SuSy is encoded at least by two genes (Sh1 and Sus1). These genes show distinct expression patterns and a potential for modulation of the expression patterns dependent on genetic, environmental and biochemical factors (Springer et al., 1986; Koch et al., 1992; Chourey and Taliercio 1994). At the nucleotide level, Sh1 and Sus1 mRNA are highly similar and expressed sequence tags (ESTs) for both genes are available (Maize Genome Database: p5C11D10, Sh1; pCSU886, Sus1). We used these sequences to generate riboprobes for an in situ hybridisation analysis to investigate the pattern of Susy transcripts within the maize shoot apex.
2. Results

2.1 SuSy accumulates in all leaf tissues except the phloem in young leaves

Hybridisation of maize apices revealed no specific accumulation of SuSy transcripts in the meristem, with signal first detectable in the leaf primordium (Fig. 31a). This pattern is distinguished from the high accumulation of transcripts on the flank of the meristem in tomato hybridised with a probe for LeSus4 (Fig. 31b, and Pien et al., 2001b). Figure 31c shows a hybridisation of a maize meristem with a probe for the homeobox gene KN1. As expected (Jackson et al., 1994), there was a high and specific accumulation of transcripts in the meristem, indicating that the lack of signal seen in Fig. 31a is not due to loss of RNA integrity. Due to the anatomy of the maize apex, sections of the meristem also include sections of surrounding leaves, which form a developmental gradient of concentrically older structures outwards from the meristem. Analysis of SuSy transcript accumulation in these structures revealed a specific and surprising pattern. As can be seen in Fig. 31d, in the young leaf, SuSy transcripts first accumulated in patches on the epidermis. As the leaves matured, these patches expanded to include all of the leaf tissue with the exception of approximately circular areas within the leaf that marked the areas of presumptive vascular formation. As the vascular tissue matured (Fig. 31d, e), SuSy transcripts accumulated in the region of xylem differentiation, but were still excluded from the opposite pole of presumptive phloem. Later in development (Fig. 31f), as vasculature took on its mature structure, SuSy transcripts accumulated in the xylem and supporting tissue and in a band of cells in the region of the differentiating collenchyma, but again did not accumulate to any appreciable extent in the region of the phloem. Similar patterns were observed using probes generated for Sh1 and Sus1. The high sequence similarity of these probes makes cross-hybridisation with their respective substrates highly likely, so that the lack of signal observed in the phloem reflects a lack of transcripts for both genes. Positive signal may reflect transcript accumulation for either Sh1 and Sus1. Hybridisations performed with sense probes gave no signal (Fig. 31g).

The specificity of the SuSy transcript pattern was confirmed by hybridisation using a probe encoding RBCS, the gene for ribulose-1,5-bisphosphate carboxylase/oxygenase. As expected (Langdale et al., 1988), RBCS transcripts accumulated in the bundle sheath cells around the xylem and phloem (Fig. 31h), indicating the integrity of the RNA within the tissue. In addition, to ensure that the patterns observed with SuSy probes did not reflect the distribution of total RNA in the sections, RNA was visualised using acridine orange (as described in...
Fleming et al., 1993). As shown in Fig. 31i, RNA was present at high levels throughout the vascular tissue, including phloem.

To investigate whether the SuSy transcript pattern led to similar distribution of the encoded protein, we performed an immunohistochemical analysis (as described in Weiss et al., 1993) using antibodies raised against SuSy (a kind gift from Prem Choure). The results indicated that SuSy protein accumulated throughout young leaf tissue but was present at much lower levels in the differentiating vascular tissue (Fig. 31j). Similarly, in older leaf tissue, SuSy protein was present at relatively high levels around the vasculature but to only a low level in the phloem (Fig. 31k). The distribution of total protein in the tissue was revealed by Ponceau S staining (Fig. 31l). This indicated a high level of protein throughout the vascular tissue.
Fig. 31 Localisation of SuSy transcripts and protein in cross sections of developing leaves of maize (Zea mays) and tomato (Lycopersicon esculentum). (a) Maize meristem hybridised with an antisense probe for Shr. Signal (blue/violet) can be seen in the leaf primordium but not in the meristem. (b) Tomato longitudinal section of meristem hybridised with an antisense probe for LeSus4. Signal is concentrated on one flank of the meristem. (c) Maize meristem hybridised with an antisense probe for KN1. Signal is localised to the meristem. (d) Maize leaves hybridised as in (a). A developmental gradient of vascular differentiation (arrows) in successive leaves is shown. (e) Detail from (d) showing differentiating vascular bundle. Signal is absent from the pole opposite the xylem. (f) A relatively mature vascular bundle of maize hybridised as in (a). Signal is absent from phloem. (g) Cross section as in (d) but hybridised with a sense probe for Shr. (h) A relatively mature vascular bundle of maize hybridised with an antisense probe for RBCS. Signal is localised to the bundle sheath cells. (i) Acridine orange-stained vascular bundle of maize to demonstrate total RNA distribution (orange signal). (j) A young leaf of maize in which SuSy protein distribution has been visualized by immunocytochemistry. Signal (blue) is seen in a ring around the differentiating vascular tissue. (k) An older maize leaf treated as in (j). (l) As in (k) but stained with Ponceau S to visualise total protein distribution. Signal (red) is seen throughout the vascular tissue. m Meristem, l leaf, x xylem, pp presumptive phloem, p phloem, c collenchyma, v vasculature. Scale bars in (a) = 100 μm, in (b) = 60 μm, in (c) = 150 μm, in (d) = 400 μm, in (e, f, h) = 250 μm, in (g) = 500 μm, (l – l) = 200 μm.
3. Discussion

3.1 Different SuSy expression patterns in leaves might reflect source and sink activity

The data reported here indicate a co-ordinated pattern of SuSy gene expression during early leaf development and, in particular, indicate no or very limited expression of Susy in phloem tissue. This is in contrast to previous reports showing that SuSy is specifically localised to phloem in leaf tissue. How can these divergent observations be reconciled?

On one hand, our data provide a direct assay of SuSy transcript and protein distribution whereas previous investigations have largely dependent on the interpretation of promoter-β-glucuronidase (GUS) fusions in transgenic plants. Although promoter-GUS fusions are very powerful tools, there are potential limitations on their interpretation (Sieburth and Meyerowitz, 1997). To our knowledge there is only one previous report in the literature where SuSy protein has been localised in leaf tissue (Nolte and Koch, 1993) and these authors found a phloem localisation in mature maize leaves. In addition, experiments utilising transgenic plants expressing a cytosolic pyrophosphatase in the phloem disrupted sucrose transport in a manner consistent with the presence of a phloem-localised SuSy (Lerchl et al., 1995). This resulted in stunted growth and increase in starch synthesis in source leaves probably due to a lack in carbohydrate loading.

The differences between the pattern of SuSy expression reported previously and in this investigation might reflect a difference in the developmental age of the tissue under examination and, in particular, the status of the tissue as either carbon sink or source. In such a scenario, SuSy expression in phloem would be linked with sucrose loading and retrieval in source tissue (mature leaf). This would also prevent futile cycles between sucrose phosphate synthase (SPS) (which in association with sucrose phosphate phosphatase (SPP) drives sucrose synthesis) and SuSy activity in non-phloem cells in the mature leaf (Frommer and Sonnewald, 1995). SPS is thought to accumulate rather late during leaf development and might, together with the predominantly phloem localised SuSy, participate in sucrose loading and retrieval in source tissue.

Non-phloem expression of SuSy on the other hand would be linked with sink tissue activity (young leaves) where SPS is lowly expressed (Frommer and Sonnewald, 1995). Observations on potato tubers and roots (sink tissues) using reporter-gene constructs under the control of SuSy promoter elements support this contention (Fu and Park 1985) in that in these tissues a high level of SuSy gene expression was found in non-vascular tissue. Moreover, transgenic
potato plants expressing SuSy antisense RNA in tubers resulted in a reduction of starch synthesis in tubers and decrease in tuber growth indicating declined sink strength (Zrenner et al., 1994). In the case of potato parenchyma, it has been suggested that the high accumulation of SuSy functions to direct the metabolism of incoming sucrose to the synthesis of storage starch. Although starch does not accumulate to appreciable levels in young maize leaf primordia (data not shown), it is possible that there is a transient starch accumulation occurring around the vascular tissue. Alternatively, since SuSy has been implicated to be membrane-associated and putatively involved in cellulose synthesis in concert with cellulose synthase, UDP-glucose might be rather fed into cell wall synthesis in young leaf primordia than starch. Investigation of the expression patterns of genes encoding enzymes involved in starch or cell wall biosynthesis could be informative in this context.

In conclusion, although SuSy is probably present in mature phloem (where it could function in sucrose loading and retrieval), it does not accumulate to any appreciable level in the phloem of young maize leaves. The actual physiological function of SuSy in young leaves remains to be clarified, but the observed accumulation of SuSy transcripts and protein in tissue around the phloem in sink leaves would fit with SuSy functioning in the metabolism of sucrose supplied via the vasculature. This would maintain a supply of fructose and UDP-glucose for the cell and a gradient for sucrose flux towards these cells from the phloem.
4. Materials and Methods

4.1 Plant material and growth conditions
Maize (Zea mays L. cv. Lh82, a gift from P. Stamp, ETH-Zürich) seedlings were grown for 4 weeks in a growth chamber (16 / 8h light / dark cycle, 100 μmol photons m⁻² s⁻¹) at 24°C before sampling for in situ hybridisation or immunohistochemical analysis. Tomato (Lycopersicon esculentum Mill. Cv. Moneymaker, a gift from M. Bucher, ETH- Zürich) seedlings were grown as described by Pien et al. (2001).

4.2 In situ hybridisation
In situ hybridisation was as described by Pien et al. (2001). Briefly, samples were fixed in 4% (w/v) formaldehyde in PBS, and embedded in paraffin. Sections (8μm) from the shoot apex of maize seedlings (see Results Fig. 30) were treated with proteinase K for 30 min at 37°C, then with acetic anhydride, dried in ethanol, and then hybridised with the appropriate digoxigenin (DIG)- labelled riboprobe overnight at 50°C. After washing with 0.2 x SSC at 55°C, the sections were treated with RNase for 30 min at 37°C, washed again at 55°C with 0.2 x SSC, then processed to reveal the DIG antigen. This involved blocking with DIG- blocking reagent and BSA, followed by incubation with an anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics), washing with blocking reagent, and incubation in nitro blue tetrazolium and bromochloroindolyl phosphate for periods of 4h to 2 days to reveal the colour. Acridine orange staining was as described previously (Fleming et al., 1993) using 0.5% acridine orange for 3 min at room temperature before washing in 50 mM CaCl₂.

4.3 Immunolocalisation
Immunohistochemistry was as described by Weiss et al. (1993). Samples were fixed in ethanol/acetic acid (3:1, v/v) before embedding in paraffin. Sections were treated with a blocking solution [10% (w/v) BioRad blotting blocker in TBS, 0.5% (w/v) Tween-80]. Following incubation in the blocking solution with an antibody raised against maize SuSy (a gift from Prem Chourey, University of Florida, USA), slides were washed three times in TBS, 0.5% (v/v) Tween before colour development for 20 – 45 min with nitro blue tetrazolium and bromochloroindolyl phosphate.
VII  General Discussion

1. The role of the leaf margin in leaf development

In the first part of this study we reported on the identification and characterisation of an enhancer trap line which shows reporter gene expression in the margin of developing leaves. Coupled with this expression pattern, the line shows an altered pattern of margin cell differentiation and altered leaf phenotype. We have termed this mutant hepatica. Our data suggest that changes in leaf shape could result from mechanical and/or functional alteration in leaf margin cells. The former is supported by TEM data, which reveal that the leaf margin cells are smaller, have thinner outer epidermal cell walls, and the structure of the cell wall itself is abnormal in HEP mutant plants. The crinkles, which are predominantly formed in the absence of normal robust margin cells provide hints that margin cells might stabilize the planar form of the leaf. Smaller epidermal cells at the margin provide more potential breaking points along the proximo-distal axis than long margin cells and this might be related to the tendency of the leaves to bend downwards. Moreover, differential growth of the margin might be related to overall growth of the leaf via biophysical forces. The robust appearing margin complex might be expected to be highly resistant to any physical stress imposed on it. Hypothetically, leaf margin cells might be involved in suppressing compression forces generated in the leaf blade underlying margin tissue. Application of cell wall loosening agents (such as expansin proteins) could result in weakening of the margin cell complex leading to an imbalance of forces between the inner and outer tissues, which could result in changes to leaf growth and shape. Data in support of that concept is provided by local application of expansin on the margin of leaf primordia, which indeed changed leaf shape in tobacco (Pien et al., 2001a).

Besides a mechanical role, leaf margin cells might have a functional role (in the form of a organizing center) in coordinating lamina growth. Our histological data indicated the accumulation of small undifferentiated cells in proximal parts of young hep leaves, where normally larger more differentiated cells are located in this region. The accumulation of small undifferentiated cells in hep might reflect a lack in cell expansion. Reduction of cell expansion might be caused by various means. Firstly, the smaller size of cells in HEP mutant leaves could be due to a delay or change in the differentiation program. HEP might be
involved in promoting differentiation in a cell non-autonomous way via the leaf margin. However, our data show that KNAT1 (which is involved in repressing differentiation) is not ectopically expressed in hep leaves. Consistent with these data, delayed senescence (which is usually a characteristic of ectopic KNAT expression in association with higher cytokinin levels) was not observed in hep leaves. Secondly, the inhibition of cell expansion might be a reflection of an alteration of cell proliferation. Cells in wild-type leaves usually stay small during proliferation but increase in size following the arrest of division, when further growth is accommodated by cell expansion (Donnelly et al. 1999). In the first stages of leaf development, cells are rather small due to continued proliferation accompanied by growth. Cell expansion and cell division arrest starts at the tip of young leaves and moves basipetally as leaf growth proceeds. At intermediate time points, this leads to a gradient of cell size in which cells at the distal end of the leaf are larger and more differentiated than cells in more proximal parts of the leaf.

The gradient in cell size is very steep in developing hep leaves compared with the wild-type situation. Cells at the proximal part of the leaf remain extremely small and densely cytoplasmic, whereas large, vacuolated cells are present in the distal area of the same leaf. This suggests that the cell cycle arrest front could be delayed in hep leaves compared with wild-type. Therefore HEP could play a role in making cells more sensitive to a signal to exit from the cell cycle via the margin. Continuous proliferation of the cells in hep leaves could make them less susceptible to expansion and differentiation. Recently, we have initiated a transcriptional profiling project using RNA amplified from dissected leaf margins to identify genes which are expressed specifically in the margin (Müller and Fleming, unpublished). One of the genes which was specifically up-regulated in the margin encoded a TCP protein. A member of the TCP family, CINCINNATA, has been shown to be involved in the timing and shape of the arrest front in Antirrhinum (Nath et al., 2003). Moreover, downregulation of TCP members results in a crinkled leaf phenotype in Arabidopsis (Palatnik et al., 2003). Hypothetically, HEP might work with such genes to regulate the timing of the arrest front via the margin.

1.1 Outlook
In this section I would like to give a description of current or planned experiments in our lab concerning the HEP mutant and the leaf margin in general. Clearly, the HEP locus determines aspects of leaf morphogenesis. Coupled with the specific pattern of reporter gene expression observed in this enhancer trap line, the mutant phenotype
raises a number of intriguing possibilities with respect to how altered gene expression in one cell type can influence the development of a whole organ. A major aim of current experiments is to identify the HEP locus. As reported here, although the mutation seems to be due to the insertion of the enhancer trap at a single genetic locus, the location of the T-DNA insert in the genome could not be identified by inverse PCR techniques. Two approaches are currently underway to tackle this problem. Firstly, a phage library with hep genomic DNA has been constructed using genomic DNA digested with Spel (which does not cut within the T-DNA or transformation vector). Screening of plaque lifts from this library with a labelled T-DNA probe should identify inserts containing T-DNA flanked genomic fragments. In parallel, the location of the HEP mutation is being identified by mapping techniques. Linkage analysis of the hep phenotype with various gene markers will be measured in F2 progenies from an outcross of hep (Columbia) to Landsberg erecta. Molecular mapping will be based on the CAPS- (cleaved amplified polymorphic sequences) technique (Konieczny and Ausubel, 1993). Although rather time-consuming, the mapping technique should allow the identification of a genomic fragment containing annotated ORFs which can be tested for their ability to complement the HEP mutant. Sequence and expression analysis of the HEP gene should provide an indication of which of the possible interpretations given in the previous section is correct.

During the cloning of the HEP gene, further analysis of the hep phenotype is planned. For example, to investigate if the timing and shape of the cell division arrest front is altered in the mutant, hep plants have been crossed with cyc1At::GUS transgenic lines (Donnelly et al., 1999). The expression pattern of the GUS gene in transgenic plants reflects the expression of a B-type cyclin, which is most abundant during G2 and M phases of the cell cycle, i.e., provides a visual marker for cell division. Comparison of the pattern of GUS expression in the hep and WT backgrounds will provide a more precise, dynamic visualisation of the changes in proliferation patterns monitored in hep by our histological analysis.

Auxin may be transported to or synthesized at the leaf margin and seems to be gradually distributed within the leaf blade (Himanen et al., 2002; Mattsson et al., 1999; Ljung et al., 2001). Therefore, auxin might be involved in regulating leaf development. In some areas in HEP mutant leaves, the margin-like cells seem to be missing. Since the leaf margin is a putative source or sink for auxin, this could have an impact on auxin distribution and, hence, result in a change of leaf shape. Therefore, crossing the HEP mutant with a transgenic plant engineered to provide visualisation of auxin distribution (e.g., DR5::GUS plants) might indicate whether local changes in auxin levels occur in the presence of the HEP mutation.
Since the margin is the boundary of ad- and abaxial domains, and since in the *HEP* mutant the margin is disrupted, it might be worth investigating the expression of ad- and abaxial fate markers (e.g., YABBY, KANADI) in *hep* by in situ hybridisation (Kerstetter et al., 2001; Siegfried et al., 1999). However, *hep* leaves seem to be polarized and lamina outgrowth is only slightly inhibited, suggesting that at least some degree of ad- abaxial polarity is maintained in these plants.

Finally, the results of our analysis, coupled with work in other groups, support the idea that the leaf margin is a special tissue which might play a key role in leaf development. Identifying the gene products involved in the acquisition of margin cell identity could provide a novel insight into a key developmental process. Therefore, as mentioned above, we have initiated a transcriptional profiling project using RNA amplified from dissected leaf margins to identify which genes are margin- specifically expressed or down-regulated. Characterisation of these genes will provide a first insight into the molecular basis of their unique pattern of differentiation.

2. Microinduction in Arabidopsis: Proliferative tissue does not respond to total or local induction using the CLX-GUS system

In the second part of this work, we tried to establish a microinduction system in Arabidopsis based on the CLX (Cre/Lox) system using the GUS reporter gene (Zuo et al., 2001). This system is induced upon application of estradiol. Similar to the Tet-system in tobacco, we would use the CLX system as a tool to manipulate locally the expression of various genes at the leaf margin, and therefore test their impact on leaf development in Arabidopsis (Pien et al., 2001a). Potential target genes might be genes involved in cell cycle regulation, cell expansion or genes identified from an enhancer- and gene trap screening showing activity in the leaf margin in Arabidopsis.

It turned out, though, that neither total- nor micro-induction experiments led to induction of the CLX system in young leaves or in meristematic tissue. It might be that double stranded breaks, caused by recombination or transposons, are highly suppressed in the apical meristem to restrict DNA damage, which could otherwise lead to premature cessation of plant development. The integrity of genomic DNA might be highly controlled in proliferating tissue, since at cell cycle checkpoints DNA repair mechanisms work which prevent DNA damage being transferred to daughter cells. In this aspect it is interesting that a surveillance system seems to function in the shoot apical meristem so that only certain mRNAs traffic into the shoot apical meristem (Foster et al., 2002; Ruiz-Medrano et al., 1999).
might have evolved as a barrier for RNA viruses to infect the stem cells in the meristem, which otherwise might transfer viruses to the plant progeny. Another handicap of the system was the position dependent effect of the T-DNA insertion and leakiness. Namely 4 out of 13 induced transgenic lines indicated patchy GUS expression patterns. In addition, ~16% of mock-inoculated transgenic plants were “escapes” showing GUS expression mainly in hydathodes of the leaves. Putatively, oestrogen-like acting compounds (iso-flavones) might be present in this tissue and activate the CLX system. (Zuo and Chua, 2000a; Valtuena et al., 2003). In conclusion, these results indicate that the CLX system is not a convenient tool to be used as microinduction system, at least not for young tissue.

2.1 Outlook
How could we perform manipulations of the leaf margin without the use of microinduction system? One possibility is the use of margin-specific promoters. For instance, PRESSED FLOWERS is a homeodomain transcription factor expressed exclusively in cells at the margin of lateral organs (Matsumoto et al., 2001). The fusion of its promoter to any target gene might be a useful tool to direct gene expression to the leaf margin. Alternatively, Jim Haseloff's Gal4-enhancer trap system could be used. This system is based on two transcriptional units. Firstly, a GAL4 transcription factor cDNA is fused to a minimal CaMV 35S promoter (see also Material and Methods). Secondly, multiple GAL4-responsive elements (GRE) are fused to GFP. When the construct is introduced into plant tissue, GAL4 expression can be activated by adjacent endogenous promoter elements. Therefore GAL4 expression reflects the activity of any endogenous promoter nearby. GAL4 expression itself is visualised by GFP expression, since GAL4 transcription factors bind to the GRE upstream of the reporter gene. We have several Arabidopsis lines containing the Haseloff enhancer trap construct which display activity in the leaf margin. Exchanging GFP in these lines with any target gene (cell cycle modulators or genes involved in leaf margin differentiation) would be a potentially powerful tool to do manipulations at the leaf margin.

3. Marker genes for leaf development: Sucrose synthase is absent from the phloem in young maize leaves
Sucrose synthase (SuSy) catalyses the readily reversible conversion of sucrose to fructose and UDP-glucose (Geigenberger and Stitt, 1993). SuSy transcripts (and transcripts of other genes involved in carbohydrate metabolism) accumulate in the meristem of tomato at the site of
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incipient leaf formation (Pien et al., 2001b). Therefore, we wanted to test whether this pattern was also observed in the meristem of monocotyledonous plants, i.e., can it be used as a general early marker of leaf initiation? Therefore we investigated the SuSy expression pattern in maize. In contrast to tomato, our data did not show a specific accumulation of SuSy in the maize meristem. However, interestingly, according to RNA and protein levels SuSy is excluded from the phloem of sink tissue (young leaves). This is in contrast to previous reports which claim a specific localisation of SuSy expression to phloem cells in leaf tissue (Yang and Russell, 1990; Martin et al., 1993; Fu and Park, 1995).

The differences between the pattern of SuSy expression reported previously and in this investigation might reflect a difference in the developmental age of the tissue under examination and, in particular, the status of the tissue as either carbon sink or source. The predominantly phloem localised SuSy could participate in sucrose loading and retrieval in source tissue (mature leaves). Data indicating a role of SuSy in phloem loading comes from transgenic plants overexpressing a cytosolic pyrophosphatase (Lerchl et al., 1995). This resulted in stunted growth and increase in starch synthesis in source leaves probably due to a lack in carbohydrate loading.

Our observed non-phloem expression of SuSy, on the other hand, could be linked with sink tissue activity. Observations on potato tubers and roots (sink tissues) using reporter-gene constructs under the control of SuSy promoter elements support this contention (Fu and Park 1985) in that in these tissues a high level of SuSy gene expression was found in non-vascular tissue. In the case of potato parenchyma, it has been suggested that the high accumulation of SuSy functions to direct the metabolism of incoming sucrose to the synthesis of storage starch. Although starch does not accumulate to appreciable levels in young maize leaf primordia, it is possible that there is a transient starch accumulation occurring around the vascular tissue. Alternatively, since SuSy has be implicated to be membrane- associated and putatively involved in cellulose synthesis in concert with cellulose synthase, UDP-glucose might be rather fed into cell wall synthesis in young leaf primordia than starch. Investigation of the expression patterns of genes encoding enzymes involved in starch or cell wall biosynthesis could be informative in this context.

3.1 Outlook

Our analysis of the expression pattern of Susy in maize has shed an insight into the potential role of this enzyme in leaf development but leaves the question open of the significance of the specific pattern observed in the apex of tomato. Directed alteration of the expression pattern
of Susy within the meristem of tobacco (using a microinduction system) or Arabidopsis (using region specific promoter elements) would provide evidence on the function of this specific gene expression pattern. In addition, techniques to visualise the distribution of metabolites within intact plant tissue would provide evidence as to the metabolic significance of the pattern of gene expression observed in the tomato meristem.
VIII References


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