## *Studies on sterol biosynthesis mutants of Arabidopsis*

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# Studies on sterol biosynthesis mutants of *Arabidopsis*

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## 4.4 Discussion

### 4.4.1 The *hydra* mutant phenotype has disrupted pattern definition in regions of p*HYD1*::GUS reporter activity

#### 4.4.1.1 Morphology of the *hydra* mutants suggests anomalies in aspects of cellular coordination across the radial axis

The *hydra* mutant phenotype is characterized by problems in the coordination of longitudinally organized cell files in both the epidermis and vasculature. The integrity of the stele is variably compromised within the sibling population, ranging from minimal strand separation in some examples to a complete duplication of the longitudinal axes in others. Full axis duplication in *hydra* allows a greater maintenance of the integrity of internal cell layers, whereas partial strand duplication is associated with aberrant patterning of the vascular meristem and ground tissue, resulting in a loss of both radial layer integrity and coordination within longitudinal cell files. Mutant leaves also develop isolated xylem sections, particularly in secondary vasculature, and show variable examples of poor cellular coordination around the primary midvein.

The *hydra* mutant plant body retains a basic organization with a defined longitudinal axis throughout the sibling population, even in examples where the development of cotyledon primordia is much reduced. This is in contrast with *gnom* in which longitudinal integrity is variably present or absent, due to a randomization of intracellular polarity caused by compromised vesicle trafficking (Mayer *et al.* 1993, Steinemann *et al.* 1999). The consequence of the *gnom* mutation is that polarized gradients within the plant body, such as are established by PIN-mediated auxin fluxes, cannot coordinate within cell

files and between layers. The result is a compromised elongation growth in all tissues comprised of longitudinally aligned cell files. This implies that pattern anomalies found in *hydra* mutants are not 'apical-basal', even though the consequences of these pattern defects result in a compromised morphology along the longitudinal growth axis.

The *hydra* sibling population share anomalies in various aspects of pattern coordination across the radial axis. The mesophyll layer is organized across the radial axis by patterning events at the meristem margins. In the shoot, specialized palisade cells lie adjacent to the adaxial epidermis of vegetative lateral organs in most dicotyledons including *Arabidopsis*. Within the mesophyll, the vascular strands have a collateral arrangement in *Arabidopsis*, with phloem towards the outer abaxial surface and xylem towards the inner adaxial surface. Mesophyll differentiation in *Arabidopsis* follows a basipetal progression from the apex and margins towards the midvein, mirroring the cessation of procambial cell fate initiation as revealed p*AthB8*::GUS activity (Scarpella *et al.* 2004). The regular longitudinal 'striped' appearance of the *Arabidopsis* root epidermis also has a radial organization, with trichoblasts forming over cell junctions of the cortex layer beneath (Dolan *et al.* 1993). Disorganization of the mesophyll was noted in *hydra* mutant leaves and in the hypocotyl cortex layers, and misplacement of hair cells in the epidermis was found to be associated with cell proliferation of cortex cells. As the pHYD1::GUS reporter was active in the root epidermal cells, the *hydra* phenotype therefore confirms a role for *HYD1* gene activity in epidermal development within these radially organized tissues.

The vascular strands comprise another functional tissue with a radial organization in relation to the plant growth axis. The *hydra* mutants exhibit multiple vascular developmental defects, often associated with a perpetuation of cell division activity. These are most obvious in association with areas of disjuncture the cotyledon and true leaf xylem traces, and co-occur with mis-expression of the provascular marker p*AthB8*::GUS, often in regions of variable

cell morphology. Various anomalies in epidermal patterning were found in vegetative leaves of the rosette, concerning the placement of marginal cell files and formation of clustered trichomes and stomates. However in most mutant leaves, excepting those with a grossly distorted morphology, trichomes did appear over the adaxial lamina.

No epidermal patterning problems were found in mutant inflorescence lateral organs; cauline leaves and sepals had normally patterned trichomes and stomata, and correctly placed marginal cells. All leaves were shortened in the longitudinal axis, although this defect was not noted in floral lateral organs. Silique elongation was reduced, possibly in association with a lack of viable pollen (which also showed strong p*HYD1*::GUS activity). The vascular patterning of cauline leaves and sepals showed an enhanced xylem 'noise' in relation to wild-type, although had substantially improved coherence in comparison to vascular traces from rosette leaves. The over-expression of the HD-Zip III transcription factor AthB8 shows a similar vascular phenotype (Baima *et al.* 1995, 2001), and the reporter for transcription of the *AthB8* gene has enhanced activity in *hydra*. These observations, considered with the transient expression of p*HYD1*::GUS at times of coordinated differentiation in the mesophyll layer, imply a role for *HYD1* in the fine-tuning of cell differentiation during mesophyll organization. The differences between mutant rosette and inflorescence leaves suggests either a differential balance of signalling cues at work during pattern formation in these structures, or a partial redundancy in function between the *HYDRA* loci and other gene products.

The *hydra* mutants have anomalous cell division activity (as revealed by the *CYC1At*::CDB::GUS reporter), including a perpetuation of division events in shoot tissues beyond the point at which this activity has ceased in wild-type. In *hydra* roots, cell division is slower to establish upon germination, has a more erratic pattern in the root meristem region than in wild-type, and is seen simultaneously in multiple adjacent cells within the same cell file. In wild-

type, such synchronous division in adjacent root cells is unusual. Wild-type post-germination cotyledons and hypocotyls have no mesophyll or ground tissue cell division, and only a few events in the epidermis associated with stomatal ontogeny (Tsukaya et al 1994, Gendreau *et al.* 1997). In contrast, *hydra* cotyledons show multiple cell division events throughout the cotyledon tissues, with a lesser but enhanced number of ectopic cell division events in hypocotyl tissues. Throughout the mutant shoot tissues, isolated cell divisions perpetuate in regions where discontinuities are seen in the xylem tissues. The patterning of these cells (i.e. epidermis, ground tissue and vasculature) are coordinated across the radial layers at the plant apical meristems, and require synchronous development across this axis in order to produce a normal morphology in the developing seedling.

#### 4.4.1.2 *HYD1* gene activity is implicated in the coordination of cellular differentiation in the epidermis and ground tissue layers

A transverse section across the radial axis of the wild-type *Arabidopsis* root bisects cells of a similar age and developmental stage; precise timing of differentiation in the epidermis show slight variation, although the entry of the ground tissue layers into expansion is tightly synchronized between cells, so preserving the structural integrity of the tissues.

In *Arabidopsis,* trichomes differentiate whilst pavement cells are still dividing, and as the surrounding cells undergo cytokinesis, the trichomes progress through rounds of endoreduplication, after which branches initiate (Oppenheimer 1997). Stomata differentiate in true leaves after the onset of trichome formation, and are never seen in adjacent positions to these hair cells. Both of these functional cell types differentiate earlier in the leaf epidermis than the mesophyll. Their position corresponds to associated patterning in cells of the mesophyll layer, establishing internal air spaces

beneath stomates and clusters of organized cells comprising the trichome socket.

In roots, atrichoblast cell files exit cell division earlier than trichoblast cell files, where the persistence of cell division generates a greater number of cells in the file (Berger *et al.* 1998b). The root epidermis is coordinated with its ground tissue in a similar arrangement to seen in the shoot; the 'exchange' cells (stomata and root hairs) form over boundaries between cells of the layer beneath. An increased number of cortical cells re-specifies the cell fate decisions of the epidermal layer, and so continuing the coordination between epidermis and ground tissue. Variation in the developmental timing of p*HYD1*::GUS seen between adjacent epidermal cell files corresponds with these differences in maturation rate, the atrichoblast/trichome 'cassette' preceding the root hair/stomata 'cassette'.

Examples of *hydra* mutants with additional numbers of root hair cells and disorganized arrangements of the trichoblast cell files were examined in transverse section, and were found to have a similar disorganization in the cortex layer. These data indicate that the mechanism which coordinates the relationship between the cortex and epidermis is still functional, but the means of controlling cell numbers in these layers is disrupted in *hydra*. The positional expression of *HYD1* reporter activity, and defects in the *hydra* mutant phenotype, implicate *HYDRA* protein function in the coordination of cell files within the root epidermal layer. These observations of the mutant phenotype also imply the operation of a mechanism in which a patterning signal from the epidermis affects cortical cell numbers.

The series of cell divisions by which stomata arise take place later than trichome initiation in epidermal development. Several types of mutant stomatal clusters have been noted in *Arabidopsis*. Mutations in the *TMM/SDD1* pathway produce adjacent stomates from cells adopting meristemoid cell fate, and failing to divide away prior to differentiation (Geisler *et al.* 2000, Nadeau

& Sack 2002, von Groll *et al.* 2002). The *FLP* gene appears to function to suppress cell division in GMCs. The *flp* mutant clusters arise from guard mother cells, i.e. at a later stage of differentiation than the stomatal patterning mutant *tmm* and *sdd1*. These clusters are different in character from those of *tmm* and *sdd1*, in that a range of stomatal patterns appear in the epidermis; *flp* plants have unpaired guard cells, and stomatal 'foci' ranging in size from one to eight cells. *FLP* encodes an R2-R3 MYB protein paralagous to *MYB88*, and double mutants at these loci produce an exaggerated phenotype, resulting in clusters of up to 12 or more cells (Lai *et al.* 2005). The stomatal lineage behaves in some ways like a stem cell population; as with root trichoblasts, stomatal precursor cells become committed to their cell fate but continue to divide for some time (Larkin *et al.* 2003).

The *hydra* stomatal clusters are associated with several phenomena.

1. Cells were noted in the mutant epidermis which appeared to adopt satellite meristemoid fate, but differentiated as a GMC adjacent to established stomata, as can be seen in Fig. 4.71; A-C.
2. Clusters formed as a result of asymmetric divisions placing meristemoids adjacent to other meristemoids, or ready differentiated stomates as in Fig. 4.71; D-G.

Both of these examples imply that the lateral inhibition preventing meristemoids from adopting guard mother cell fate adjacent to established stomata is not functioning normally, and the mutant phenotype shares these characteristics of stomatal patterning with *tmm* and *sdd1*. Also in common with *tmm*, the resultant clusters have a pronounced abaxial distribution, with larger clusters seen on the abaxial lamina, detailed in Fig. 4.69: A and B.

3. Third, the derivation of stomatal clusters such as that in Fig. 4.67; B, with variable lineages that can result in unevenly sized cells and occasional unpaired guard cells.

These defects encompass phenomena associated with *flp*, and with *flp-myb88* double mutants (Lai *et al.* 2005).

These patterning defects in the *hydra* mutant epidermis can be explained by considering the mutants as compromised in their ability to differentiate their cells synchronously. If the *HYD1* gene is a transcriptional target for MYB proteins involved in epidermal patterning, then the participation of *HYDRA* in the MYB signal reception process could induce differentiation and the accompanying symplastic isolation required in these targeted cells. The MYB gene *FLP* is proposed to be required in order to make a timely transition from cell division to differentiation in the stomatal precursor cells (Lai *et al.* 2005). Were this not to take place, as in the *hydra* mutant phenotype, then the symplastic autonomy stage is not reached, or is not induced in the normal sequence. It could be envisaged that a lack of induction of this timed event could result in the over-stimulation of the patterning mechanism, and an enhanced production of other pattern components such as TTG and the R-homologue GL3. The result would be that a 'focus' for stomatal initiation, which resolves a single stoma in wild-type plants, is not defined clearly in *hydra* mutants. In the absence of a positive reinforcement-lateral inhibition mechanism, several adjacent cells could commit to stomatal cell fate, hence resolving a cluster. Alternatively, clusters could form because these cells continue to divide beyond the time point when wild-type cells exit the cell cycle and differentiate.

### 4.4.1.3 The *HYD1* gene may produce a sterol-based signal in stipules which coordinates both laminar dorsiventrality and meristem maintenance

Stipule function is compromised in *hydra*, with very few siblings producing a viable reporter signal from the DR5::GUS transgene, although morphologically these structures appeared to be present in some siblings. This implies that the initial definition of stipule morphology in the first true leaf pair is

independent of *HYD1* activity. Stipule differentiation in hydra was severely limited in association with expanding leaves developed from primordia initiated post-germination. Siblings with DR5::GUS activity in stipules were rare in *hyd1* (associated with the few siblings with an apparently normal phyllotaxy of the first true leaf pair), and were virtually absent in *hyd2*. The *hyd1* siblings with functional stipules on the first true leaves did not continue to produce stipules through the primordial succession; none were seen in subsequent true leaves. This lack of stipules was associated with an inability to correctly position subsequent primordia around the SAM. This suggests three conclusions. First, *HYDRA* gene activity is required for the morphological resolution of correctly spaced primordia and the differentiation of functional stipules at their margin boundaries. Second, as primordial initiation is dependent upon auxin transport, that correct auxin signalling for lateral organ development cannot function in the *hydra* SAM even in the presence of a normal vascular pattern definition at the hypocotyl-cotyledon transition. Third, the stipules associated with the first true leaf pair, which differentiate during embryogenesis in wild-type plants, are occasionally present in *hyd1* mutants, which means that their formation is dependent upon different factors (or gene activities) in pre- and post-germination growth.

The significance of the stipule structure is unclear. One mutant, *pressed flower* (*prs*), has a morphological phenotype which includes a lack of stipules, and an absence of lateral sepals in the flower; the morphology of the abaxial and adaxial sepals are normal, but cell files at the lateral margins are missing (Matsumoto & Okada 2001, Nardman *et al.* 2004). The *PRS* gene was also isolated as a *WUSCHEL*-like gene, designated *WOX3* (Haecker *et al.* 2004), and is a homologue of the maize genes *NARROW SHEATH1* and *2* (Scanlon *et al.* 1996), whose mutant phenotype causes the deletion of a lateral domain that includes the lower leaf margins.

A model of leaf zonation predicts that dorsiventrally flattened dicotyledon leaves are subdivided into a large upper leaf zone which develops as the

lamina and petiole, whilst a greatly diminished leaf zone defines the leaf base and lateral stipules; in contrast the monocot leaf is developed almost entirely from the lower leaf zone, while the upper leaf zone defines a tiny region at the apex (Kaplan 1973). Accordingly the *prs* phenotype in *Arabidopsis* is reported to lack any leaf shape or patterning anomalies (Nardmann *et al.* 2004). This morphological deletion does not preclude the adoption of stipule cell function in morphologically indistinct tissues, as the embryonic expression of *PRS* defines a lateral domain in cotyledons during the torpedo stage of embryogenesis, here in the absence of a morphologically distinct region (Nardmann *et al.* 2004, Haecker *et al.* 2004). In addition, this lateral domain develops stipule morphology on cotyledons in the absence of *LEC1* function during embryogenesis (Tsukaya *et al.* 2000).

It is notable that Kaplan's model of leaf zonation places the lower leaf zone in dicotyledons as an intervening region between the meristem transition zone and the developing primordium, making a conceivable model of this tissue as a 'gate', or symplastically distinct region, controlling communication between the leaf and the meristem periphery via plasmodesmata. This zone shows persistent expression of the p*HYD1*::GUS reporter in the only morphologically distinct structures to derive from the lower leaf zone, i.e. the stipules at either side of the petiole base. The *PRS* expression domain in both cotyledon and true leaf primordia define epidermal cells that will eventually form the margins of the *Arabidopsis* lamina. The morphological phenotype of *prs* places this domain at a boundary between the abaxial and adaxial domains, and the expression of *PRS* mRNA coincides with the timing of abaxial and adaxial positional definition by *YAB* and *REV* (Sawa *et al.*1999, Siegfried *et al.* 1999, Otsuga *et al.* 2001, Matsumoto & Okada 2001).

The stipule-based p*HYD1*::GUS signal, the absence of functional stipules from most *hydra* siblings, and the misplacement of marginal cell files in *hydra* mutant cotyledons and leaves suggests a strong correspondence between *HYD* function and a stipule-based definition of the centrolateral (transverse) leaf

axis. Primordia of *hydra* mutants are compromised in the positioning of dorsiventral cues as revealed by reporter activity of *YAB, PHB* and *REV,* with some examples having a skewed distribution around the primordium relative to the SAM. This skewness could correspond with the skewed alignment in primordial expansion noted in many *hydra* mutant apices. Marginal cell file misplacement across the *hydra* rosette organ lamina had profound effects upon leaf shape development, and suggests that a dorsiventral signal, mediated perhaps to the lamina via the marginal cell files, is crucial in the coordination of centrolateral expansion.

However it is unclear whether mis-positioning of marginal cells results from modified abaxial-adaxial signalling cues, or whether their absence or mis-positioning results in the modulation of response to these cues around the *hydra* shoot apex. As clear marginal definition is compromised in *rev* mutants (Talbert *et al.* 1995), adaxial cues are required for margin placement. The *prs* mutant phenotype is reported to have no effect on leaf shape and epidermal patterning in *Arabidopsis* other than the loss of morphologically defined stipules (Nardmann *et al.* 2004), although the *prs* mutant is a partial transcript, and may retain some functionality. *PRS* gene expression is localized to the L1 layer at the boundary between abaxial and adaxial cell fates (Matsumoto & Okada 2001). A putative *HYD1*-implicated stipule-supplied signal, possibly a sterol, would therefore be ideally positioned to be directed into the L1 symplast, either affecting the primordium, the meristem L1 layer, or both.

#### 4.4.1.4 The *hydra* mutant phenotype displays phenomena characteristic of compromised auxin transport, and may implicate sterols in boundary definition at the meristem periphery

The morphology of *hydra* mutants includes a number of features in common with other mutants characterized by altered auxin responses. The *hydra* mutants have mis-coordination in their longitudinal cell files; a phenomenon noted in *monopteros*, which like *hydra*, has a disjunct vasculature (Berleth & Jürgens 1993). Similarly, vascular islands noted in the primary and secondary vasculature of cotyledons in the *scarface* mutant correlate with a heightened auxin sensitivity (Deyholos *et al.* 2000). As the action of both of these genes is to induce coordinated longitudinally aligned vascular cell files, then the main effects of their mutation may be a modulated auxin transport efficiency downstream of PIN-mediated intercellular transport, resulting in poor intercellular coordination of vascular cell files at the differentiation stage. The patterning of vasculature in the *hydra* leaf traces is also reminiscent of that seen in the *lop1/trn1* mutation, compromised in auxin transport (Carland & McHale 1996, Cnops *et al.* 2000). The *lop1* leaf has a bifurcated primary midvein and reduced tertiary and quaternary vasculature, which results in a reduced network, where much of the lamina appears unvascularized (Carland & McHale 1996).

Treatment of wild-type embryos with exogenous auxin produced a seedling with a cup-shaped region around the apex, and signs of vascular dissociation within the hypocotyl (Furutani *et al.* 2004), in a manner reminiscent of that seen in many *hydra* siblings. The formation of a broadened vascular cylinder has been noted in plants with auxin transport-inhibited roots, along with differentiation of multiple xylem strands (Mattsson *et al.* 1999, Sabatini *et al.* 1999), suggesting that a build-up of auxin in these regions caused the radial modulation of vascular patterning. These characteristics are common in the *hydra* sibling population. Pattern anomalies in *hydra* may result from a general elevation of auxin in a normal auxin transport environment; this would result in a less pronounced resolution in transport-mediated gradients within the embryo. As vascular differentiation progresses, the establishment of disjunct xylem sections in *hydra*, resulting from a poor coordination between

cells exiting the cell cycle, would disrupt auxin transport locally, and so exacerbate local auxin accumulation. It is conceivable that the same outcome could occur in a normal auxin environment where the auxin transport efficiency was compromised, resulting in an uneven auxin distribution, interpreted within the cell mass as defining a wider region of procambium. This scenario could explain the enhanced xylem 'noise' and the over-expression of the auxin up-regulated p*AthB8*::GUS reporter. If stochastic factors defining the sites of xylem initiation are also considered, this could explain the appearance in *hydra* of distinct apical longitudinal cues, resolving a duplicated embryonic longitudinal axis and multiple apical hydathode foci in emergent seedling leaf organs.

Plants grown in the presence of auxin transport inhibitors that interfere with the (PIN1-mediated) efflux mechanism, result in a distinctive xylem patterning; these leaves have multiple strands in the midvein region, and a concentration of vascular differentiation around the leaf margin (Mattsson *et al.* 1999). This is not the characteristic vascular patterning found in *hydra.* Neither do *hydra* inflorescences resemble those of the *pin1* mutant (Okada *et al.* 1991). These observations imply that the auxin transport defects of these mutants are not typified by mis-function of the PIN1-mediated efflux mechanism. The enhanced vascular 'noise' of *hydra* mutant xylem traces are more reminiscent of those observed in *ifl1/rev* mutant leaves (Zhang *et al.* 1997), proposed as resulting from lateral diffusion of auxin out of the vascular bundles. Similar additional ectopic xylem cells have been noted in *pin1* inflorescence stems near the cauline axils, and also presumed to result from diffusion of auxin from the source in the leaf, and causing nearby procambial cells to differentiate (Gälweiler *et al.* 1998).

The *hydra* mutants develop clustered primordia, reminiscent of those resulting from localized ectopic application of auxin to *pinoid* (*pid*) apices. Mutants of *pid* are compromised in auxin transport in a manner independent of PIN1, (shown by the additive phenotype of *pin1* and *pid* mutants; Reinhardt *et al.*

2003, Furutani *et al.* 2004). The *PID* gene is induced by auxin, and is a positive regulator of polar auxin transport (Bennett *et al.* 1995, Benjamins *et al.* 2001). Furutani *et al.* (2004) found that *PIN1* and *PID* fulfil overlapping and synergistic functions in the establishment of bilateral symmetry and cotyledon outgrowth during embryogenesis; the double mutants produced sibling variability encompassing multiple reduced cotyledons and a complete lack of organs in the most severe phenotypes. These authors suggest that PIN1 is involved in partitioning of auxin into the regions of developing primordia, whilst *PID* is induced by auxin and reinforces the boundary distinction between the meristem flanks and developing organ, through a promotion of auxin transport into the primordium.

Primordial cluster formation in enhanced in *hydra* siblings in association with severe disjunction in xylem traces at the hypocotyl-cotyledon transition. PIN1-mediated auxin transport in the L1 layer is critical for dorsiventral primordial patterning (Reinhardt *et al.* 2004). The similarity in primordial cluster formation between *hydra* and locally auxin treated *pid* apices suggests that a reduced efficiency of auxin transport contributes to the formation of clustered primordia in *hydra,* by an inefficient focusing of the auxin maximum in the region of primordial initiation.

Both *hydra* mutants (Souter *et al.* 2002) and *smt1* (Willeemsen *et al.* 2003) have variable PIN protein positioning in cells involved with directional auxin transport. This could result in several adjacent 'foci' of auxin accumulation, or a broad region of cells experiencing an auxin maximum at the meristem flank, causing the initiation of multiple primordia reminiscent of auxin-treated *pid* apices. The proximity of such maxima would subsequently affect the invasion of provascular strands into developing primordia. Differences in the resolution between such adjacent auxin foci could explain the dissociation of the primary vascular strands in *hydra* leaves, i.e. the wider apart the foci, the more severe the primary strand dissociation. Wider foci could thus result in the emergence of fused adjacent primordia, each with its own vascular trace.

This disruption of the vasculature would modify auxin transport around the SAM, and result in increasing disruption of t he phyllotactic pattern as development proceeded. This concept of multiple auxin foci, and the interdependence of primordial formation and vascular patterning in the hypocotyl-cotyledon transition region, also explains the correlation between the most extreme examples of clustered primordia over the shoot apex, and the chaotic differentiation of xylem vessels beneath.

Stipules are L1-layer associated, and originate from the same tissues that provide founder cells for the marginal cell files (Matsumoto & Okada 2001). As the *HYD1* reporter defined a peak of gene expression in stipules, this suggests that a modulation of sterol production, perhaps resulting in an increase in specific sterols in the L1 layer, could facilitate vesicle trafficking in this layer around the meristem PZ, and also contribute to an enhanced primordial-meristem boundary definition. The absence of ectopic primordia on *hydra* leaves suggests that the meristem-lateral organ boundary definition mechanism is working correctly in these mutants. However the initiation of primordia around the base of expanded true leaves means that the time limitation for primordial initiation in cells of the meristem periphery is not operating correctly. This again is suggestive of a problem with the cellular transition to differentiation.

#### 4.4.1.5 The *hydra* sepal phenotype suggests a possible role for *HYDRA* gene activity in the definition of organ boundaries in light-responsive tissues around the floral apex

Another radial patterning system is in operation in inflorescences, which defines the positioning of organ boundaries around the shoot apex. Clonal analysis of sectors in *Arabidopsis* flowers indicate that sepals and carpels are initiated by a line of eight cells, petals by a pair of cells, and stamens by four cells in a block. Sector boundary analysis of ABC mutants indicates that where

one whorl identity is replaced by another, then this mutant whorl initiates from the same precursor cells as the organs they replace, implying that the shapes of organ primordia are independent of the ABC system (Bossinger & Smyth 1996).

Petals lacked any signs of p*HYD1*::GUS activity. Petals are the only flattened lateral organs in *Arabidopsis* which do not develop stomata or trichomes (Bowman 1994). The whole petal lamina, including the epidermal layer, contains plastids which re-differentiate into colourless leukoplasts, producing a mature petal with a white appearance, although some green chloroplasts are seen around the primary midvein region (Pike & Page 1998). This contrasts with the leaf epidermis, where all cells other than stomatal guard cells lack chloroplasts, as this would interfere with light penetration to the mesophyll layer. Pike & Page (1998) comment that no coloured or green petalled *Arabidopsis* mutants have been found, implying that leukoplast differentiation must be a default pathway for cells of the petal blade. This 'reversion to default' also suggests an explanation for the white leaf sectors that are often seen in plants from mutagenesis screens.

As both p*HYD1*::GUS transcription and the *hydra* mutant phenotype suggest an absence of *HYD* gene activity in petals, this implies that *HYDRA* may be a component of the modification of this default route in photosynthetic lateral organs. This transcription in photosynthetically-competent organs may be light induced, and a putative light-responsive 'I-Box' motif is present within the first -800bp upstream of the *HYD1* transcriptional start. The variable boundary spacing within the sepal whorl in *hydra* implicates *HYD1* gene activity in radial marginal positioning in sepals, although this function in other whorled organs appears normal. However the persistence of xylem 'noise' in sepals and other organs associated with p*HYD1*::GUS activity, suggests that *HYDRA* gene activity plays a role in cell file coordination, particularly affecting the differentiation of xylem. As xylem differentiation follows the phloem traces, itself following the provascular strand in wild-type (Scarpella *et al.*

2004), this patterning could be checked in *hydra* by examining the phloem traces in aniline blue stained leaves in association with the p*AthB8*::GUS signal.

The severe phenotype of *hydra* mutant rosette leaves is correlated with misplacement of marginal cell files in these organs. The sepal morphology phenotype, in conjunction with rosette leaf pattering, and the persistence of xylem noise in all of these organs (defined by boundaries between abaxial and adaxial cues), implies a role for *HYDRA* in the specification of radial boundaries.

#### 4.4.1.6 The *hydra* mutant floral phenotype implies independence of *HYDRA* function and ABC-based radial pattern formation in inflorescences

Patterning anomalies found in flattened lateral organs of the rosette were not replicated in lateral organs of the hydra mutant inflorescence, even though these structures showed activity of the p*HYD1*::GUS reporter (cauline leaves, sepals, silique valves). However these organs displayed some 'noise' in the xylem traces of mutant plants, although at reduced levels compared to that seen in rosette leaves, and had coherent xylem traces without vascular 'islands'. No patterning anomalies were found in functional cells of the epidermis in these organs. Whorls of floral organs appear to have a generally normal morphology in both *hydra* mutants, although placement of the sepal organ boundaries was indistinct in some flowers, resulting in an unevenly divided whorl with partially fused margins.

Differences exist in the molecular-genetic definition of radial patterning in *Arabidopsis* vegetative and inflorescence structures. Floral lateral organ radial pattern formation is governed by a flower-specific set of homeodomain transcription factors, the MADS proteins, which pattern the determinate floral

apex by overlapping domains of expression in the so-called ABC system and the *SEPALLATA* genes (Bowman *et al.* 1991, Weigel & Meyerowitz 1994, Guiterrez-Cortines & Davies 2000, Peltaz *et al.* 2000). The action of MADS transcription factors is thought to involve the formation of homo- and hetero-dimers which define the four whorls of the flower according to the ratio of one to another. Nectaries are independent from this system, arising abaxially to the third whorl, and formed from receptacle tissues through the action of the *YABBY* gene *CRABS CLAW*, in conjunction with other unknown signals (Baum *et al.* 2001, Siegfried *et al.* 1999). Although the transcriptional activity of p*HYD1*::GUS implies gene activation in sepals and anthers, this activity of *HYD1* appears to be independent of ABC mediated development.

### 4.4.1.7 Architectural differences between wild-type and *hydra* primary and lateral root apices imply the activity of differential patterning processes in these organs

Lateral roots form post-embryonically after the point of transition from primary to secondary growth, i.e. through an activation of the lateral meristems (Steeves & Sussex, 1989). Cells proliferate locally in the pericycle layer in positions adjacent to the protoxylem pole, just beyond the elongation zone proximal to the primary root apex. Their initiation is prompted by auxin accumulation beyond a critical threshold in tissues adjacent to the xylem pole, as a result of basipetal transport in peripheral tissues (Casmirio *et al.* 2001). The efficiency of this transport may influence the longitudinal spacing of lateral root primordia, although the precise signalling mechanism is not clear (Casmiro *et al.* 2001). The first division of the pericycle cells is polarized and asymmetric, and again auxin transport dependent. These cells form a layered, radially organized structure, requiring at least three cell layers for viability and generation of the root cap and meristem region (Laskowski *et al.* 1995). Procambial cells then 'invade' from the established

vasculature (as in shoot primordia), developing acropetally into the primordium from the primary root axis.

In *hydra* mutants, the growth of the primary root apex is compromised, particularly in the *hyd2* sibling population, and a reduced auxin response is implicated in the stages preceding death of the *hyd2* primary root meristem (Topping *et al.* 1997, Souter *et al.* 2002). In contrast, post-germination derived lateral roots showed a greater viability, particularly in the *hydra-ein2* double mutants, and the enhanced longevity of *hyd2-ein2* compared with *hyd2* correlated with an enhanced growth of the lateral root system which persisted long after death of the primary root meristem (data not shown). This growth was particularly noticeable in anchor roots, initiating from the root-hypocotyl transition. In wild-type plants (carrying the p*HYD1*::GUS transgene), anchor roots showed the least morphological modification to exogenous auxins, implying a general distinction in signalling regime between these root types.

As *hydra* appears compromised in auxin transport activities at the shoot apex, this implies that a variable amount of auxin may be delivered to the primary root apex by PIN-mediated polar transport. This could mean that the improved viability of *hydra* lateral roots may result from an innately lower auxin requirement in laterals, or a lesser dependency upon auxin for their development than primary root apices. This difference in signalling physiology could explain the greater viability of *hydra* laterals, i.e. through locally tempering the general auxin hypersensitivity noted in the mutants by Souter *et al.* (2002). Reduced lateral root auxin sensitivity in wild-type plants may also be the reason for the greater tolerance of cell file variations in lateral roots (as compared to primary roots) of wild-type plants, noted by Dolan *et al.* (1993).

### 4.4.1.8 The *hydra* phenotype displays phase related problems which are distinct from defects in *lec1*

In addition to the compromised longitudinal expansion growth which affected roots, stems, and all vegetative lateral organs, the *hydra* mutant phenotype exhibits a number of developmental phase-related phenomena. Successive wild-type rosette leaves undergo a developmental transition from juvenile to adult phases, manifest as a reduction in petiole length coupled with increasing elongation of the lamina. These more lanceolate leaves have more complex vascular network and a greater number of marginal hydathodes, associated with marginal 'teeth' (Tsukaya *et al.* 2000). Epidermal patterning is also modified, the later leaves having abaxial trichomes (Hülskamp & Schnittger 1997). In *hydra*, no rosette leaves examined were clearly of later adult phase, both in terms of the distribution of trichomes (which were all adaxial) and vascular patterning. Placement of hydathodes was varied; in addition to many rosette leaves having two of these functional regions in close proximity at the leaf apex, adjacent hydathodes could also be found in lateral positions. No leaves examined, including cauline leaves, had more than three hydathode regions; this mimicked the arrangement in the most juvenile of wild type rosette leaves (Tsukaya *et al.* 2000). This could be interpreted as a requirement for *HYDRA* activity to make the 'juvenile to adult' phase transition.

The presence of cotyledon trichomes in *hydra* raises a further question over phase-related phenomena in the mutant phenotype. The appearance of cotyledon trichomes is reminiscent of mutations at the *LEC1* locus (Meinke 1992, West *et al.* 1994), interpreted as unable to suppress the true leaf identity in cotyledons during embryo development. The *lec1* phenotype also demonstrates varied cotyledon vascular patterning and organ shape, often with more than one cotyledon hydathode (Tsukaya *et al* 2000). However *hydra* mutants do not produce trichomes during embryogenesis, in contrast to

*lec1*, suggesting that *hydra* cotyledon trichomes result from mis-positioned cues during post-germination differentiation in the cotyledon epidermis.

Tsukaya *et al.* (2000) also noted the presence of rudimentary stipules at the base of *lec1* cotyledons, of which 25% expressed a reporter construct active in hydathodes and stipules. No putative stipule structures were found in association with *hydra* cotyledons, and although putative stipule structures were distinguishable morphologically in occasional *hydra* mutants, their functionality (shown by DR5::GUS) was compromised, with the signal diminishing in later initiating leaves. This phenotype is in contrast with that of *lec1*. The *hydra* mutant phenotype, and the stipule-specific signal associated with p*HYD1*::GUS reporter activity, suggests a fundamental role for *HYD1* in the morphological maintenance and function of stipules which is independent of the phase-related phenomena associated with *LEC1*.

## 4.4.2 Embryonic axis definition and longitudinal cell file coordination anomalies in *hydra* siblings

### 4.4.2.1 Axis definition problems during *hydra* embryogenesis implicate sterols in an axial patterning mechanism

The p*HYD1*::GUS reporter is active in the globular embryo, when radial definition takes place within the plant body (Helariutta *et al.* 2000, Nakajima *et al.* 2001, Di Laurenzio *et al.* 1996), and when the first patterning anomalies are found in *hydra* mutants (Topping *et al.* 1997, Schrick *et al.* 2000). Embryonic procambial traces in *hydra* are variably defined, and expression of p*AthB8*::GUS shows a range of activities, resulting in a pattern which resolves upon differentiation as a range of degrees of axis dissociation or full duplication of the vascular stele. The axial problems in the mutant plant body

are associated with the upper (apical) portion of the plant, and appear to be in place upon germination, i.e. result from embryonic defects.

Axial definition is minimal in the early globular embryo, as shown by the even distribution of PIN1 proteins and auxin (Steinemann *et al.* 1999, Reinhardt *et al.* 2003) However there is an outer-inner distinction, defined by the position of the START domain protein AtML1 in the protoderm (Lu *et al.* 1996). This provides the opportunity for the resolution of an outer and inner domain. The auxin flux machinery defines a longitudinal gradient in the globular embryo in relation to the suspensor (Friml *et al.* 2002, 2003). This then defines an apical region where *STM* and *WUS*, and their interacting pattern components define a bilateral axis by initially stochastic means. Patterning at the apex is resolved in association with the START-domain proteins REV, PHB, PHV and CNA, which define the apical and central zones of the embryo (Prigge *et al.* 2004). The REV protein domain extends not just across the apex into the adaxial regions of the primordia, but also is found in the centre. As this process begins, transcription of a second START domain protein, AthB8, is initiated in the pre-procambium (Scarpella *et al.* 2004, Prigge *et al.* 2005), and advances acropetally from the base of the embryo adjacent to the differentiating hypophysis, in synchrony with the timing of SCR-mediated endodermal definition (Wysocka-Diller *et al* 2000). These START domain genes therefore provide an axis differentiation mechanism which is based upon 'outer' to 'inner' patterning; this is then modulated by the longitudinal auxin flux which is involved in the establishment of an 'apical-basal' developmental axis.

Evidence for disruption of a sterol-implicated process in *hydra* development is suggested by the mis-positioning of reporters for several START domain genes, p*AthB8*::GUS, p*REV*::GUS and p*PHB*::GUS, in *hydra* mutant embryos. The p*REV*::GUS signal, seen apically distributed in wild type embryos, was disrupted in *hydra* with a strongly enhanced reporter expression throughout the embryo body and occasional reversal of the apical distribution. The p*PHB*::GUS reporter, seen in the procambial strand and adaxial tissues of wild-

type heart and torpedo stage cotyledons, showed a randomised and diffuse distribution in *hydra* embryos, with examples of activity maxima in apical, basal or lateral positions. These 'central' or 'apical' domains appeared to have a random distribution in *hydra*. The p*AthB8*::GUS provascular marker showed a diffuse distribution throughout wild-type heart and torpedo stage embryos, resolving to the provascular strands as the embryos matured. In *hydra* embryos, a range of p*AthB8*::GUS activities were observed including an enhanced activity throughout the embryo body, to a weak activity in provascular traces in conjunction with ectopic local peaks of reporter expression.

The apparent random distribution of these markers of radial differentiation in *hydra* contrasts with the correct apical-basal positioning of other reporters of embryo polarity, as noted in these mutants by Topping & Lindsey (1997). These results therefore imply a potential modified distribution in *hydra* mutants of START domain gene activity involved with radial axis definition, and could indicate the mis-functioning of a sterol-mediated radial patterning signal. The anomalous behaviour of all three of HD-Zip III reporters also did not appear to bear any relationship to the range of *hydra* mutant embryonic morphology. This suggests that the morphological development of *hydra* mutant embryos is independent of radial patterning. However interpretation of these data must be cautious however as this promoter-GUS activity produces neither mRNA nor protein of the actual genes, i.e. the components with the START sequence targeted respectively by micro-RNAs and putative sterol ligands.

Radial patterning of the embryonic shoot apex is known to be compromised in *hydra* mutants; Schrick *et al.* (2000) found that *STM* RNA appeared in *hyd2/fk* at random locations around the apex, or at multiple positions. The expression of p*HYD1*::GUS throughout the embryo proper during the globular and heart stages (as presented in chapter 3) and the mis-positioning of promoter-reporter expression for the START domain genes *AthB8, REV* and *PHB*

promoter-reporters in *hydra* embryos, both implicate sterols in the maintenance of the embryonic internal environment. The reversals of longitudinal distribution seen in from reporter activity for *REV, PHB* and *YAB*, in *hydra* embryos also raise the possibility that the radial patterning mechanisms which are disrupted in these mutants have implications for the maintenance of the embryonic apical-basal axis.

### 4.2.2.2 Variation in the apical definition of the *hydra* longitudinal axis may result from intercellular communication problems

The duplication or dissociation of the *hydra* apical longitudinal axis and its morphological consequences is reminiscent of some of the results seen from 'classical' surgical experiments on the meristem (reviewed by Steeves & Sussex 1989). In these experiments, a surgical incision into the central zone of the SAM resulted in the generation of new meristems from the peripheral zone, producing an apex with multiple growth axes from that point. A full bisection of the meristem results in a regeneration of each half separately. These experiments indicate that a collection of cells in the meristem zone can self-organize into a viable SAM.

Reinhardt *et al.* (2003) demonstrated that the capacity for the meristem to self-organize is dependent upon inter-layer communication within the shoot apex. Ablation of the central region displaces *WUSCHEL* expression to the periphery, and provokes the establishment of a new meristem centre. Laser ablation of a primordium initiation point which included several of the subtending layers, resulted not only in adjacent cells adopting primordial fates, but also a displacement of the SAM position (Reinhardt *et al.* 2004). These observations do not fit comfortably with the idea of a defined apical-basal axis in the plant. Rather they suggest a dynamic signalling situation with minimal functional patterning differences between the seedling SAM and the

'upper' region of the globular embryo, where a three-dimensional signalling environment allows the establishment of boundaries of mutually excluding gene activities. The functioning of the post-embryonic SAM appears to retain all of these attributes. Reinhardt *et al.* (2004) also noted that a functional L1 layer was crucial in the establishment and maintenance of a dorsiventral axis across the emerging primordia.

The variable integrity of adjacent tissue layers in the *hydra* mutant shoot in the light of the above information, suggests that poor communication between layers of the upper region of the globular embryo could result in similar effects to the mechanical separation of regions of the SAM from each other. The disorganized cell division seen in *hydra* is linked to the mutant's inability to efficiently control the cell cycle and coordinate the adoption of cell fate within coherent layers. The result is an apical region without a clear layered organization, in which subsets of the cell population may have a partial symplastic isolation from other cell subsets, and so establish meristematic cues independently within these regions. This would result in either an absence of a clear apical meristem cue, or multiple apical cues over the radial axis. Such a signalling disruption would distort the recruitment of procambium (mediated by *AthB8*- and *SCR*- expressing cells), beginning in the basal region of the embryo and consolidating the radial arrangement of vascular tissues acropetally (Wysocka-Diller *et al.* 2000, Baima *et al.* 1995, 2001). Further mis-positioned cell division events in *hydra* may then compound the patterning anomalies by exacerbating tensile tissue stress between the poorly organized radial cell layers.

### 4.4.2.3 Anomalies in the coordination of leaf vascular differentiation in *hydra* imply a role for sterols in the resolution of cell fate within the mesophyll, and the transition to expansion growth

Analysis of the xylem vascular traces in *hydra* mutants, and the expression profile of the p*AthB8*::GUS procambial cell fate reporter, reveal a variable pattern which is subordinate to strand disjuncture or dissociation of the primary midvein. Leaf organs with lateral lobes were seen to have dissociated primary veins, or duplicated primary axes with additional hydathodes. Where this occurred, aberrant cell expansion behaviour was found to exacerbate the vascular anomalies in later leaf development. This implies that integrity of the primary midvein is not only required for the integrity of subsequent vascular patterning, but also that the vascular pattern influences leaf shape through influencing cell patterning and expansion in the *hydra* epidermis. In wild-type plants, xylem strand positioning is coordinated with the phloem trace, which is in turn defined by the recruitment of mesophyll cells to procambial cell fate (Esau 1977, Carland *et al.* 1999, Scarpella *et al.* 2004). This coordination appears to function minimally in *hydra*, and the callose deposition associated with phloem development is found ectopically in mutant leaves in regions where xylem vessels have the most severe anomalies in strand coordination.

Kang & Dengler (2002) note that cell division in the lamina is associated with activity of p*AthB8*::GUS, which highlights provascular strand identity, and the cessation of division that first occurs near the leaf apex correlates precisely with a cessation of provascular pattern definition. These authors found that the regression of p*AthB8*::GUS activity in the leaf (prior to later expansion) began in marginal positions near the apex, and progressed in a basipetal fashion, resolving finally to the midvein before fading. Scarpella *et al.* (2004) examined the cessation of p*AthB8*::GUS activity in relation to the appearance of other genetic markers of mesophyll cell identity, and found that vascular pattern formation is terminated temporally by the onset of mesophyll fate identity in the surrounding cells, i.e. recruitment to procambial cell fate continues until the temporal change to expansion and maturation in the leaf. At this point, uncompleted vascular loops cannot extend further, and so form free ending veinlets.

If *hydra* mutants are interpreted as unable to coordinate the transition from cell division to differentiation, then this presents an explanation for the appearance of 'vascular islands' of isolated xylem, and ectopic callose deposition in mutant rosette leaves. Poor and incomplete differentiation would result in an exacerbation of the disjointed nature of the xylem strand, which follows the trace established by the phloem. This is also confirmed by the enhanced and persistent p*AthB8*::GUS activity. Although the vascular network defined by this reporter was mostly coherent in *hydra,* its expression was enhanced, suggesting additional cells from the mesophyll were being recruited to procambial cell fate. This could result from either a poorly resolved auxin signal between adjacent cells, or an overall heightened level of auxin in the tissues. The presence of ectopic callose suggested incoherence in phloem trace differentiation in *hydra* leaves, which would perpetuate the poor auxin resolution. The inability of mutant leaves to undergo longitudinal expansion growth may therefore be associated with a persistence of auxin in these organs, which would also explain the continued persistence of the auxin-upregulated p*AthB8*::GUS reporter.

Vascular strand integrity varies over the *hydra* plant body and between siblings, with the most extreme patterning defects resulting from mis-coordination of xylem differentiation in post-embryonic lateral organs and the cotyledon-hypocotyl transition zone. Cotyledons and true leaves of the rosette have a high degree of 'noise' in the coordination of xylem strands, and show multiple (though varied) examples of strand dissociation within the traces, culminating in disjunct sections of xylem, or 'vascular islands'. Pattern anomalies in the higher order vasculature of *hydra* mutant lateral organs appear to be subordinate to the patterning of the primary midvein, and are most extreme in regions of the leaf where this primary trace is mis-positioned, dissociated or duplicated. These effects appear to determine the shape of *hydra* mutant leaves, and have implications for the direction of their later expansion growth.

### 4.4.2.4 Polarly expanding cells in *hydra* mutants show defective functioning of the cortical microtubules

The *hydra* mutant phenotype demonstrates a range of cell shape defects. The longitudinally shortened cells of the *hydra* root epidermis include trichoblasts where the hair bifurcates into two, and/or with an excessive swelling in the region of tip growth initiation. Less frequently, cells were noted with multiple root tips initiating along their length, and rarely, large inflated hairs protruding from the surface of the trichoblast. In longitudinally aligned epidermal cell files of the root and hypocotyl, cells were often of irregular shape, some of which were excessively large and encroached upon neighbouring cell files so that longitudinal alignment was disrupted. Epidermal cells from rosette leaves demonstrated variable size and morphology. Trichomes often had an inflated appearance in the early stages of cell expansion, and the direction of branching, which aligns with the leaf longitudinal axis in wild-type, appeared variable. Areas with clusters of unusually small cells were seen in regions of active stomatal ontogeny. Elsewhere, large isodiametric cells formed in regions of inter-digitating tip growth, or protruded from the surface of longitudinally aligned cell files as they crossed the mutant lamina.

A number of similar cell shape defects have been noted in mutations which affect the functioning of the plant cytoskeleton, or in cells treated with chemical modulators of cytoskeletal activity. Treatment of anisotropically growing cells with microtubule depolymerising herbicides or stabilizing substances results in isotropic growth, implicating microtubules in polar growth directionality (Mathur & Hülskamp 2002, Wasteneys & Galway 2003, Bibikova *et al.* 1999). Mutants in microtubule-associated genes have a range of cell morphologies which can include abnormal swelling of diffusely growing cells such as trichomes and root hair cells, sinuous or branching root hairs, and skewed, right- or left-handed bending of roots and hypocotyl cells (reviewed

by Mathur 2004). Links to the microtubular cytoskeleton in conjunction with cell wall synthesis defects have also been proposed in the mutant phenotype of the *ANGUSTIFOLIA* gene, compromised in longitudinal organ expansion (Kim 2002, Folkers *et al.* 2002). The similarities between many of these cell shape characteristics and phenomena noted in the *hydra* phenotype, suggest that these mutants have a modulated organization of the microtubular cytoskeleton.

Organization of the microtubular cortical arrays in expanding cell was investigated in *hydra* using the GFP::TUA6 construct (Ueda *et al.* 1999). Cortical arrays in actively expanding cells of the wild-type hypocotyl epidermis have a uniform and coordinated polarity. In contrast, cells of the *hyd1* hypocotyl epidermis revealed a variable polarity, with individual cells demonstrating varied expansion axes both within the same cell files and between adjacent cells. Non-polar cells such as guard cell pairs and pavement cells of the lamina epidermis had a normal microtubular arrangement. This suggests that the maintenance of polarity in cells within longitudinally aligned tissues requires a controlled membrane sterol environment. Proteins such as PIN1 have a polar distribution in these cell types, and require accurate intracellular vesicle trafficking to maintain their positioning (Steinemann *et al.* 1999, Geldner *et al.* 2003). These vesicles have distinctive sterol profiles, and are distributed in association with the actin cytoskeleton (Grebe *et al.* 2003). Both *hydra* and *smt1* mutants have variable PIN protein localization in polarly aligned cells (Souter *et al.* 2002, Willeemsen *et al.* 2003), and a highly modified sterol profile (Souter *et al.* 2002, Diener *et al.* 2000, Willeemsen *et al.* 2003). These data imply an interaction between sterols and cytoskeleton function which is modified in *hydra* and other mutants with altered sterol profiles.

## 4.4.3 Coordination between radial layers in the *hydra mutant plant* body

### 4.4.3.1 Coordination between radial layers of the *hydra* root

#### 4.4.3.1.1 The proliferation of root hair in *hydra* mutants is associated with an increased number of cortical cells

Examination of *hydra* mutant roots '*in situ*', grown on microscope coverslips in a film of agar, revealed that not all epidermal cells in the *hyd1* and *hyd2* mutant roots differentiate as trichoblasts, including those which appear in trichoblast cell files. This finding is in contrast to the interpretations of Souter *et al.* (2002). Examination of transverse sections of *hydra* roots revealed a proliferation of cortical cells, giving smaller and more numerous cells within the layer than seen in wild-type roots. Due to this proliferation, epidermal cells occasionally were in contact with two cortical cell junctions, although the present study did not notice an affect on the resulting morphology of the hair cell. Many *hydra* trichoblasts initiated more than one growing tip, which contributed to the appearance of excessive root hair in the mutants.

Increased numbers of cortical cells results in an increase in the positional cues which promote hair cell fate, i.e. the presence of an epidermal cell over an anticlinal boundary between cortical cells. This means that the enhanced root hair proliferation seen in both *hydra* mutants is associated with increased cell numbers in the cortical layer. The observation that not all trichoblast cells within the cell file differentiate as root hairs has two possible explanations. These are firstly that the delayed differentiation associated with the *hydra*

phenotype interferes with the process of cell fate commitment, and second that the proliferation of cortical cells disrupts the order of cell file arrangement in this layer, so changing the patterning cues received by adjacent epidermal cells.

#### 4.4.3.1.2 Definition of the cortex and endodermal layers in *hydra* varies between shoot and root tissues, and implicates sterols in maintenance of the integrity of the pericycle in shoots

The *hydra* phenotype shows differential radial coordination between the root and hypocotyl, with a greater proliferation of ground tissue in the hypocotyl than the root. Although *hydra* roots were found to have a proliferation of ground tissues (i.e. within the cortex layer), the histological consequences are less disruptive over the root radial axis than in the shoot. As p*HYD1*::GUS activity showed localization to the whole meristem periphery in the root (i.e. in the root cap and epidermal tissues) but was confined to stipules in proximity to the shoot apex, these histological differences may reflect the differences in rotational symmetry of development across the radial axis at these meristems. The differences in *HYDRA* gene activity between the root and shoot apex also suggests that these developmental differences may be mediated by a sterol-based signal.

The SCR::GFP protein fusion reporter highlights cell identity in both the root and shoot tissues of wild-type *Arabidopsis* (Wysocka-Diller *et al.* 2000). This layer definition appeared normal in *hydra* roots, but was not observed in hypocotyls of mutant seedlings with strong SCR::GFP root expression. The definition of a single cell layer with SCR::GFP expression in *hydra* roots with duplicated steles (where endodermal layers from the adjacent steles were in contact) indicates that this cellular identity mechanism operates independently of the tissue physical pattern. Differences in the activity of

this reporter in roots and hypocotyls therefore suggest that endodermal cell identity can be established in the *hydra* root independently of tissue pattern, although this mechanism is not operating in the mutant shoot. The *hydra* mutant hypocotyl lacks a coherent radial layered organization in the hypocotyl cortex. It is possible that the transient *pHYD1*::GUS reporter signal seen in the cortex layer is indicative of a sterol-mediated signal from the peripheral tissues which allows correct development of the adjacent endodermal layer in shoots.

A single-layer recruitment of cells to SCR expression independently of the established tissue pattern was noted in regenerating roots of maize after excision of the apex (Lin *et al.* 2000). Here, a SCR::GFP signal displaced itself distally to the first complete cells adjacent to the cut surfaces, and 'recruited' its way beneath the stele, adopting cells until an uneven though complete layer was formed over the apical end of the root. Upon completion, a coordinated asymmetric cell division produced additional cells which comprised an additional outer layer; a process which continued until the regeneration of the root cap and meristem was complete.

The SCR protein is produced in response to production of the SHORTROOT (SHR) protein in the pericycle and stele (Helariutta *et al.* 2000, Sena *et al.* 2004). Movement of SHR from the stele into the adjacent cells prompts SCR production, and the presence of SCR then limits further movement of SHR. When *SHR* is expressed in *Arabidopsis* root epidermal tissues under the control of the *WER* promoter (i.e. confining transcription to the epidermis), supernumerary cell layers resulted, which depended upon SCR function (Sena *et al.* 2004). SHR ectopic expression driven by the *GL2* promoter, which appears at the point of cell exit from the meristem in epidermal tissues, did not produce supernumerary cell layers (Sena *et al.* 2004). This mechanism therefore has a time-related component, namely it requires SHR expression within the meristem. In combination with the patterns of SCR::GFP expression in maize roots (Lin *et al.* 2000), it would appear that the SCR-inducing signal

(i.e. SHR) displaces itself outwards until limited by a signal from the periphery.

The *hydra* phenotype suggests that a peripheral signal also affects the positioning of SCR. If the endodermal layer represented the position of the outer limit of the SHR-SCR signal, then the double adjacent 'endodermal' layers between the two steles seen in the *hydra* root shown in Fig. 4.32 E would have included activity of SCR::GFP. Instead, a single cell layer defined by SCR::GFP expression encircled the outer margin between steles. These results suggest instead that competence to induce SCR by SHR may depend upon interaction with an additional signal or signals associated with peripheral tissues. This unknown signal appears to function normally in the *hydra* mutant root, but is disrupted in the hypocotyl.

The data from Sena *et al.* 2004 and Lin *et al.* (2000), along with the observed behaviour of SCR::GFP in *hydra* roots with a duplicated longitudinal axis, suggest SCR as a strong candidate for mediating between central and peripheral signals at the root meristem. In the absence of feedback from the periphery, SCR would act to promote division in the pericycle layer, generating additional cell layers until there are sufficient peripheral layers for an outer signal to resolve. This offers a means both to limit cell division across the radial axis, and regenerate meristematic tissues at the apex. This could explain the presence of SCR::GFP in the QC, where the signal originates during embryogenesis (Wysocka-Diller *et al.* 2000). An absence of SCR, as in the *scr* mutant, produces an identical phenotype to that obtained by expressing SHR in the root epidermis, i.e. a root with many supernumerary radial layers (all with apparent layer integrity).

In most examples of the *hydra* mutant siblings, the root appears to retain intact cell layers in the stele and pericycle, which allows the movement of SHR protein out to a position where a coherent boundary forms in response to a peripheral signal. However the *HYDRA1* protein is not expressed in the root

meristem; rather *HYD1* gene activity is confined to the root cap and the differentiating epidermis as it emerges from the lateral root cap. At this transverse section across the root growth axis, the SCR-dependent division of cortical initials to form the cortex and endodermal cell layers has already taken place. The peripheral signal therefore is likely to originate in tissues showing *HYD1* gene activity, i.e. in the root cap and epidermis; however the coherence of the SCR::GFP signal in *hydra* roots suggests independence between this mechanism and the *hyd* mutant phenotype.

In contrast, the *HYD1* reporter highlighted gene activity in the hypocotyl ground tissues, where a SCR::GFP signal was not resolved in *hydra* mutants. This suggests that the positional cues which define the endodermal cell layer are unable to function in the environment of the *hydra* mutant shoot. Hypocotyl tissues are embryonically derived and undergo differentiation during post-germination growth (Gendreau *et al.* 1977, Busse & Evert 1999b). This means that upon seedling germination, SCR expression must establish in the hypocotyl within tissues not associated with meristematic cues. These data imply that *HYD1*-mediated sterol biosynthesis is not directly implicated in the SHR-SCR mechanism of endodermal establishment, but the sterol environment may have a modulating effect upon SCR activity throughout the plant. This was also implied by the findings of Müssig *et al.* (2003), who note that SCR::GFP activity was increased in the brassinosteroid-deficient mutant *dwf1-6* (Müssig *et al.* 2003).

### 4.4.3.2 Coordination across the shoot radial axis

#### 4.4.3.2.1 Defects in xylem pattern definition over the vascular transition zone could be responsible for precocious primordial initiation across the *hydra* mutant SAM

The patterning of the hypocotyl and cotyledon vascular trace in wild-type plants is laid down in the formation of the procambial strands during embryo development (Baima *et al.* 1995). Vascular patterning of the young seedling shoot then differentiates after germination, resolving an alternate arrangement of xylem and phloem in the hypocotyl stele and a collateral pattern in the cotyledon primary traces (Busse & Evert 1998a, b), i.e. with phloem on the outer abaxial side and xylem on the inner adaxial side. Phloem traces differentiate first, the strands bifurcating in the upper hypocotyl just beneath the incipient SAM and extending into the cotyledons. Xylem differentiation follows this in sections, the last vessels to appear are those which connect the strands across the transition zone at the hypocotyl-cotyledon junction (Busse & Evert 1998b). Subsequent vascular traces to the true leaves form in connection with these traces, resulting in a paired vascular branching pattern with a clear periodicity in successive primordia, generating the phyllotactic spiral.

The p*HYD1*::GUS reporter showed diffuse transient activity in the ground tissues of the hypocotyl and cotyledons, and strong persistent activity in the stipules by 4 dae. This expression pattern implies a strong gene activity in the vicinity of the hypocotyl-cotyledon transition zone in synchrony with the differentiation of both phloem (4-5 dae) and xylem (6-7 dae) as noted by Busse & Evert (1998a, 1998b). Patterning of the *hydra* shoot apex also demonstrated problems in coordinated differentiation over this region from the earliest stages of SAM inception. The bifurcation of the stele in the mutant upper hypocotyl is variable, with siblings showing dissociation of stele vasculature below, near or above the predicted branch point. Some plants have multiple vascular branching events at different points over the longitudinal axis. Where a clear branching event occurs in the hypocotyl-cotyledon transition, the mutants derive a SAM, and where more than one branching event occurs within this zone, multiple SAM formation follows. In examples where branching of the stele is not clearly defined, a widened meristem may result, or a single meristem with precocious primordial initiation. No meristems or

precocious primordia were observed in any *hydra* mutants outside of the hypocotyl-cotyledon transition region, suggesting that the symplastic limits of this domain are intact, but the signals which define central and peripheral regions of the SAM are indistinct.

The *hydra* mutant phenotype, and *HYD1* reporter profile, suggests that *HYDRA* gene activity is necessary for coordinated differentiation over this hypocotyl-cotyledon transition, both for establishment of the leaf traces and meristem positioning. Whatever the cues that define pattern in this region, the close correlation of vascular branching and precocious SAM or primordial development implies that a common mechanism defines these phenomena. The transient p*HYD1*::GUS signal between 4 and 7 dae in the hypocotyl-cotyledon transition zone, in combination with the strong reporter activity in stipules, implies a possible role for HYD1-modified sterols in the coordinated differentiation of the vasculature in this region. This in turn provides cues to the apex through modulation of auxin distribution. The p*HYD1*::GUS stipule signal, which appears prior to the timing of stipular expression of DR5::GUS in wild-type seedlings, suggests that *HYDRA* activity in these highly metabolically active cells, appears prior to the auxin response. This behaviour of *pHYD1*::GUS is distinct from the transient signals seen in differentiating functional epidermal cells.

These data suggest a role for sterols in the coordination of the differentiation signal required between the hypocotyl and cotyledons in order to connect the xylem traces. As gene activity is evident in proximity to these differentiating cells, it is possible that sterols produced in the stipules participate in a mechanism involving a putatively sterol-interacting protein such as the START domain HD-Zip III, AthB8. Alternatively, *HYD* gene activity the lower leaf domain, from which stipules are derived (Kaplan 1973), forms a plate of cells between the expanding leaf and the meristem that may mediate peripheral feedback signals involved in meristem maintenance. Again the *hydra* mutant phenotype suggests that this function is not operating correctly, as ectopic

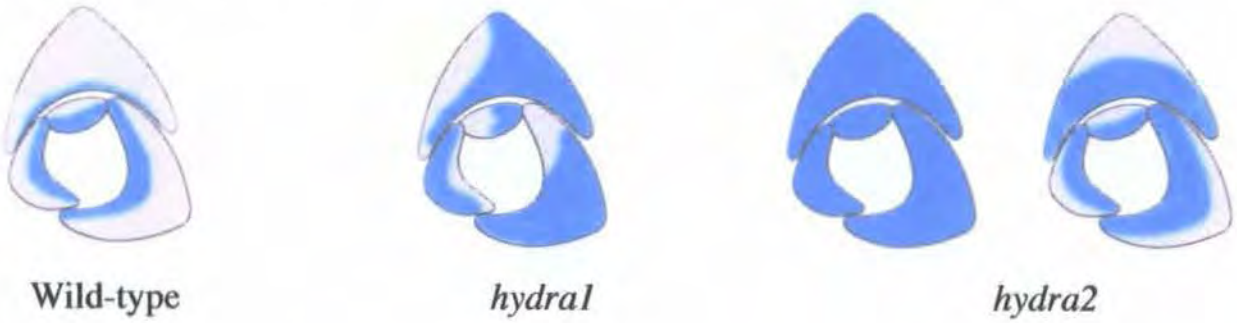
primordia emerge around the bases of established leaves in a region of the PZ which is no longer competent for primordial formation in wild-type apices.

Mis-coordination of vascular branching occurs in the *hydra* mutant stele below the meristematic region, where vascular traces extend towards incipient primordia in wild-type plants in conjunction with primordial expansion. Varying degrees of severity in adjacent primordial initiation were observed in the sibling populations of both *hydra* mutants, some plants showing indiscriminate clusters of primordia right across their apices. These extreme examples are typically positioned over a xylem trace in which few if any of the vessels are connected. As the xylem cells are implicated in the basipetal transport of auxin in the shoot, this suggests that multiple or indiscriminate auxin thresholds are perceived by the SAM above. The vascular strands of wild-type plants form in continuity with the existing traces and invade into the developing primordia, transporting auxin away from the meristem (Reinhardt *et al.* 2000, 2003). A mis-coordination of vascular branching as seen in *hydra* could therefore affect primordial initiation by modifying the positioning of auxin maxima, and disrupting thresholds for patterning cues in these tissues.

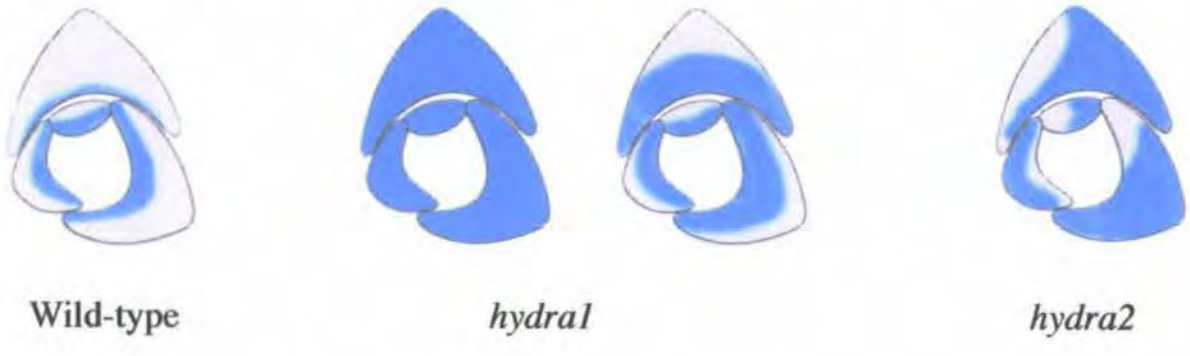
#### 4.4.3.2.2 *hydra* mutant primordia demonstrate variable and anomalous definition of dorsiventral orientation and mesophyll organization

The reporter constructs which demonstrated abaxial (p*YAB*::GUS) and adaxial (p*REV*::GUS and p*PHB*::GUS) positional cues in wild-type plants revealed a range of anomalies in the placement of these cues in the *hydra* mutants, summarised in Diagram 4.5. The p*REV*::GUS reporter was variably reversed (i.e. abaxial) or skewed towards one lateral margin in primordia of *hyd1*, whilst activity in *hyd2* was so strongly enhanced that the signal obscured any

**p*REVOLUTA*::GUS**




**p*PHABULOSA*::GUS**




**p*YABBY*::GUS**

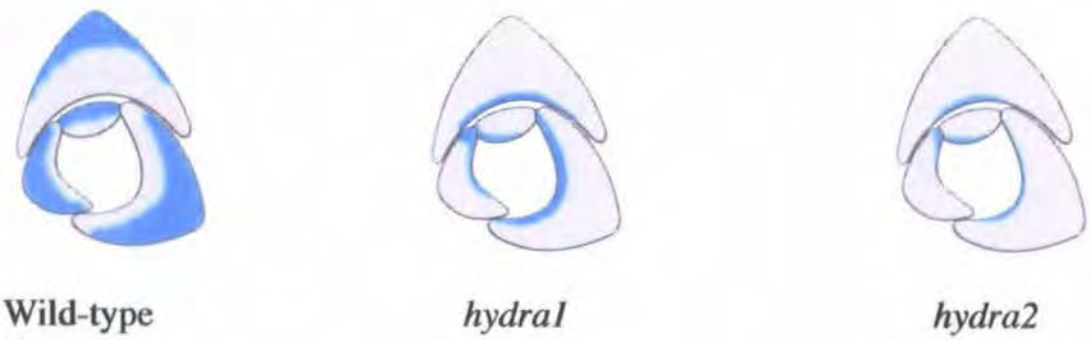



Diagram 4.4 Localization of *PHB*, *REV* and *YAB* promoter-reporter transcription in wild-type and *hydra* mutant seedlings

axiality. An opposite pattern was found in response to the p*PHB*::GUS reporter, with the variably reversed signal evident in *hyd2* whilst primordia of *hyd1* were obscured by high levels of reporter activity. The abaxial marker p*YAB*::GUS was also variably reversed, although minimally expressed in both *hyd* mutants, with a particularly weak signal in *hyd2*. Prong-shaped structures which developed around the meristem carried a p*YAB*::GUS signal, unless xylem differentiation was visible, in which instance an axiality was conveyed upon the structure, although not always correctly positioned in relation to the meristem. Extensive clusters of multiple primordia over severely disjunct xylem in the hypocotyl-cotyledon transition zone have stronger p*YAB*::GUS expression than non-clustered primordia, and in these clusters, where organs have expanded to reveal a lamina, they appear to have a normal abaxial location.

An interpretation of the reporter signals is not straightforward when considering the *hydra* mutant phenotype. Abaxial-adaxial definition is undoubtedly compromised, as shown by the lack of clear palisade mesophyll differentiation in many mutant leaves. Some *hydra* siblings have morphological features of both abaxialized and adaxialized tissues, occasionally as adjacent structures at the same shoot apex (e.g. Fig. 4.42; H). The over-expression of adaxial tissue markers could suggest an adaxialization of organ primordia. Gain of function mutations at the *REV* and *PHB* loci produce morphological phenotypes which appear to have dorsiventral reversal (Emery *et al.* 2001); however this phenotype is based upon the modification of mRNA degradation rates prior to translation (Emery *et al.* 2003). The consequences to reporter gene activity (which lack the regulatory StART cleavage domains) have not been investigated in *rev* and *phb* mutant organs.

The *rev* loss of function mutants, interpreted as having an abaxialized morphology, have swollen margins and indistinct mesophyll dorsiventrality (Talbert *et al.* 1995), which is reminiscent of the *hydra* phenotype. This suggests that p*REV*::GUS and p*PHB*::GUS over-expression may result from an

attempt to restore the ratio between abaxializing and adaxializing signals; similarly the p*YAB*::GUS response could be down-regulated for the same reason. Triple mutants of *phb, phv* and *cna* produce extra cotyledons, enlarged SAMs and fasciated stems as in *hydra*, along with a number of floral defects (Prigge *et al.* 2005) which were not observed in *hydra* inflorescences. Whatever modulates these signals in *hydra* rosette primordia, this agent is not in operation in cauline leaves and flowers, as *hydra* floral dorsiventral morphology is apparently normal; this contrasts strongly with the *rev* mutant phenotype in florescence bolts (Talbert *et al.* 1995).

One interpretation of the *hydra* phenotype could consider that the agent or agents which mediate the boundary between dorsiventral gradients or domains is missing or non-functional. Examples are known from animal development where gradients of small diffusible molecules induce the expression of genes that mutually repress their expression, and so define a boundary within an otherwise apparently uniform cell mass (Briscoe *et al.* 2000). The undifferentiated mesophyll layer within the developing leaf primordium, could be considered as such a cell mass, and mutually exclusive domains appear to pattern the shoot apical meristem. Some members of the HD-Zip III family have a mutual antagonism, e.g. the *AthB8* and *CNA* genes antagonize *REV* function (Prigge *et al.* 2005) and the balance between the HD-Zip IIIs and their antagonists are proposed to control the position of the vascular meristem tissue, i.e. at the boundary between the (abaxial) phloem and (adaxial) xylem (Emery *et al.* 2003).

Other agents are also involved in dorsiventrality. The *AUX1* gene is associated with the abaxial L1 layer, and has been suggested to function as an agent concentrating auxin into this cell layer from adjacent tissues, so 'sharpening' the boundary definition between layers (Reinhardt *et al.* 2003). MicroRNAs which target and cleave the START domain of *PHV* mRNAs in tobacco are abaxially expressed (McHale & König 2004), defining the lower leaf zone, and antagonizing adaxial signals. The *KANADI* genes also interact with adaxial

signals, apparently independently of the miRNA mechanism (Emery *et al.* 2003). These, along with other currently unknown factors, are influential in the definition of abaxial-adaxial boundaries in the developing leaf. They suggest that the balance which defines lateral organ patterning is a complexity of signals which act at transcriptional and translational levels. Micro-RNAs are interpreted as the target molecules for START domain protein expression, but their activity is at the mRNA level. This does not exclude the possibility of a sterol-mediated signal, presumably disrupted in the *hydra* mutants, which interacts with translated proteins and modulates their function.

# Chapter 5

# Phenotypic rescue by the *ein2* mutation

# 5.1 Abstract

Many characteristics of the *hydra* phenotype exhibit phenomena associated with ethylene effects. Souter *et al.* (2002, 2004) implied that aspects of the *hydra* mutant phenotype may be attributable to an enhanced ethylene responses in cells and tissues during pattern definition. The hypothesis that many of *hydra's* patterning defects are attributable to ethylene was examined in the context of the epidermal and radial defects presented in chapter 4 of this thesis. The *ein2* mutation, conveying a systemic ethylene resistance through the elimination of a signalling relay step between the cytoplasm and the nucleus (Guzman & Ecker 1990), was used to create an ethylene-independent environment in the *hydra* mutant plant body, and ethylene effects were discerned through a comparison of *hydra* single and *hydra-ein2* double mutants.

This analysis revealed only a partial rescue of *hydra* cell and tissue patterning, which does not include pattern definition resulting from embryogenesis. Seedling root and shoot viability was improved, and integrity of longitudinally aligned cell files including true leaf vasculature were increased. However phyllotaxy and the mis-positioning of radial cues were unaffected, although reporter intensity was modulated slightly. Phytohormone signalling responses, revealed as anomalous in single *hydra* mutants, were variably modulated by the introduction of *ein2*, whilst the *ein2* controls themselves showed no or little change in reporter activity compared with wild-type. Specifically, differences in cytokinin response between *hyd1* and *hyd2* sibling populations were modulated to an equivalent level of mis-regulation, by introduction of the *ein2* mutation.

These phenomena are discussed in the context of current knowledge of ethylene-associated effects upon signalling cross-talk, auxin transport and cellular patterning.

# 5.2 Introduction

## 5.2.1 Ethylene is an agent of signal transduction in plant development

Ethylene ($C_2H_4$), a simple gaseous hydrocarbon, was first identified as a plant 'hormone' through its role as a promoter of fruit ripening in greenhouse cultured tomatoes. Early 'classical' and later molecular-based approaches to the study of this and other hormonal activities have established that ethylene influences a number of complex physiological processes throughout the life cycle of the plant, from the germination of the seed through vegetative growth, flowering, fruit ripening and senescence, and is also implicated in a range of stress responses (Abeles *et al.* 1992, Bleecker & Kende 2000). Its role in vegetative growth concerns the induction of asymmetric stem and petiole elongation, and the promotion of agravitropic root responses (Bleecker & Kende 2000). One of the most dramatic effects of ethylene on morphology is the classical 'triple response' of dark grown germination seedlings; this is characterised in *Arabidopsis* by exaggerated curvature of the apical hook, radial swelling of the hypocotyl, and inhibition of longitudinal hypocotyl and root growth (Guzman & Ecker 1990). Ethylene has been implicated in the regulation of hair cell fate commitment in the radially-organized root epidermis (Tanimoto *et al.* 1995, Bleecker *et al.* 1998), and in the regulation of swelling across the radial axis of the hypocotyl and stem (Kieber *et al.* 1993, Larsen & Chang 2001).

Cell size is affected by ethylene signalling; constitutive or hypersensitive ethylene response mutants show reduced cell and organ size (Kieber *et al.* 1993, Hua & Meyerowitz 1998, Woeste & Kieber 2000). The *ethylene insensitive-2* mutation has reduced numbers of (enlarged) cells (Lake *et al.* 2002) although organ size is not affected. Ethylene treatment of wild-type roots results in a rapid reorientation of the microtubular cytoskeleton in a

cell-dependent manner in differentiating cells of the epidermis (Le *et al.* 2004), and promotes the induction by auxin of root hair initiation in lettuce seedlings (Takahashi *et al.* 2003).

Ethylene is produced in by plant tissues in response to internal signal relays during development, triggered by environmental stimuli such as pathogen attack, wounding, hypoxia, ozone, chilling and freezing. Ethylene is released rapidly in plant tissues in response to these stimuli through the oxidation of its precursor ACC, via an ACC Oxidase enzyme (Hamilton *et al.* 1991, Spanu *et al.* 1991). ACC is itself produced from methionine, the final step in this biosynthesis being catalysed by a an ACC Synthase (Sato & Theologis 1989). Both of these enzymes are encoded by multigene families, and are regulated by a complex network of developmental and environmental cues. ACS gene transcripts are short lived, and are thought to be negatively regulated by some unknown labile repressor(s) (Liang *et al.* 1992); in addition it is possible that ACS is regulated post-translationally (Vogel *et al.* 1998b, Tatsuki & Mori 2001).

### 5.2.2 Ethylene triggers a developmental signalling relay pathway which affects the expression of many genes

In *Arabidopsis,* ethylene is perceived by a small family of putatively membrane-anchored receptors (Guzman & Ecker 1990, Chang *et al.* 1993, Hua *et al.* 1998, Sakai *et al.* 1998) that possess sequence similarity with bacterial two-component histidine kinases, and have genetic orthologues in a range of other plant species (Goff *et al.* 2002, Yu *et al.* 2002, Shibuya *et al.* 2002, Bassett *et al.* 2002, Tiemann *et al.* 2000). These receptors were identified in *Arabidopsis* through gain-of-function and loss-of-function mutagenesis screens, and are classified into two subfamilies based on these criteria (Chang *et al.* 1993, Hua *et al.* 1998, Sakai *et al.* 1998).

The cellular response to ethylene is thought to involve a signal relay from the membrane to the nucleus, where response genes are actively transcribed upon receipt of a positive signal. Ethylene binds to its

receptors in a manner dependent upon a copper co-factor, which causes a conformational change in the protein that activates a signal cascade (Rodriguez *et al.* 1999). The ER-localized receptor ETHYLENE RESPONSE1 (ETR1) interacts with another ER-anchored protein, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) (Clark *et al.* 1998, Kieber *et al.* 1993, Chen *et al.* 2002, Gao *et al.* 2003). Binding of ethylene switches the receptor interaction off, depriving them of the ability to activate CTR1, which then relieves downstream repression by CTR1, resulting in a 'positive' ethylene signal. The nature of this signal is unclear, although the similarity of CTR1 with other known protein kinases imply a possible activation of a MAP kinase (MAPK) cascade (Kieber *et al.* 1993). An ethylene mediated MAPK cascade has been shown to exist in *Arabidopsis* (Ouaked *et al.* 2003). Genetically downstream of *CTR1,* the *EIN2* gene of *Arabidopsis* encodes a positive regulator of ethylene signalling, which comprises a novel, plant-specific protein of unknown biochemical function (Alonso *et al.* 1999). The two predicted functional domains of this protein imply a role in respectively sensing the activity of upstream agents, and transmitting a signal to downstream components (Alonso *et al.* 1990). Beyond EIN2, EIN3 (and possibly also a family of EIN3-like (EIL) proteins) mediate gene transcription through direct interaction with two F-box transcription factors EBF1 and EBF2 (Chao *et al.* 1997, Guo & Ecker 2003, Potuschak *et al.* 2003, Gagne *et al.* 2004). Microarray experiments have shown that hundreds of genes are ethylene-regulated (Alonso *et al.* 2003, Zhong & Burns 2003), although this data is 'static', in that it does not distinguish between cell types, resolve developmental differences in cells and tissues, or illustrate gene expression responses to environmental conditions.

### 5.2.3 Ethylene interacts with other signalling systems affecting pattern definition, morphology and growth

The ethylene signal transduction pathway operates in a context of complex hormonal interplay; plant signalling systems interconnect and 'cross-talk' in an integrated manner which is currently conceived at an only elementary level. Other phytohormones interact with ethylene signal transduction, and

with each other. Light and sugar affect these interactions and affect morphogenesis, as does the nutrient status of the growing medium. Individual cell types within tissues then have the ability to respond differentially to these stimuli, e.g. the greater sensitivity of immature cells in root trichoblast cell files predisposes them to adopting root hair cell fate (Cao *et al.* 1999). This web of interacting mechanisms constitute the means by which the plant fine-tunes its environmental responses and physiology.

Auxin can induce ethylene biosynthesis (Yu & Yang 1979) whist it has been a long standing hypothesis that ethylene plays a role in the regulation of auxin transport (Morgan & Gausman 1966, Goldsmith 1977). Many processes including cell elongation, the number and size of root hairs, and aspects of leaf morphology, are affected by both ethylene and auxin (Davies 1995). In addition a number of mutants isolated for their impaired auxin response or auxin transport capacity also show a clear modulation of ethylene response (Swarup *et al.* 2002, Alonso *et al* 2003b, *Larsen & Cancel* 2003). The ethylene resistant mutants *ein2* and *etr1* have a mild auxin-resistant phenotype, proposed to result from a reduced auxin-mediated ethylene biosynthesis rather than a general auxin defect (Hobbie 1998).

GA is required for the induction by ethylene of curvature of the apical hook (Vriezen *et al.* 2004). This interaction may be mediated via the DELLA proteins, as null mutations in several DELLA genes (gai-t6 and rga-24) result in an attenuated response to low levels of ethylene (Achard *et al.* 2003).

Two mutants from screens for ABA hypersensitivity and ABA resistance respectively were found to be allelic to *ein2* and *ctr1* (Beadoin *et al.* 2000, Ghassemian *et al.* 2000). Strong ethylene insensitive mutants (etr1-1, ein2 and axr2) were also affected in their ABA responses (Beadoin *et al.* 2000, Ghassemian *et al.* 2000), implying the existence of an ethylene threshold below which ABA response is impaired.

Su & Howell (1992) recovered a cytokinin resistant mutant, ckr1, which was also allelic to *ein2*; its phenotype in the presence of cytokinin was found to result from an insensitivity to cytokinin-stimulated ethylene biosynthesis. Another mutant, cin5, that failed to produce the ethylene-mediated triple response in the presence of low levels of cytokinin, was found to be allelic to the ACS5 gene (Vogel *et al.* 1998).

Genetic screens for glucose response mutants have similarly highlighted unanticipated interactions between glucose, ethylene and ABA (Leon & Sheen 2003).

Plant signal transduction pathways can also converge at the promoter level to regulate the transcription of common target genes, e.g. the pathogen defence gene PDF1-2 (Pennickx et al 1996) and the expression of the transcription factor ERF1 (Lorenzo *et al.* 2003) are both induced by ethylene and jasmonate.

### 5.2.4 Heightened ethylene response is a component of the *hydra* mutant phenotype

The *hydra* primary root meristem demonstrated an improved viability and a more normal epidermal patterning in response to ethylene signal inhibition (Souter *et al.* 2002, 2004). The *hyd2* primary root meristem remained viable and retained its root cap columella starch granules (shown by lugol staining) both when grown in the presence of silver ions, and in combination with the *ethylene resistant-1* (*etr1*) mutation (Souter *et al.* 2002, 2004, Bleecker *et al.* 1988, Chang *et al.* 1993). Pattern formation in the *hydra* mutant root, characterised by prolific root hair induction and excessive hair tip lengthening, showed a reduced hair cell initiation and shorter hair length in the presence of silver and *etr1* (Souter *et al.* 2002, 2004). However the inhibition of ethylene biosynthesis using AVG did not affect the *hydra* phenotype, and ethylene levels in the mutants were not excessively high (Souter *et al.* 2002), implying an enhanced response to ethylene in the *hydra* mutant plant body.

## 5.2.5 Rationale

The data presented in this chapter was compiled with the aim of distinguishing ethylene-mediated and sterol-mediated effects upon longitudinal co-ordination, radial pattern development and phytohormone signalling cues in *hydra* mutant plants. This approach compares *hydra* single and *hydra-ein2* double mutants to define ethylene dependent and independent aspects of the *hydra* phenotype.

Treatment with exogenous silver ions, and the *etr1* mutation as used by Souter *et al.* (2002) to effect a rescue of the *hydra* root meristem, both target root tissues. Some of the patterning anomalies found in *hydra*, particularly the formation of clustered stomata in the shoot epidermis, have been noted previously in wild-type plants grown in an enhanced ethylene environment (Serna & Fenoll 1997), therefore ethylene effects in *hydra* require attention throughout the seedling. Mutation of the *EIN2* gene, situated downstream of *ETR1* and other members of the ethylene receptor family in the ethylene signalling pathway in *Arabidopsis* (Alonso *et al.* 1999), convey a systemic ethylene resistance, i.e. targeting both root and shoot tissues (Guzmán & Ecker 1990).

The EIN2 protein, proposed as a nuclear membrane anchored 'relay', was found to be required for all ethylene responses studied, and so constitutes a critical step in ethylene signal transduction (Alonso *et al.* 1999). The *ein2* mutation was therefore chosen as a means of studying the consequences of constitutively removing ethylene responsiveness in *hydra* seedlings. As Souter *et al.* (2002) reported an allele dosage effect for *hydra-etr1* double mutants, both *hyd1* and *hyd2* were studied as sibling populations derived from *ein2* homozygous parents. Reporter constructs used in Chapter 4 were again examined in the double mutant siblings, to reveal the positioning of cell division events and the positioning of radial cues. In addition, a comparison of markers for phytohormone response in *hydra* single and *hydra-ein2* double mutants was made in early (3-12 dae) seedling development.

# 5.3 Results

## 5.3.1 Anatomical effects of *ein2* on *hydra* mutant morphology

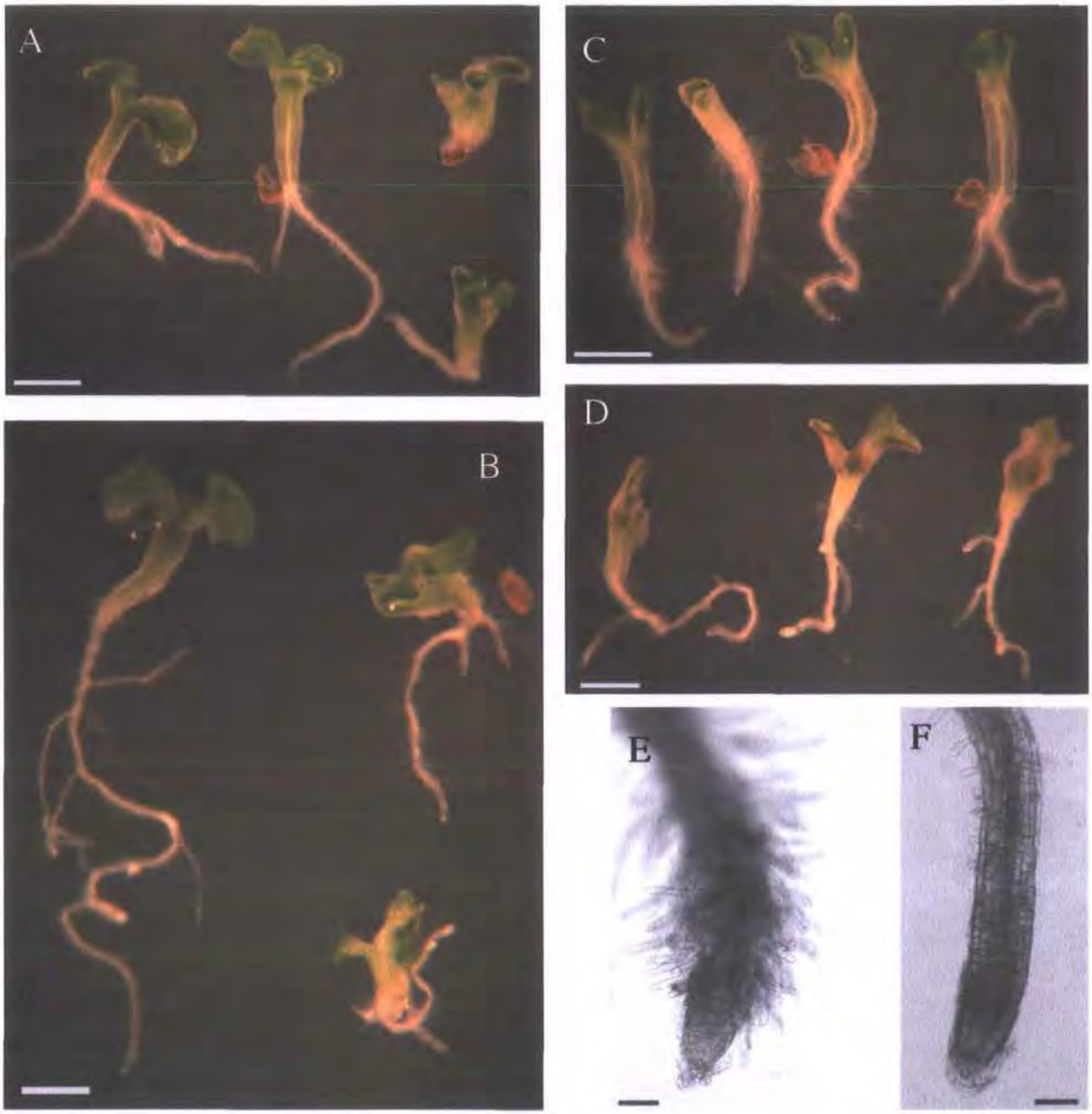
### 5.3.1.1 Sibling variation and axis duplication

#### 5.3.1.1.1 *hydra-ein2* double mutants show aspects of phenotypic rescue, but retain their inter-sibling variation

Fig. 5.1 presents a representative range of siblings from 7 dae *hyd1*, *hyd1-ein2*, *hyd2* and *hyd2-ein2* populations for comparison.

In *hyd1-ein2* (Fig. 5.1; B) compared to *hyd1* (Fig. 5.1; A), the most noticeable change is an improvement in root elongation in certain individuals, such as the siblings visible towards the right of photograph B. The other two seedlings in Fig. 5.1; B have radially-swollen hypocotyls; in these individuals, root elongation is improved compared with similar examples from the *hyd1* single mutant population. A dramatic reduction in root hair results from the introduction of *ein2* into the hydra background (Fig. 5.1; E and F).

In the *hyd2* population, the *ein2* mutation produces a more consistent though less dramatic improvement in root elongation (Fig. 5.1; C-D). A more rapid development of cotyledons is also noted in the *hyd2-ein2* plants, and a similarly improved epidermal patterning in the root (not shown). This reduction in root hair is similar to the improvement seen in root epidermal patterning by introduction of the *etr1* mutation, conferring reduced ethylene sensitivity, into the *hyd2* mutant background (Souter *et al.* 2002).




## Figure 5.1 Partial phenotypic rescue of *hydra* mutants by introduction of the *ein2* mutation

A; *hyd1*, B; *hyd1-ein2*, C; *hyd2*, and D; *hyd2-ein2*, all at 7 dae, bar = 1mm. E, *hyd1*, and F, *hyd1-ein2* primary root tips from 7 dae plants grown in agar, bar = 100$\mu$m.

Introduction of the *ein2* mutation, which abolishes the ethylene signal perception relay throughout the plant body, results in a partial rescue of the phenotype of both *hyd1* and *hyd2* mutant seedlings. Most noticeable are the improved rates of primary root growth, and root hair patterning (E, F). However the examples shown demonstrate that within the sibling populations, those seedlings with less severe distortion of their morphology are those individuals which demonstrate the most dramatic rescue. Siblings with more extreme morphologies show a less dramatic rescue of root elongation. Overall the rescue appears more pronounced in *hyd1* siblings than in the *hyd2* mutant population.

From the photographs in Fig. 5.1; A-D, it is clear that general growth rates are slower in both *hyd2* populations, as compared to the *hyd1* populations. An assessment of the effects of *ein2* on lifespan of the *hydra* mutants was made, scoring double mutant plants for longevity (as described for single mutants in section 4.3.1.1). Table 5.1 shows that for *hyd2*, the presence of *ein2* results in a doubling of the median lifespan for the sibling population. A dramatic improvement appears to have resulted from *ein2* in *hyd1*, although as such a high proportion of the population survived beyond the time course of the experiment, this is not possible to quantify. However the reduced ethylene perception appears to have abolished very young seedling death in the *hyd1* sibling population, and reduced its occurrence in *hyd2*.

| Lifespan (dae) | *hyd1-2* (n = 94, median = 36-42) | *hyd1-ein2* (n = 125, median = 51+) | *hyd2* (n = 97, median = 22-28) | *hyd2-ein2* (n = 160, median = 43-50) |
|---|---|---|---|---|
| 1-6 | 2.1% | | 9.3% | |
| 7-13 | 17.0% | | 21.6% | 5.0% |
| 14-21 | 11.7% | | 18.6% | 1.9% |
| 22-28 | 8.5% | | 15.5% | 9.4% |
| 29-35 | 10.6% | 10.4% | 17.5% | 8.8% |
| 36-42 | 16.0% | 10.4% | 2.1% | 16.3% |
| 43-50 | 4.3% | 6.4% | - | 18.8% |
| 51+ (surviving) | 28.7% | 72.8% | 14.4% | 40.0% |

Table 5.1; Longevity in *hydra* and *hydra-ein2* siblings

In all single and double *hydra* mutants, seedling death was preceded first by death of the primary root meristem, followed by signs of accelerated senescence in the rosette. Occasional rosettes showed senescence in cotyledons prior to primary root death, although this was more common in *hyd2* than in *hyd1* or in any of the double mutant populations. Death of the primary root meristem preceded seedling death with different rapidity between the mutant populations. This was between 2-3 weeks in *hyd1*, by

one week or less in *hyd1-ein2*, by around 2 weeks in *hyd2*, and around 4 weeks in *hyd2-ein2*. The *hyd1-ein2* population were prone to rapid death upon loss of primary root viability, although the majority of the *hyd1* double mutants survived for the duration of the experiment. The extended gap between primary root meristem death and seedling death in *hyd1* and *hyd2-ein2* resulted from reliance upon anchor and lateral root growth supporting continued growth of the shoot. In the *hyd2* mutant population, shoot growth is much slower and is typically maintained for the shorter life of these seedlings at the expense of root growth. Some individuals of *hyd2* did not appear to develop any root growth beyond that which could be expected from post-germination elongation (data not shown).

#### 5.3.1.1.2 Duplication and dissociation of the *hydra* longitudinal axis is unaffected by *ein2*

Fig. 5.2 shows examples of *hyd1-ein2* and *hyd2-ein2* double mutant seedlings, stained and cleared to allow visualisation of the vascular strands in the hypocotyl stele. As with the *hydra* single mutant sibling populations, double mutant seedlings showed multiple cotyledons, radialized 'prong'-shaped structures, and variable dissociation or duplication of the stele.

Siblings were scored for axis dissociation against the three categories described in section 4.1.2 (above the SAM, within the hypocotyl, or affecting the full longitudinal axis); this data is presented alongside the data from section 4.1.2, in Table 5.2 below. From these figures, no significant differences are evident between the *hyd* and *hyd-ein2* populations. It is presumed that differences relate to random differences between samples taken from these highly variable sibling populations, and possibly to an increased proportion of sibling survival upon germination, causing a slight increase in the proportion of seedlings in the middle category.

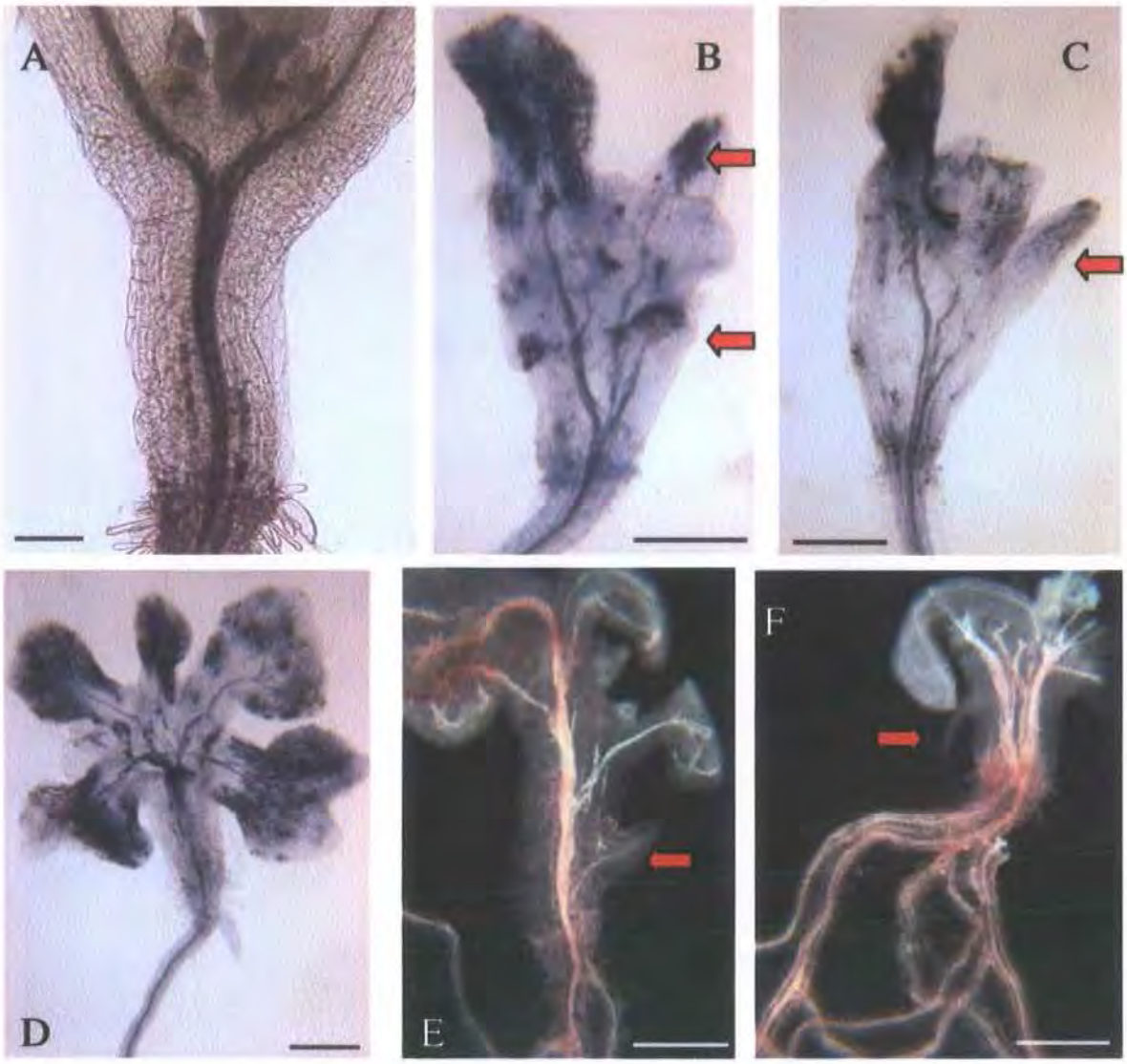



Figure 5.2 Longitudinal axis dissociation and duplication in hypocotyls of *hydra-ein2* double mutant siblings

A-C, *hyd2-ein2* at 8 dae. D, *hyd1-ein2* at 8 dae; E, F, *hyd1-ein2 at* 18 dae. D has true leaves removed, and F has most of the vegetative lateral organs removed to allow visualisation of the hypocotyl region. Bar = 0.5mm.

Although resulting in improved root patterning and elongation, the introduction of *ein2* into a *hydra* background does not change the unusual morphology of young seedlings, and affects neither the incidence nor severity of longitudinal axis duplication.

The plant in A has a coherent stele in the hypocotyl; in more mature *hydra* siblings such as the 18dae plant shown in E, single axes often show secondary vascular strand dissociation as the lateral meristem develops, resulting in further patterning anomalies. Plant D shows a dissociated hypocotyl vascular trace branching into five cotyledons. The duplicated shoot and root axis of seedling F resulted in a similar pattern of super-numerary cotyledons. Both of these phenomena parallel observations from the development of *hydra* mutants in the absence of *ein2*.

Radialized 'prongs' of tissue are still evident in *hydra-ein2* double mutants, indicated by arrows in B, C, E and F. These structures are common in *hydra* siblings, and may or may not include a differentiated xylem trace.

| | *hyd1-2* (n = 221) | *hyd1-ein2* (n = 135) | *hyd2* (n = 178) | *hyd2-ein2* (n = 189) |
|---|---|---|---|---|
| Strand dissociation above the SAM only | 54 (24.4%) | 25 (18.5%) | 48 (27.0%) | 40 (21.2%) |
| Dissociation within the hypocotyl stele | 134 (60.7%) | 92 (68.2%) | 120 (67.4%) | 140 (74.1%) |
| Full axis duplication | 33 (14.9%) | 18 (13.3%) | 10 (5.6%) | 9 (4.7%) |

Table 5.2; Axis duplication and dissociation in *hydra* and *hydra-ein2* siblings

### 5.3.1.1.3 Patterning of procambial strands in *hydra-ein2* mutant embryos is indistinguishable from the patterning phenomena seen in *hydra* embryos

The p*AthB8*::GUS reporter of pre-procambial cell identity (Baima *et al* 1995) shows an expression pattern in *ein2* mutant embryos (Fig. 5.3; A, B) which is indistinguishable from that in wild-type embryos expressing this transgene. Similarly, expression of p*AthB8*::GUS in *hydra2-ein2* embryos (Fig. 5.3; C-F) is indistinguishable from that seen during *hyd2* embryogenesis (Fig. 4.3). The double mutant embryos demonstrate heightened reporter activity, precluding cell specific definition (Fig. 5.3; C, D), with peaks of expression in specific locations (Fig. 5.3; E, F).

There is no clear evidence to date that the *EIN2* gene is active during embryogenesis. As procambial patterning in the embryo determines the route of the differentiating vascular network in the post-germination seedling, it would appear from the data in Table 5.2, and the results from the p*AthB8*::GUS reporter in Fig. 5.3, that the *ein2* mutation does not affect embryogenesis, either in terms of patterning phenomena, or in the modulation of gene expression.

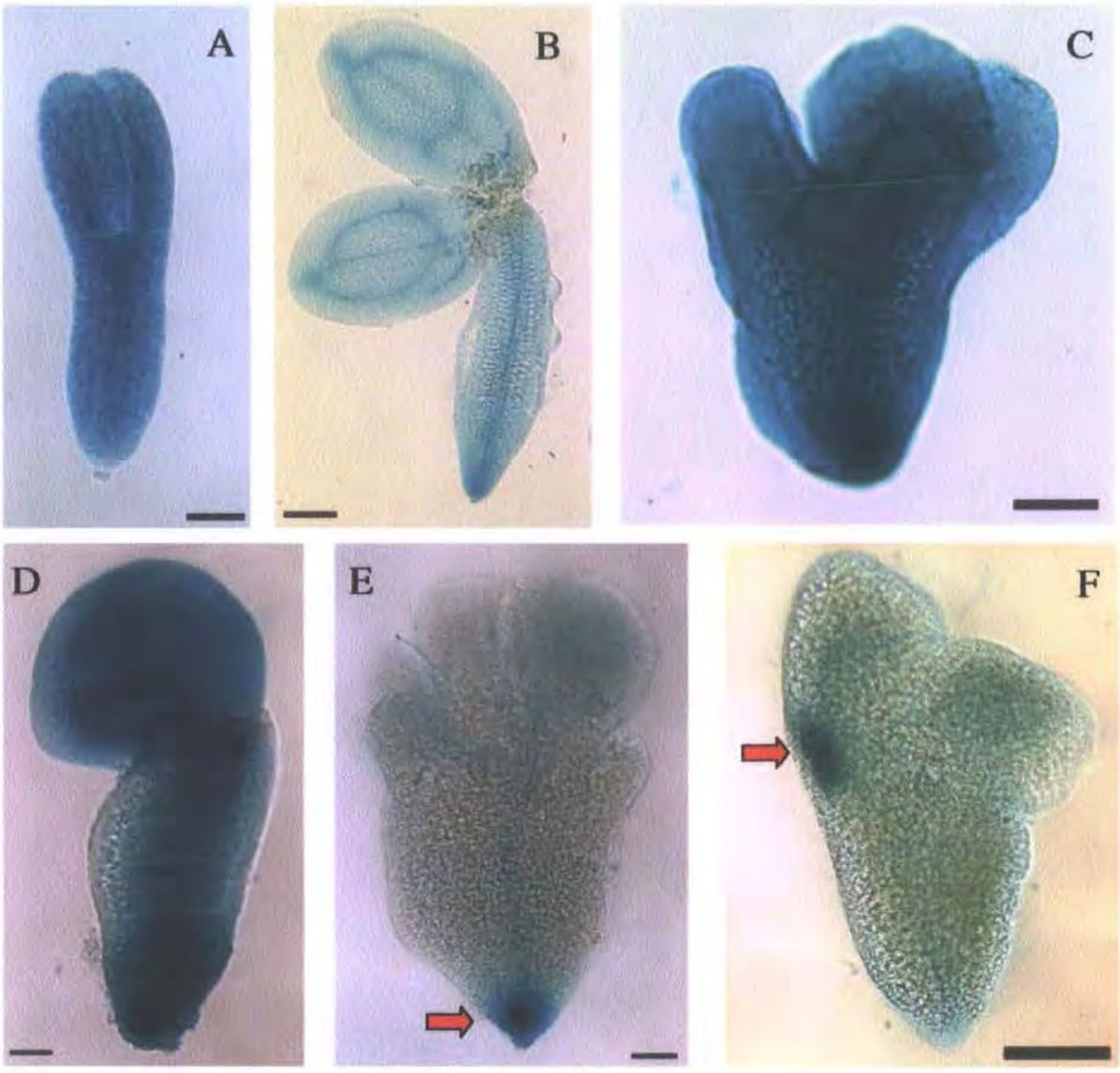
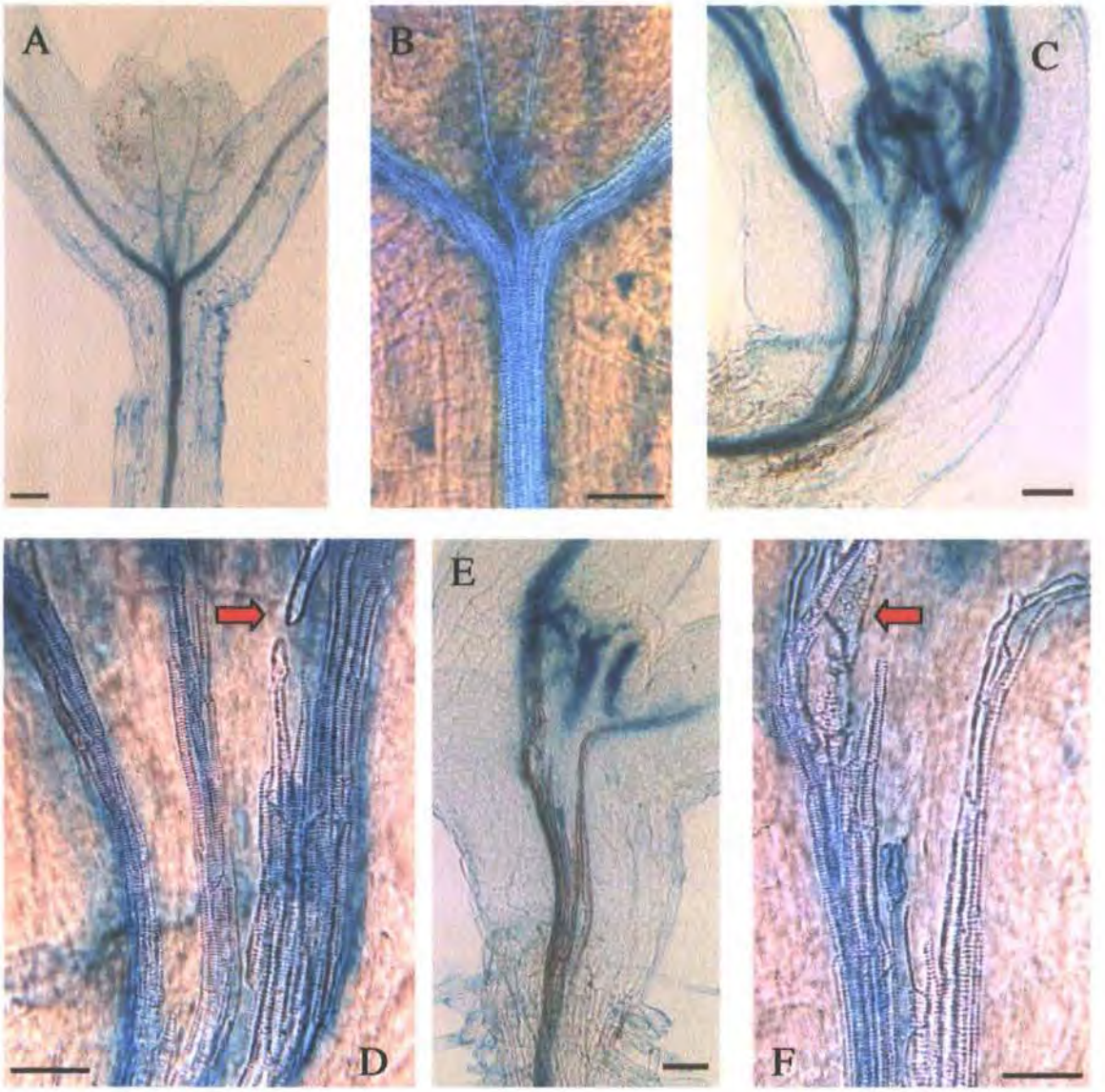



Figure 5.3 Provascular traces in *hydra-ein2* double mutant embryos

The expression of the p*AthB8*::GUS provascular tissue marker in *ein2* shows an expression pattern indistinguishable from that observed in wild-type plants carrying this construct. A; torpedo stage, and B; bent cotyledons stage embryos from plants homozygous for the *ein2* mutation.

Likewise, embryos of *hydra2-ein2* (C-F), show similar patterns to those observed in single mutants. These include indistinct traces in pro-hypocotyl or cotyledon tissues (C, D), longitudinal dissociation (F), strand divergence in the upper hypocotyl region producing multiple cotyledons (E) and unusual localised peaks of marker expression (E, F, arrows).

B, bar = 100$\mu$m; A and C-F, bar = 50$\mu$m.




## Figure 5.4 Longitudinal integrity of hypocotyl vascular strands in *hydra-ein2* double mutants

A; *ein2* at 5 dae, carrying the p*AthB*8::GUS marker of provascular tissue identity (bar = 100$\mu$m). B; detail of vascular strand formation in the upper hypocotyl, taken from the same plant shown in A (bar = 50$\mu$m). C (detail D) and E (detail F), *hyd2-ein2* double mutant seedlings at 5 dae showing activity of the p*AthB*8::GUS reporter. C, E, bar = 100$\mu$m; D, F, bar = 50$\mu$m.

The pre-provascular and vascular trace of *ein2* mutant plants (A, B) as shown by this reporter is indistinguishable from wild-type.

The *hydra* mutation in an *ein2* background shows a greater coherence of xylem differentiation in hypocotyl primary vascular strands than is observed in single *hyd* plants. However where axis dissociation occurs (C, E), cell files may show anomalies in xylem coherence (D, arrow) and unusual cell shapes or sizes (F, arrow). Provascular traces are coherent as in *hydra* single mutants, but similarly, may be dissociated.

### 5.3.1.2 Vascular strand coherence and integrity of longitudinally oriented cell files in *hydra-ein2* double mutants

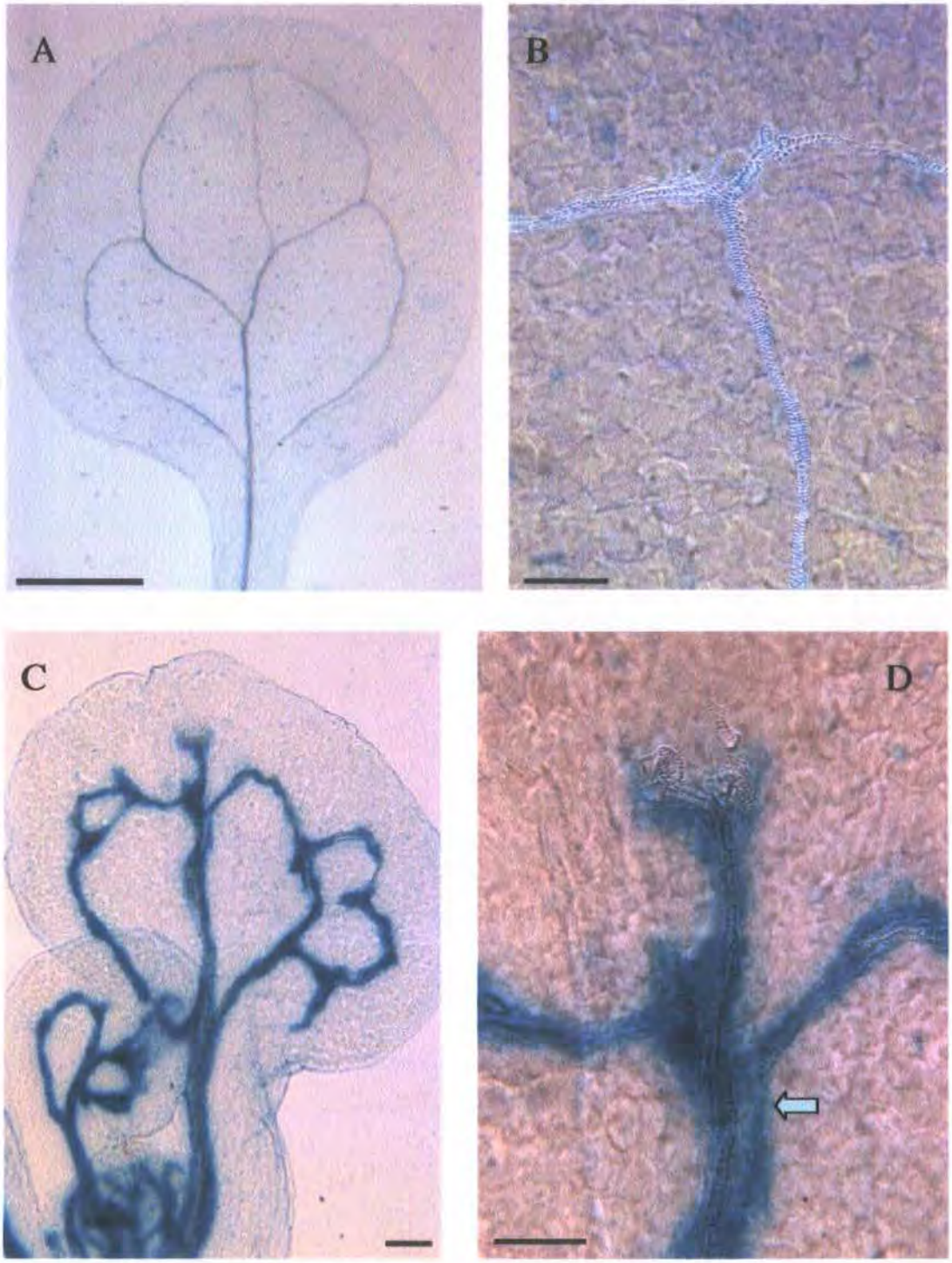
#### 5.3.1.2.1 Introduction of the *ein2* mutation into *hydra* results in a greater coherence of xylem strands in the hypocotyl

Fig. 5.4 shows seedlings of *ein2* and *hyd2-ein2* at 5 dae, expressing the p*AthB8*::GUS procambial cell identity reporter. The activity of this transgene in *ein2* seedlings (Fig. 5.4; A, B) is indistinguishable from wild-type. Similarly, the patterns of differentiating xylem traces in wild-type and *ein2* show no distinguishing characteristics. This may indicate that wild-type ethylene mediated auxin signalling is independent of auxin-upregulated HD-Zip gene activity such as seen with *AthB8*.

In the *hyd2-ein2* examples shown in Fig. 5.4; C and E (with detail in D and F respectively), a greater strand coherence is observed than was seen in *hyd2* single mutants (Fig.s 4.4 and 4.5). In these double mutants, an improved xylem integrity, and a xylem pattern more closely corresponding to the procambial trace, appears to be achieved by a greater expansion of fewer xylem vessels, resulting in some unusual cell morphologies such as indicated in Fig. 5.4; F (arrow). The xylem trace is not completely coherent in these mutants, shown by the disjunct xylem indicated in Fig. 5.4; D (arrow).

#### 5.3.1.2.2 The *ein2* mutation allows the development of a more coherent primary vascular strand in *hydra* cotyledons

As in hypocotyl tissues, the xylem traces of cotyledon primary vascular strands in *hydra-ein2* mutants (Fig. 5.5; C, detailed in D) are more coherent than observed in *hydra* single mutants (Figs. 4.8 and 4.9). Less 'noise' is

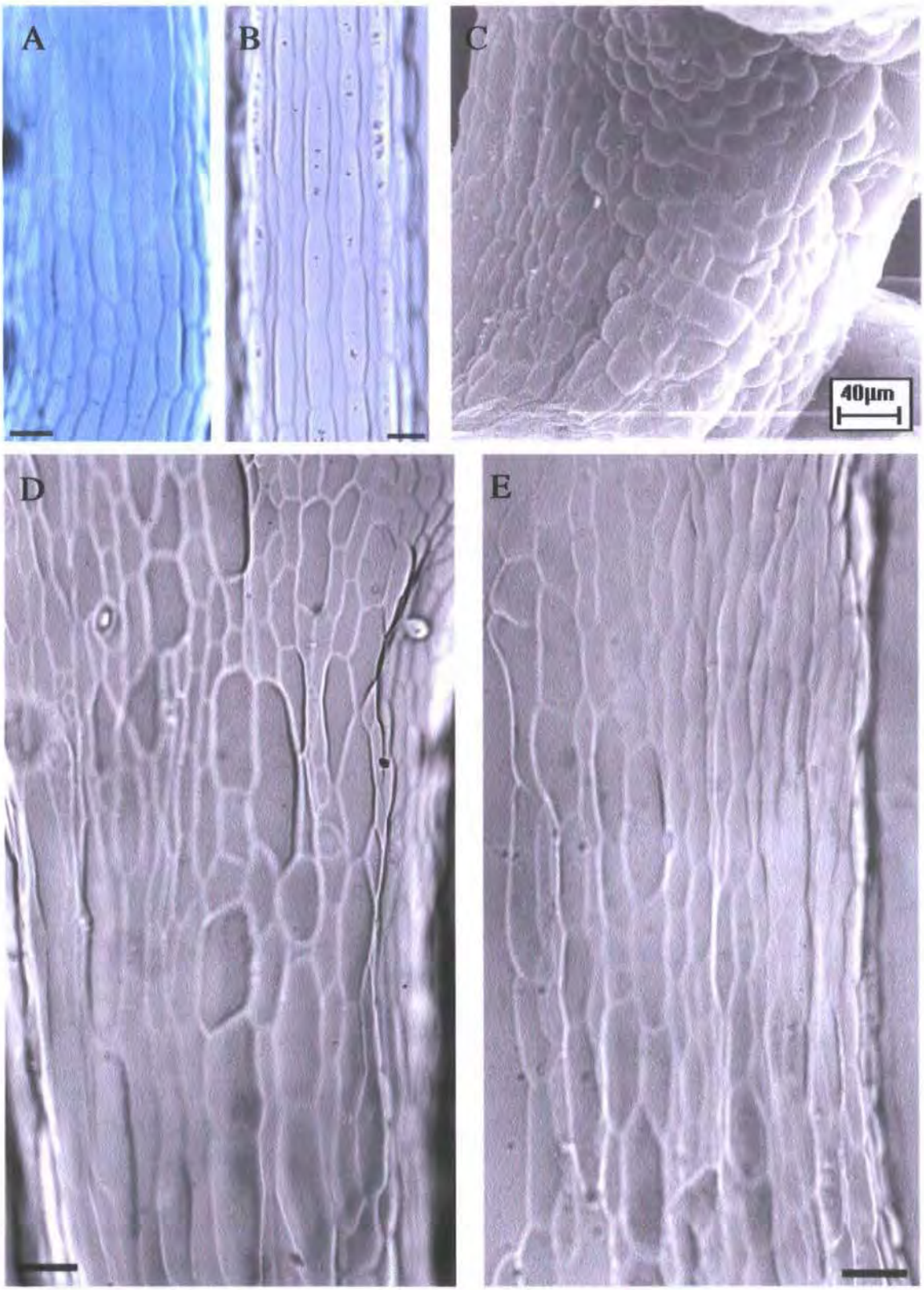



## Figure 5.5 Cotyledon primary vasculature in *hydra2-ein2*

A; ***ein2*** cotyledon at 5 dae, showing expression of the p*AthB8*::GUS provascular tissue marker construct. B, detail of *ein2* cotyledon hydathode; these nodes are found in the apex of the organ, and represent the point of most 'noise', i.e. strand incoherence, in differentiated cotyledon tissues.

The two cotyledons from ***hyd2-ein2*** shown in C have better laminar expansion and a more normal vascular trace. There is a greater coherence of the primary vascular strand, with less 'noise' in xylem strands at the hydathode and primary midvein than seen in single mutants (see figure 4.1.4). However vascular coherence in *hyd2-ein2* is much less, and xylem noise greater, than Ws or *ein2* plants with wild-type genes at the *HYDRA* loci.

A-D taken from 5 dae plants. A, bar = 0.5mm; C, bar = 100$\mu$m; B and D, bar = 50$\mu$m.




## Figure 5.6 Hypocotyl epidermal cell files in *hydra1-ein2* double mutants

**The *ein2* mutant hypocotyl epidermis has similar patterning to wild-type but has a reduced number of larger cells per unit area; this can be seen using agarose epidermal prints of Ws (A), and *ein2* (B) at 6 dae (bar = 50$\mu$m). The epidermis of *hyd1* mutants shows some compromised longitudinal integrity of cell files, and a range of cells sizes (C). The *hydra1-ein2* hypocotyl epidermal pattern can vary between siblings (D and E, bar = 50$\mu$m), though shows an improvement in longitudinal integrity compared with single mutant *hydra* seedlings. A similar improvement is seen between *hyd2* and *hyd2-ein2* (data not shown).**

evident within the xylem, and the vessel trace more closely follows the procambial signal defined by p*AthB8*::GUS.

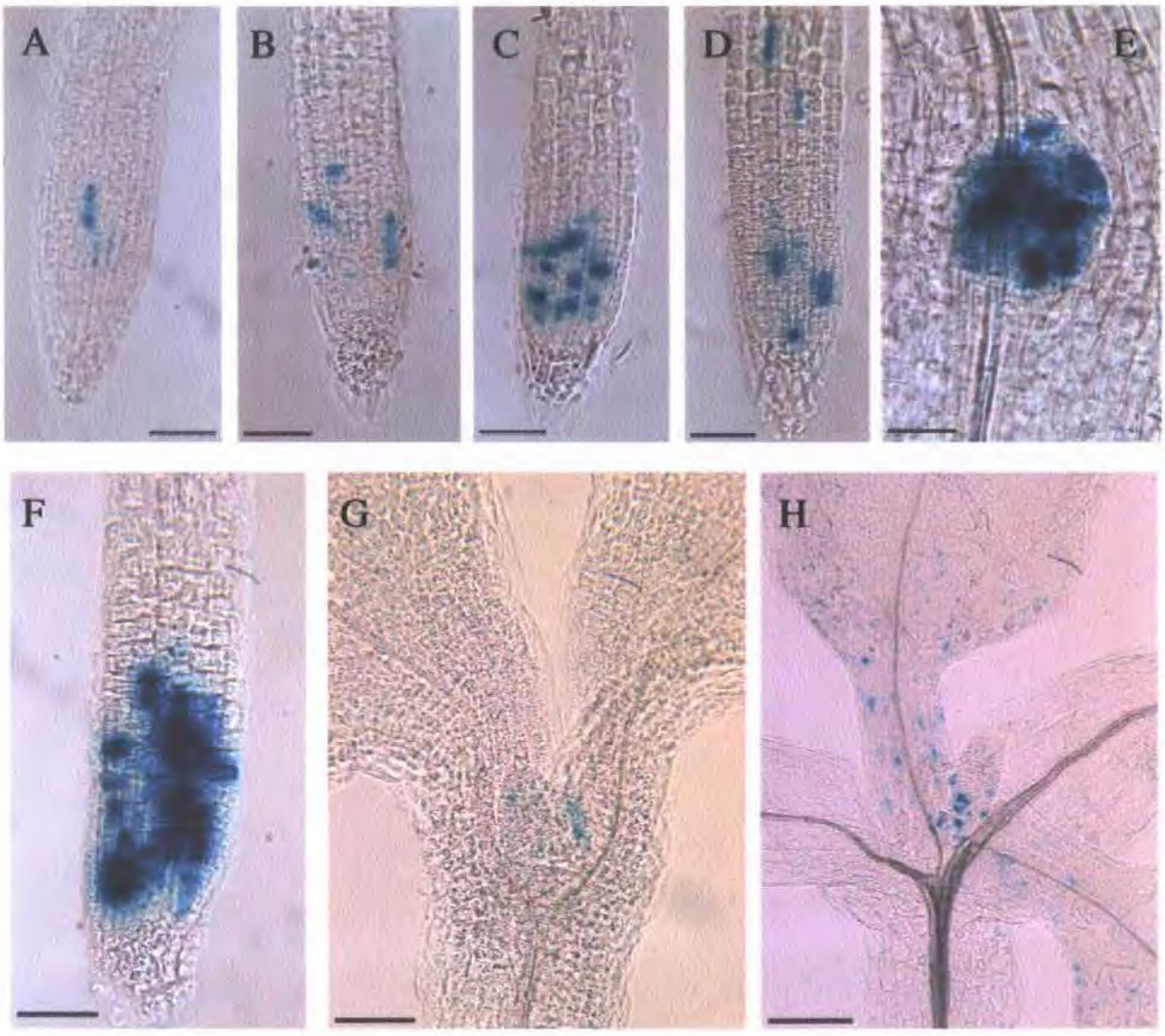
#### 5.3.1.2.3 Hypocotyl epidermal cell files in *hydra-ein2* double mutants show improved longitudinal integrity and greater cell expansion than in *hydra* single mutants

Fig. 5.6 contrasts wild-type and *ein2* hypocotyl epidermal patterning (shown respectively in Fig. 5.6; A and B). The cells of *ein2* mutants are larger than wild-type; this size difference is evident as an enhanced expansion along the longitudinal axis. The two control seedlings have similar integrity of the longitudinal cell files. Epidermal patterning in the hypocotyl of *hyd1* (Fig. 5.6; C) also contrasts with that of the *hyd1-ein2* hypocotyl epidermis (Fig. 5.6; D and E). It appears from these pictures that the double mutant has greater integrity of longitudinal cell files, in association with an improved cellular longitudinal expansion and a reduction in cell numbers per unit area.

A reduction in cell numbers has been observed previously in *ein2* (Lake *et al.* 2000). This means that the improved integrity of these cell files (along with the improved xylem integrity described in sections 5.3.1.2.1 and 5.3.1.2.2) are attributable in part to a modulation of the cell cycle towards earlier differentiation.

## 5.3.1.3 Cell division and expansion in longitudinal cell files

#### 5.3.1.3.1 A reduced number of cell division events is evident in both *ein2* and *hydra-ein2* mutant seedlings

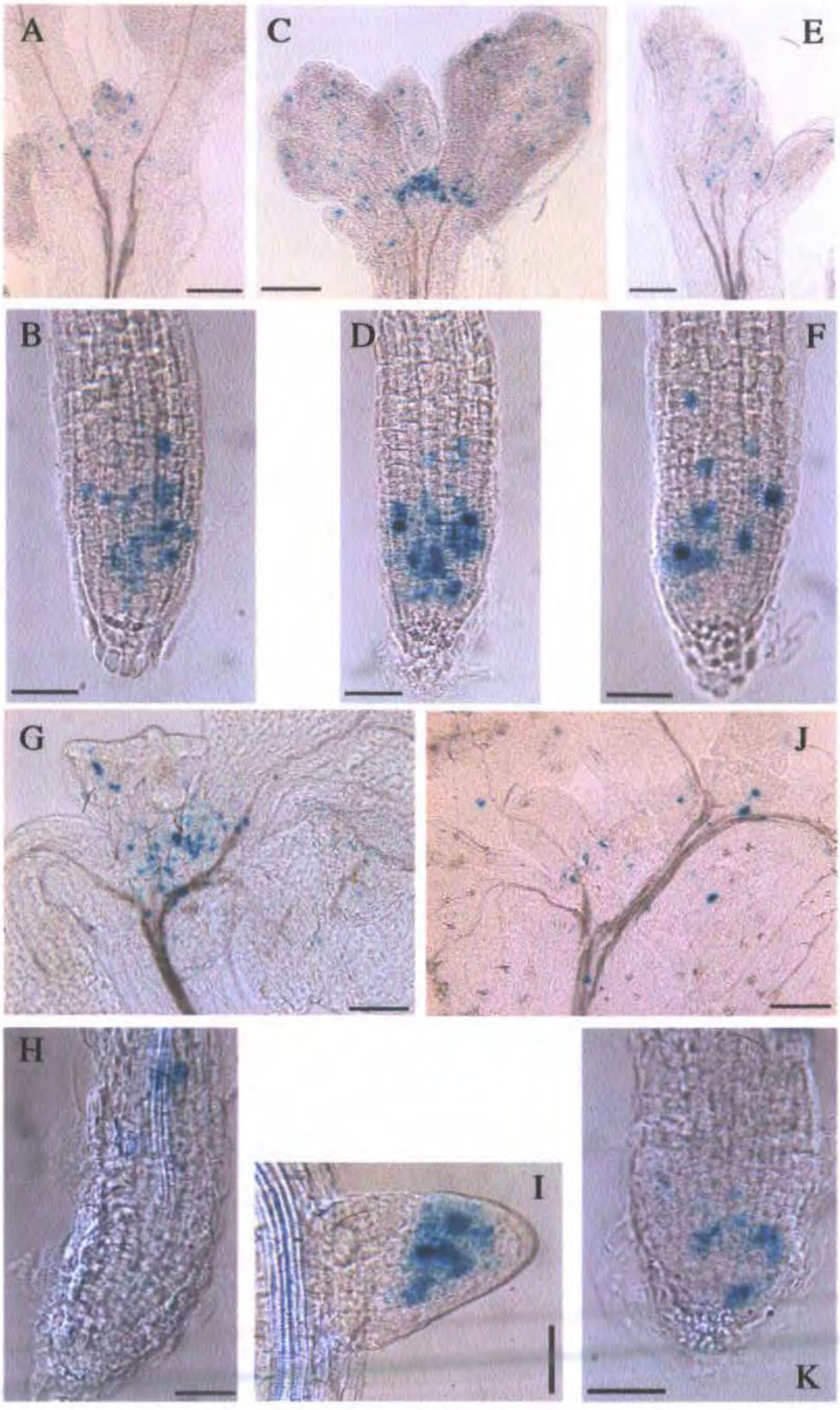



## Figure 5.7 Cell division in *ein2* seedlings

A-D; primary root apices, and E; initiating lateral (anchor) root from 3dae *ein2* seedlings expressing the p*CYC1At*::CDB::GUS reporter. F; *ein2* seedling root apex at 7dae. Bar = 50$\mu$m.

The establishment of cell division events in the emerging primary root of *ein2* seedlings appears much slower than in wild-type plants expressing this reporter construct. As the *ein2* roots are not compromised in length, this suggests a greater reliance upon cell expansion for growth at this stage, made possible by the reduced cell numbers and greater cell size found in these seedlings. However emerging laterals (E) appear to have a normal activity. The establishment of laterals appears to progress more rapidly in *ein2* than in wild-type roots. A more normal pattern of cell division is established in the*ein2* root apex by 7dae (F), though with some seedling roots having areas of unusually dense reporter activity, as in the example shown. A more regular pattern is observed in wild-type (Fig. 4.2.1). This may suggest that the *ein2* mutant is modified in the way it regulates cytokinesis patterns, and seems to undergo a more rapid progression to differentiation.

G and H; seedling shoot apices at 3dae (G, bar = 100$\mu$m) and 7dae (H, bar = 200$\mu$m). Shoot expression of the reporter is similar to that seen in wild-type (Fig 4.2.1), although *ein2* initiates and expands its rosette leaves at a more rapid rate (H), again through a more rapid transition to differentiation and cell expansion.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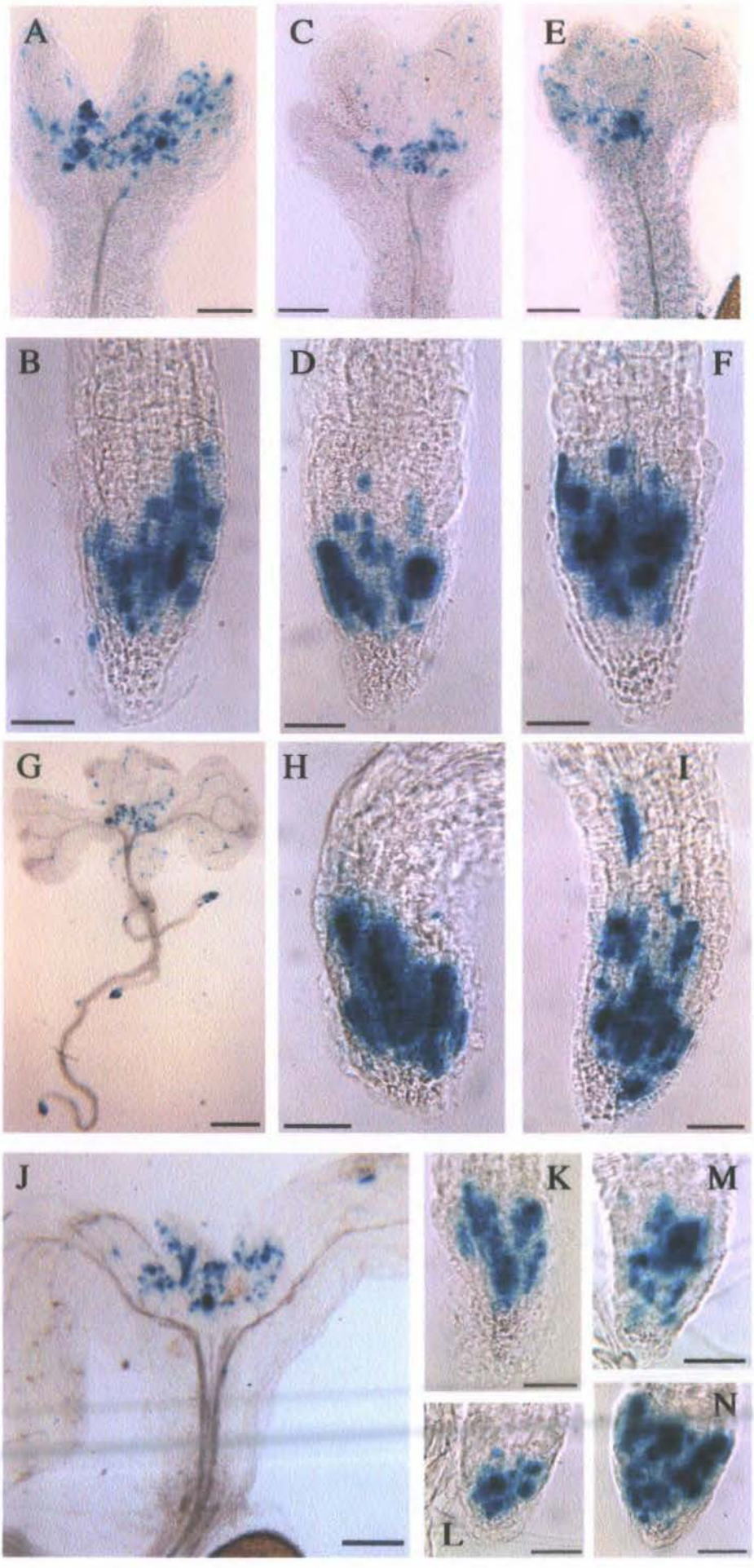
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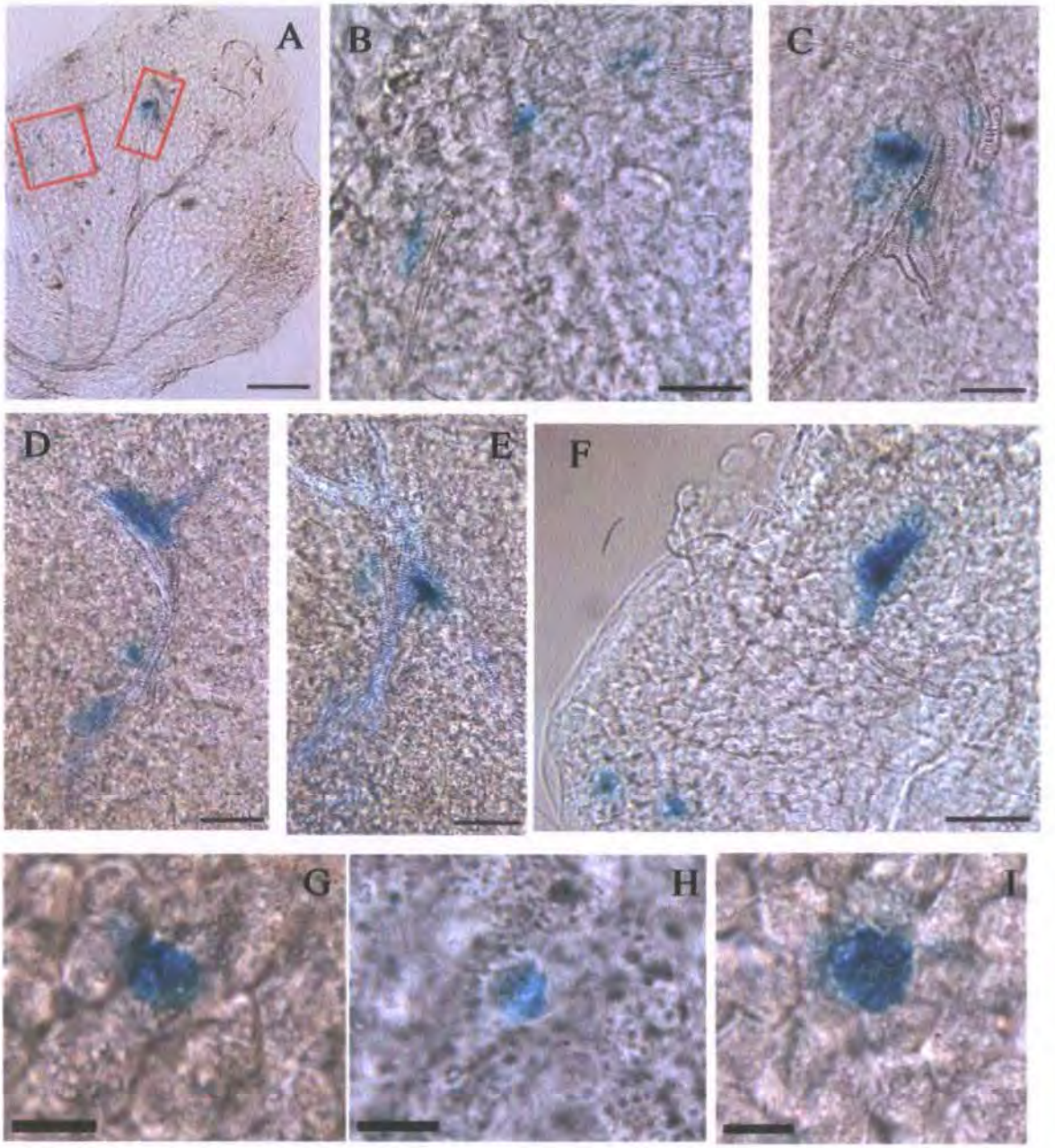
The initiation of cell division events at the root apex (Fig. 5.7; A-D) and in emerging true leaf primordia (Fig. 5.7; G, H) occurs more slowly in *ein2* than in wild-type post-germination seedlings. As expansion growth appears to proceed more rapidly in both root and shoot tissues of *ein2*, this implies a more rapid developmental transition from division to differentiation and elongation in these mutants. Lateral root apices of *ein2* (Fig. 5.7; E and F) appear to have a cell division pattern similar to that observed in wild-type laterals, suggesting a differential regulation of these processes in primary and lateral root apices.

In both shoot and root primary apices at 3 dae, *hyd1-ein2* had a greater number of cell divisions than its *ein2* background, as shown by the *CYC1At*::CDB::GUS reporter in Fig. 5.8; A-F. There are minimal differences between shoot cell division activity in these seedlings and the *hyd1* single mutants of the same age class, (Fig. 4.19; A and B), although some reduction in cell division in root apices like that observed in *ein2* is visible in these examples (Fig. 5.8; B, D, F). A greater reduction in cell division is visible in root and shoot apices of *hyd1-ein2* at 7 dae (Fig. 5.8; G-K), in contrast to that of the *ein2* background, although a *hyd1-ein2* retains more cell division events in lateral roots than *ein2* primary roots. These observations suggest that the reduction in cell division and an emphasis on cell elongation in *hyd1-ein2* is combining the mutations in an additive manner.

In contrast to both *hyd1-ein2* and *ein2*, the *hyd2-ein2* primary apices at 3 dae reveals a heightened GUS activity from the *CYC1At*::CDB::GUS reporter in both roots (Fig. 5.9; B, D, F) and shoots (Fig. 5.9; A, C, E). This enhanced activity is retained in *hyd2-ein2* 7 dae seedlings, bringing the shoot activity into a similar level as observed in *hyd1-ein2* and maintaining a more viable primary root apical meristem than in *hyd2* single mutants. Lateral root apices of *hyd2-ein2* at 7 dae appear to have a more normal cell division activity than that seen in *hyd1-ein2* laterals, although this may be misleading, as numerous large and irregularly shaped cells in the process of dividing are highlighted in the epidermis by this reporter (Fig. 5.9; L-N).




## Figure 5.10 Ectopic cell division events in *hyd2-ein2* cotyledons

A-I; *hyd2-ein2* cotyledon detail from 7 dae seedlings, showing ectopic expression of the p*CYC1A*t::CDB::GUS marker of cell division events. B and C; detail of the areas of vasculature highlighted in the cotyledon shown in A. D; vascular branch point from a different cotyledon. E and F; isolated 'islands' of xylem vasculature from different *hyd2-ein2* cotyledons. G-I; epidermal cell division events. A, bar = 200$\mu$m; B-F; bar = 50$\mu$m; G-I, bar = 20$\mu$m.

Although *ein2* results in fewer cell division events and an increased cell size, *hydra-ein2* cotyledons continue to show ectopic expression of this reporter, mostly near anomalies in xylem strand integrity as in single *hyd* mutants, as in C and D. B shows an area of incomplete secondary loop formation from cotyledon A; here, ectopic cell divisions are taking place in an area where the outlines of organized cell files connecting the two xylem ends are just visible. This pattern is in contrast to both wild-type and *ein2*, where no cell division occurs in the post-germination cotyledon mesophyll. G and H show division events producing stomatal guard cell pairs from a guard mother cell parent; stomates differentiate in wild-type cotyledons around 7 dae, but is already over in *ein2* by this point. I shows a cluster of dividing cotyledon epidermal cells without obvious cell fate.

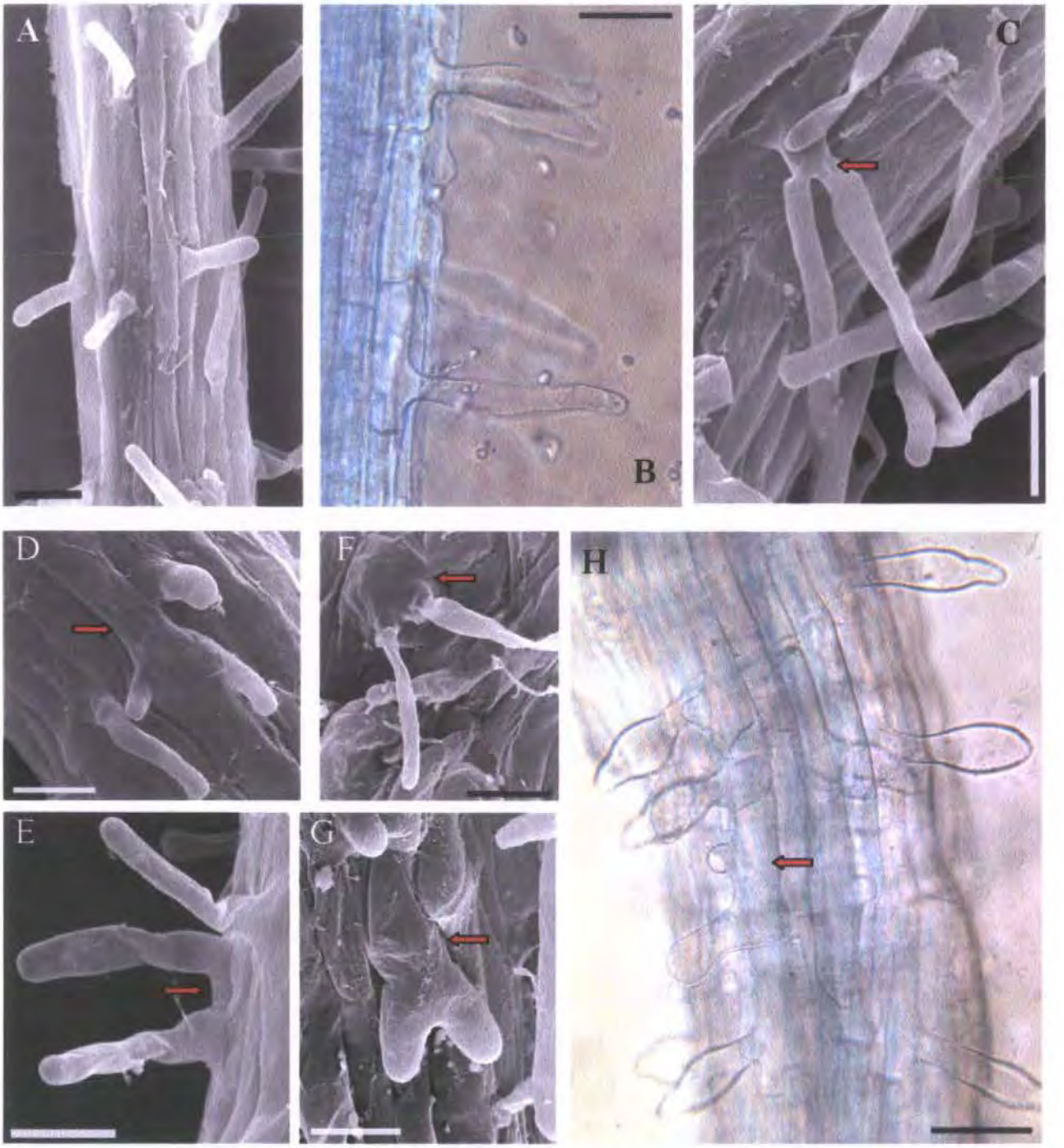
### 5.3.1.3.2 The reduced cell division events seen in the *ein2* background are not sufficient to abolish ectopic cell division in *hydra-ein2* cotyledons

Fig. 5.10; A, shows a 7 dae *hyd2-ein2* cotyledon expressing the *CYC1At*::CDB::GUS transgene. As in *hydra* single mutants, ectopic cell division events are visible in the double mutant cotyledon mesophyll; a tissue type which does not undergo post-germination cytokinesis in wild-type (Tsukaya *et al.* 1994) or *ein2* (Fig. 5.10; G). Ectopic cell division events in *hydra* double mutants were found in similar positions as in single mutants, in association with compromised xylem integrity (Fig. 5.10; C, D), vascular islands (Fig. 5.10; E, F) and in association with late-differentiating xylem (Fig. 5.10; B).

Other occasional cell division events were noted in the double mutant cotyledon epidermis (Fig. 5.10; G-I). Wild-type 7 dae cotyledons showed few epidermal cell division events in association with differentiating guard cell pairs, although these were not found in 7 dae *ein2* seedlings, which undergo a more rapid tissue maturation. In *hyd2-ein2*, these epidermal cell divisions highlighted guard cell pairs (as in Fig. 5.10; H), or clusters of cells (Fig. 5.10; G, I) that may give rise to clustered stomata.

### 5.3.1.3.3 Morphological anomalies in root hair development are retained in the *hydra-ein2* double mutants

The development of root hairs in *ein2* is reduced in relation to wild-type (Su & Howell 1992) attributed to the loss of ethylene perception in these mutants (Tanimoto *et al.* 1995, Dolan *et al.* 1997). The *ein2* root epidermis (Fig. 5.11; A, B) has elongated cells, with root hair growth that is slightly shorter than wild-type under normal growth conditions (Su & Howell 1992). The longitudinal file of trichoblast cells in Fig. 5.11; B, shows a cell which




## Figure 5.11 Root hair initiation in *ein2* and *hydra-ein2* double mutants

The reduced ethylene environment of the *ein2* mutation confers a reduction in the numbers of cells in trichoblast cell files that initiate root hairs (A), but does not affect the longitudinal position of the emerging root tip is as observed in wild-type plants. B shows young hair cells from an *ein2* root grown in agar on a coverslip; this 'in vivo' example shows normal hair morphology.

C-G; root hair morphology in *hyd2-ein2* seedlings includes multiple tip initiations (arrows) across the radial axis (C, D, F and G), and also some examples of longitudinal tandem root hairs emerging from a single trichoblast (E).

H shows a *hyd1-ein2* lateral seedling root grown in agar under the same conditions as the *ein2* root shown in B. The arrow highlights a trichoblast cell with two points of root hair initiation. Note also the unusually inflated morphology of these growing tips.

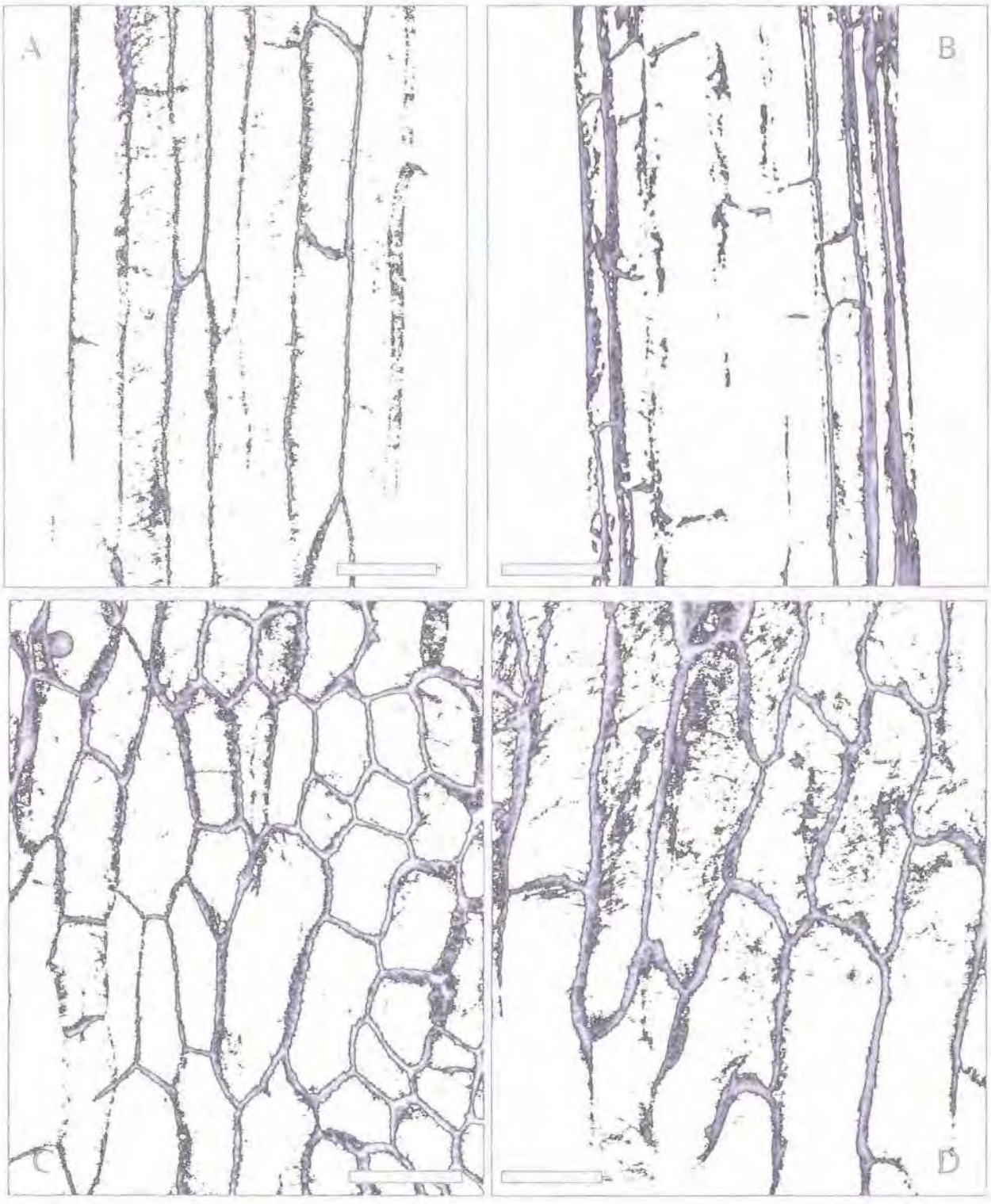
A-H, bar = 50$\mu$m.

has not adopted hair cell fate, amongst adjacent hair cells. The reduction in root hairs found in the *ein2* mutant therefore appears to result from a lesser number of trichoblast cells opting for hair cell fate. Polarity of hair cells in *ein2* appears normal.

Root hair production in *hydra-ein2* is reduced (Fig. 5.1; F). However, the formation of root hairs in *hyd1-ein2* and *hyd2-ein2* demonstrates a similar spectrum of morphological anomalies as in *hydra* single mutants. These include reversal of hair cell polarity, swelling of the root tip emergence region, branched root hairs (Fig. 5.11; C, D, F, G), or initiation of multiple tip growth positions in alignment with the cell's longitudinal axis (Fig. 5.11; E, H). As in *hydra* (Fig. 4.25; E, F), cells in the root epidermis have compromised expansion growth in the longitudinal axis, although this is less severe in double than single mutants (Fig. 5.11; H). Some swelling of the cells is evident, both at the base of the hair initiation point (as in Fig. 5.11; F), and in the expanding hairs themselves, shown in the agarose-grown sample in Fig. 5.11; H. This was not observed in the controls (e.g. Fig. 5.11; B).

### 5.3.1.3.4 The double mutant hypocotyl epidermis has a more coherent alignment of cellular expansion axes, and more regular arrangement of cortical microtubular arrays

Fig. 5.12 compares expanding hypocotyl cells from wild-type (Fig. 5.12; A) with *ein2* (Fig. 5.12; B), *hyd1* (Fig. 5.12; C) and *hyd1-ein2* (Fig. 5.12; D). A similar directionality of the cortical microtubular arrays is evident in the two controls. A slightly oblique angle in the *ein2* sample corresponds to the angle of the transverse walls within each cell file. In contrast, a substantial difference between *hyd1* and *hyd1-ein2* is apparent, both in the greater cohesion of cell files in the double mutant, and the overall improved regularity of microtubular arrays relative to the longitudinal expansion axis.




## Figure 5.12 Microtubule orientation during active expansion in hypocotyl epidermal cell files of *hyd1* and *hyd1-ein2*

A; Wild-type and B; ein2 plants carrying the GFP::TUA6 reporter construct, highlighting the cortical microtubular arrays in the zone of expanding hypocotyl cells of 5 day seedlings. C; *hyd1*, and D; *hyd1-ein2* double mutants, showing sub-cellular localization of the same reporter.

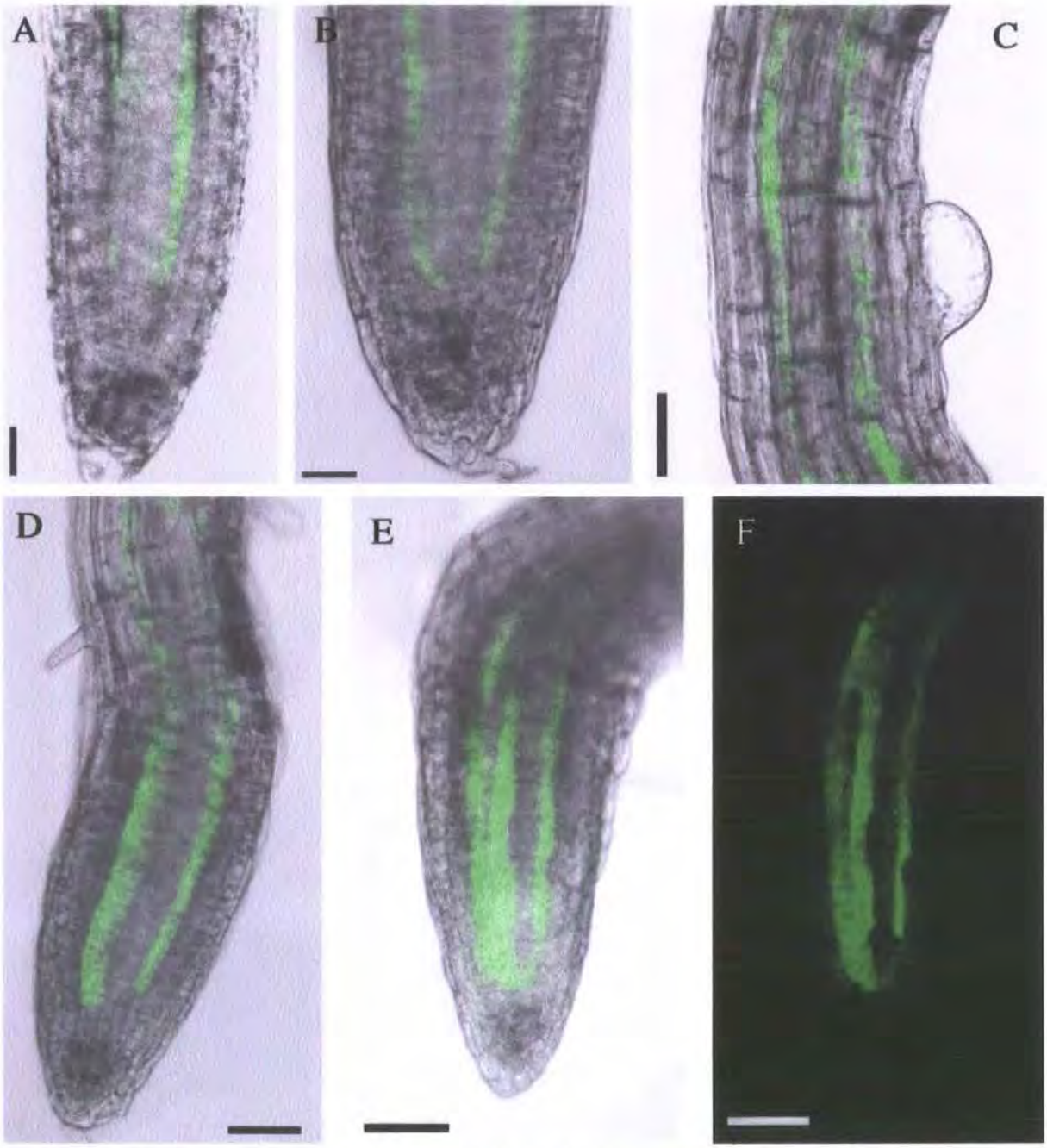
A-D, bar = 50μm.

## 5.3.1.4 Cell layer identity and integrity across the radial axis

### 5.3.1.4.1 Integrity of the endodermal cell layer in the *hydra* root is marginally improved by the presence of *ein2*

The *ein2* root apex shows expression of the SCR::GFP reporter (Fig. 5.13; A) in a similar manner to that observed in wild-type apices (Fig. 4.32; A). In *hydra-ein2* double mutants a pattern comparable to that in *hyd* single mutants was seen, although expression was slightly more intense in the doubles, and had a reduction of diffuse GFP fluorescence such as seen in some *hydra* single mutant roots (e.g. Fig. 4.32; B). The improved epidermal cell file patterning of the double mutant roots appeared to correspond with an improved integrity in the endodermal cell files, which showed SCR::GFP further up the root away from the tip in some siblings (e.g. Fig. 5.13; B and C). Note the reduction in root hair cells, and the enlarged epidermal cell visible in C.

Cell files with a better longitudinal integrity expressed a stronger GFP signal. A stacked Z-series of a whole *hyd2-ein2* mutant root apex is shown in Fig. 5.13 with (E) and without (F) the transmission image overlain. The distinct set of three traces in Fig. 5.13; E, which appeared at first as a possible axis duplication, was the result of certain longitudinal cell files more strongly expressing endodermal identity. These cell files within the root appear unusually large, which both explains the dominance of their SCR::GFP signal, and suggests that reduced rates of cell division in *hyd-ein2* results in larger cells and a more contiguous function of cell files.




## Figure 5.13 Expression of the *SCR::*GFP reporter in *ein2* and *hyd2-ein2* roots

A-F, 3dae seedlings; bar = 50$\mu$m. The *ein2* root apex in A shows SCR::GFP expression in a similar manner to that observed in Ws (as shown in Fig. 4.32).

As with *hydra* single mutants, the *hyd2-ein2* siblings show some variation in the activity of this reporter. B and C show respectively the root apex and a section of mid-root from the same *hyd2-ein2* seedling. An improvement in radial alignment is evident in comparison with *hyd2* single mutants. Here, the better organisation of the root apex allows a less diffuse expression of *SCR*::GFP in what appears to be a single cell layer. The *hyd2-ein2* root apex in D shows a similar pattern. The *hyd2-ein2* root depicted in E and F shows a stacked z-series across the uppermost radial half of the root from the central stele region to above the endodermis. The GFP signal (F) shows expression only in certain longitudinal cell files, whereas the expression in wild-type and *ein2* resolves a signal in all cells of this layer across the root. It seems that the better aligned cell files of the endodermis, i.e. those with greater longitudinal integrity, produce a stronger GFP signal.

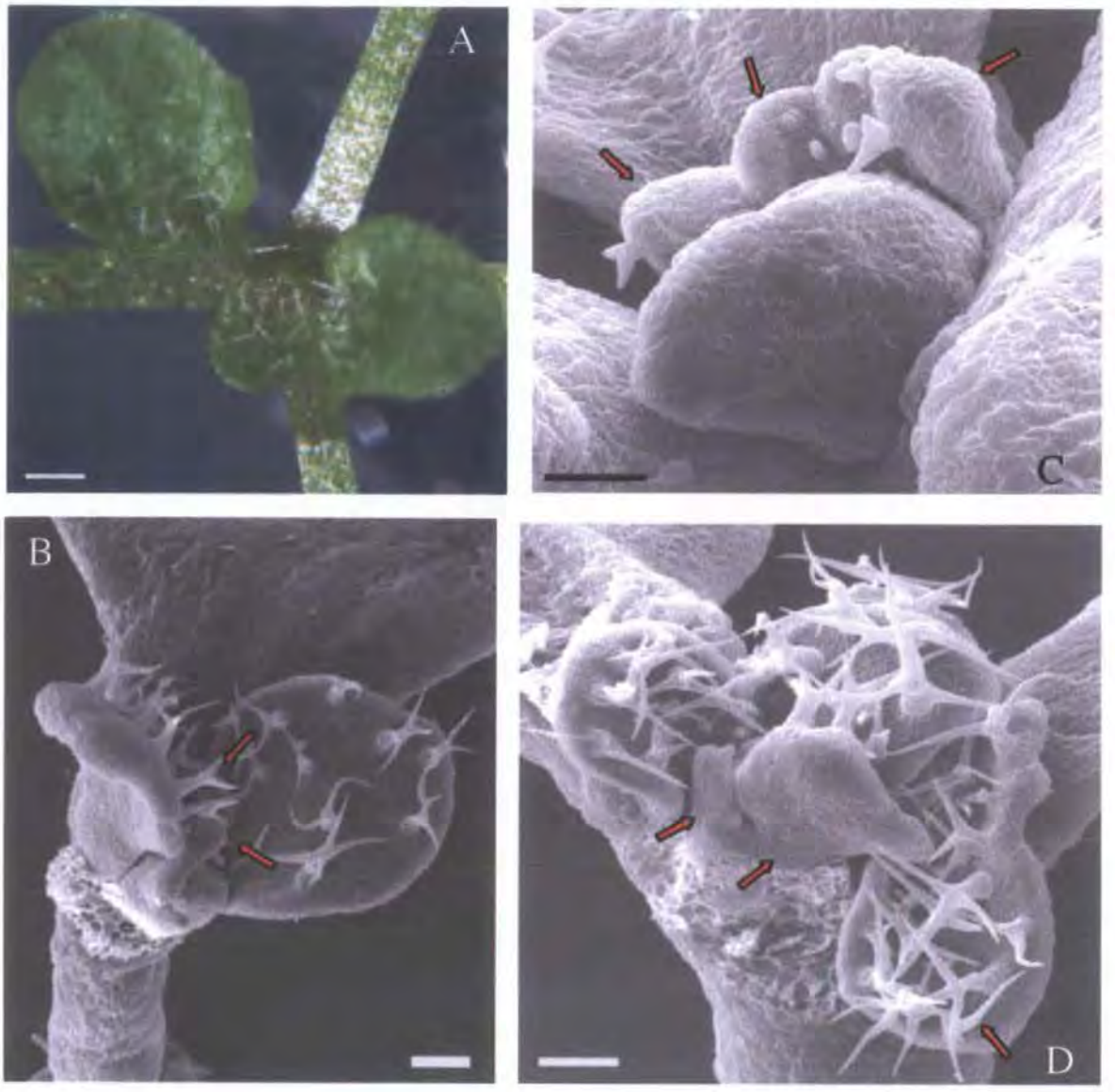
## 5.3.1.5 Phyllotaxy

### 5.3.1.5.1 The rate of leaf initiation is not significantly modified by the introduction of *ein2* into the *hydra* background

The *ein2* mutation promotes a more rapid development and expansion of leaves of the rosette, resulting in a slightly increased number of rosette leaves, and larger laminae (Ogawara *et al.* 2003). Leaf initiation rate did not appear to be modified between *hydra* and *hydra-ein2* double mutants, although most siblings showed a more rapid leaf expansion as well as accelerated root growth. However improved expansion is relative; as can be seen from Fig. 5.14; B-D, the double mutant true leaves do not reach a size comparable with the controls. The rate at which true leaves appear around the SAM was counted for *ein2* control plants alongside the *hyd-ein2* double mutants, and average leaf numbers are presented below along with the wild-type and *hyd* single mutant data, summarised from Appendix 6.

| Ws (n = 20) | *ein2* (n = 20) | *hyd1* (n = 68) | *hyd1-ein2* (n = 89) | *hyd2* (n= 61) | *hyd2-ein2* (n = 68) |
|---|---|---|---|---|---|
| 5.10 | 6.25 | 9.35 | 10.19 | 7.67 | 7.72 |

These data show that the increased leaf number between wild type and *ein2* is greater than the differences between *hydra* and *hydra-ein2*. Ogawara *et al.* (2003) demonstrated that elevated ethylene levels associated with the *eto* mutation cause a reduced number of leaves (less than wild-type) as well as a reduced leaf area, and the *ein2* mutation increased wild-type leaf area by around two thirds, whilst marginally increasing numbers of rosette leaves. The data shows that *ein2* has a minimally additive effect on primordial initiation in *hydra*, which already has a greater rate of leaf initiation. It is possible that the increased leaf number recorded may be due entirely to improved primordial expansion, and hence visibility, during data collection.




## Figure 5.14 Primordial orientation around the *hydra-ein2* SAM

A, *ein2* (bar = 0.5mm), 12 dae plant; B, *hyd1-ein2*; C and D, *hyd2-ein2* double mutants, all 8 dae; all bar = 50$\mu$m.

A; the initiation position and orientation of new primordia around the *ein2* mutant SAM appears morphologically indistinguishable from wild type, although the rate of leaf expansion is greater in *ein2* seedlings.

B-D shows *hyd-ein2* double mutant plants, selected for the presence of only two cotyledons and apparently one apical meristem. The *hyd1-ein2* plant in B has one cotyledon removed. The first pair of new primordia appear correctly positioned and orientated, but subsequent true leaves are emerging without proper alignment around the central SAM. The primordia indicated (arrows)have adaxial surfaces facing at an oblique angle to the putative origin of the phyllotactic spiral, although their position is not obviously wrong. A similar situation is seen in *hyd2-ein2*, shown in D (arrows). In these leaves, some expansion in the centrolateral plane appears to be taking place, rather than in the longitudinal axis. The *hyd2-ein2* seedling in C appears to have initiated three new primordia simultaneously (arrows); these leaves are partially fused, and show variable orientation of their adaxial (trichome bearing) surfaces.

### 5.3.1.5.2 Expanding primordia around the *hydra-ein2* double mutant SAM are mis-oriented in relation to the phyllotactic origin as in single *hydra* mutants

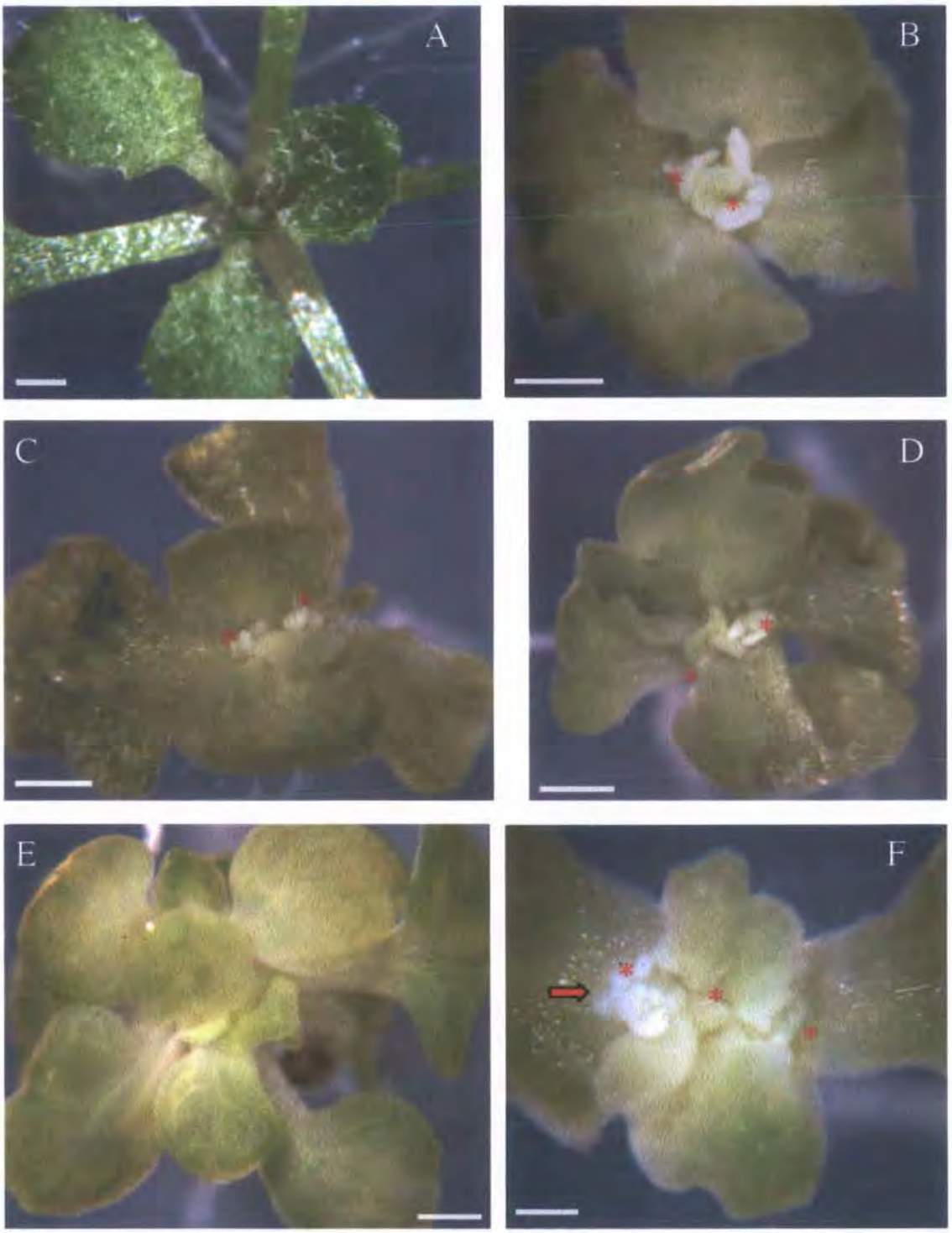
Fig. 5.14; A shows the shoot apex of a 12 dae *ein2* plant, where the phyllotactic spiral is patterned as in wild-type, and the leaves are positioned with their centrolateral axes at right angles to the leaf longitudinal axis, so that all adaxial surfaces of emergent primordia face in towards the meristem.

This alignment is disrupted in both *hydra* mutants (Fig. 4.34) and in *hydra-ein2* (Fig. 5.14; B-D). These examples show seedlings with misaligned centrolateral axes, independent of phyllotactic position around the SAM. Misaligned laminae are found in plants with correctly positioned leaves (Fig. 5.14; B), incorrectly positioned and excessive numbers of leaves (Fig. 5.14; D), and in the components of composite organs originating from several fused primordia (Fig. 5.14; C).

### 5.3.1.5.3 The *ein2* mutation neither modifies *hydra* phyllotaxy, nor reduces multiple and ectopic primordial initiation in the cotyledon-hypocotyl transition zone

The *ein2* phyllotactic series is as seen in wild-type plants (Fig. 5.15; A). A selection of *hydra-ein2* plants, chosen at 4 dae for the presence of two approximately equally sized cotyledons, were grown on horizontal plates until 12 dae, and harvested for examination as for single *hydra* mutants (section 4.3.5).

A similar range of phenomena were observed in *hydra-ein2* double mutants as were apparent in the single mutant siblings. The *hyd1-ein2* seedlings shown in Fig. 5.15; B-F demonstrate multiple SAM formation; between two and three SAMs have formed at the hypocotyl-cotyledon transition zone (indicated by asterisks). Similar phenomena was found in *hydra2-ein2*,

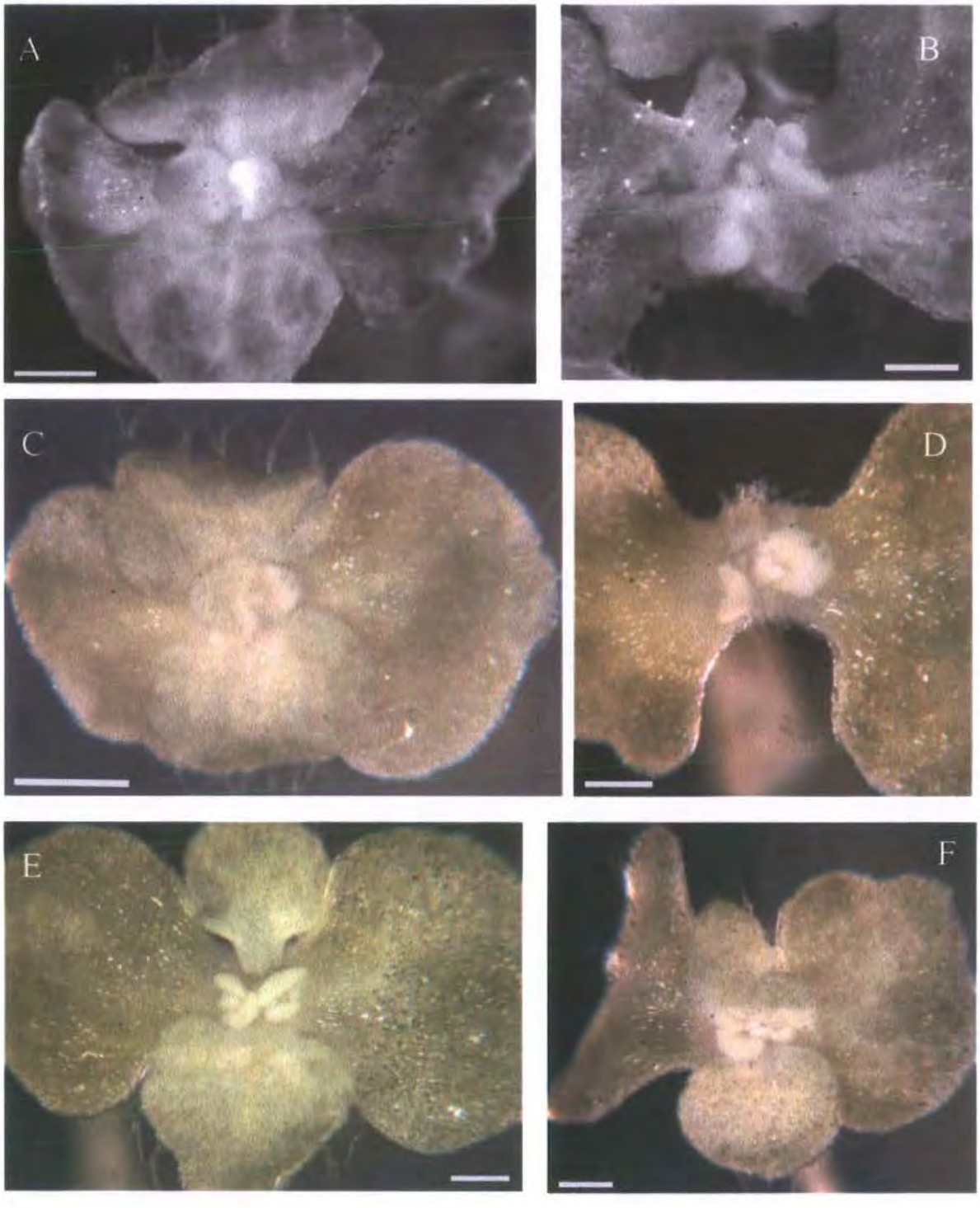



## Figure 5.15 Phyllotaxy and meristem initiation in *ein2* and *hyd1-ein2*

A, *ein2;* B-F, *hyd1-ein2*, all 12dae plants. A-E, bar = 0.5mm; F, bar = 0.25mm.

The *ein2* seedling shown in A has a single shoot apical meristem, and initiates primordia in a predictable spiral pattern indistinguishable from wild-type plants.

*B*-F; *hyd1-ein2* double mutants, selected at 5 dae for the presence of two cotyledons and apparently one apical meristem. Multiple points of primordial initiation are visible in all these seedlings (asterisks) except E, where leaf expansion is unusually rapid and has obscured the apex. Even in in this example, the point of origin of these leaves is evidently variable. The shoot region in seedling F has three obvious SAMs, one centrally in the expected position, and the others on the base of the cotyledon petioles. The ectopic SAM indicated by the arrow does not have an obvious regular phyllotaxy, with multiple primordial structures evident.




## Figure 5.16 Phyllotaxy and ectopic meristem formation in *hyd2-ein2*.

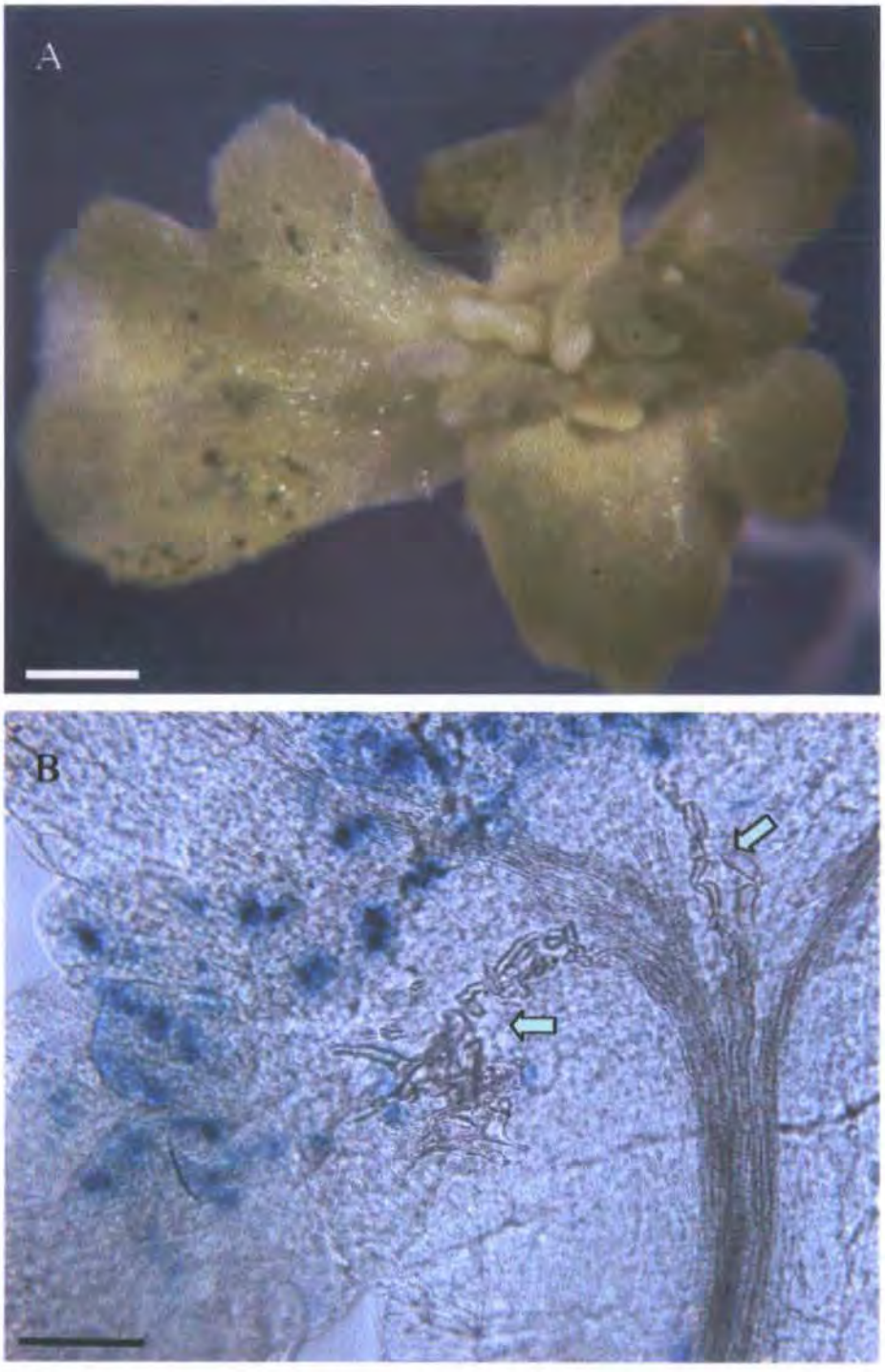
**A-F; *hyd2-ein2* seedlings at 12 dae, selected as 5 dae plants with two cotyledons and only one apparent SAM. B shows seedling A with true leaves removed to expose the meristem, likewise D is the plant shown in C with its true leaves removed. E shows clearly its two SAM regions, formed at the locations where true leaf primordia 3 and 4 would be expected to initiate. The plant visible in F appears to have an enlarged SAM region, extending bilaterally between the two cotyledons.**

**A, C, E-F, bar = 0.5mm; B and D, bar = 0.25mm.**

shown in Fig. 5.16; A and C; both of these seedlings appear to have a single phyllotactic origin, and a normal positioning of the first four true leaves. Removal of the expanded true leaves from these plants (shown in Fig. 5.16; B and D respectively) reveals the central SAM region (asterisk) and other ectopic primordial initiation points, indicated by arrows. The example shown in Fig. 5.16; E has formed two adjacent SAMs between the first pair of true leaves, whilst the seedling in Fig. 5.16; F has a widened SAM region where multiple primordia are emerging.

As in the *hydra* single mutants, the majority of seedlings which resolved a pair of expanding first true leaves, correctly positioned this pair relative to the two cotyledons. It appears that in *hydra*, the positioning of the post-germination SAM is not solely defined by the patterning events of embryogenesis; these examples have resolved a variable number of meristems and ectopic primordia independently from patterning cues derived from the first pair of true leaves. The first true leaf pair are resolved as rudimentary structures during embryogenesis, positioned relative to the embryonic cotyledons. These data imply a difficulty in *hydra* seedlings in the maintenance of the post-germination SAM, defined in seedling growth by mutually excluding gene expression domains across the radial axis.

Additional SAM formation in *hydra* single mutants was predictable relative to bifurcation points in the zone of hypocotyl-cotyledon transition (section 4.3.5.5). A similar situation was found in most seedlings of *hydra-ein2* (not shown). A modulation of this pattern was found in situations where the transition zone did not differentiate a coherent vasculature. Fig. 5.17; A and B show seedlings with a very similar morphology, where multiple primordia initiate from a single enlarged SAM in a disorganised fashion. Fig. 5.17; A was chosen for its paired cotyledons at 4 dae; this plant has modified its original morphology during early seedling growth, and developed primordia across the breadth of a widened hypocotyl apex. In Fig. 5.17; B, the behaviour of the xylem vasculature in the cotyledon-hypocotyl transition zone is visible. Vasculature of the stele bifurcates into




## Figure 5.17 Primordial cluster formation and xylem disjuncture at the *hyd2-ein2* hypocotyl-cotyledon transition zone

Ectopic meristem formation is associated with branch points in the cotyledon-hypocotyl transition region in *hydra-ein2* double mutants in the same way as seen in *hydra* single mutant seedlings (Fig. 4.37). Extreme examples of ectopic SAM formation, such as the elongated zone of primordia visible on seedling A (*hyd2-ein2* at 12 dae, bar = 0.5mm), are associated with more extreme levels of vascular disorganisation.

Another 12dae *hyd2-ein2* seedling with similar SAM morphology is shown in B (bar = 100$\mu$m); the primordia are highlighted by expression of the p*CYC1At*::CDB::GUS cell division reporter. Beneath the primordial initiation zone is an area of chaotic xylem vessel formation (arrow). Another disorganised xylem strand is visible in the upper right of this picture.

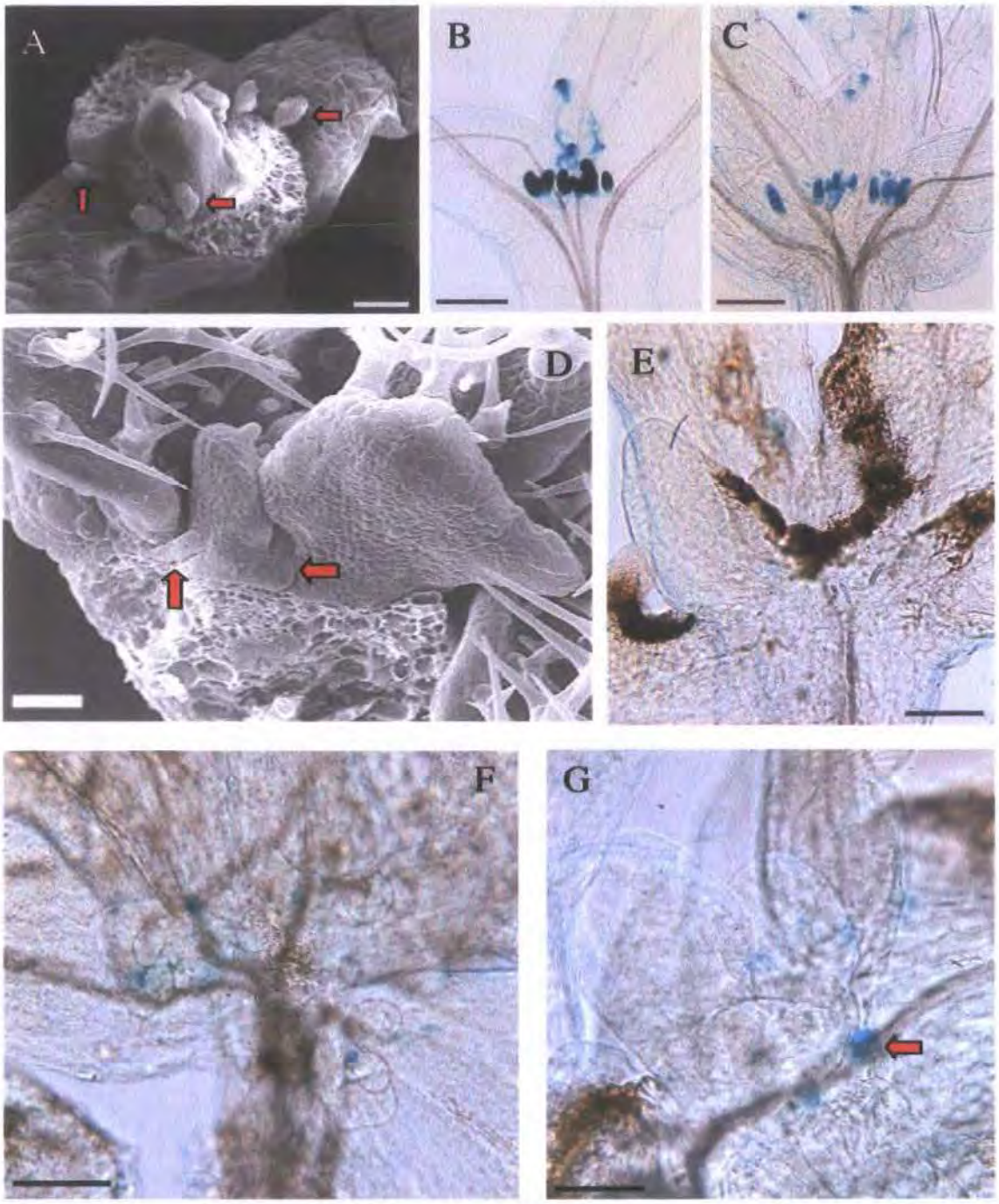
the cotyledon traces, but differentiation has resolved highly mis-aligned vessels along the traces into the first pair of true leaves (arrows). Multiple young primordia have formed above the disjunct xylem in this seedling (highlighted here by the *CYC1At*::CDB::GUS reporter revealing clusters of cell division events in developing primordia across the apex). These observations imply a role for the xylem trace in maintenance of lateral inhibition cues around the meristem periphery, and hence limitation of primordial numbers.

## 5.3.1.6 Dorsiventrality

### 5.3.1.6.1 Putative stipule structures are present morphologically in some *hyd-ein2* mutants, although their functionality is unclear.

The *ein2* mutant has normally-positioned stipules at the base of its leaves (Fig. 5.6.1;A), and shows a stipule signal with the DR5::GUS reporter (Fig. 5.6.1; C), although this is not as strong as the DR5 signal in wild-type stipules (Fig. 5.6.1; B).

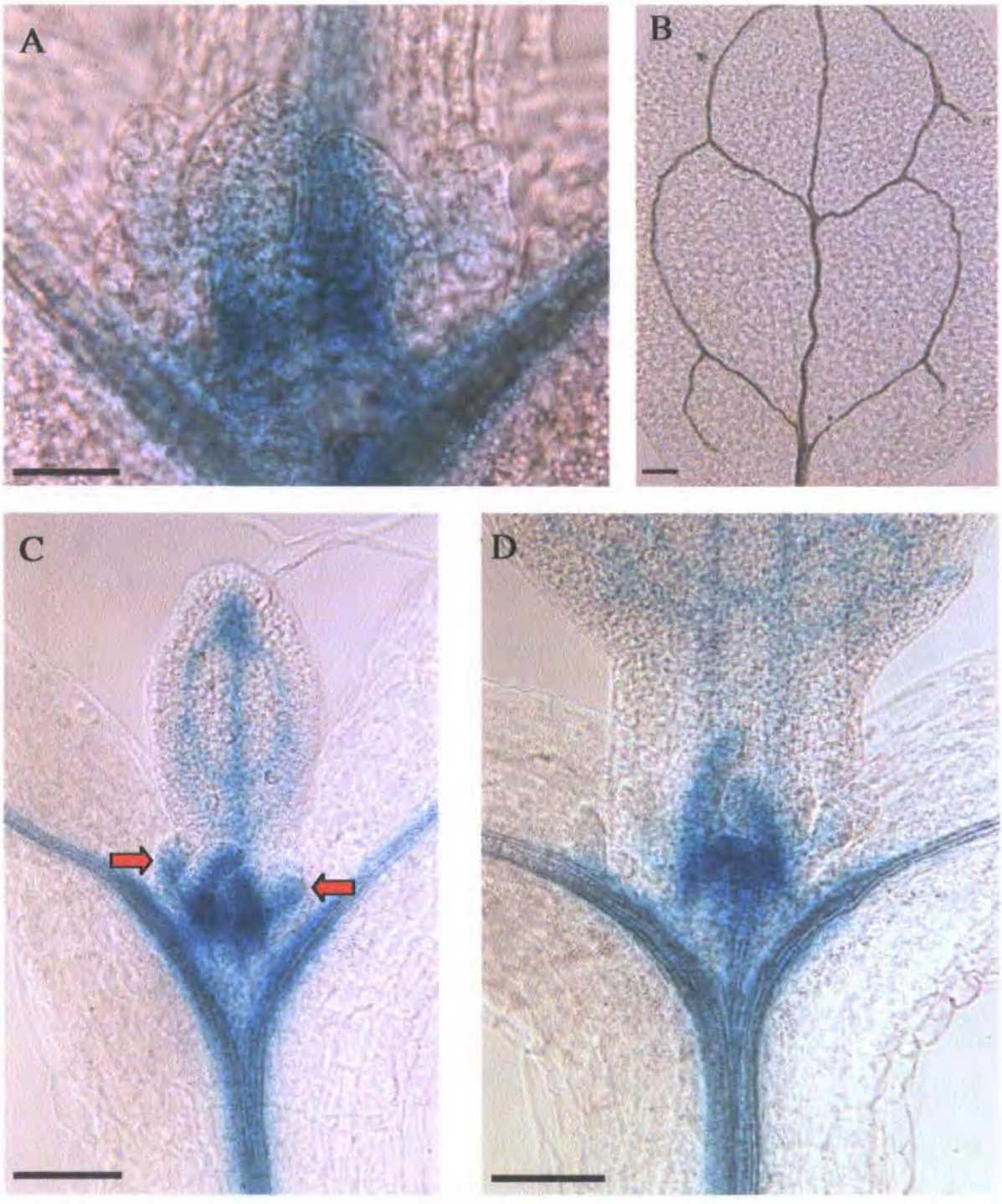
The DR5::GUS stipule signal was rarely seen in *hyd1*, and no *hyd2* siblings were found with a stipule signature (section 4.3.6.2). In *hyd2-ein2*, stipule-like structures were morphologically distinguishable in some siblings, as in Fig. 5.18; D (arrows). 10 dae seedlings of *hyd2-ein2* showing strong root activity for the DR5::GUS reporter were examined for signs of functional stipules (i.e. the resolution of a DR5 signal). In a sample set of approximately 40 seedlings of this age class, only one plant was found with a putative stipule signal, shown in Fig. 5.18; G, detailed in H. Other seedlings had a weak DR5::GUS expression in the shoot tissues, but no putative stipules (as in the examples shown in Fig. 5.18; E and F).




## Figure 5.18 Stipules in *ein2* and *hyd2-ein2*

A; ***ein2*** shoot meristem region from 11 dae plant, with most of the true leaves removed, bar = 50$\mu$m. Arrows indicate stipules, formed at the margins of expanding true leaves. B and C; 10dae wild-type (B) and ***ein2*** (C) shoot apices expressing the DR5::GUS reporter, which highlights stipules. Reporter activity confirms the presence of functional stipules, although reporter intensity is reduced in the ***ein2*** mutants, suggesting that the function indicated by DR5::GUS is down-regulated slightly in the ***ein2*** mutant plant body.

D; ***hyd2-ein2*** seedling at 11dae, with leaf removed and showing detail of the apical region. Structures which may represent stipules are indicated by the arrows. Bar = 30$\mu$m. E-G; apical regions of ***hyd2-ein2*** seedlings at 10dae, bar = 200$\mu$m. All of these samples showed obvious root expression of DR5::GUS. E and F have some shoot expression of the reporter, but no putative stipules are highlighted around the meristem region. The seedling shown in G has a structure, indicated by the arrow, which may be a stipule. Note that this plant shows improved primordial expansion, when compared to its siblings in E and F. This seedling was exceptional, as no other ***hyd2-ein2*** plants examined appeared to have stipule-like structures defined by this reporter.




## Figure 5.19 Expression of the p*REV*::GUS reporter in *ein2*

A, C, D; SAM, and B; cotyledon detail from 7 dae *ein2* seedlings carrying the p*REV*::GUS marker of vascular and primordium adaxial tissue identity. C has one of the first true leaves removed, allowing a clearer view of the SAM region. A; bar = 50$\mu$m, B-D; bar = 100$\mu$m.

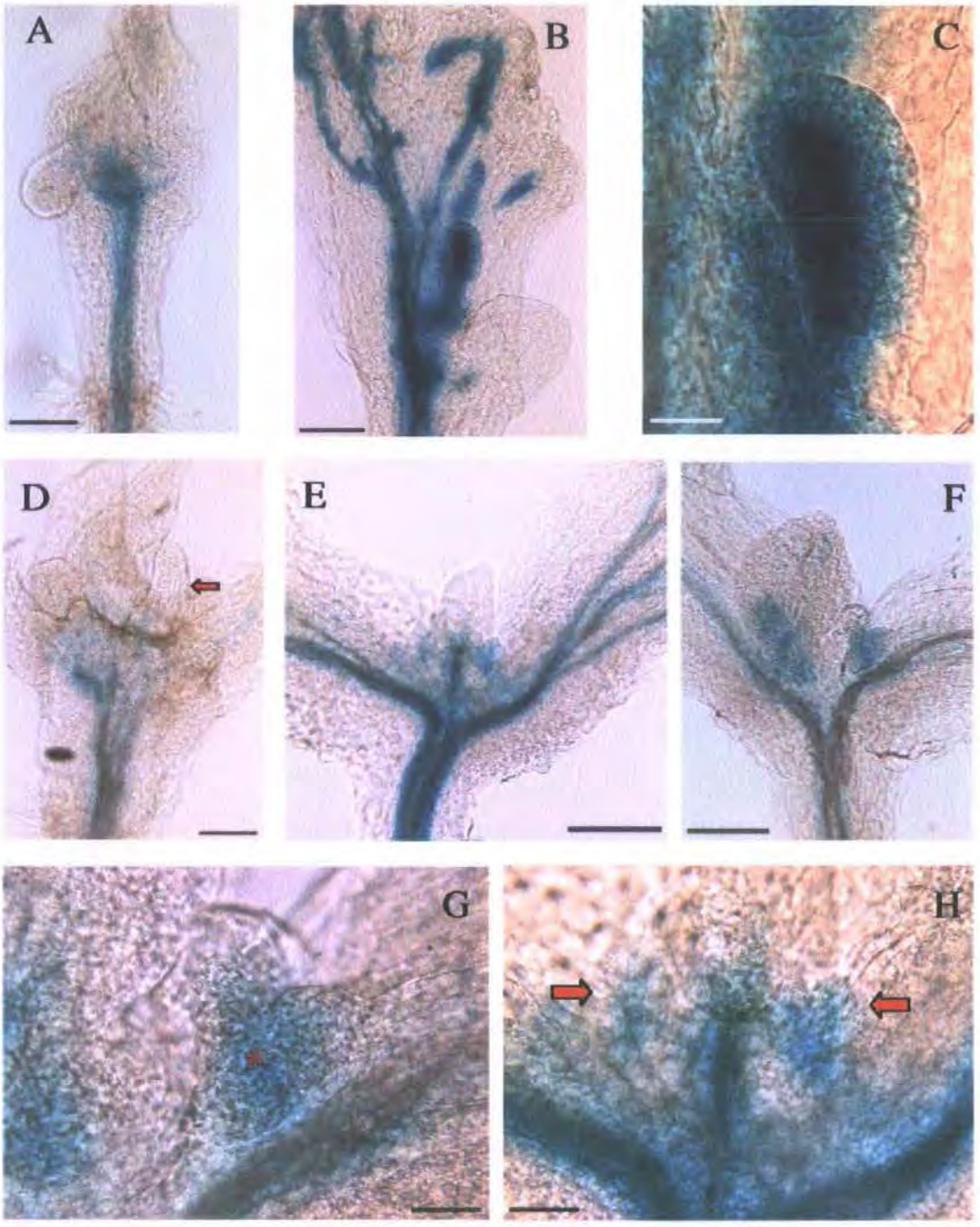
Expression of p*REV*::GUS is similar in *ein2* and wild-type plants; true leaf primordia and vascular tissues all show the same positional cues. However, in addition to the wild-type pattern, the stipules of young expanding true leaves in *ein2* seedlings show a transient expression (C, arrows), corresponding to the point at which p*REV*::GUS highlights the first two loops of secondary vasculature in the expanding lamina. This signal fades (D) as the leaves expand.

### 5.3.1.6.2 The p*REV*::GUS reporter has a slightly reduced activity in primordia of *hyd2-ein2* revealing a correctly positioned adaxiality, whilst in *hyd1-ein2* signal mis-positioning was as seen in *hyd1*

The p*REV*::GUS reporter showed a similar GUS activity in the *ein2* background found in wild-type plants; young primordia showed an adaxial localization, along with activity in vascular tissues of the hypocotyl stele, procambium and differentiating vascular traces of the true leaf primordia (Fig. 5.19; A-D). The only discernible difference between wild-type and *ein2*, was that the stipules of very young primordia carried a transient GUS activity, seen at around the stage when the first loops of secondary vasculature were visible (Fig. 5.19; C, arrows).

Siblings of *hyd1* and *hyd2* resolved a differential positional localization of p*REV*::GUS. A dorsiventral axis was defined in *hyd1* by p*REV*::GUS, in which patterning had variably normal, skewed or even reversed adaxial orientation. In *hydra1-ein2*, a similar range of positional localization for p*REV*::GUS as was seen as in *hyd1* single mutants (data not shown).

A strong p*REV*::GUS reporter activity was evident throughout young primordia of *hyd2*, obscuring any dorsiventral differentiation, and was upregulated in the cotyledon primary traces (as described in section 4.3.6.3). In contrast, siblings of *hyd2-ein2* had a reduction in the intensity of p*REV*::GUS activity, which reduced the histochemical signal sufficiently to reveal a correctly positioned adaxial signal in young primordia (Fig. 5.20; A-E). Normal adaxial positioning was seen; the example shown in Fig. 5.20; H, has a single SAM in which the primordial positioning appears normal relative to the phyllotactic origin, and adaxial identity is evident in all of these primordia. Where misplacement of the shoot meristems promoted ectopic SAM formation on the flanking regions of the apex, clusters of primordia such as in Fig. 5.20; G showed a co-ordinated adaxiality around a




## Figure 5.20 Activity of p*REV*::GUS in *hydra2-ein2*

A-H; *hyd2-ein2* seedlings carrying the p*REV*::GUS marker. A, 3 dae, and B, D, E and F, 7 dae seedlings, all bar = 200$\mu$m. C shows detail of the enlarged primordium visible in B, G shows the SAM region from plant E, and H shows primordia from F; all bar = 50$\mu$m.

Expression of p*REV*::GUS in *hyd2-ein2* siblings demonstrates reduced histochemical activity in comparison to *hyd2* single mutants, although some plants (C) still show stronger expression than that observed in wild-type. As in *hyd2*, the histochemical signal appears more rapidly in post-germination seedlings (B), and persists longer in the cotyledon vascular trace than in either wild-type or *ein2* (visible in E). Where a phyllotactic axis is evident (F/H), adaxiality is visible in the primordia (H, arrows). Where leaf primordia initiate in clusters (E/G, asterisk), the adaxial signal is visible in the centre of the cluster, suggesting an orientation around the position of the presumptive ectopic SAM. Post-germinative 'prong' structures (D, arrow) are not highlighted by this reporter.

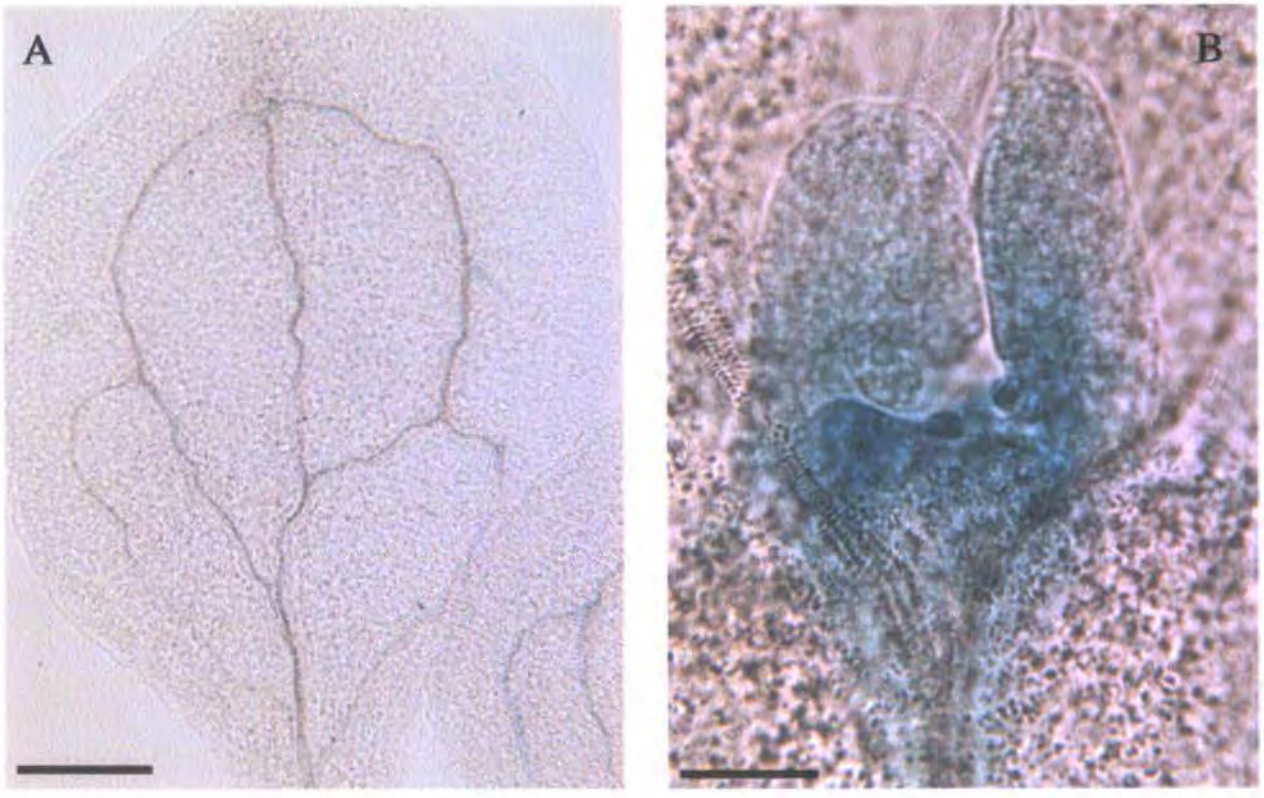
central point (marked by an asterisk), even though a SAM region is not obvious within this primordial cluster.

### 5.3.1.6.3 The activity of the p*PHB*::GUS reporter is reduced in *hyd1-ein2* and reveals a correctly positioned adaxial pattern,

The p*PHB*::GUS marker of adaxial tissue identity shows positioning and activity in the *ein2* background which is indistinguishable from the reporter expression observed in wild-type plants (Fig. 5.21; A-B).

Section 4.3.6.4 described a strong up-regulation of the p*PHB*::GUS reporter in *hyd1* mutant apices, producing a strong signal throughout young primordia, with no clear adaxial resolution. In seedlings of *hyd1-ein2* at 3 dae, no differences were seen in GUS expression from that observed in *hyd1* single mutants (Fig. 5.22; A-C). By 7 dae, the reporter showed some reduction in activity, which allowed the visualisation of a correctly positioned adaxial signal in most primordia (Fig. 5.22; F-I). (This phenomenon is similar to the reduction in *pREV*::GUS activity in *hyd2-ein2*, although the reduction in intensity of the *PHB* reporter in *hyd1-ein2* was not as pronounced.) The p*PHB*::GUS reporter positioning appeared to remain in the correct orientation to the *hyd1-ein2* meristem, and was distorted in some samples in conjunction with a morphological distortion of the meristem region.

In *hyd2*, an abaxially-positioned (i.e. reversed) signal was seen in the cotyledons of 3 dae seedlings (described in section 4.6.4), and in primordia from 7 dae seedlings, although the GUS activity was very weak in most examples. Although most had a reversed adaxiality, some primordia of *hyd2* showed reporter expression throughout the young leaf organs, without dorsiventral resolution. In seedlings of *hyd2-ein2* (Fig. 5.23; A-G), a slightly enhanced GUS activity was seen, with a similar abaxial positional mis-localization in cotyledons (Fig. 5.23; A-C) and true leaves. Some primordia carried abaxially-positioned signals (Fig. 5.23; F, detail G), whilst others




## Figure 5.21 Activity of the p*PHB*::GUS reporter in *ein2*

A; cotyledon, and B; SAM detail from *ein2* seedlings at 3 dae, showing expression of the p*PHB*::GUS reporter.

Positional localisation of the histochemical signal is indistinguishable between *ein2* and wild-type seedlings, with a slight increase in the diffuse transient signal seen in expanding true leaves.

A, bar = 200$\mu$m; B, bar = 50$\mu$m.

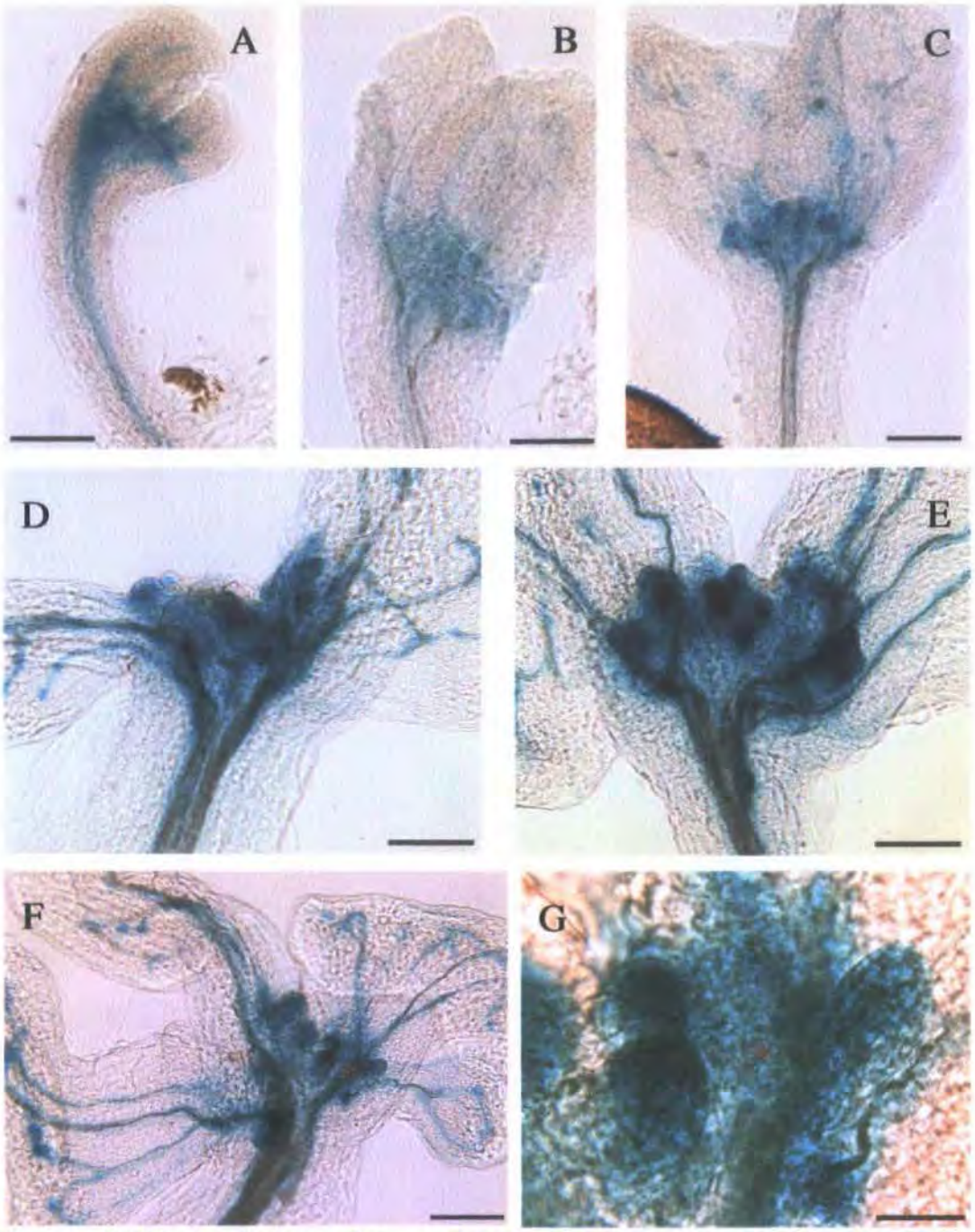
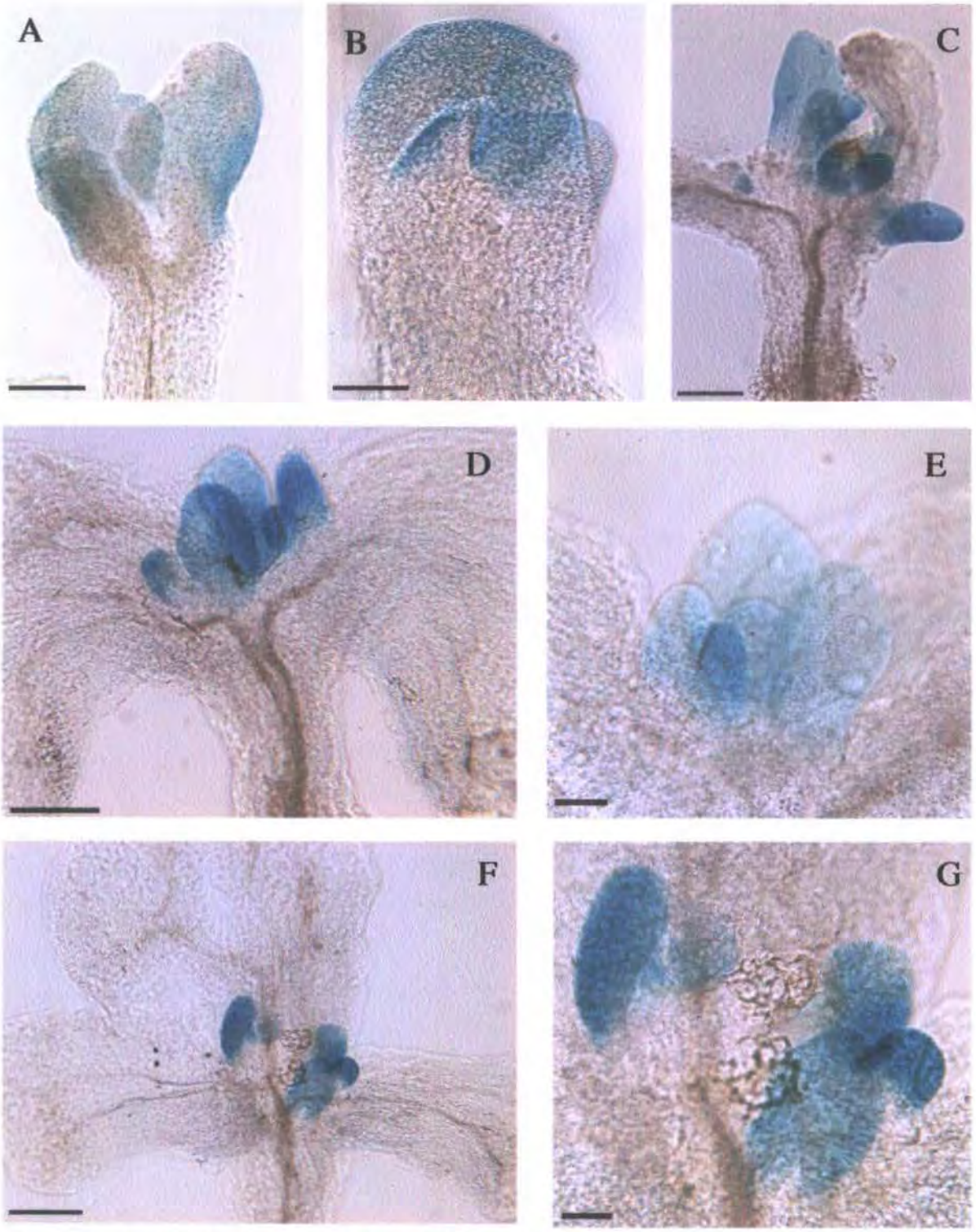



Figure 5.22 Activity of the p*PHB*::GUS reporter in *hyd1-ein2*

A-C, p*PHB*::GUS activity in *hyd1-ein2* at 3 dae; D-F; *hyd1-ein2* seedlings at 7 dae; bar = 200$\mu$m. G shows detail of the SAM region from F; bar = 50$\mu$m.

Histochemical localisation of this reporter is similar to the pattern observed in *hyd1* single mutants at 3 dae; in both single and double *hydra* mutants, higher levels of staining are visible around the young primordia, with little identifiable distinction between abaxial and adaxial surfaces.

In contrast, the presence of *ein2* in the *hyd1* background modulates expression of the p*PHB*::GUS reporter at later stages, reducing histochemical intensity around the SAM by 7 dae, and increasing the signal in cotyledon vasculature. Around the SAM, this down-modulation makes it possible to distinguish some dorsiventral axes in certain young primordia. The asterisk in F, detailed in G, is positioned at the presumptive centre of a phyllotactic axis, around which the primordia show correct adaxial patterning. Where the SAM region is expanded, or ectopic SAMs appear, (visible in D-F), primordial orientation is variable.




## Figure 5.23 Activity of the p*PHB*::GUS reporter in *hyd2-ein2*

A-G; *hyd2-ein2* seedlings at 3dae (A, B), and 7 dae (C-F), with detail of the SAM region of plant F shown in G. A, C, D, F, bar = 200$\mu$m; B, bar = 100$\mu$m; E and G, bar = 50$\mu$m.

Activity of the p*PHB*::GUS reporter is similar in *hyd2* and *hyd2-ein2*, although with an increased intensity evident in the double mutants. Cotyledons of 3 dae seedlings carry an abaxial diffuse staining as seen in *hyd2*. Newly initiating primordia show strong GUS activity across the radial axis of young true leaf primordia, but lack an obvious adaxial localisation as observed in wild-type and *ein2*.

appeared to express a uniform GUS activity across the dorsiventral axis (Fig. 5.23; D, detailed in E).

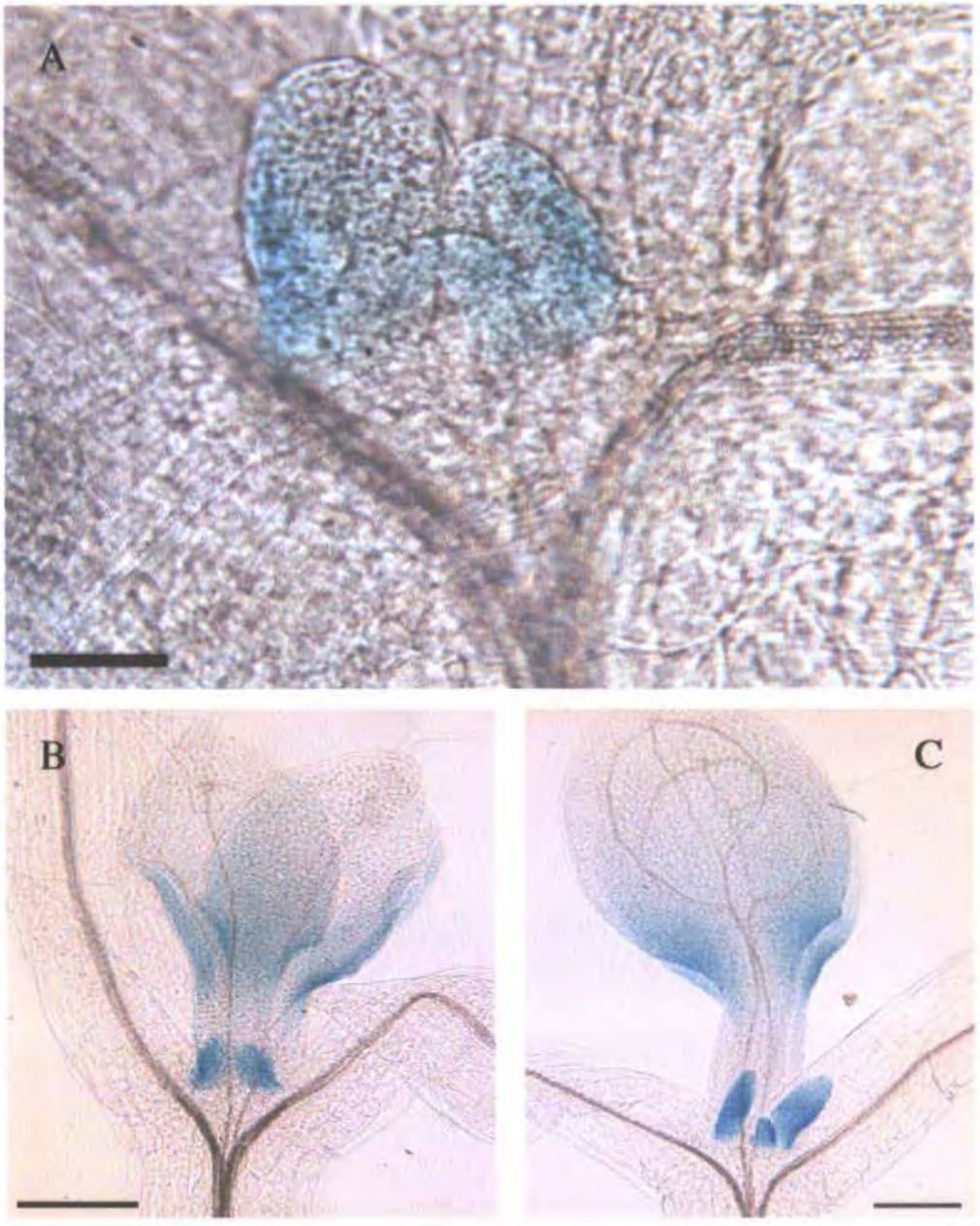
### 5.3.1.6.4 The presence of *ein2* modulates the intensity of p*YAB*::GUS activity in *hyd2*, but does not alter the occurrence of reversed dorsiventrality seen in some *hyd1* siblings

The positioning of the p*YAB*::GUS reporter activity in *ein2* was indistinguishable from that observed in wild-type (Fig. 4.24; A-C). In contrast, an increased expression was seen in both *hyd1* and *hyd2* in the presence of *ein2*.

Section 4.6.5 described the dorsiventral positioning of p*YAB*::GUS as variably reversed in primordia of *hyd1*; some examples showed the expected abaxial positioning, whilst others carried an abaxial signal on adaxial surfaces. A similar pattern was seen in examples of *hyd1-ein2*, (Fig. 5.25; A-G) with anomalous reporter activity on adaxial surfaces, or throughout the primordium.

The improved organ expansion in *hyd1-ein2* allowed some clearer resolution of radialized structures. In the absence of xylem differentiation (Fig. 5.26; C, E, G), these structures appear to be fully abaxialised. Where xylem strands differentiate, an axiality appears (Fig. 5.26; A). As in *hyd1*, a prolonged expression of the reporter was seen variably in proximity to leaf apices or ectopically around leaf margins in *hyd1-ein2*, beyond the age at which expression in the lower lamina had ceased in the controls (Fig. 5.26; F-H).

Reporter activity in *hyd2* was too weak to see in most siblings, but the increased intensity of signal in *hyd2-ein2* was slightly increased, becoming most apparent in apices with multiple primordia (Fig. 5.27; A-C).. In Fig. 5.27; C, the primordia are positioned over an area of unconnected xylem vessels, just visible in the picture. In these and the other *hyd2-ein2* apices,

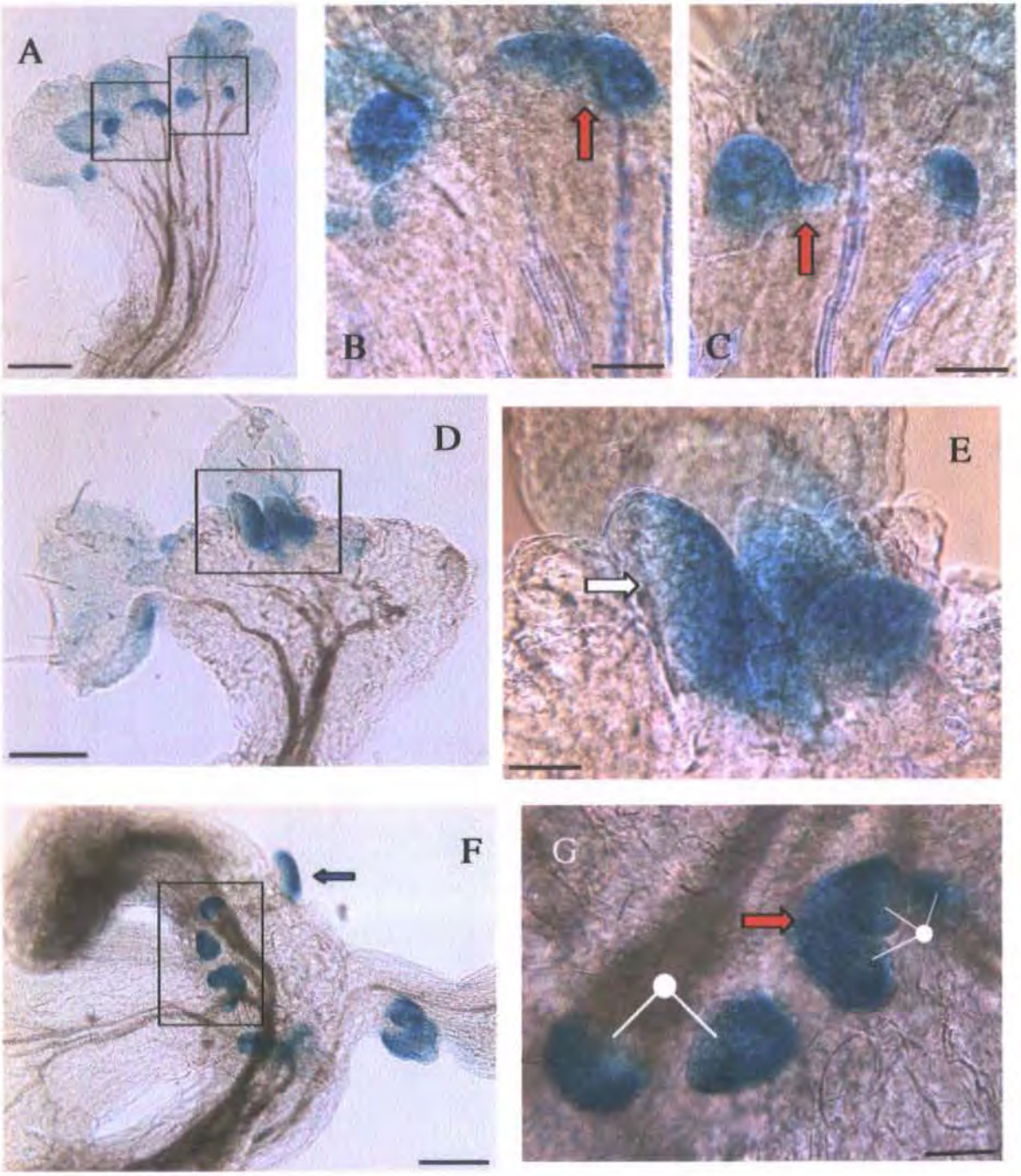



## Figure 5.24 p*YAB*::GUS expression in *ein2*

A-C, detail from *ein2* plants at 7 dae, showing expression of the p*YAB*::GUS reporter. Seedlings of *ein2* demonstrate the same reporter activity as that seen in wild-type plants, although the signal appears slightly stronger in these mutants.

A shows very young primordia with the abaxial lamina carrying a GUS signal, bar = 50$\mu$m. B and C show the histochemical signal that persists in the basal leaf abaxial epidermis during active expansion of the lamina; bar= 200$\mu$m.

The true leaves in B have completed differentiation of the primary vascular xylem strand, and the first of the secondary loops are visible in C. New primordia with strong GUS activity are present around the SAM region in both seedlings.

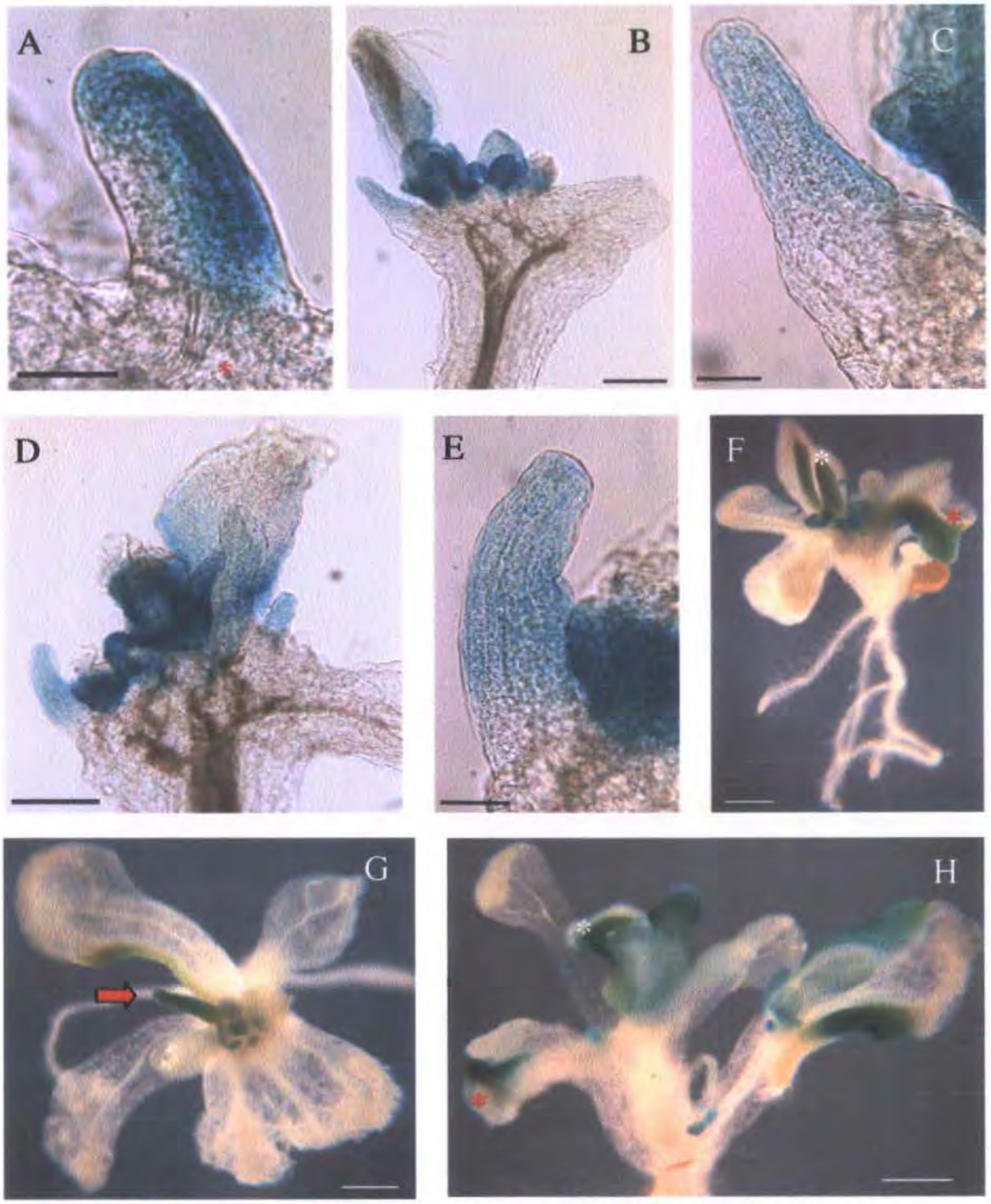



## Figure 5.25 Activity of p*YAB*::GUS in *hyd1-ein2* seedlings at 7 dae

The *hyd1-ein2* 7 dae plants in A, D and F (bar = 20$\mu$m) show SAM detail as indicated in the images to the right (B and C from A, E from D and G from F, bar = 50$\mu$m).

An increased p*YAB*:: GUS intensity is observed in both *hydra-ein2* double mutants; as with single mutants, the strongest signal is in plants with disturbed vascular coherence in the upper hypocotyl stele region. Activity of p*YAB*::GUS is enhanced in *hydra-ein2* double mutants relative to *hydra* single mutants. Anomalies in p*YAB*::GUS reporter positioning include a signal throughout the young primordia (in B and C), and GUS activity on the adaxial side of an expanding primordium in E (white arrow).

G shows detail of ectopic primordia with apparently two adjacent phyllotactic origins (white circles and lines). Note the primordia which have initiated as either adjacent or fused pairs in B, C and G (red arrows).

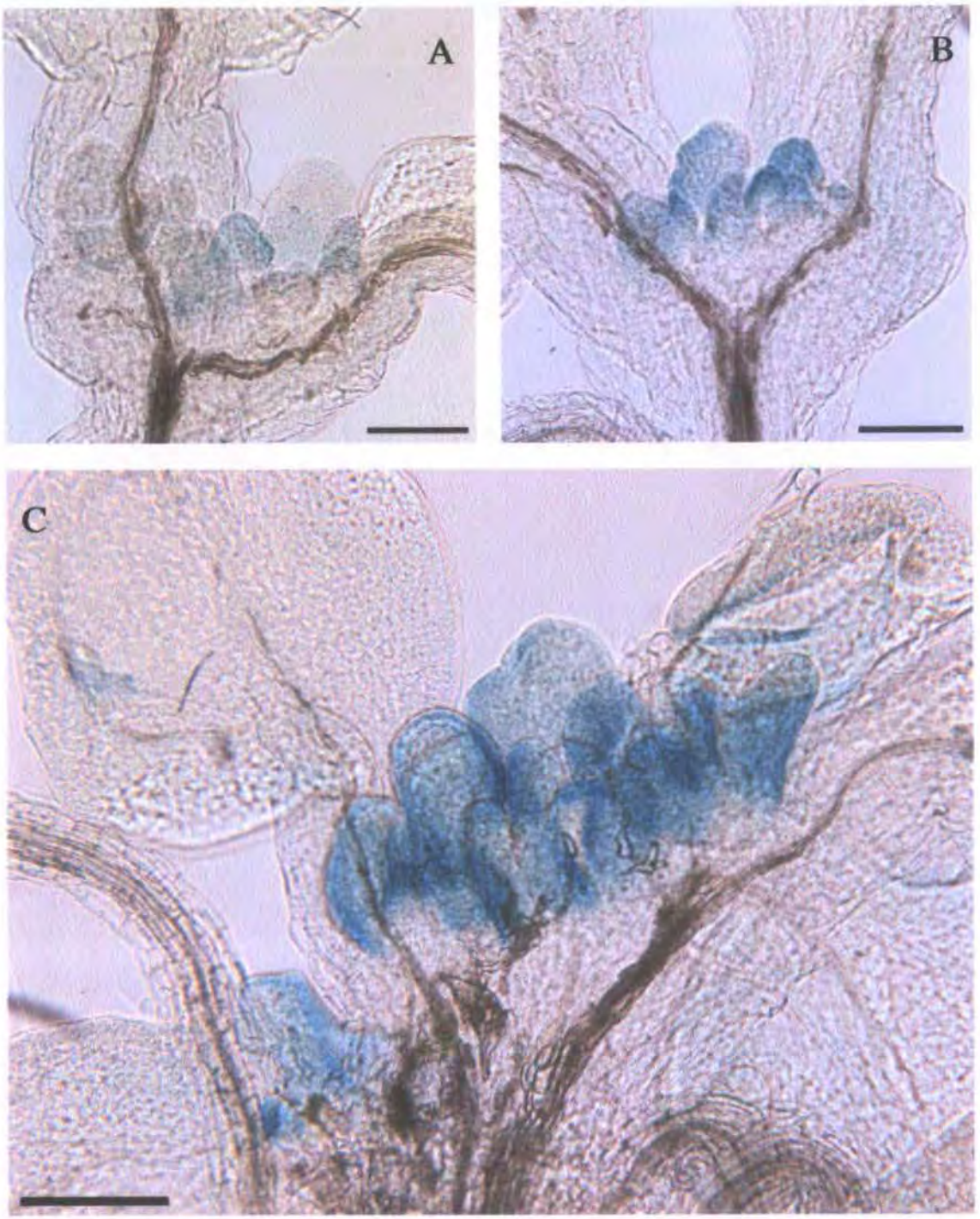



## Figure 5.26 p*YAB*::GUS activity in radialized structures and mature seedlings of *hyd1-ein2*

The 'prong' shape shown in A is from the seedling in Fig. 5.25; F, its position indicated by the blue arrow in that figure. Note the asymmetric distribution of p*YAB*::GUS activity in this structure, and the invading xylem strand visible at the base (asterisk). This organ appears to be undergoing some dorsiventral expansion. Other prong structures (C from the seedling shown in B, and E from seedling D) do not have an obvious xylem strand, and show an abaxialized radial expression around the primordium. Their expansion axis appears to be longitudinal.

F-H, *hyd1-ein2* 13 dae plants, show ectopic expression in cotyledons (G, arrow; red asterisks in F and H), and true leaves (F and H, white asterisks).

A, C, E, bar = 50$\mu$m; B and D, bar = 200$\mu$m; F and G, bar = 0.5mm; H, bar = 1mm.




## Figure 5.27 Detail of p*YAB*::GUS activity in clustered primordia of *hyd2-ein2*

A-C; ***hyd2-ein2***, 7 dae plants showing expression of p***YAB***::GUS, bar = 200$\mu$m.

The ***hyd2*** single mutant siblings carrying this reporter produced such low levels of expression that little or no signal evident in most siblings even when the heterozygous parental line was homozygous for the reporter insert. The presence of *ein2* increased GUS expression in these double mutants, so that the abaxial signal is more evident. As with *hyd1*, the expression pattern is weakest in plants with good vascular coherence in the upper hypocotyl stele (A), and increases as the xylem strands become more dissociated (B) or disjunct (C). Over disjunct xylem, the *hydra-ein2* seedlings produce clustered primordia without a clear SAM region. In example C, these expanding leaves appear to have self-organized an independent dorsiventral axis, as the many organs are visible with apparent correct p*YAB*::GUS localization but in a range of orientation.

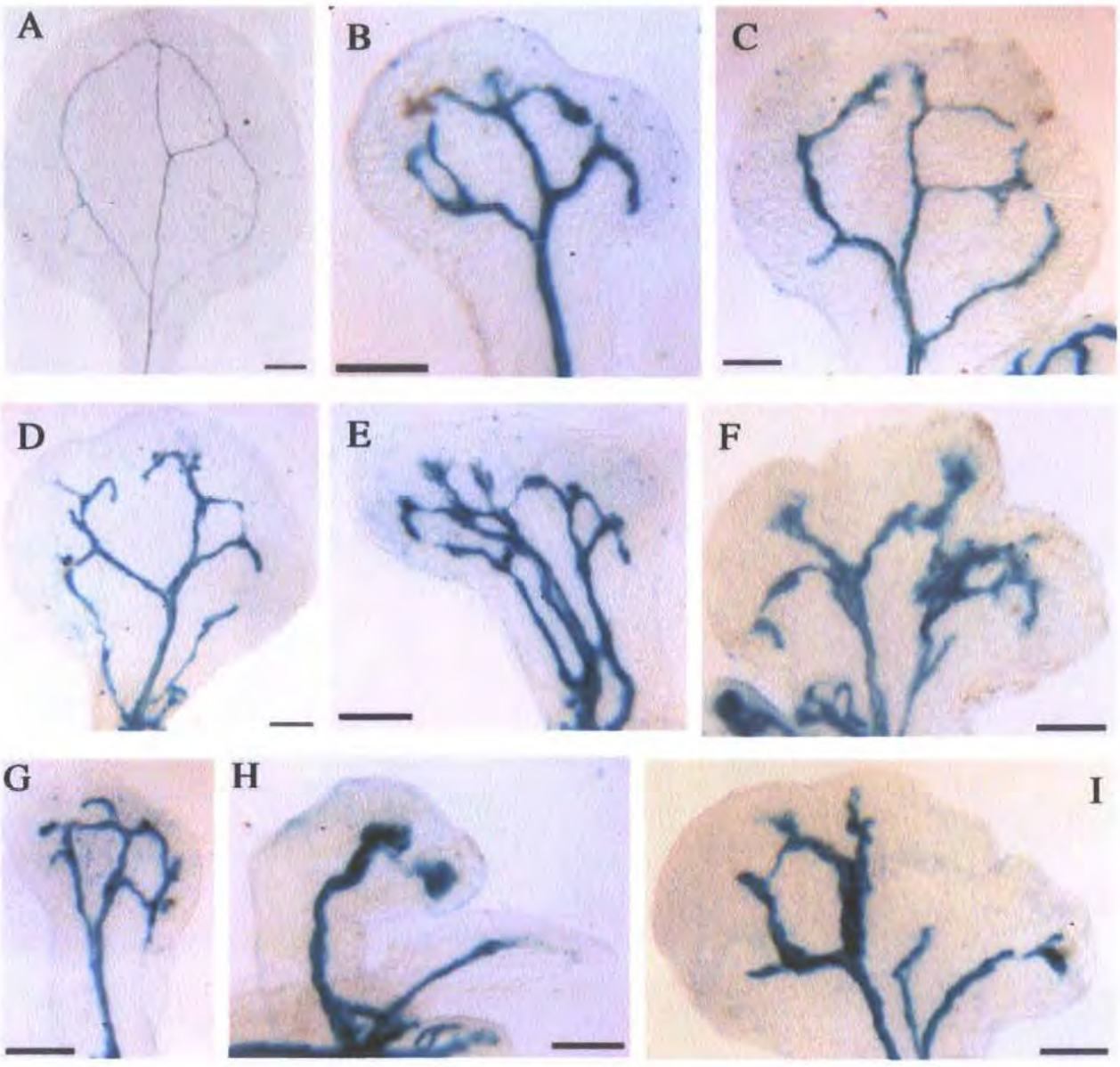
shown in Fig. 5.27; A and B, primordia carry a correctly positioned abaxial signal.

## 5.3.1.7 Co-ordination between cells during lateral expansion

### 5.3.1.7.1 Cotyledons and true leaves of *hydra-ein2* mutants have improved vascular patterning and laminar expansion, but retain anomalies in organ morphology

The vascular traces in cotyledons (Fig. 5.28;A) and true leaves (Fig. 5.29; A) of *ein2* are indistinguishable from wild-type vascular patterns. The *ein2* first true leaves did continue to increase in complexity during expansion, developing tertiary and also quaternary venation (not shown).

Cotyledon vasculature of the *hydra2-ein2* siblings shown in Fig. 5.28; B-I, demonstrate a variety of patterning phenomena. The traces shown in Fig. 5.28; B-D and G, have a pattern which approximates to wild-type, although with a thicker and less regular strand formation; such examples as these are found within a mostly regular-shaped lamina. The examples in Fig. 5.28; E-I have a much more variable lamina shape. All of these include a dissociation of the primary vascular trace. A partial dissociation as in Fig. 5.28; E, has resolved two apical hydathodes (asterisks) and a distorted lamina with additional vascular strands. The primary traces in Fig. 5.28; F and I have separated further towards the SAM, away from the organ apex, resulting in an enhanced 'noise' and a very irregular pattern, along with a distortion of the lamina. In Fig. 5.28; H, a more complete separation of the primary strands has resolved two organs, neither of which have a flattened lamina.

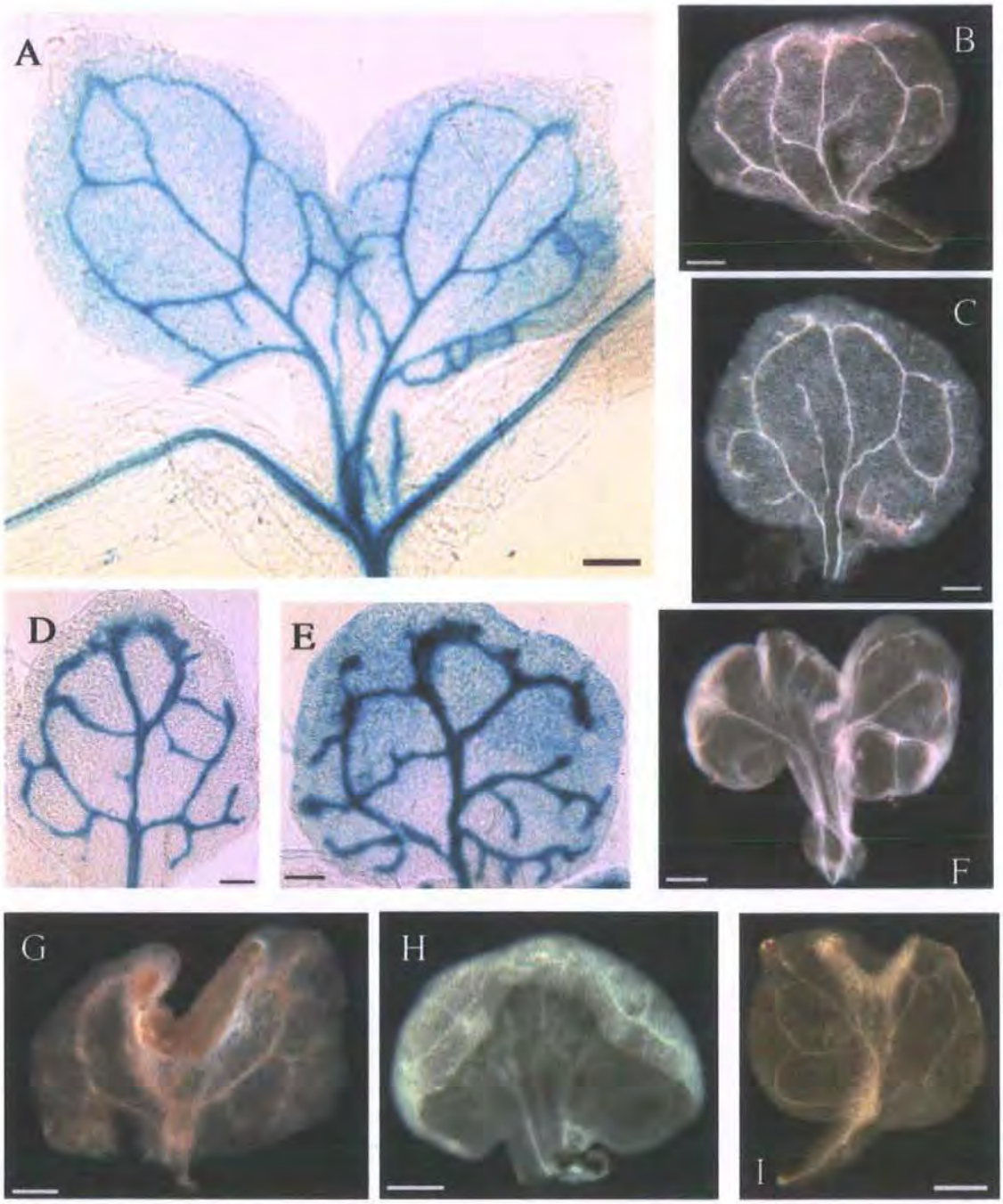



## Figure 5.28 Morphology and vascular traces in*ein2* and *hydra-ein2* double mutant cotyledons

A; *ein2* B-I; *hyd2-ein2* cotyledons from 10dae plants, expressing the p*AthB8*::GUS provascular marker, bar = 0.25mm.

The cotyledon vascular traces of *ein2* are indistinguishable from the pattern observed in wild-type plants (A). In *hydra2-ein2* cotyledons where the vascular trace approximates to that found in wild-type and *ein2*, leaf shape is approximately normal (B-D).

The cotyledons shown in E-I have various degrees of primary strand dissociation, giving an irregular, often lobed cotyledon morphology with increased numbers of open loops in the secondary vasculature. It appears that the closer to the SAM region that this dissociation occurs, the greater the effect on leaf shape. F and I, whose primary strands dissociation very close to the SAM, have two lobes and much less vascular coherence than E and G, where the primary strand diverges further into the cotyledon lamina. In the extreme example in H, the strands separate at or immediately next to the meristem, forming two separate structures which have not undergone expansion in the centrolateral axis.




## Figure 5.29 Morphology and vasculature of *ein2* and *hydra-ein2* true leaves

A; *ein2* , and D, E; *hyd1-ein2* first true leaves from 10 dae plants expressing the p*AthB8*::GUS construct. B, C, and F-I; *hyd2-ein2* first true leaves from 18 dae plants stained with safranin. A, bar = 100$\mu$m; B-I, bar = 0.25mm.

The *ein2* young true leaves shown in A are developing a vascular trace indistinguishable from that of wild-type. These leaves develop a more complex vascular pattern as they expand, with higher order branches developing.

The provascular trace of the *hyd2-ein2* true leaves in D and E appear similar to the control, though do not increase in size beyond this, and have a less complex differentiation of xylem than this pattern would predict, as seen in the mature first true leaves B and C from *hyd1-ein2*. The simple xylem traces may be due to the limited laminar expansion seen in the *hydra* mutants. Other morphological phenomena are observed, associated with a divergent primary vascular trace; these include lobing (F, G), cup-shaped leaves (H) and secondary growths in a plane other than that of the original lamina (I). The lobed leaf morphologies of F-I are associated with bifurcation of the primary midvein.

The true leaves of *hydra* single mutants (described in section 4.3.7.2) rarely produce a vascular pattern with more than second order complexity. In the first true leaf pairs of *hydra1-ein2* (Fig. 5.29; D and E), a more complex pattern is evident, with putative tertiary veins visible as in true leaves of the same stage from *ein2* (Fig. 5.29; A). This increased vascular complexity in *hydra-ein2* may be associated with an increased laminar size resulting from improved laminar expansion in the presence of *ein2*. No examples of greater complexity than this were found in all *hydra-ein2* double mutants, examined, including older material, suggesting that laminar size is developmentally limited in the mutants. This also suggests that the size of the lamina itself may exert a limitation on the vascular complexity that may develop in its tissues.

A further difference between *ein2* and the *hydra1-ein2* leaves concerns the pattern itself, which is less regular in the *hydra* background, particularly in the example shown in Fig. 5.29; E, and contains a higher proportion of free vein endings. The improvement in the vascular traces was not paralleled clearly in xylem differentiation; Fig. 5.29; B and C show similarly shaped leaves from 18 dae plants, where a simple xylem trace is visible. This implies that xylem differentiation may require a greater spacing between veins within the mesophyll layer than procambium or phloem definition, and this space is not generated during the limited laminar expansion seen in *hydra* mutant leaves.

Although the procambial pattern may extend to tertiary branching, the xylem traces of *hyd2-ein2* (Fig. 5.29; B-C, F, I) showed a similar simple patterning. These examples have modified laminar morphologies, producing lobed, hood-shaped and invaginated forms. All of these examples have a dissociated primary vascular trace at various points close to the meristem.

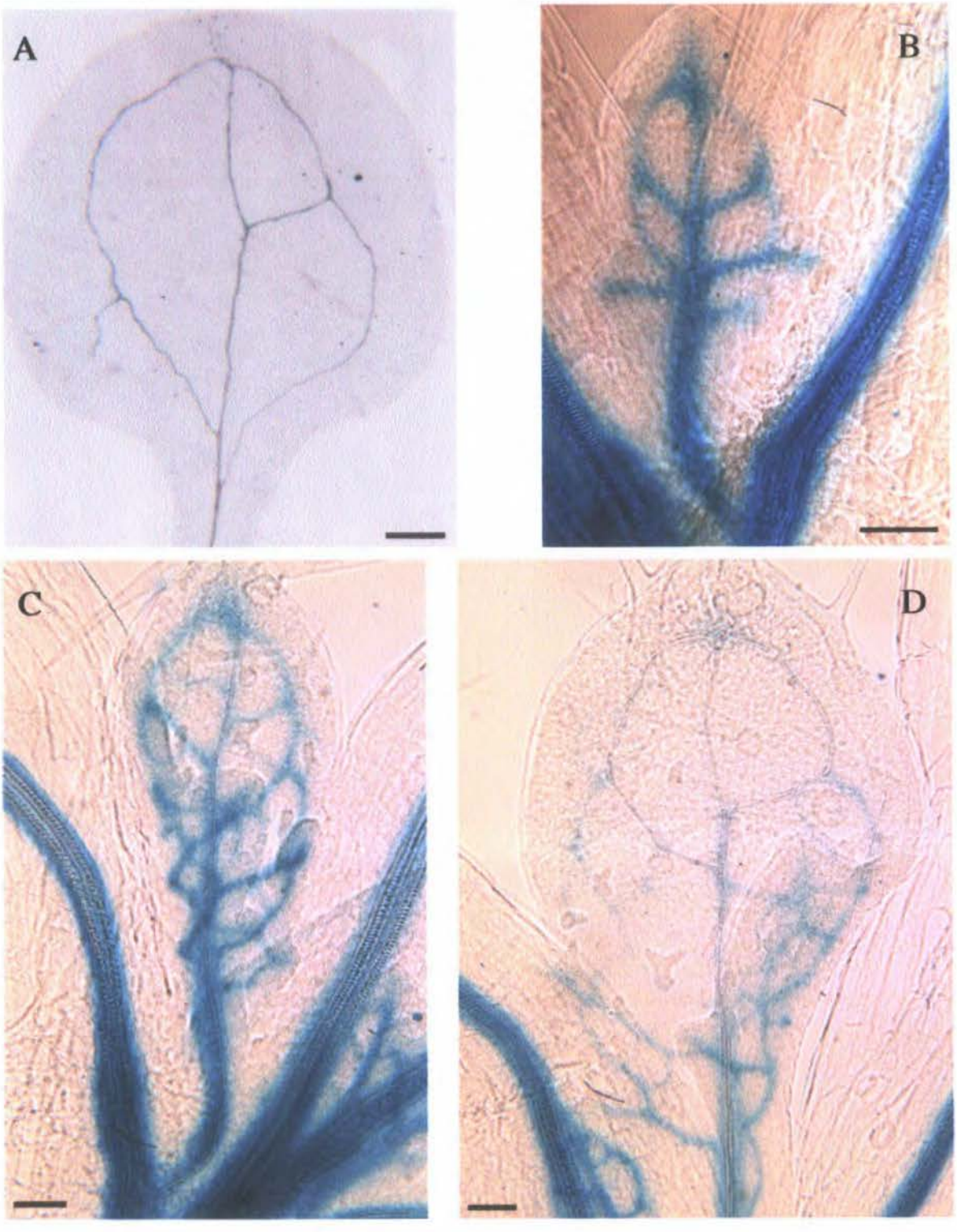
### 5.3.1.7.2 Xylem differentiation in *hydra-ein2* double mutant siblings includes greater strand integrity, a closer correspondence between xylem and procambial traces, and a reduced ectopic deposition of callose.

As in wild-type, the pre-provascular pattern in *ein2* defined by expression of the p*AthB8*::GUS reporter, highlights the route of procambial strands, and prefigures first phloem and then xylem differentiation (Baima *et al*, 1995, 2001). Xylem differentiation proceeds first with definition of the primary strand, followed by secondary loops in cotyledons (Fig. 5.30; A), and in leaves, a higher order vasculature producing tertiary and quaternary branches (Fig. 5.30; B).

The primary vascular strand of the *hyd2-ein2* cotyledon shown in Fig. 5.31; A is detailed in Fig. 5.31; B. Here the xylem trace appears coherent, and follows the route defined by expression of p*AthB8*::GUS. This cotyledon primary strand reveals a more coherent xylem than seen in *hydra2* (section 4.3.7.5), implying that the problems in co-ordinating xylem differentiation are reduced, at least in part, by the presence of the *ein2* mutation.

Similarly, in true leaves of *hydra-ein2* double mutants, a closer examination of the xylem, in association with the procambial pattern, reveals a stronger correlation between the provascular trace and the co-ordinated differentiation of xylem vessels (Fig.s 5.31; C, detailed in D and E, also 5.32; A, detailed in B-E). Vascular noise is still apparent, particularly around the apical hydathode (Fig. 5.32; E), whist elsewhere the strand co-ordination can appear normal (Fig. 5.32; B).

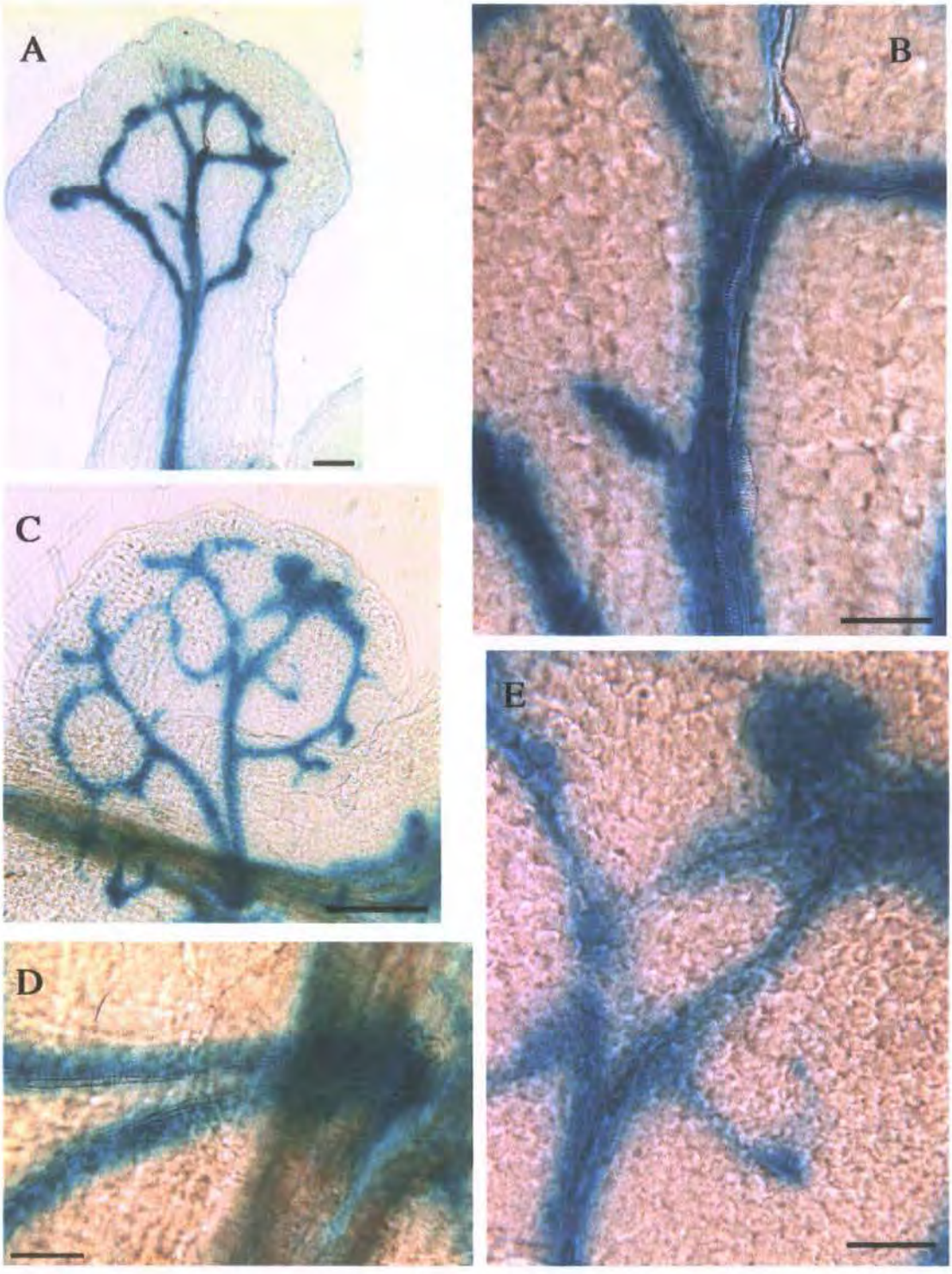
Fig. 5.31; D and E show two sections of the primary midvein of the true leaf indicated in Fig. 5.31; C. Primary strand dissociation has taken place in this leaf at or near the cotyledon-hypocotyl boundary, and two weakly differentiated xylem strands emerge into the lamina. In this leaf, the relative hierarchy in size between primary and secondary xylem is not




## Figure 5.30 Vascular pattern differentiation in *ein2*

A; cotyledon (bar = 0.25mm) and B-D;young true leaves (bar = 5$\mu$m) from 10dae *ein2* seedlings carrying the p*AthB8*::GUS provascular tissue marker.

In *ein2*, vascular differentiation proceeds as in wild-type, with first the definition of the provascular trace (B), followed by differentiated primary strands (C), then secondary loops (D). Higher order vasculature forms simultaneous coherent xylem traces section by section in a basipetal fashion as the leaf organs expand.




## Figure 5.31 Vascular pattern differentiation in *hyd2-ein2* cotyledons and true leaves

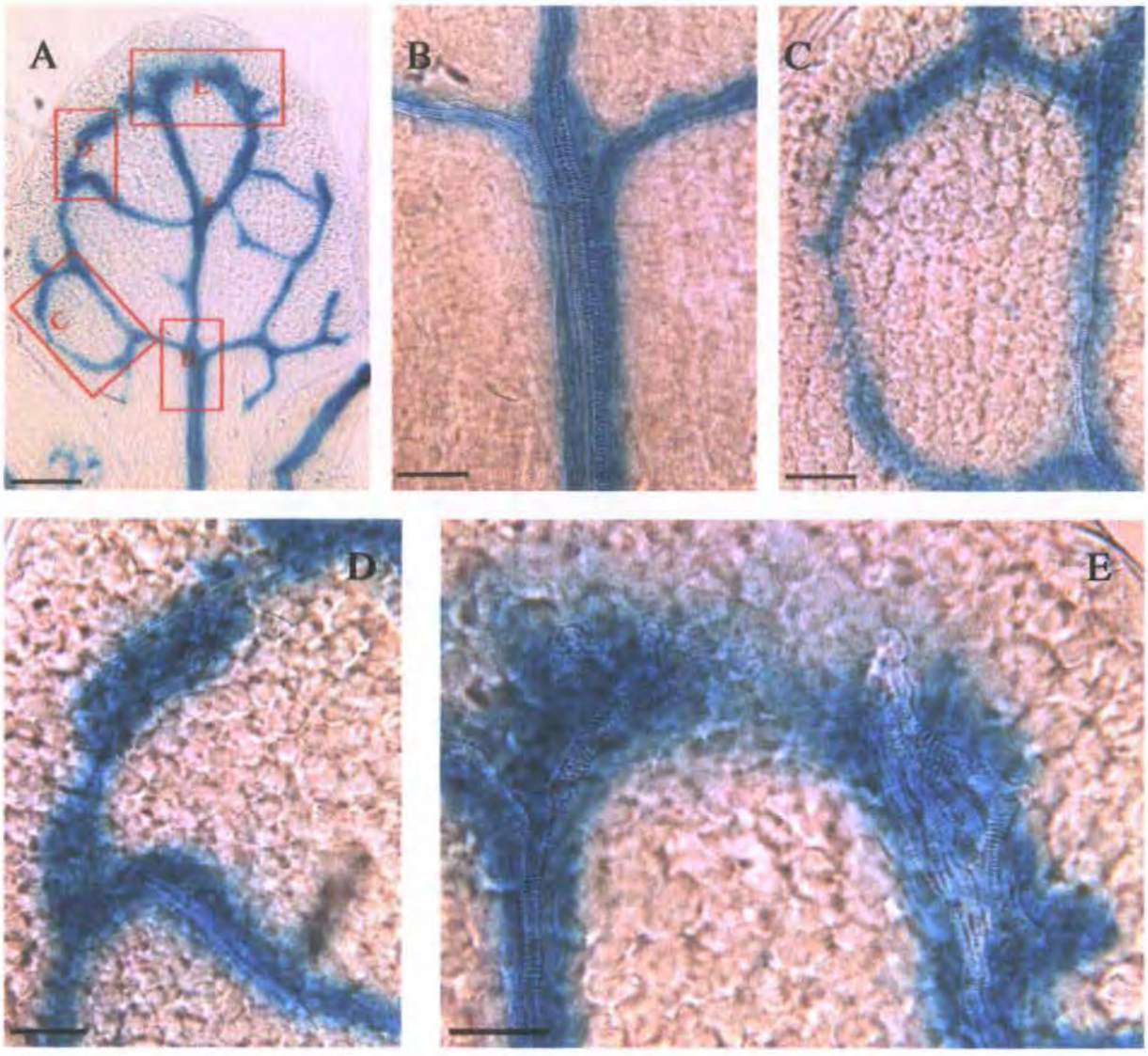
**A; whole cotyledon (bar = 0.25mm) with B; detail of the mid-vein region, (bar = 50$\mu$m), from a *hyd2-ein2* plants expressing p*AthB8*::GUS. The primary xylem strands are slightly more coherent than seen in examples from *hyd2* single mutants, and more closely align with the provascular trace. Note that expression of the provascular tissue marker is still strong here at 10 dae, though in the Ws and *ein2* controls has faded in cotyledons.**

**D and E; mid-vein details of the *hyd2-ein2* true leaf shown in C. D shows dissociation of the primary strand as the vascular trace enters the true leaf lamina. E details weak xylem differentiation in conjunction with indistinct provascular cues. C, bar = 20$\mu$m; D and E, bar = 50$\mu$m.**

obvious. Near the apical hydathode (Fig. 5.31; E), reporter mis-expression is associated with a further anomaly in the vascular trace, resulting in a twin-hydathode signal. In the other true leaf from the same seedling in Fig. 5.32; A, the midvein dissociates two thirds of the way up the lamina (asterisk), producing a region of heightened vascular noise near the leaf apex (Fig. 5.32; E). In contrast, further down the lamina the primary midvein has a strong integrity (Fig. 5.32; B), and secondary xylem appear to differentiate in a near-simultaneous fashion (Fig. 5.32; C) as in wild-type and *ein2*.

Phloem-associated deposition of callose correlated with the xylem trace in cotyledons and true leaves of *ein2* (Fig. 5.33 A, B) in a manner indistinguishable from wild-type (section 4.3.7.5). In cotyledons of the *hydra-ein2* double mutants, a mis-localization of callose similar to that observed in single *hydra* mutants was noted (Fig. 5.33; C-E). Areas with higher levels of xylem 'noise', as in Fig. 5.33; E, were associated with a greater deposition of callose in the lamina. Other areas with a more normal xylem patterning, (e.g. Fig. 5.33; C) showed a greater association between xylem and phloem, but still not as close as that from wild-type and *ein2*. In contrast, true leaves of *hydra-ein2* had a consistently greater coherence of xylem and phloem associated callose (Fig. 5.33; F-G). This corresponds to a greater xylem coherence in the true leaves of double mutants, although vascular pattern anomalies are still present in these leaves. Considering the sibling double mutant population, ectopic callose deposition was reduced but not eliminated in post-germination lateral organs of the rosette.

The increased integrity of vascular strands in many of the *hydra-ein2* double mutants suggests a modulation of intercellular communication with regard to vascular strand formation. As a greater degree of vascular integrity can be seen in leaf sectors next to areas of poor xylem coherence in the same organ, these observations suggest that the integrity of the lower order vasculature has implications for the patterning and differentiation of higher order vascular strands. Two true leaves from the



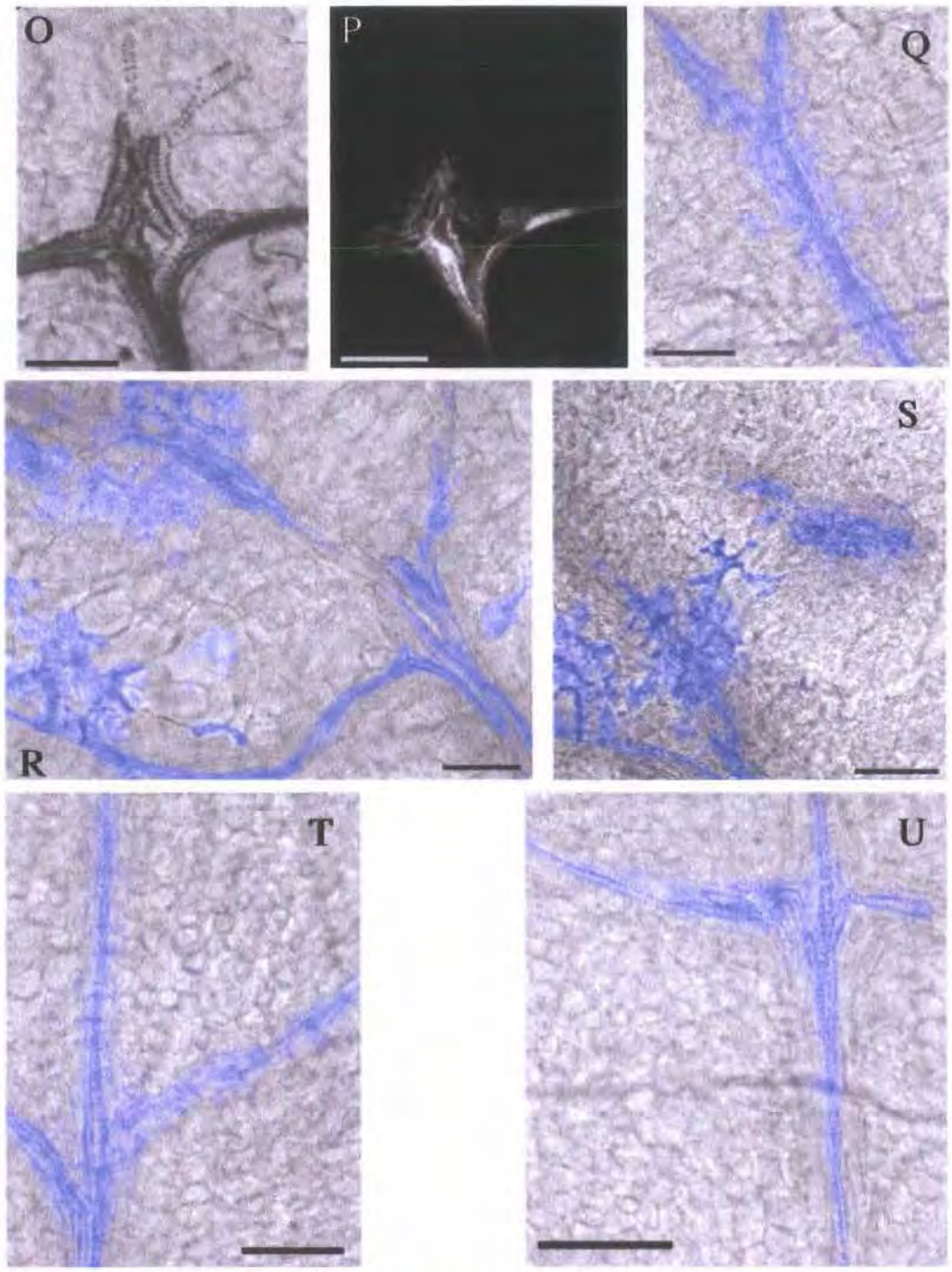

## Figure 5.32 Detail of procambial and xylem strand coherence in *hyd2-ein2*

B-E; detail from the *hyd2-ein2* second true leaf shown in A, as annotated. This leaf does not dissociate its midvein until close to the apex of the leaf organ (asterisk).

B; detail of the lower midvein region, where the strands appear very coherent. Parallel with this region, the higher order vascular traces of the lower lamina appear to be differentiating normally; C shows a secondary vein with its xylem strand, adjacent to a tertiary loop where differentiation has not taken place yet.

Nearer to the apex of the lamina, above the region where the midvein dissociates, E shows the high degree of 'noise' in xylem deposition associated with the two apical hydathodes. D shows the secondary loop adjacent to this region; here xylem vessels are differentiating in isolated sections.

A, bar = 200$\mu$m; B-E, bar = 50$\mu$m.




## Figure 5.33 Correspondence of xylem traces and phloem-associated callose in *ein2* and *hyd1-ein2*

**A and B; light-field and UV images respectively of the hydathode region from an 8 dae *ein2* cotyledon, stained with Aniline Blue to highlight phloem callose. In wild-type, as seen here in *ein2*, the fluorescence trace is associated with coherent vascular strands, and the isolated xylem vessels visible in A do not correspond to phloem callose fluorescence (B) in this area of enhanced xylem 'noise'. Bar = 50$\mu$m**

**C-G; overlain light-field and fluorescence images of 8 dae *hyd1-ein2*. C-E show cotyledon vascular traces, where deposits of callose are evident both ectopically (mostly near isolated xylem sections) and in the expected association with the xylem strands. There is evidence of further 'noise' in phloem differentiation, as the cotyledon primary strand in C shows fluorescence diffusely associated with the mid-vein region. The primary mid-vein traces from *hyd1-ein2* first true leaves F and G show reduced or absent ectopic deposition of callose, and a correlation between xylem and fluorescence traces. C-G, bar =100$\mu$m.**

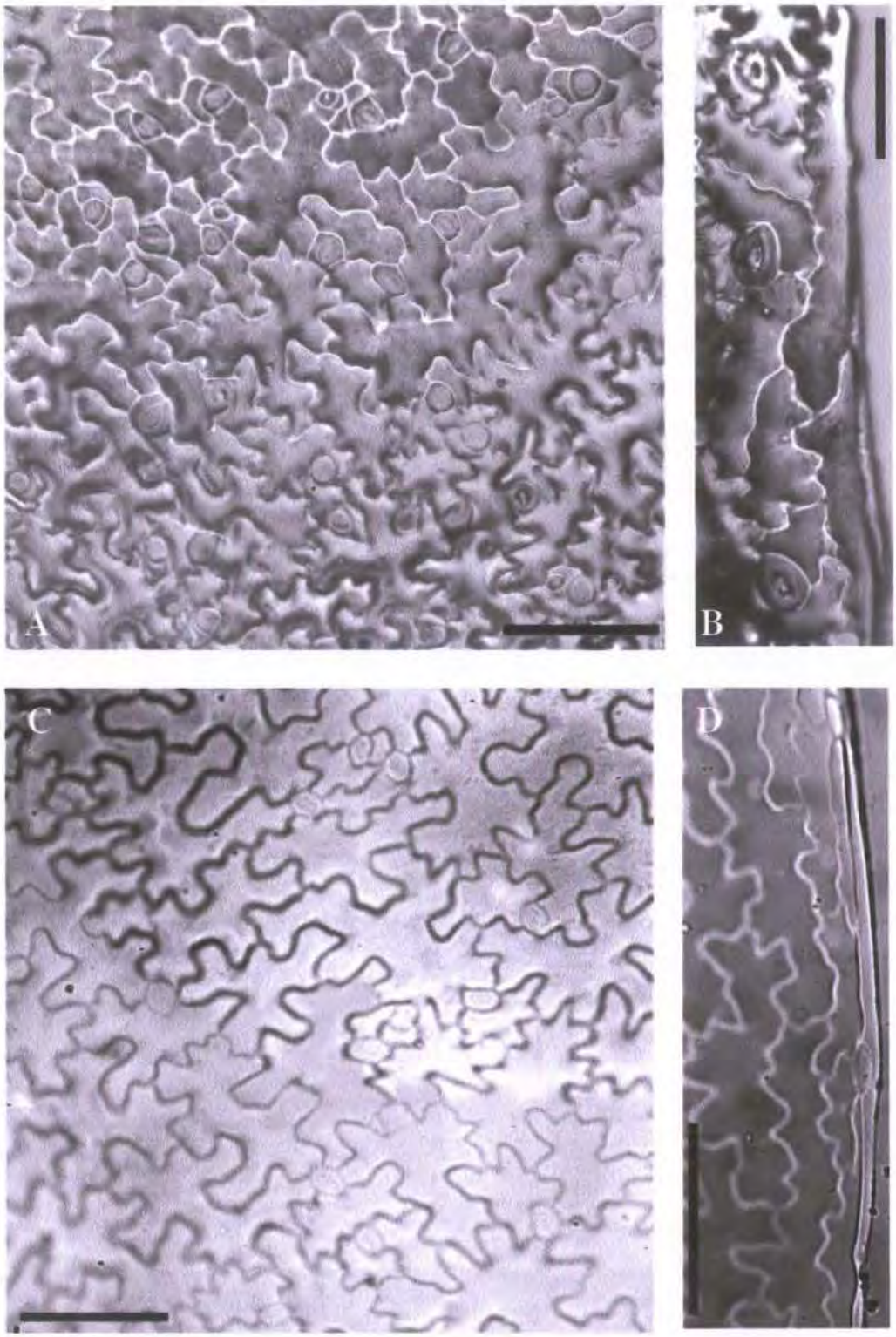
seedling, shown in Fig. 5.31 and 5.32, have a dissociation of the primary xylem strand at different points in the lamina, and an associated differential modulation of vascular pattern formation. Intercellular signalling is certainly modified in *hydra-ein2* post-germination true leaves, as indicated by a the better coordination between callose deposition and xylem integrity. Xylem 'noise' persists however even in these organs, and regions of isolated xylem vessels could still be found.

### 5.3.1.7.3 Improved laminar expansion associated with the *ein2* mutation reveals a close association between misplacement of marginal cell files and the distortion of organ shape in *hydra* cotyledons

The epidermis of *ein2* has reduced numbers of larger cells per unit area than wild-type (Fig. 5.34; A and C), along with greater elongation of the marginal cells (Fig. 5.34; B and D), and a flatter lamina from reduced epinasty. This effect is additive with the *hydra* phenotype, resulting in a better laminar expansion in the cotyledons, and a more even surface which is easier to analyse over a sequential series.

Figure 5.35; A and B show composite images of agarose impressions of the whole epidermal surface of the same *hyd1-ein2* cotyledon, at respectively 5 dae and 8 dae. This example is striking in that two 'domains' or regions of the lamina are visible; one side with correctly positioned marginal cells, and the other with a more confused arrangement, comprising a number of elongated cells. The boundary between these areas is indicated as a dashed line in this figure. Between 5 and 8 dae, these domains show a pronounced difference in expansion behaviour.

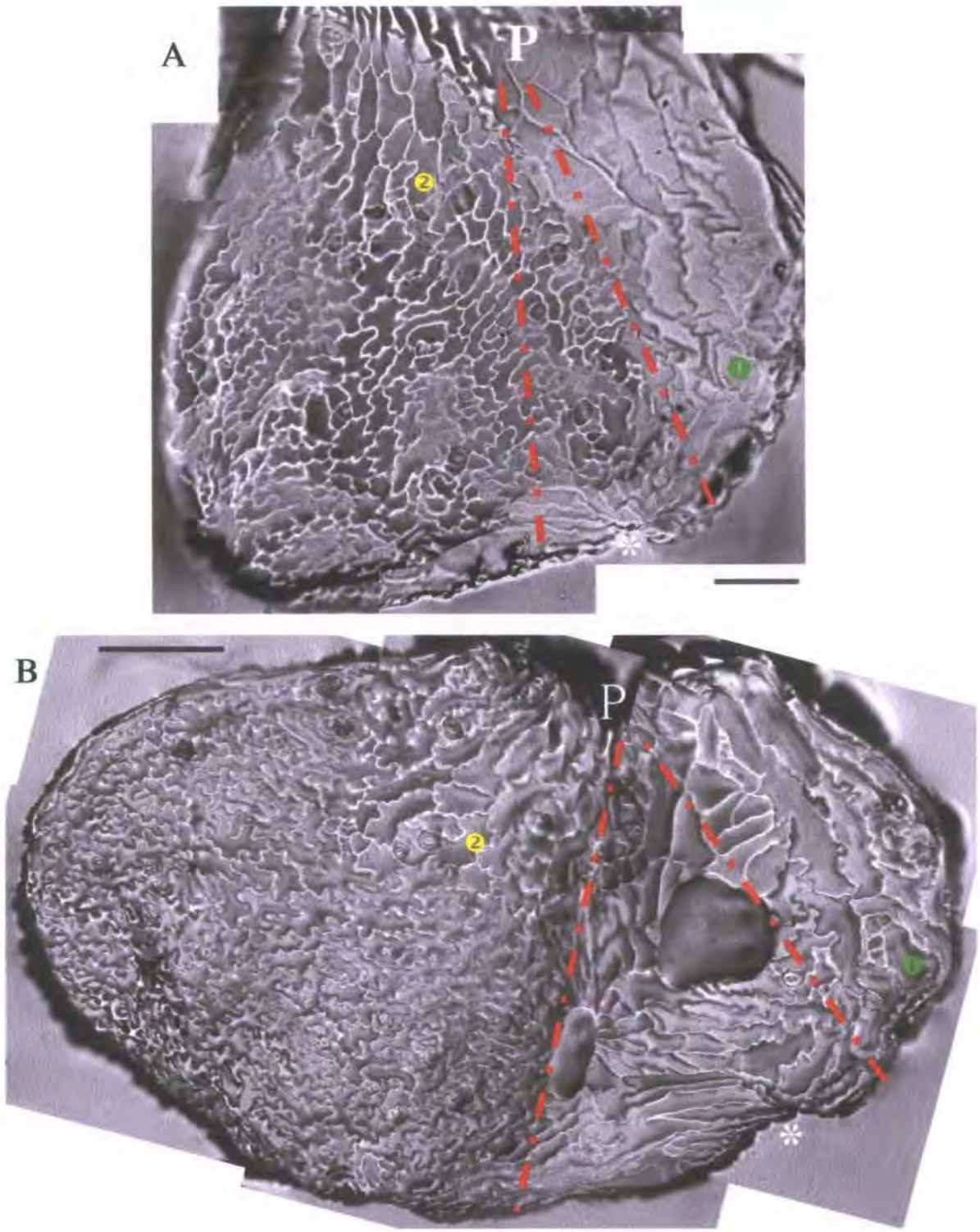
The domain with relatively normal marginal positioning to the left of the dashed line in Fig. 5.35; A, undergoes a relatively normal expansion in which pavement cells develop inter-digitating margins, and stomatal complexes form from meristemoid mother cells. Although some stomatal




## Figure 534 Patterning in the Ws and *ein2* cotyledon epidermis

**A, Ws and C, *ein2* epidermal patterning from the centre of the cotyledon adaxial lamina at 8 dae. B, Ws and D,*ein2* marginal cell patterning from the widest point of the fully expanded cotyledon lamina. A-D, bar = 100$\mu$m.**

**In *ein2* mutant cotyledons, epidermal adaxial patterning is similar to that observed in wild-type plants; a broad field of epidermal cells differentiate as either stomatal guard cells or pavement cells, bounded by elongated cell files at the laminar margin. The differences between them concern cell size and numbers; the *ein2* epidermis contains fewer cells which are larger in size than those found in Ws. Cotyledon surface area is the same in both Ws and *ein2*, and so the *ein2* mutant has adapted to its reduced cell number by effecting greater cell expansion during laminar development.**




## Figure 5.35 Patterning of the *hyd1-ein2* cotyledon epidermis

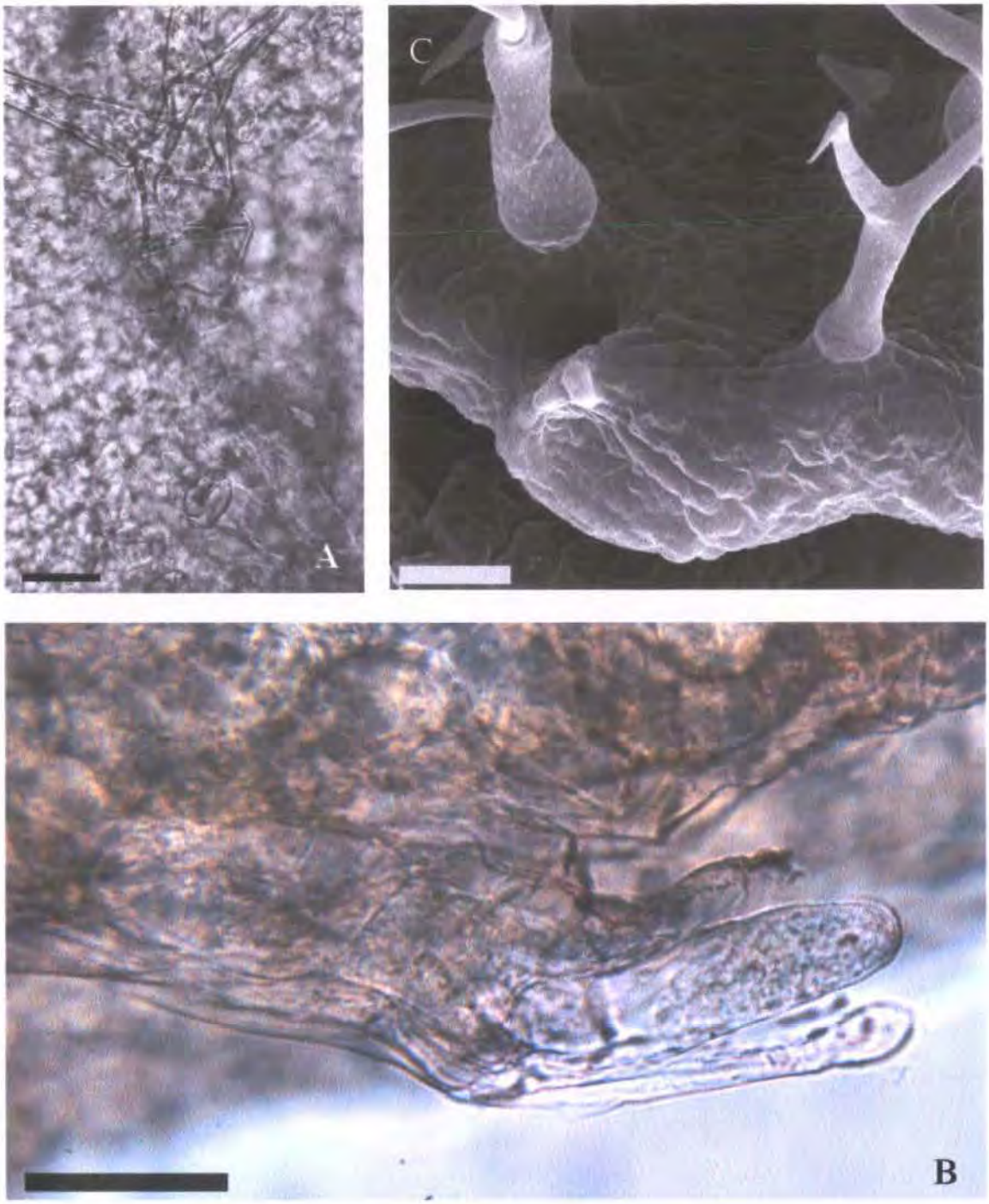
**A and B, composite images of agarose impressions from the same *hyd1-ein2* mutant cotyledon adaxial surface at 5dae (A, bar = 100$\mu$m) and 8 dae (B, bar = 200$\mu$m).**

**The junction between the cotyledon petiole and lamina is marked with the letter P, and the presumptive most acropetal point of the laminar surface marked with an asterisk in both images. These pictures show that the distorted morphology of the 8 dae lamina is associated with expansion in the centrolateral direction, including anomalous expansion of cells in the central lamina between the dashed lines. To the left of this region, the expanding lamina has undergone stomatal ontogeny and resolved a relatively normal mosaic of stomata and pavement cells. To the right, the lamina comprises giant cells, formed by expansion without cell division. Numerical annotations mark individual cells recognizable in both images.**

clusters are evident, as found in *hydra* single mutants, this domain presents one of the better examples of laminar expansion seen in the *hydra* mutants. The second domain, to the right of the dashed line, comprises elongated cells oriented on the axis between the petiole (p) and the putative cotyledon apex (asterisk), undergoes a very different expansion growth between 5 and 8 dae. The relative position of the cotyledon apex, shown by the asterisk in Fig. 5.35; A and B, indicates that most laminar expansion has taken place in a limited zone originally lying across the 'centre' of the lamina between the petiole and apex. Within this zone, cells near the apex have elongated further in the centrolateral direction than cells nearer to the petiole. The result is that close to the apex, a number of elongated cells are aligned across the leaf in line with the centrolateral axis, and near the base are enlarged cells with long axes running in a longitudinal direction. Between these extremes, a range of cellular orientations is visible, including two exceptionally large, almost oval shaped cells without a clear expansion axis. The margin of the lamina to the right of this region, contains enlarged cells which appear to have retained a pavement cell identity, and amongst which stomatal complexes are differentiating. This implies that the central region, with its unusually enlarged cells, is a distinct zone of cell identity (possibly comprised of misplaced marginal cells) separating two relatively normal areas of cotyledon epidermis.

Fig. 5.36; A-C shows detail of epidermis from *hyd2-ein2* first true leaves. In Fig. 5.36; A and C, elongated cell files were seen over the lamina adaxial surface, in association with a folded and invaginated leaf morphology. Fig. 5.5.36; A shows a trichome in these misplaced cell files, at the edge of a concave section of adaxial lamina. Fig. 5.36; B show the termination of a set of elongated cell files, which have formed a structure protruding from the laminar surface.

Both wild-type and *ein2* true leaves have elongated cell files in the adaxial surface above the primary midvein, running between the apex of the leaf and the base, where they are in continuity with longitudinal cell files of the




## Figure 5.36 Marginal cell file detail in *hyd2-ein2*

**A-C; *hyd2-ein2* epidermal patterning from first true leaves, showing misplaced marginal cell files. A and B, bar = 50$\mu$m; C, bar = 30$\mu$m.**

**A and B; images from safranin-stained and cleared tissues, 18 dae. A shows a section of adaxial true leaf epidermis where the marginal cell files cross the laminar surface, associated with a folded and invaginated leaf morphology. The cells visible in B are protruding from marginal cell files which leave the laminar margin and cross part way over the adaxial surface, terminating in the structure shown here.**

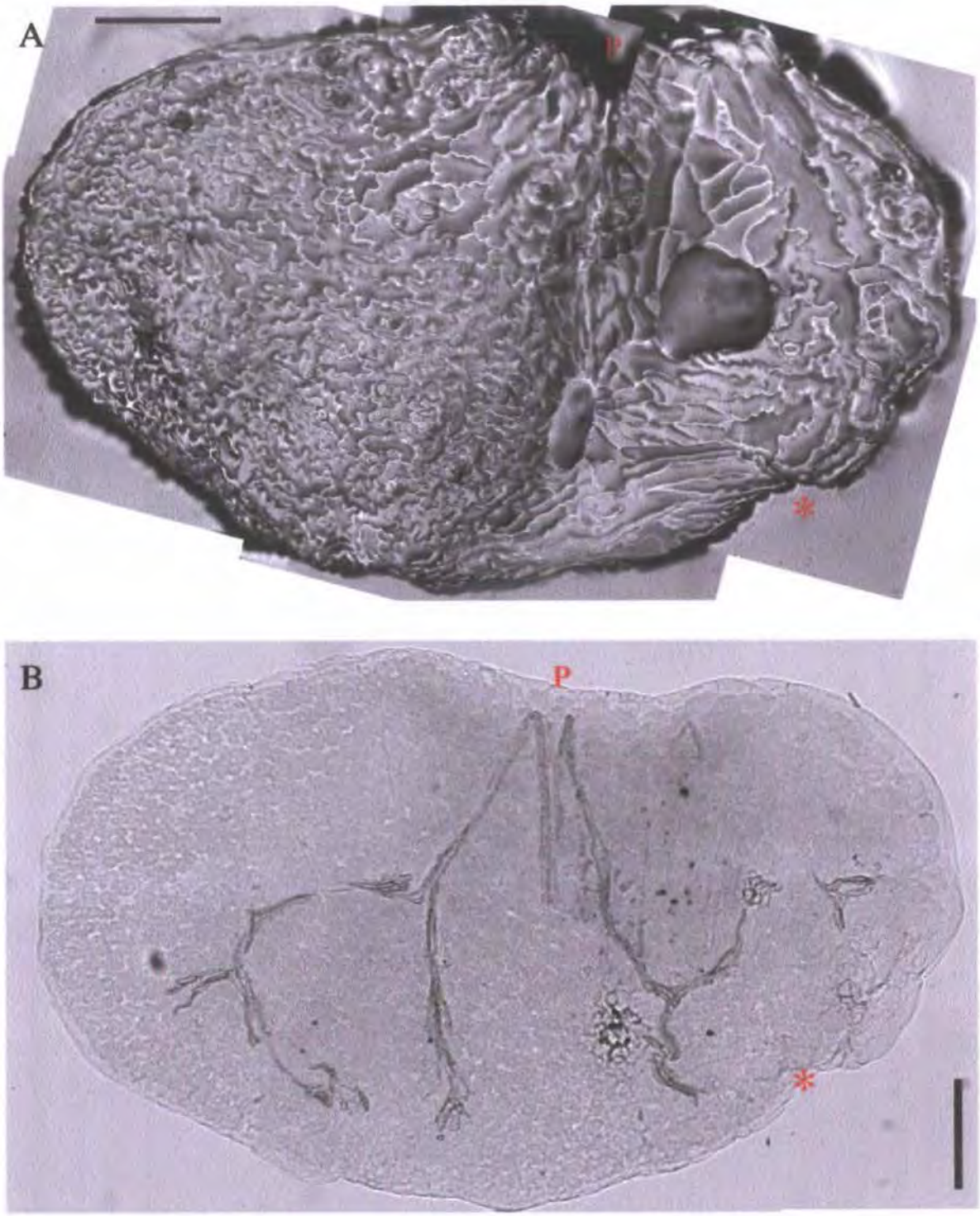
**C; detail of a first true leaf apical margin in the anticipated position of the apical hydathode (at the end of the primary vein ) taken from a *hyd2-ein2* plant at 8 dae. The adaxial surface is visible, with trichomes initiating from adjacent epidermal cells visible in the top right corner. Marginal cell files do not define a clear boundary to this lamina, which is misshapen and invaginated.**

petiole epidermis. As in single *hydra* mutants, misplaced longitudinally oriented cell files on true leaves could also originate from these central epidermal cell files. This suggests that the misplaced cell files detailed in Fig. 5.36; A and C could derive from either central or marginal longitudinal cells.

### 5.3.1.7.4 The distortion of the cotyledon lamina from Fig. 5.35 is associated with separation of the primary midvein, accompanied by misdirected expansion growth in the epidermis and mesophyll.

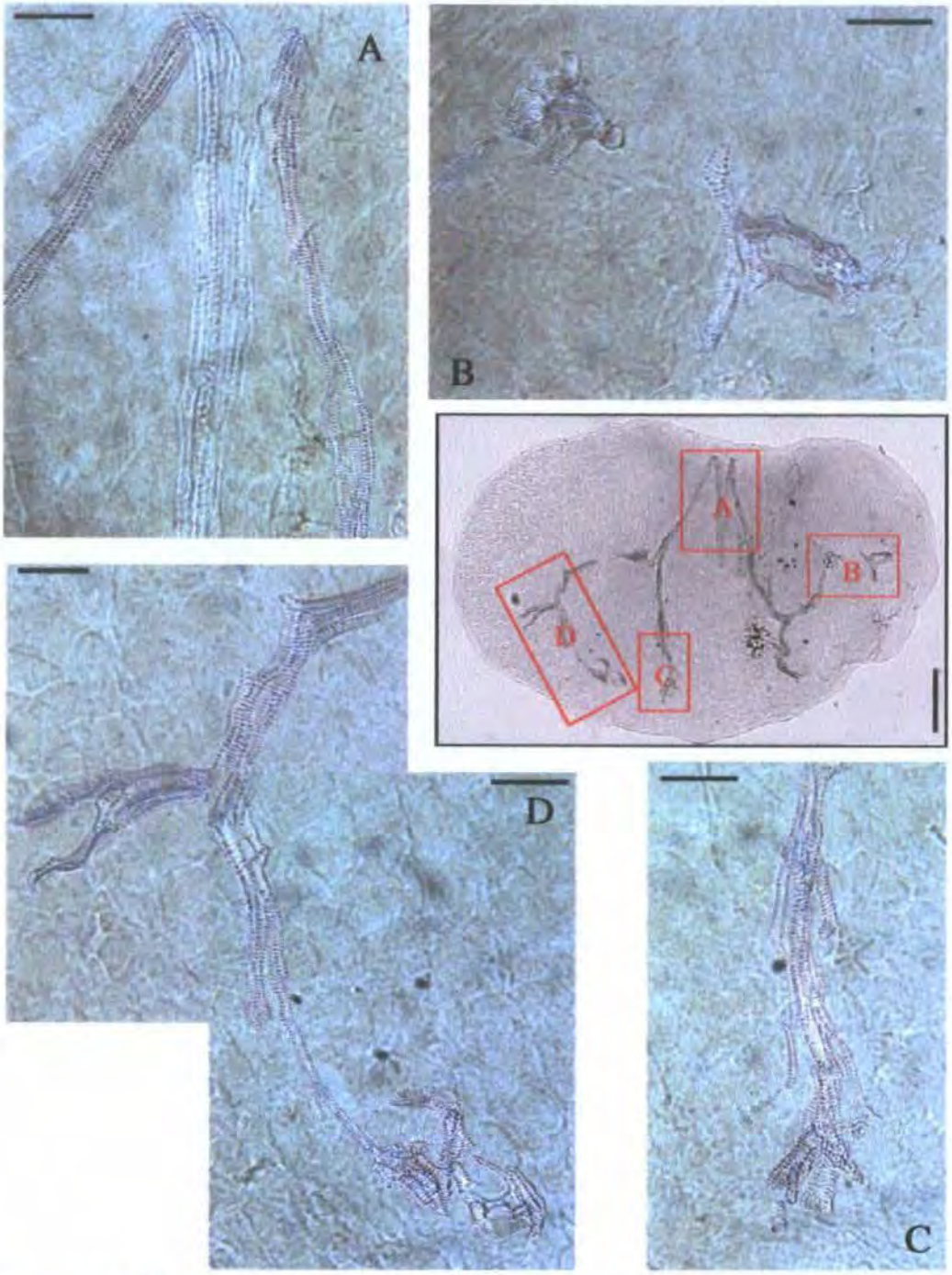
Fig. 5.37; A, shows the 8 dae cotyledon epidermis from Fig. 5.35; B, alongside the leaf organ used to make the epidermal print, cleared in chloral hydrate to reveal the xylem trace (Fig. 5.37; B). Along with the data from section 5.3.1.7.3, this suggests that the zone of anomalous cell expansion seen in this lamina corresponds to the epidermis above the region of separation between the xylem strands of the primary midvein.

The separation of the midvein in Fig. 5.37;B extends from the petiole-laminar boundary towards the leaf apex. In the mounting of this sample, the petiole has bent beneath the lamina, and hence the petiole xylem appear to be extending into the leaf; this is a mounting artefact. The basal region of the petiole is shown in more detail in Fig. 5.38; A. This image shows that prior to strand divergence (i.e. in the petiole), xylem noise is minimal in the trace. The left side of the lamina in Fig. 5.37; B, corresponding to the region of abnormal epidermal pattern, is associated with an extreme level of xylem noise, culminating in vascular islands at the end of the strand (Fig. 5.38; B). The relatively normal region of lamina to the right shows a vascular trace where xylem noise is exacerbated relative to below the bifurcation point, but does not include disconnected xylem vessels (Fig. 5.38; C and D).




## Figure 5.37 Coordinated vascular and epidermal patterning anomalies in *hyd1-ein2* associated with leaf expansion

**A; composite image of epidermis from the developing cotyledon shown in Fig. 5.35. B, the same 8dae cotyledon, cleared with chloral hydrate to reveal the xylem trace; bar = 200$\mu$m. Note that the petiole is folded under the lamina in B, and dissociation of the primary midvein in this cotyledon begins in the petiole. The central-right zone which is populated by giant epidermal cells lies above the region between the two dissociated primary vascular strands; the resultant large central area of un-vascularized tissue is reminiscent of vascular patterning in the *lop1/trn1* mutant.**




## Figure 5.38 Cotyledon xylem detail from *hyd1-ein2*

A-D; Nomarski images showing detail of the ***hyd1-ein2*** cotyledon vascular trace presented in Fig. 5.37; bar = 50$\mu$m. The position of these images in the vascular trace is shown in the inset.

The primary mid-vein dissociates in the cotyledon petiole before entering the lamina (A). The lamina to the right of the mid-vein contains vascular islands (B), beneath an epidermis which carries a field of elongated cells and giant cells, some or all of which may have marginal cell file identity. The left side of the lamina shows a high degree of 'noise' in the co-ordination of xylem vessels into linear strands (C and D), but does not have disjunct sections of xylem. Elongated marginal cell files are correctly located at the edge of the lamina in this region of the cotyledon, and the adaxial epidermis above consists of a field of pavement cells and stomatal guard cells (Fig. 5.37), i.e. shows a more normal patterning than the right side. This implies that the substantial mis-patterning of the epidermis is correlated with severe vascular disjuncture in the mesophyll.

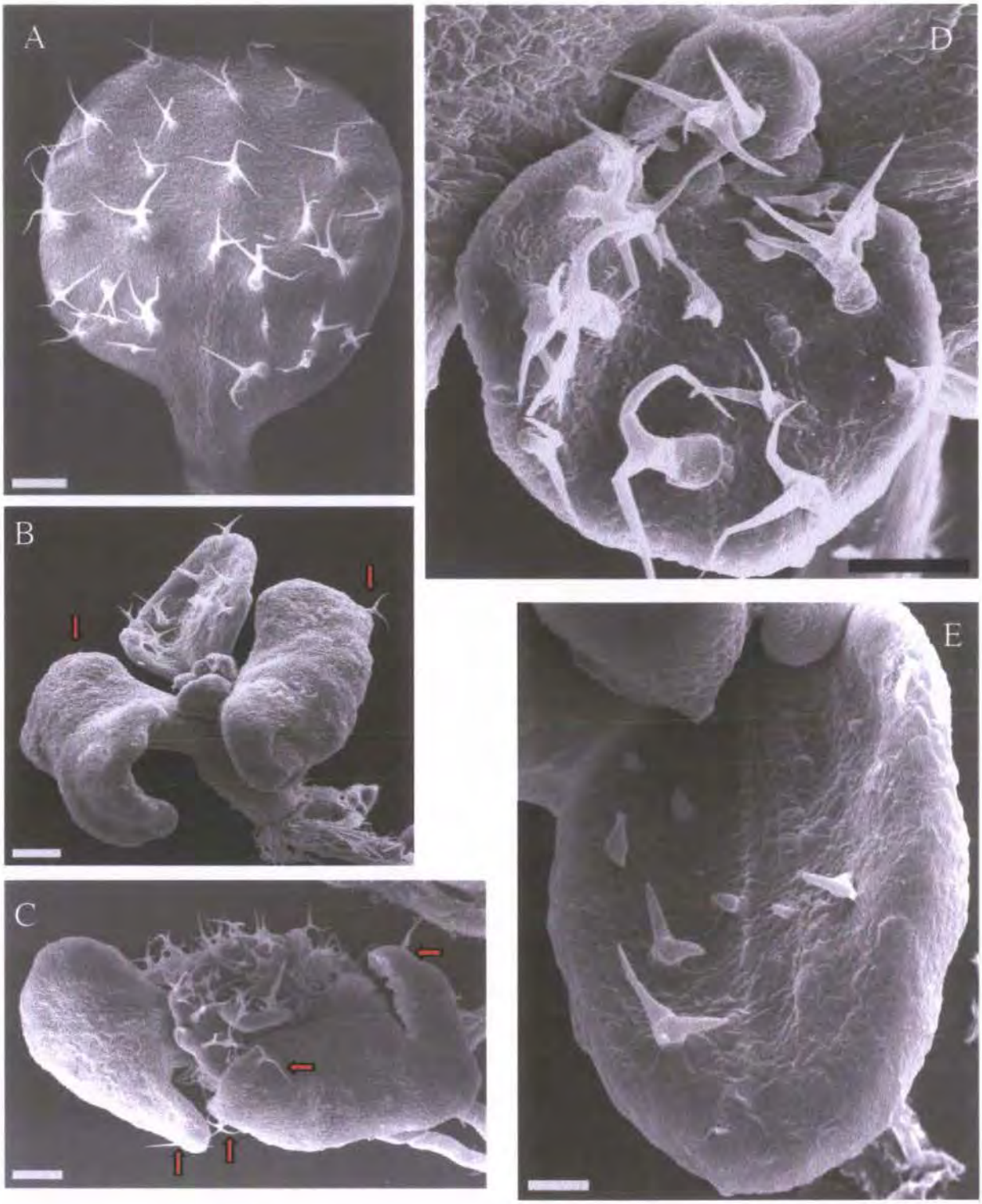
This implies that integrity of the primary midvein is not only required for the integrity of subsequent vascular patterning, but also that the vascular pattern is intimately linked to leaf shape through the controlled positioning of cell expansion in the *hydra* mesophyll and epidermis.

### 5.3.1.7.5 Trichome spacing, morphology and associated patterning of basal cells are similar in *hydra* and *hydra-ein2*

The epidermis of *ein2* lateral organs contains a reduced number of cells, which maintain organ size by greater expansion growth as shown in Fig. 5.39; A-D. This reduction in cell numbers does not seem to affect the trichome spacing process; the *ein2* lamina in Fig. 5.39; A shows an even distribution of trichomes over the surface of this young first true leaf. It is not known whether overall trichome numbers are reduced in *ein2* leaves as in the mutant root epidermis, although the Ws true leaf shown in Fig. 4.62; D appears to have a similar numbers of hair cells as the *ein2* leaf in Fig. 5.39; A. No trichomes were observed on cotyledons from *ein2*. In the expanding epidermis, trichomes showed a normal morphology and normal development of basal cells (Fig. 5.40; A).

Cotyledons from *hydra2-ein2* seedlings, shown in Fig. 5.39; B and C, carry occasional ectopic trichomes, as in the *hydra* single mutants. Trichome spacing in the double mutants was also similar to *hydra* (Fig. 5.39; D), although differentiation appeared to progress at variable speeds in different leaf organs (compare the leaves in Fig. 5.39; D and E). Within the epidermis, examples were seen of adjacent cells in pairs adopting trichome cell fate (Fig. 5.40; B and C), although as in *hyd* single mutants, most of the population of trichomes developed from single cells.

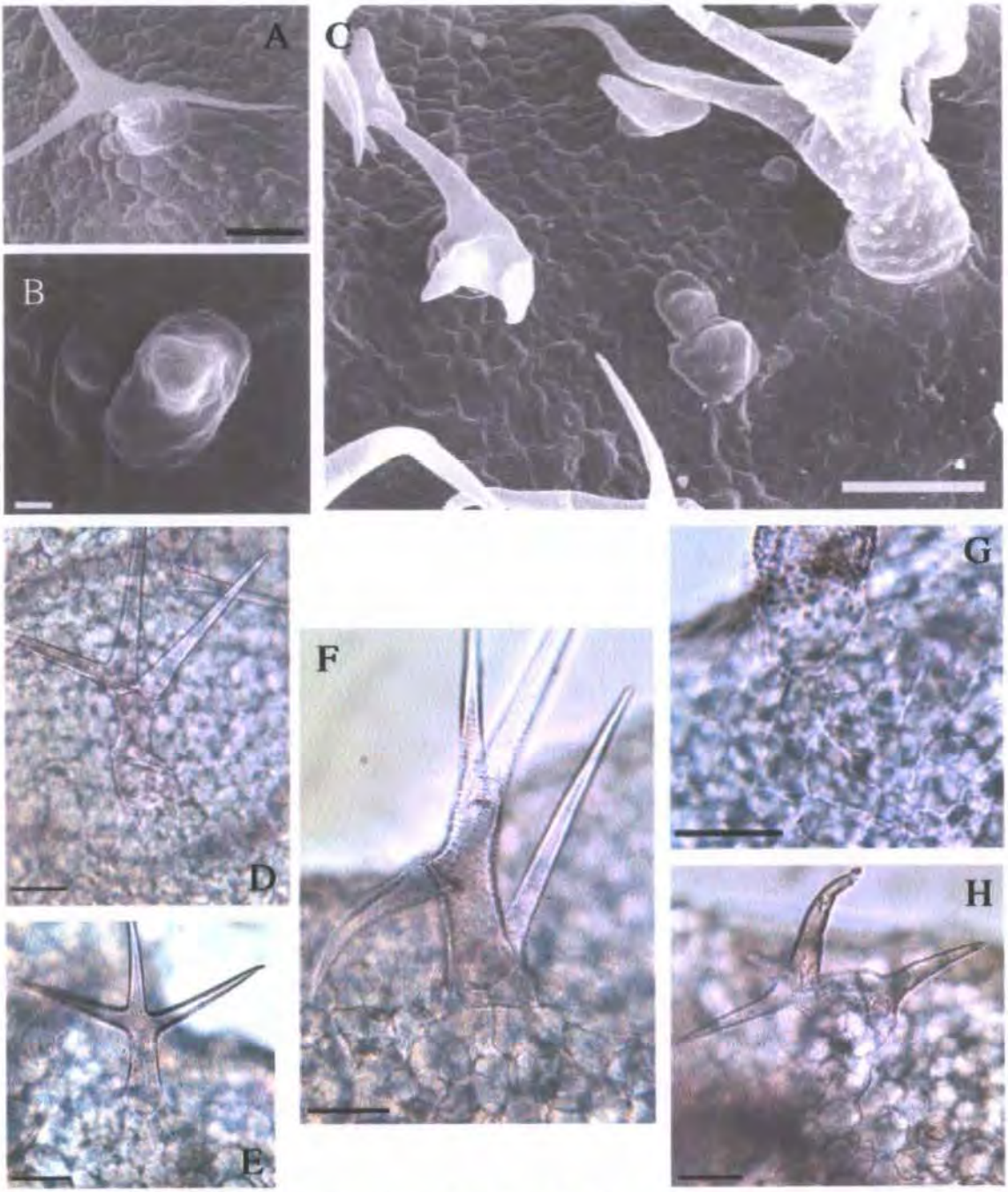
The patterning of basal cells in *hydra-ein2* was also variable, again as in *hydra* single mutants. Patterns included a normal arrangement (Fig. 5.40; G), an arrangement of small isodiametric cells in rows along the




## Figure 5.39 Trichome distribution in *ein2* and *hyd2-ein2*

**Trichome distribution in *ein2* (A) and *hyd2*-ein2 (B-E), showing true leaf detail from 8dae plants. A-C; bar = 200$\mu$m, D and E; bar = 30$\mu$m.**

**The distribution of trichomes over *ein2* true leaves is essentially similar to that observed in wild-type plants (A). In *hydra-ein2* double mutants, anomalies observed in the single mutant phenotype are still prevalent. B and C show trichomes on cotyledons (arrows). The general pattern of trichome spacing is similar to that seen in single *hyd* mutants (D, E).**




## Figure 5.40 Trichome patterning in *ein2* and *hyd2-ein2*

A; young trichome from an ***ein2*** plant showing the arrangement of elongated basal cells; this pattern is indistinguishable from that found in wild-type plants (bar = 30$\mu$m). B and C; detail from ***hyd2***-ein2 8 dae plants showing initiation of trichomes from adjacent epidermal cells (B) and the production of two growing hair tips from a single enlarged cell (C). Many of the other trichomes visible in C also have unusual morphology. B, bar = 5$\mu$m; C, bar = 30$\mu$m.

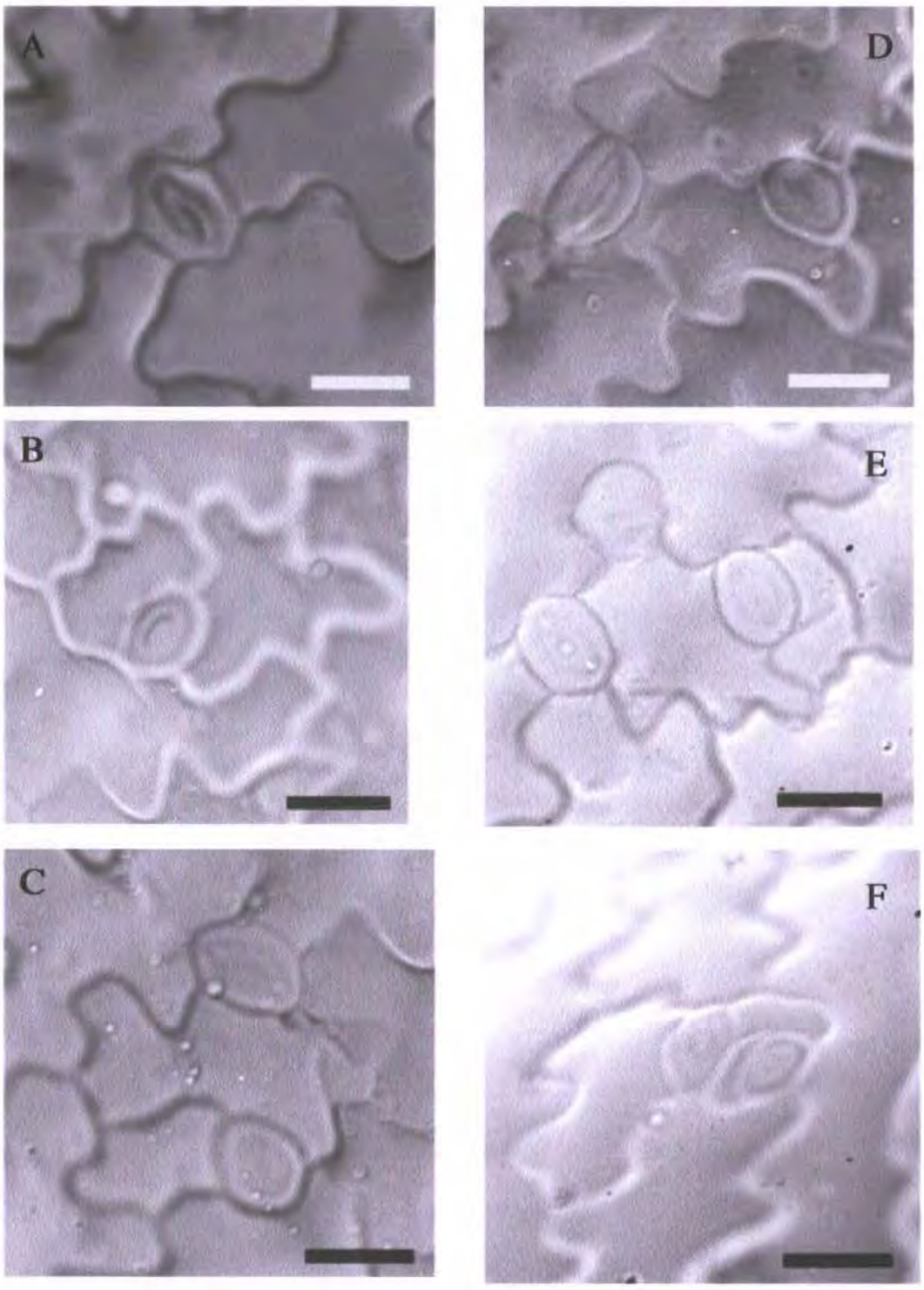
D-H; detail from ***hyd2***-ein2 12 dae plants stained with safranin, bar = 50$\mu$m. The trichome shown in D has elongated basal cells that have extended upwards from the plane of the leaf, producing an unusually high platform for the trichome. In E, the basal cells have divided producing rows of unusually isodiametric cells around the trichome. In F, the basal cells have elongated but are not oriented towards the trichome. The trichomes shown in G have initiated from adjacent epidermal cells, though the arrangement of basal cells appears normal. Trichome H is formed from a single, unusually inflated cell. One of the basal cells is just visible; the cell wall furthest from the trichome base appears to be developing inter-digitating tips with adjacent pavement cells, a phenomenon not observed in wild-type.

anticipated elongation axis of the basal cells, i.e. in a circle radiating in towards the trichome (Fig. 5.40; E), a group of elongated cells with variable orientations around the trichome base (Fig. 5.40; F), and variably sized and patterned elongated cells which appear to have elevated the trichome above the laminar surface (Fig. 5.40; D and H). These latter phenomena may have been present in *hydra* single mutants, but were not obvious due to the lower levels of cellular expansion. The basal cell indicated by an arrow in Fig. 5.40; H, has a mixed identity and appears to have been incompletely recruited to trichome basal cell fate. A pointed 'end' almost reaches the base of the hair cell, whilst at the other extreme of its axis, this cell forms an inter-digitating cell wall with adjacent pavement cells.

### 5.3.1.7.6 The reduction in cell numbers associated with the *ein2* mutation reduces but does not eliminate the formation of adjacent stomates in the cotyledon epidermis

In *ein2,* the reduction in cell numbers in the cotyledon adaxial epidermis appears to be associated with a minimal development of stomatal complexes (Fig. 5.41; A-F). In the samples studied, many stomates differentiated from the meristemoids directly, without the subsequent divisions associated with complex formation that produce the characteristic smaller accessory cells. This is in contrast with wild-type, where between 2 and 3 stomata form within a complex, derived from a single meristemoid mother cell, and satellite meristemoids differentiate from other unspecified epidermal cells.

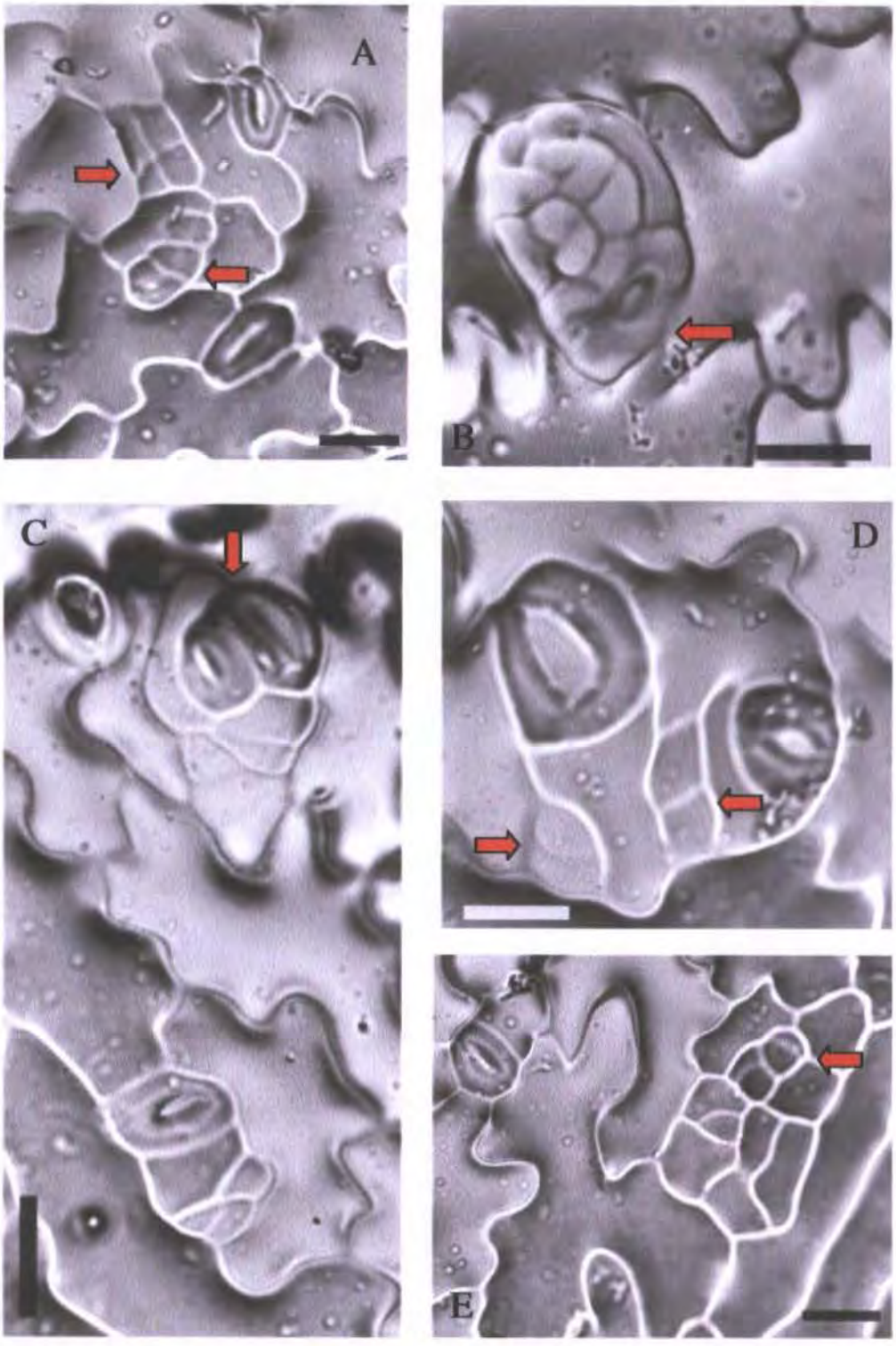
In the *hydra-ein2* samples studied, the extreme stomatal clusters found in *hydra* single mutants were not found, although stomatal clusters of between 2 and 4 adjacent guard cell pairs were evident (Fig. 5.42; C, and Fig. 5.43; A, B). As with *hydra* single mutants, these clusters were more developed on the abaxial epidermis of the cotyledon than the adaxial surface. The extent of stomatal complex formation similarly progresses




## Figure 5.41 Stomatal development in *ein2* mutant cotyledons

**A-F; *ein2* cotyledon adaxial stomata, as epidermal impressions in agarose from 8dae seedlings, bar = 20$\mu$m.**

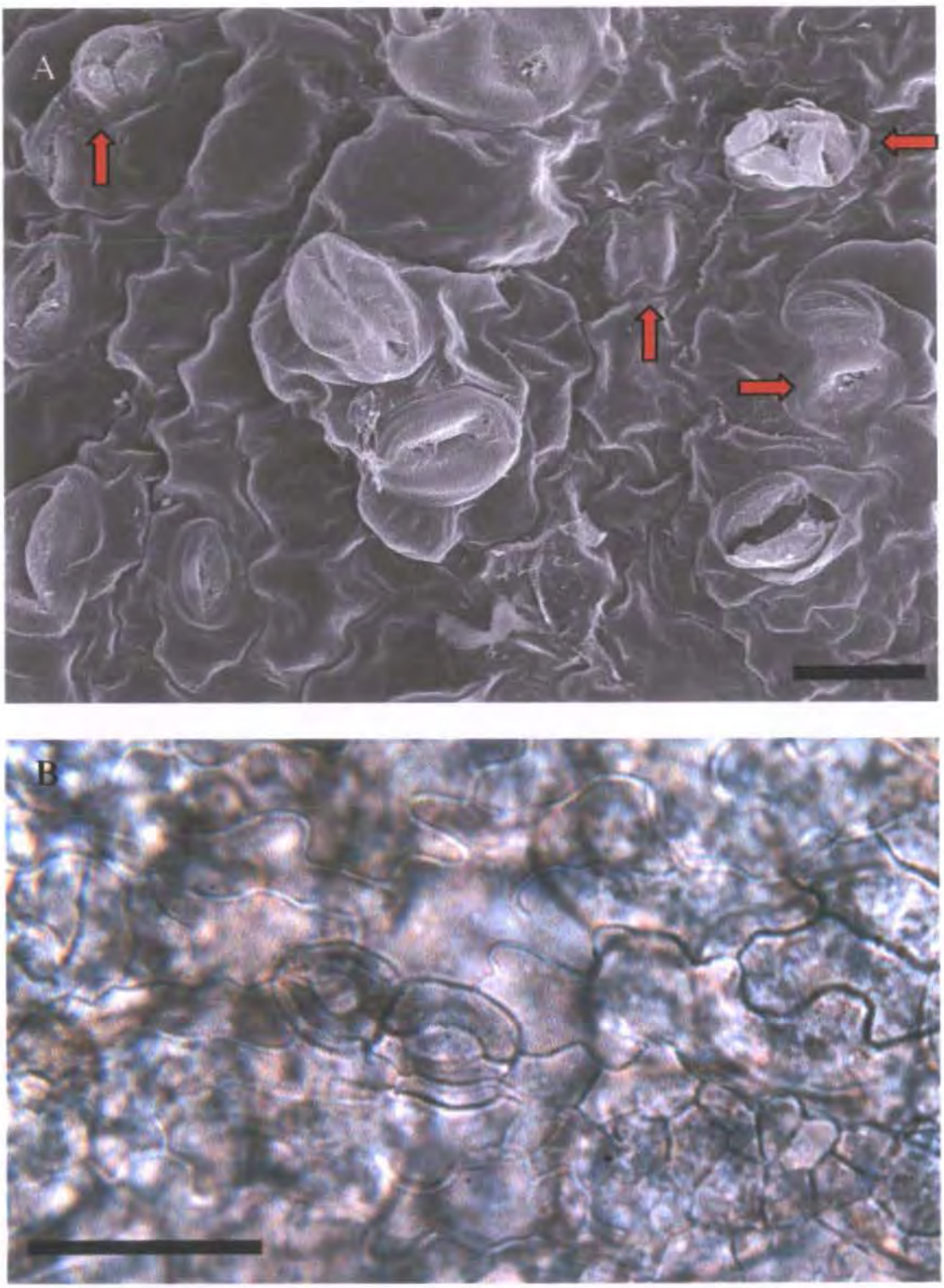
**Relatively few of the cells adopting meristemoid mother cell identity in the *ein2* cotyledon epidermis form stomatal complexes. A, B, D and E above appear to have developed from individual meristemoids without further division or satellite meristemoid formation. The cells shown in F are taken from the petiole-laminar boundary, where local cell division events associated with meristemoids are seen last during laminar development. It appears that two cells adopted meristemoid mother cell identity, and one of these has made an asymmetric division to separate the cells prior to adopting guard mother cell fate. A developmental event such as this would result in a pattern similar to that shown in C.**




## Figure 5.42 Stomatal pattern development in *hyd1-ein2*

G-K; ***hyd1-ein2*** cotyledon adaxial stomata from 8 dae seedlings, bar = 20$\mu$m.

Unlike *ein2* where cells appear to lose totipotency early, the *hyd1-ein2* double mutant undergoes frequent cell division in the cotyledon epidermis, producing clusters of small cells which may be associated with an obvious stoma (as in B), or may proceed through multiple divisions prior to stomatal differentiation (as in A and E). The cells in E marked by the arrow appear to be undergoing asymmetric divisions to produce small meristemoidal cells which do not contact other meristemoids. Cells in A and D appear to be dividing to produce approximately isodiametric daughters (arrows). Adjacent or clustered stomata also form (B and C, arrows).




## Figure 5.43 Stomatal patterning in *hyd2-ein2*

A, B; *hyd2-ein2* cotyledon abaxial (A) and adaxial (B) stomata from 8 dae seedlings. A, bar = 10$\mu$m; B, bar = 50$\mu$m.

As with the *hyd1-ein2* double mutant, stomatal clusters are also seen in *hyd2-ein2*. The adaxial epidermis contains frequent adjacent stomata (B) as in *hyd2* single mutants. Clusters of small cells are also present, as in the lower right of this image. The abaxial epidermis shows a more pronounced development of stomatal clusters (again as in *hydra* single mutants), although the extent of cluster formation is not as severe. Several clusters are evident in A (arrows), along with other unusually large stomates in the centre of the picture.

further over the abaxial surfaces of wild-type and *ein2* cotyledons and true leaves, (although wild-type, and *ein2* mutants, do not produce adjacent stomata).

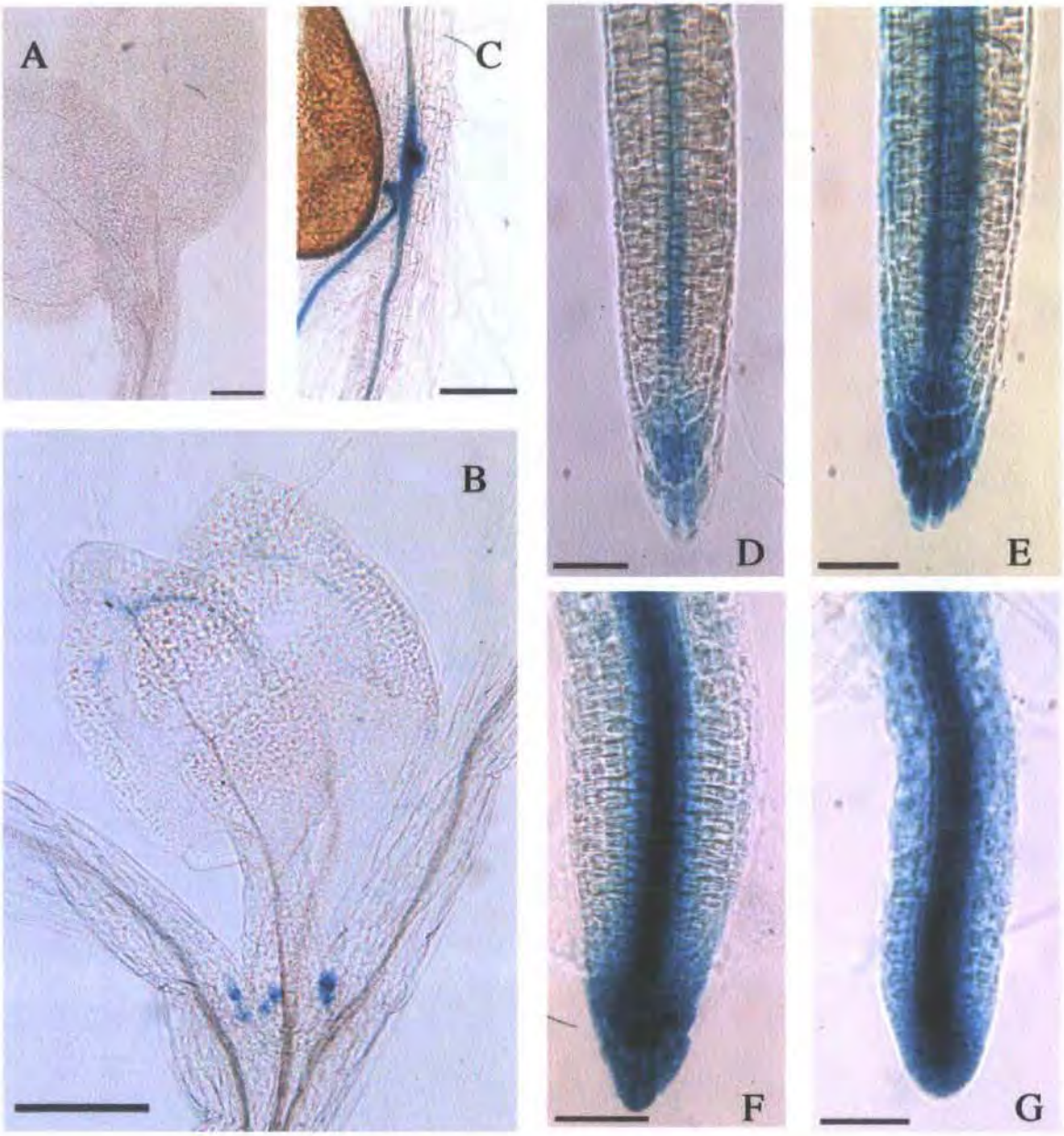
The agarose impressions shown in Fig. 5.42; A, B and E, show stomatal complexes at various stages of differentiation, where division events have produced a range of cell sizes and shapes. In Fig. 5.42; A (arrows) and B, the epidermis shows signs of cell proliferation that has resulted in evenly-sized cells, rather than the asymmetrically distinguishable meristemoids and companion cells of wild-type and *ein2*. In contrast, the cells of the stomatal complexes in D and E appear to be resolving more normally, and meristemoid divisions have produced potential guard mother cells with a corrected spacing pattern. In all of these examples, the sizes of the differentiated stomates are highly variable.

## 5.3.2 Signalling responses in *hydra* and *hydra-ein2* double mutants

### 5.3.2.1 Auxin response

#### 5.3.2.1.1 The *hydra* mutant phenotype has heightened auxin response in shoot tissues and primary root apices, reduced response in lateral root apices, and accumulates auxin near isolated or dissociated xylem

The early auxin response *AUX/IAA* gene *IAA2* is induced strongly and specifically by endogenous auxin (Abel *et al.* 1995, Abel *et al.* 1996), and shows an asymmetric distribution in gravi-stimulated roots in wild-type plants (Luschnig *et al.* 1998). The p*IAA2*::GUS reporter (Swarup *et al.* 2001) therefore acts as a marker for early auxin-induced gene expression




## Figure 5.43 Expression of p*IAA2*::GUS in wild-type seedlings

A-G; wild-type seedlings showing the expression of p*IAA2*::GUS.

A-C; shoot tissues, bar = 200$\mu$m. A; seedling shoot apex at 3dae, and B; at 12dae. C; root-hypocotyl junction at 12dae.

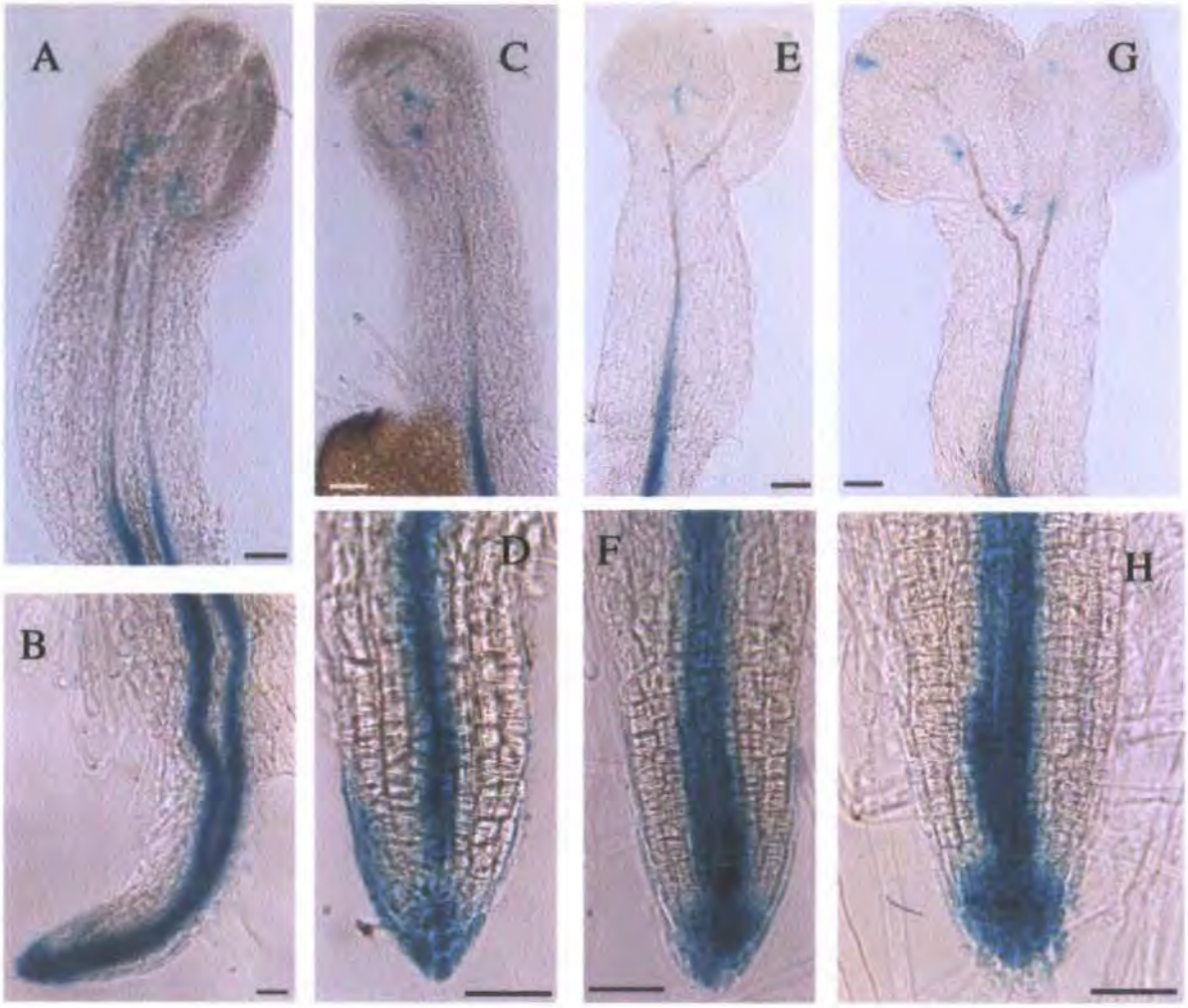
D-G; root tissues expressing the same construct, bar = 50$\mu$m. D; emergence primary root apex, E; root apex at 3dae. F; primary root at 12dae, and G; anchor lateral root apex at 12dae.

Expression of pIAA2::GUS appears early in root development in association with the developing stele and root cap C, D, E), but does not appear in the shoot tissues until emergence of the first true leaves at around 7dae, when a stipule signal appears simultaneously with the definition of the primary strand. As the young leaves develop, reporter activity is seen in the vascular traces prior to xylem differentiation (B). In the established seedling, the primary root apex maintains a higher level of signal in the root cap tissues (F) than is seen in mature lateral root apices (G).

and indicates the presence of active auxins within the plant body. In wild-type plants, expression of p*IAA2*::GUS appears first in the root cap columella and root stele at around 3 dae, becoming more intense in these tissues between 5 and 7 dae (Fig. 5.43; D-F). A less pronounced columella signal is seen in mature wild-type lateral root apices (Fig. 5.43; G). No expression is seen in the hypocotyl stele, and GUS activity is absent from the root stele where cell maturation is complete, reappearing in association with initiating lateral roots (Fig. 5.43; C). In the shoot, no signal is discernible in cotyledons (Fig. 5.43; A). As true leaves begin to emerge between 5 and 7 dae, a stipule signal appears, followed by transient definition of the leaf vascular traces, prior to differentiation of the xylem vessels (Fig. 5.43; B).

Seedlings of both *hyd1* and *hyd2* at 3 dae show a similar ectopic expression of p*IAA2*::GUS in shoot tissues. GUS activity, confined to the root stele in wild-type, extends part-way into the mutant hypocotyl stele, and appears as a localised ectopic signal in the cotyledons (Fig. 5.44; A, C, E, G). In older *hydra* shoot tissues, ectopic cotyledon p*IAA2*::GUS activity persists in the vicinity of late-differentiating xylem, vascular islands, and in regions where the xylem trace shows poor coherence (most noise) in the strand, particularly at the hydathodes (Fig. 5.46; A-C). This ectopic expression was present in most cotyledons, although no signal was found in radialized cotyledon structures (Fig. 5.45; F). In true leaves, both *hydra* mutants show an enhanced p*IAA2*::GUS expression in the vascular traces during laminar development (Fig. 5.45; A-C), a signal persisting longer in the mutants than in wild-type, and still visible at 12 dae.

In 3 dae root apices, p*IAA2*::GUS activity in *hyd1* (Fig. 5.44; B and D) was closer to wild-type expression than in *hyd2* (Fig. 5.44; F and H). In *hyd2*, the root cap columella appears less regular in shape than *hyd1*, and reporter activity in the stele near the root apical meristem shows a wider band of expression than in the differentiated vasculature further from the apex. This suggests a stronger reporter activity in the 3 dae *hyd2* root apex, compared with *hyd1* roots, indicating a more diffuse localization of



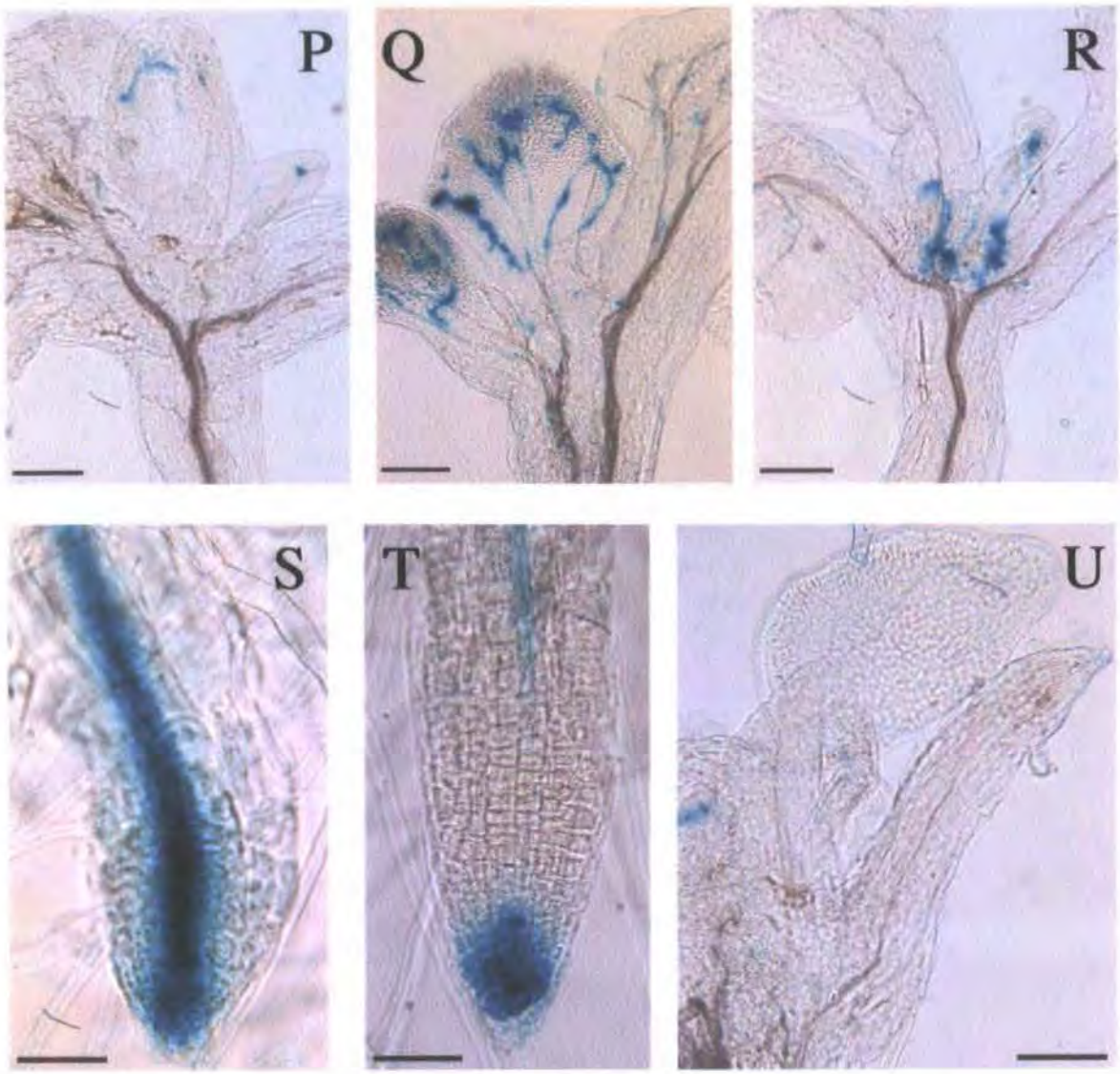

## Figure 5.44 Activity of p*IAA2*::GUS in 3 dae *hydra* seedlings

A-B, C-D; *hyd1* seedling shoot and root apices from the same 3 dae seedlings. E-F and G-H; shoot and root apices from the same *hyd2* 3 dae seedlings, all showing expression of p*IAA2*::GUS. A, C, E, G, bar = 10$\mu$m; B, D, F, H, bar = 50$\mu$m.

Root expression of p*IAA2*::GUS in *hydra* is as wild type at 3dae, following the vascular traces, although with a more diffuse expression where the stele is broader, in association with separated xylem strands (e.g. B and F), or in the region of provascular strand formation as in H.

In wild type plants, reporter activity in the stele stops abruptly at the root-hypocotyl junction, whereas in *hydra*, the signal extends up the hypocotyl towards the SAM, to varying degrees in different siblings. This does not appear to be through strand separation, as the phenomenon is still observed in siblings where the axis is duplicated (A).

The wild type activity of p*IAA2*::GUS is not seen in post-germination cotyledons. In contrast, both *hyd1* and *hyd2* have ectopic cotyledon expression from between 2 and 3 dae, visible in A, C, E and G above.

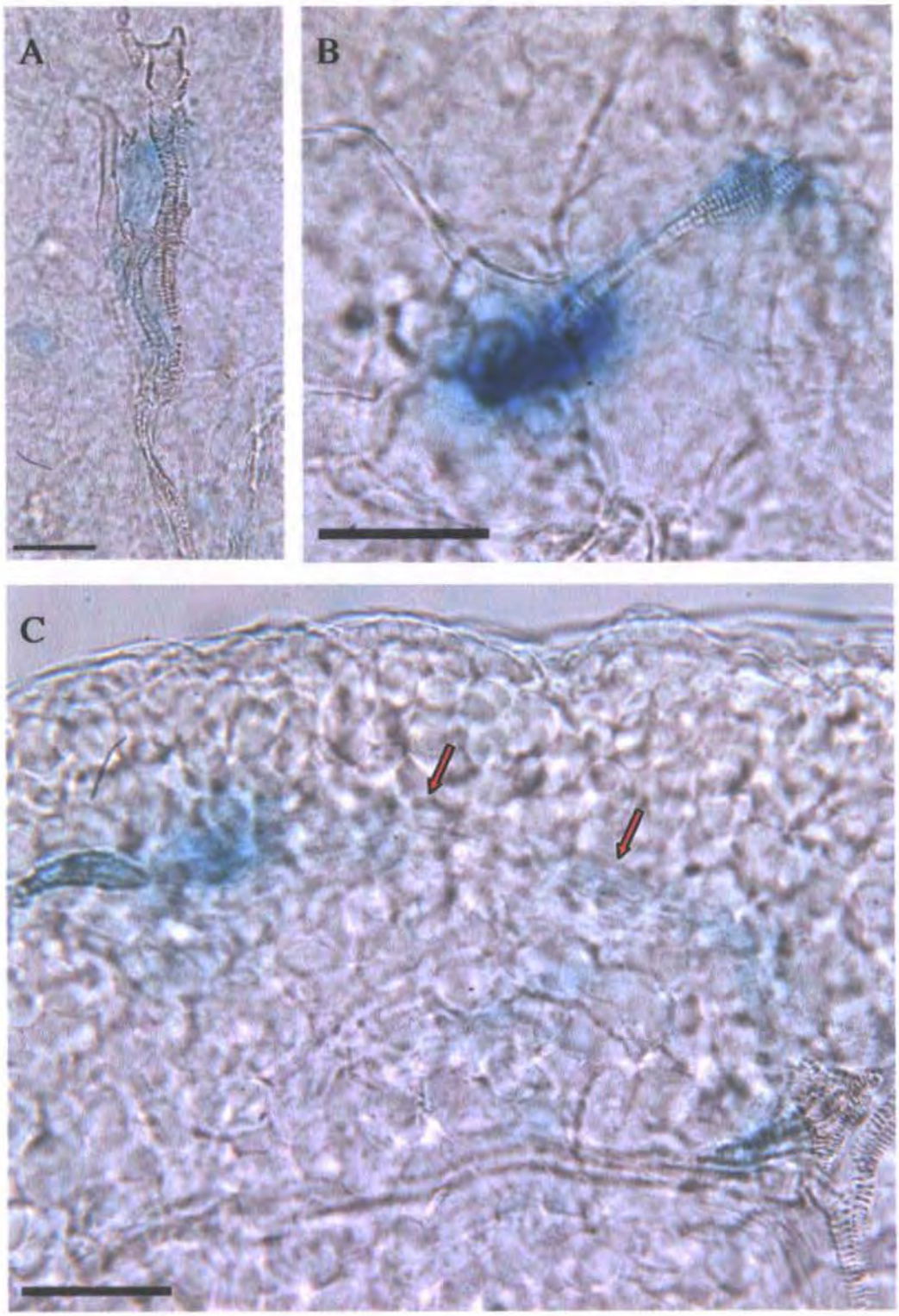



## Figure 5.45 Activity of p*IAA2*::GUS in 12 dae *hydra* seedlings

A-F; ***hydra*** seedling shoot and root apices from 12 dae plants expressing the p*IAA2*::GUS reporter. A, C and F; *hyd1*, and B; *hyd2* seedlings, bar = 200$\mu$m. D and E show the primary (D) and lateral (E) root apices from the seedling shown in A; bar = 50$\mu$m.

12 dae shoot tissues from both *hyd1* and *hyd2* demonstrate persistent ectopic cotyledon expression, although this was not seen in radialized structures, such as the prong-shaped cotyledon in F. Where expansion of true leaves takes place, as in A and B, the developing vascular network becomes highlighted, although this is often via stronger expression than in wild type plants, and persists for longer. Primordia compromised in expansion growth show peaks of diffuse signal (C). No stipule expression was observed from this reporter in any of the *hydra* seedlings examined.

The *hyd1* primary root apex in D is typical of the pattern seen in both *hyd1* and *hyd2* root apices at 12 dae; the signal is less distinct in the root cap and exacerbated in the provascular region. The lateral apices show a reduced staining, though retain definition of the root cap, as in the anchor root tip shown in E.




## Figure 5.46 Ectopic expression of p*IAA2*::GUS in association with disjunct xylem vasculature in *hydra1*

A-C; xylem-associated ectopic and mistimed expression of p*IAA2*::GUS from the same 12 dae *hyd1* plant, bar = 50$\mu$m.

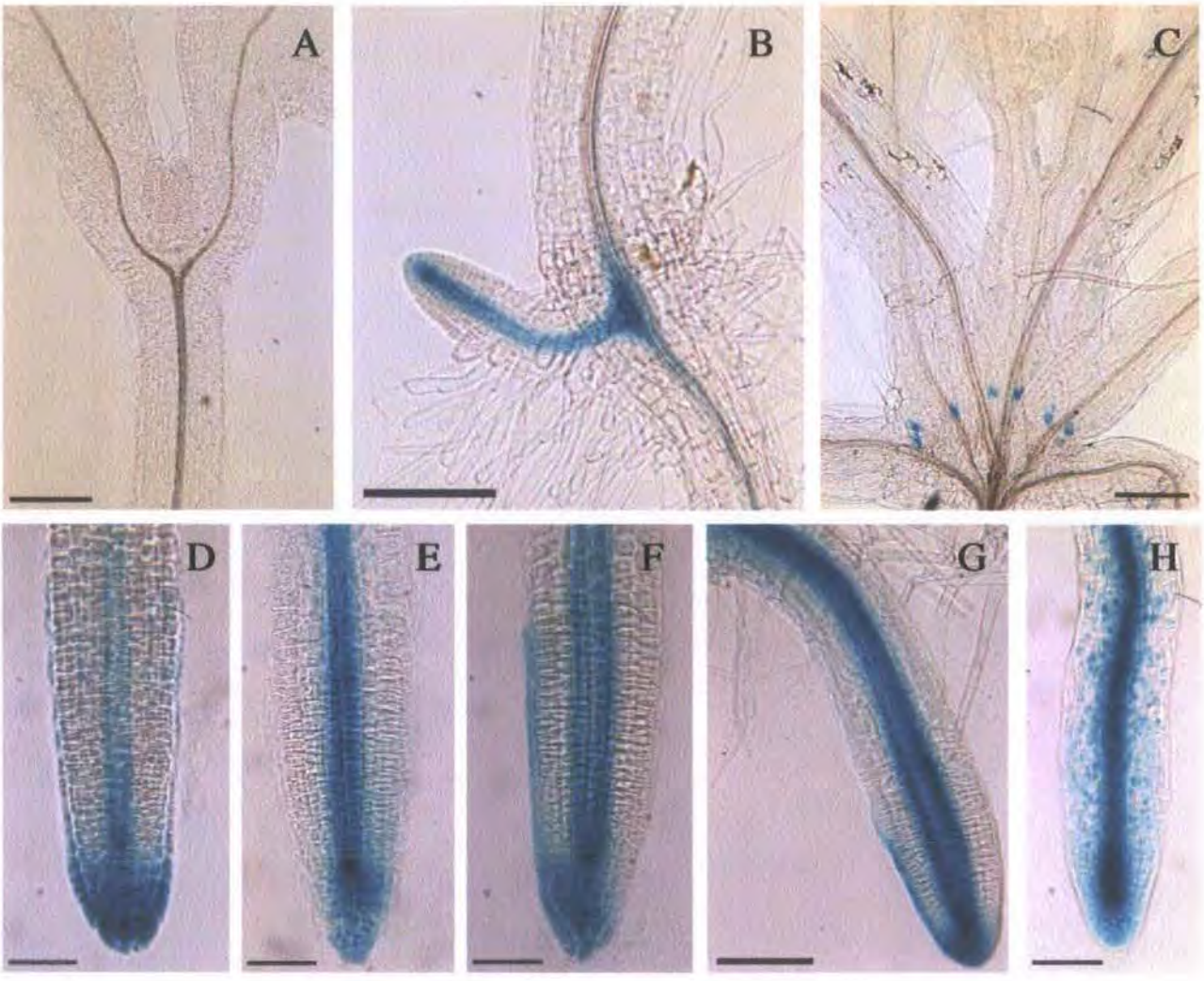
A shows the cotyledon primary strand hydathode region. The vascular island in B is from a position expected to contain secondary cotyledon vasculature. The discontinuous xylem from a first true leaf shown in C highlights an area where the condensation of a second order loop is incomplete. Lines of provascular cell files are just visible (arrow).

active auxin. This difference between *hydra* mutants does not persist in the developing seedlings. Root expression in 12 dae seedlings of both *hydra* mutants was similar; primary apices retained a strong GUS activity but lost clear definition of the columella, as seen in younger root primary apices of *hyd1* (Fig. 5.45; D). In lateral roots, a less pronounced signal was observed in both *hyd1* and *hyd2*, with a reduced GUS activity in the stele compared to *hydra* primary root apices, and a diffuse reporter expression around the root cap columella (Fig. 5.45; E).

These observations suggest that auxin is elevated in *hydra* cotyledons, hypocotyls and root apices of germinating seedlings, and is poorly localized in the developing vasculature of both young leaves and roots. This implies either a heightened auxin response, or a reduced auxin transport efficiency. The persistence of ectopic p*IAA2*::GUS activity in the vicinity of discontinuities in the vascular xylem suggests that auxin transport is compromised by a lack of coherence between vessel elements, allowing a build-up of local ectopic peaks of auxin. Differences between *hyd1* and *hyd2* upon germination suggest that compromised auxin transport is more severe in *hyd2*, perhaps reflecting a slower rate of vascular differentiation in the *hyd2*-modified sterol environment.

#### 5.3.2.1.2 The slight reduction in auxin responsiveness conveyed by *ein2* reduces the intensity of auxin response in *hydra*, but does not affect the mis-positioning of signalling cues

Expression of the p*IAA2*::GUS auxin-responsive reporter showed similar positional signals in wild-type and *ein2* mutant seedlings (Fig. 5.47; A-H). Overall intensity of reporter expression was slightly reduced in *ein2* relative to wild-type, although the signal in young lateral root apices was slightly enhanced (Fig. 5.47; G). This modulation was lost upon maturity of the lateral root (Fig. 5.47; H). This suggests that auxin positional localization functions normally in *ein2*, but that the differences in auxin sensitivity between primary and lateral root apices are reduced.

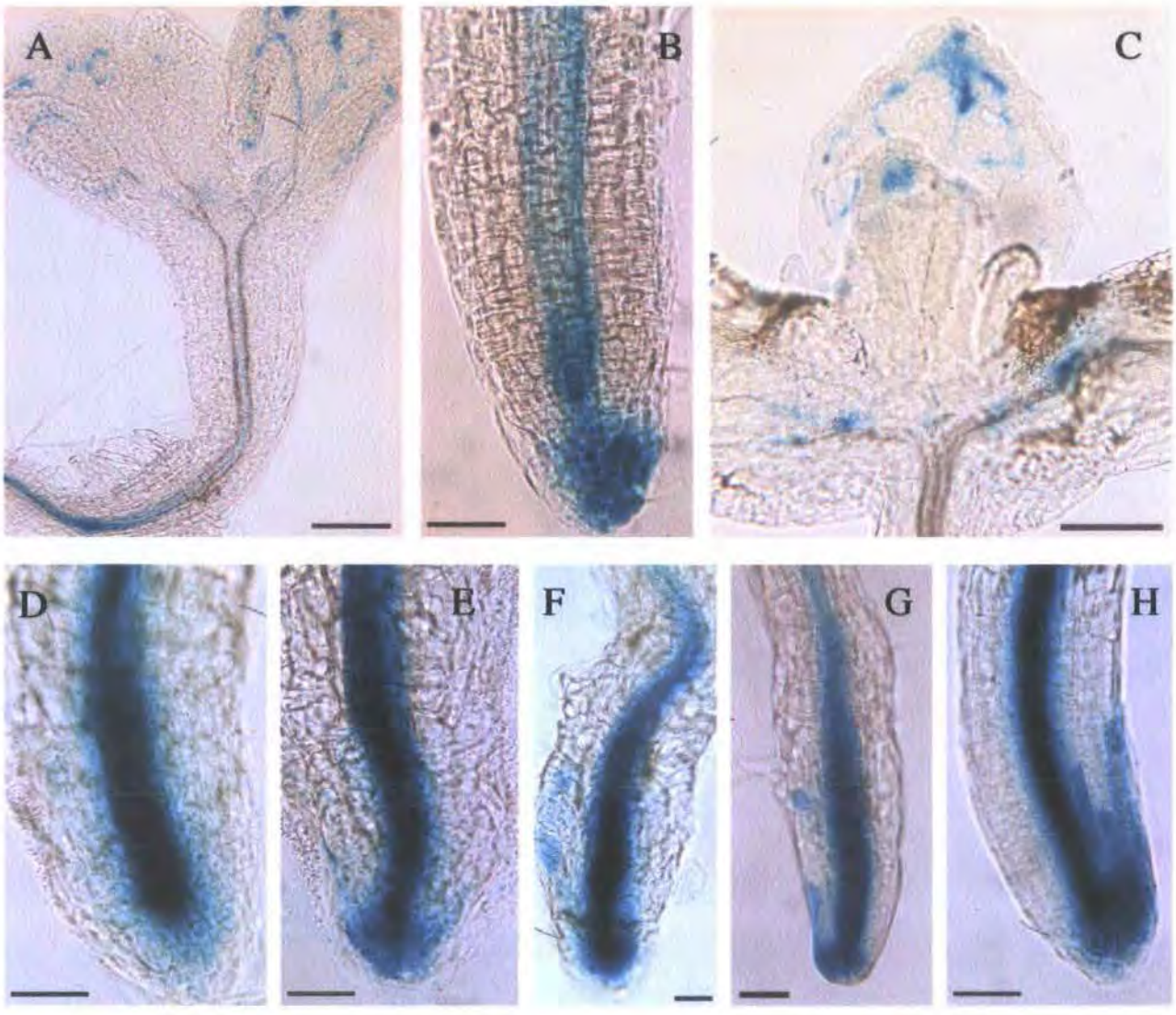



## Figure 5.47 Expression of p*IAA2*::GUS in *ein2*

A: shoot region and B; root-hypocotyl junction of 3 dae *ein2* seedling showing expression of the p*IAA2*::GUS auxin responsive reporter. C; *ein2* shoot region at 12 dae. A-C, bar = 200$\mu$m.

D, *ein2* 3 dae primary root apex. E-H, root tips from the same 12dae *ein2* seedling. E, primary root apex; F, anchor root apex; G, young lateral from main root; H, mature lateral apex. D-H, bar = 50$\mu$m.

Positional expression of p*IAA2*::GUS is seen in a similar position in both *ein2* and wild-type plants, although GUS activity in *ein2* appears slightly less intense. Note that *ein2* seedlings develop more rapidly than wild-type under these culture conditions, with some siblings having bolted by 12 dae, as in C.




## Figure 5.48 Expression of p*IAA2*::GUS in *hyd2-ein2*

A-H; ***hyd2-ein2*** seedlings expressing p*IAA2*::GUS. A; shoot and B; root apex at 3 dae. C; shoot from 12 dae plant, D and E; primary root apices, F; anchor root apex, G; young lateral from main root and H mature lateral. A and C, bar = 200$\mu$m; B, D-H, bar = 50$\mu$m.

Shoot expression in *hydra-ein2* double mutants is similar to that seen in single mutants,though with a slight reduction in staining intensity.

Primary root expression in younger seedlings is similar to that seen in single mutants. By 12 dae, there is some sibling variation in *hyd2-ein2* as shown in D and E, where certain seedlings retain the root cap signal for longer, possibly in association with variation in meristem viability between siblings. Lateral root apices also show increased viability (F-H). The asymmetrically distributed histochoemical signal seen in G and H, present in epidermal cell files at the root apex, and also observed in some *ein2* laterals, marks the downward side of the developing root, and may be representative of an improved gravity perception effected in *hydra* by the presence of *ein2*.

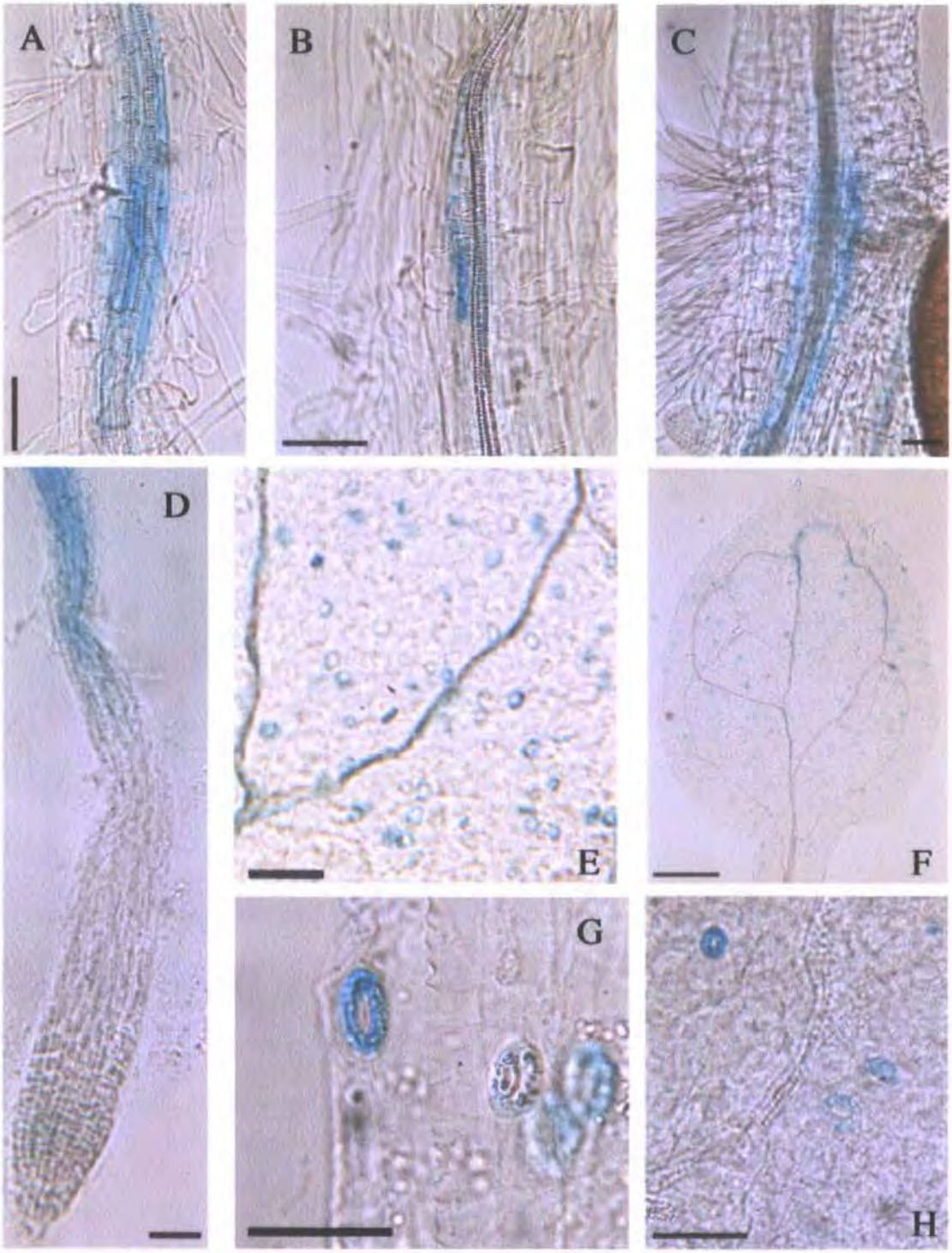
The activity of p*IAA2*::GUS in both *hydra-ein2* mutants (shown for *hyd2-ein2* in Fig. 5.48; A-H) demonstrated enhanced GUS activity in the root stele relative to the control, particularly in younger seedlings of *hyd2-ein2*, as seen in *hydra* single mutants. Ectopic expression in cotyledons appeared similar to that in *hydra* single mutants, and likewise occurred in association with xylem disjunctures. The 3 dae *hyd2-ein2* root apex established a GUS signal in the stele and root cap (Fig. 5.48; B). A clearer transient vascular definition of true leaves was found in some seedlings (e.g. Fig. 5.48; C) in tandem with *ein2*-associated improvement of leaf expansion. Variation in *hyd2-ein2* primary root apices at 12 dae (Fig. 5.48; D and E) can be explained in terms of a different viability of the RAM between seedlings; as the meristem loses function, auxin gradients within the apex diminish, and reporter activity is lost in the root cap columella. Double mutant lateral apices retained viability, and appeared to have an enhanced auxin response.

These observations imply that *ein2* has a slight reduction in its overall auxin response, though the auxin balance between primary and lateral apices is shifted slightly. The behaviour of p*IAA2*::GUS in *ein2* and *hydra-ein2* imply that auxin-related effects appear to be conveyed to the *hydra* mutants in an additive fashion.

## 5.3.2.2 Ethylene Biosynthesis

### 5.3.2.2.1 Expression of the p*ACS1*::GUS reporter in *hydra* is enhanced in roots and reduced in shoot tissues, and varies between mutant siblings

The p*ACS1*::GUS reporter shows transcriptional activity for the *ACS1* gene from *Arabidopsis* (Rodriguez-Pousada *et al.* 1993). *ACS1* in *Arabidopsis* is a pseudogene, producing a non-functional homodimer of ACC synthase, the enzyme catalysing the release of ethylene from its endogenous precursor




## Figure 5.49 p*ACS1*::GUS expression in wild-type plants

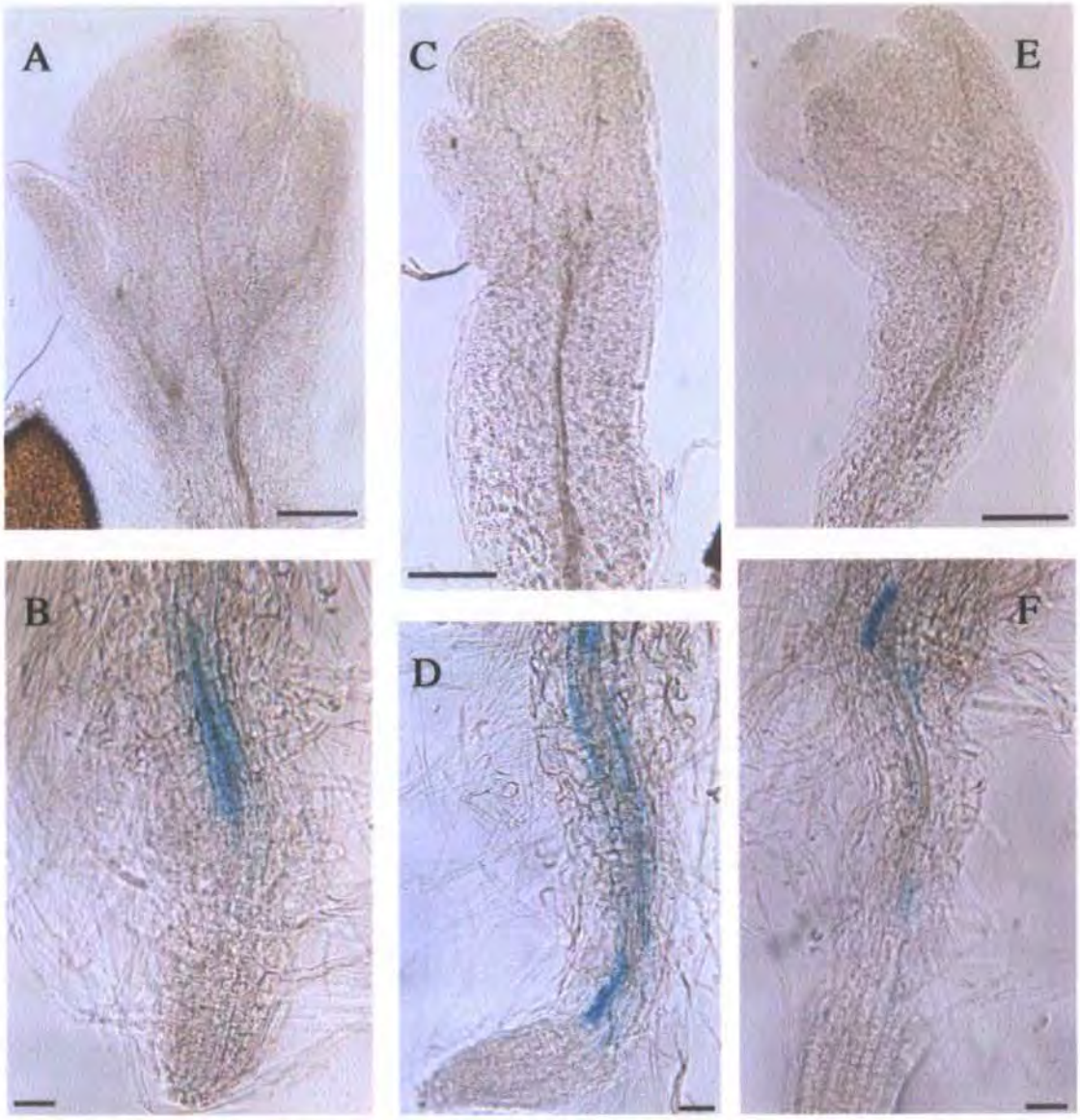
A-H; expression of the p*ACS1*::GUS construct in root and shoot tissues of wild-type plants at 5dae (A, B, G) and 12dae C-F, H). A-E, G, H; bar = 50$\mu$m. F; bar = 200$\mu$m.

Expression of the *ACS1* reporter is first seen in sections of the primary root, here in the 5dae seedling mid-root (A), at points of lateral initiation (B) and in the guard cells of certain stomata in the cotyledons and hypocotyl (G). As the seedling becomes established, expression is seen in the inner cell layers of mature sections of root (C, D), but not around the root tip. As cotyledons mature, guard cell staining increases (shown for the adaxial surface in E and H), and is also seen in guard cells and (transiently) in association with the maturing vasculature of expanding true leaves (F) although staining intensity in these cells varies between plants, and even between the two cotyledons of an individual plant, under these growth conditions.

ACC (Arabidopsis Genome Initiative 2000, Yamagami *et al.* 2003). The other eight members of the ACS multigene family form functional heterodimers (Arabidopsis Genome Initiative 2000, Yamagami *et al.* 2003), and all nine show precise and differential tissue-specific transcription throughout development (Tsuchisaka & Theologis 2004). Transcription of the p*ACS1*::GUS reporter is positively induced by the addition of the ethylene precursor ACC in both root and shoot tissues when grown in the light (Rodriguez-Pousada *et al.* 1999), suggesting that its expression indicates sites of ethylene biosynthesis within the plant body.

Expression of the p*ACS1*::GUS reporter in wild-type plants, showed GUS activity in initial cells of lateral roots (Fig. 5.49; B) and in association with the stele of maturing root tissues (Fig. 5.49; A, C, D). In the shoot, GUS activity was limited to guard cells of mature stomata in the cotyledons, true leaves and hypocotyl, and to mature vascular tissues of the cotyledons and true leaves (Fig. 5.49; E-H).

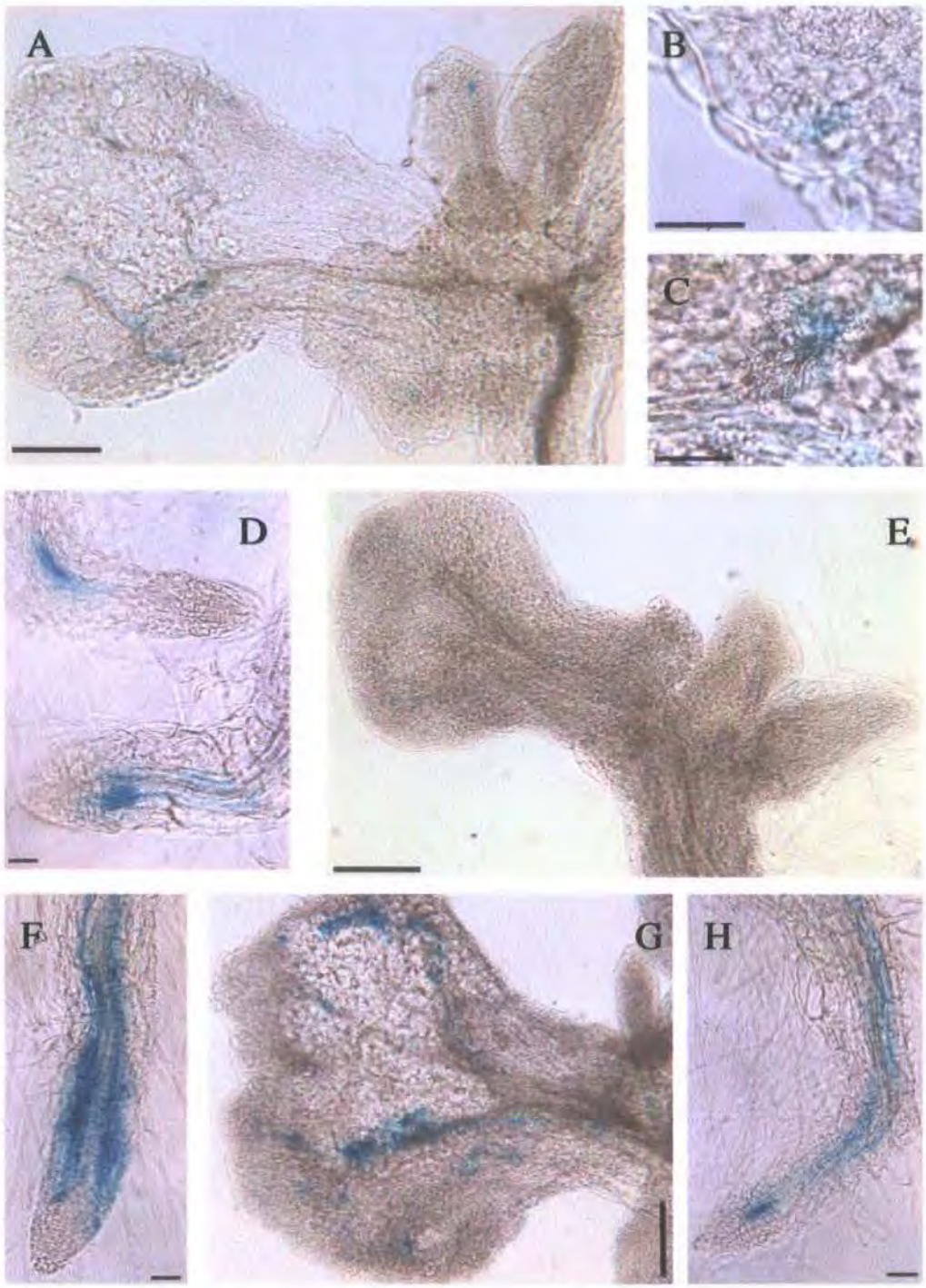
In 5 dae siblings of both *hyd1* and *hyd2*, the p*ACS1*::GUS reporter was observed in similar positions in parts of the root stele and pericycle as seen in wild-type plants, although with slightly elevated levels of GUS activity (Fig. 5.50; B, D, F). No shoot GUS activity was visible in this age class, in contrast to wild-type plants, where the first mature stomata of the cotyledons already carried a reporter signal at this age. By 12 dae, a much stronger root expression in the mutants, most enhanced in primary roots, exceeded the wild-type levels significantly (Fig. 5.51; D, F, H). Most mutant siblings showed GUS activity in their cotyledon vasculature, although the distribution of signal was uneven, being elevated in relation to wild-type in some individuals, whist almost absent in others (Fig. 5.51; A-C, E, G). Areas of heightened signal appeared to be in association with late xylem differentiation, and areas of disjunct xylem (Fig. 5.51; C). Stomatal p*ACS1*::GUS reporter signals were absent from all *hydra* mutant seedlings examined.




## Figure 5.50 p*ACS1*::GUS expression in 5 dae *hydra2* seedlings

A-B, C-D and E-F; shoot and root tissues from the same *hyd2* seedlings at 5 dae, expressing the p*ACS1*::GUS reporter. A, C, E; bar = 200$\mu$m, B, D, F; bar = 50$\mu$m.

Root expression of the ***ACS1*** reporter in *hydra* mutants at 5 dae is similar to that observed in wild-type plants, again with sibling variability. In contrast, no guard cell staining was observed in mutant cotyledons or hypocotyls in this age class.




## Figure 5.51 p*ACS1*::GUS expression in *hyd2* at 12 dae

**A & D, E & F and G & H; shoot and root detail from the same *hyd2* seedlings at 12 dae, showing activity of the p*ACS1*::GUS reporter. A, E, G, bar = 200$\mu$m; B-D, F and H, bar = 50$\mu$m. B shows guard cell detail from A, and C shows cotyledon vascular-associated mesophyll, in the vicinity of a section of disjunct xylem. D displays the primary root apex below a lateral apex, again from A.**

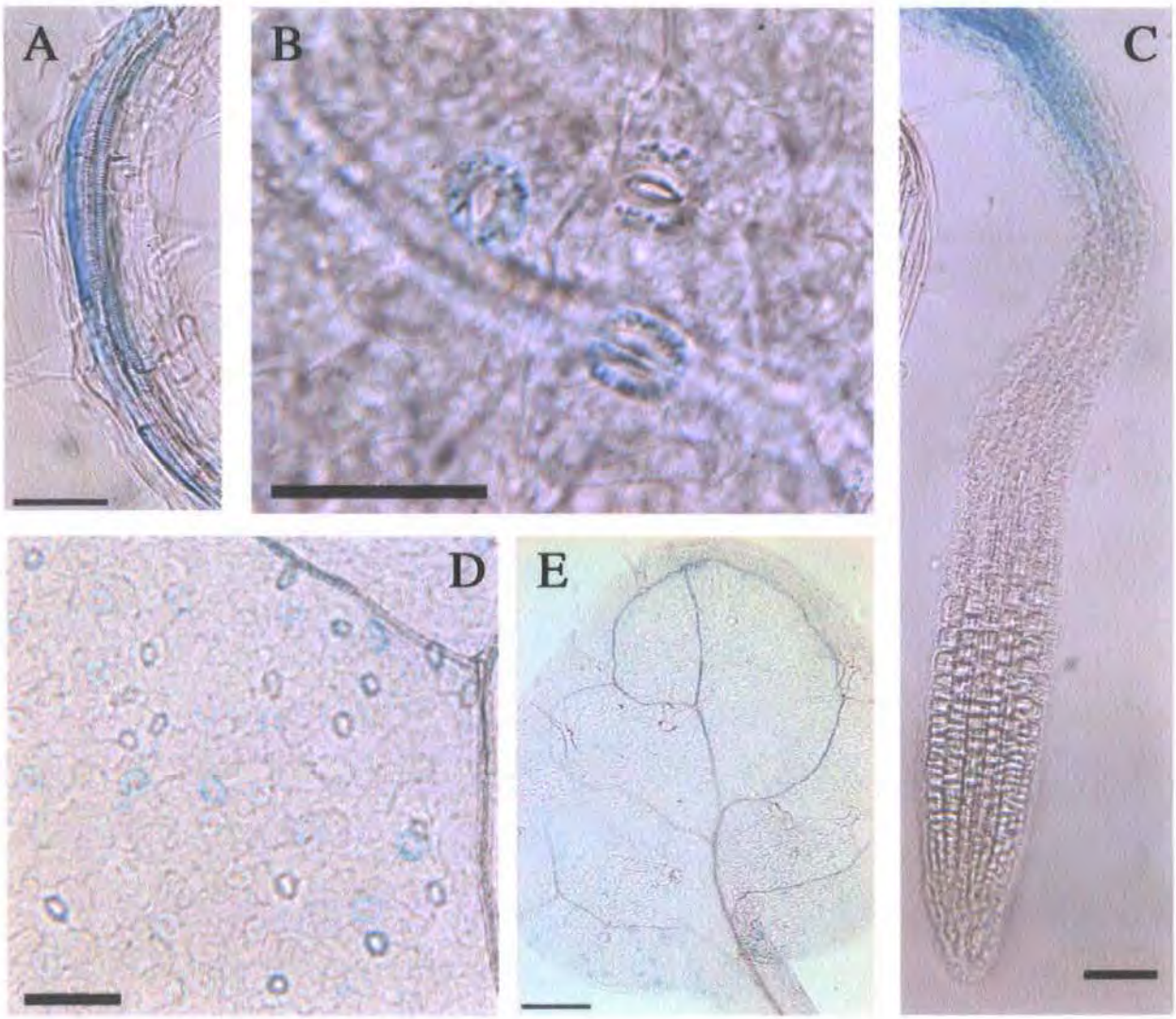
**At 12 dae, root expression in *hyd2* is both of a stronger intensity than in wild-type, and is closer to the root apex. Sibling variation is evident (D, F, H), and where lateral roots are present (D, upper portion of picture), the GUS activity is further from the root apex than in primary roots (D, lower portion). Few stomata carry p*ACS1*::GUS expression; the stoma in B (from A), was the only discernible guard cell pair with a histochemical signature in this seedling. A sibling-variable vascular-associated mesophyll signal in cotyledons ranges from none (E), through weak (A, detailed in C) to strong (G).**

These data suggest that a transient production of ethylene seen in roots of wild-type is more persistent in *hydra* mutants, perhaps indicating slower differentiation in these cells. The ACS1-associated signal in guard cells does not establish in *hydra,* and may indicate the mis-function in these mutants of either the stomates themselves, or a stomatal signalling mechanism.

### 5.3.2.2.2 The p*ACS1*::GUS reporter shows an enhanced and more persistent activity in *hydra-ein2*, which is modulated in lateral roots.

Activity of the p*ACS1*::GUS reporter in *ein2* mimicked the expression seen in wild-type plants, although was modulated by the more rapid development of *ein2* seedlings (Fig. 5.52; A-E). This implies that ethylene biosynthetic activity (as indicated by *ACS1* transcription) is unaffected by ethylene signal transduction at or beyond *EIN2*. It also appears that *ACS* gene activity is under a tight developmental regulation, and transcription is probably affected by other signal transduction systems.

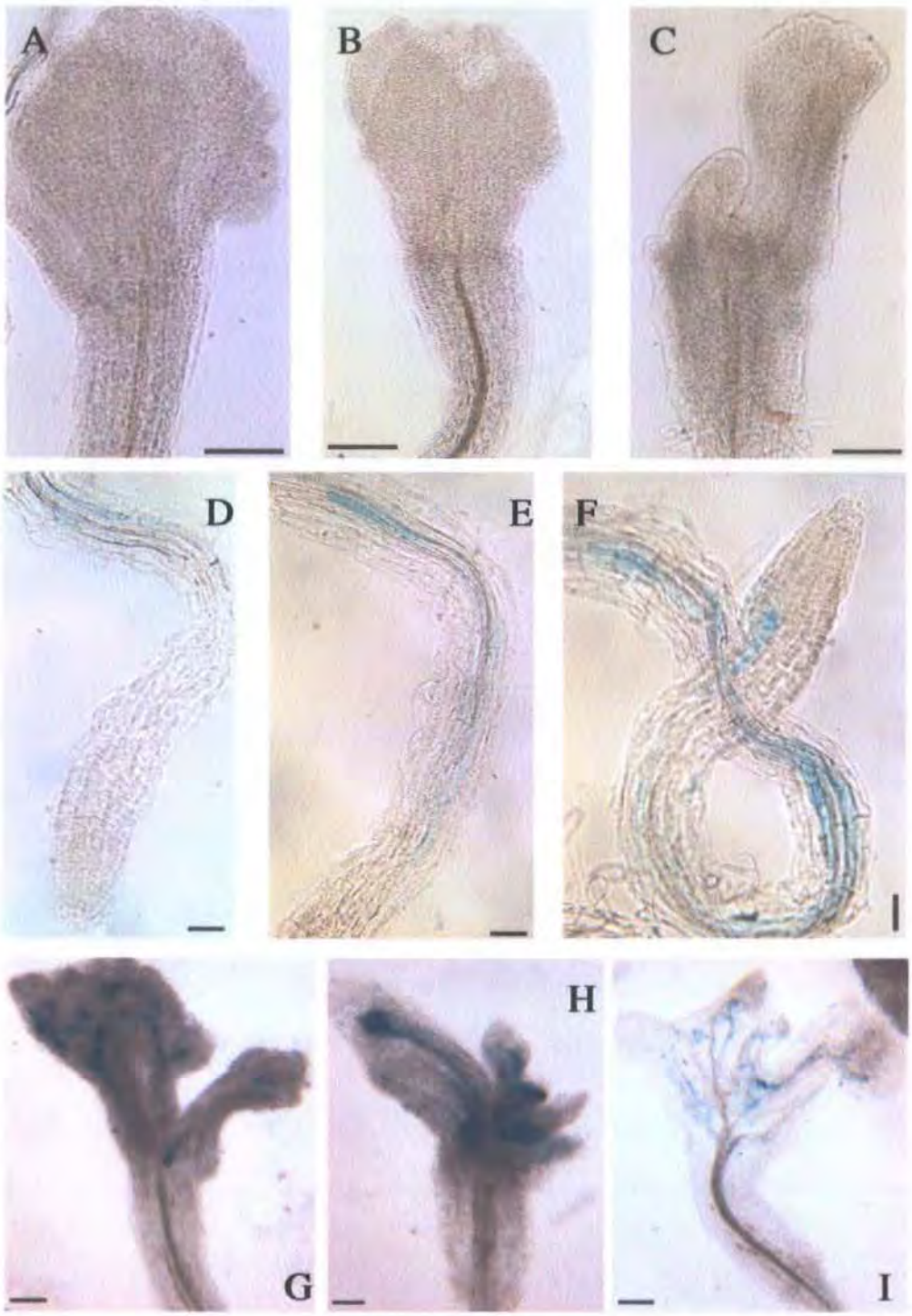
In *hydra-ein2* double mutants (illustrated in Fig. 5.53 and Fig. 5.54 for *hyd2-ein2*), root expression of p*ACS1*::GUS was slightly increased relative to both controls, as seen in *hydra* single mutants (Fig 5.53; D-F), although was slower to establish in early post-germination growth. Although mature double mutant primary roots had GUS activity levels similar to *hydra* single mutants (Fig. 5.54; E), the position of the *ACS1* signal was modified in both primary and lateral root apices (Fig. 5.54; E-G). This positional shift, resolving the histochemical signal further away from the apex, implies that these tissues have reached a stage of 'maturity'; i.e. in wild-type and *ein2,* this signal is visible in tissues after the cessation of longitudinal expansion. This may reflect either a change in auxin activity in these tissues, and/or an activation of the lateral mersitems associated with secondary growth. In contrast, laterals formed along the primary root (Fig. 5.54; G) had an un-




## Figure 5.52 Expression of p*ACS1*::GUS in *ein2*

A-E; activity of the p*ACS1*::GUS construct in ein2 mutant seedlings. A, primary mid-root and B, cotyledon adaxial detail from 5dae *ein2* seedlings; bar = 50$\mu$m. C, primary root; D, cotyledon adaxial detail, and E, true leaf detail from *ein2* seedlings at 12dae. C and D, bar = 50$\mu$m; E, bar = 200$\mu$m.

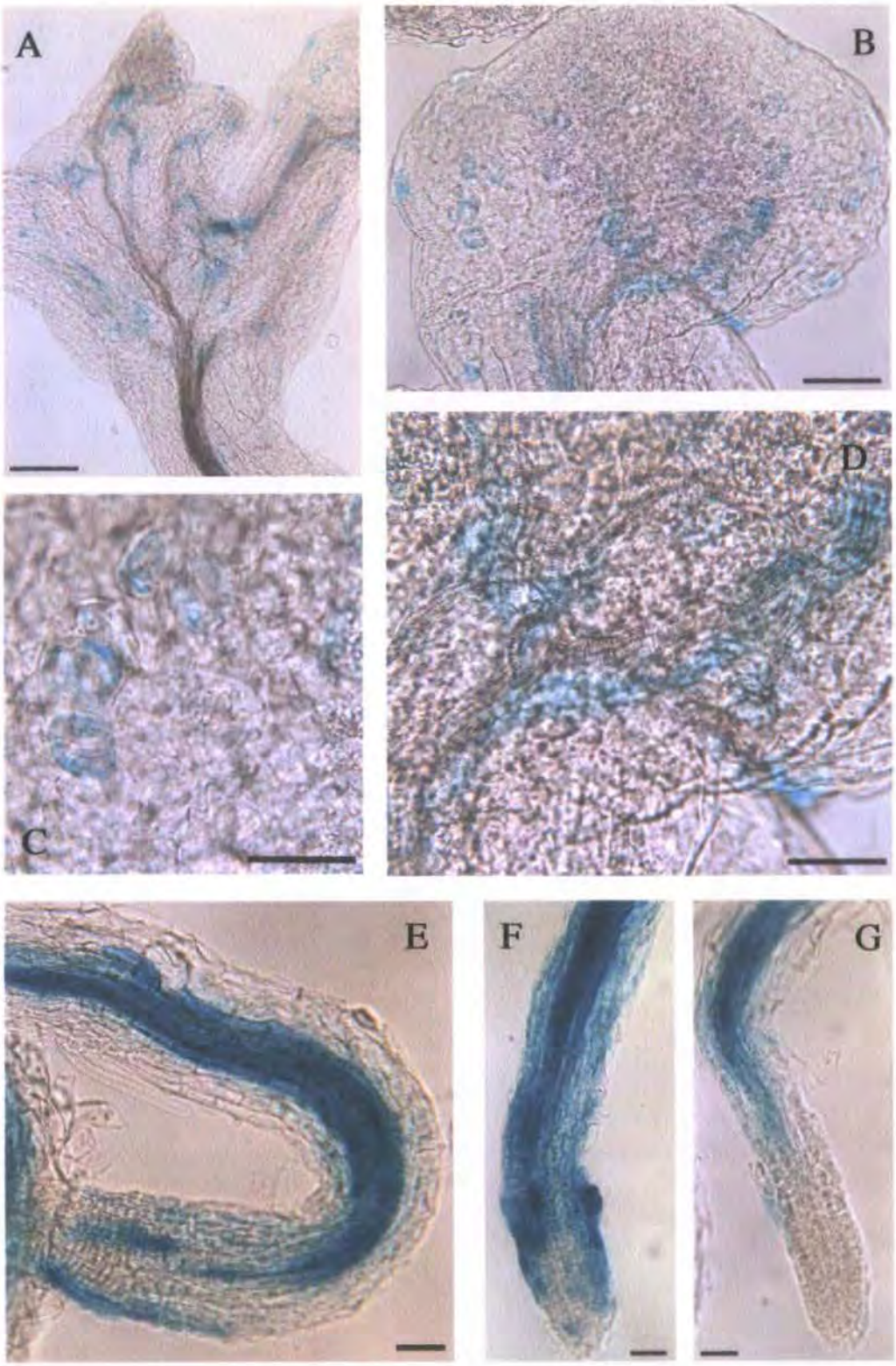
Histochemical activity from this reporter in *ein2* has a similar positional distribution to that observed in wild-type plants. In roots, the length of stained sections of mid-root appear longer in *ein2*, but as the roots themselves are longer than wild-type, this may be an expression of the greater rate of growth seen in *ein2* mutant seedlings. The onset of staining commences at a similar distance from the root apex in wild-type and *ein2*. In the shoot, guard cell staining in cotyledons and true leaves, and transient vascular-associated staining of true leaves, is present though at a lesser intensity than in wild type. Again, this may be the result of more rapid cellular maturation in these tissues.




## Figure 5.53 Activity of p*ACS1*::GUS in *hyd2-ein2* at 5 dae

A-I; activity of the p*ACS1*::GUS construct in *hyd2-ein2* mutant seedlings. A & D, B & E and C & F show shoot and root images from the same seedlings at 5 dae. G-I show expression in shoot regions of three siblings at 12 dae. A-C and G-I, bar = 200$\mu$m; D-F, bar = 50$\mu$m.

At 5 dae, shoots of *hyd2-ein2* have a lack of discernible reporter activity as observed in *hyd2* single mutants (Fig.s 5.50-5.51). Root expression is slightly stronger, and may reflect the more rapid growth seen in double mutant roots. By 12 dae, shoot expression has developed to substantially stronger levels than in single mutant *hyd2* siblings, the reporter highlighting both cotyledon and true leaf vasculature.




## Figure 5.54 Activity of p*ACS1*::GUS in *hyd2-ein2* at 12 dae

A-G; activity of the p*ACS1*::GUS construct in shoot and root tissues from the same 12dae *hyd2-ein2* mutant seedling. A, B, bar = 200 $\mu$m; C and D, bar = 100 $\mu$m; E-G, bar = 50 $\mu$m.

Young true leaves (visible in A) have vascular-associated expression as in cotyledons (B, detailed in D). Guard cells carrying reporter expression are evident over sections of the cotyledon lamina (C). In roots, the primary root apex shows stronger staining, and in a more proximal position than *ein2* (E), and a similar pattern is also observed in anchor root apices (F). In contrast, laterals from the main root (G) although carrying a greater intensity of GUS activity, show similar positional localisation to that seen in wild-type and *ein2*.

modified positional distribution of p*ACS1*::GUS relative to *hydra* single mutants.

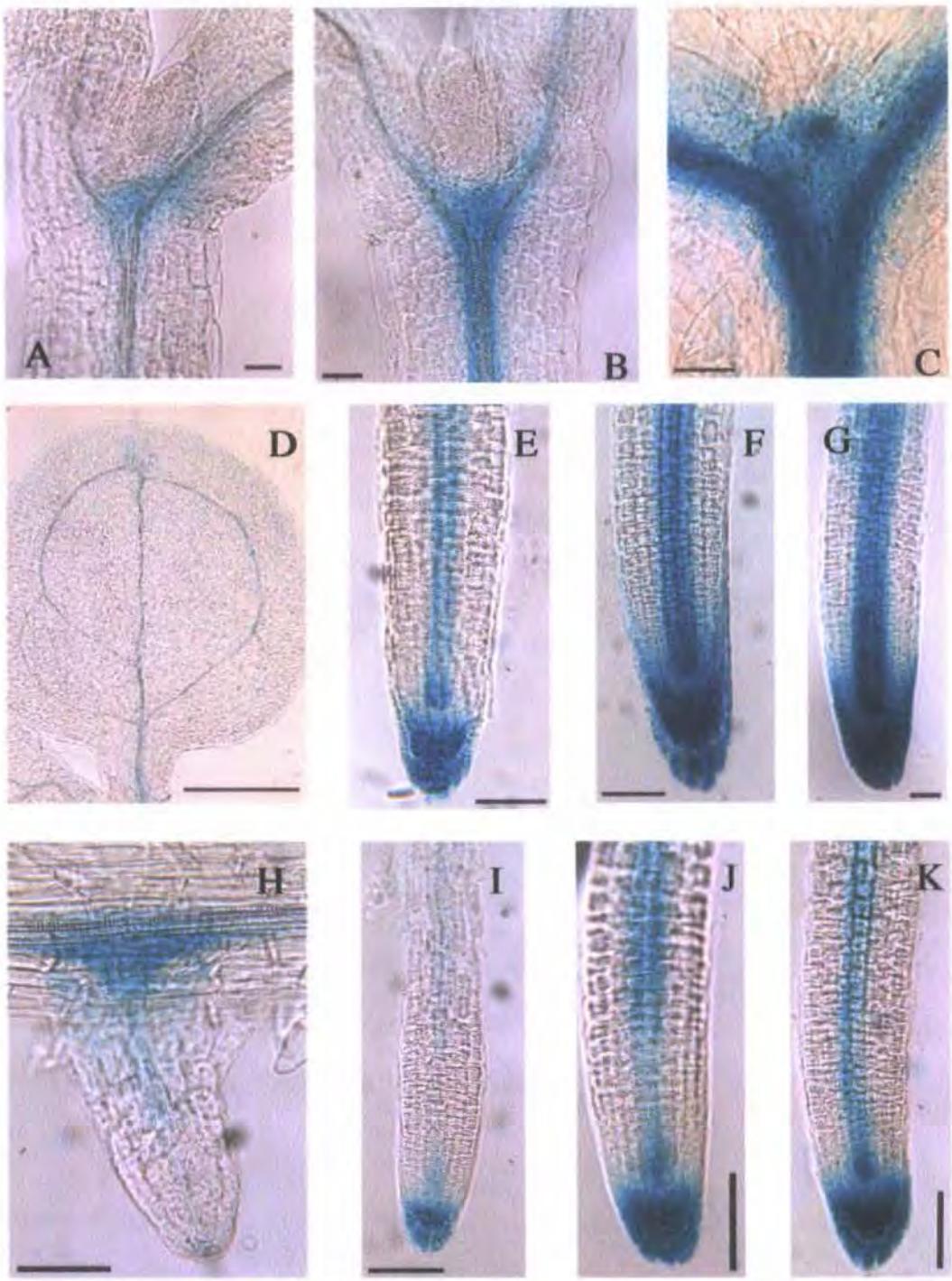
Shoot expression of p*ACS1*::GUS was absent from young *hyd2-ein2* seedlings as from *hydra* single mutants, although the activity appearing in more mature tissues of single mutants was enhanced in *hydra-ein2* (Fig. 5.54; A-D). Reporter activity was found in vascular traces of both cotyledons and true leaves, at levels enhanced in relation to the wild-type and *ein2* controls, and persisting for longer. Also visible in these 12 dae tissues are stomatal guard cells with a reporter expression (Fig. 5.54; B and C); this phenomenon, found in both controls, was not seen in *hydra* single mutants.

These observations suggest that the modulation of *hydra* development by *ein2* restores some aspects of signal transduction in specific tissues, e.g. in primary root meristems and (to a limited degree) in stomata. This implies that the physiological problems in these *hydra* mutant tissues are exacerbated by signalling via the *EIN2* pathway.

### 5.3.2.3 Cytokinin response

#### 5.3.2.3.1 Expression of the cytokinin-responsive p*ARR5*::GUS reporter has differential positoning in *hyd1* and *hyd2*.

The p*ARR5*::GUS construct in *Arabidopsis* reports the transcription of the *ARR5* cytokinin primary response gene (D'Agostino *et al.* 2000). Reporter expression in wild-type plants of the *hydra* mutant backgrounds appeared first in the root cap and then the root stele of emergent seedlings, followed rapidly by an accumulation in the pith tissues beneath the SAM (Fig. 5.55; E and A). GUS activity increased in intensity over the first 7 dae, developing a strong signal in the upper hypocotyl stele, cotyledon primary vascular strands and hydathode, root cap, in the root stele near the apical meristem, and in regions along the main root in the vicinity of emerging laterals (Fig. 5.55; A-G). Lateral root apices showed a faint GUS




## Figure 5.55 p*ARR5*::GUS expression in wild-type seedlings

A-K; wild-type seedlings expressing the p*ARR5*::GUS cytokinin-responsive reporter in root and shoot tissues at 3 dae (A, D, E), 5 dae (B, F) and 7 dae (C, G-K). A-C, E-K, bar = 50$\mu$m; D, bar = 200$\mu$m.

Reporter activity establishes first in the root apex in association with the root cap and stele (E), becoming more intense between 5 and 7dae (F, G). Laterals emerging from the main axis (H-K) have a faint signal in the developing stele, and show strong root cap expression as these cells differentiate. In mature laterals (J) and anchor root apices (K), histochemical activity is consistently lower than that seen in the primary root apex (G).

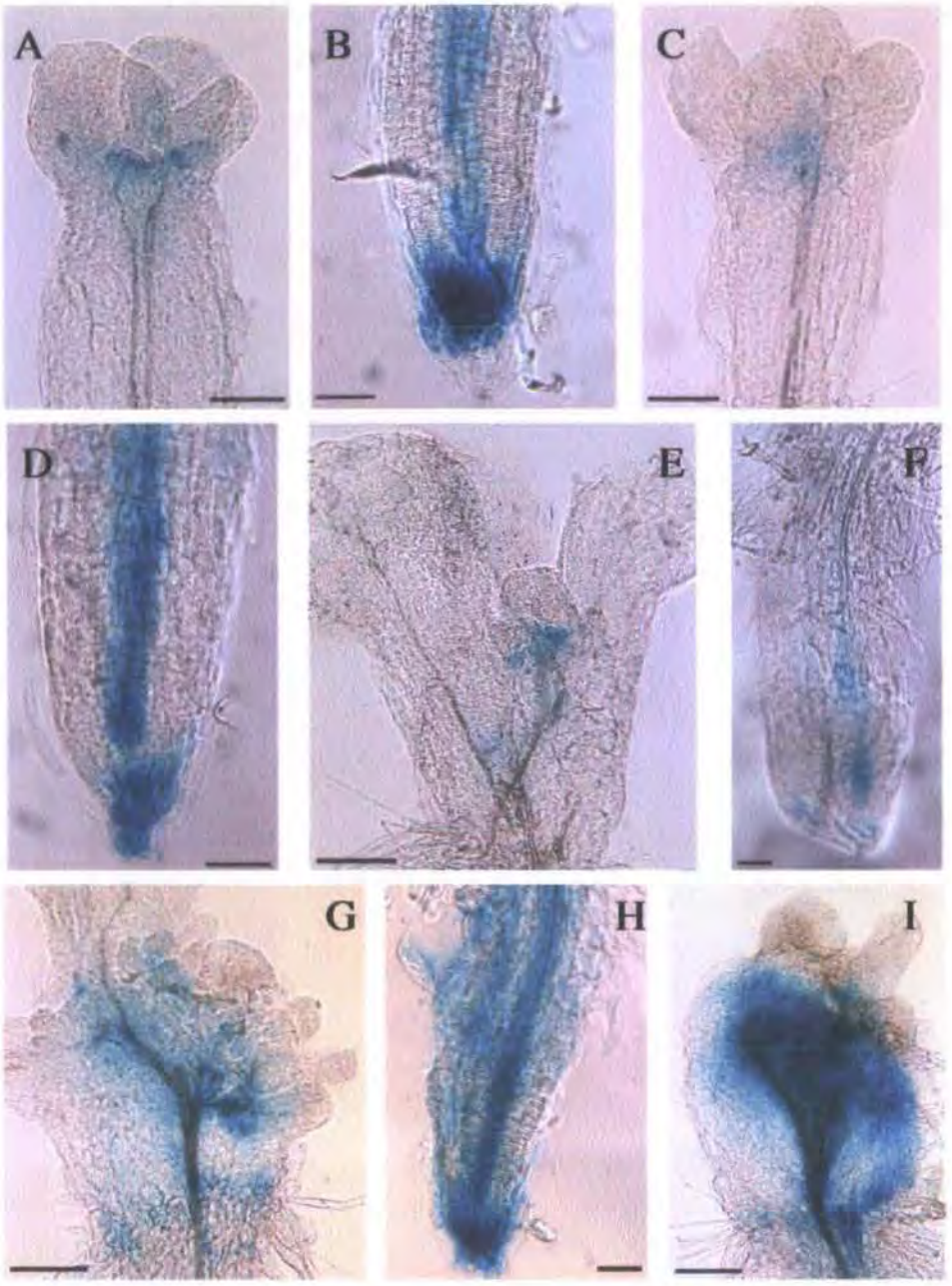
Shoot expression of *ARR5* begins around 3dae in the upper hypocotyl above the point of vascular strand branching, beneath the developing SAM (A). As expression becomes established, the signal expands into the upper hypocotyl stele (B; SAM at 5dae, and C; 7 dae), and is seen transiently during expansion growth in the primary axes of cotyledons from the apical hydathode to the meristem (visible in the 3 dae cotyledon shown in D).

activity in the procambial strands prior to differentiation of the root cap and meristem (Fig. 5.55; H). As the lateral root meristem differentiated, GUS activity in the root cap appeared first, followed by an intensifying signal in the stele (Fig. 5.55; I-K). Lateral root apices did not show the intensity of signal seen in primary apices, suggesting a reduced cytokinin response in lateral roots as compared to primary roots.

A differential transcription of the p*ARR5*::GUS reporter was observed between the sibling populations of *hyd1* and *hyd2*. Reporter activity in *hydra* single mutants showed a strong differential response. In *hyd1*, shoot expression of p*ARR5*::GUS was enhanced in association with dissociation of the hypocotyl stele, and root expression appeared either unaffected in normally patterned roots, or reduced in the presence of axis duplication. In *hyd2*, the opposite patterning was seen, with shoot tissues developing minimal GUS activity, whilst strong reporter expression was present in both the root stele and the peripheral tissues.

In 3 dae *hyd1* seedlings (Fig. 5.56; A-J), GUS activity became established rapidly in the root cap tissues, although sibling variation was apparent (Fig. 5.56; B, D, F). Seedling root apices with better integrity in the root cap and stele showed most rapid induction of reporter activity. In instances where the root axis was duplicated (Fig. 5.56; F), this effected a profound reduction in establishment of the signal of both root cap and stele, although the activation of transcription in the zone beneath the shoot apical meristem appeared normal in this age class. By 5 dae, a similar or slightly enhanced root signal was apparent, with a mild ectopic expression in peripheral tissues of some individuals (Fig. 5.56; H). GUS activity in the shoot varied between plants, although was consistently elevated in relation to wild-type, and was enhanced substantially in siblings showing dissociation of vascular tissues in the hypocotyl stele (Fig. 5.56; G, I).

In the *hyd1* 7 dae seedling detailed in Fig. 5.57; A-D, root expression is similar to wild-type, with a slightly less intense GUS activity in the lateral root apex. In the shoot, GUS expression is enhanced beneath a broadened

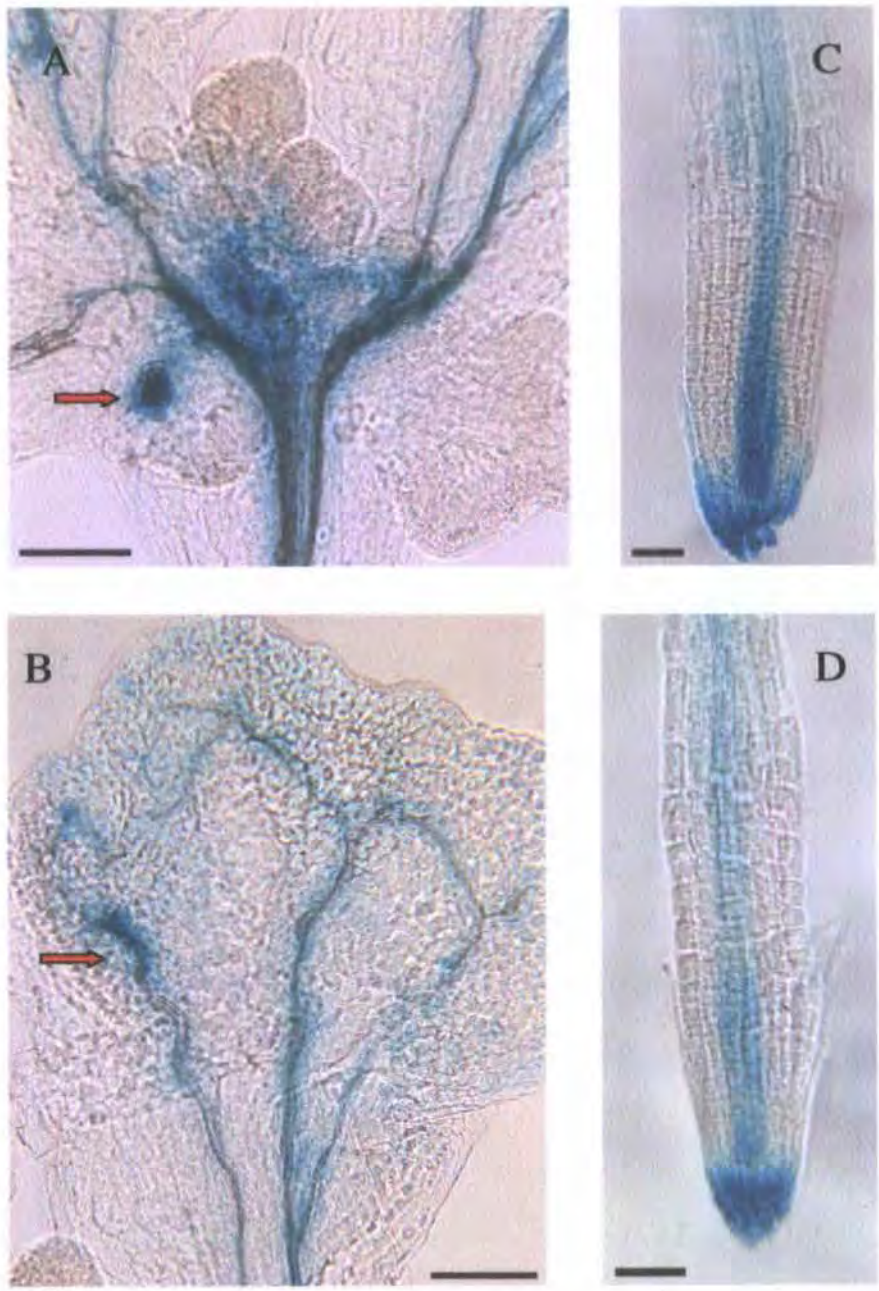



## Figure 5.56 p*ARR5*::GUS expression in *hydra1* at 3 and 5dae

A-J; *hyd1* seedlings showing root and shoot tissues from the same siblings expressing p*ARR5*::GUS at 3 dae (A-B, C-D, E-F) and 5 dae (G-H, I-J). A, C, E, G, I, bar = 200$\mu$m; B, D, F, S, U, bar = 50$\mu$m.

Plant E-F has axis duplication in both shoot and root apices. These images show that vascular dissociation, but not axis duplication, results in an elevation of reporter activity.

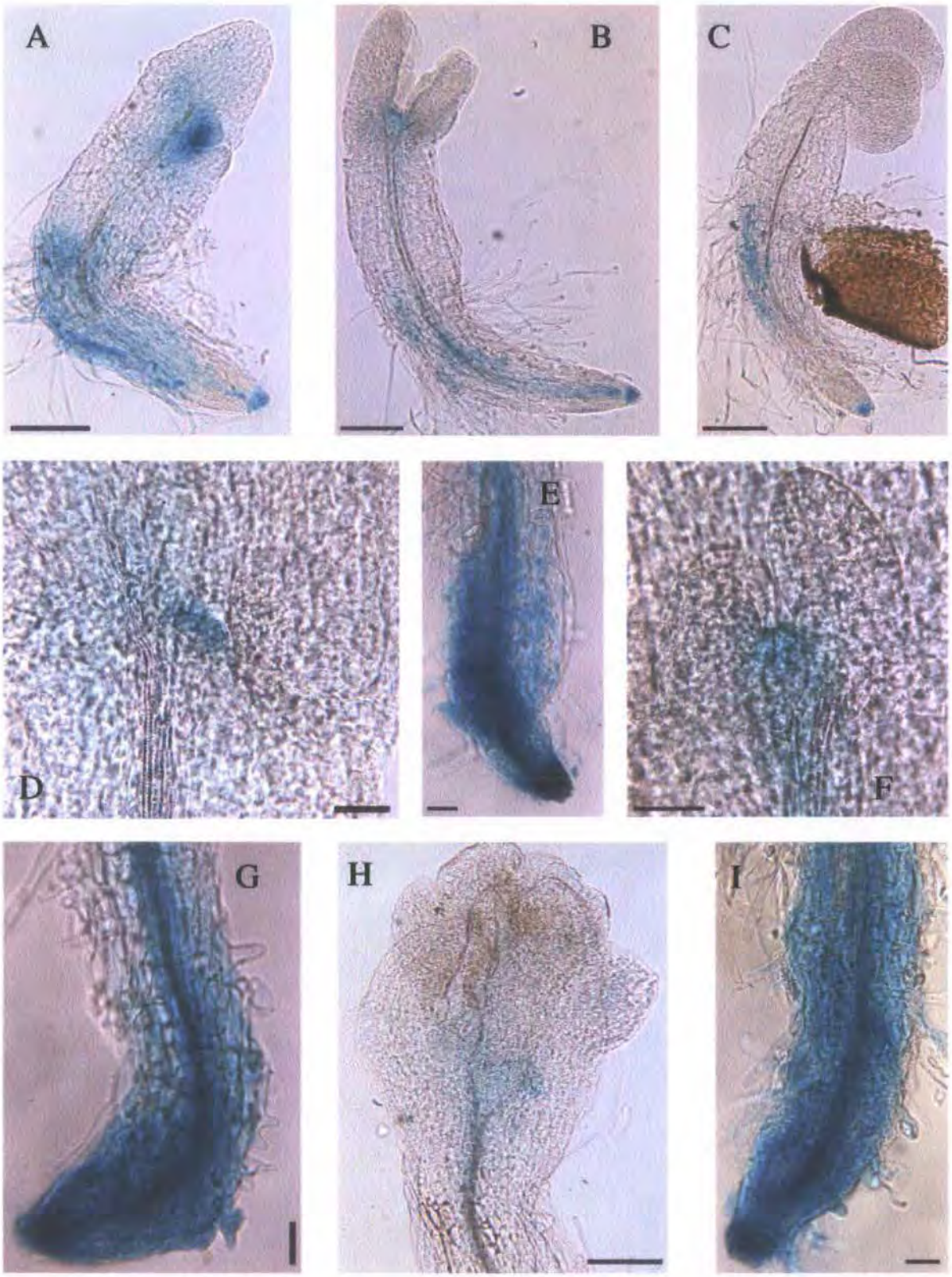
At 3 dae (A-F), reporter activity in *hyd1* shows similarities to wild-type in root apices, with some exaggerated staining in shoot tissues. Older 5 dae seedlings have exaggerated shoot expression (stronger than in wild-type), with wide variation in shoot GUS activity between siblings; the most intensely staining examples (G and I) corresponding to vascular dissociation in the upper hypocotyl stele.




## Figure 5.57 p*ARR5*::GUS expression in *hydra1* at 7 dae

A-D; p*ARR5*::GUS expression in the SAM (A), cotyledon (B), primary root apex (C) and anchor root apex (D) from the same *hyd1* seedling at 7dae. A, B, bar = 200$\mu$m; C, D, bar = 50$\mu$m.

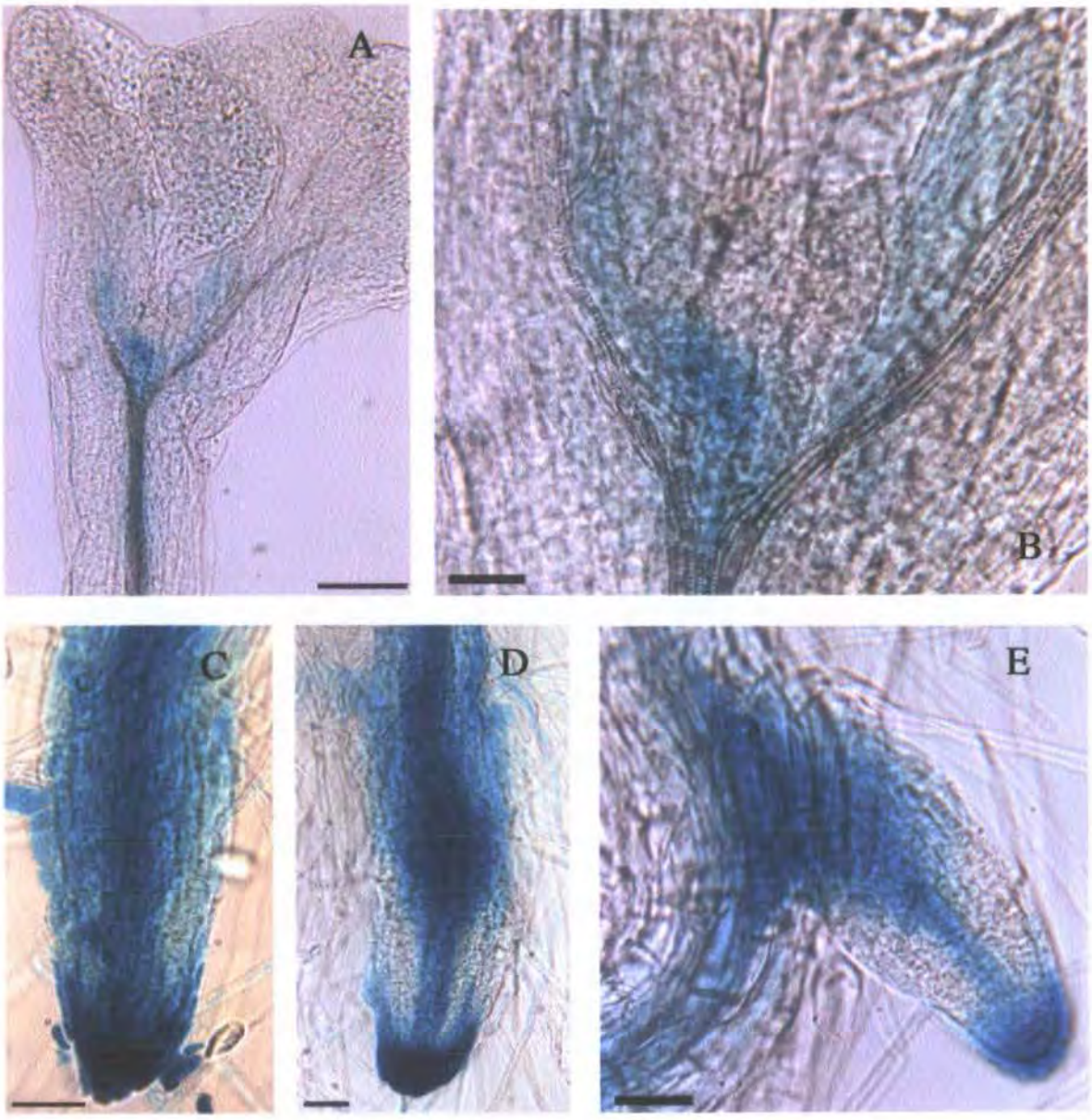
The 7 dae seedling pictured A-D has exaggerated reporter expression in vascular-associated mesophyll cells of the cotyledon lamina, with peaks in areas where xylem strands show obvious discontinuities (A, B; arrows). In contrast, root expression appears similar to wild-type, though often with a less intense GUS signal in root cap tissues of both primary and lateral apices (C, D).




## Figure 5.58 p*ARR5*::GUS expression in *hydra2* seedlings at 3 and 5 dae

A-I; p*ARR5*::GUS expression in *hyd2* at 3dae (A-C), and in the SAM region and root apices of 5dae seedlings (D-E, F, G, I). A-C, H, bar = 200$\mu$m; D-G, I, bar = 50$\mu$m.

At 3 dae, reporter activity in *hyd2* is similar to *hyd1*, both of which show stronger shoot expression than wild-type seedlings of the same age. In contrast to *hyd1*, seedlings of *hyd2* from 5 dae onwards show minimal shoot expression, and strongly enhanced root activity.




## Figure 5.59 p*ARR5*::GUS expression in *hydra2* at 7 dae

A-E; p*ARR5*::GUS expression in shoot and root tissues from the same *hyd2* seedling at 7dae. A, shoot; B, SAM region; C, primary root apex; D, anchor root apex; E, young lateral root. A, bar = 200$\mu$m; B-E, bar = 50$\mu$m.

Mutants of *hyd2* have minimal shoot expression, and strongly enhanced root activity for the p*ARR5*::GUS reporter. This figure illustrates reporter activity at 7 dae, where primary roots (C), anchor roots (C ) and laterals from the main root (E) show exaggerated expression.

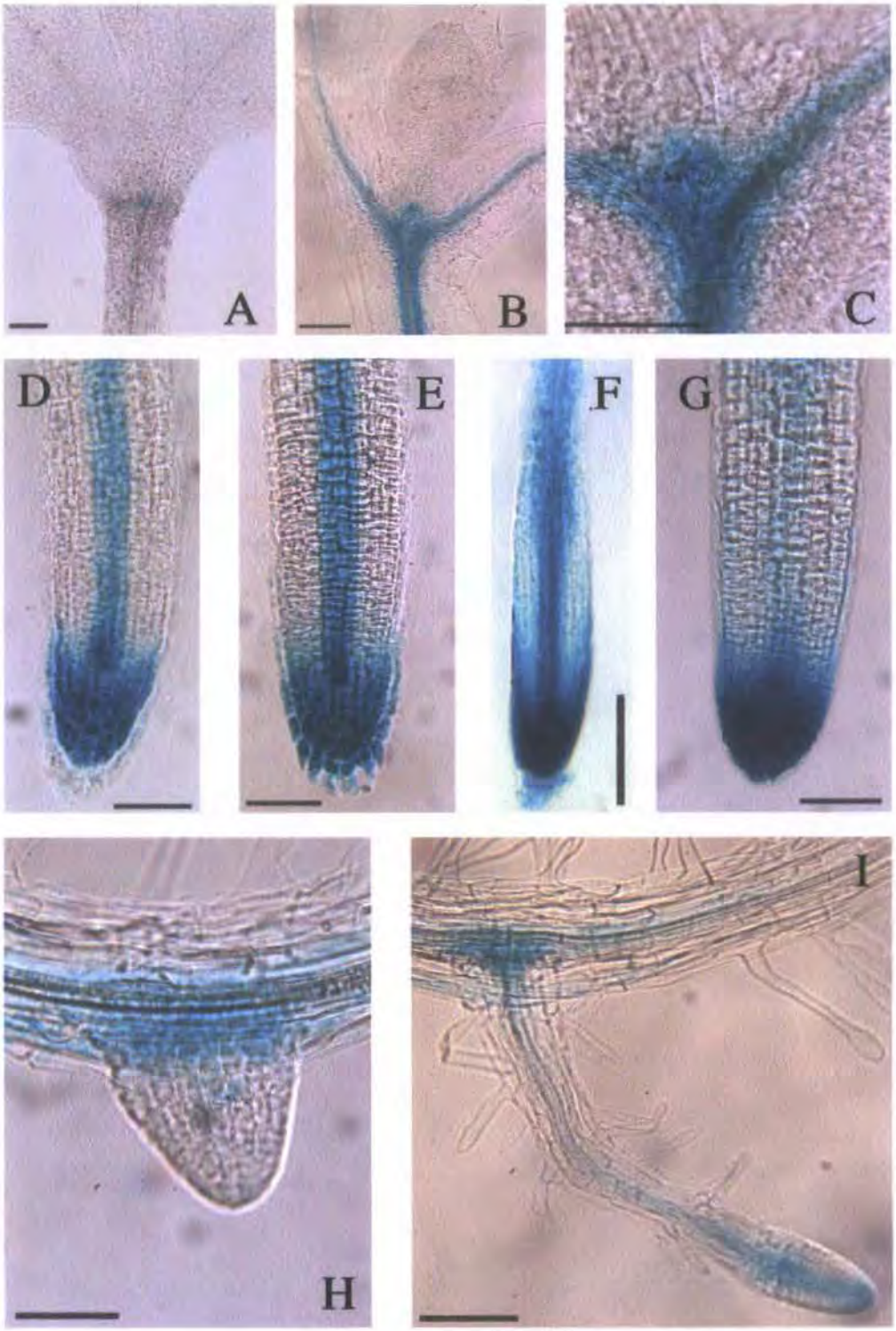
SAM, and in vascular associated mesophyll cells of the cotyledon lamina. Peaks of intense activity are visible in association with discontinuities in the xylem; arrows indicate a vascular island (Fig. 5.57; A) and a discontinuous secondary trace (Fig. 5.57; B).

In *hyd2*, activity of p*ARR5*::GUS shows an enhanced signal in root peripheral tissues from 3 dae. By 5 dae, most siblings exhibit an indiscriminate GUS activity throughout the entire root by 5 dae, with a histochemical peak in the root cap; this implies an exaggerated cytokinin response in peripheral tissues of the *hydra2* root. Shoot GUS activity is not enhanced in these same individuals, and the signal is localised to the SAM, in a manner similar to wild-type (Fig. 5.58; A-I). A similar pattern is seen in the shoot and primary root of the 7 dae seedling shown in Fig. 5.59; A-E. In this seedling, the lateral and anchor root apices have a reduced GUS activity, reflecting the relative differences seen between primary and lateral roots of control plants (Fig. 5.55; G-K). However the p*ARR5*::GUS signal is consistently stronger throughout the root if *hyd2* than in wild-type.

These observations imply differences in the cytokinin responses of mutants at the *hydra* loci. This distinction was not noted in mutants expressing p*IAA2*::GUS, implying that the modulated activity of p*ARR5*::GUS is not caused by auxin.

### 5.3.2.3.2 Activity of the p*ARR5*::GUS reporter is differentially modulated in root and shoot tissues of *hyd1-ein2* and *hyd2-ein2*.

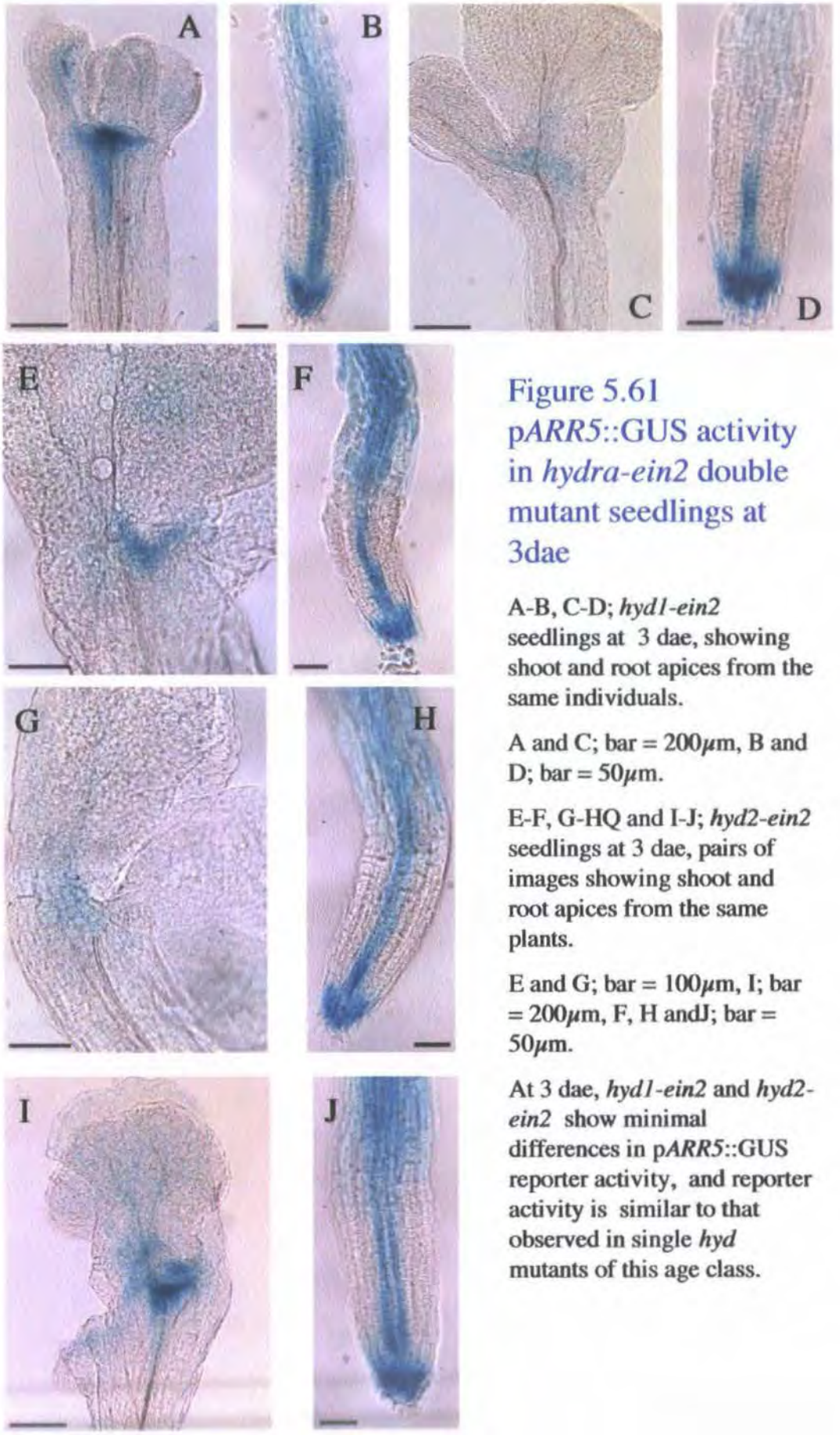
Few differences were apparent in p*ARR5*::GUS activity between wild-type and *ein2* seedlings (Fig. 5.60; A-I). As in wild-type, expression appeared first in the root cap, followed by the root stele and shoot apex, developing over 7 dae into a strong stele-associated signal in the root and hypocotyl, peaking beneath the SAM in the region of stele bifurcation, but absent from leaf primordia. The *ein2* signal in the shoot apex appeared slightly slower to develop, but positional definition was indistinguishable from wild-type. The 7 dae primary root of *ein2* showed a slight reduction in cellular




## Figure 5.60 Expression of pARR5::GUS in *ein2*

A-I; *ein2* seedlings showing expression of the p*ARR5*::GUS cytokinin-responsive reporter in shoot apices (A; 3dae, B; 5dae and C; 7dae), and roots (D; 3dae, E; 5dae, F-I; 7dae). A-C, bar = 100$\mu$m; D-I, bar = 50$\mu$m.

Positional information demonstrated by activity of this reporter is similar in wild-type and *ein2* plants, although shoot staining appears slower to establish in the *ein2* mutants. Intensity of staining in the root cap and stele of primary root apices (D-F) appears as in wild-type, although the cell-specific localisation of the reporter signal less distinct by 7dae (F). Anchor root apices (G) have a similar diffuse signal, with slightly less staining in the stele. Laterals (H and I) have a reduced signal in the stele and root cap.




## Figure 5.61 p*ARR5*::GUS activity in *hydra-ein2* double mutant seedlings at 3dae

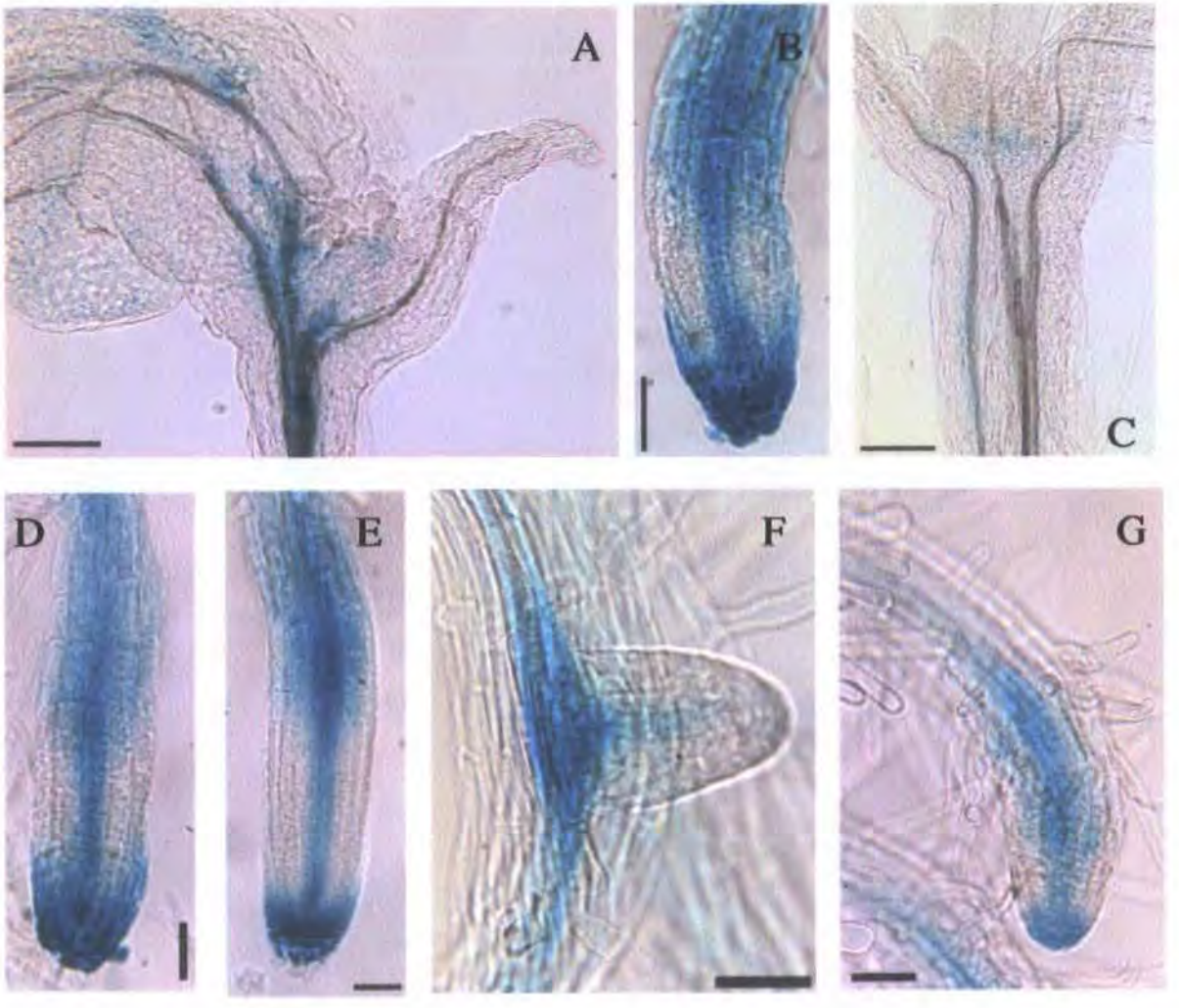
A-B, C-D; *hyd1-ein2* seedlings at 3 dae, showing shoot and root apices from the same individuals.

A and C; bar = 200$\mu$m, B and D; bar = 50$\mu$m.

E-F, G-HQ and I-J; *hyd2-ein2* seedlings at 3 dae, pairs of images showing shoot and root apices from the same plants.

E and G; bar = 100$\mu$m, I; bar = 200$\mu$m, F, H andJ; bar = 50$\mu$m.

At 3 dae, *hyd1-ein2* and *hyd2-ein2* show minimal differences in p*ARR5*::GUS reporter activity, and reporter activity is similar to that observed in single *hyd* mutants of this age class.

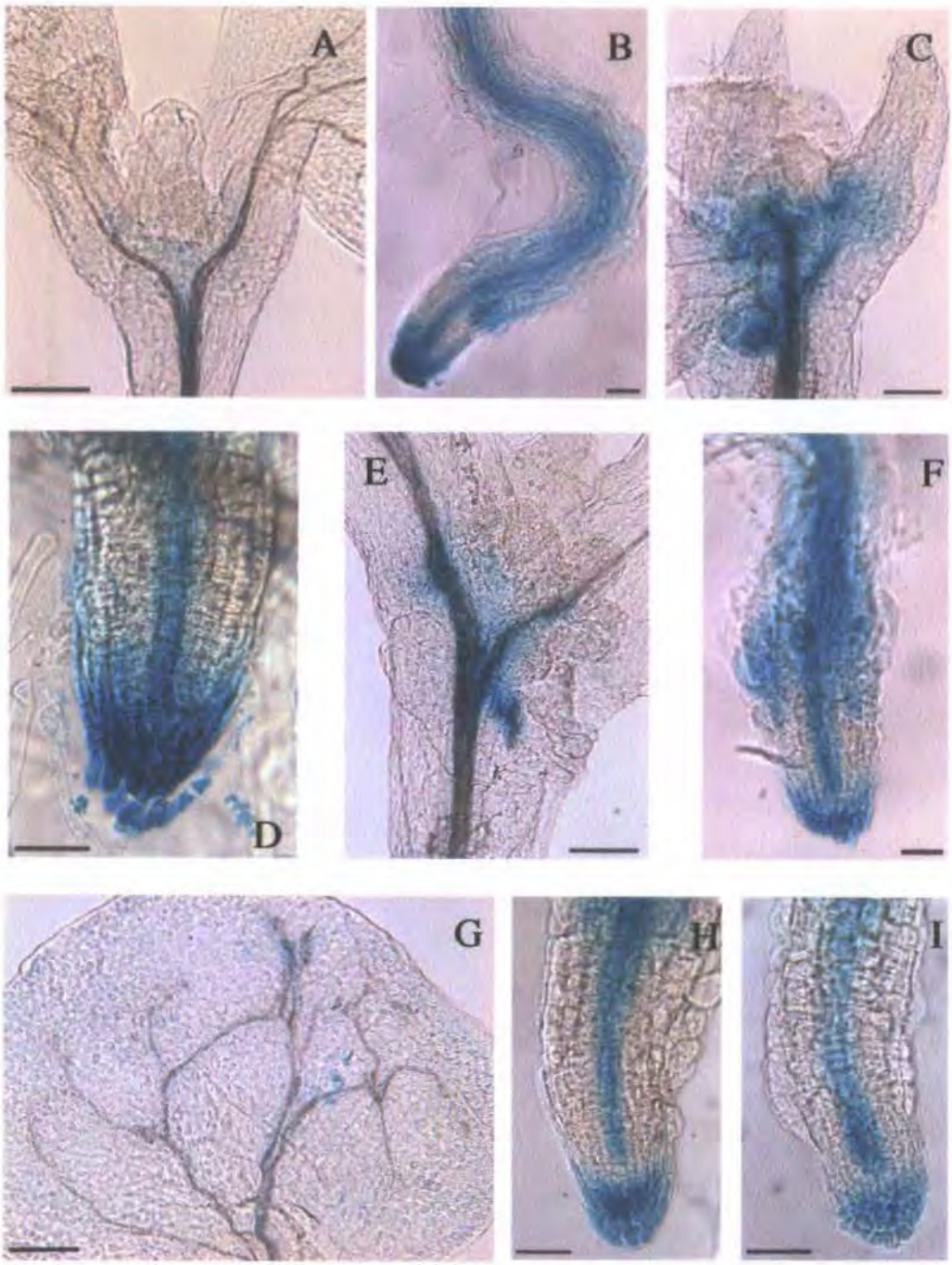



## Figure 5.62 Expression of p*ARR5*::GUS in *hydra1-ein2* at 7 dae

A-B and C-G; root and shoot tissues from the same *hyd1-ein2* seedlings at 7 dae. A and C, bar = 200$\mu$m; B, D-G, bar = 50$\mu$m.

In contrast to the 3 dae *hyd-ein2* double mutants, older age classes of seedlings show a modulated expression of p*ARR5*::GUS. The exaggerated expression in *hyd1* siblings with dissociation of vascular strands in the upper hypocotyl is much reduced in *hyd1-ein2* (A). Other siblings show similar to or lesser staining around the SAM than both wild-type and *ein2* plants. Histochemical activity is strongly reduced in siblings where the longitudinal axis is clearly duplicated (C), and the definition of the stele in the upper hypocotyl is lost in these siblings.

The presence of the *ein2* mutation in the *hydra* background substantially modulates the behaviour of the p*ARR5*::GUS reporter, in a manner which was not seen in *ein2* single mutants. Root expression in *hyd1-ein2* is stronger in primary root apices (B, D) than in *hyd1* single mutants. The diffuse nature of the root cap staining, seen in *ein2* at this age of seedling development, is not found in the *hydra* double mutants. Positional localisation in anchor roots (E) and laterals from the main root (F, G) have similar expression intensities to that seen in wild-type roots.




## Figure 5.63 Expression of p*ARR5*::GUS in *hydra2-ein2*

A-B, C-D, E-I; root and shoot tissues from *hyd2-ein2* siblings at 7dae, showing expression of the p*ARR5*::GUS reporter. A, C, E and G, bar = 200$\mu$m; B, D, F, H-I; bar = 50$\mu$m.

In *hyd2-ein2* (A-I) at 7 dae, expression is remarkably similar to that of *hyd1-ein2*. In this case, the exaggerated root expression from the pARR5::GUS reporter has been modulated down in the roots, and up in the shoot apex, by the presence of *ein2*. This contrasts with a modulation of reduced expression in shoots and increased activity in roots by the introduction of *hyd1* into an *ein2* background. The *ein2* mutation therefore alleviates the inter-allelic differences in reporter activity seen between these *hydra* mutants, and modulates reporter activity to a similar level (itself enhanced relative to the controls).

B, D and F show primary root apices, H is an anchor root apex and I the top of an established lateral from the main root. Note that ectopic expression in parts of the cotyledon shown in G are in association with sections of vascular primary strand dissociation and disjunct xylem vessels.

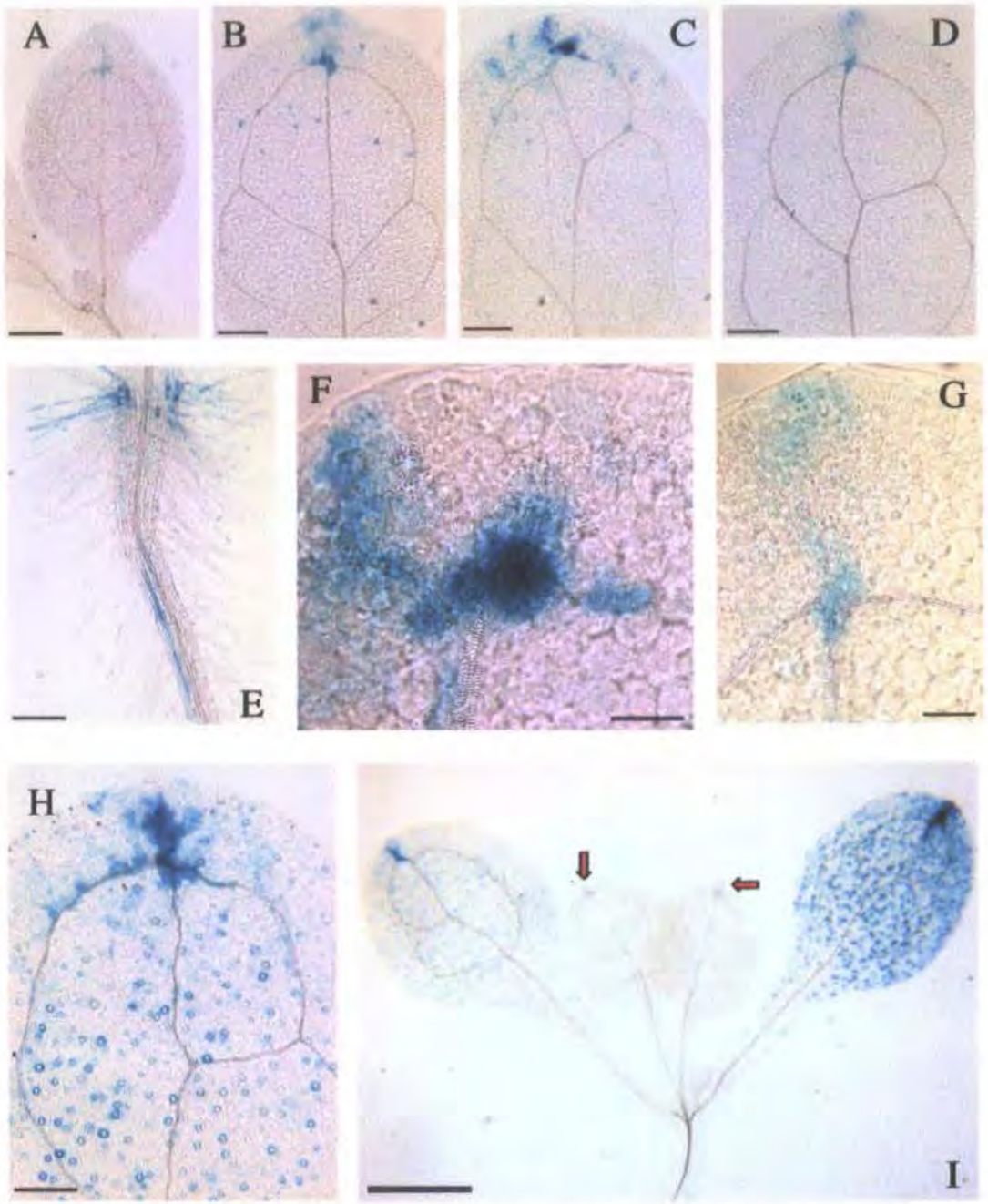
definition around the root cap, with the appearance of a diffuse GUS activity in peripheral tissues further up the root (Fig. 5.60; F).

In 3 dae *hydra-ein2* double mutants, activity of p*ARR5*::GUS was similar in both *hyd1* and *hyd2* backgrounds. The substantial differences seen in later (7 dae) seedlings of the single mutants were also modulated to give a much more uniform reporter expression between the two populations of *hydra-ein2* double mutant siblings. This resulted in a down-regulation of shoot expression, and up-regulation of root expression in *hyd1-ein2* (Fig. 5.61; A-D, Fig. 5.62)relative to *hyd1*. The opposite situation (increased shoot expression and reduced root expression) was seen in *hyd2-ein2* relative to *hyd2* (Fig. 5.61; E-J and Fig. 5.63). The expression of p*ARR5*::GUS in peripheral tissues of the *hyd2* root was substantially reduced in *hyd2-ein2* (Fig. 5.63; B, D and F). This may be in association with an improved elongation near the root apex, as reporter activity is displaced into peripheral tissues in *ein2* (Fig. 5.60; F) in a manner which is reminiscent of the patterning seen in *hyd-ein2* double mutant primary root apices.

These data imply that differences in the transcriptional response of *ARR5* between *hydra1* and *hydra2* mutants involves ethylene signalling via the *EIN2* pathway. Other factors may also be implicated, as the intensity of GUS signal in *hydra-ein2* double mutants appeared greater than that observed in *ein2*. However this appearance may be exacerbated by the smaller cell sizes and hence greater cytoplasmic density in *hydra* than in control plants, in which case the *ein2* mutation could be considered as abolishing the cytokinin response anomalies in the *hydra* mutant plant body.

## 5.3.2.4 Gibberellin signalling

### 5.3.2.4.1 The p*GASA1*::GUS GA-responsive reporter showed elevated expression in *hydra* cotyledons, and ectopic expression in mutant hypocotyls.



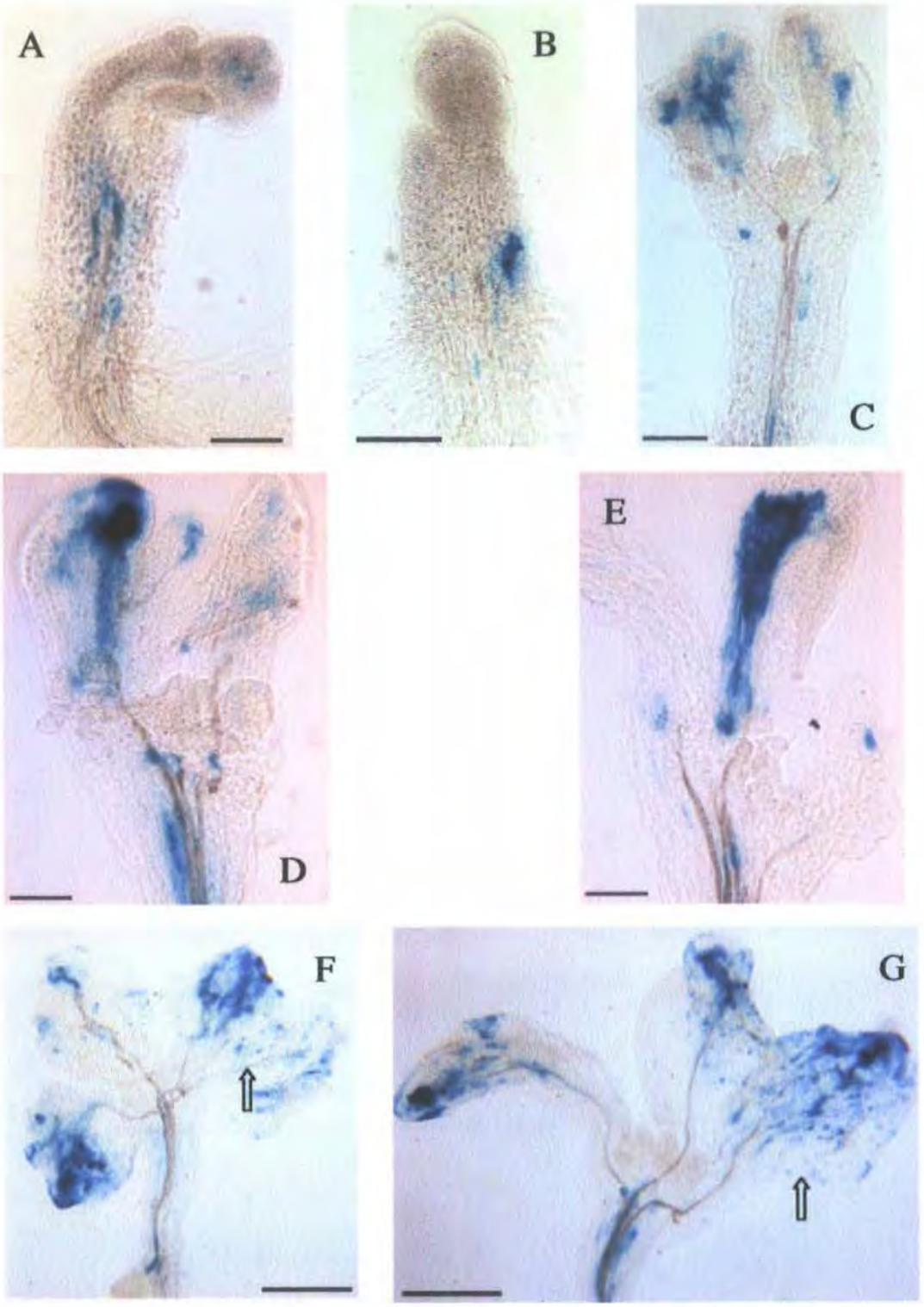

## Figure 5.64 Expression of p*GASA1*::GUS in wild-type seedlings

A-I; wild-type plants showing activity of the p*GASA1*::GUS gibberellin-responsive reporter. A; 2dae cotyledon. B; cotyledon and E; root-hypocotyl junction at 3dae. C and D; cotyledons at 5dae, with the apical hydathode regions from these leaves detailed below in F and G respectively. H; cotyledon from 7 dae plant, showing a stomatal signal. I; shoot region from a 10dae seedling; the two cotyledons can vary in histochemical intensity, a phenomenon observed in many individuals, although localization of the signal was unaffected. This plant shows the first signs of staining in the apical hydathodes of the first pair of true leaves (arrows). A-H, bar = 200$\mu$m; I, bar = 1mm.

Expression appears at emergence in mesophyll cells of the cotyledon apical hydathode, and in stomatal guard cells as they differentiate. The apical and lateral hydathodes of true leaves also develop a signal in older seedlings, after the establishment of secondary vasculature in the expanding lamina. Root expression of this reporter was found to be minimal, and varied between seedlings with a few individuals highlighting certain cell files of the inner cell layers of the main root, and in a radial fashion in the epidermis around the root hypocotyl junction, in association with emerging anchor lateral primordia.

The GA-responsive *GASA1* gene from *Arabidopsis* is from a gene family related to the *GAST1* gene of tomato (Herzog *et al.* 1995). The activity of the p*GASA1*::GUS reporter, like *GASA1* mRNA accumulation, is induced by endogenous and exogenous GA, and repressed by ABA (Raventos *et al.* 2000). It is unclear whether GASA1 is a primary GA or BR response gene (Boquin *et al.* 2001). Accumulation of *GASA1* mRNA is reduced by BR, although activity of p*GASA1*::GUS is unaffected, implying that BR repression is active via elements either within the *GASA1* gene, or at a post-transcriptional stage (Boquin *et al.* 2001). Therefore the p*GASA1*::GUS reporter is indicative of the GA:ABA balance, and highlights GA-mediated transcriptional activity.

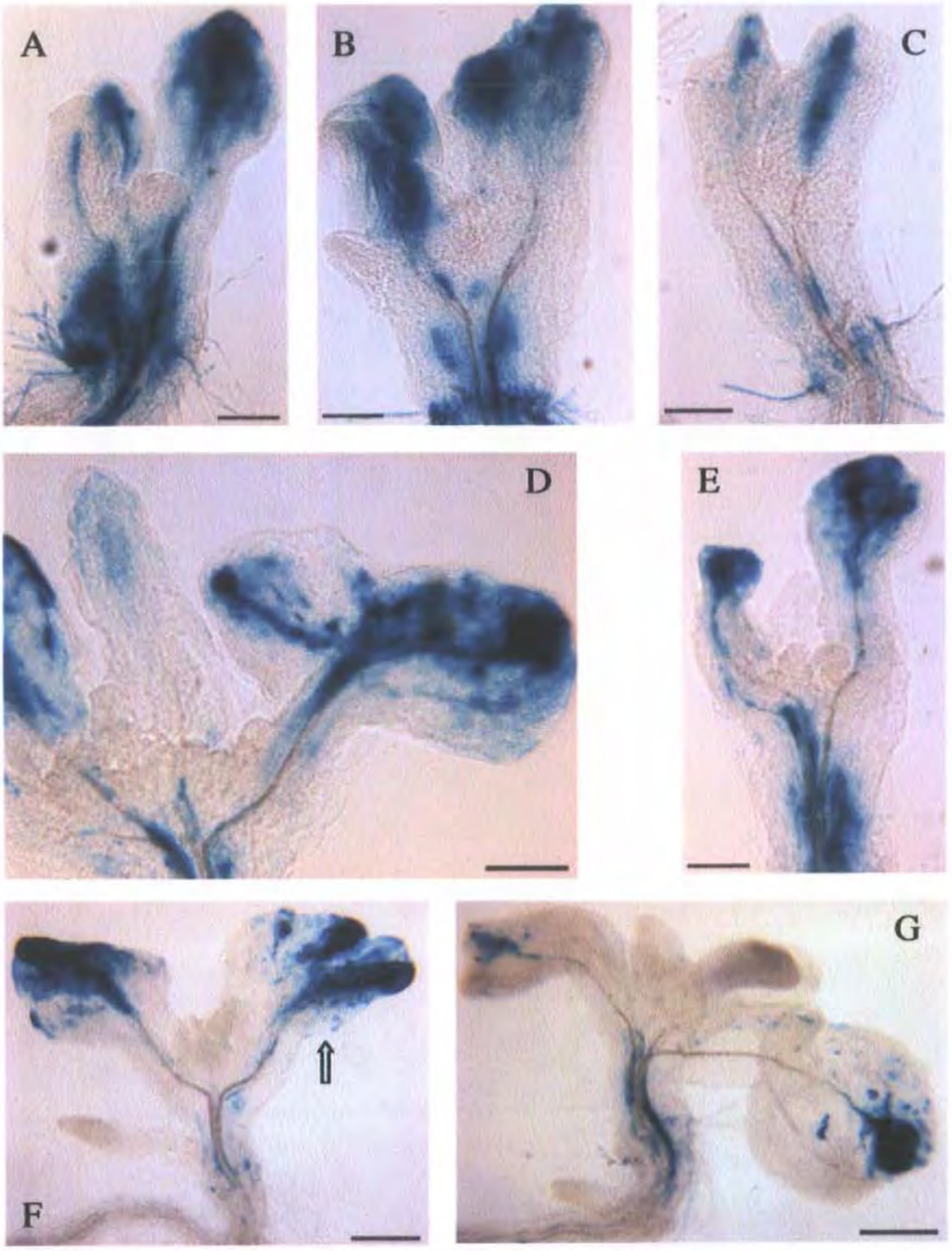
Wild-type seedling shoots expressing the p*GASA1*::GUS reporter developed strong GUS activity in cotyledon hydathodes very early after germination, from 2 dae (Fig. 5.64; A). This signal intensified to a maximum at around 7 dae (Fig. 5.64; B-D, H). From 3 dae, differentiating stomata in the cotyledon epidermis also showed GUS activity, beginning with the first maturing cells in the cotyledon apex. By 7 dae, the majority of guard cells in these organs showed reporter activity (Fig. 5.64; H). The intensity of the apical hydathode signal showed some variation within different cotyledons, with an enhanced expression in those examples with a greater degree of xylem 'noise' (Fig. 5.64; C and D, detailed in F and G). The signal intensity varied also in the stomates from these leaves, and could vary between organs from the same plant, as shown in the 10 dae seedling in Fig. 5.64; I. In this example, the apical hydathodes of the first true leaf pair are just beginning to develop GUS expression, after differentiation of the third loop of secondary xylem, i.e. close to the completion of pattern definition in these organs. No reporter activity was observed in wild-type hypocotyls. Although Raventos *et al.* (2000) report GUS activity in the root from this construct, minimal reporter expression was found in the roots of wild-type plants. Some seedlings showed a signal in the root stele, but many seedlings with strong shoot expression lacked any reporter activity in their roots. The clearest signal was seen in occasional seedlings around the root-




## Figure 5.65 Activity of the p*GASA1*::GUS reporter in *hydra1*

A-G; expression of p*GASA1*::GUS in *hyd1* seedlings. A-C, 3 dae; D-E, 5 dae and F-G, 7 dae. A-E, bar = 200$\mu$m; F-G, bar = 0.5mm.

Reporter activity in *hyd1* shows a greater intensity than that observed in wild-type, with ectopic expression appearing in some hypocotyl cell files from 3 dae (A-C). Cotyledon expression is stronger than in wild-type plants and less localised; in many individuals the signal extends from the apical hydathode region over a much broader section of the lamina (D, E). Guard cell expression appears normal, and is visible in cotyledon epidermis of 7 dae plants (F, G). Roots showed some staining in *hyd1*, in a similar pattern to that observed in wild-type (not shown). Arrows indicate guard cells highlighted by GUS activity.




## Figure 5.66 Activity of p*GASA1*::GUS in *hydra2*

A-G; expression of p*GASA1*::GUS in *hyd2*. A-C, 3 dae, D and E, 5 dae, and F, G, 7 dae; bar = 200$\mu$m.

Reporter activity in *hyd2* shoots shows a similar positioning of ectopic expression to that seen in *hyd1*, although at a greater intensity. Activity differed from wild-type in having ectopic expression in hypocotyl cell files, with an enhanced signal from the cotyledon hydathode, extending over much of the lamina. In examples of seedlings where the cotyledon has a dissociated primary midvein, the two apical hydathode regions were highlighted by the reporter, as in D and F.

As in *hyd1*, root expression of this reporter in *hyd2* was similar to that observed in wild-type plants (not shown).

hypocotyl junction, as in Fig. 5.64; E. This difference may be attributable to the different growth conditions; all samples for this analysis were grown on $^1/_2MS_{10}$, whilst Raventos *et al.* (2000) used full strength $MS_{10}$ media.

Expression of p*GASA1*::GUS showed positioning in *hyd1* and *hyd2* similar to wild type. However the histochemical activity was increased in both mutant populations, and was substantially elevated in *hydra2* relative to *hydra1*. As few *hydra* seedlings showed root expression, and the reporter activity present in *hydra* roots was very similar to that seen in the wild-type background, root tissues are not described further in this analysis.

In *hyd1* seedlings at 3dae, ectopic expression of the p*GASA1*::GUS reporter was evident in hypocotyls in association with the stele (Fig. 5.65; A) or peripheral tissues (Fig. 5.65; B), or in patches in the cotyledons across the lamina (Fig. 5.65; C). Cotyledon expression was further intensified in 5 dae seedlings (Fig. 5.65; D and E), by this point a substantial GUS activity was evident in the apical hydathodes and extending into the lamina of some organs, whilst absent from others within the same seedling. The 7 dae *hyd1* siblings shown in Fig. 5.65; F and G have a substantial although poorly localised GUS activity in the hydathode regions, with some signal evident in stomates, and a persistent ectopic expression in the hypocotyl. Cotyledon-like structures lacking a differentiated xylem trace (as in the radialized organ to the left of the photograph in Fig. 5.65; E), showed minimal or no GUS activity, suggesting an association of reporter expression with the presence of differentiated xylem vasculature.

In *hyd2* seedlings, the p*GASA1*::GUS reporter showed substantially heightened activity in both cotyledon apices and hypocotyls (Fig. 5.66; A-G). Expression was positionally similar to the phenomena observed in *hyd1*, although at a greater intensity. Again an association with xylem differentiation appears to correspond to the presence of the reporter signal; the cotyledons in Fig. 5.66; C show poor xylem differentiation and have less GUS activity than seedlings of the same age class with better xylem differentiation, as in Fig. 5.66; A and B. The radialized cotyledonary

organs visible in Fig. 5.66; D and E, have no expression in the absence of xylem (E) and minimal expression with a rudimentary xylem trace visible towards the base of the organ (D). GUS activity in *hyd2* appeared to resolve a greater tissue-specificity between 3 dae (Fig. 5.66; A-C) and 7 dae (Fig. 5.66; F and G). In cotyledons where two hydathode regions have resolved, as in Fig. 5.66; D and F, accordingly show expression maxima in both positions.

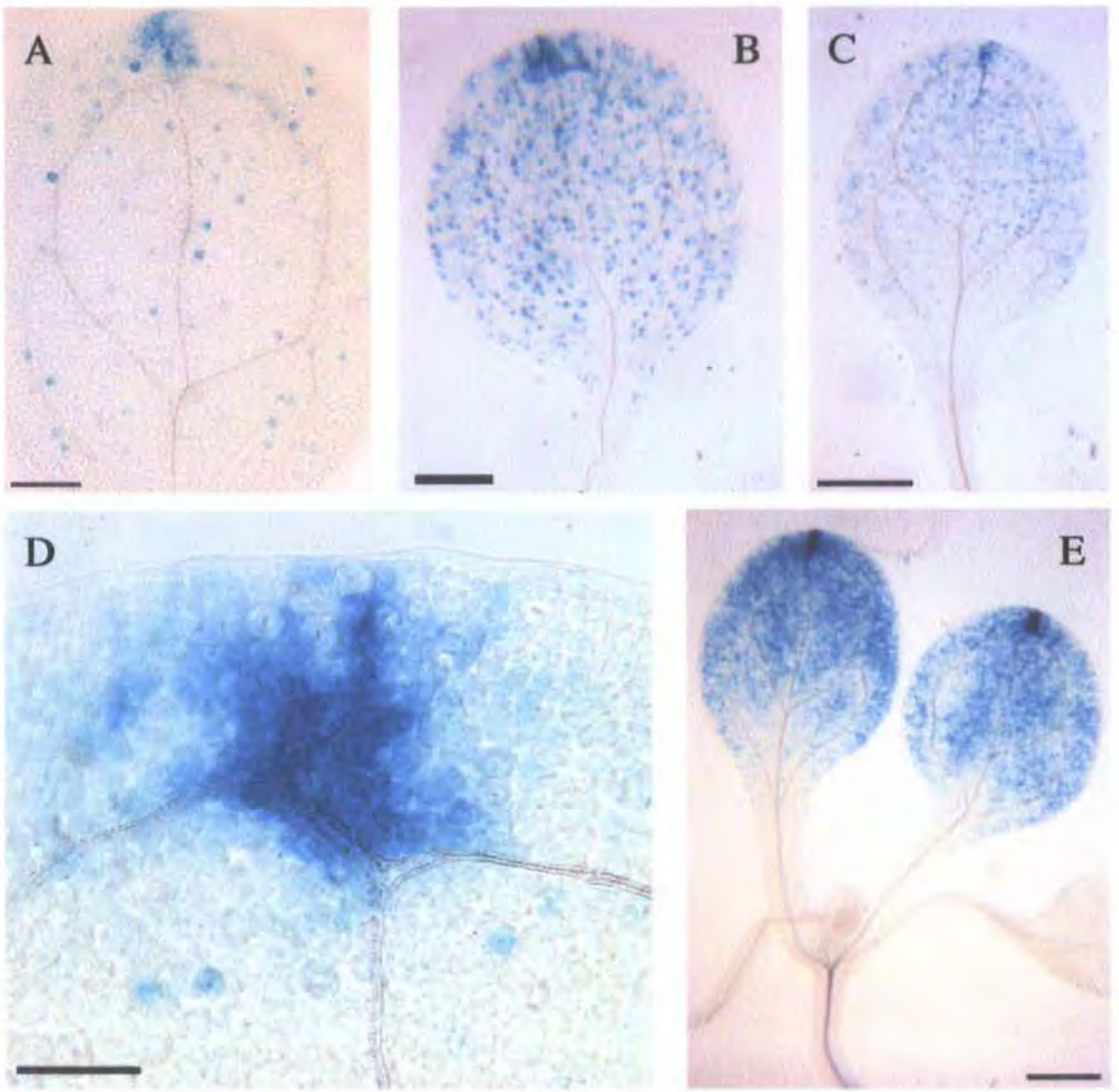
Some activity from p*GASA1*::GUS was visible in some (though not all) 7 dae siblings of both *hyd1* (Fig. 5.65; F, G) and *hyd2* (Fig. 5.66; F). Differentiated guard cells are evident in the adaxial epidermis of cotyledons in this age class. However the reporter does not highlight all cells; guard cells from individual stomates appear defined only in certain sectors of the laminae in the samples depicted in Fig.s 5.65; F-G and 5.66; F. No clustered stomata showed reporter activity, (although such clusters appear to be ubiquitous throughout the *hydra* sibling populations) suggesting a link between putatively functional stomates and p*GASA1*::GUS expression in these mutants.

These observations suggest that GA signalling may be enhanced in the *hydra* mutants relative to wild-type plants. This phenomenon is more pronounced in *hyd2* than in *hyd1*, and is exacerbated in both by the dissociation of the vascular strands. An enhanced GA response in these mutants would also imply the converse, that ABA signalling is down-regulated in the *hydra* mutant plant body. The association of exacerbated p*GASA1*::GUS activity with vascular dissociation implies that the modulated signalling phenomena indicated by this reporter is subordinate to the vascular anatomy of the mutant sibling under examination, suggesting that the disruption of the GA-ABA system is not a primary causing factor in the *hydra* mutant phenotype, but is a consequence of anomalous *hydra* development.

### 5.3.2.4.2 Expression of the p*GASA1*::GUS reporter is elevated in *ein2* but is differentially modulated in the *hyd1-ein2* double mutants according to the xylem integrity.

Expression of p*GASA1*::GUS in *ein2* (Fig. 5.67; A-E) appeared in similar positions to the signal seen in wild-type plants. Reporter activity appeared first at the cotyledon hydathode, resolving into guard cells as stomates differentiated in the epidermis. As development and hence differentiation is more rapid in *ein2*, the timing of the stomatal signal appeared slightly ahead of wild-type. In contrast to wild-type plants, *ein2* developed an intense GUS activity in mesophyll of the cotyledons in older seedlings, shown in a 10 dae plant in Fig. 5.67; E. No reporter activity was seen in hypocotyls, and root expression was indistinguishable from wild-type (not shown).

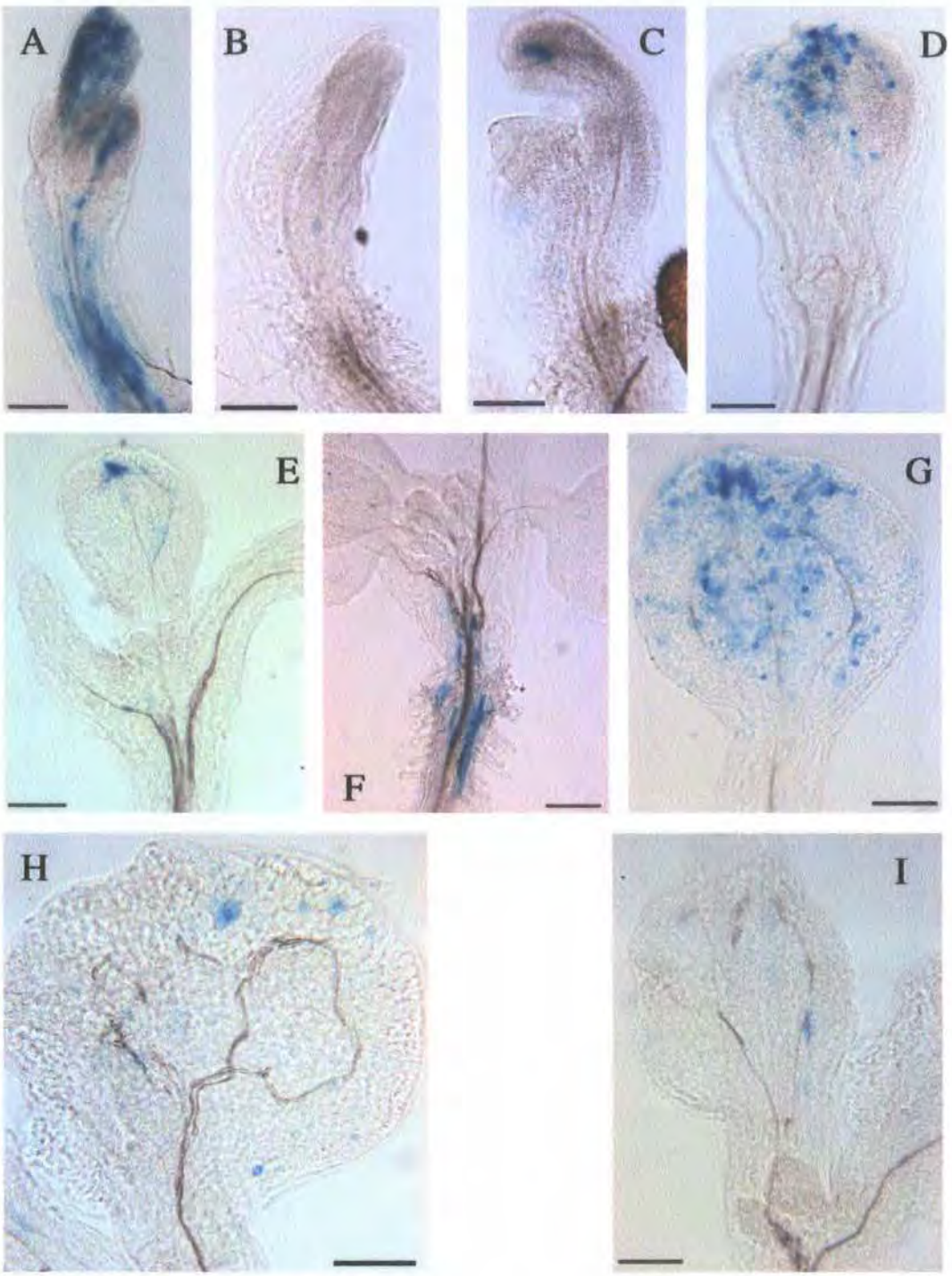
Activity of the p*GASA1*::GUS reporter was up-regulated in both *hydra* mutants, particularly in *hyd2*. Both mutants showed a substantial increase in signal in association with the cotyledon apical hydathode, and ectopic expression variably in hypocotyl tissues. In the *hyd1-ein2* seedlings shown in Fig.s 5.68 and 5.69, this expression throughout development was slightly modulated by the introduction of *ein2*. The ectopic expression seen in single *hydra* mutant hypocotyl tissues is still visible in some seedlings, particularly at early developmental stages, though is not present in all 3 dae siblings showing reporter activity (Fig. 5.68; A-D). As the xylem traces of *hyd1-ein2* cotyledons are generally improved, it was possible to distinguish a qualitative improvement in the localization of the cotyledon hydathode signal in tandem with greater integrity of the xylem vasculature (as visible in Fig. 5.68; E, and differentiated between the cotyledon traces in the two organs visible in Fig. 5.69C; Q). Other seedlings with poor vascular definition appear to lose the apical hydathode signal (Fig. 5.68; H and I). Stomatal differentiation and the associated GUS activity appears to proceed in a manner similar to that observed for *hyd1*. Older *hyd1-ein2* seedlings (e.g. Fig. 5.69; A-C) resolved a signal visible in the cotyledon




## Figure 5.67 Expression of p*GASA1*::GUS in *ein2*

A-E; activity of the p*GASA1*::GUS reporter of gibberellin response in *ein2* mutant seedlings. A, 3 dae; B, 5 dae and C, 7 dae cotyledons, bar = 200$\mu$m. D shows detail of the apical hydathode region from a 5 dae cotyledon, bar = 100$\mu$m. E; 10 dae seedling with enhanced GUS activity in certain mesophyll cells within the cotyledon lamina in addition to the stomatal signal, bar = 1mm.

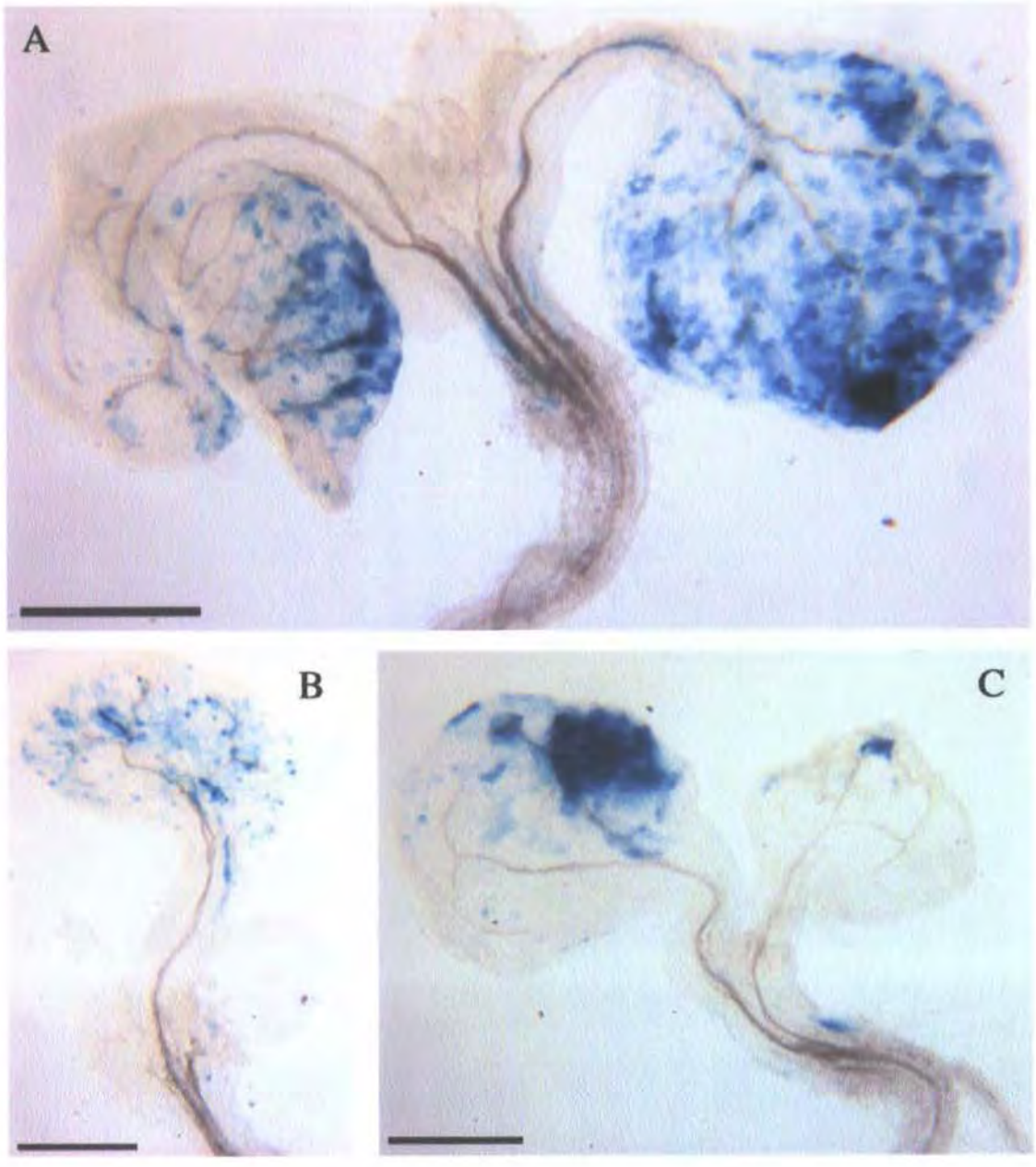
Positioning of reporter activity was broadly similar in wild-type and *ein2*, although expression in cotyledon hydathodes and stomata of *ein2* mutants developed more rapidly than in wild-type between 3 and 7 dae (A-C, D). Positional information in young cotyledons were indistinguishable from wild-type, although in older seedlings, a strong signal in cotyledon mesophyll cells was observed in many individuals (E). As with wild-type, intensity of cotyledon GUS staining could vary between the two cotyledons from an individual seedling. No differences were observed between wild-type and *ein2* plants for root expression (not shown).




## Figure 5.68 Expression of p*GASA1*::GUS in *hyd1-ein2* at 3 and 5 dae

A-I; seedlings of *hyd1-ein2* at 3 dae (A-D) and 5 dae (E-I) showing expression of the p*GASA1*::GUS reporter. G shows the cotyledon from seedling F. H and I are cotyledons from the same 5 dae plant. A-I, bar = 200$\mu$m.

Variation between siblings at 3 dae encompasses the same range of ectopic expression in cotyledons as seen in *hyd1*, although the 5dae signal intensity appears less severe than in *hyd1* single mutants. The presence of *ein2* promotes a more rapid differentiation and expansion, shown by the young true leaf in the 5 dae plant shown in E, with GUS activity in the apical hydathode. In cotyledons with a mostly or fully coherent vascular trace (L), an approximately normal staining pattern is observed. In cotyledons where vascular coherence is compromised (H, I) the seedling lacks reporter definition of the apical hydathode region.




## Figure 5.69 Expression of p*GASA1*::GUS in *hyd1-ein2* at 7 dae

A-C; seedlings of *hyd1-ein2* at 7 dae; bar = 0.5mm.

Older seedlings of *hyd1-ein2* showed less GUS activity in cotyledon mesophyll cells than observed in the *ein2* control background. The overall pattern and intensity of histochemical activity in these double mutants is similar to that observed in the *hyd1* single mutants at this age class expressing the p*GASA1*::GUS reporter. This suggests that the less intense signal seen in 5 dae *hyd1-ein2* mutants as compared with *hyd*, may reflect differences in developmental rate caused by *ein2* rather than a direct modulation of reporter activity by *ein2* in the *hydra* background. Coherence of the xylem traces does appear to affect reporter behaviour locally.

mesophyll, in a manner similar to that observed in single mutants. This phenomenon varied both between siblings and between organs, with localization to certain sections of the lamina in some seedlings (such as that shown in Fig. 5.69; C). The extremes of GUS activity extending the length of the cotyledon lamina from the apex to the meristem junction seen in some *hyd1* examples (Fig. 5.65; D and E) were not observed in the double mutants, suggesting that there has been some overall reduction in reporter activity as a consequence of the *ein2* mutation. Expression in *hyd-ein2* double mutant stomates followed a similar pattern to that seen in hyd1 single mutants.

These observations imply that the p*GASA1*::GUS elevated transcriptional response seen in both *hydra* mutants is modulated in intensity by ethylene via the *EIN2* pathway. Introduction of *ein2* into *hydra* down-modulated this signal slightly, although in *ein2* mutants an elevation of reporter transcription appeared in mature tissues. Part of the *hydra* ectopic p*GASA1*::GUS response, associated with vascular patterning anomalies, was therefore partly ameliorated by *ein2*, in association with greater xylem integrity. This suggests that GA signalling in *hydra* is modified by a range of inputs, possibly involving auxin and ethylene.

## 5.3.3 Summary of Results from Chapter 5

Abolition of ethylene signal transduction via *ein2* effects a partial rescue of the *hydra* mutant phenotype by reducing cell division activity and promoting cellular elongation. In association with this, vascular integrity was improved, and polarly-organized epidermal cells showed a more normal expansion growth as revealed by microtubular cortical arrays. However vascular dissociation anomalies in both phyllotaxy and dorsiventral positional signalling cues were unaffected, resulting in a similarly varied population of sibling morphologies as seen in *hydra* single mutants.

The basis of morphological phenotypic rescue by *ein2* appears relative, and concerns a reduced cell proliferation with increased expansion, along with a mild reduction in auxin responsiveness. Introduction of the *ein2* mutation into *hydra* did not affect duplication and dissociation of the longitudinal axis, the integrity of leaf primary vascular strands, leaf trichome and root trichoblast morphology, the development of ectopic meristems or the initiation of clustered primordia.

The use of reporters for different phytohormone responses revealed a considerable modification of various signalling cascades within the *hydra* mutant plant body. The presence of *ein2* modulated aspects of this anomalous signalling activity in *hydra*, whilst reporter positioning was unaffected by the *ein2* mutation alone. Variation was noted in the position of phytohormone responsive cells between siblings of both *hydra* mutants, and the *hydra-ein2* doubles, in a manner which reflected the variable sibling morphology in these seedling populations.

Double mutants of *hydra-ein2* show improved longitudinal cell file integrity, greater (but not complete) vascular cohesion, a reduction in the adoption of trichoblast cell fate in potential root hair cells, a slightly increased expansion growth in lateral organs, and an increased longevity.

Stomatal clusters were reduced, although not eliminated, and appeared to have a greater viability (for transpiration) as shown by p*ACS1*::GUS activity. The intensity of adaxial positioning cues were reduced slightly in *hydra* by *ein2*, allowing a better resolution of abaxial patterning cues, and the morphological (if not functional) resolution of stipules in some siblings.

Disjunctures in the xylem patterning of *hydra* mutant cotyledons attracted persistent localized expression of reporters indicating auxin response and ACC biosynthesis. Ectopic expression of the *IAA2*::GUS reporter was seen particularly in shoot tissues, with irregular peaks in the vascular traces of expanding true leaves in the vicinity of disjunct xylem vessels. This activity was unaffected by the presence of *ein2*. Transcription of the ACC synthase reporter p*ACS1*::GUS did not mirror p*IAA2*::GUS activity. The positioning of p*ACS1*::GUS varied substantially between siblings, with some plants developing enhanced root expression. Vascular disjunctures were also associated with persistent ectopic p*ACS1*::GUS activity. The p*IAA2*::GUS auxin responsive reporter showed a slight reduction of activity in *hydra-ein2* double mutant roots, and the p*ACS1*::GUS reporter showed increased transcription, possibly via a negative feedback in the absence of *EIN2*-mediated ethylene response. This suggests that ethylene contributes to, but does not cause the problems in auxin signalling found in *hydra* mutants.

Opposite extremes of transcription from the cytokinin responsive p*ARR5*::GUS reporter in *hydra* siblings, with peaks seen in respectively shoot and root tissues of *hyd1* and *hyd2*. Activity of p*ARR5*::GUS was heightened around dissociated and discontinuous xylem strands near the SAM in both mutants, but was minimally expressed where complete axis duplication had occurred. Full axis duplication similarly diminished the root cap and root stele signal. The *hyd2* sibling population showed an enhanced p*ARR5*::GUS transcription in roots, suggesting an elevated cytokinin level in peripheral tissues. This differential cytokinin response between *hyd1* and *hyd2* was modulated to a similar (though enhanced) expression by *ein2* in both mutants, implying that part of the differential

component of their cytokinin responses is mediated via ethylene. Alternatively, the reduction in p*ARR5*::GUS expression may result from an improved vascular integrity in the double mutant sibling populations, making the root stele of *hyd2* and the hypocotyl stele of *hyd1* more coherent.

Activity of the GA-upregulated p*GASA1*::GUS reporter was substantially enhanced in leaf tissues throughout the sibling populations of both *hydra* mutants, although this was particularly strong in *hyd2*. Individual patterning was variable, even between lateral organs from the same seedling. Mis-expression of p*GASA1*::GUS was particularly noted in hypocotyls, associated with the ground tissue (cortex and endodermal) cell layers. Heightened activity was also seen in some cotyledons and true leaves around the hydathode regions, although different organs within the same seedling could show opposing extremes of expression. Ethylene is also implicated in GA signalling, as *ein2* reduced the ectopic expression of p*GASA1*::GUS in hypocotyls and cotyledons of both *hydra* mutants. This modulation was closely correlated with the integrity of the xylem strands in the double mutant lateral organs.

Reporter activity of p*ACS1*::GUS suggested a reduced function in stomata. GUS expression from p*ACS1*::GUS in guard cells (as seen in the wild type) was rarely evident in the mutant siblings. Some mutant siblings developed areas of cotyledon or true leaf epidermis in which single stomates showed p*GASA1*::GUS activity; although the signal never highlighted clusters or closely spaced stomata; this expression may indicate regions of functional transpiration. Stomatal function appeared to be partly restored in *hydra* by the presence of *ein2*, and was again related to general organ morphology.

## 5.4 Discussion

### 5.4.1 The effects of *ein2* on *hydra* mutant morphology and development

#### 5.4.1.1 The *ein2* mutation results in only partial rescue of the *hydra* phenotype

The inhibition of ethylene signal transduction in *hydra* by the *ein2* mutation resulted in partial rescue of aspects of the *hydra* mutant phenotype. No effects were observed upon *hydra* embryogenesis in the *ein2* mutant background. The incidence of morphological anomalies in *hydra* siblings was unaffected by the introduction of *ein2*, and the development of duplicated longitudinal axes, vascular dissociation, precocious cotyledon and primordial development were unaffected. The *hydra-ein2* siblings did however show improvement in the elongation of radially organized tissues (i.e. the root and hypocotyl) but not dorsiventrally flattened lateral organs of the shoot. Increased viability of the root apical meristem (as reported under conditions of ethylene signalling inhibition by Souter *et al.* 2002) was seen in the double mutants, and was associated with an improved seedling longevity.

Ethylene appears to contribute to, but does not cause, the longitudinal cell coordination defects in *hydra*. The integrity of xylem strands were not altered in cotyledons, but were improved in true leaves of the *hyd-ein2* doubles, as compared to single *hydra* mutants, along with an improved coherence of phloem associated callose in these organs. However vascular defects were not eliminated, and the anomalies in hydathode patterning and distortions of leaf shape in *hydra* were unaffected. Although ethylene is implicated in various

epidermal pattering processes, particularly concerning proliferation of root hair and stomata (i.e. the same epidermal patterning 'cassette' in the root and shoot), *ein2* reduces but does not eliminate these patterning anomalies in *hydra*. Similarly, the *hydra-ein2* double mutants reveal a modulated intensity but not positioning of radial patterning cues in the abaxial and adaxial domains of shoot lateral organs. These observations imply that the primary defects of *hydra* mutants are not dependent upon ethylene, but are exacerbated by a heightened ethylene response.

### 5.4.1.2 Reduced ethylene perception conveyed by the *ein2* mutation modifies cell division activity

In the present study, the *ein2* mutation was found to be associated with lower rates of cell division and greater cell elongation than that observed in wild-type plants, in accordance with the findings of Kazama *et al.* (2004) that transient exposure to ethylene promotes local cell division events. Introduction of the *ein2* mutation into *hydra* had a modulating effect on the cell division rate in an additive manner, and so permitted a more normal cell division pattern in *hydra2* root apices, but did not prevent ectopic cell division. This means that ethylene signalling is only a component of the poor spatial control of division activity in the *hydra* mutants. The persistence of *CYC1At*::CDB::GUS activity around the disjunct xylem in *hydra-ein2* double mutant cotyledons implies an alternative cause for this persistent cell division activity. One possible candidate is the phytohormone auxin, which may accumulate locally in tissues in the vicinity of *hydra's* disrupted xylem vessels, and result in a localized modulation of cellular signalling responses, including cell division.

### 5.4.1.3 Effects of the *ein2* mutation in *hydra* may be auxin related

The activity of *ein2* in *hyd-ein2* double mutants appears to convey a reduced auxin responsiveness by increasing the rate of auxin transport, a noted consequence of reduced ethylene activity (Morgan & Gausman 1996, Suttle 1988, Alonso *et al.* 1999). Auxin over-producing mutants in *Arabidopsis* have been noted to also have elevated ethylene levels of between 2.5 - 4 times that of wild-type plants (Romano *et al.* 1993). As auxin overproducers may have apparent auxin transport difficulties due to overwhelming of the native transport capacity, this is likely to result in local auxin peaks, which could promote the localized biosynthesis of ethylene in response to these auxin levels. The increased ethylene levels in *hydra* reported by Souter *et al.* (2002) may therefore result from local increases in ethylene activity; not from an excess of auxin in the *hydra* plant body, but rather as a consequence of an under-functioning auxin transport system.

An auxin-related effect seen in the *hydra* plant body is the deposition of ectopic callose in the developing cotyledon and leaf mesophyll, in contrast to wild type leaves where the phloem-associated callose is confined histologically to the vascular trace. The presence of the *ein2* mutation partly ameliorated the deposition of ectopic callose in *hydra* mutant true leaves, and resulted in a less intense expression and greater coherence of the auxin-upregulated pAthB8::GUS reporter in true leaves but not cotyledons. As there is no evidence to date that the *EIN2* gene is active during embryogenesis, and the cotyledons are embryo-derived structures, this means that cotyledon patterning would be unaffected in the *hydra-ein2* double mutants. The cotyledon mesophyll undergoes only cell differentiation after germination in *Arabidopsis* (Tsukaya *et al.* 1994), indicating that these organs and specifically this cell layer are direct products of embryogenesis. As the cotyledon vascular tissues are derived entirely from the mesophyll layer, these observations imply that the *ein2* mutation has no effect on the developing *hydra* embryo, and

imply that ethylene signalling, at least that mediated via *EIN2*, does not affect either pattern definition or morphology during embryogenesis.

Peaks of p*AthB8*::GUS over-expression were noted in the hydathode regions of *hydra* leaves, an effect which was reduced but not abolished by *ein2*. Over-expression of p*REV*::GUS in *hyd2* and the closely related p*PHB*::GUS in *hyd1*, were down-modulated by the introduction of the *ein2* mutation, implicating these adaxial-promoting genes in ectopic cell division in the *hydra* mesophyll layer. Both *AthB8* and *REV* (but not *PHB*) are known to be transcriptionally auxin responsive, and the reduced auxin signal in the *ein2* plant body could down-regulate the ectopic transcription of these genes, helping to ameliorate the effects of their over-expression in *hydra*.

Xylem noise and phloem-xylem coordination are improved in *hydra-ein2* double mutants; an effect which is likely to result from a modulated auxin signalling. Ethylene stimulates cell division (Kazama *et al.* 2004), and inhibits lateral and basipetal auxin transport (Morgan & Gausman 1966, Suttle 1988). The reduction of ethylene responses by the *ein2* mutation (Alonso *et al.* 1999) would therefore accelerate the removal of auxins (via enhanced transport) from the differentiating tissues by promoting cell differentiation and expansion over division, and facilitating an improved longitudinal coherence of vascular strands by reducing cell numbers.

Alternatively, vascular anomalies may be promoted in the *hydra* mutants by over-expression of *AthB8* and *REV*, both of which result in the promotion of vascular cell differentiation through the precocious differentiation of procambial cells into xylem (Baima *et al.* 2001, Zhong & Ye 1999). Studies in many cell types have shown that cell division and differentiation are inversely correlated processes, and the transcription factor *REV/IFL1* has been implicated in the balance between them (Zhong & YE 1999, Ratcliffe *et al.* 2000). The AthB8, REV and PHB proteins each carry a START domain, which offers an alternative interpretation; either a lack or over-abundance of sterol-

mediated binding, or unique sterols found in the *hydra* mutants interfering with normal sterol binding, resulting in a modulation of START protein activity.

The initiation of primordia at the shoot apical meristem has been attributed to auxin accumulation, resulting in pin-mediated auxin transport in the L1 layer of the SAM (Reinhardt *et al.* 2003, 2004). Although vascular integrity shows some improvements in *hydra-ein2*, neither the mis-positioning of vasculature in the hypocotyl-cotyledon transition zone, nor the incidence of precocious SAM and primordial initiation are affected by the presence of *ein2*. These data, in the light of the strong p*HYD1*::GUS transcription in stipules (which precedes the activity of the auxin responsive p*IAA2*::GUS and DR5::GUS reporters in stipules of wild-type plants), suggests that a *HYDRA*-mediated sterol effect pre-empts auxin-mediated patterning in these tissues, perhaps in an L1-dependent manner.

### 5.4.1.4 Mis-positioning of abaxial and adaxial tissue identity reporters in *hydra* mutants are unaffected by ethylene signalling

The intensity of p*REV*::GUS and p*PHB*::GUS expression was reduced in *hydra-ein2* double mutants, and the p*YAB*::GUS signal was enhanced. This effect is not caused by ethylene, as positioning of these reporters was unaffected in the *ein2* mutants, although their histochemical intensity was very slightly increased relative to wild-type. This indicates the radial distribution of these reporters in the *hydra* plant body was unaffected by the presence of the *ein2* mutation. These data therefore imply that a signalling system which is modulated by ethylene, e.g. auxin (Luschnig *et al.* 1998) is affecting the relative intensity of gene expression between adaxializing and abaxializing signals in wild-type plants. This process appears to be independent of the

causes of radial patterning defects in the *hydra* mutants, suggesting that dorsiventral radial cues may be independent of auxin-related signalling.

The *ein2* mutation does not affect the positioning of the p*REV*::GUS reporter in *hydra* mutants, but does modulate the intensity of their expression. These results suggest a significant modulation of the positioning of *REVOLUTA* gene expression, encoding a START domain transcription factor protein. However the reporter reveals transcription, and does not indicate activity of the putative sterol-interacting protein. This suggests that the positioning of REV across the *hydra* mutant radial axis is disrupted by the altered sterol environment of *hyd1* but not *hyd2,* in advance of any putative sterol interaction by the REVOLUTA protein. This positioning is independent of *ein2*, but is modulated in intensity in *hydra* in the reduced ethylene response environment.

The abaxial and adaxial promoting signals in leaves are thought to act in an antagonistic fashion, and over-expression studies with a number of these genes result in abaxialization or adaxialization of primordia (Emery *et al.* 2001, 2003). As *REV* transcription is known to be auxin responsive (Zhong & Ye 1999), this implies that the modified auxin response of ethylene insensitive mutants (Luschnig *et al.* 1998) may be responsible for the modulation of the intensity between abaxial and adaxial cues. This would explain the enhancement of p*YAB*::GUS activity in both *hydra* mutants, resulting from a reduction of the intensity of transcription of adaxialising genes such as *REV* and *PHB*.

The positioning of the *YAB, PHB* and *REV* reporter activity in the *hydra* mutants broadly correspond with this model of mutually exclusive abaxial and adaxial domains in developing primordia. The exception to this pattern, i.e. the apparently normal abaxial positioning of p*YAB*::GUS in the *hyd2-ein2* examples shown in Fig. 5.20, must be considered in the context of the *hyd2* sibling population. Even in the presence of *ein2*, the p*YAB*::GUS reporter

signal was so weak in most *hyd2* siblings that dorsiventral positioning could not clearly be described. Fig. 5.27; C shows clustered primordia with an apparently normal morphology but not position, in the absence of a clearly defined SAM region. The few examples in which p*YAB*::GUS activity is visible may be only those siblings with the most compromised SAM organization, in which case the primordia 'self-organize' a dorsiventral axis and develop according to it. This would explain the phenomena visible in Fig 5.20, whereby the random placement of these primordia resolves an abaxial p*YAB*::GUS signal relative to its own morphology, in the absence of a SAM.

These data imply that the positioning of the boundary between abaxial and adaxial molecular domains, but not the definition of the dorsiventral axis itself, are modulated by *ein2*. This can be proposed to result from increased auxin transport efficiency; both a reduction in ethylene signalling (Luschnig *et al.* 1998), and the increased integrity of longitudinal cell files found in the *hydra-ein2* double mutants, could contribute to improved auxin movement. This means that whilst controlled transport of auxin may be the main agent controlling SAM morphology via the directing of primordial radial position (Reinhardt *et al* 2003, 2004), the *hydra* mutant phenotypes indicate that dorsiventral coordination requires other, additional factors, possibly sterols.

### 5.4.1.5 The coordination between polarly expanding hypocotyl cells is partially rescued in *hydra* by the *ein2* mutation

The poor organization of cortical microtubular arrays in expanding hypocotyl cells, as revealed by the GFP::TUA6 construct (Ueda *et al.* 1999), was improved, but not restored to wild-type by the introduction of *ein2* into the *hydra* background. This implies that ethylene exacerbates the effects of poor longitudinal intercellular coordination, but does not cause it. Cell shape defects in the *hydra* mutant epidermis also appeared to function

independently of ethylene effects mediated via *ein2*. Not only were the same patterning anomalies found in *hydra-ein2* double mutants, but the reduced rates of cell division conveyed by *ein2* promoted cell expansion, so that irregularly- or isodiametrically-expanding cells were exaggerated in size.

Kazama *et al.* (2004) found that transient ethylene stimulation of cell division could alter cell fate and polarity in hypocotyl epidermal cells of *Cucumis sativus*, resulting in local proliferation of cells competent for division such as stomatal subsidiary cells and developing trichomes. The consequences to epidermal patterning were that trichomes were variably branched, and stomates appeared in clusters. Whilst continuous ethylene treatment suppresses cell division, the release from ethylene suppression can have a stimulatory effect, as though high ethylene levels maintained cells in a division-competent condition (Dan *et al.* 2003). This suggests that the *ein2*-mediated abolition of ethylene perception partly ameliorates the reduced differentiation signal in the *hydra* mutants.

An additional component to the cell polarity signal could be contributed by tensile tissue stress within the compound structure of the cell mass. This was shown by Hejnowicz *et al.* (2000) in sunflower hypocotyls to cause a modulation of the orientation of cortical microtubules in expanding epidermal cell files. As the *hydra* mutants have excessive cell division activity, the appearance of additional cells within hypocotyl peripheral tissues is likely to cause variable unusual tensile stresses within the hypocotyl structure. This could result in the persistent orientation anomalies in individual cells which were not implicated in the ethylene responses of *hydra* mutants.

### 5.4.1.6 The *ein2* mutation may result in better longitudinal cell alignment in *hydra* double mutants by promoting differentiation in the absence of the *HYD*-mediated mechanism.

The abolished ethylene response of the *ein2* mutation (Alonso *et al* 1999), with its reduced cell division rate (Kazama *et al.* 2004), partly ameliorates the *hyd* phenotype, and allows a greater cohesion between longitudinal cell files. If the *hydra* mutants are cell division-compromised, then the result of the *ein2* mutation is to promote differentiation throughout the mutant plant body, thereby allowing a greater synchronization between longitudinally aligned cell files of both the epidermis and vascular system.

An improved ability to differentiate would also produce a more normal timing of symplastic isolation in *hydra-ein2* functional epidermal cells. Stomatal clusters prevailed in the *hydra-ein2* epidermis, although were not as severe as in the *hyd* single mutants, and no partly-divided stomates were seen in the samples examined, although paired trichomes were still present. The interpretation of the *hydra* defect as a problem in the coordination of cell differentiation suggests that the ultrastructural phenomena seen in *hydra,* namely the formation of incomplete cell walls, may result not from a problem with cellulose biosynthesis as proposed by Schrick *et al.* (2004), but from a partial cell division event. Although incomplete cell walls were found in both *hydra* and *smt1*$^{cph}$ mutants (Schrick *et al.* 2004), these were in a minority of the cell population, which already contained a greater multiple of cells than wild-type, suggestive of a substantially increased demand for cellulose synthesis. In a system where cells cannot readily differentiate, the high metabolic cost of partitioning available carbon into cell wall components could produce a carbon-limitation constraint to cell wall biosynthesis. Schrick *et al.* (2004) point out that there is no direct correlation between the levels of sitosterol (implicated in cellulose biosynthesis) and cellulose levels amongst the *hyd* and *smt1*$^{cph}$ mutants examined, suggesting that cell wall defects do not result directly from these mutations.

### 5.4.1.7 The *ein2*-induced reduction in cell division activity does not confer any increased integrity to the *hydra* shoot endodermis

The *ein2* mutants showed normal SCR::GFP behaviour, and the presence of *ein2* in the *hydra* background, whilst not modifying the position of the GFP signal, appeared as a more clearly defined signal in roots, with less diffuse fluorescence around the endodermis than in single *hydra* mutants. In some siblings, the root signal was strongly resolved into certain endodermal cell files which had a more obvious longitudinal integrity, and was present at a lower level in adjacent cell files of the endodermal layer with a more compromised longitudinal alignment. The SCR::GFP signal also resolved more strongly in the more mature root tissues of *hydra-ein2* double mutants, However no SCR::GFP was noted in hypocotyl tissues of these doubles, implying that the lack of endodermal layer integrity in the *hydra* shoot is independent of ethylene-enhanced cell division in these tissues.

### 5.4.1.8 The *ein2* mutation conveys a reduction in root hair density, but does not modulate anomalies in hair cell morphology

The *ein2* mutation substantially reduced the root hair density of the *hydra* seedlings in comparison to single mutants, in a manner which mimicked the reduction in root hairs seen when comparing the wild-type and *ein2* backgrounds. The range of root hair morphological anomalies found in *hydra* single mutants were still present in the *hydra-ein2* double mutants. The *ein2* mutation had no effect on the bulging morphology of the developing hairs in *hydra*, and examples were still seen of multiple root tips emerging from the same cell bulge, and multiple tip initiation along the longitudinal axis of the hair cell. It therefore appears that root hair density but not morphology is affected by ethylene-implicated processes in the *hydra* mutant root.

It has been proposed that the position-dependent differentiation of trichoblasts may be regulated by ethylene, transported from the inner root through the apoplast (Tanimoto *et al.* 1995). This idea was supported by the observations that root hair production was enhanced by ethylene, ACC and the constitutive ethylene response mutant *ctr1*, along with inhibition of root hair production by ethylene inhibitors (Dolan *et al* 1994, Kieber *et al.* 1993, Tanimoto *et al.* 1995). However later work demonstrated that no inhibition of root hair formation took place in the presence of ethylene inhibition unless root hair formation was stimulated by other hormonal or environmental cues (Cho & Cosgrove 2002).

Schmidt & Shikora (2001) found that phosphorus deficiency increased root hair density of *Arabidopsis* plants even in the presence of ethylene antagonists. Ma *et al.* (2001) examined the histological basis of the increased root hair in plants grown on phosphorus-deficient media, and found that on average the cortical cell number increased from the usual 8 to 12, resulting in 12 trichoblast cell files and subsequently more root hair. Zhang *et al.* (2003) examined the interrelationship between ethylene and phosphorus in the control of root hair production, and found that low phosphorus conditions induced the formation of smaller and more numerous cortical cells, hence increasing trichoblast development in the epidermal layer. Mutation- or chemical-mediated inhibition of ethylene response in the low phosphorus environment did not affect the proliferation of these cortical cells, with one exception. This was the *eir1* mutation, which abolishes the activity of a root-specific auxin efflux protein. Mutants of *eir1* strongly resisted the low phosphorus-induced change in radial anatomy, indicating a requirement for auxin transport, rather than ethylene, in the cortex cell proliferation process (Luschnig *et al.* 1998, Zhang *et al.* 2003).

However ethylene inhibition did reduce root hair cell fate commitment; ethylene insensitive mutants showed a lesser proportion of epidermal cells forming hairs in response to low phosphorus (Zhang *et al.* 2003). Low

phosphorus-induced root hair development could be mimicked by adding the ethylene precursor ACC to high phosphorus media, and inhibited by adding ethylene inhibitors to low phosphorus media. The results of Zhang *et al.* (2003) imply that low phosphorus increases cortical cell number and hence the density of root hair cell files, whilst ethylene promotes hair cell fate commitment in these cell files. These authors also noted that the *ein2* mutation had even greater reductions in the percentage of trichoblast cells forming hairs under low phosphorus conditions than under high phosphorus conditions, both confirming a role for ethylene in root hair induction, and indicating an additive effect between ethylene and low phosphorus stress.

These findings raise the possibility that the high levels of root hair production in *hydra* mutants, which have proliferated cortical cells in the root (and hypocotyl), may do so as a result of a perceived phosphorus deficiency rather than ethylene hypersensitivity. As the *hydra* mutants are grown on nutrient rich media, this means that the altered sterol environment of the mutant roots result in a problem with either perception or uptake. Souter *et al.* (2002, 2004) attributed the rescue of *hydra* root patterning to an ethylene effect, although their image data show *hydra-etr1* root apices which still appear broader across the radial axis, and with a greater root hair density than wild-type.

The *exordium* (*exo*) promoter trap mutation was found to convey a partial rescue upon the *hydra* root phenotype; cellular patterning and overall root architecture was improved, although again the double mutant root apex was broader than wild type (Farrar *et al.* 2003). *EXO* is a homologue of *PHI-1* from tobacco (Farrar *et al.* 2003), a gene proposed to play a role in causing phosphate-starved cells to re-enter the cell cycle in response to phosphate addition (Sano *et al.* 1999). The function of *PHI-1* and *EXO* are not equivalent in the two species however, as *EXO* is regulated in a cell-cycle dependent manner, in contrast to *PHI-1* (Farrar *et al.* 2003, Sano *et al.* 1999). This suggests that the *exo* mutation may repress a phosphate starvation response

linked to cell cycle control, which in *hydra* roots results in an improved pattern response through a reduced promotion of cell cycle activity. These data also suggest that the *hydra* phenotype may be mediated, at least in part, by a modification of the perception of environmental cues.

## 5.4.2 Signalling systems in the *hydra* mutant plant body are variably modulated by ethylene

### 5.4.2.1 Mutants at the *hydra* loci have markers of modified auxin response which suggest that auxin transport is compromised

Souter *et al.* (2002) reported an enhanced auxin response in *hydra* mutants, as a result of modulated expression of the p*ACS1*::GUS and DR5::GUS reporters in individual cells, and by the mutant's enhanced callus response to exogenous auxin. The behaviours of these reporters implied that individual cells or regions of cells in the shoot and hypocotyl have an up-regulated auxin response, and these findings were interpreted by Souter *et al.* (2002) as indicative of an enhanced auxin responsiveness in the *hydra* mutant plant body.

The auxin-responsive p*IAA2*::GUS transgene which reveals the positioning of endogenous active auxin (Abel *et al.* 1995, 1996), showed a modified behaviour in *hydra* mutants, and revealed 'leakage' in the mutant vascular strands. Reporter activity highlighted isolated diffuse regions of auxin response, particularly around newly differentiating vasculature in the shoot, and persistent expression in the regions of xylem 'noise', disjuncture or around vascular islands. In the root, the initial auxin signal in 3 dae seedlings appeared normal around the apex, but revealed an additional build-up of auxin in the lower hypocotyl stele. This phenomenon was noted in seedlings

with a normally patterned vascular stele in this region, and persisted in *hydra-ein2* 3 dae seedlings. However the double mutants retained a GUS signal in the roots at later stages which was lost in some siblings of *hyd2*. Differences were also noted between primary and lateral root apices; of the surviving siblings at 12 dae, the *hyd* single mutants had a stronger p*IAA2*::GUS activity in primary roots and a minimal signal in laterals. In contrast the double mutants had a stronger signal in lateral apices, with not only a more normal signal around the stele, but evidence of the characteristic asymmetric distribution seen from this reporter in gravistimulated root apices (Luschnig *et al.* 1998).

The *ein2* mutation conveys a reduced auxin responsiveness through increased rates of auxin transport (Morgan & Gausman 1966, Suttle 1988, Alonso *et al.* 1999), and hence some auxin resistance. As auxin flow, or flux, is required for vascular development (Sachs 1991), this could explain the a better coordination of files of longitudinally aligned cells in vasculature and the epidermis in *hydra-ein2*, both of which are auxin transport implicated (Mattsson *et al.* 1999). The p*IAA2*::GUS construct is a marker for early auxin-induced gene expression (Swarup *et al.* 2001), and its activity suggests that auxin-related patterning processes in *hydra* have less clear boundary definition in regions where auxin flow becomes 'canalized', such as during vascular differentiation in developing leaves and at the root apex. Whilst the improved coherence of phloem callose deposition and xylem traces in *hydra-ein2* double mutants can be attributed to a reduced auxin responsiveness in the tiddues, the p*IAA2*::GUS signal implies difficulties in *hydra* mutants for the coordination of auxin transport rather than an enhanced auxin sensitivity. This possibility does not exclude the idea of differential auxin responsiveness within cells which are auxin starved, or auxin overloaded, as a result of reduced auxin movement within the plant body.

### 5.4.2.2 Persistent ectopic activity from the auxin responsive p*IAA2*::GUS and p*ACS1*::GUS reporters may indicate localized auxin-induced transient ethylene peaks near disjunct xylem in *hydra* cotyledons

Local activity of both p*IAA2*::GUS and p*ACS1*::GUS reporters around disjunct xylem in the *hydra* mutant cotyledon traces was unaffected by introduction of the *ein2* mutation, indicating that these phenomena are independent of the *EIN2*-mediated signalling pathway. An alternative explanation for these phenomena could consider the implications of local auxin peaks at the cellular level. High local thresholds of auxin can result in ethylene production (Kang *et al.* 1971, Sakai & Imaseki 1971). These observations suggest that an ethylene 'flush' could act as part of a phase-shifting signal in some cells, i.e. after the time for auxin response has diminished. This could be involved with the transition to differentiation, or from primary to secondary meristem activation in roots, or may be differentiation associated and indicative of symplastic isolation in certain cells or tissues.

Kazama *et al.* (2004) observed that transient exposure of cucumber hypocotyls to ethylene causes locally delayed differentiation, locally enhanced division, altered cell division polarity, and resulted in clustered stomata and anomalous trichome morphology. If ectopic local peaks of auxin appear in regions of vascular noise and disjuncture where polar transport is compromised, these could cause local ethylene production, and stimulate the ectopic transcription of the p*IAA2*::GUS and p*ACS1*::GUS reporters at these points. This situation would persist until the auxin peak is diminished, perhaps by assisted diffusion, which also allows the locally produced ethylene to disperse rapidly. Upon release from the auxin-induced high ethylene levels, cells of the vascular cambium in these regions of xylem disjuncture would then undergo ectopic cell division; such activity in these regions has been already noted in *hydra* mutants, as highlighted by activity of the *CYC1At*::CDB::GUS reporter.

### 5.4.2.3 Expression of the p*AtACS1*::GUS reporter in *hydra* mutants may indicate an elevated cytokinin:auxin ratio in root tissues, and may implicate *EIN2*-mediated signalling in the transition to differentiation

Transcription of the p*ACS1*::GUS reporter is thought to indicate sites of ethylene biosynthesis within the plant body, and is modulated by endogenous signalling systems (Rodriguez-Pousada *et al.* 1999). The p*ACS1*::GUS reporter is auxin-responsive, increasing expression slightly in young shoot tissues whilst decreasing it significantly in young roots, and both endogenous levels of auxin and ethylene exert a negative-feedback control on transcription in mature shoot tissues. The stability of ACS proteins are also increased by cytokinin (Chae *et al.* 2003). Exogenous cytokinins also result in a significant increase of p*ACS1*::GUS root transcription, an effect which may suggest an induction of ethylene biosynthesis by cytokinin in root tissues (Cary *et al.* 1995). An elevated cytokinin:auxin ratio could explain the reduced shoot signal and slightly elevated root signal from p*AtACS1*::GUS in *hydra* at 3 dae, and the substantially elevated root signal by 12 dae.

GUS activity persisted in the cotyledon vasculature of some siblings, particularly in regions of xylem disjunction. This may result from an impairment of negative-feedback control on p*ACS1*::GUS transcription in mature shoot tissues by endogenous auxin and ethylene. This activity is seen most strongly in the presence of discontinuities in the xylem vasculature, suggesting that auxin levels may be elevated in some cells as a result of impaired auxin transport, and the ectopic expression is due to auxin-promoted reporter transcription in the shoot. This explanation would also explain ectopic activity of p*IAA2*::GUS and the associated stimulation of cell cycling (i.e. transcription of the *CYC1At*::CDB::GUS reporter) by auxin at the ends of these isolated xylem vessels.

Activity of the *ACS1* reporter in the *ein2* control background appeared indistinguishable from wild-type, suggesting that ethylene biosynthesis via ACS activity does not have a negative feedback mechanism in relation to *ein2*. However p*ACS1*::GUS up-regulation in *hydra* mutant shoot vasculature was enhanced in *hyd2-ein2*, when the reduced auxin response and enhanced transport conferred by the *ein2* mutation could be expected to have the opposite effect in these tissues. An alternative explanation could be that a sterol-mediated impairment of differentiation in the *hydra* mutants causes these p*ACS1*::GUS-responding cells to be held at a more premature stage or phase of cell fate commitment than the differentiated cells around them, so retaining an auxin-responsive capacity for longer.

The introduction of *ein2* into the *hydra* mutants resulted in a modulation of p*ACS1*::GUS activity which implied a more normal development in certain tissues, specifically in the primary root stele after the cessation of longitudinal elongation growth, and in the stomata. The stomatal signal is particularly difficult to interpret, as stomates appear of variable size in the *hydra* and *hydra-ein2* mutant epidermis. The *ACS1* transcription signal may not relate to a direct promotion of ethylene biosynthesis in these cells, but to the indirect activation of this pathway. Both auxin and cytokinin inhibit ABA-induced stomatal closure by increasing ethylene biosynthesis in guard cells (Takama *et al.* 2005, 2006). The enhanced transcription of *ACS1* therefore would indicate the onset of transpiration. The stomatal p*ACS1*::GUS signal was absent from the *hydra* mutant epidermis, but was restored in some stomata in the *hydra-ein2* double mutants, implying that some transpirational activity may take place in certain sectors of *hydra-ein2* mutant leaves.

Although ethylene inhibition is reported to delay differentiation in diverse tissues (Zhang *et al.* 2003, Kazama *et al.* 2004), the results of *ein2* mediated inhibition have been shown in this thesis to slow cell division rates in *hydra-ein2* double mutants. This suggests that the *EIN2* pathway is independent of the mechanisms which shift cells between division and differentiation. These

mechanisms may involve ethylene, but in an *EIN2*-independent manner. The formation of giant cells in the *hydra* epidermis are exacerbated in *hydra-ein2* double mutants. This suggests that *ein2* may promote endoreduplication at the expense of both division and differentiation. This could explain the reduced numbers of correspondingly larger cells in *ein2* mutant organs as reported by Lake *et al.* (2000), and the absence of satellite meristemoid formation in the *ein2* cotyledon epidermis as observed in this thesis. This therefore suggests the hypothesis that the shift between cell division and differentiation has two stages; firstly a shift to endoreduplication (i.e. division is switched off), and then to a final differentiation stage where DNA duplication and associated cell expansion is switched off.

### 5.4.2.4 The *hydra* mutants show differential elevated cytokinin responses which are ethylene-dependent

Mutation of the *HYDRA* loci appears to result in elevated cytokinin response. Jang *et al.* (2000) isolated an allele of *fk/hyd2* using a screen for putative constitutive cytokinin response mutants. He *et al.* (2002) showed that *fk* root explants callus more rapidly in the presence of high auxin-low cytokinin, and callus showed diminished shoot regeneration on high cytokinin-low auxin, from which they conclude that *fk/hyd2* is hypersensitive to auxin and insensitive to cytokinin.

The p*ARR5*::GUS reporter highlighted a differential signalling response between *hydra1* and *hydra2* throughout both sibling populations. Gus activity was elevated in the shoot meristem and hypocotyl region of *hyd1*, whilst the root signal appeared similar to wild-type. In contrast, *hyd2* had elevated root responses, and a minimal signal in the shoot region. However in the presence of *ein2*, the activity of this cytokinin-responsive reporter was modulated to similar levels in both mutants, and devolved a more equivalent GUS activity in

shoot and root tissues (although still enhanced in relation to wild-type). This suggests that the differential response between the two *hydra* mutants is mediated by ethylene. Alternatively, the differences in *ARR5* transcription resulting from introduction of the *ein2* mutation into the *hydra* background may result indirectly from the ethylene reduction, possibly via promoting more efficient auxin transport. The *ein2* mutation has a moderately cytokinin-resistant phenotype and has been isolated in screens for cytokinin resistance (Cary *et al.* 1995, Su & Howell *et al.* 1992). This implies that the *hydra-ein2* double mutants could be interpreted as having their anomalous cytokinin responses abolished by disabling *EIN2*-mediated signal transduction.

### 5.4.2.5 pGASA1::GUS transcription in *hydra* suggests a modulation of GA levels in the mutant plant body

The pGASA1::GUS reporter is expressed in response to GA, and repressed by ABA (Raventos *et al.* 2000). However the expression signal is also decreased in response to brassinosteroids, and it is unclear whether the reporter activity is primarily regulated by GA or BR (Boquin *et al.* 2001). In *hydra,* ectopic reporter activity was seen in seedling hypocotyls from 3 dae, particularly in the *hyd2* sibling population. Wild-type p*GASA1*::GUS expression showed maxima in the apical hydathodes of cotyledons and true leaves. In *hydra,* reporter activity was strongly up-regulated in these regions. The different siblings showed a range of GUS activities, from expression around the apical hydathode as in wild-type although including a wider area, to a signal extending from the apex down the length of the cotyledon lamina. Reporter activity was increased overall in *hyd1* in relation to wild-type, and was substantially enhanced in *hyd2*, with particularly dense activity in the hypocotyl region.

Although the elevated levels of pGASA1::GUS reporter activity in *hydra* suggests that GA-mediated signalling is modified in the mutants, the way in which the altered sterol environment impacts GA signalling is difficult to interpret.

a.) Elevated levels of pGASA1::GUS reporter activity could suggest a strongly enhanced GA response in *hydra* mutant tissues. The *HYD1* promoter contains putative recognition motifs for MYC, MYB and MYB-related families of transcription factors which may modulate expression by GA and ABA; this implies a possible lack of a sterol-mediated GA response route in the mutants, which could result in GA upregulation and so enhancement of the pGASA1::GUS reporter.

b.) Alternatively, ectopic expression of pGASA1::GUS may indicate GA insensitivity, perhaps resulting from a modified BR signalling. A decreased sensitivity to GA in the *ga1* mutants has been shown to elevate levels of the *GA1* transcript, indicating a lack of feedback control (Hedden & Croker 1992). The p*GASA1*::GUS reporter does not show a decreased activity in response to BR, although *GASA1* mRNA levels are reduced (Boquin *et al.* 2001). In wild-type plants, the GA5 protein, a key component of gibberellin biosynthesis, regulates GA levels via a negative feedback control on its transcription (Philips *et al.* 1995, Xu *et al.* 1995), and is expressed in hypocotyls, along with other GA biosynthesis enzymes GA1 and GA4 (Silverstone *et al.* 1997, Meier *et al.* 2001). Also, in the BR-insensitive mutant *sax1*, gibberellin-insensitive cell elongation is restricted to the hypocotyl, and is reversible by application of BRs (Ephrithine *et al.* 1999). As it appears that the hypocotyl tissues are major sites of GA biosynthesis, and p*GASA1*::GUS reporter activity is enhanced ectopically in this region in *hydra*, this response may indicate that the mutants are insensitive to GA, resulting in an enhanced overproduction of this phytohormone in the hypocotyl region.

Whichever scenario is in operation in the *hydra* mutants, other signalling systems such as ABA and cytokinin feedback are likely to also be modulated.

### 5.4.2.6 Altered GA and other responses may result from modified protein-protein interactions by affecting the availability of sterols to membrane 'lipid rafts'

It is also possible that other agents mediating signalling responses may have altered functionality in the *hydra* mutants. The *SPINDLY* (*SPY*) gene encodes a protein with sequence similarity to animal N-acetylglucosamine transferases, which modify the nuclear or cytosolic localization, phosphorylation, interactions or stability of other proteins (Greenboim-Wainberg *et al.* 2005, Wells *et al.* 2001). The SPY protein inhibits GA response and promotes cytokinin signal transduction (Greenboim-Wainberg *et al.* 2005). Over-expression of SPY in *Arabidopsis* and petunia produced phenotypes consistent with reduced GA activity (Swain *et al.* 2001, Izhaki *et al.* 2001). Interactions of proteins such as SPY have been suggested as agents involved in substrate recognition and/or in the generation of active complexes, mediating interactions between diverse proteins. In the *hydra* mutants, function of SPY may be impaired, perhaps by alteration of the local sterol environment in lipid rafts in the membrane, known as sites of protein interactions in animals and plants (reviewed by Martin *et al.* 2005). The *spy* phenotype has a shortened hypocotyl, smaller leaves and deviant phyllotaxy, implicating the protein in cellular processes other than GA-related signalling (Swain *et al.* 2001, Greenboim-Wainberg *et al.* 2005).

Lipid rafts were originally noted from animal studies, in examinations of the polarized distributions of sphingolipids in epithelial membranes; further research revealed that membrane-anchored GPI (glycosyl-phsphatidylinositol) proteins were preferentially partitioned into a low-density sphingolipid and cholesterol enriched membrane fraction (Brown & Rose 1992). Lipid rafts

have now been isolated as detergent resistant membrane (DRM) fractions from *Arabidopsis* callus and tobacco leaves and suspension cells; they were found to be sterol- and sphingolipid-enriched, but glycerol depleted (Mongrand *et al.* 2004, Borner *et al.* 2005, Peskan *et al.* 2000). The *Arabidopsis* raft contains three putative hypersensitive-response proteins (Borner *et al.* 2005). The tobacco rafts were found to recruit NADPH oxidases and G protein regulators (Mongrand *et al.* 2004), which suggests that many functions may take place in these regions, including guard cell signalling and root hair polar growth (Mittler *et al.* 2004). The cell expansion regulator COBRA is a GPI-anchored protein, and Borner *et al.* (2005) suggest that lipid rafts may determine its position in lateral membranes of root epidermal cells. Similarly they suggest that lipid rafts could help explain the mis-localization of PIN proteins and disrupted vesicle-mediated auxin transport found in the sitosterol deficient *smt1*$^{orc}$ mutant. This means that disruption of the internal sterol environment, as found in *hydra,* has the potential to interfere with multiple protein-lipid interactions, and multiple cellular processes such as directional auxin transport, and localization of Rops to tip growing regions of root hair cells.

### 5.4.2.7 Altered cytokinin and gibberellin activity in concert may result in mis-functioning of KNOTTED transcription factor activity in *hydra* mutants

The KNOTTED class of homeodomain transcription factors are part of the mechanism which keep cells of the SAM indeterminate, whilst cells of the organ are determinate (Endrizzi *et al.* 1996). Also, ectopic expression of KNOX genes in leaves result in a more indeterminate pattern of growth, affecting leaf size, shape, and the distribution of cell fate identities (Jackson *et al* 1994, Hake *et al.* 1995, Chan *et al.* 1998). A molecular reporter of *KNAT2* expression was noted to have reduced activity in *fackel* mutants, and the knox gene *STM* was variably positioned across the mutant apex (Schrick *et al.* 2000), implying a modification of meristem function. *STM* is a class-I KNOTTED1-like

homeobox transcription factor (Long *et al.* 1996), a group of transcriptional activator proteins which promote meristem function partly through repression of GA biosynthesis (Rosin *et al.* 2003). Jasinski *et al.* (2005) found that KNOX function is also mediated by cytokinin, which is known to promote cell division and meristem function (Riou-Khamlichi *et al.* 1999, Chaudhury *et al.* 1993, Rupp*et al.* 1999). Jasinski *et al.* (2005) found that constitutive GA signalling in combination with reduced cytokinin levels is detrimental to the functioning of the meristem. Expression of KNOX1 proteins induced increased mRNA levels of cytokinin primary response genes, and rapid and dramatic increases of cytokinin levels are induced by STM (Yanai *et al.* 2005). These authors suggest that KNOX proteins act as general regulators of growth homeostasis by simultaneously activating cytokinin and repressing GA biosynthesis in the meristem region. As *STM* expression was shown to vary in extent and position in *hyd2/fk* embryos, (Schrick *et al.* 2000), this may result from heightened GA signalling, resulting in reduced expression of *KNOX* genes, and a reduced cytokinin response. In this scenario, the heightened activity of the cytokinin responsive p*ARR5*::GUS may indicate overproduction within a cytokinin-insensitive mutant. He *et al.* (2002) considered *fk/hyd2* to be insensitive to cytokinin, based on the responses of root explants on callus-inducing and shoot-inducing medium. Further work is required to elucidate exactly what the cytokinin response modulation is by the *hydra* mutant phenotype.

# Chapter 6

# Towards a definition of the *hydra* mutant phenotype

## 6.1 Abstract

The investigation of *hydra* mutants from the view of a radial patterning phenomenon has revealed several previously overlooked aspects of the phenotype. The gene expression pattern of *HYD1* and the corresponding morphology in the *hydra* mutant tissues suggest a role for *HYDRA*-mediated sterols in the transition to differentiation at the cellular level, and both intercellular patterning and organ boundary definition at the tissue level. Many of the morphological anomalies seen in *hydra* mutants then result from difficulties in intercellular coordination during expansion growth. The disrupted sterol environment of the mutant plant body has implications for cell polarity, the balance between division and differentiation, and the cohesion of transport and signalling networks. Anomalies in pattern definition across the shoot radial axis implicate a sterol signal in adaxial tissue identity definition, possibly mediated via START domain transcription factors. Several mechanisms are discussed which propose a role for sterols in these patterning processes, and some ideas for future work are suggested.

## 6.2 Discussion

### 6.2.1 Sterols are implicated in the targeted differentiation of functional epidermal cells

#### 6.2.1.1 The *HYD1* gene appears to act at the point of differentiation

Activity of the *HYD1* reporter suggests an association with the onset of differentiation, which occurs in plants over a protracted developmental sequence, and is reversible by a modification of positional cues until later in the process. Two expression 'modules' appear to function in *HYDRA* transcriptional activity;

1. an intense, cell-specific signal, associated with functional epidermal cells at or near the time point of symplastic isolation, which may involve the definition by differentiation of a 'target cell' in a patterning process involving lateral inhibition mediated by MYB transcription factor cascades

2. a diffuse signal defining fields of cells, such as the root cortex and endodermis, the hypocotyl cortex, or the leaf mesophyll, after differentiation of functional cells and tissues in this region. The purpose of this signal appears to be to coordinate a transition from cell division to differentiation, and to allow the coordinated expansion of the entire organ, maintaining the relationships between radial layers.

### 6.2.1.2 The *hydra* epidermal phenotype suggests problems in the demarcation of cell division, endoreduplication and differentiation

Many aspects of the *hydra* phenotype can be considered to result from a lack of controlled coordination in the transition from division to differentiation. In *hydra* root and shoot apical meristems, cell division events are enhanced relative to wild-type, and are associated with distortions in embryonic and seedling gross morphology. Cell division also persists in leaf organs, particularly in the vicinity of vascular mis-patterning, and results in excessive and uneven thickening of rosette leaves as they mature. Patterning phenomena in the *hydra* epidermis include clusters of small cells, trichomes developing from adjacent epidermal cells, the presence of unevenly sized 'giant' cells without an obvious polarity, and various stomatal patterning anomalies. Some stomates which appear to result from either a synchronous cell division and differentiation, or a post-differentiation cell division. These anomalies could result from a poor coordination of the balance between division and post-differentiation endoreduplication.

Endoreduplication is an alternate version of the cell cycle that is found in a wide variety of organisms (Nagl 1976, Traas *et al.* 1998). In the wild-type leaf epidermis, trichomes differentiate ahead of the surrounding field of cells, and proceed through endoreduplication cycles at the same time and at the same rate as surrounding cells (Hulskamp *et al.* 1994). MYB proteins and other transcription factors involved in epidermal cell patterning influence this process. Mutations at the *TRY* locus increase endoreduplication, trichome branching and trichome cell size as well as affecting lateral inhibition of trichome cell fate in adjacent cells (Hulskamp *et al.* 1994, Schellmann *et al.* 2002). Conversely, mutations at the *GL3* locus reduce endoreduplication, trichome branching and final cell size (Hulskamp *et al.* 1994). The *GL1* gene may also affect the

endoreduplication process, although the published evidence is contradictory (Schnittger *et al.* 1998, Szymanski & Marks 1998).

The *siamese* (*sim*) mutant of *Arabidopsis* produces multicellular trichomes, which often occur as clustered 'twins', dependent upon the timing of the first division (Walker *et al.* 2000). The cells of *sim* mutants contain altered quantities of DNA compared to wild-type trichome cells, indicating that endoreduplication was not proceeding as normal. All *sim* clusters were found to be clonal in origin. The molecular genetic basis of the *sim* phenotype is not yet known, but appears to fit with models indicating that the endocycle is regulated at both the G1/S and G2/M transitions (Walker *et al.* 2000, Meijer & Murray 2001). Unlike *sim, hydra* does not produce multicellular trichomes, which suggests that trichome pairs result from an incomplete co-ordination of the transition from division competence to differentiation competence in the *hydra* trichome upon adoption of cell fate.

### 6.2.1.3 *HYDRA* gene activity may participate in a MYB-based Turing mechanism of tissue pattern resolution in the *Arabidopsis* epidermis

The *HYD1* promoter contains a number of MYB recognition sequences, implying that transcriptional activity seen in these cell types is at least in part a result of MYB-stimulated activity. Activity of p*HYD1*::GUS is associated with stomata and trichomes from early in their differentiation, and root atrichoblasts and trichoblasts in a distinct temporal sequence. Both trichome and atrichoblast cell fates are dependent upon activation of the *GL2* gene (Massucci *et al.* 1996, Ohashi *et al.* 2002), suggesting a common pathway for differentiation which temporally targets these epidermal cells first as a 'cassette'. This activation is dependent upon two MYB proteins; WEREWOLF in roots, and GLABRA1 in the leaf (Lee & Schiefelbein 2001). The *WER* gene is transcribed first in root atrichoblasts,

actively defining these cell files from the torpedo stage of embryogenesis (Lee & Schiefelbein 1999), and *GL1* transcription has been noted in stipules as well as trichomes (Oppenheimer *et al.* 1991). These locations correlate neatly with sites of p*HYD1*::GUS early transcription in both shoots and root apices, and also define cells with patterning difficulties in the mutant phenotype.

Sequential symplastic isolation during progressive stages of differentiation in plant cells allows the developmental control of cell fate and function; this permits groups of cells (e.g. phloem) to fulfill specific functions in the plant body (Oparka *et al.* 1999, Pickard & Beachy 1999). In the root, trichoblast cells show full symplastic isolation upon entering the expansion phase and initiating root hair tip growth (Duckett *et al.* 1994). In the shoot, guard cells and surrounding epidermal cells are symplastically coupled until the guard cells become isolated at maturity (Palevitz & Hepler 1985). Trichome and atrichoblast cells are patterned by the same 'cassette' of MYB transcription factors, under the master control of the exchangeable WER and GL1 proteins (Lee & Schiefelbein 2001), and activating the common relay of *GL2* expression upon commitment to cell fate. This would suggest that trichomes are also symplastically isolated at this differentiation stage. In roots, the expression of WER in atrichoblasts appears first in the torpedo stage embryo (Lee & Schiefelbein 1997). *GL2* is similarly active during embryogenesis in epidermal cell files before emergence from the root meristem (Lin & Schiefelbein 2001), suggesting that this differential and active promotion of non-hair cell fate has temporal precedence over trichoblast differentiation in the same way that trichomes are developmentally ahead of stomata in the shoot.

The growing body of evidence concerning the activities of MYB transcription factors in the *Arabidopsis* epidermis adds weight to the idea of a 'positive reinforcement-lateral inhibition' process at work. This idea was first described by Turing (1952), and further developed by several authors, drawing on computer-generated modelling to offer a common mechanism for biological pattern formation processes (Green 1989, Koch & Meinhardt

1994, Dulos *et al.* 1996, Franks & Britton 2000). However the mechanism has not yet been fully elucidated.

In the *Arabidopsis* epidermis, cells which activate the 'positive reinforcement' component of the system are those of the trichome-atrichoblast cassette, targeting positive transcription of the *GL2* gene. Atrichoblasts are cytologically distinct from the adjacent trichoblast cell files at the point of exit from the root apical meristem (when the epidermis emerges form the sheath of the lateral root cap), and have vacuolated cells which are less densely stained in histological sections than the adjacent presumptive atrichoblasts (Dolan *et al.* 1993, Lee & Schiefelbein 1999).

Two possibilities arise for involvement of the *HYDRA* gene products in differential signalling.

1. As one of the downstream products of the HYD enzymes are bulk membrane sterols, it appears likely that early reporter activity in epidermal cells is indicative of protein activity for membrane production. This cytological evidence suggests that *differentiation itself* could be a component of the 'positive reinforcement' mechanism.
2. Another possibility involves the putative role of a sterol acting as a direct signal. GL2, a START domain epidermally active transcription factor (Ponting & Avarind 1999), is one candidate for a positive reinforcement agent; this gene is actively transcribed in trichomes and atrichoblasts. Positive reinforcement could involve the interaction of a sterol metabolite (modified by HYDRA protein activity) with the START domain of this protein. Alternatively the START-associated sterol could inhibit GL2 activity once the protein had reached a critical threshold level. A lack of feedback inhibition caused by an absence of the critical sterol(s) in the *hydra* mutants would then result in enhanced activity of the patterning protein.

Either or both of these mechanisms would promote cell differentiation, and would involve the activation of *HYD1* transcription by MYB transcription

factors, possibly WER or GL1. As p*HYD1*::GUS reporter activity appears in the epidermis whilst still within the root meristem (i.e. within the confines of the lateral root cap), the rapid *HYD1*-associated promotion of differentiation in atrichoblasts (and so positive reinforcement in the patterning mechanism), could be differentiated from the laterally inhibited neighbours very rapidly after derivation of the epidermal and lateral root cap cells from the division of their initial cells. This hypothesis involves a *HYD1*-mediated differentiation mechanism for individual cells within a tissue ahead of the main differentiation stage for the main body of cells. This would imply a mechanism for pattern formation involving a resolution of 'target cells', as proposed by Osbourne (1984).

### 6.2.1.4 A lack of *HYD* transcriptional response may result in overproduction of components of the epidermal patterning machinery

The *hydra* mutant phenotype demonstrates certain characteristics which are reminiscent of maize R-protein over-expression. Trichome and anthocyanin production are both enhanced in the *hydra* mutant shoot, (as shown in Fig. 4.7.4; B and F in conjunction with other epidermal patterning anomalies), and root hair production is enhanced (Topping *et al.* 1997, Souter *et al.* 2002). Anthocyanins are promoted by the same genes that promote trichome formation. These include transcription factors with a basic helix-loop-helix (bHLH) domain, which share homology with the DNA binding and protein dimerization motif of the MYC family of mammalian transcriptional regulators (Lloyd *et al.* 1994). The maize R protein contains acidic and basic helix-loop-helix (HLH) domains, and when over-expressed in A*rabidopsis* results in the production of clustered trichomes, excessive root hair and elevated levels of anthocyanins (Lloyd *et al.* 1994). Two of these genes are known in *Arabidopsis*; GL3 and EGL3, and they physically interact with other epidermal patterning components (Bernhardt *et al.* 2003).

The correct spacing and development of trichomes and stomates involves multiple patterning inputs from both MYB and MYC proteins, along with other transcriptional activation systems such as WRKY (Johnson *et al.* 2002). A WRKY transcription factor, TTG2, has been found to act downstream of the trichome initiation genes *TTG1* and *GL1*, and shares functions with *GL2* in controlling trichome outgrowth (Johnson *et al.* 2002). WRKY proteins comprise a large multi-gene family, all previously known to operate in stress response cascades (Eulgem *et al.* 2000). Johnson *et al.* (2002) describe TTG2 as the first WRKY protein to be involved in 'morphogenesis' rather than 'stress signalling', although trichome function could be implicated in stress responses such as photo-response and herbivory attack. Trichome density increases in *Quercus* species in response to elevated UVB exposure along with the cytoplasmic density of UVB absorbing flavonoid compounds (Liakura *et al.* 1997). Trichomes may also contribute to water conservation; adaxial trichome density increased in *Cassia angustifolia* under drought stress conditions (Ratnayaka & Kincaid 2005). Drought stressed *Arabidopsis* plants produce both extra trichomes and anthocyanins, suggesting that the participation of TTG2 in a stress cascade appears likely. Anthocyanins are also noted as a stress response marker; these light-quenching pigments are induced by phytochrome stimulation in individual cells in a stochastic manner (Nick *et al.* 1993). The *HYD1* promoter includes MYB and MYC binding sequences, along with several WRKY sites. It is possible that *HYD1* mediates cellular progression to differentiation in the epidermis, and in addition may be a component of negative feedback in the stress response cascade.

### 6.2.1.5 The transition from cell division to differentiation may include interaction between *HYD1* gene activity and retinoblastoma-related proteins

Retinoblastoma (Rb) proteins are known from mammalian systems as tumour suppressors, and regulate cell division, differentiation and programmed cell death in specific cell types. During the cell cycle, Rb

proteins regulate the G1/S phase transition by binding to and repressing the activity of E2F transcription factors, i.e. the Rb functions to inhibit cell division, and so allow differentiation. This Rb-mediated inhibition is prevalent in differentiated tissues, and release of this inhibition through disruption of Rb function results in tumorogenesis in animal systems (Weinberg 1995, Müller *et al.* 2001). In plants, loss of Rb activity leads to ectopic cell division in major organs which correlates with a delay in cell differentiation and increased endoreduplication, indicting that these processes are coordinated by Rb activity (Park *et al.* 2005).

An extraordinary degree of conservation has been noted in the interaction between Rbs and the E2F target proteins; even the canonical DNA sequence (TTTCCCGC) recognized by the E2F transcription factors of animals and plants is identical (de Jager *et al.* 2001). The *Arabidopsis* genome encodes six E2F genes, some activators and others repressors of the Rb target (reviewed by Inze 2005). One of these proteins, E2Ff/DEL3, has been shown to play a possible role in repressing cell wall biosynthesis during cell elongation in differentiated cells (Ramirez-Parra *et al.* 2004), whilst E2Fe/DEL1 appears to control endoreduplication (Vlieghe *et al.* 2005). Both incomplete cell wall formation and multinucleate cells have been noted in mutants of *hydra* and *smt1* (Schrick *et al.* 2004).

Silencing of the tobacco Rb gene in the tobacco shoot epidermis resulted in clustered stomata and abnormal trichome differentiation (Park *et al.* 2005); manifest as raised clusters of proliferating cells in the modified leaf epidermis, which closely resemble the raised areas seen in *hydra* mutants (e.g. Fig. 5.7.6; H). Over-expression of the *ORF13* T-DNA gene from *Agrobacterium* in tobacco plants resulted in an excess of this Rb-inhibiting protein, which produced ectopic cell division patterns throughout expanded leaf primordia and in the vicinity of the QC in the root (Stieger *et al.* 2004). The cell division activity shown in transgenic plants by these authors resembled the ectopic division events seen in *hydra* cotyledons, and in close proximity to the QC region in 7 dae root apices (Fig. 4.3.1; N, T and V). The similarity between these characteristics and the cell division

phenomena in *hydra* mutants implies a direct or indirect interaction between *HYDRA*-mediated cell differentiation and Rb activity.

## 6.2.2 A putative role for sterols in the coordination of differentiation and radial patterning

### 6.2.2.1 The *hydra* phenotype, and *HYD1* reporter activity, imply a role for sterols in the coordination of radial patterning at the shoot meristem periphery

The analysis of pattern formation in the *hydra* mutant phenotype, in the light of the positional localization of *HYD1* reporter gene expression, revealed an association with patterning around the radial axis, in a symmetrical manner in the radially symmetrical root, and asymmetrically in the definition of the stipule basal leaf domain in rosette and cauline leaves. In both shoot and root tissues, *HYD1* gene activity appears to be associated with patterning processes involving the L1 layer, forming the epidermis. Although the mutant phenotype had relatively few defects in lateral organs of the inflorescence stems, some patterning anomalies were found, concerning the definition of organ lateral boundaries. These findings imply a role for a *HYDRA*-mediated signal, possibly a sterol, which is influential in defining and maintaining this domain, necessary for coordinated transverse (centrolateral) expansion in lateral organs.

During *hydra* primordial formation, the dorsiventral signals in emerging organs around the meristem are variably positioned, and can be skewed relative to the phyllotactic origin, or reversed. Reporters of adaxial definition are over-expressed, suggesting either an enhanced function or a lack of negative feedback regulation. This balance is modulated by auxin, and the predicted improved auxin transport associated with the *ein2* mutation may modulate the expression intensity from these reporters. The *hydra* phenotype suggests a difficulty with lateral boundary definition in

primordia throughout the shoot growth axis, although the severity of this disruption is minimal in cauline leaves and inflorescence organs, suggesting a different balance of gene activities and radial cues in this phase.

Reporters of the transcription of START domain transcription factor genes *AthB8, REV* and *PHB* during *hydra* embryogenesis appeared to have a random expression pattern which was unrelated to the morphology of the embryo. In contrast, Topping & Lindsey (1997) found correct localization of reporter gene expression indicating apical-basal positioning. This suggests that the formation of the variable morphology of *hydra* mutant embryos and the establishment of radial cues around the meristem are independent processes during embryogenesis. It is unclear from these data whether radial patterning mechanisms are required to *establish* the (apical-basal) longitudinal growth axis, but they are required for its *maintenance* through the onset of cellular differentiation and expansion growth. The 'apical' definition found in *hydra* embryos is variably re-established at the onset of post-germination growth, with some mutant apices failing to differentiate a clear central meristematic zone.

### 6.2.2.2 Sterols are required for the maintenance of cell shape and polarity across the radial axis

Establishment of primordia in correct positions around the SAM requires polar auxin transport in the L1 layer (Reinhardt *et al.* 2003, 2004). Evidence in the present study from promoter-reporter constructs highlighting auxin responsive cells in *hydra,* suggest that the mutants have a problem with establishment and maintenance of routes for polar auxin transport, resulting in multiple patterning anomalies in longitudinally aligned cells across the radial axis. This suggests a role for *HYDRA* function in the maintenance of coordination between longitudinal cell files, possibly by allowing efficient sterol-mediated vesicle trafficking of PIN proteins.

Most of the cell shape anomalies seen in *hydra* mutants (in root hairs, longitudinally aligned cell files and young trichomes) involve cells with a precisely regulated internal polarity, implicating *HYDRA*-mediated sterol production in coordinated polar growth. Other more localized anomalies affected single cells without an obvious polar axis, resulting in giant epidermal cells in the midst of regions of smaller pavement cells and stomates. This de-regularization of cell size may imply a role for *HYDRA* in polar diffuse growth. Both of these phenomena are found in mutants modulated in the function of Rop GTPases (reviewed by Fu & Yang 2001). Constitutively active Rop mutants show non-polarized isotropic growth, whilst a loss of Rop function results in inhibited polar expansion. Rop function has been studied in pollen cells and root hairs, and is implicated both in polar diffuse growth and directionally-growing cells, via an actin-dependent mechanism (Fu & Yang 2001, Xu & Scheres 2005).

Root hair development requires the establishment of two separate polar sites; firstly a swelling near the apical end of the trichoblast, and secondly a site for tip growth at the apex of this swelling; sub-cellular localization of Rop proteins is seen at both swelling sites (Molendijk *et al.* 2001). Over-expression of Rop2 results in multiple swellings from the same epidermal cell, multiple sites of tip growth from each swelling giving fork-shaped hairs, and repeated initiation of tip growth from the same hair giving branched hairs (Jones *et al.* 2002). Fu & Yang (2001) also report depolarized cell expansion in hypocotyl epidermal cells in response to constitutively active Rop2, resulting in isotropically growing cells in this region usually characterized by anisotropic growth.

These characteristics of Rop2 over-expression are reminiscent of the range of hair cell morphologies seen in *hydra*, implying that Rop proteins may be over-active in *hydra* epidermal cells, and that sterols are implicated in Rop-mediated cell polarity establishment. The localization of Rop to growing tips of pollen or root hair cells is inhibited by Brefeldin A, which inhibits the activator of the ARF GTPase involved in directional vesicle trafficking (Molendijk *et al.* 2001, Geldner *et al.* 2003). This same GNOM-

dependent mechanism is required for the polar localization of the PIN1 auxin efflux carrier, and has also been implicated in the establishment of polarity in *Fucus* zygote development (Geldner *et al* 2003, Shaw & Quatrano 1996).

Sterol-dependent vesicle transport is implicated in the establishment of cell polarity in *Caenorrhabiditis elegans* (Michaux *et al.* 2000), and patterning of the *Drosophila* wing (Ingham 2001); also defective cholesterol trafficking has been shown to cause several human genetic disorders (Ioannou 2001), suggesting a highly conserved mechanism. As in animals, plant biosynthetic sterol transport occurs from the endoplasmic reticulum via the Golgi apparatus to the plasma membrane; however in contrast to animals, this process in plants has been shown to be actin-dependent (Grebe *et al.* 2003). The *hydra* mutation may interfere with this process by modifying the availability of sterols for the production of early endosomes, resulting in a modification of vesicle trafficking and a variable cell polarity.

However this process is at least partly functional in *hydra* mutants, as their phenotype does not mimic that of *gnom; hydra* mutants are able to establish a growth axis in alignment with the suspensor-embryo proper longitudinal axis. As PIN protein positioning has been noted to localize variably correctly and incorrectly in both *hydra* and *smt1*$^{orc}$ (Souter *et al.* 2002, Willeemsen *et al.* 2003), this indicates a similarly variable efficiency of vesicle transport in the three distinct modified sterol environments of these mutants. Although PIN1 trafficking has been shown to be actin-dependent (Geldner *et al.* 2001), the *smt1*$^{orc}$ mutant was able to respond to the actin-disrupting drugs Cytochalasin B and Latrunculin B (Willeemsen *et al.* 2003), it appears that these cell polarity defects cannot be explained by gross defects in the actin cytoskeleton.

The *hydra* phenotype reveals a varied individual cellular polarity, and so interpretation of cell morphology and polarity has to consider the interactions of these individual cells within the cell mass. Mechanical stresses induced by ectopic cell division are likely to result in an uneven

pressure between and within radial layers, and where epidermal cells are in receipt of these stresses, then the cell's situation could dictate the available expansion axis. This would mean that the orientation of growth in hypocotyl or leaf epidermis is dependent at least in part from mechanical forces, determined by the relationship between cells in adjacent radial layers.

### 6.2.2.3 Aspects of the *hydra* mutant phenotype appear in tissues not associated with *HYDRA* gene activity, implying a requirement for sterols remote from their sites of biosynthesis

*HYD1* gene activity as shown in this study, and promoter-reporter constructs for other known sterol biosynthetic genes from Domain A of the *Arabidopsis* isoprenoid pathway (as defined by Lindsey *et al.* 2003), have specific transcriptional localization within the plant body (Diener *et al.* 2000, Willeemsen *et al.* 2003, Kim *et al.* 2005, Jang *et al.* 2000, Schrick *et al.* 2000). Considering that sterols are ubiquitous components of cell membranes throughout the plant, this within-tissue localization is curious, as activity of the entire metabolic pathway is required for the production of the diversity of sterol end products. An ubiquitous requirement for sterols throughout the plant would predict that transcription of these genes should appear in all developing tissues. Instead, the *HYDRA* genes are active peripherally, whilst *SMT* genes are expressed centrally or in association with the vascular tissues (Diener *et al.* 2000, Willeemsen *et al.* 2003). In *hydra* mutants, the mis-differentiation of vascular tissues highlights defective patterning in cell types which do not have *HYDRA* transcriptional activity in wild-type plants. This implies that *HYDRA*-mediated sterols or sterol intermediates have a role in cell differentiation which is remote from their sites of biosynthesis.

The *HYD1* gene encodes a predicted protein of 223 amino acids, with a molecular weight of 251.47 Kda. The HYD1 protein sequence has high homology to the Emopamil-binding protein of mammals, known to be

targeted within the cell to the nuclear membrane and endoplasmic reticulum (Souter *et al.* 2002, Dussossoy *et al.* 1999). Movement of a protein of this size between cells via plasmodesmata is unlikely due to its predicted membrane-targeting (Crawford & Zambryski 2001). The cell-remote activity of the gene or gene products therefore suggests a requirement for either intercellular mRNA transport, or the movement of sterol intermediates between cells. Movement of mRNA via plasmodesmata has been shown to occur for the maize transcription factor *KNOTTED1,* in a manner dependent upon the KNOTTED1 protein (Lucas *et al.* 1995). It is also possible that sterol intermediates produced in some cell types may move via these plasmodesmatal connections. These channels are highly dynamic in plant cell development, and are much more extensive in young leaves (Oparka *et al.* 1999), i.e. when patterning mechanisms are resolving cell identities within developing tissues.

Sterols or their intermediates may be produced in one part of the developing organ, and then transported to other cell types, where further processing could take place. This would result in a local modulation of sterol content in certain cells, perhaps at a critical point in developmental timing which allows the precipitation of a lateral inhibition mechanism within the tissue. This could involve interaction with sterol-receptive proteins such as the START domain transcription factor family, and makes it possible to conceive of a mechanism for the early differentiation of these cell types within a field of undifferentiated cells using sterol-mediated signalling. Considering that the definition of procambial tissue within the mesophyll cell mass is associated with expression of the putative sterol-binding START domain protein AthB8 (Baima *et al.* 1995, 2000, Scarpella *et al.* 2004), this could implicate sterol biosynthesis downstream of *HYDRA* activity as a requirement for the early stages of cell fate commitment during vascular differentiation. Later vascular coordination (i.e. of differentiating xylem vessels) also requires START domain transcription factors (Zhong *et al.* 1999).

Elongating polarized higher plant cells, such as are found in longitudinal cell files in the plant root epidermis and vasculature, differ fundamentally from all other eukaryotic cells in that their polarity is marked by the presence of a pair of non-expanding domains at the end poles of the cell surface. In this manner they superficially resemble prokaryotic bacteria (Baluska *et al.* 2003). These end-poles are enriched with actin and depleted of microtubules (Baluska *et al.* 1992, 1997, 2001a, 2001b, Inada *et al.* 2000), whilst microtubules are associated with the lateral plasma membrane (Kost & Chua 2002, Wasteneys 2002). Plasmodesmata were found to be abundant at these cellular end poles in longitudinal cell files of the maize root apex, whilst their density in the side walls was significantly lower (Juniper & Barlow 1969). These end-poles therefore mediate the transport of proteins and RNA preferentially along the root's longitudinal axis within the individual cell file (Tirlapur & König 1999).

Within longitudinally aligned files of cells, F-actin, known to mediate exocytotic vesicle movements, is localized at the end poles (Ryu *et al.* 1995, Baluska *et al* 1997, 2001b). Plant cells are also equipped with the molecular machinery for performing receptor-mediated endocytosis (Holstein 2002). This process, typically associated with signal relay between animal cells (Bernstein *et al.* 1998, Morlaes *et al.* 2000), has been observed in association with the auxin transport proteins PIN1 and AUX1 (Swarup *et al.* 2001, Steinemann *et al.* 1999, Geldner *et al.* 2001, 2003, Grebe *et al.* 2002). The semi-autonomous symplast within longitudinal cell files, and the high density of plasmodesmata at their cellular end poles, suggest that the cell files may function as conduits for relay signals. Baluska *et al.* (2003) hypothesize that the actin-dependent exocytosis at the end poles is effectively balanced by endocytotic events, and comprise a mechanism which might be considered as a form of 'plant synapse', involved in rapid signal relay.

A common characteristic of the *hydra* mutants and other known Domain A sterol biosynthetic mutants in *Arabidopsis* (Diener *et al.* 2000, Willeemsen *et al.* 2003, Kim *et al.* 2005, Jang *et al.* 2000, Schrick *et al.* 2000) is their

compromised polar organization and coordinated elongation of their longitudinal cell files. These mutants also exhibit variable mis-positioning of PIN proteins in their polarly-organized cells (Souter *et al.* 2002, Willeemsen *et al.* 2003). This suggests that sterols could have a critical role in a signal relay involving sterol-enriched vesicle-mediated transmission. In addition, this movement of vesicles would transfer specific sterol-enriched membrane fractions between cells. Alternatively, sterols themselves, in sterol-enriched vesicles, could effect a signal relay.

A sterol-mediated signal relay in plants would require a sterol receptor to facilitate endocytosis. In animals, sterol binding proteins are known which not only regulate the concentrations of general available steroids and their metabolic clearance rates, but also function in steroid signal transduction through interacting with specific receptors on the plasma membrane of target cells and regulating cell growth and differentiation (Rosner *et al.* 1999, Breuner & Orchinik 2002). Yang *et al.* (2005) have identified an *Arabidopsis* membrane-anchored sterol binding protein, MSBP1, which binds various sterol molecules including stigmasterol and the brassinosteroid 24-epibrassinolide with different affinities *in vitro*. The *MSBP1* gene, one of four *Arabidopsis* genes encoding a putative sterol binding protein, has a role in the negative regulation of hypocotyl elongation in phototropic growth (Yang *et al.* 2005). Gene homologues of sterol binding proteins are also present in rice (Yang *et al.* 2005). These authors found that altered expression levels of *MSBP1* affected the regulation of other developmental and morphogenesis genes along with a total of 18 genes sterol metabolism including components of the isoprenoid biosynthetic pathway. It therefore appears that the required components for a sterol-mediated signalling mechanism are present in the higher plants.

Around the shoot meristem, expanding primordia have longitudinally-organized epidermal (L1 layer derived) cell files which are retained at the margins throughout leaf development; these cells therefore form a 'loop' of cell files in proximity to the stipules at the petiole base, between which the expanding lamina epidermis is patterned by non-longitudinal cells.

Strong activity of p*HYD1*::GUS in stipules suggests that these structures, comprised of highly metabolically active cells, are sites of HYDRA-mediated sterol production along with other signalling molecules such as auxin.

Auxin transport between plant cells effects pattern definition at the tissue level, and is known to be involved in both correct primordial positioning, and the coordination of primordial expansion in association with the invasion of a coherent procambial strand (Reinhardt *et al.* 2003, 2004, Scarpella *et al.* 2004). Development of the leaf vascular network is then dependent upon auxin transport between cells in a manner which is coordinated at the tissue level. Auxin is trafficked within longitudinally-aligned cells in association with sterol-enriched endosomes, is enriched in the L1 layer, and is transported towards the apices of cotyledon primordia in the embryo proto-epidermis using a vesicle-associated PIN1-mediated mechanism (Reinhardt *et al.* 2003, 2004; Friml *et al.* 2002, 2003). Strong symplastic connections within the longitudinally aligned marginal epidermal cells could therefore result in acropetal vesicle transport of both auxin and HYD1-mediated sterols or sterol intermediates from the stipules into the marginal cell files, enriching these cells with both sterols and auxin.

Auxin and sterol-enriched marginal cells could provide a target which directs the differentiation of pre-provascular cell files, known to advance in the direction of an auxin source (Sachs 1981, 1991). Where the vascular network contacts a leaf marginal region, hydathodes are found. These sub-regions of the vascular network have a cellular patterning which diverges form the coherent vascular form. Hydathode-associated 'noise' in both phloem and xylem differentiation may result from a high tissue-level of auxin, caused by close proximity to the margin 'source', which results locally in an indistinct auxin gradient in the presence of a high auxin threshold. These conditions could explain the formation of these indistinct 'nodes' within the vascular pattern. Basipetal auxin transport from the hydathode regions into the procambial tissues could then result in the establishment of a route for sterol intermediates to be relayed from the margins into the vascular tissues. This model therefore proposes a possible

route for sterol movement within the developing leaf organ which could translocate metabolic intermediates between diverse cellular locations.

### 6.2.2.4 Towards an integrated model of the *hydra* mutant phenotype

Auxin can be considered as a morphogen, defining pattern within tissues via the generation of a spatial concentration gradient between a source and a target (Jones 1998, Friml *et al.* 2002). Recent analyses of data from animal studies, on the possible mechanisms by which morphogens form spatial concentration gradients while moving away from a restricted source of production, support the idea that movement of morphogens could also occur via vesicle trafficking between cells (reviewed by Entchev and Gonzalez-Gaitan 2002). This implies an innate requirement for sterol production at or near the sites of morphogen synthesis, in order to allow correct movement of the signalling compound(s), establishment of a morphogen gradient, and subsequent pattern definition. Other patterning models, such as the 'positive reinforcement-lateral inhibition' MYB-based signalling cascade that defines the trichome patterning mechanism also rely on a protein signal production and relay from a source (the trichome cell) over a gradient of decreasing signal concentration (Schellmann *et al.* 2002), and may require a sterol-enriched vesicle-associated intercellular transport mechanism.

A sterol-dependent vesicle-mediated signal relay model, in conjunction with the current observed variations in transcriptional behavior of the *HYDRA1* gene throughout development, offers a means of integrating the many diverse aspects of the *hydra* mutant phenotype. This model proposes *HYDRA* transcription in response to phytohormone and MYB-associated cues, resulting in the production of sterols or sterol intermediates which are associated with vesicle-mediated transport (of auxin and other morphogens) to remote tissues. Here, in tandem with auxin and other morphogen-associated patterning events, these sterols either act directly,

or are further metabolized to produce active sterol compounds which combine with target proteins including certain of the START domain transcription factor family.

1. Axis definition and radial patterning during embryogenesis
Sterol-enriched PIN7-mediated vesicular auxin transport from the basal cell into the apical cell of the divided zygote (Friml *et al.* 2003), possibly utilizing auxin from maternal tissues. At the point where *HYDRA* gene transcription begins, in the globular stage, the embryo proper appears to become independent of the maternal tissues in terms of its auxin and sterol supply, as suggested by the onset of pattern definition problems in the *hydra* phenotype (Topping *et al.* 1997). Vesicle-dependent auxin flux establishes a longitudinal axis (Friml *et al. 2003,* Geldner *et al.* 2003), and then a low auxin threshold at the presumptive SAM position stimulates auxin production which activates the multiple signalling cascades that pattern the radial axis.

In *hydra* mutants, an absence of correct sterols, or the presence of an inappropriate cocktail of sterol intermediates, could disrupt vesicle-mediated auxin transport away from the embryonic apex, and possibly subsequent auxin production in the presumptive SAM. This could result in uneven and erratic auxin distribution within the embryo, causing a range of pattern definition problems emanating from the SAM region. Across the shoot radial axis, this would affect the placement of primordial marginal boundaries and the number of initiating cotyledon primordia. Misplaced cotyledon primordia would exacerbate anomalies in auxin distribution around the presumptive SAM. Uneven radial auxin transport could then affect patterning of the longitudinal axis, resulting in the observed range of *hydra* mutant seedling morphologies; these morphologies range from mild degrees of strand dissociation with little or slight divergence in the upper hypocotyl, through a spectrum of stele widening and vascular dissociation extending towards the root, culminating in complete axis duplication in a subset of the sibling population.

2. Phyllotaxy

Formation of the stipules of the first true leaf pair takes place during late embryogenesis, and may rely on correct radial placement of the marginal expression domains of the *WOX5/PRS* transcription factor (Haecker *et al.* 2004). Subsequent formation of stipules in post-germination true leaves requires correct HYDRA function. Auxin-responsive reporters such as p*IAA2*::GUS and DR5::GUS highlight stipules (Sabatini *et al.* 1999, Luschnig *et al.* 1998). These structures may be biosynthetic sites for both auxin and HYDRA-mediated sterol production. As the pHYD1::GUS reporter is active in stipules prior to these reporters, sterols may even be required ahead of the onset of auxin biosynthesis. An inability to transport auxin in *hydra* due to an absence of the correct vesicular sterol components, could inactivate the auxin signalling mechanism and prevent the stipules acing as auxin source tissues. Maintenance of a correct phyllotaxy appears to be dependent upon HYDRA-mediated sterols, perhaps via a 'positive feedback-lateral inhibition' mechanism which defines the stipule position and marginal domains in the developing primordium, and supplies sterols in conjunction with auxin to the L1 layer of the SAM via vesicle-mediated relays.

Cotyledons undergo mostly expansion-associated growth in post-germination *Arabidopsis* seedlings (Tsukaya *et al.* 1994), their procambial patterning having been defined during embryogenesis (Baima *et al.* 1995). However the onset of their vascular differentiation occurs developmentally at the same point as the p*HYD1*::GUS stipule signal appears at the meristem margins, around 4 dae. Both xylem and phloem traces have problems with strand coherence in *hydra*, suggesting that their coordinated differentiation is dependent upon a sterol signal originating in the stipules. Final differentiation of the xylem vessels which join the traces in the cotyledons and hypocotyl across the transition domain beneath the SAM takes place around 7 dae (Busse & Evert 1999b), at which point the basipetal transport of auxin from the seedling shoot towards the root has become established. Perhaps this final stage of strand differentiation marks the capacity to create a sufficient auxin 'low' in the hypocotyl-cotyledon transition to facilitate auxin transport out of the cotyledons and

into the hypocotyl stele, so promoting both the coordination of xylem traces between these structures, and allowing an auxin flux around the meristem margins to initiate the phyllotactic patterning mechanism.

Variable degrees of radial axis coordination between *hydra* siblings in the upper hypocotyl-cotyledon transition, through a modulation of the sterol environment, according to this model would inhibit auxin transport, and result in a local peaks of auxin in the L1 layer which would precipitate a highly variable phyllotaxy. As the misplaced markers of radial pattern definition in *hydra* mutants suggest, it appears likely that sterol distribution via vesicle-mediated transport (in tandem with auxin movement) affects both the placement of primordia, and the correct orientation of their marginal (stipular) domains. This provides a route implicating sterol interaction with START domain proteins and possibly other unknown sterol-receptive agents.

3. shoot vascular development

The invading procambial strand extends from the established cotyledon traces, and enters the primordium through mesophyll cells situated between the stipules; this may mean that either the high auxin concentration resulting from the stipules is balanced locally, preventing the perception of a lateral gradient as the procambial strand proceeds into the expanding primordium, or symplastic isolation of the stipule-associated L1 layer, and active vesicle-mediated transport, keeps the auxin gradient stronger in the direction of the leaf margin. Development of a marginal hydathode provides a route for auxin and sterols away from the marginal cells and into the procambial strand, precipitating differentiation, perhaps via subsequent processing of the sterol intermediates.

As the leaf expands, auxin transported acropetally in the marginal cells and basipetally in the vascular strands would keep auxin levels low towards the leaf apex, but would build up in the more basal marginal cells ahead of higher order vascular differentiation in these regions. This would promote later differentiation of secondary vascular loops and associated hydathodal

nodes as the lamina expands. Phase-related modulation of transcription of the *HYDRA* genes through the juvenile-adult transition in rosette leaf development could alter the rates of vesicle traffic and hence the local intensity of auxin gradients within the leaf, so modifying the complexity of the vascular network. As the primordium expands, sterol metabolites move from the stipules into the vascular tissues, where they are substrates for further metabolism by other sterol biosynthetic enzymes such as SMT2. Disruption of the sterol supply to differentiating vascular tissues in *hydra* mutants prevents the coordinated differentiation of these cells, and the subsequent pattern formation processes (such as START-domain radial signalling relays) which use these cells as reference points.

4. Epidermal patterning

The association of p*HYD1*::GUS activity with differentiating functional epidermal cells, and the disruption of pattern definition in these cells in the *hydra* mutants, implies a role for sterols in epidermal patterning.

Pattern definition in shoot lateral organs is modified across the radial axis, as shown by the phase-specific asymmetric distribution of trichomes between laminar surfaces of the Arabidopsis rosette. Tissue pattern in lateral organs has been shown to be coordinated at the whole-organ level (Schnittger *et al.* 1998, Bean *et al.* 2002). Within this pattern, trichomes invariably differentiate ahead of stomata in the adaxial epidermis (Glover 2000), suggesting that proximity to the meristem may influence the switch between these fates in expanding *Arabidopsis* true leaf primordia. Trichomes are limited to the adaxial surfaces of juvenile rosette leaves (Hulskamp & Schnittger 1997), implying that the mechanisms of radial pattern definition affecting primordial growth around the meristem periphery (e.g. the adaxial-promoting START domain transcription factors REV, PHB and AthB8) may also influence cell fate decisions in the epidermis. START domain protein distribution, which is both strongest in the young leaf domain region closest to the meristem periphery, and is adaxially limited due to miRNA-targeted mRNA degradation in the abaxial domain of developing primordia, thus provides a putative adaxial sterol-

interacting target which could influence cell fate decisions. If such sterol interactions are disrupted in *hydra,* subsequent placement of cues would be mis-timed during development. This could explain the appearance of trichomes in meristem-proximal sections of *hydra* cotyledons.

If vesicle-mediated transport between cells is a common mechanism of intercellular communication, and not specifically associated with auxin movement, then the modification of the sterol environment in *hydra* mutants would also affect other signalling systems. The positive reinforcement-lateral inhibition hypothesis, which allows the mathematical modelling of pattern formation within the epidermis (Koch & Meinhardt 1994, Green 1999), depends upon the movement of signalling molecules outward from a 'source', in order to produce a morphogenic signal gradient. Such a system is proposed for the activity of MYB and MYB-related transcription factor cascades in epidermal patterning, e.g. where the TRYPTICHON and CAPRICE proteins move from a source (trichomes and atrichoblasts in shoot and root tissues respectively) into adjacent cells, there inhibiting the adoption of this cell fate in adjacent cells (Schellmann *et al.* 2002). In this circumstance, vesicle-mediated movement in a non-polar fashion may be required for the resolution of cell fate adoption by one cell relative to another.

A DR5::GUS signal is also resolved in trichomes (Aloni *et al.* 2003*)*, implicating auxin in the possible precipitation of the signalling mechanism within the field of undifferentiated epidermal cells via stochastic differences between adjacent cells. These authors do not report a stomatal signal, although the auxin-upregulated p*ACS1*::GUS reporter was noted in this thesis to have transient expression in guard cells as they mature. The auxin levels in guard mother cells may be much lower than in trichomes, as they occur at a later stage of leaf development when auxin in that region of the lamina would be predicted to be much lower than during trichome pattern definition. Reaching of the auxin threshold in an epidermal cell such as a trichome/atrichoblast (or later in a guard mother

cell/trichoblast) may then facilitate vesicle-mediated transport, as is suggested by the appearance of p*HYD1*::GUS transcription in these cells.

In the laminar epidermis, plasmodesmatal connections are not directed to a particular section of the cell wall, suggesting an initial even export of auxin into all adjoining cells (including those of the mesophyll beneath the differentiating trichome or stomate). In the case of trichome precursors, the surrounding basal cells in the epidermis do subsequently appear to polarize in wild-type, forming an encircling ring of elongated cells. This mechanism was compromised in the *hydra* siblings examined in the present study. The polarization mechanism does not proceed further within the wild-type epidermis, perhaps because auxin production in the single trichome cell is insufficient to produce an effective gradient of auxin beyond the adjacent cells in the L1 layer. This encircling polarization of cells does not occur for trichomes formed in the marginal cell files at the hydathode points (i.e. marginal 'teeth'), as here the directional movement of auxin is away from the trichome cell into the tissue layers 'beneath', i.e. towards the hydathode and vascular region.

This model would predict that in *hydra*, where vesicle-mediated transport is compromised, intercellular communication in the expanding lamina would proceed unevenly, resulting in disruption of the pattern within the field of cells, and variably paired or clustered trichomes and stomata. In the hypocotyl epidermis, clustered stomata do differentiate in *hydra* within the (disrupted) longitudinal pattern of cell files. This phenomenon is more extreme in the upper hypocotyl, in which the post-germination 'wave' of acropetal expansion occurs later than in the lower hypocotyl (Gendreau *et al.* 1997). Disruption is not as pronounced in the *hydra* root epidermis, perhaps because intercellular transport is strongly directional in the semi-autonomous longitudinal cell files of the root.

5. Root development

The root apex, undergoing unidirectional polar growth, has a well-characterized auxin circulation involving vesicle associated PIN protein

trafficking from inner vascular tissues through the root cap in distal and lateral directions, and then away from the apex in the epidermal layer (Friml 2002a, b, 2003, Geldner *et al.* 2001, 2003). Subtle shifts in the balance of this transport effect a bending of the root in the elongation zone (Rashotte *et al.* 2000), i.e. the cells of the epidermal files which would be in immediate receipt of basipetally-transported auxin arriving from the differentiation zone, where p*HYD1*::GUS transcription was at its maximum. A compromised availability of sterols for vesicle-mediated transport would disrupt both the expansion growth ability of the root and this gravity response, as seen in *hydra* mutants (Souter *et al.* 2002). The direction of PIN-mediated polarity in these cells is also disrupted in *hydra* (Souter *et al.* 2002), as are the epidermal cell files themselves.

Cortical cell proliferation can result from phosphate starvation (Zhang *et al.* 2003), and compromised auxin transport (Luschnig *et al.* 1998). The *hydra* mutants have excessive proliferation of cells in the cortex layer, mimicking this physiological response. The partial rescue of *hydra* root growth by *exo* (Farrar *et al.* 2003) suggests that a phosphate response may be implicated in this phenomenon. Although it has not yet been shown experimentally whether *hydra* mutants are unable to perceive phosphate in their environment, it is conceivable that the cortex layer, possibly the 'phosphate sensor', is in receipt of a phosphate-inhibited signal from the epidermis which limits cell division. In the absence of this signal, under phosphate starvation, a mechanism is triggered causing additional cell proliferation in the cortex increases the number of anticlinal cortical cell walls perceived by the epidermis, resulting in more epidermal cells adopting trichoblast cell fate, and thereby creating a greater absorptive surface area for nutrient uptake. As *hydra* mutants are grown on phosphate-enriched media, this would imply that the signal itself is not being transmitted to the cortical layer by the epidermis, due to an absence or correct sterol availability, or perhaps a reduced auxin transport capacity. Once more, a compromised vesicle-mediated intercellular communication can be proposed to account for the resultant mutant phenotype.

## 6.3 Future work

### 6.3.1 Further experimental investigation of *HYDRA* gene activity

#### 6.3.1.1 The link between differentiation and *HYDRA* gene activity

*HYD1* gene expression appears at the time when vacuolation becomes visible in the cell cytoplasm. Future work could address the nature of the change at this time point by correlating the expression signal with changes in the sterol composition in these tissues, perhaps by using synchronized cell cultures at the division-differentiation transition, and comparing HYD1 mRNA levels with the cellular sterol profile. Alternatively, controlled transgenic activation of the wild-type *HYDRA* genes in a null mutant background could help to demonstrate the requirements for *HYDRA* gene activity at different developmental stages, and the resulting morphological consequences.

The transcription factor *REV/IFL1* has been implicated as a factor controlling the balance between cell division and differentiation (Zhong & Ye 1999, Ratcliffe *et al.* 2000). The *REV*-mediated system of adaxial pattern definition may not function in *hydra* mutants (suggested by p*REV*::GUS reporter mis-localization) because of the alteration of the internal sterol environment; this means that in addition to supplying membrane components for vacuolation, there could also be a sterol signaling molecule interacting with REV via the START domain, which functions as a positive regulator of differentiation. As REV is associated with the adaxial domain of developing leaves, this suggests the possibility of a tissue level of resolution for differentiation, possibly involving a tissue-specific requirement for sterol production. It would be possible to modulate the sterol composition of certain cells tissues in the plant body by cell- or tissue-specific transcription of components sterol biosynthesis. The very different distribution of p*HYD1*::GUS transcription and that of *SMT*

reporter genes suggest that this may be the case. This also implies that certain products of sterol biosynthetic enzymes produced in some tissues may be further modified in other, remote tissues. Such an effect is suggested by the vascular phenotype of *hydra* mutants, even though the transcription of *HYD1* was not seen in vascular strands or their precursors. These tissue-specific effects could be investigated by a transgenic tissue-specific direct manipulation of the relative abundance of these genes in a controlled system.

### 6.3.1.2 *HYD1* as a potential target of MYB-mediated cell definition

The *HYD1* gene may participate in a positive reinforcement-lateral inhibition Turing-style patterning mechanism in the epidermis. The proposed mechanism involves promotion of early differentiation in targeted cells in response to transcription by MYB proteins involved in the definition of epidermal patterning.

In the absence of the 'master' genes *WER* and *GL1*, and the subsequent downstream MYB proteins of the cascade, the early promotion of *HYD1* and would be predicted to be reduced, or present at a low level without signs of differentiation between adjacent cell files in the root epidermis. To investigate this further, it is necessary to identify the putative MYB protein which activates *HYD1* transcription. One way of achieving this is to introduce the *HYD1* reporter into phenotypic backgrounds of null mutants for the currently known MYB genes, i.e. *wer, cpc, try, gl1* and *flp*. Another option, necessary in the case of *HYD1* activation through multiple MYB proteins, would be to remove activity of these genes in combination, using an RNAi transgene cassette or by using multiply (double, triple, quadruple) mutant backgrounds. Candidates for transcriptional activation of *HYD1* or *HYD2* could then be investigated further by using a system of artificially induced MYB expression, e.g. via an alcohol inducible transgenic system), accompanied by a time-course for RNA induction.

### 6.3.1.3 Deregulation of the control over cell size in the *hydra* epidermis

The factors which result in the cessation of extension growth in epidermal cells appear not to operate in the *hydra* mutant epidermis. Cell size is related to its DNA content throughout the angiosperms (Jovtchev *et al.* 2006). Guard cells do not undergo endoreduplication, unlike other cells in the *Arabidopsis* epidermis. The appearance in the *hydra* epidermis of giant stomates, and occasional stomata appearing to comprise several fused guard cells, implies that these cells are either the products of partial divisions in already differentiated cells, or result from a continuation of endoreduplication after differentiation. As both *hydra* mutants have such variable cell sizes throughout the epidermis, it is possible that at least a subset of the guard cell population may endoreduplicate in these mutants. This could be ascertained by examination of the nuclear content in these anomalously sized cells, via DAPI staining and examination under fluorescence.

### 6.3.1.4 The non-cell autonomous activity of sterols across the radial axis

A role has been proposed for a *HYD1*-mediated sterol signal which resolves 'outer' from 'inner' in the globular embryo and at later stages of differentiation. The *HYD1* reporter appeared to be active throughout the embryo, whereas the *HYD2/FK* mRNA signal, though similar in the globular embryo showed a later displacement into the root region (where it was present throughout the radial axis), and in the cotyledons in the maturation stages (Schrick *et al.* 2000). A closer examination of the *HYD1* positional distribution in the early establishment of pattern, in conjunction with *HYD2*, may show that either the *HYD1* domain is similarly resolved, or that there are distinctions between the expression profiles of the two genes. As a detailed analysis of the p*FK*::GUS reporter as presented in this thesis for p*HYD1*::GUS has not been conducted by either Jang *et al.* (2000) or He *et*

*al.* (2003), a more detailed comparison of expression profiles throughout development would help to clarify both the extent of overlap of these domains, and provide indicators for a more refined examination of the mutant phenotypes. As recent research reveals an increasing emphasis on post-transcriptional modulation of gene activity (e.g. through small RNAs), a comparison of GFP-tagged HYD proteins would establish a more accurate portrayal of the sites of sterol-mediated activity in the multiple patterning processes which define plant form.

### 6.3.1.5 Sterols as participants in the maintenance of shoot radial layer integrity

The *HYD1* reporter and the *hydra* phenotype implicate a role for these genes in the maintenance of layer integrity in the ground tissues of the shoot. Behaviour of the SCR-GFP reporter in mutant roots implied that the position of the endodermis requires an 'outer' signal boundary as well as the inner signal originating from the pericycle to define the single layer. This definition may be the result of two interacting signals which diffuse through the radial layers and form a boundary at a particular ratio of the two 'morphogens'. The *hyd* mutants showed a compromised expression of SCR-GFP in shoot tissues, implying either that the boundary signals are not in operation in this region, or that the cell integrity in *hydra* is insufficient to support the metabolic cost of GFP production. A comparison of the positioning of a GUS-derived SCR reporter or an *in situ* mRNA signal in tissue sections from mutants with a range of longitudinal axis definition problems would clarify this matter.

### 6.3.1.6 Potential disruption to sterol-rich lipid rafts by mutations at the *hydra* loci

Functional proteins such as Rops and the PIN-mediated auxin efflux machinery may have a variable polar localization in *hydra* mutants, as a result of altered membrane sterols. Also the transcriptional activation of

*HYD1* in response to diverse phytohormone signals requires clarification. This could be achieved by assessment of the transcriptional response of *HYD1* to diverse phytohormone signalling cues by RNA induction over a controlled time course. If a MYB-mediated signal is found to induce *HYD1* gene expression, this cue could then be used as a means of assessing the changes in cell sterol profile, particularly regarding the composition of lipids and sterols in detergent-resistant fractions.

## 6.3.2 Towards a new framework for the evaluation of developmental mutants

### 6.3.2.1 Pattern phenomena at the *hydra* mutant shoot apex are not explained by current ideas of pattern definition around the SAM

The variability in establishment of a central meristematic region in post-germination *hydra* siblings raises a question over the nature of axis definition at the shoot apical meristem. The *hydra* mutants' displaced radial cues are associated with a phenotype which is compromised in longitudinal expansion growth. Auxin, required for vascular definition and primordial placement at the SAM, has been reinterpreted by Reinhardt *et al.* (2000, 2003, 2004) as an agent of radial pattern definition at the meristem. Their data revealed that the laser-ablation of primordial at the periphery resulted in a shift of the central growth axis, suggesting that the placement of this central domain is itself a component of the radial axis.

The expression of certain patterning genes, particularly the *WUS-CLV3-CLV1* feedback loop, have been typically interpreted as an apical-basal phenomenon. This is because *WUS* transcripts appear first in the inner cell layer in the apical half of the globular embryo (Laux *et al.* 1996, Mayer *et al.* 1998), i.e. just after the radial distinction between cell layers has been established by *AtML1*. The transcription factor *STM* is then expressed across the apex, encompassing the *WUS* domain and extending into the L1

(proto-epidermal) layer (Long & Barton 1998). The activity of the *CLV* genes commences after *STM* activity has established this central (non-primordial) position, resulting in a feedback loop which is above and overlapping with *WUS* gene activity (Laux *et al.* 1996, Schoof *et al.* 2000, Brand *et al.* 2000).

In *hydra, STM* RNA appears at random locations around the apex, or at multiple positions (Schrick *et al.* 2000). If the positioning of patterning genes such as *WUS* and *STM* are interpreted as an outer-to-inner signal, rather than apical-basal, then it is possible to conceive of a single patterning mechanism in operation in a dome-shaped tissue (the SAM) where stochastic factors define the initial point of *WUS* and subsequently *STM* activity. Considering the tight anatomical conservation of the tunica-corpus arrangement of the SAM, this would imply that the L1-L3 layers are a radial 'plate', domed over an apex which is possibly shaped by physical constraints within the cell mass. Additional patterning factors (possibly the CLV feedback loop) act to constrain cell division at a defined point (i.e. nominally central) to stop proliferation of the outer layers in this region. This would make the conception of the shoot and root meristem much more analogous than the current model; there the CLV feedback is not implemented in the same way, so the domed radial plate produces initials evenly over its surface, allowing the production of a root cap tissue. Auxin transport then modifies this arrangement, and hence cell identity, over both apices.

This conceptual approach permits the integration of multiple patterning processes, so far considered as functioning independently. This framework offers a route to conceiving auxin-mediated patterning as a single process, integrating the coordination of longitudinally aligned cell files and their associated expansion growth with radial pattern definition and primordial initiation, i.e. the differentiation of vascular tissues and the positioning of primordia both derive from a common mechanism. Other factors then modify this process, possibly sterols. If PIN-mediated auxin transport requires a specific sterol supply for vesicle trafficking, then *HYD1*-mediated

sterol products manufactured in the stipules and released into the meristem L1 layer could diffuse laterally and make auxin transport more active in the vicinity of established primordia. Thus a sterol distribution in conjunction with an auxin signal could be the means of 'lateral inhibition', proposed by Snow & snow (1931) which defines the position of subsequent primordia. The asymmetry of these signals which over time resolve the phyllotactic spiral around the developing SAM are not in operation at the root apex. Instead, a balanced activity of *HYD1* around the root meristem and radial axis (in the root cap and epidermis) supply sterols to these cells evenly, therefore facilitating the vesicle-mediated basipetal polar auxin transport which occurs in these (L1 equivalent) cells.

6.3.2.2 The requirement for a re-evaluation of current models of plant development

This thesis examined the *hydra* mutants with particular reference to pattern definition over the radial axis. Whist this approach has yielded new and useful information in interpretation of the phenotype, a satisfactory definition of the developmental problem caused by these mutations remains elusive. The consideration of *hydra* as a radial patterning problem is possible if a number of characteristics conventionally attributed to apical-basal patterning are considered as components of radial patterning. This suggests an inadequacy in the current framework for the assessment of developmental mutants such as *hydra*.

When examining the body organization of plants beyond the dicotyledonous taxa, the definition of apical-basal and radial axes becomes complex, as many plant groups do not oblige with a symmetrical growth form, either in embryogenesis or in post-germination growth. Whilst there are certainly growth axes in plants, these are developmentally plastic phenomena, determined by complex cues from the internal and external environment, and their modulation allows survival of the individual in a changing habitat. Patterning processes at the apices, considered as functioning in a radial

and apical-basal axis are inappropriate for processes which are cohesive, and therefore not directionally defined. The apical-basal axis of a plant is modulated by internal and external cues, as shown by Reinhardt *et al.* (2003, 2004). Also in embryogenesis, cues which establish longitudinal patterning appear more reliant on cues from the embryo's immediate environment than on the establishment of an innate axis as is found in animal development.

The idea of a distinct 'apical-basal' and 'radial' axis in plant pattern definition may now be an outmoded model, to be assigned to the list of ideas from animal development which are misconceptions when applied to plants (such as the apical, central and basal domains of Mayer *et al.* 1991, by which *hyd2/fk* was originally defined as a central domain deletion). The purpose of a model is as a framework for thinking, and the life of any such model is finite. Models are useful, and necessary, until thinking progresses to a stage where a new model is required. The difficulties experienced in the satisfactory definition of defects in *hydra* and other developmental mutants imply that plant pattern formation is not so much "A concept in search for a molecular mechanism" (Mayer & Jürgens 1998), but a mechanism in need of a new conceptual framework.

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## Appendix 6: Rates of leaf initiation in Ws, *ein2*, *hydra* and *hydra-ein2* double mutants.

NB; this data was obtained by visual light microscope examination of fresh material, selected at 4 dae, transplanted onto vertical plates and scored for visible expanded leaf primordia at 12 dae, by visual examination using a compound dissecting microscope with top illumination.

| Number of true leaves | Ws<br>n = 20<br>mean = 5.10 | *hyd1*<br>n= 68<br>mean = 9.35 | *hyd2*<br>n=61<br>mean = 7.67 | *ein2*<br>n=20<br>mean = 6.25 | *hyd1-ein2*<br>n = 89<br>mean = 10.19 | *hyd2-ein2*<br>n = 68<br>mean = 7.72 |
|---|---|---|---|---|---|---|
| 5 | 18 | | 6 | 2 | | 7 |
| 6 | 2 | 2 | 10 | 11 | 4 | 15 |
| 7 | | 6 | 16 | 7 | 12 | 13 |
| 8 | | 10 | 11 | | 11 | 16 |
| 9 | | 15 | 9 | | 14 | 5 |
| 10 | | 13 | 5 | | 10 | 4 |
| 11 | | 5 | 1 | | 9 | 3 |
| 12 | | 13 | 3 | | 15 | 3 |
| 13 | | 2 | | | 4 | 1 |
| 14 | | | | | 5 | 1 |
| 15 | | | | | 1 | |
| 16 | | | | | 1 | |
| 17 | | | | | 1 | |
| 18 | | | | | 2 | |

# Appendices

# Appendix 1: Plant Growth Media

## Murashige and Skoog basal medium (Murashige & Skoog, 1962)

| Component | mg/litre |
|---|---|
| Ammonium nitrate | 1650.0 |
| Boric acid | 6.2 |
| Calcium chloride (anhydrous) | 332.2 |
| Cobalt chloride.$6H_2O$ | 0.025 |
| Cupric sulphate.$5H_2O$ | 0.025 |
| EDTA (disodium) | 37.26 |
| Ferrous sulphate.$7H_2O$ | 27.8 |
| Magnesium sulphate | 180.7 |
| Manganese sulphate.$H_2O$ | 16.9 |
| Molybdic acid (sodium salt).$2H_2O$ | 0.25 |
| Potassium iodide | 0.83 |
| Potassium nitrate | 1900.0 |
| Potassium phosphate monobasic | 170.0 |
| Zinc sulphate.$7H_2O$ | 8.6 |
| Glycine (free base) | 2.0 |
| Myo-inositol | 100.0 |
| Nicotinic acid (free acid) | 0.5 |
| Pyridoxine-HCL | 0.5 |
| Thiamine-HCL | 0.1 |

## $^1/_2$ $MS_{10}$ media

The seed germination medium $^1/_2MS_{10}$ was prepared by dissolving MS basal medium (half concentration; 2.2g/l) and 1% sucrose in distilled water, adjusting the pH to 5.65 using 0.5M KOH, measured using a newly-calibrated pH meter (Jenway 3020). The medium was solidified with 0.8% w/v Bacto-Agar (Difco, UK) and sterilised by autoclaving at 120°C for 20 minutes.

# Appendix 2: Primers

Primer sequences used to clone differential lengths of the *HYDRA1* promoter from *Arabidopsis thaliana* ecotype Wassileskia, are shown below. Their positional locations are annotated onto the HYD1 promoter sequence, shown in Figure 3.1.

## Reverse

CGG**GGATCC**CTCCTTTCTCTGTGTGT
(BamHI)

## Forward; -834bp

CCCAAGCTTCACATGTGCCTCTCGTGC
(Hind III)

## Forward; -482bp

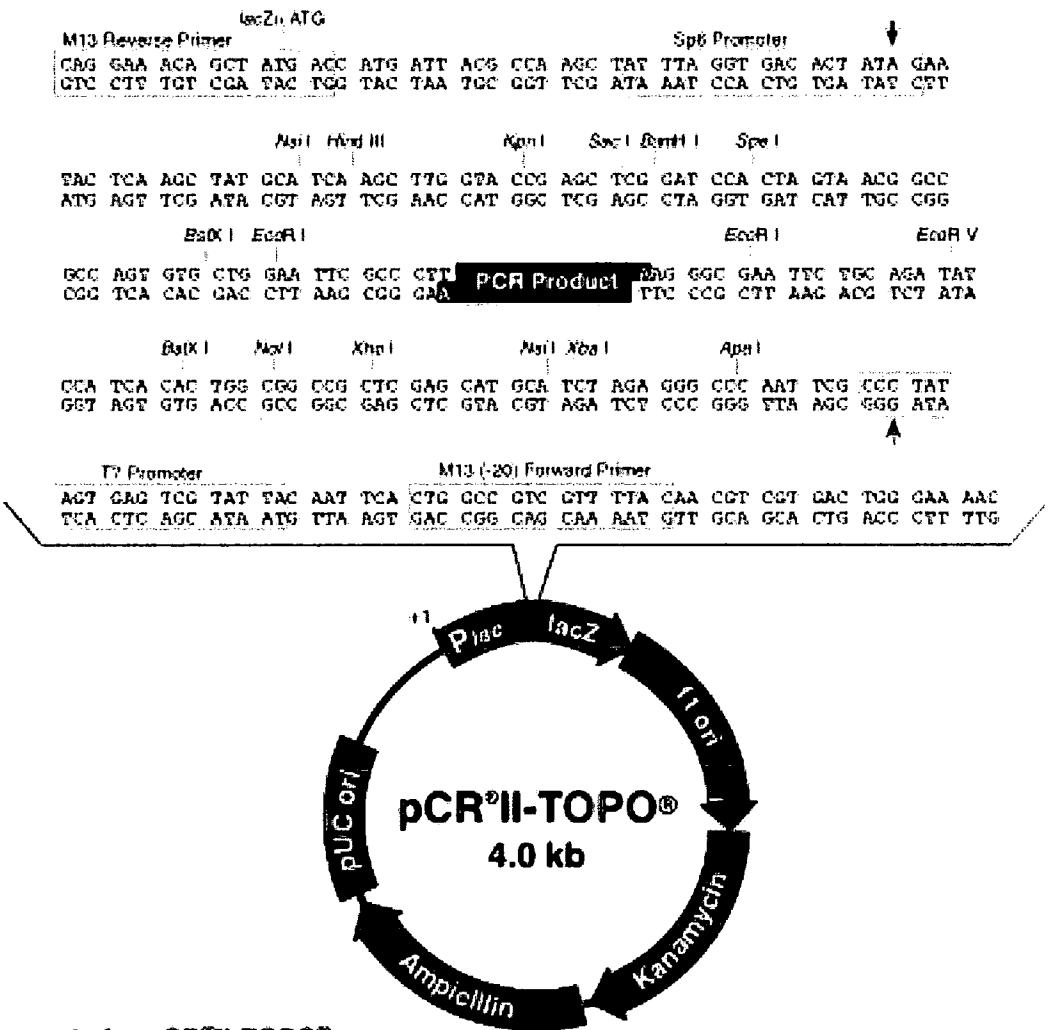
CCCAAGCTTCTCCAGAACTAATACATCA
(Hind III)

## Forward; -215bp

CCCAAGCTTGACGTTCCTGGTTGTAAA
(Hind III)

# Appendix 3: Vectors

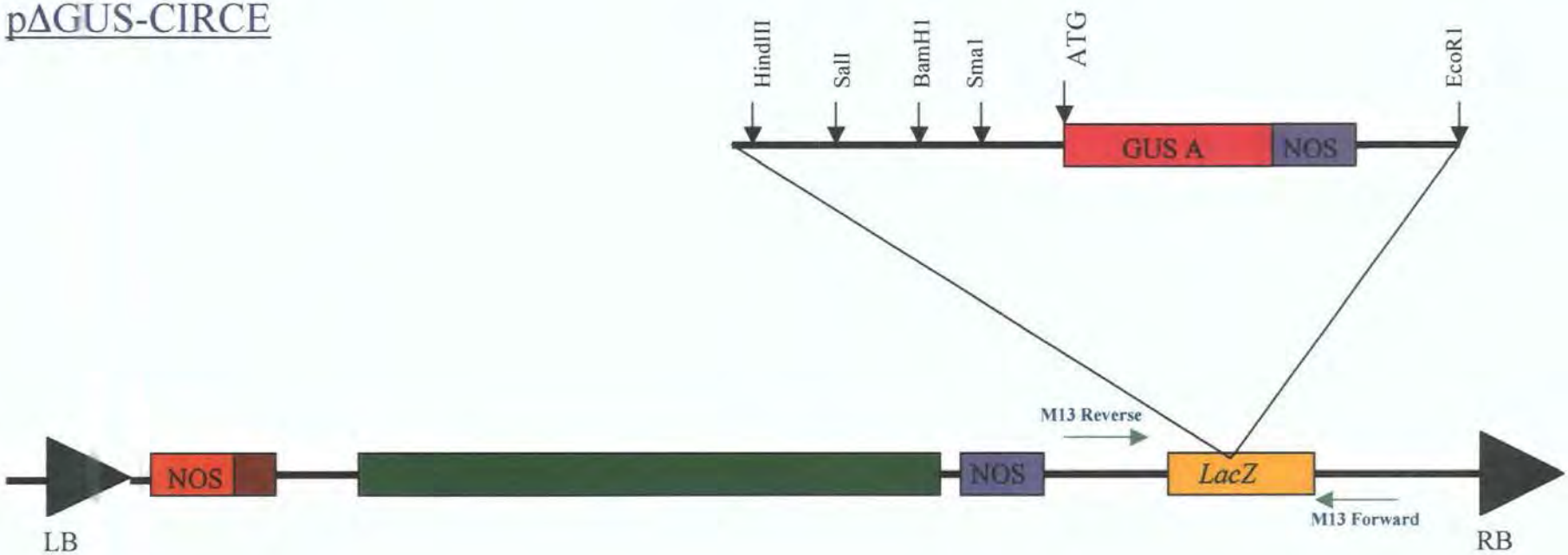
## 1. PCRII-TOPO (Invitrogen)




**Comments for pCR®II-TOPO®**
**3973 nucleotides**

*LacZα* gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

# pΔGUS-CIRCE




Promoter from the *NOS* gene

Leader sequence from TMV

Terminator and polyadenylation sequences from the *NOS* gene

# Appendix 4

## Putative patterning and signaling recognition sequences in the –2kb *HYD1* promoter from Ws

AAGCTTTTTATAACCCTTTTAATGGATTAGAAAAAAAGGGGAAAAAGCCGAAAAAACC

CTCATTTATTTTTAATTTGGCCGTTTAATATCTATACTTTTAAAATTGGAAGAAAATTAC

ATAAGTTAATTTTGATTGTCTACTAAAACCCTGGTCTTTTAAATATTAGGTATTTTCTGC

GCCCGAGTTAACGACTGTTAGTGTTGGTAACGGAACTACTAACTGCAATAACTCTCG

GTATTTCCACATATATATTCAATCTCTAATAACTATCTCACGAATTAATTGTCTTACCCA

AGAAGTGGGGTGAAATTTCATCCATATCCGGGTTCGAACCCAACAGTATACTTTTTGA

CGTTTTTTCTTTCTTTTTTTGTCTTAATTTAATTGAAGAAGGAAGAATGGAGGTGGTGG

TGGTGGAGAGGACGACGGAGATGGCACANCAACGGGTATTCTCAACCACCAGATCT

GAAAACTTTTTCTATGCATAACCTCGACGAGAAACTTATGTGTAGATTCTGAGATTTGA

TTTGATATTTTGTGGACTTTTACTTTCTTCTCTGTAGATACATGTTTAANATTTGTATCCA

AATTGGAATTTATTAAAATTCAAATTGGATTTGTTTTCCAATCCGAATTTGTTGCTTATG

TTGTGTTGTGAGAAAAGAGAGTTTGGTGAGAAAAGAGAGTTTGGTGAAGAANAANAA

GTCNAGTTAAAAAGAAGAAAAAAGGAAAAAAGAAAAACGTCTTTTGCTGGTGGTAGG

ACTTGAACTCGGGTATANATAAAAAATAAACCTACATCTAACCACCTGACTGANACAA

CATAATCGGTAGGTTATTAAAAGAGATTGTATATATATGTGGAAATACCATGAGTTATG

TTTTAACCACAGTTAGTGGTTCCGTTACAACATTAACAGTCGTTAACTCGGGAGTAAG

AAATATCCAACATTTAAAAGACTAGAGTTTTAGTAGACAATCAAAATTAACTTATGTATT

TTTCTTCCAATTTTAAAAGTATTGGTATTAAACGGCCAAATTAAAAATAAATAAGGGTT

TTTCCGGTTTTTTTCCCGAAAAAAAAAGAATAGATGGGGCAAAATTATACTCGGATCT

AGTCCGGCTAGAGAAAGGATATGGATACCCGAATCGATTTTATTGATTTTCACATGTG

CCTCTCGTGCTTTCATTCCTTTTGCAATTGTAGACCAAAGTATTTGAATTTATATGCCA

Ws upstream sequence absent from Col

-834bp primer

CCACGAGGTTGAACGCCAGGCTATCTTGGCAAGTTTTAATTCAAANTAGGTCAAT

ACCAGCACGTTTATCATACATAGTTTGTTAGTTATAACAATTTACCTCGAGTATAT

TAATTTGATTTAGTTAATTTGATAAATAATATTTTTGGTGTTTTAAAAATCATTTTGA

TTTATACTTTAAAGATAACAAATTTTTATTATTATTATTGTTAAGTAAGTAATTGTAA

TTAAGTGTTAACTACCATTAAAATAGATAAGTAAACAAAATAGCAAATGAATTATTA

ACTCCAGAACTAATACATCAAAGATTATATTAGTGGAGAACAGATGGGCCAATAA -482bp primer

TCCAAATTTAACTAATATTTAGAAAAGCCCATAAATATATTCCTGACCTTGGTGAA

GACACTTTATCTTAGAAAAGCCCATGAATATATTCCTGACCTTGGTGAGGACACT

TTATCCAAGAAAAACAAAACTTATGTCAGAAGTGGGGTTTGAACCCACGCCCTCTT

ACGAGGACCAGAACTTGAGTCTGGCGCCTTAGACCACTCGGCCATCCTGACGTT

CCTGGTTGTAAATCTCATAGTATTATTAACTTGGACAATTTAGTTTGACCAATTATT -215bp primer

TTATTACCAAACAAAAGACACTTGAGTGAATGAATCGCCGGGATCGAGGAGATTA

GTAGTAGTAAGTCATCATCACTCAGATCTAGTAGTTCCACAAATCACATTGGGTC

GGATCGTTGACCAGAAAAACACACAGAGAAAGGAGAAAAACATG

## Legend

CAAT defines a consensus motif of an upstream activating sequence (CAAT box), close to a TTATTT (TATA box), which acts as a recognition sequence for the binding for RNA Polymerase II; both of these are upstream of the ATG translational start.

GATA, the GATA motif, specifies trichomes, vascular, epidermal and mesophyll cells.

AAAAG is a typical Dof binding site

**AGAAA** is a pollen signal

TGACGT is a cotyledon-specific signal

ATATT is a root motif.

AAAGAT and CTCTT are also root-specific elements, associated with nodulation signals and mycorrhizal organ-specific expression.

ACACTTG from carrot binds bZIP transcription factors, is an embryo-specific signal that can also be induced by ABA.

CANNNTG is a consensus binding sequence for MYC transcription factors, involved in ABA induced gene expression.

TAACCA (MYB1 from *Arabidopsis*), GTTAGTT (MYB1 from tomato); along with the GA responsive element TAACAA from *Arabidopsis*, comprise components of a GA responsive complex (GARC); the Dof pyrimidine box CTTTT is also implicated in GA signaling.

TAACTG and TAACGG are MYB2 sites from *Arabidopsis*.

GGATA is an alternative MYB recognition sequence.

TTGACC is a WRKY transcription factor binding domain implicated in negative regulation of GA.

TGACT is a WRKY site, present in the Ws upstream unique sequence, and implicated in sugar responses.

NGATT binds ARR1, a transcriptional activator of cytokinin response genes.

AATTCAAA is an ethylene responsive motif.

GATAAG ia an I-box, implicated in light response.

ACTTTA is a motif from the rolB gene promoter which may be involved in the response to auxin.

TGACG is a recognition motif for ASF1, implicated in auxin and salicylic acid response.

## Appendix 5: Longevity of *hydra* and *hydra-ein2* double mutants.

All seedlings were germinated on horizontal plates, on $^1/_2MS_{10}$, and transferred to vertical plates at 4dae. 'Death' was determined as the point referring to death of the entire seedling, although this progressed in stages over a number of days, beginning in most siblings with death of the primary root meristem.

| Lifespan (dae) | *hyd1-2* (n = 94) | *hyd1-ein2* (n = 125) | *hyd2* (n = 97) | *hyd2-ein2* (n = 160) |
|---|---|---|---|---|
| 1-6 | 2 | | 9 | |
| 7-13 | 16 | | 21 | 8 |
| 14-21 | 11 | | 18 | 3 |
| 22-28 | 8 | | 15 | 15 |
| 29-35 | 10 | 13 | 17 | 14 |
| 36-42 | 15 | 13 | 2 | 26 |
| 43-50 | 4 | 8 | - | 30 |
| 51+ (surviving) | 27 | 91 | 14 | 64 |