PROTEIN INTERACTIONS OF PHANTASTICA HOMOLOGUES IN
Antirrhinum AND Arabidopsis.

Peter Newton

A thesis submitted in partial fulfillment of the requirements for the degree of

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I declare that this is my own work.

Any contribution made by other parties is clearly acknowledged.
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The leaves of *Antirrhinum* provide a good model system to study the mechanisms that specify formation of axes during plant development because mutation of the *PHANTASTICA* gene (*PHAN*) causes more complete disruption of these features than does mutation of its homologues in *Arabidopsis* or maize. *PHAN* encodes a Myb homologue and is expressed in the whole of the zone of developing lateral organs yet the mutant phenotype suggests that it has functions restricted to the dorsal domain as well as in meristem maintenance. Mutants of *PHAN* in *Antirrhinum*, *Arabidopsis* and maize all missexpress *knox* genes so a related function of *PHAN* homologues is repression of meristem genes in lateral organs. The yeast 2-hybrid assay was used here to isolate PULP a novel potential modifier of PHAN protein function which, based on expression pattern in plant tissue has potential to interact with PHAN in vivo. The yeast 2-hybrid assay suggested that the interaction between PHAN and PULP is conserved because *Arabidopsis* PHAN and PULP homologues, AS1 and T20403, interact in the same way. Mutagenesis indicated that the interaction is biologically relevant because a putative T20403 mutant enhanced the *as1-1* phenotype causing it to resemble that of *phan*. PULP expression pattern suggested that it may function in collaboration with PHAN in lateral organs but independently of PHAN in stem tissue to repress *knox* genes. The yeast 2-hybrid assay also revealed potential homotypic and heterotypic interaction between PHAN, AS1 and PHANL1 which could provide a mechanism for PHAN and AS1 repression of *knox* expression, but taken together with the function of *knox* gene and Myb homologues in animals and the nature of the genetic pathways important to regulation of growth of the tissue where they are expressed, a degree of functional homology between PHAN and cMyb or aMyb in animals is also suggested. The importance of this during specification of the adaxial-abaxial axis of lateral organs in plants may be that juxtaposed adaxial and abaxial cell types which are required for development of the mediolateral axis of lateral organs may not be formed in the absence of PHAN due to failure of cells to proliferate and or differentiate correctly.
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<tr>
<td>AML</td>
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<tr>
<td>OD\textsubscript{600}</td>
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<td>SAP</td>
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<td>SD</td>
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1. INTRODUCTION

1.1 A GENERAL INTRODUCTION

Although the *Antirrhinum PHANTASTICA* (*PHAN*) gene and its homologues in *Arabidopsis* and maize are known to be important for lateral organ development only some functions of *PHAN*-like genes have been demonstrated and their precise role in organogenesis is still unclear. The mutant phenotype in *Antirrhinum* suggests that *PHAN* is important for axis specification because two axes of lateral organ development are disrupted in *phan* mutants. For this reason lateral organ development and *PHAN*-like gene function have been considered recently in terms of axis specification. However, the mutant phenotypes observed for functional homologues of *PHAN* in other species suggest no role - or roles in specification of fewer axes than in *Antirrhinum*. Therefore it is unclear to what extent *PHAN* homologues are required specifically for axis specification. Additionally, because of the complex interaction between meristems - the generative part of plants - and lateral organ development, *PHAN* function may be linked to processes of cell proliferation and differentiation or determinacy of tissues during lateral organ development. Therefore general aspects of plant development relevant to this theme will be discussed before *PHAN* and its function is covered in more detail.

1.2 THE RELATIONSHIP BETWEEN POST EMBRYONIC PRIMARY AXIS DEVELOPMENT AND LATERAL ORGAN DEVELOPMENT

The bulk of the aerial parts of higher plants is generated outside of embryogenesis by activity of meristems which produce organs in regular sequence and position relative to other components in repeated "phytomers". A phytomer consists of a node with lateral organs, axillary meristems and internodes separating organs. Changes in meristem identity occur throughout the life of the plant, the most obvious of which are associated with reproduction. These can involve changes in the phyllotaxy, identity of lateral organs, determinacy and size as well as cell division rate of the meristem. For example in *Antirrhinum*, the vegetative meristem usually produces an opposite pair of leaves at each node separated by long internodes. Meristems in the axils of leaves behave in the same way as the primary meristem. On transition to flowering, the meristem assumes inflorescence identity and initiates a single bract at
each node in a spiral phylotaxy, separated by shorter internodes. Meristems in the
axils of bracts produce flowers with the characteristic floral lateral organ identities of
sepals, petals, stamens and the carpel which are arranged in concentric whorls with
little or no internodal tissues. Floral meristems also differ from vegetative and
inflorescence meristems in being determinate as the stem cell population is lost
during formation of the carpel. Clonal analysis in Arabidopsis and maize has
revealed that components of each phytomer are derived from the same population of
initial cells (Furner and Pumfrey, 1992; Irish and Sussex, 1992; Sharman, 1942;
McDaniel and Poethig, 1988). This suggests that their development might first
involve specification of a phytomer which is then sub-divided into lateral organ,
node and internode initials. Initiation of internodes has been observed in some dicot
meristems such as Silene where each internode appears to arise from a disc two cells
deep which separates organ primordia at adjacent nodes along the apical-basal axis,
(Lyndon 1990).

1.3 SPECIFICATION OF LATERAL ORGAN AXES

Fig 1. Above shows the axes that are recognised in plant development. The primary
axis of the shoot is described as the apical-basal axis but a second axis, the
inside-outside axis, is recognisable along the radius of the stem from the centre to the
epidermis. The primary axis of lateral organs is described as the proximodistal axis
but a second axis develops perpendicular to this called the mediolateral axis. Lateral organs also have distinct upper and lower parts, specialized for light and gas interaction, which are designated adaxial and abaxial respectively. These may also be referred to as dorsal and ventral as if an organ is being considered in isolation from the plant. Because the differences in the adaxial and abaxial parts reduce the number of planes of symmetry this has also been referred to as dorsoventral asymmetry.

1.3.1 THE PROXIMODISTAL AXIS

Lateral organs develop from the flanks of meristems where axes are already specified and are apparent from histology. Perhaps the most important axis for lateral organs is the proximodistal axis which is the first to be observed in generation of a new organ and is probably pre-specified by the inside-outside axis of the meristem. The inside-outside axis of the plant body-plan is maintained throughout the life of the plant from embryogenesis and is apparent by the 8 cell stage of the embryo when a change in the orientation of cell division from anticlinal to periclinal forms an outer, protoderm, cell layer (Mayer et al., 1991). The mechanisms involved in specification

Fig. 2. Establishment of the inside-outside axis first becomes visible at the eight-cell stage of the embryo (top left) when cell division orientation changes from anticlinal (blue) to periclinal (red) producing the first protoderm cells in the 16 cell stage embryo (top right). The cell layer organization of the shoot apical meristem and lateral organs is maintained by restriction in the orientation of cell division throughout the life of the plant (bottom right). The epidermal layer (L1) and subepidermal layer (L2, in dicots) constitute the tunica whilst the corpus includes the inner cell layers (L3). Lateral organ primordia are produced sequentially and numbered by convention so that the first observable has the lowest number; (P1), (P2), (P3), primordia one to three.
of the axis of the first periclinal division is unknown but might involve signals from outside the embryo or polarity within the cells of the eight cell stage embryo itself. Along the inside-outside axis of the meristem can be recognised two clonally distinct cell layers in monocots and three layers in dicots that are maintained by constraints on the orientation of cell division Fig 2. (Satina et al., 1940; Poethig, 1987; Tilney Bassett, 1986). The outermost (L1) layer consists of a single layer of cells that forms the epidermis which is maintained by an almost exclusive pattern of anticlinal divisions from embryogenesis onwards. The underlying L2 layer is also one cell thick and divides only anticlinally in the meristem whereas the corpus (L3 inwards) contains cells which divide in many planes. Clonal analysis has revealed that the L1 continues to divide anticlinally outside of the meristem to give rise to the epidermis, except at petal margins where it can also contribute to internal tissues (Vincent et al., 1995). Restrictions on division planes in the L2 are lost outside the meristem, so the L2 and the corpus contribute to formation of internal tissue of the stem and lateral organs (Poethig and Sussex, 1985a, b). Clonal analysis has shown that all histogenic cell layers of the meristem contribute to the developing lateral organ primordia in maize and Arabidopsis. (Poethig, 1984; Poethig and Symkowiak, 1995; Furner and Pumfrey, 1992; Irish and Sussex, 1992).

The earliest sign of lateral organ initiation is a change in the rate or orientation of cell division resulting in swelling to form a primordial bulge at the site of organ initiation. Periclinal cell divisions in the L1 predict the site of leaf primordia formation in maize (Smith, 1996). In the vegetative apex of pea the rate of cell division remains the same as sites not destined to produce a leaf primordia, but the proportion of periclinal divisions in L2 and L3 increases significantly (Lyndon 1990). In Solanum tuberosum localised increases in cell division can be observed in all three layers of the initiation site. In the L1 only anticlinal divisions occur whilst in underlying cells both anticlinal and periclinal divisions are seen (Sussex, 1955). In Silene, however, the rate of cell division in the shoot apex is highest at the site of leaf initiation but there is no apparent change in the orientation of cell division. Pea and Silene therefore seem to exemplify two extremes in the way that the axis of growth are changed during leaf formation (Lyndon, 1990).
1.3.2 THE MEDIOLATERAL AND ADAXIAL-ABAXIAL AXES

In many species lateral organ primordia initiate as peg like outgrowths with radial symmetry (Steeves and Sussex, 1989) but soon exhibit mediolateral and adaxial-abaxial axes by flattening of the primordium. In Arabidopsis experiments that mark the location of mitotic cells during leaf development by placing a GUS gene under control of a cyclin D promoter revealed that growth of the mediolateral axis occurs early in leaf initiation through activity of a "marginal meristem". The marginal meristem soon ceases to be active and gives way to intercalary cell division so does not contribute significantly to width of the lamina (Donnelly et al., 1999). Clonal analysis has revealed absence of a marginal meristem during later leaf development and that intercalary growth is most significant in development of lamina width and shape in tobacco also (Poethig and Sussex, 1985a).

The mediolateral axis is postulated to be dependent on juxtaposition of adaxial and abaxial cell types because when genes that specify these are mutated, and the cell types are visibly absent in the epidermal layer, lateral organs are frequently produced that are of one epidermal cell type and are radially symmetrical (Waites and Hudson, 1995; McConnell and Barton, 1998; Siegfried et al., 1999). This suggests that in dicots the mediolateral and adaxial-abaxial axes are connected (see section 9).

The mediolateral axis of lateral organs may be specified slightly differently in monocots and establishment of the mediolateral axis (of growth) may be more robust than in dicots because a greater proportion of the leaf width is established by specification of leaf initials in a crescent shape around the circumference of the meristem. This is also apparent when the expression pattern of meristem and lateral organ specific genes are analysed, as described below.
1.4 CELL PROLIFERATION, DIFFERENTIATION AND MERISTEM FUNCTION

Beside the physical process of axis formation, lateral organ development requires differentiation and cell proliferation of both the meristem and lateral organ initial cells. The influence of cell division rate on primordia emergence is as variable as that of cell division orientation with regard to species (see section 1.3.1). For example in Pea the cell division rate at the site of primordia remains the same as sites not destined to produce lateral organs. In other species such as *Solanum* and *Silene* changes in cell division rate do appear to contribute to primordia emergence.

Because cells are displaced from the centrally located group of slowly-dividing "stem" cells towards the flanks of the meristem, where they contribute to formation of lateral organs, a balance is required between replenishment of stem cells in the CZ and recruitment of organ founder cells in the PZ. Organ founder cells must assume different fates which enable them to progress along paths of development distinct from those of their neighbours.

![Diagram](image.png)

Fig 3. The progress of cells from the centre of the meristem to the peripheral zone. The shoot apical meristem (SAM) consists of a centrally located group of slowly-dividing stem cells which produce daughter cells that are displaced towards the flanks of the meristem and interior of the stem. Here they assume different fates as initials of the lateral components and primary axis tissues. Three zones can be distinguished within the meristem on the basis of cell division rates and histology. The central zone (CZ) consists of slowly dividing, cytoplasmically dense cells that provide a pool of stem cells for replenishment of the meristem. The peripheral zone (PZ), where lateral organs arise, surrounds the central zone in the form of a doughnut and contains more rapidly dividing cells which are less cytoplasmically dense than those of the CZ. A third zone, the rib zone, is located internally, basal to the CZ, and contains cells that are destined to differentiate into stem tissue (Steeves and Sussex, 1989; Medford, 1992). Lateral organs originate from the PZ and are named by convention with increasingly mature primordia being named $P_1$, $P_2$... and groups of initial cells at sites destined to form primordia being named $I_1$, $I_2$ etc $I_n$ is also referred to as P0 because it is often the primordia associated with the next plastochron to be elaborated (see figure 4 for P0).
1.4.1 MERISTEM GENES

Two sets of genes are known that are important for the process of meristem maintenance. The first includes genes of the CLAVATA (CLV) signal transduction pathway which act as negative regulators of WUSCHEL (WUS), a homeobox transcription factor gene. The second consists of the knotted1-like homeobox (knox) genes.

Maintenance of the size of the stem cell population in the CZ of Arabidopsis requires activity of WUS and the CLV signalling pathway. Loss of function mutations in the three known CLV genes (CLV1 to CLV3), causes an increase in size of the CZ. CLV3 encodes a potential signalling peptide which is expressed in the CZ (Fletcher et al., 1999). CLV1, which is expressed more generally in the meristem, encodes a transmembrane kinase capable of binding CLV3 and therefore is likely to be its receptor. CLV2 encodes a related receptor that lacks a kinase domain, but is able to associate with CLV1 (Fletcher et al., 1999; Clark et al., 1997; Jeong et al., 1999). A model for CLV function entails CLV3 signalling from the CZ to other cells of the meristem which functions to restrict the size of the CZ. One target of this signalling appears to be activity of WUS (Laux et al., 1996; Schoof et al., 2000). WUS is normally expressed only in internal cells of the meristem in a region approximating the rib zone and is required to promote activity of the meristem and CLV3 expression (Mayer et al., 1998). In clv mutants, the domain of WUS expression increases, but when CLV3 is expressed ectopically this leads to repression of WUS and a wus-like phenotype. The feedback loop between WUS and CLV might therefore provide flexible control of CZ size. WUS and STM seem to act in independent pathways because WUS expression is independent of STM activity (Mayer et al., 1998) but they have similar functions in specifying meristem characteristics and may have common targets.

The second set of genes important for meristem function are knotted1-like homeobox (knox) genes. knox genes can be divided into two classes on the basis of their sequence and expression patterns (Kerstetter et al., 1994). Whereas Class II knox genes have general expression patterns and unknown functions, Class I knox genes are expressed primarily in meristems in cells at the apex and internode initials, but
not usually in organs or organ founder cells within the PZ (Reiser et al., 2000). This suggests that they may be part of a mechanism that distinguishes meristem cells from organ founder cells. Two common expression patterns are distinguishable for Class I knox genes. The first is exemplified by Kn1. Kn1 mRNA expression in wild type plants is confined to the corpus whilst its protein is found in both the corpus and L1 (Jackson et al., 1994) but it is down regulated in regions corresponding to lateral organ initial cells. The second pattern is like that of Rs-I which is expressed at the border between undetermined meristem tissue and lateral organ primordia initials (Reiser et al., 2000) (see Table 1.) In Arabidopsis the functional orthologue of knl is SHOOT MERISTEMLESS (STM) (Smith et al., 1992; Jackson et al., 1994; Long et al., 1996). STM expression first appears in a single cell in the globular stage of the embryo which contributes to the SAM. Later in development expression occurs continuously in an expanded domain and resembles that of Knl as it is down-regulated in the lateral organ primordia (Long et al., 1996; Vollbrecht et al., 1991). Knl appears to act non-cell autonomously because it is misexpressed only in internal leaf cells but affects development of epidermal cells (Sinha and Hake, 1990; Jackson et al., 1994) and is known to be trafficked through plasmodesmata (Lucas et al., 1995). Knox genes such as KNAT1 in Arabidopsis are expressed both within meristems in tissue that will become the internodes and also later in the internode (Lincoln et al., 1994) and may contribute to specification of stem fate or specify boundaries within the SAM (Ori et al., 2000).

Homozygous loss-of-function stm mutations prevent initial formation of the SAM during embryogenesis but occasional leaves are formed later without any recognisable meristem structure or phylotaxy. Two weak stm alleles also lack a fully functional embryonic SAM but have a small number of characteristically embryonic cells at the normal site of the embryonic SAM. They produce several leaves and extra meristems in the axils of existing leaves but shoots terminate with the final organ formed in the centre of the meristem. Flowers that are eventually formed are incomplete, lacking inner whorls and frequently form fused or mosaic organs (Clark, 1996; Endrizzi et al., 1996). Based on these loss-of-function mutations in STM its function is postulated to be maintenance of cells in an undifferentiated state or to
promote meristem identity by repressing organ fate in the CZ of the meristem (Kerstetter et al., 1997; Barton and Poethig, 1993).

Several genes have been implicated in regulation of STM expression or function. **CUPSHAPED COTYLEDON1** (**CUC1**) and **CUC2** (Aida et al., 1997; Takada et al., 2001) are related to **NAM** of Petunia (Aida et al., 1997; Souer et al., 1996). Due to a block of embryonic meristem formation in **cuc** loss-of-function mutants **CUC** genes are thought to positively regulate **STM**. Consistent with this **CUC1** expression can be detected in the globular embryo at the site of meristem formation. Furthermore, ectopic **STM** mRNA can be detected when **CUC1** is expressed from the 35S promoter and the rosette leaves of transformed plants have characteristics of **knox** misexpression such as leaf lobing and formation of ectopic meristems on the adaxial leaf surface (Takada et al., 2001). A second gene called **PINHEAD** (**PNH**) which is allelic to **ZWILLE** (**ZLL**) (McConnell and Barton, et al., 1995; Moussian, et al., 1998), and is in part functionally redundant with **ARGONAUT** (**AGO1**; Bohmert et al., 1998) is expressed in vascular precursor cells underlying the embryonic SAM, in the SAM, adaxial organ primordia and also appears to be required for **STM** expression (Moussian, et al., 1998; Lynn et al., 1999). **ZLL** has homology with mammalian translation initiation factors and **PIWI** which is required for the non-autonomous maintenance of stem cells in the Drosophila germ line. A **ZLL** homologue also is implicated in this role in *C. elegans* (Cox et al., 1998). **pnh ago** double mutants fail to accumulate detectable STM protein and fail to attain bilateral
symmetry during embryogenesis suggesting a role for both genes in regulation of STM at the point of bilateral symmetry specification (Lynn et al., 1999).

The plant hormones, gibberellins and cytokinins, are implicated in the phenotypes caused by ectopic knox gene expression. Elevated cytokinin levels and delayed senescence characteristic of increased cytokinin activity are observed in plants ectopically expressing knox genes, suggesting that knox genes positively regulate cytokinin levels (Ori et al., 1999; Hewelt et al., 2000; Kusaba et al., 1998). Similar evidence has been obtained for the involvement of gibberellins. Inhibitors of GA biosynthesis or GA biosynthetic mutations suppress the effects of knox gene misexpression in Arabidopsis (Miltos Tsiantis, pers. comm.) suggesting that GA biosynthesis is regulated by knox genes or required for some of the consequences of ectopic expression. However, there is evidence that knox gene expression represses GA synthase genes (Sakamoto et al., 2001). Therefore the relationship between ectopic knox gene expression and hormones, and how these might reflect their roles in the wild-type meristem, are not yet clear.

1.5 SPECIFICATION OF LATERAL ORGAN FATE

Expression of MYB-like and AP2-like transcription factor genes is initiated in organ founder cells at the same time that knox gene expression is down-regulated. The MYB-like PHANTASTICA (PHAN) gene in Antirrhinum (Waites et al., 1998) and the AP2-like AINTEGUMENTA (ANT) gene of Arabidopsis (Klucher et al., 1996) both might have roles in specifying organ identity or organ formation but ANT appears to be required for cellular proliferation in lateral organs as well as ovules because reduced cell number correlates with ovule and organ size in ant mutants (Klucher et al., 1996; Mizukami and Fisher, 2000). The function of PHAN in repression of knox genes in lateral organs is conserved with its Arabidopsis ortholog ASYMMETRIC LEAVES1 (ASI; Byrne et al., 2000) and maize ortholog Rough sheath2 (Rs2; Tsiantis et al, 1999; Timmermans et al. 1999). However, the repressed knox genes are not orthologous (see Table 1.). In Antirrhinum PHAN activity is required to repress expression of the knox gene, HIRZINA (HIRZ, Tsiantis et al, 1999; Timmermans et al., 1999), which is one of two STM-like genes present in
Antirrhinum (Golz and Hudson, 2002), but PHAN does not repress orthologues of the Arabidopsis KNAT1 genes. In Arabidopsis AS1 is needed to repress KNAT genes but not STM. The evolutionary distance between maize and the two dicot species hinders identification of orthologous genes in this monocot, but Rs2 is required to repress Rs1, which appears most similar to KNAT1 of Arabidopsis (Reiser et al., 2000), and two related Class I knox genes, Liguleless3 and Knotted1. Although Rs2 is required to repress knox genes in maize leaves, it appears not to be instrumental in knox down-regulation in organ initials, because this occurs normally in the organ initials of even strong rs2 mutants (Schneeberger et al., 1998). This also applies to other PHAN-like genes such as AS1 (Chuck et al., 1996; Ori et al., 2000) and over expression experiments suggest that the initial down-regulation may function at the level of RNA turn-over in Arabidopsis and barley (Chuck et al., 1996; Williams-Carrier et al., 1997).

<table>
<thead>
<tr>
<th>PHAN</th>
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<th>RS2</th>
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<td>hirzina</td>
<td>+</td>
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<tr>
<td>AmStm2</td>
<td>-</td>
<td>STM</td>
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<td>Snap1</td>
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<td>Knat6</td>
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<td>Snap20</td>
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Table. 1. The expression pattern and repression of knox genes in Antirrhinum, Arabidopsis and maize by PHAN-like genes. Symbols are as follows; - no repression by PHAN-like genes; + repressed; ? repression unknown. Expression pattern classes shown in the left column for each species are based on the review by Reiser et al., (2000) Andrew Hudson and John Golz (pers. comm). Knox genes in the same row are approximately orthologous.
1.5.1 LOSS OF FUNCTION MUTATIONS OF PHAN-LIKE GENES SUGGEST THEY PROMOTE EPIGENETIC REPRESSION OF KNOX GENES

The PHAN family genes encode MYB transcription factor homologues, so the mechanism by which they repress knox genes could be direct or indirect. However, maize rs2 and phan mutants suggest that knox genes are imperfectly silenced; maize rs2 mutants are sensitive to environmental conditions and display clonal sectors where knox gene protein accumulates (Timmermans et al., 1999). In phan mutants abaxial tissue appears on the adaxial surfaces of leaves and petals in patches resembling clonal sectors. One explanation for this mosaic mis-expression is that PHAN-like genes are necessary for an epigenetic process that maintains repression of knox genes in dividing organ cells. Evidence for epigenetic regulation of knox genes exists in Arabidopsis where the phenotype of knox gene misexpression due to as1 or ASYMMETRIC LEAVES 2 (as2) mutations (as2 has a similar phenotype to as1 but involves an unrelated gene; Semiarti et al., 2001,) can be enhanced in presence of the serrate (se) or gymnos/pickle (gym) mutations. as, se or as, gym double mutants phenocopy strong misexpression of KNAT1 from the 35S promoter. Therefore SE and GYM are postulated to act as repressors of KNAT1 and KNAT2 knox genes, or their targets, and to act independently of AS1 and AS2 (Ori et al., 2000). Because neither gym nor se expression pattern are affected by as mutations or vice versa. GYM encodes a member of the CHD class of chromatin remodelling factors (Eshed, et al., 1999; Ogas et al., 1999). se mutants show similar pleiotropic effects to gym mutants, including slow growth and se mutations enhance the phenotype of gym mutants, suggesting that SE and GYM might be involved in a similar process.

1.5.2 THE CONSEQUENCES OF KNOX GENE MISSEXPRESSION DUE TO LOSS OF REPRESSION BY PHAN HOMOLOGUES

Mutations in PHAN homologues are known in Antirrhinum, Arabidopsis (AS1) and Maize (Rs2). In each case the mutation appears to affect various aspects of axis development, boundary specification or cause reiteration of developmental processes. rs2 mutants exhibit variable defects including knotted-like out-growths of aberrantly differentiated tissue and wider leaves with extra veins but also narrower or semi-bladeless leaves. The most penetrant and consistent phenotype however, is
disruption of the blade-sheath boundary with proximal sheath and ligule features being displaced distally into the blade (Schneeberger et al., 1998).

In dicots such as *Antirrhinum* and *Arabidopsis* mutation of PHAN and AS1 both cause the early leaves to develop a wider form than wild type which suggests disruption of the mediolateral axes. The most striking feature of *phan* mutants is that later leaves as well as bracts and petal lobes have disrupted dorsoventral asymmetry, which is apparent from the radially symmetrical (needle-like) organs with epidermal cells that resemble those of the abaxial midrib or stem (Fig. 5; Waites and Hudson 1995).

The *as1-1* mutation leads to formation of cabbage-like or lobed rosette leaves and lobed cauline leaves on the inflorescence but petals are not noticeably affected (Redei, 1965). The *as1*-magnifica allele results from chromosomal rearrangement at the *AS1* locus which causes a similar leaf phenotype to *as1-1* but also has callus-like growths on the leaf lamina with occasional ectopic shoots on the adaxial surfaces of the petiole. In wild type leaves the abaxial epidermis of the petiole and lamina is comprised of elongated cells but in *as1-1* there are multiple bundles of elongated cells extending into the leaf blade (Byrne et al., 2000). These features may be interpreted as disruption of the mediolateral or proximodistal axes but like monocots the adaxial-abaxial axis does not appear to be affected.

Fig 5. Abaxialization of *phan* leaves. (Lower left) a transverse section of a wild type leaf showing adaxial outgrowth of the lamina that produces the wild-type leaf shape (above left); (below right) a severely abaxialised *phan* mutant leaf has failed to produce a lamina and results in the leaf shown above right. The adaxial domain lacks parenchyma and vasculature typical of the adaxial domain and has epidermal cells that resemble those of the abaxial domain or stem tissue (Waites and Hudson, 1995).
MISSEXPRESSION OF KNOX GENES LEADING TO DISRUPTION OF LATERAL ORGAN AXES AND SHIFTS OF LATERAL ORGAN FEATURES

Misexpression of knox genes in dicot leaves has been discovered or achieved by transgenic approaches but leads to a variety of different effects. Consistent with the role of PHAN homologues in repression of knox genes some of the phenotypic features of knox misexpression resemble those of PHAN homologue mutants. The rounded leaf phenotype exhibited by early leaves of Antirrhinum phan mutants is also caused by the gain of function Hirz mutation which allows ectopic expression of HIRZ (Fig. 6; John Golz, pers. comm.). The rounded leaf phenotype also occurs in normally lanceolate tobacco leaves when tobacco knox genes are expressed from the actin promoter (Nishimura et al., 2000).

As in Rs2 mutants knox misexpression in monocots causes shifts of proximal sheath characters distally into the leaf blade. This is exemplified by mutations in the maize knotted1 (kn1) gene which was first identified on the basis of gain-of-function mutations caused mainly by transposon insertions or DNA rearrangements in non-coding regions. These mutations cause kn1 misexpression in cells surrounding veins and to the displacement of sheath, ligule and auricle tissues to distal positions, as well as shortened internodes (the ligule and auricle are normally formed at the junction between proximal sheath and distal blade tissue) (Freeling and Hake, 1985). Gain of function mutations have also been identified for the related maize knox genes, Liguleless3 (Kerstetter et al., 1997) and Rough sheath1 (Schneeberger et al., 1995). Rs1 Lg3 and Kn1 condition similar phenotypes but in Lg3 mutants tissue with sheath characteristics extends in to the leaf blade eliminating the ligule Fig. 6.

To explain the common consequence of knox misexpression in monocots, Frilling (1992), formulated a time dependent “maturation schedule” hypothesis. The hypothesis postulates that leaf founder cells acquire regional identity in the meristem. During this time the maturation schedule is initiated which manifests itself as sequential movement of regional tissue domains through windows of competencies. This occurs early in leaf development because tissue identities begin to be apparent in leaf primordia P2. Competency to respond to developmental signals depends on progress of regional domains through the maturation schedule not position at the time when the signal occurs. Subsequently, cell proliferation occurs to produce the
full size leaf during which cells are recruited based on their position. Because of the basipetal pattern of differentiation in leaves, knox gene misexpression retards the leaf maturation schedule and domains further up the primordium are competent to respond to developmental signals.

A simpler explanation for the distal shift of features in monocot leaves is that they are caused by changes in positional information which enable leaf blade cells to respond to sheath signals (Hake et al., 1995).

In some circumstances and at higher levels of transgenic knox gene expression more severe phenotypes have been observed in monocots. Expression of Knl in Barley under control of the ubiquitin promoter causes an ectopic meristem to form on adaxial tissues at the lemma/awn transition zone (Williams-Carrier et al., 1997). This feature is also found in the barley mutant Hooded which causes misexpression of the Barley knl homologue hvknox3 (Mueller et al., 1995). Ectopic meristems also occur in monocot leaves, Senko et al., (2000) described formation of ectopic meristems on rice leaves as well as completely bladeless leaves in plants misexpressing the rice knox genes OSH15 or OSH71 from the strong Act1 promoter. Occasional ectopic meristems are also seen, on adaxial leaf surfaces, when knl or other knox genes are expressed at higher levels in Arabidopsis and tobacco (Lincoln et al., 1994; Chuck et al., 1996; Sinha et al., 1993; Nishimura et al., 2000). Expression of meristems on leaves has been considered as a distal shift of proximal features (Hake et al., 1995).
Fig. 6. Phenotypes of *knox* misexpressing *Arabidopsis*, *Antirrhinum* and maize plants due to misregulation or loss of repression in *PHAN*-homologue mutants. (A) Leaves and flowers of wild type, *Hirz* and *phan* mutant *Antirrhinum* plants, *phan* leaves are wider at node 1 like *Hirz* but later leaves and petals are increasingly radial; instead of *phan* radial petals *Hirz* produces an ectopic spur see section 1.6.1; (B) like *as1-1*, misexpression of *KNAT1* using the 35S promoter in *Arabidopsis* causes rosette and cauline leaves to be wider or lobed. Two *as1* rosette leaves are shown because of the variable phenotype; (C) a wild type maize leaf showing the junction between the sheath and blade, (s) sheath, (b) blade, (m) midrib, (a) auricle, (l) ligule; (D) *liguleless3* mutant showing the shift of sheath into the blade; (E,F) show the similar phenotypes of *Kn1* and *Rs2* mutants respectively (see section 1.6). Not to scale.
1.6.1 KNOX GENE MISSEXPRESSION LEADING TO ECTOPIC BOUNDARY FORMATION OR REITERATION OF DEVELOPMENTAL PROCESSES

A second phenotype associated with knox misexpression is lobing in leaves. This could be interpreted as reiteration of developmental processes or developmental processes responding to ectopic boundary specification. The mildly serrated spoon shaped leaf of Arabidopsis develops deep lobes when misexpressing knl or KNAT1 (Lincoln et al., 1994; Chuck et al., 1996). The serrations initiate as dorsiventral primordia and have flanking stipules in the sinuses which are normally associated with the stem and axil at the leaf base. This together with basipetal differentiation of cells in the lobes suggests that the lobes retain leaf as opposed to stem characteristics. The lobes however appear to form by failure of growth in the sinus region which show the highest accumulation of knox transcripts. Ectopic meristems also form in adaxial parts of the sinuses and this correlates with the severity of lobing rather than expression level so it has been suggested that meristem formation is dependent on the period of time that immature tissue is exposed to signal (Chuck et al., 1996). KNAT1 and KNAT2 which normally mark the meristem or stem are also expressed in the sinuses of lobed leaves found in as se double mutants which correlates with the meristematic characteristics (Ori et al., 2000). Because KNAT1 is expressed in a ring in the peripheral zone of the SAM in wild type plants, where it could specify a boundary between indeterminate cells and primordia, the lobed leaf phenotype of ectopically expressing KNAT1 plants has been interpreted as resulting from ectopic boundary formation specified by KNAT1 (Ori et al., 2000). Other knox genes have also been suggested to predict boundaries within the meristem (Jackson et al., 1994). When maize knl is expressed from the 35S promoter in tobacco this causes the normally entire leaf to form three lobes at low expression levels and reduced leaf size with ectopic shoot formation at increased expression levels (Sinha et al., 1993). Because the displacement of meristems and stipules in KNAT1 misexpressing plants can be interpreted as proximal-distal shifts, and this feature is held in common with the knox misexpression phenotype of monocots, it has been suggested that the phan needle-like leaf is also derived by the same process with the interpretation that the radial leaf represents petiolised tissue (Tsiantis et al., 1999). However, petiole has a
clear adaxial-abaxial axis and demonstrates differentiated adaxial as well as abaxial tissue types unlike phan radially symmetrical leaves.

Another explanation for subdivision or lobing of leaves is that misexpression of knox genes causes reiteration of developmental process. This is exemplified by misexpression of knl in tomato. The tomato leaf is normally compound and although knox genes are down-regulated in its initials, they are expressed again later in the developing leaf. However, the level of dissection increases 100 fold when knl or a tomato knox gene are expressed from the 35S promoter (Hareven et al., 1996) or where a knox gene has been brought under the control of strong constitutive promoter by chromosomal rearrangement (Chen et al., 1997). Reiteration of features may also be seen in Antirrhinum Hirz mutants which ectopically express HIRZ leading to outgrowth of the corolla tube in a novel axis (Fig. 6. Golz, et al., 2002). A further example of this may be seen in the barley Hooded mutant phenotype which results from misregulation of hvknox3 (Muler et al., 1995) or when maize Knl is expressed under control of the ubiquitin promoter (Williams-Carrier et al., 1997). In both cases an additional inflorescence consisting of a lemma, palea and rudimentary awn are initiated from the existing awn.
1.7 PHENOTYPIC COMPARISON OF PHAN HOMOLOGUE MUTANTS AND KNOX MISSEXPRESSING PLANTS WITHIN AND BETWEEN SPECIES

1.7.1 COMPARISON OF PHAN HOMOLOGUE MUTANTS AND KNOX MISSEXPRESSING PLANTS WITHIN SPECIES

Because PHAN-like genes act as repressors of knox genes in leaves of Antirrhinum, Arabidopsis and maize, at least some aspects of the phan-homologue mutant phenotypes are expected to result from ectopic expression of knox genes. The difference between PHAN homologue mutants and knox overexpression phenotypes might be expected to be informative about the role of PHAN homologues that are additional to knox regulation. However, which ever way the knox misexpression phenotypes due to mutation of PHAN homologues or misregulation of knox are compared there are sufficient unknown factors to limit useful conclusions from being drawn.

phan mutants have heart-shaped early and needle-like later leaves suggesting that PHAN is required for lamina outgrowth in later leaves - but in Hirz the heart shaped or rounder leaves are produced throughout the plant and presumably PHAN is active in this tissue. However, in Hirz phan double mutants conversion to either heart-shaped or needle-like leaves occurs throughout the plant suggesting that PHAN is not required for the enhanced lamina growth in Hirz. Unlike the needle-like phenotype of both later leaves and petals in phan mutants the wider leaf phenotype of Hirz does not occur in petals - instead a novel axis of growth occurs in the corolla tube (Golz et al., 2002). The absence of needle-like leaves and petals in Hirz demonstrates that ectopic knox gene expression is not linked to ventral fate and argues against the possibility that knox genes specify the ventral identity of leaves during primordia specification in the meristem. It could also however be due to differences to strength, position or timing of misexpression of knox genes. The difference between PHAN and Hirz phenotypes does also suggest a function of PHAN which is independent of knox repression and is most obviously required, at least conditionally, to facilitate lateral organ axis development in the later stages of the Antirrhinum life cycle.
The cabbage-like or lobed rosette leaves and lobed cauline leaves on the inflorescence of asl mutants have some similarities to those observed in 35S::KNAT1 plants and although the phenotype is less severe it correlates with loss of KNAT1 repression in asl mutants. 35S::KNAT1 plants show some evidence of disruption of the adaxial-abaxial axis because the adaxial palisade parenchyma cell layer is reduced to resemble the abaxial spongy mesophyll layer (Chuck et al., 1996). Whether this feature is present in asl mutants has not been reported.

In contrast to the phenotypes of PHAN and Hirz all aspects of the rs2 leaf phenotype can be explained on the basis of ectopic accumulation of knox proteins in leaves because the mutant phenotype resembles entirely that seen in dominant knox expressers such as knl and rs1 or lg3 which Rs2 represses (Tsiantis et al., 1999).

1.7.2 COMPARISON OF PHAN HOMOLOGUE PHENOTYPES IN ANTIRRHINUM, ARABIDOPSIS AND MAIZE

There are a few notable differences between PHAN homologue mutant phenotypes in Antirrhinum, Arabidopsis and maize. Whilst phan shows disruption of the adaxial-abaxial and mediolateral axes by its needle-like and heart shaped leaves and the proximodistal axis is often reduced, asl mutants appear affected mainly in the mediolateral axis (wider leaves) and the proximodistal axis (lobed leaves, possibly caused by ectopic distal specification of boundaries within the leaf). In contrast the phenotype of Rs2, and knox misexpression in other monocot species, resembles mainly disruption of aspects of the proximodistal axis by the shift of proximal characters distally into the leaf blade or reduction of the proximodistal axis to meristematic characteristics (Sentoku et al., 2000). The adaxial-abaxial axis is not normally affected in monocots (except in some genetic backgrounds in maize; Schneeberger et al., 1998).

There are several possible explanations for these differences, the first is that differences between the phan mutant phenotype (which includes loss of adaxial-abaxial asymmetry), and those of asl and rs2 (which appear not to) might reflect variation in the respective functions the knox genes that are misexpressed in each case. For example PHAN represses Hirz which is a meristem gene of the type
expressed throughout the meristem (see table 1), whilst ASI represses KNAT1 which is expressed at the border of lateral organs and may specify such positions as boundaries (Timmermans et al., 1999; Byrne et al., 2000; Reiser et al., 2000). This could account for the lobed leaves of asl-1 and is supported by overexpression of STM in Arabidopsis which causes needle-like leaves but is less easily interpreted for PHAN and Hirz because the wider leaf phenotype and ectopic corolla of Hirz is distinct from the phan phenotype. If maize is considered in this way it is seen that misexpression of Rs1 (which has an organ margin expression pattern similar to KNAT1) and kn1 or lg3 (which are expressed throughout the meristem) primarily cause disruption of the proximodistal axis and do not lead to lobing or radially symmetrical leaves. However it is unclear which of the maize knox genes can be considered orthologous to the dicot knox genes (Reiser et al., 2000).

One possibility for the lack of a phan-like phenotype in maize is that establishment of the mediolateral axis of growth in monocot leaves may be more robust than dicots because a proportion of the leaf width is established through specification of initials in the meristem. Consistent with this neither initial-cell recruitment nor knox gene down-regulation appear dependent on Rs2 function (Schneeberger et al., 1998; Chuck et al., 1996; Williams-Carrier et al., 1997).

Alternatively some PHAN functions might be redundant in other species (Timmermans et al., 1999). In maize a second Rs2-like gene may exist due to the duplicated nature of the maize genome (Schneeberger et al., 1998). Other genes may also play a role, this is supported because in monocots as in dicots mutants have been found that cause radially symmetrical leaves. The leaf bladeless1 mutant of maize is one example which causes completely radially symmetrical organs (Timmermans et al., 1998). In Arabidopsis the argonaut (AGO1) mutant also has radially symmetrical leaves (Bohmert et al., 1998). Interestingly, AGO has overlapping functions with PINHEAD/ZWILLE (PNH) (Lynn et al., 1999). Both AGO1 and PNH are homologous to rabbit eIF2C translation initiation factor and are partially redundant with one another but do not affect translation generally suggesting a role in gene specific translation initiation (Lynn et al., 1999). However, AGO is required for post transcriptional gene silencing and has homology to genes required for similar processes in yeast and nematodes suggesting conservation of this function.
from an early common ancestor. No morphological defects are apparent in yeast and nematode mutants (Lynn et al., 1999) but based on the phenotype of *argonaut* it could have a role in regulation of gene expression during plant development by PTGS (Fagard et al., 2000).

Of the models proposed to account for the various consequences of *knox* misexpression due either to loss of repression caused by mutation of repressors or ectopic expression (Freeling 1992; Hake et al., 1995; Tsiantis et al., 1999) perhaps the most insightful is provided by Hake et al., (1995) who speculate that formation of meristems on leaves may represent the most profound proximodistal shift possible. Meristems may be the most proximal position in plants because in some cases the transition between leaves and stems cannot be distinguished. In support of this the stem differentiates acropetally whilst leaves differentiate basipetally but they both differentiate towards the meristem. This is consistent with the evidence that *kn1*-like *knox* genes specify indeterminate "stem" cells (Sinha et al., 1993) in the central and peripheral zone of meristems and *PHAN*-homologues repress them in leaves.

This concept to some extent unifies the differences observed between monocot and dicot *PHAN*-homologue mutant phenotypes as well as *knox* misexpression phenotypes and supports a hypothesis that a function of *PHAN*-homologues is to mediate transition of tissue from indeterminate towards differentiated or determinate states. The various phenotypes in monocots and dicots can then be explained as follows. In monocots *knox* misexpression due to reduction of *RS2* function or misexpression of *knox* genes due to gain of function mutations reduces determinacy of lateral organ tissue which perturbs differentiation allowing distal organ tissue to respond to proximal signals. At higher levels of *knox* gene expression meristems are formed indicating a further reduction in determinacy. In rice completely bladeless sheaths and sheaths bearing meristems at their distal end may form because leaf blade development fails when indeterminate meristem characteristics are ectopically specified. *knox* induced meristem identity in monocots may cause incorporation of leaf initial cells into leaf primordia to fail leading to narrow or bladeless leaves. Similarly in dicots lobing of leaves in *asl* or *magnifica* mutants may result from reduced determinacy allowing reiteration of process or ectopic boundary formation.
due to reduction of determinacy caused by KNAT1 misexpression (Byrne et al., 2000). Heart shaped or rounded leaves observed in dicots may result from reduced determinacy which allows cells to proliferate and contribute excessively to the mediolateral axis. Needle-like leaves like those appearing in phan mutants may represent tissue that is insufficiently determinate or has not differentiated sufficiently for the mediolateral and adaxial-abaxial axis to be specified correctly.

1.8 INTERACTIONS BETWEEN MERISTEMS AND LATERAL ORGAN PRIMORDIA THAT CONTRIBUTE TO ASPECTS OF DEVELOPMENT

1.8.1 SIGNALS BETWEEN MERISTEM AND PRIMORDIA

Several pieces of evidence suggest that signals from meristems are required for specification of the adaxial-abaxial axis in lateral organs. Adaxial and abaxial domains in organ anlagen can be seen as positions along the radial axis of the SAM and could enable adaxial or abaxial identity to be specified by a mechanism that relies on proximity to the meristem (Bowman, 2000). This is consistent with surgical experiments in which leaf initials of a number of species were separated from the meristem by insertion of impermeable barriers. The isolated primordia often developed into radially symmetrical organs lacking adaxial cell types which suggested that signals from the centre of the meristem are required for specification of adaxial cell fate. Any signal must operate early in organ development, because older primordia were able to develop autonomously into phenotypically normal leaves (Sussex 1954, 1955; Snow and Snow 1959). Similarly, in Arabidopsis pinhead (pnh) mutants (also known as zwille; ZLL), organs that occasionally arise from the centre of the meristem lack adaxial-abaxial asymmetry and form radially symmetric trumpet or pin-like structures with abaxial tissue on the outside (Lynn et al., 1999). A simple explanation for this is that the primordium is symmetrically positioned on the meristem and therefore cannot sense radial polarity. Mechanisms that specify the adaxial-abaxial axis of floral organs may be specified differently because in the Arabidopsis mutant petalloss petals are formed with their adaxial-abaxial axis orientated apparently at random (Griffith et al., 1999). In the presence of an apetala3 mutation, which affects organ identity but not dorsoventral asymmetry, petalloss causes all second whorl sepaloid organs to form with their
adaxial-abaxial axis rotated by 180°C both morphologically and with respect to asymmetric gene expression (Siegfried et al., 1999). Petals were suggested to perceive their orientation dependently on the petal-loss gene supported redundantly by B class genes. One explanation for the reversed orientation of second whorl organs in plants lacking B class domains is that radial signals sufficient for specification of the adaxial-abaxial axis originate from a second source, or are interpreted differently (Griffith et al., 1999).

1.8.2 SIGNALS FROM PRIMORDIA TO MERISTEMS
1.8.2.1 SIGNALS THAT MAINTAIN PHYLOTAXY

Classical models of the mechanism underlying shoot architecture suggest that signals from existing organs determine the positions at which later organs subsequently initiate. For example surgical separation of primordia in Lupinus or ablation of initials in the fern Dryopteris, which has widely spaced primordia, cause subsequent primordia which are destined to be formed near to the disrupted primordia being shifted closer to it. The wide spacing of Dryopteris initials enabled physical contact to be discounted as a mechanism for the primordia placement. This inspired a model whereby older primordia or initials produce an inhibitor which affects positioning of new primordia and could provide a mechanism for normal spacing of lateral organs. The inhibitor theory is supported by further experiments with Dryopteris which isolated primordia with flanking radial incisions, the isolated primordia outgrew others that were older and larger suggesting that the inhibitor had been removed (Snow and Snow, 1931; Wardlaw, 1949).

1.8.3.2 SIGNALS THAT INFLUENCE MERISTEM ACTIVITY

Signals from organ initials and primordia also appear necessary to maintain activity of the apical meristem and for formation or activity of axillary meristems. The signals probably emanate from adaxial organ tissue because in situations where adaxial identity is increased meristem development is often enhanced (McConnell and Barton, 1998) but mutant plants in which adaxial tissue is absent generally have defects in meristem maintenance. Formation of ectopic meristems on leaves of the mutant and transgenic plants discussed above or those found naturally in some plant
species (see Sinha, 1993) occurs exclusively from the adaxial leaf tissue. Consistent with this in dicots axillary buds appear to form directly on the subtending leaf base (Talbert et al., 1995) and the bud in Arabidopsis is clonally related to the subtending leaf (Furner and Pumfrey, 1992; Irish and Sussex, 1992). Also when the subtending leaf primordium is surgically removed in Epilobium hirsutum the axillary meristem can fail to form (Snow and Snow, 1942). In Arabidopsis phabulosa-1d (phb-1d) mutants, which convert leaves to radialised adaxial tissue, axillary meristems form around the circumference of the leaf base in addition to the normal position in the axil. Occasional ectopic meristems are also seen, on adaxial leaf surfaces, when knl or other knox genes are expressed at higher levels in Arabidopsis and tobacco (Lincoln et al., 1994; Chuck et al., 1996; Sinha et al., 1993; Nishimura et al., 2000). In phb-1d mutants the size of the meristem is also increased and the effects of sim mutations that reduce meristem size and activity are suppressed further suggesting that there is a positive influence of adaxial tissue on meristem formation (McConnell and Barton, 1998).

Conversely reduction of adaxial tissue identity causes defects in meristem formation or maintenance. For example ubiquitous expression of YABBY genes which promote abaxial cell identity in Arabidopsis organs leads to leaves with intermediate or mixed tissue identity on the adaxial surface and frequently to arrested meristems (Siegfried et al., 1999). Reduction in activity of other genes also affects both adaxial tissue and meristem function. For example, in pnh mutants the SAM is arrested, enlarged and composed of vacuolated cells (Moussian et al., 1998; McConnell and Barton, 1998) suggesting they have differentiated inappropriately, ago pnh double mutant embryos produce abaxialized lateral organs and also fail to form functional meristems (Lynn et al., 1999) suggesting that PNH functions redundantly with AGO in organ polarity but has AGO-independent functions in the meristem (Siegfried et al., 1999). These roles are consistent with the expression domain of PNH which includes the meristem and adaxial domain of lateral organs. Finally, phan mutants which lack dorsal tissue in apical lateral organs are defective in meristem maintenance throughout development when a cold sensitive pathway is removed at non-permissive temperatures. Since PHAN is expressed in lateral organ initials and primordia a non-cell-autonomous PHAN-dependent signal must be required to maintain activity (Waites et al., 1998).
1.8.4 SIGNALS WITHIN ORGAN PRIMORDIA

Interactions intrinsic to lateral organ primordia also appear to be important for their development. In *Antirrhinum* lateral growth of the leaf lamina is postulated to depend on interaction of adaxial and abaxial cell types along their boundary. This is demonstrated by the radially symmetrical later leaves of *phan* mutants which consist of ventral tissue only and consequently lack an adaxial-abaxial boundary (see below). Also ectopic boundaries between adaxial and abaxial cell type sectors in *phan* heart shaped leaves produce novel laminal outgrowths (Waites and Hudson, 1995). Similar loss of an adaxial-abaxial boundary and lamina growth is also found in the *phb-ld* mutant which gains, rather than loses, dorsal identity. (McConnell and Barton, 1998).
1.9 HYPOTHESES FOR PHAN FUNCTION IN LATERAL ORGAN DEVELOPMENT

The impetus for this work was based on the information available at the time which led to a hypothesis that \textit{PHAN} was required for aspects of lateral organ axis specification. The function of \textit{PHAN} was unclear because characterisation of \textit{PHAN}-homologues in \textit{Arabidopsis} and maize revealed differences in their contribution to lateral organ axis specification. These differences may have resulted from varying function of \textit{knox} genes that were repressed by \textit{PHAN}-homologues in each species, may have been due to differences in timing and position or expression of the \textit{knox} genes or been caused by other factors which may mask the features of the \textit{PHAN} phenotype in other species, (section 1.7.2, table 1.). However, from examination of wild type and \textit{PHAN} mutant leaves it appeared that \textit{PHAN} was required to specify adaxial cell identity and lamina outgrowth contingent on interaction of adaxial and abaxial tissue (Fig. 7). Additionally, transverse sections of wild type and \textit{PHAN} mutant leaves suggested that the lamina develops from the adaxial domain of the primordia (Fig. 5; Waites and Hudson, 1995). \textit{PHAN} mRNA is transcribed evenly throughout the lateral organ initials and early primordia when the adaxial-abaxial axis is being elaborated but the leaf phenotype suggested a restricted requirement for \textit{PHAN} to specify adaxial cell fate. Therefore, other spatially restricted factors were expected to interact with \textit{PHAN} to specify the difference between the adaxial and abaxial leaf domains.

![Fig. 7. A model for PHAN function in specification of lateral organ axes. (A) PHAN mRNA is expressed throughout the radially symmetrical lateral organ primordia but at (B) the time when it becomes flattened and the adaxial abaxial axis is being specified \textit{PHAN} protein might be modified to specify the difference between the adaxial and abaxial domains. The radial abaxialized leaves of severe \textit{phan} mutants and patches of tissue with abaxial identity in \textit{phan} heart shaped leaves suggests that an abaxial fate is the default. Therefore \textit{PHAN} protein may be modified in the adaxial domain to directly regulate adaxial identity or a gene downstream of \textit{PHAN} may specify the adaxial domain. Since \textit{PHAN} was the only known gene for this process modification of \textit{PHAN} was the obvious place to start this enquiry. (C) several pieces of evidence support the idea that lamina outgrowth is promoted by interaction of tissue with adaxial and abaxial identities (see sections 1.7.2 and 1.8.4)](image-url)
Modification of \textit{PHAN} expression might occur at the level of translation of \textit{PHAN} mRNA but its expression throughout the primordia might equally reflect a requirement for \textit{PHAN} protein in both domains. Therefore, the function of \textit{PHAN} protein might be modified in a spatially restricted manner and this possibility was pursued because it had greater potential to reveal information about \textit{PHAN} function and was easier to test than transcriptional modification.

\textit{Mybs} and other proteins in plants have previously been shown to interact and provide an example of how interaction and transcriptional activation may spatially modify \textit{PHAN} protein function. The best characterised example comes from developmentally regulated expression of anthocyanins in maize by the \textit{Mybs C1 and Pl} which interact in an obligate fashion with bHLH proteins \textit{R} and its homologues \textit{B}, \textit{SN} or \textit{LC} to enable transcriptional activation. Interaction occurs between the MYB domain of \textit{C1} with the N-terminal domain of \textit{B} (Goff \textit{et al.}, 1992). A second example of interaction with a plant \textit{Myb} is that of \textit{GLABROUS1 (GL1)} which regulates trichome formation in \textit{Arabidopsis} (Szymanski \textit{et al.}, 1998). Experiments with the yeast 2-hybrid assay suggest that a bHLH protein, \textit{GL3}, mediates interaction between \textit{GL1} and \textit{TRANSPARENT TESTA GLABROUS} which contains WD40 repeats (TTG, Walker \textit{et al.}, 2000; Payne \textit{et al.}, 2000). \textit{GL1} is required for trichome cell expansion, branching and differentiation and is regulated by gibberellic acid (Perazza \textit{et al.}, 1998).

Like the majority of plant \textit{Mybs} \textit{PHAN} has two \textit{Myb} repeats consisting of approximately 50 amino acid residues in a helix-turn-helix (HTH) DNA binding motif. This motif is well conserved between animals plants and yeast (Rosinski and Atchley, 1998) but also occurs in other DNA binding proteins such as the homeodomain. The main examples of 3-repeat \textit{Mybs} in vertebrates are \textit{aMyb}, \textit{bMyb} and \textit{cMyb} which are all 3-repeat \textit{Mybs}. Phylogenetic analysis of \textit{Mybs} in animals and plants suggests that the plant \textit{Mybs} evolved from a three repeat ancestor by deletion of repeat 1 (Lipsick, 1996; Jin and Martin, 1999). The majority of plant \textit{Mybs} therefore consist of repeats 2 and 3 and are referred to as \textit{R2R3 Mybs}. The
R2R3 Mybs have radiated to control numerous plant specific processes including secondary metabolism and differentiation (Jin and Martin, 1999) and the number of Mybs in a plant species may exceed 97 (Kranz et al., 1998). Three repeat Mybs also exist in plants (Kranz et al., 2000; Braun and Grotewold, 1999) but their function has not yet been fully characterised. In animals the number of Myb genes is low and examples with 1 or 3 Myb repeats exist but R2R3 examples have not been found.

Comparison of PHAN with other Mybs gave little reason to suppose that PHAN-homologues functioned similarly to the animal Mybs because of the presence of 3-repeat Mybs in plants as well as low amino acid sequence homology between animal Mybs and plant R2R3 Mybs outside of the Myb domain. There also appeared to be little conservation of secondary structure based on α-helix forming or breaking predictions and hydropathy plots as shown in (Fig. 8). However, the structure of the Myb domain is conserved and interactors with the C-terminal domain may have undergone co-evolution in plants and animals as have plant and animal components of polycomb complexes which interact by similarly positioned domains that bear little sequence homology in animals and plants (Spillane et al., 2000). Consequently the interactions of animal Mybs may also provide examples for protein modification that provide a model for modification of PHAN protein function.

In aMyb and bMyb the C-terminal domains appear to act as negative regulatory domains and may be regulated by phosphorylation which would entail interaction with kinases (Lane et al., 1997; Ziebold et al., 1997a,b; Sala et al., 1997; Aziz et al., 1995). A similar domain is present in cMyb which includes a motif called the EVES motif. The EVES motif apparently undergoes intramolecular interaction with the cMyb DNA binding domain. This interaction may compete with a co-activator protein, p100, which binds the DNA binding domain and also contains an EVES motif. A model for p100 function entails disruption of intramolecular interaction between the Myb EVES motif and DNA binding domain which enables transcriptional activation by binding of CBF (Dash et al., 1996; Ness, 1996). The EVES motif is not particularly well conserved within other animal Mybs so could be a specific regulator of cMyb alone (Aziz et al., 1995; Aziz et al., 1993). cMyb and aMyb also have a centrally located transcriptional activation domain which is shifted
Fig. 8. Comparison of Myb proteins from animals and plants based on α-helical forming or breaking plots (red; Chou and Fasman, 1978); and hydrophathy plots (black; Kyte and Doolittle, 1982). α-helix forming and breaking are above and below the line respectively. Plots have the same scales as for PHANL1. (x-axis) amino acid residues; (y-axis) hydrophobicity. The plots of plant Mybs are aligned with those of animal Mybs based on homology of the R2 and R3 repeats.
more C-terminally in bMyb (Weston and Bishop, 1989; Weston, 1998). The core binding factor histone acetyltransferase (CBF/p300) binding site encompasses the transcriptional activation domain of cMyb and aMyb and potentiates their transactivation (Hernandez-Munain, et al., 1994) as it does a number of other transactivators involved to different extents in hematopoiesis such as C/EBPα, PU.1 and Ets-1 (see Ward, et al., 2000). The C-terminal negative regulatory domain of cMyb has been postulated to form a leucine zipper (Biedenkapp et al., 1988; Kanei-Ishi et al., 1992) which mediates dimerization at higher protein concentrations and prevents cMyb binding to DNA (Nomura, et al., 1993). In support of this deletion of the C-terminal domain increases transcriptional activation activity of cMyb (Sakura et al., 1989; Weston et al., 1989). Other evidence that protein interactions occur with the negative regulatory domain come from characterisation of cMyb in yeast where cMyb is transcriptionally active. This suggests that transcriptional repression in animals required species specific factors (Chen and Lipsick, 1993; Seneca et al., 1993). The existence of the cMyb leucine zipper has been challenged however based on circular dichroism spectroscopy measurements of α-helical content (Ebneth et al., 1994) despite this the region including the putative leucine zipper is required for protein-protein interaction of some kind because substitution of the leucine residues to either proline or alanine abrogates the interaction with two proteins, p67 and p160 which can be isolated with cMyb by pull-down experiments from hematopoietic cell line extracts (Favier and Gonda, 1994). The function of p67 binding to Myb is unknown however. A second domain called the FAETL domain (Fu and Lipsick, 1996) which is highly conserved in cMyb and aMyb and less so in bMyb proteins, encodes a determinant involved in proteolysis so protein-protein interaction with this region may be required for regulated Myb degradation (Bies and Wolff, 1997).

The numerous protein interactions including transcriptional activation and protein degradation that occur with Mybs in plants and animals demonstrate potential for modification of PHAN activity that may bring about spatially restricted modification of its function. The simplest approach to finding interactors with PHAN was via an in vitro method or better still the yeast 2-hybrid assay because of its utility and efficiency and that was the approach taken here.
2 YEAST METHODS

2.1 THE YEAST TWO HYBRID ASSAY

The yeast 2-Hybrid assay has been described previously by Fields and Song (1989). The system used here utilises the GAL4 transcriptional activator and Matchmaker vectors manufactured by CLONTECH Laboratories. The assay depends on the properties of the GAL4 transcriptional activator which can be divided into two functional domains: the GAL4 DNA binding domain (GAL4-DB) and the GAL4 activation domain (GAL4-AD), (Brent and Ptashne, 1985; Keegan, Gill and Ptashne, 1986; Ma and Ptashne, 1987). Transcriptional activity can be restored \textit{in vivo} if the two domains of the GAL4 transcriptional activator are tethered by fusing them to interacting proteins (Ma and Ptashne, 1988)

Fusion proteins with the GAL4-DB and GAL4-AD were made in the yeast shuttle vectors pGBT9 and pACT2 or pGAD424 respectively, (Bartel \textit{et al.}, 1993; Li \textit{et al.}, 1994). Each has a polylinker 3' of the region encoding the GAL4 domain enabling sequences to be cloned in-frame for expression of fusion proteins. Fusion proteins with the GAL4-DB were termed "baits" (Gyuris \textit{et al.}, 1993) because the DNA incorporated in the plasmid construct encoded a protein with which interaction was being sought (Figure 2.). Fusion proteins with the GAL4-AD were termed "preys" because they represent part or all of an unknown protein which was being sought. pGBT9 and pGAD424 or derivatives were also used to test for interaction between the products of known genes and the same terminology was used for convenience. Because the stoichiometry of interaction is not revealed by the yeast 2-hybrid assay, terminology appropriate to the lowest order of oligomerization possible (that is 1:1 or dimerization) will be used here.

2.2 YEAST STRAINS USED IN THE YEAST TWO HYBRID ASSAY

Yeast host strains used currently in the yeast 2-hybrid assay are modified to enable selection for plasmid retention. Additionally detection of fusion protein interaction is made possible by two or more chromosomally located reporter genes. Consequently, the copy number of reporter genes required to detect interactions remains stable regardless of the fusion protein combination.
The *Saccharomyces cerevisiae* strain Hf7c (Feiloter et al., 1994) was mainly used as a host in this work. Hf7c is auxotrophic for leucine and tryptophan due to deletion of the *TRP1* and *LEU2* genes needed for their biosynthesis. This enables selection for transformation with bait and prey vectors carrying *TRP1* or *LEU2* markers on media lacking tryptophan or leucine, respectively. Fusion protein interaction is detected by activation of a *HIS3* gene under control the *GAL1* upstream activator (UASG, also called GAL4 UAS) which enables transformants with GAL4 activity to grow on media lacking histidine (shown in Fig. 9. as 'prey X'; Chien et al., 1991; Durfee et al., 1993).
Fig. 9. The yeast 2-hybrid assay. Bait and prey fusion proteins are expressed in yeast from plasmids that can be selected for with tryptophan (w) and leucine (L) biosynthesis markers expressed by the plasmids. The yeast strain is auxotrophic for these as well as histidine (H). Interaction is detected on media lacking LW H when fusion proteins are present that can interact. This reconstitutes the GAL4 transcriptional activator by bringing the DNA binding and activation domains together (interactor prey x and PHAN bait). To help discriminate prey fusion proteins that can activate the HIS3 reporter gene by bypassing interaction and binding the activating element directly (prey y), a second reporter (β-galactosidase) gene is present under control of a variant of the GAL4-UAS.
To help eliminate false interactors that activate *HIS3* by binding directly to the GAL4 UAS (Fig. 9. ‘prey y’) the second reporter gene, β-galactosidase, is under control of a trimer of the 17 bp GAL4 consensus binding site (Giniger *et al.*, 1986) and the weak minimal promoter of the cytochrome C1 gene. Transformants in which GAL4 activity has been restored by genuine protein-protein interaction should show activation of both reporter genes regardless of the differences in DNA sequence of the activator and promoter regions but in some strains the level of activity is different.

### 2.3 AUTOACTIVATION BY BAIT FUSION PROTEINS

Because bait fusion proteins are tethered to the reporter gene promoters by the GAL4-DB domain, any activation potential within the bait may confound the screening process. For this reason bait constructs were tested for autoactivation before use. To identify baits with activation potential yeast were transformed and plated on -trp media. Colonies that grew, indicating that they had retained the bait construct, were then streaked on -trp -his media, to test for autoactivation of *HIS3* and on -trp plates for a paper lift β-galactosidase assay.

### 2.4 TRANSFORMATION OF YEAST

To enable efficient screening of the prey library, yeast were transformed sequentially: HF7c was transformed with the bait vector alone before being sub-cultured and retransformed on a large scale with the prey library.

#### 2.4.1 SMALL SCALE TRANSFORMATION OF YEAST

Routine transformations of yeast were done by the method of Klebe *et al.*, (1983). This allowed competent yeast to be stored for up to one year at -80°C ready for transformation. Alternatively transformations were done by the method of Gietz *et al.* (1992). Briefly, a colony of the strain to be transformed was grown in 10 ml of appropriate medium at 30°C overnight, 50 ml of medium was inoculated with this to OD$_{600}$ = 0.01 and grown with shaking at 30°C to an OD$_{600}$ between 0.5 and 1.0. The culture was then pelleted at 6900 x g in a benchtop centrifuge for 5 minutes. The cells were then resuspended in 1.5 ml of 10 mM Tris.HCl pH 7.5, 1 mM EDTA, 100 mM LiAc, and pelleted at 13000 x g in a microfuge for 10 seconds. The cells
were then resuspended in 250 μl TE, LiAc as above and 50 μl aliquots used for each transformation. To each aliquot was added 1 μg of transforming DNA, 50 μg of denatured salmon sperm carrier DNA and 300 μl of transformation media (40% PEG, 10 mM Tris.HCl pH 7.5, 1 mM EDTA) before vortexing for several seconds and incubation with agitation at 30°C for 30 minutes. The cells were then heat shocked at 42°C for 15 minutes. After heat shock the cells were pelleted briefly in a microfuge, resuspended in 100 μl TE, plated on selection plates and grown at 30°C for two to five days.

2.4.2 LARGE SCALE PREY LIBRARY TRANSFORMATION

Large scale transformations for library screens were done by the lithium acetate, heat shock method described by Geitz et al., (1995), but modified as described below to optimise the heat shock and cell culture of HF7c containing PHANTASTICA bait plasmids. 100 ml of special yeast medium consisting of 50% SD-W and 50% YPDA was inoculated with several colonies of HF7c carrying pGBT9-PHAN and allowed to grow overnight at 30°C with shaking at 250 rpm. Based on the standardisation curve of OD₆₀₀ for HF7c harbouring PHAN-bait constructs a volume of culture that would yield 2.5 x 10⁹ cells was pelleted in a 50 ml Falcon tube for 4 min at 2333 x g and washed in 10 ml dH₂O. The cells were used to inoculate 1 litre of pre-warmed SD-W medium in two 2 l flasks containing 500 ml of medium each. These were grown at 30°C as above to OD₆₀₀ = 0.68 to provide approximately 1x10¹⁰ cells in log phase growth. The cells were harvested at 3000 x g for 5 min in a GSA Sorval centrifuge and washed in a total volume of 11 dH₂O. They were then pelleted and washed in a total volume of 500 ml of dH₂O. The cells were then resuspended in a total volume of 80 ml 0.1M LiAc and distributed to three 50 ml Falcon tubes for pelleting in a benchtop centrifuge at 2333g for three minutes. Each tube of cells was then resuspended in 20 ml of 0.1M LiAc. 2ug/ul of salmon sperm carrier DNA with an average size of 7 kb was then boiled for 5 minutes before chilling on ice. Meanwhile the cell suspension was pelleted and the supernatant removed. Each tube received in the following order; 8 ml of 50% polyethylene glycol 5000, 1.2 ml of 1M LiAc pH7.5, 833 μl of 2 μg /μl boiled carrier DNA, 17 μl of library DNA and the volume made up to 12 ml with dH₂O. This was vortexed, incubated at 30°C for 30 minutes,
heat shocked at 42°C for 56 minutes with gentle mixing at 5 minute intervals before being pelleted to remove the supernatant and resuspended in 4.83 ml of water per tube. 1 μl of transformation was removed and diluted 100-, 500- and 1000-fold and 100 μl of each dilution plated on -LW agar to determine the co-transformation efficiency. 250 μl of transformation was plated on each of sixty 14 cm diameter -LWH selective plates with or without 5 mM 3-aminotriazole (3AT) and incubated for 10 to 15 days.

This method met the following criteria which have been found to increase the transformation efficiency of yeast (Geitz et al., 1995). First, the cells had undergone at least two cell divisions in fresh media and were therefore in log phase growth. Second it was hypothesised by Geitz et al. that double stranded DNA is absorbed specifically and that single stranded DNA prevents it binding to sites on the yeast cell surface where it is not absorbed. The protocol ensured that single stranded carrier DNA and double stranded library DNA were in the optimum proportion. Thirdly LiAc, which promotes uptake of DNA by yeast cells, and polyethylene glycol, which is known to precipitate DNA onto the yeast cell surface, were both present in the optimum concentration for transformation. Fourthly the heat shock, which appears to have a significant effect on the transformation process had been optimised for the plasmid constructs and yeast strain used in this work. Heat shock proteins act as molecular chaperones to protect important proteins from stress induced degradation (Ellis and van der Vies, 1991). Heat shock induced changes in the levels or activities of specific proteins might affect either the transport of plasmid DNA from the cell membrane to the nucleus or eliminate a cellular component which degrades it (Geitz et al., 1995). Finally a suitable plating density had been used for a screen using HF7c without 3AT in the medium. 3AT acts as an inhibitor of the histidine pathway (Kishore and Shah, 1988) and is used with some strains of yeast to suppress leaky expression of the HIS3 reporter gene. In Hf7c the HIS3 gene is under much tighter control, so 3AT is not necessary at low plating density. The plating density used here was low enough that background growth supported by nutrients derived from dead cells does not cause weak interactors to go undetected because they are indistinguishable from background. Avoiding use of 3AT in the media increased the sensitivity of the screen since 3AT might suppress growth of weak interactors.
2.5 SCREENING OF POSITIVES FROM THE YEAST TWO HYBRID ASSAY

Two methods were used to test colonies that were growing under histidine selection for activation of the β-galactosidase reporter gene. All colonies from the first two screens were tested using an X-gal overlay assay described below. In subsequent screens, colonies that had achieved a diameter greater than 1.5 mm and continued to grow after 5 days on -LWH medium were selected as potential positives. Initially these colonies were re-streaked on -LWH plates containing 10 mM 3AT to ensure that growth was independent of background sources of histidine.

2.6 TESTS FOR AUTOACTIVATION BY THE PREY FUSION PROTEINS

To ensure that activation of the yeast reporter genes by prey plasmids was bait dependent, prey plasmids were tested alone for activation of the reporter genes in yeast. Yeast were transformed with the isolated prey plasmids and grown on -L medium to select for plasmid retention. Colonies were then tested for reporter gene auto-activation by streaking on -L medium for the β-galactosidase assay and on -LH to test for histidine reporter gene activation.

2.7 β-GALACTOSIDASE REPORTER GENE ASSAYS

2.7.1 X-GAL OVERLAY ASSAY

Colonies derived from library transformations were tested in situ for β-galactosidase activity using an X-gal overlay assay (Micheline Fromont, unpublished). The overlay was prepared in 250 ml batches in heavy glass bottles that would stay submerged in a water bath. Each bottle contained 2 g of Bacto agar that had been autoclaved in 105 ml of dH2O. To each was added 125 ml of 0.5 M phosphate, pH 7.0 (86.5g Na2HPO4 with 2ml of orthophosphoric acid in a final volume of 500 ml dH2O), 2.5 ml 10% (w/v) SDS and 15 ml DMF. The overlay was then maintained at 50°C in a water bath and 2.5 ml of 2% (w/v) X-gal in DMF added just before pouring. The overlay was poured in a fume hood onto a region of each plate lacking colonies and allowed to spread evenly across the whole plate. When the overlay had set, the plates were sealed and incubated at 30°C for several days. Blue colonies were recovered by streaking onto -LWH agar.
2.7.2 FILTER LIFT X-GAL ASSAY

Alternatively activation of the β-galactosidase reporter gene was tested by a method modified from Breedon and Nasmyth (1985). Yeast were grown on Whatman filter paper circles which had been sterilized by exposing them to 40 J/cm² of ultraviolet light in a transilluminator and laid on selective media plates. Up to six separate yeast colonies were streaked onto the surface. The cells were allowed to grow at 30°C for several days. The assay was then done by soaking a 9 cm diameter filter paper with 1.8 ml of z-buffer (60 mM Na₂PO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, freshly prepared with 1 mg/ml X-gal, 38 mM β-mercaptoethanol) in a 15 cm Petri dish. Yeast samples supported on the filter paper were separated from the growth medium and permeabilized by freezing in liquid nitrogen for 15 seconds before being placed on the z-buffer filters to thaw. The Petri dish was sealed with Parafilm and incubated at 30°C from half an hour to over-night for development of the assay.

2.7.3 COMPARATIVE YEAST 2-HYBRID ASSAYS

Because the amount of growth observed in assays can be proportional to the amount of cells initially applied to the media, measures were taken to ensure similar amounts of cells were streaked on plates in comparative assays such as that shown in Fig. 10. All the yeast strains used in an assay were grown fresh on selective media. Colonies were then suspended in a sterile ependorf in water. The OD₆₀₀ was then measured by placing the ependorf in a specially fashioned carrier cuvette and the cell concentration adjusted to within 0.1 OD unit of OD₆₀₀= 1.0. Cells were then all steaked onto large media plates from this suspension with sterile toothpicks before being incubated for 3 or 4 days at 30°C.

2.8 RECOVERY OF PREY PLASMIDS

Prey plasmids were recovered from yeast colonies by preparing bulk DNA by the method of Hoffman and Winston (1987). A large colony from a plate or 1.5 ml of overnight culture was pelleted in a microfuge, 0.2 ml of yeast lysis buffer (2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris.HCl pH 8.0, 1.0 mM EDTA) was added along with 0.2 ml of phenol:chloroform and 0.3 g of crushed glass beads. This was centrifuged in a microfuge for 5 minutes at 13000 x g.
before vortexing and centrifuging again. The supernatant was transferred to a new tube and the DNA precipitated at room temperature with 0.1 volume of 3M sodium acetate pH 5.0 and 2 volumes of ice cold ethanol followed by centrifugation for 10 minutes at top speed in a microfuge. The pellet was then washed with 70% ethanol to remove salt before drying and dissolving in 50 μl of TE.

*E. coli* HB101 were then transformed by electroporation with 1 μl of the DNA preparation and plated on M9 plates lacking leucine and containing ampicillin as described in section 2.9. Only HB101 containing prey vector plasmids can grow under these conditions due to complementation of the bacterial *B6* leucine pathway gene by *LEU2* and ampicillin resistance conferred by the vector. Plasmids were then purified from HB101 for further analysis by general methods as described by Maniatis *et al.* (1982).

### 2.9 YEAST MEDIA

Yeast complete media (YPDA) contained 10 g Difco Bacto yeast extract, 20 g Difco Bacto peptone, 20 g glucose, 0.02 g adenine sulphate per litre.

Yeast minimal media (YMM) contained 6.7 g yeast nitrogen base without amino acids (T. Becton Dickinson, Oxford), and 20 g of glucose per litre.

Selective yeast media (SD) was made by adding 400 mg of amino acid dropout powder (described in Table 3.) to 500 ml of yeast minimal media and making it approximately pH 5.8 with 1 ml of 2 M NaOH. If solid media was being made 0.02 g/ml of Difco Bacto agar was added before autoclaving at 117°C for 20 minutes. The following filter sterilized amino acid stocks in dH2O were then added after autoclaving as required.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>volume added to 500 ml</th>
<th>final concentration</th>
<th>inclusion when Selecting for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>bait</td>
</tr>
<tr>
<td>250 mg/50 ml L-Tryptophan</td>
<td>4 ml</td>
<td>40 μg/l</td>
<td>-</td>
</tr>
<tr>
<td>750 mg/50 ml L-Leucine</td>
<td>2 ml</td>
<td>60 μg/l</td>
<td>+</td>
</tr>
<tr>
<td>1 g/50 ml L-Histidine·HCl·H₂O</td>
<td>1 ml</td>
<td>40 μg/l</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 3. Proportions of amino acids included in amino acid dropout powder.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Adenine hemisulphate</td>
<td>0.4 g</td>
</tr>
<tr>
<td>salt</td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Arginine.HCl</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0.6 g</td>
</tr>
<tr>
<td>L-Lysine.HCl</td>
<td>0.6 g</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>1.0 g</td>
</tr>
<tr>
<td>L-Proline</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Serine</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>0.6 g</td>
</tr>
<tr>
<td>Uracil</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Valine</td>
<td>1.5 g</td>
</tr>
</tbody>
</table>
3 MOLECULAR METHODS

3.1 SCREENING OF CDNA LIBRARIES IN BACTERIOPHAGE λ

Bacteriophage λ cDNA libraries were screened as described by Benton and Davis (1977). For culture of phage, plating cells were prepared by picking single colonies of C600 hflA- into two 5 ml bottles of LB with 10 mM MgSO₄ and 0.2% maltose. They were then grown overnight before being pelleted in a single 10 ml bottle and resuspended in 10 mM MgSO₄ to a final OD₆₀₀ = 1.2. The cells could then be stored at 4°C for up to two weeks.

Phage plaques were produced on lawns of E. coli by inoculating 100 μl of plating cells with 1 μl of phage. These were left at 37°C for 15 minutes for infection to occur. The cells were then suspended in 3.5 ml of BBL agar which was previously melted and made 0.2% maltose, 10 mM MgSO₄ and cooled to 46-48°C in a water bath. The cell suspension was rapidly mixed by swirling and plated on LB plates before being allowed to set and incubated at 37°C overnight. When plaques had achieved a suitable size the plates were chilled for 1 hour to prevent lifting of the bacterial lawn. Phage DNA was transferred to nitrocellulose filters by laying them on the surface of the plates. The plates and filters were marked for reorientation by stabbing three times asymmetrically through the periphery with a hypodermic needle. The filters were left on the plates for a maximum of two minutes before removal and denaturing of the phage DNA in DNAT (1.5 M NaCl, 0.5 M NaOH) for 5 minutes. They were then neutralised in NEUT (1 M Tris.HCl pH 8.0, 1.5 M NaCl) for 5 minutes, washed in 2 x SSC (1 x SSC is 50 mM NaCl, 5 mM Na₃citrate, pH 7.0) for 5 minutes to remove cell debris and dried in air on Whatman 3MM paper. To fix the DNA to the nitrocellulose the filters were baked in a vacuum oven at 80°C for 45 minutes. Filters were then prehybridised to prevent non specific binding of the probe to the filter, for one hour in prehyb. (4 x SSC, 1% (w/v) SDS, 0.5% (w/v) dried skimmed milk) with shaking at 65°C. Hybridization with 32P labelled DNA probes (made as described below) was done at 65°C with shaking overnight. The next day probe solution was removed and stored frozen at -20°C for further use. Filters were washed once for 5 minutes and one to two times for 20 minutes at 65°C to remove background signal in 2 x SSC, 0.5 % SDS. Autoradiography was done using Cronex 4 medical X-ray film exposed at -70°C with intensifying screens before developing in
an automatic developer. A core of each positive plaque was picked with a 0.8 mm diameter glass capillary tube and phage eluted for seven hours in 200 μl of 10 mM MgCl₂. A 1 μl aliquot from a 1/50 dilution was then used to re-infect plating cells and the process repeated until single pure plaques were obtained.

3.2 PURIFICATION OF λgt10 DNA

The titre of phage from single plaques was determined by plating 1/50, 1/100 and 1/500 dilutions of phage eluate as described above. 3 x 10⁶ phage/ml were then used to infect 50 ml of C 600 hflA⁺ freshly grown overnight in LB with 10 mM MgCl₂ and 0.2% maltose. The culture was then left shaking at 37°C until lysis occurred. To lyse any remaining cells the culture was then incubated as above for 20 minutes with 1% chloroform. Further steps were done using a Qiagen Lambda DNA mini prep kit with the following modification. Phage particles were collected by adding 100 μl of buffer L1 followed by solid polyethylene glycol (PEG) 6000 and NaCl to 6% and 0.6% (w/v) respectively. The NaCl and PEG were allowed to dissolve and the mixture incubated at 4°C for 20 minutes before centrifugation at 10 000 x g in 15 ml Corex tubes to pellet bacteriophage particles. All further steps, from addition of buffer L3, were done as described by Qiagen. cDNA inserts were then recovered from positive clones by PCR using primers λGT10 FWD and RVS (Table 5.) and purified phage DNA template. PCR products were gel-purified, their ends polished with T4 DNA polymerase and cloned into Eco-RV-digested pBluescript for sequencing.

3.3 IN VIVO EXCISION OF cDNA CLONES FROM λ ZAP AND λ ZAP EXPRESS

cDNA clones in the pBluescript II phagemid (λ ZAP) and pBKCMV phagemid (λ ZAP EXPRESS) were excised as described in the λ ZAP EXPRESS instruction manual, but E. coli JM109 and R408 helper phage (STRATAGENE) were used in place of the XLOR strain and Exassist helper phage. In vitro excision is possible because the initiator DNA placed 5' of the phagemid sequence within λ ZAP provides a signal for proteins encoded by the M13 R408
helper phage to nick a single DNA strand and commence single stranded DNA replication of the phagemid. DNA replication is terminated 3' of the phagemid sequence at the terminator signal. The single stranded phagemid DNA is then circularised by the M13 gene II product, packaged into M13 particles and secreted from the *E. coli* cells.

To make use of these processes JM109 cells were co-infected with both λ and M13 helper phage as follows: a plug from a positive λ plaque was eluted overnight in 500 μl of SM buffer (100 mM NaCl, 10 mM MgSO₄, 50 mM Tris.HCl pH 8.0) with 20 μl of chloroform to lyse remaining *E. coli*. A colony of JM109 was grown with shaking at 37°C overnight in 5 ml of LB, 10 mM MgSO₄ and 0.2% maltose before being resuspended in 10 mM MgSO₄ to OD₆₀₀ = 1.0. In a 10 ml Falcon tube, 250 μl of the eluted λ phage in SM and 1 μl of R408 helper phage (titre greater than 1x10⁶ plaque forming units/μl) were added to 200 μl of the JM109 preparation. This was then incubated at 37°C for 20 minutes to allow infection. Next 3 ml of 2x YT was added and incubated for three hours with shaking at 37°C to allow packaging and secretion of M13 phagemid particles. The culture was then heated at 70°C for 20 minutes to destroy JM109 cells and phage λ before being spun in a benchtop centrifuge at 1000 x g for 15 minutes to pellet cell debris. To make double stranded phagemid, 200 μl of the supernatant containing the M13 phagemid was used to re-infect 100 ml of JM109 cells for 15 minutes at 37°C before pelleting the cells at low speed and plating them in 100 μl of 2x YT on LB plates containing appropriate antibiotic selection. pBluescript phagemid and pBKCMV were selected using 100 μg/ml of ampicillin or 30 μg/ml of kanamycin sulphate, respectively.

### 3.4 SOUTHERN BLOTTING AND HYBRIDIZATION WITH ³²P LABELLED DNA PROBES

To enable DNA to be analysed by Southern hybridisation it was transferred to nylon filters (Hybond-N, Amersham Pharmacia Biotech) by Southern blotting (Southern, 1975; Wahl et al., 1979). DNA in 7 x 10 cm meniscus gels was depurinated by treatment in 0.25 M HCl for five minutes. It was then incubated with shaking for 20-25 minutes in DNAT. This both denatured the DNA to enable
efficient hybridisation of the probe to target and fragmented it at depurinated sites to reduce the size of fragments and so promote transfer of DNA from the gel. The pH was then reduced by incubation with gentle shaking for 25 minutes in NEUT. DNA was transferred from the gel to the filter by sandwiching the filter between the gel and Whatman 3MM paper backed by several layers of paper towel so that capillary flow of the buffer from the gel into the paper wadding would carry the DNA onto the filter. This process was done overnight at room temperature. DNA was then cross-linked to the Hybond-N by exposing the filter to 40 J/cm² of ultraviolet light in a transilluminator.

To block non-specific binding of probe to the filter it was prehybridised for one hour in prehybe (4 x SSC, 1% (w/v) SDS, 0.5% (w/v) dried milk) with shaking at 65°C. Hybridization was done overnight in plastic boxes at 65°C shaking with ³²P labelled DNA probe in prehybe solution. The next day, the probe was removed and stored frozen at -20°C for further use. Filters were then washed once for 5 minutes and one or two times for 20 minutes at 65°C in 2 x SSC, 0.5% SDS to remove background signal. Autoradiography was done using CRONEX 4 medical X-ray film with intensifying screens at -70°C overnight or longer as for phage plaque-lifts (above).

3.5 PRODUCTION OF ³²P LABELLED DNA PROBES FOR HYBRIDISATION

³²P labelled probes were generated by random oligonucleotide priming (Hodgson and Fisk, 1987). 25 ng of template DNA in 15.5 μl of dH₂O was boiled for 5 minutes to make it single stranded and then chilled on ice before addition of 6 μl of oligo labelling buffer (250 mM Tris.HCl pH 8.0, 25 mM MgCl₂, 5 mM β-mercaptoethanol, 2 mM each of dATP, dTTP, dGTP, 1 M HEPES pH 6.6, 1 mg/ml random hexamers), 1 μl of bovine serum albumin (10 μg/ml) and 1.5 μl of Klenow fragment of DNA polymerase I (1.5 units). This mixture was vortexed and collected in the bottom of the Eppendorf tube by briefly spinning in a microfuge before adding 30 μCi (3 μl) of [³²P]dCTP (3 Ci/μmole, Amersham) and incubating at 37°C for 45 minutes. Labelled DNA was then separated from unincorporated labelled nucleotide on a Sephadex G50 (Pharmacia) column in TE. The column was made in a 1 ml disposable syringe fixed in a retort and blocked inside the tip with Whatman 3MM
paper. About 30 μl of 5% (w/v) blue dextran, 0.5% (w/v) orange G was added to the labelling reaction and the whole applied to the Sephadex column. The labelling reaction was allowed to fully enter the Sephadex before washing it through the column with TE. The labelled probe typically eluted with the blue dextran while unincorporated [α^{32}P]dCTP, co-migrating with orange G, was left on the column for disposal. Before use the eluted probe was denatured by heating at 100°C for five to ten minutes.
3.6 EXTRACTION OF NUCLEIC ACIDS

3.6.1 PLANT GENOMIC DNA EXTRACTION

Antirrhinum and Arabidopsis genomic DNA was isolated by the method of Coen et al. (1986). 5-10 grams of leaf material was ground in a mortar and pestle with a pinch of ground glass and 1-2 ml of extraction buffer. For this purpose DNA extraction buffer consists of 150 mM NaCl, 15 mM sodium citrate, 0.1 M EDTA, 0.1 M sodium diethyldithiocarbamate and was made to 2% (w/v) SDS just before use. When all visible particles of plant material had been ground up, 1 ml the homogenate was transferred to an Eppendorf tube. 500 μl of chloroform was added and the sample vortexed for 40 seconds. After centrifugation at 13 000 x g in a microfuge for 5 minutes, the aqueous phase was recovered and extracted with an equal volume of 1:1 phenol:chloroform. After centrifugation as above, 750 μl of the aqueous phase was recovered so that the nucleic acids could be precipitated from it with an equal volume of ethanol. Nucleic acids were recovered by centrifugation at 13 000 x g for 10 minutes, the ethanol was removed and the pellet dissolved in 50-100 μl of TE. To remove RNA, 2-4 μg of RNAase A (Sigma) was added and the reaction allowed to proceed at 37°C for 15 minutes. The solution was then vortexed briefly, to ensure DNA had dissolved, and centrifuged at 13 000 x g for 5 minutes to pellet any insoluble material. The supernatant was removed to a new tube for precipitation of DNA. To each 100 μl of sample were added 14 μl of 5 M NaCl and 114 μl of 2% (w/v) CTAB in 50 mM Tris.HCl pH 7.5, 10 mM EDTA. The DNA and CTAB solutions were mixed by inversion and the resulting DNA precipitate recovered by centrifugation for 5 minutes at 13 000 x g. All CTAB solution was removed from the DNA pellet which was then washed at 4°C overnight in 500 μl of 70% (v/v) ethanol, 30% 15 mM NaCl. Finally the DNA was dissolved in 50 μl of TE pH 8.0.

3.6.2 SMALL SCALE PURIFICATION OF PLANT GENOMIC DNA

For screening of individual Arabidopsis plants, genomic DNA was isolated by the method of Edwards et al. (1991). 1-2 leaves were ground to a powder in an Eppendorf tube with liquid nitrogen and a disposable plastic pestle. 400 μl of Edwards buffer (200 mM Tris.HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% (w/v) SDS) was then added and the tube vortexed for five seconds. The tubes
were then centrifuged at 13 000 x g for one minute and 300 µl of supernatant recovered to a new tube. Nucleic acids were precipitated from this by adding 300 µl of isopropanol, mixing and leaving at room temperature for two minutes before centrifuging for five minutes at 13 000 x g in a microcentrifuge. The supernatant was then removed and the pellet dried briefly in a vacuum desiccator before being dissolved in 50 µl of TE. One µl was then used as template in a 10 µl PCR reaction.

3.6.3 ISOLATION OF BACTERIAL PLASMID DNA

Bacterial colonies containing plasmid clones were screened by picking single colonies into 1.5 ml of LB with appropriate antibiotic (section 3.20) and allowing them to grow overnight at 37°C with shaking. A 50 µl aliquot of this overnight culture was stored in a sterile microtitre plate at 4°C for larger scale culture of positive clones. Small amounts of plasmid DNA were prepared from the overnight culture using the alkaline lysis method of Ish-Horovitz and Burke (1981). 1.5 ml of cells were pelleted in a microfuge at low speed (6 000 x g) for one minute and the growth media removed. 100 µl of GTE (50 mM glucose, 25 mM Tris.HCl pH 8.0, 10 mM EDTA) was then used to resuspend the cells. 200 µl of Solution 2 (0.2 M NaOH, 1% SDS) was then added and mixed by inversion to lyse the cells. The reaction was left for four minutes before neutralisation with 150 µl of 3 M potassium acetate (made pH 5.6 by the addition of acetic acid) and again mixed by inversion. Cell debris, precipitated protein and potassium dodecylsulfate were then pelleted at 13 000 x g in a microfuge. Plasmid DNA was then precipitated from the supernatant by removing it to a new tube and adding two volumes of ice cold ethanol. After incubation at room temperature for 5 minutes the DNA was pelleted, washed in 70% ethanol and dissolved in 30 µl of Tris HCl containing 100 µg/ml of RNAse A. Normally 3 µl of this preparation was sufficient for restriction analysis and 1 µl of a 1:50 dilution could be used as template in a PCR reaction.

3.6.4 ISOLATION OF PLANT TOTAL RNA

Plant total RNA was purified by a method based on that of Martin and Northcote (1981).

Samples of 100-200 mg of tissue were collected, frozen in liquid nitrogen and stored at -80°C until use. The tissue was then thawed and ground promptly in a mortar and
pestle with a pinch of ground glass and 1 ml of freshly made extraction buffer. For this protocol RNA extraction buffer consists of 40 mM Tris.HCl pH 9.0, 120 mM LiCl, 4 mM EDTA and made to 2% (w/v) SDS just before use. To remove protein 1 ml aliquots were transferred to an Eppendorf tube and extracted with 500 μl of phenol-chloroform (1:1) by vortexing thoroughly and centrifugation at 13 000 x g. 800 μl of the supernatant was recovered and the process repeated with 750 μl of phenol-chloroform. To precipitate RNA, the solution was made 2 M LiCl by adding 0.2 volumes of 12 M LiCl stock solution and incubated at 4°C overnight. RNA was then pelleted by centrifugation at 13 000 x g for 5 minutes and dissolved in 200 μl of DEPC-treated dH2O at 80°C. RNA was dissolved by this method to minimise the time that it was held in aqueous solution and susceptible to RNAase. After dissolving the sample was chilled on ice and spun for 2 minutes to remove insoluble material before RNA was reprecipitated in a fresh tube with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. The RNA was finally pelleted at 13 000 x g for 5 minutes, washed with 70% ethanol, dried in a vacuum desiccator for 2 minutes and dissolved in 100 μl of DEPC-treated water. Upon dissolving a 1 μl aliquot was removed to be examined by gel electrophoresis and a 10 μl aliquot was removed for quantitation by spectrophotometry (see quantitation of nucleic acids). Samples were then immediately refrozen in liquid nitrogen and stored at -80°C.

3.6.5 NORTHERN ANALYSIS

RNA for Northern analysis was purified using TRIZOL (Life Technologies) according to their protocol. About 0.1 g of tissue from unexpanded leaves and organs or 0.3 g of older organs such as leaves and petals was required to yield 50-60 μg of total RNA. 25 μg of each RNA sample was then precipitated with sodium acetate and ethanol. Electrophoresis was done in agarose gels made as follows: sufficient agarose for 1% (w/v) was melted in 0.8 of the final gel volume made 1.25 x MOPS buffer (0.2 M MOPS, 50 mM NaAc, 5 mM EDTA). Once cooled to approximately 60°C, 0.2 volumes of 37% formaldehyde solution was added and the gel poured promptly in a fume hood. Samples were dissolved in 25 μl of sample buffer (500 μl formamide, 175 μl formaldehyde, 100 μl 10 x MOPS buffer) and 1/10th volume of loading buffer
added (505 glycerol, 1 mM EDTA, 0.4% each of xylene cyanol and bromophenol blue). The samples were then heated at 80°C for 5 min. immediately before loading. Gels were run at 8 V/cm in 1 x MOPS buffer with a peristaltic pump to recycle buffer between the electrodes.

3.6.6 QUANTITATION OF NUCLEIC ACIDS

DNA fragments purified from gels and PCR products cleaned on Qiagen columns were quantified by comparing their fluorescence with DNA molecular mass markers (GIBCO BRL) on a 0.7% agarose gel containing ethidium bromide using the EASY gel analysis program on a gel documentation system (Herolab, GmBH). Typically 2 μl of BRL high or low mass markers were compared with 3 μl of a 30 μl preparation from a Qiagen column eluate.

DNA and RNA were otherwise quantified with a 1 cm light path quartz cuvette in a Beckman DU-64 spectrophotometer. Nucleic acid samples were diluted 1-2:100 then the concentration determined assuming that 1 mg/ml of double stranded DNA has an OD_{260} = 20 and 1 mg/ml of RNA has an OD_{260} = 25.

3.7 DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES

For the purpose of cloning, plasmid vector or insert DNA was digested with 3-5 units of enzyme per μg of DNA in a 10 or 15 μl reaction volume using enzyme manufacturers’ buffers at 37°C for 4-12 hours before being purified electrophoresis in 0.7% agarose gels.

PCR products prepared for cloning were purified using the Qiagen PCR clean up kit before further cloning steps were taken.

3.8 LIGATIONS

DNA fragments prepared for cloning were quantified as described in section 3.6.6. For intermolecular ligations, target DNA was ligated with 50-100 ng of vector DNA so that the molar ratio of target to vector DNA was ~ 3:1. For intramolecular ligations approximately 25 ng of linear DNA was ligated in a 10 μl reaction. Reactions were done with 3 units of T4 DNA ligase (Promega) in the manufacturer’s buffer at 4°C overnight.
3.9 AGAROSE GEL ELECTROPHORESIS

Small quantities of DNA such as PCR products and restriction digests were examined on 0.7-1.0% meniscus gels (12 cm x 6 cm x 4 mm) or minigels (12 cm x 11 cm x 8 cm) made with and run in 0.5 x TBE (1 x TBE is 90 mM Tris.HCl, 90 mM boric acid, 2 mM EDTA) at an appropriate voltage.

3.10 ISOLATION OF DNA FROM AGAROSE GELS

Isolation of PCR products and restriction fragments from agarose gels was done using the Qiagen gel extraction kit following the manufacturer's instructions.

3.11 ELECTRO COMPETENT BACTERIA

For general cloning and recovery of ligated plasmids E. coli DH10B (Life Technologies) were found to yield the highest number of transformants (1x10¹¹ μg⁻¹ of pBluescript SK-). Cells were made competent for electroporation using the method of Hans Sommer (pers. comm.), with slight modifications. Two 500 ml volumes of LB in 2 litre flasks were each inoculated with 10 ml of DH10B overnight culture. The 500 ml inoculates were then grown for >20 hours at 18°C with shaking at 200 rpm until an OD₆₀₀ between 0.4 and 0.6 was achieved. Cultures were then chilled on ice and maintained ice cold during all subsequent steps of preparation. The cells were harvested in four 250 ml bottles at 5 800 x g in a Sorval GSA centrifuge rotor. The cells were then washed and concentrated as follows: the cells in each 250 ml bottle were resuspended in 100 ml of ice cold dH₂O and pelleted at 5 800 x g for 30 minutes. Each pellet was then resuspended in 20 ml of dH₂O and the four suspensions combined in two 50 ml Falcon tubes. These were centrifuged at 4 000 x g for 10 minutes. The pelleted cells in each Falcon tube were resuspended in 3 ml of dH₂O with 225 μl of dimethylsulphoxide. 80 μl aliquots were then frozen in liquid nitrogen and stored at -70°C. For use, suspensions were thawed on ice and 40 μl aliquots used for electroporation with 0.5 μl of a ligation reaction or plasmid solution. The cells were pulsed at 1 800 V, 150 Ω and 50 μF in a chilled electroporation cuvette (1 mm electrode gap) with a Biorad Gene Pulser and pulse controller electroporation apparatus before being immediately suspended in 1 ml of SOC medium. The cells were then incubated at 37°C with shaking for recovery and...
antibiotic resistance marker expression before plating 100 μl and 900 μl on selective LB plates. These were incubated overnight at 37°C for colony formation.

3.12 POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) was used to amplify DNA fragments for cloning, diagnostic screens of plasmid constructs, generation of templates for in vitro transcription, generation of digoxigenin labelled DNA probes, cycle sequencing, reverse transcriptase PCR (RT-PCR) and transposon mediated mutagenesis screening. In all cases, except cycle sequencing and RT-PCR, reactions were typically done in a Rapidcycler (Idaho Technology) using 10 μl reactions sealed in borosilicate tubes of 5-30 μl capacity. A PCR buffer previously designed to optimise reactions in these conditions (Ian Oliver, pers. comm.) consisted of 50 mM Tris.HCl pH 8.3, 500 μg/ml bovine serum albumin, 0.5% Ficol 400, 1.0% sucrose, 30 mM KCl, 2 mM MgCl2 and 1 mM tartrazine. Deoxynucleoside triphosphates (dNTPs) were added to 200 μM each from a stock solution, primers to 0.2 μM each primer and Taq DNA polymerase to 0.005 units per μl. Cycling conditions were varied with each type of reaction but the following was adequate for most purposes and would amplify up to 2 kb of DNA to 60 ng/μl. Template DNA was first denatured at 94°C for 30 seconds. The following was then repeated for 35 cycles: denaturing at 94°C for 2 seconds, annealing at ≥ 45°C for 2 seconds, primer elongation at 72°C for 45 seconds. This was followed by one cycle of 3 minutes at 72°C to complete elongation. The GAL4 activation vector specific clontech primers pACTF1 and pACTR1 produced poor results in PCR directly from yeast or sequencing of isolated prey plasmids. This was possibly due to their low annealing temperature or lack of specificity. For this reason pACTF2 was designed using the Oligo program. Improved cycle sequencing reliability was subsequently obtained using 3.2 pM of either primer in place of 1.6 pM in a 10 μl sequencing reaction.

3.13 SEQUENCING

Cycle sequencing was done using a deoxyrhodamine dye terminator ready reaction kit (Perkin Elmer). The sequencing reaction was done using the following cycle conditions: one denature step of 96°C for 30s followed by 25 cycles of denaturing at 96°C for 30s, annealing at 50°C for 15s and strand elongation at 60°C for 210s.
Template consisted of 250-500 ng of plasmid DNA or approximately 10 ng of unpurified PCR product if the PCR had undergone 35 cycles of amplification and only a single major product was present. After the cycle sequencing reaction the products were precipitated with sodium acetate and ethanol to remove unincorporated nucleotides before analysis on an ABI Prism 377 DNA sequencer.

### 3.14 Screening of Transposon Mutagenized Plant Pools by PCR

#### 3.14.1 *Antirrhinum* Mutagenesis Screens

A reverse genetic screen was carried out to identify transposon-induced mutations in the *Antirrhinum* *Pulp* gene. This used pools of genomic DNA from ~28 000 *Antirrhinum* plants carrying active transposons that had been kindly provided by Enrico Coen and Rosemary Carpenter, John Innes Centre.

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<td>Tam3 5' end</td>
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<td>conserved cacta end</td>
</tr>
<tr>
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<td>gggacattwatttrtgct</td>
<td>conserved sub-terminal region of Tam 1, 2, 4 and 5</td>
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<td>5' end</td>
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<td>5' end</td>
</tr>
<tr>
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<td>PULP198#*</td>
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Table 4. primers used for transposon mutagenesis screen of *Antirrhinum PULP*. # used for Gatersleben screen.* additional *PULP* specific primers used to screen the Gatersleben collection.

Each pool contained genomic DNA from ~450 M1 parents. To reduce the number of screening reactions, stocks were combined into eight super-pools (each containing DNA from ~3 600 plants) and each screened by PCR with a gene-specific primer and
a transposon-specific primers. Gene-specific primers were designed for both strands of the *Pulp* gene, and transposon-specific primers for those transposons known to be most active in this genetic background (Table 4.). In the case of the CACTA family of transposons, a degenerate primer was used that was complementary to a sub-terminal region conserved in all known members of the family. Reactions were examined by agarose gel electrophoresis in 10 x 7 cm 1.0% agarose meniscus gels. To visualise weak bands and distinguish spurious products from gene-specific ones PCR products were Southern blotted (section 3.4) and probed with $^{32}$P or digoxygenin labelled *Pulp* sequences, as described in sections 3.4 and 3.17.

### 3.14.2 AGROBACTERIUM TRANSFORMATION OF *ARABIDOPSIS*

Agrobacterium strain GV3101 harbouring pMP90 were made competent by the method of Clough and Bent (1998) but LB was substituted for TYNG. Briefly, cells were grown overnight in 5ml LB with 50 ug/ml rifampicin and 25 ug/ml of gentamycin at 28°C and 250 rpm. 60ml of culture with the same selection was then inoculated with 1ml of the overnight and grown as above to OD$_{680}$ of 0.5-1.0 before chilling on ice and centrifuging briefly at 370g in a sorval GSA to remove clumps of cells. Cells were then harvested at 1470g for 6 min at 4 °C, resuspended in 1.2 ml ice cold 20 mM CaCl$_2$, frozen in liquid nitrogen and stored at -80 °C. For transformation 100 µl of frozen cells were thawed on ice and 10 µl of plasmid miniprep before refreezing in liquid nitrogen for 5 minutes. Cells were rethawed, 1ml of LB added and grown at 28°C for 2-4 hours with shaking. Transformants were then plated on media containing rifampicin gentamycin and 50ug/ml kanamycin until colonies appeared. Colonies were screened for by purifying DNA and retransforming *E. coli* so that adequate plasmid could be characterised. *Arabidopsis* transformation was done by growing agrobacterium colonies in 5ml LB with kanamycin and genamycin for two days, inoculating 100 ml of media with this and incubating until stationary phase was obtained harvesting the cells and resuspending them in at OD$_{600}$= 0.8 in 5% sucrose and 0.005% silwet. This was used to dip flowering *Arabidopsis* which were then maintained in humid conditions for 24 hrs.
3.15 VIRAL CO-SUPPRESSION OF PULP

3.15.1 VIRAL INNOCULATION PROCEDURE

Innoculum was provided by Stuart Macfarlane and consisted of homogenised infected tobacco tissue in water which was stored in dry ice or at -80°C in 500μl aliquotes. Aliquotes were thawed and ground to an even consistency with 5 volumes of tap water in a mortar and pestle. Plants were then inoculated by hand wearing rubber gloves by gently rubbing the diluted inoculum onto immature leaves that had been coated with a thin layer of fine carburandum powder. After several minutes the leaves were washed with water and the plants covered with newspaper over night. Plants were maintained in a growth room at 20°C under 16 hr days and grown on growers specific Antirrhinum soil (Sinclair).

3.16 ARABIDOPSIS GROWTH CONDITIONS

Plants were grown in a 2:1:1 mixture of Levington F2 compost (Fisons Horticulture, Loughborough), sand and fine grit. Long day conditions consisted of 16 hours light: 8 hours dark in either growth rooms or greenhouses with daylight and supplementary light. Growth rooms were maintained at 25°C and greenhouses at an average temperature of 20°C. Short-day conditions consisted of a 12 hour light period at a constant temperature of 25°C.

3.17 ARABIDOPSIS MUTAGENESIS SCREENS

The Sainsbury Laboratory Arabidopsis thaliana (SLAT) transposon mutagenesis collection (Tissier et al., 1999) was used to screen for a transposon insertion in AtF10M170. All plant and DNA resources were obtained from Kanu Patel at the John Innes Centre, Norwich. The plants used in this screen had been derived from parents that were heterozygous for a T-DNA consisting of a dSpm transposon (containing a phosphinotricin (PPT) resistance gene) flanked by an Spm transposase gene and a counter-selectable marker conferring sensitivity to the pro-herbicide R7402. Progeny had been selected for BASTA resistance (presence of dSpm) and survival in the presence of R7402 (absence of the original T-DNA ‘launch-pad’. Each was therefore assumed to carry a novel dSpm insertion at a position unlinked to the T-DNA, and termed a transposant.
To screen the population, a $^{32}$P-labelled f10M170 probe was hybridised to a filter loaded with an array of inverse PCR products produced by amplification with dSpm-specific primers and DNA templates derived from pools of transposants. The filter was probed as described in Section 3.4. The autoradiograph was used to identify a single pool of plants by comparing it to a map of the array. DNA corresponding to the subpool was obtained and PCR with gene-specific and transposon-specific primers and sequenced to confirm the site of insertion. Seed from the pool of 50 plants was grown and DNA purified from pools of 16 plants for further PCR screens.

3.17 SOUTHERN BLOTTING AND HYBRIDISATION WITH DIGOXYGENIN LABELLED DNA PROBES

3.17.1 DIGOXYGENIN-11-dUTP LABELLED PROBE PRODUCTION

Probes for detection of DNA in gels by Southern blotting and hybridization were made as described by Roche Molecular in their DIG System Users Guide for filter hybridisation. A standard PCR was done with a plasmid template using primers complementary to either the cloned fragment or vector-specific primers flanking the cloning site. Labelling of the PCR fragment was done by substituting 200 μM Digoxygenin labelled dNTPs (Roche Scientific) for unlabelled dNTPs in a standard PCR, and incorporation of Digoxygenin seen by slower relative migration on an agarose gel of the labelled PCR fragment compared to an identical reaction with dNTPs.

3.17.2 HYBRIDIZATION WITH DIGOXYGENIN-11-dUTP LABELLED PROBES

Nylon Filters were prehybridized for two hours at 65°C in 50 ml of prehybe solution (in this case, 5 x SSC, 0.1% (w/v) N-lauroyl sarcosine, 0.02% (w/v) SDS with 1% (w/v) dried milk powder to block non-specific binding of the Digoxygenin labelled probe to the filter. Filters were then incubated overnight with shaking at 65°C in 20 ml of prehyb. solution with 10 μl of Digoxygenin-11-dUTP labelled probe. The following day probe was stored frozen at -20°C for reuse. To remove unhybridized probe the filters were washed twice for five minutes at room
temperature in 2 x SSC, 0.1% (w/v) SDS. They were then washed twice for 15 minutes in high stringency wash (0.5 x SSC, 0.1% (w/v) SDS) with shaking at 68°C. The digoxygenin labelled probe was then detected immunologically as follows: the filters were first incubated for 30 minutes in maleic acid blocking buffer to prevent non-specific binding of the antibody. Blocking buffer is 100 mM maleic acid pH 7.5, 150 mM NaCl and 1% (w/v) blocking reagent (Roche). Filters were then incubated for 30 minutes in maleic acid buffer with blocking reagent and 0.75 units of alkaline phosphatase conjugated anti-Digoxygenin Fab fragments per ml.

Unbound antibody was then removed with two 15 minute washes at room temperature in 50 ml of maleic acid buffer (100 mM maleic acid pH 7.5, 150 mM NaCl) including 0.3% (v/v) Tween 20 as a dispersant. Filters were then equilibrated in detection buffer for 3 minutes at room temperature to raise the pH to that of functional alkaline phosphatase. Detection buffer is 10 mM Tris.HCl pH 9.5, 100 mM NaCl. The chemiluminescent detection was then done by dipping filters in a minimal volume of detection buffer containing 0.25 mM CSPD before sealing in a plastic film envelope with a bag sealer and incubating them at 37°C for 15 minutes for signal to develop. Filters were then exposed to CRONEX 4 X-ray film in a film cassette for a period of between 30 seconds and 30 minutes as necessary before developing in a film developer.

3.18 INSITU HYBRIDIZATION

Insitu hybridization was done by the method based on that of Jackson, (1991) and described by Jeff Long (1997).
3.19 CLONING

All sequence fragment and primers are named or described on the basis of nucleotide distance from the first methionine codon in the ORF of genes, except where stated otherwise.

YEAST 2-HYBRID ASSAY CLONES

Antirrhinum YEAST 2-HYBRID LIBRARY

The *Antirrhinum* whole plant cDNA prey library, provided by Brendan Davies, Leeds, UK., was made by priming cDNA synthesis with an *Xho*I linked random primer and using methylated cytosine. An *Eco*RI linker was attached at the 5' end and the whole digested with *Xho*I. This provided directed clones with an *Eco*RI site at the 5' end and *Xho*I at the 3' end for ligation into pACT2. Methylation at the cytosine residue prevents digestion internally with *Xho*I. To increase diversity a proportion of the cDNAs were partially digested with *Bgl*II and ligated into the pACT2 vector in *Eco*RI and *Bgl*II sites.

MODIFIED YEAST 2-HYBRID VECTORS

To facilitate efficient cloning the Clonetech bait and prey vectors pGBT9 and pGAD424 were modified to introduce the two extra unique restriction sites *Not*I and *Spe*I. This was done by introducing an approximately 200 bp *Pst*I fragment from the 3' UTR of a clone of T20403. Clones pGBT923 and pGAD8 had the insert orientated so that *Spe*I was 21 bp from the 3' *Pst*I site. Deletion of the approximately 180 bp fragment could be easily detected during plasmid preparation for cloning and both plasmids behaved the same as the Clonetech versions when used as controls. The junctions of the bait and prey clones derived from these were sequenced before use.

PHAN-bait

The PHAN prey vector (pY2HPHANa) construct had previously been made by Andrew Hudson using *in vitro* mutagenesis to introduce an *Eco*RI restriction site into a *PHAN* cDNA clone. This was then ligated into pGBT9 using the *Eco*RI site and 3' site in the vector.
PHAN-CT\textsuperscript{147-351} (PHAN C-TERMINUS)
pY21PHANc which encoded amino acid residues 147 to the end (351) of C-terminal
domain of PHAN was made by digesting pY21PHANa with EcoRI infilling with
Klenow, digesting with Nco I, blunt ending with mung bean exonuclease and ligating
the two blunt ends to produce an in frame fusion.

PHAN-MYB\textsuperscript{2-147}
pY21PHANb was made by digesting pY21PHANa with Nco I and Pst I, before
creating blunt ends with Klenow fragment and religating the blunt ends.

PHAN prey (AW5)
The insert was purified from PHAN-bait as an EcoRI-NotI fragment and ligated into
pACT8 using the same restriction sites.

PHANL-1-bait
The PHANL1 bait was made by Richard Waites by random cloning in Sal I sites in
pGBT9

PHANL-1 prey (AC1)
The PHANL-1 insert was amplified with primers GALDB and PHANL1not which
introduces a Not I site and includes a Sal I site from PHANL-1. The PCR product
was then purified as a Sal 1-Not I fragment and religated into pACT8.

Arabidopsis cDNA library screen
Probe template for screening of the Arabidopsis cDNA library for F10.170 clones
was made by PCR from 78G3T7 with M13 primers y1563/5. Phagemid sequences
were then removed by digesting with BamHI and EcoRI and gel purifying the 600bp
fragment.
INSITU PROBE TEMPLATES

_Antirrhinum_: pABI insert was made by cloning the insert of S1-1 into pBluescript II SK- with EcoR I and Bgl I sites in the insert and EcoR I and BamHI sites in the vector. Template was made using M13 primer sites flanking the insert and T7 promoter region.

_Arabidopsis_: A T20403 insitu probe template was made by PCR with Y1563 and T3 across the insert of pBA13 this produced a fragment with the T7 promoter 3' of the the 5' half of T20403. pBA13 was made from pKS-D3 by deleting the 3' half of T20403 between a centrally located Xba I site and and another in the 3' polylinker of the plasmid.

_Arabidopsis ETHANOL INDUCIBLE EXPRESSION CONSTRUCTS_

pAH54 was constructed using the pGreen_KanR backbone which had a BASTA resistance gene inserted in the Hpa I site for selection of plants grown on soil. The ppt cassette was known to contain a SalI site. This was obtained from Justin Goodrich. Lucia Primavesi (University of Edinburgh) had previously deleted a section of the pGreen polylinker between EcoR V and Sma I to remove EcoR I and Pst I sites. So that sites within the alcA cassette polylinker could be used for cloning after it was inserted into pGreen, the pGreen was digested with BamHI and Not I, blunt ended with Klenow fragment and religated. The resulting plasmid was named pAH5.

pUC19alcA contained the alcA fusion promotor described by Caddick et al. (1997) was obtained from Justin Goodrich. It contains a CMV 35s terminator and the GUS gene had been removed leaving restriction sites in the following order; Pst I, Sal I, BamHI, Sma I and EcoRI RI RI/Apo I. This was digested with Hind III to obtain the cassette and ligated into pAH5 that had been Hind III digested and treated with Shrimp alkaline phosphatase (SAP) (Roche) to prevent religation. The resulting clones were restriction mapped to determine the orientation of the insert. A clone (pAH54) was selected such that the alcA cassette was expressed in the opposite direction to the lacZ gene. Restriction mapping also revealed that the ppt resistance gene is orientated away from the alcA cassette.
pAS3 and pAT5: T20403 SILENCING INSERTS

By consideration of the work of Chuang and Meyerowitz (2000) it appeared that duplex encoding inserts were more potent at silencing if each part was from the 3' half of the target gene and greater than 400bp in length. Two T20403 constructs were made which had these properties. Both were derived from yeast 2-hybrid-bait clone, pU6, by exonuclease III deletion (as described by Henikoff, 1987) and called pU6-5 and pU6-11. Compatible Nhe I and Xba I sites were used to join clones at the 5' end. 148bp separated the Nhe I and Xba I sites. Three way ligations were done with pGEMT (Promega) and gel purified PCR fragments that had been digested either with Nhe I or Xba I. This could have produced 3 possible products: where long fragments are described "A", and short fragments are "B" they could ligate into pGEMT in the following combinations, AA, AB, BB. Interestingly only the AB combination was obtained which was as required. These products were then recovered from pGEMT using an Apol site which was situated 17bp into the yeast 2-hybrid bait clone and was included in the PCR product, before cloning into SAP treated pAH54. Two clones pAS3 and pAT5 were selected that had the linker part of T20403 continuous with the leading antisense part of the construct. pAS3 was made with nucleotides 1380-2020 (640bp) and pAT5 included nucleotides 1380-1877 (497bp) of T20403. The linker section of the constructs included nucleotides 1232-1380 of T20403.

pAU2: T20403 OVER EXPRESSION INSERT

the ORF of T20403 was cloned in sense orientation with the alcA promotor from pAN1 by digesting with Eco RI and blunting the product with Klenow fragment. This was then cleaned up, digested with Bam HI, and gel purified. The product was ligated into pAH54 that had been digested with Pst I and blunted with Klenow before digesting with BamHI. This produced directed clones and reduced the need for extensive screening, a clone was selected and called pAU2.

T20403 CLONES

pAN1

The insert of pAN1 which includes the whole ORF of T20403 was made by PCR with primers atpATG and atp2354 from Arabidopsis columbia genomic DNA. This
was digested with Eco RI and Bam HI for which sites are introduced by atpATG and atp2354 respectively, and cloned into pBluescriptII SK- (stratagene). This was then sequenced.

AS1 Bait (2AJ1) a PCR product made using primers AS1BAM and AS1-1112 which introduce Bam HI and Spe I sites respectively was ligated into pGBT9-23.

S1-1 Bait
The insert of S1-1 was amplified by PCR with pACTF2 and pACTR1 and purified as an EcoRI and BamHI fragment then ligated into pGBT9 digested with EcoRI and BglII.

AS1 PREY
The same PCR fragment used to make AS1 bait was ligated also into BamHI, SpeI digested pACT8.

T20403 403-764 BAIT and PREY
T20403 403-764 was made by PCR from pKS-D3 using primer ATP1198 and the M13 T7 primer site located in the vector. ATP1198 introduces an EcoRI site into T20403 in frame with the yeast 2-hybrid vector cloning site. enabling cloning into pGBT923 or pGAD8 using EcoRI and Spe I.

T20403 403-539
The T20403-S1-1 equivalent was made from T20403 using a PCR primer (ATP1198) to introduce an Eco RI site at the start of the region equivalent to S1-1 for fusion to the GAL4 domains. Once cloned into bait vectors the insert was subjected to exonuclease deletion by the method of Henikoff (1987). A clone corresponding to the S1-1 region was obtained by this method and designated T20403 403-539.

T20403 in pGEMT
A PCR product from genomic DNA was made using primer ATP1eco and P1 from the SLAT screen, this was cloned into pGEMT. ATP1eco introduces an EcoRI site. The insert could only be obtained in one orientation so it was subcloned into pBluescript II KS- to make pKS-D3: a fragment was purified from pD3 using 5'
EcoRI and a 3’ Spe I site present in pGEMT. This was ligated into pBluescript II KS- using the same restriction enzymes. This provided the T7 promoter 3’ of the gene for invitro transcription.

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<td>Y1563</td>
<td>ttgtaaaaacagcggccagt</td>
<td>M13</td>
</tr>
<tr>
<td>PACTF2</td>
<td>cgttgagacactacagggga</td>
<td>Amplify prey inserts</td>
</tr>
<tr>
<td>PACTR1</td>
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<td></td>
</tr>
<tr>
<td>DSPM1</td>
<td>cttatttcgtaagaggtctggttttt</td>
<td>dSPm specific primers for SLAT screen</td>
</tr>
<tr>
<td>DSPM11</td>
<td>ggtgcagcaaaaaacacacttctcctc</td>
<td></td>
</tr>
<tr>
<td>DSPM5.2</td>
<td>cccgacacttcttaattaactcagacactcc</td>
<td></td>
</tr>
<tr>
<td>DSPM8</td>
<td>gttttgcgacactccttacc</td>
<td></td>
</tr>
<tr>
<td>Qt</td>
<td>Ccaagtgacagctgagctgactctgactcagctf(t)_{14}vnt</td>
<td>RTPCR</td>
</tr>
<tr>
<td>Qo</td>
<td>ccaagtgacagcactgacgag</td>
<td></td>
</tr>
<tr>
<td>Qi</td>
<td>acgaggacagctgagctcaagc</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Primers used for general DNA amplification and modification.

3.20 BACTERIAL MEDIA AND ANTIBIOTICS

3.20.1 MEDIA

L Broth and L Agar

L broth used for growing most E. coli strains contained 10 g Bacto-tryptone, 5 g Bacto-yeast extract, (both from Difco Laboratories) and 10 g NaCl per litre. The pH was 7.5. Agar plates used for growing bacterial colonies and for the bottom layer of phage plates included 15 g/l of Bacto agar, also from Difco.
BBL
BBL top agar for plating phage contained 10 g Oxoid tryptone soya broth, 5 g NaCl and 6.5 g Bacto agar per litre. It was made 0.2% (w/v) maltose and 10 mM MgSO₄ before use.

2xYT
2xYT, used for growing M13 phagemid contained 10g NaCl, 10g yeast extract and 16 g tryptone per litre, it was made to pH 7.5 with NaOH before sterilization.

SOC
SOC medium, used for recovery of *E. coli* after transformation by electroporation, was 2% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose and 20 mM Mg²⁺. The glucose and Mg²⁺ were added from filter sterilized stock after the medium had been autoclaved. The Mg²⁺ stock was 1 M MgCl₂·6H₂O and 1 M MgSO₄·7H₂O.
All media were sterilised by autoclaving at 120°C for 20 minutes or by filtration through 0.2 μm pore size Acrodiscs (Gelman Sciences, Michigan, USA.)

M9 PLATES
M9 medium was made as described by Maniatis *et al.*, (1985) with addition of the following amino acids and nutrients. For 500 ml of medium, 10 g of DIFCO bacto agar was made up to 310 ml with dH₂O and autoclaved at 117°C for 20 minutes before being allowed to cool to about 65°C. To this were added 125 ml of 4 x M9 salts (6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl and 1 g NH₄Cl per litre), 400 mg amino acid dropout powder (as described in yeast methods), and sterile stock solutions to give final concentrations of 0.4% (w/v) glucose, 200 μg/ml histidine, 200 μg/ml tryptophan, 40 μg/ml proline, 50 μg/ml ampicilin and 1 mM thiamine.

SOLUTIONS
TE 10 mM Tris HCl, 1 mM EDTA.
3.20.2 GENOTYPES OF BACTERIAL AND YEAST STRAINS

C600  
F' thi-1, thr-1 leuB6, lacY1, tonA21, supE44,

HB101  
F' thi-1, hsdS20 (r_B, m_B), supE44, recA13, ara-14, leuB6, proA2,  
lacY1, rpsL20 (str'), xyl-5, mtl-1 rpsL20, supE44, galK2.  
Bolivar and Backman. (1979)

DH5α  
φ80dlacZΔM15, recA1, end A1, gyrA96, thi, hsdR17 (rk',mk'),  
supE44, relA1,deo R, Δ(lacZYA-argF)U169

JM109  
end A1, recA1, gyrA96, thi, hsdR17 (rk',mk'), relA1, supE44, Δ(lac-  
proAB), [F', traD36, proAB, lacZΔM15]

XL1 BLUE  
Δ(mcrA)183Δ(mcrCB-hsdSMR-mrr) 173 end A1, supE44, thi-1,  
recA1, gyrA96, relA1, lac [F' proAB lacZΔM15 Tn10 (Tet')]

XLOLR  
Δ(mcrA)183Δ(mcrCB-hsdSMR-mrr) 173 end A1, supE44, thi-1,  
recA1, gyrA96, relA1, lac [F' proAB lacZΔM15 Tn10 (Tet')] Su'  
(nonsuppressing) λ' (lambda resistant)

HF7c  
Matα ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112  
gal4-542 gal80-538 (Lys2::Gal1uras-G-Al1TATATATA-His3) -CyC1TATA-lacZ  
URA3::Gal4_17mer(x3)

3.20.3 ANTIBIOTIC SELECTION

Ampicillin was used at a final concentration of 100 μg/ml from a stock of 100 mg/ml  
in water. It was used to select bacteria carrying plasmids pGBT9, pGAD424, and  
pBluescript. Kanamycin sulphate was made up as a 50 mg/ml stock in water and  
used at a final concentration of 30 μg/ml to select bacteria carrying the plasmids  
pBKCMV and pGREEN. Tetracyline, used for selection of the phage host, C600 f,  
was used at 10 μg/ml from a stock of 5 mg/ml in ethanol.
RESULTS. YEAST 2-HYBRID SCREENS FOR INTERACTIONS WITH PHAN PROTEIN

4.1 PREPARATION FOR THE YEAST TWO HYBRID ASSAY

The yeast two hybrid assay is considered one of the best ways to identify eukaryotic proteins that potentially interact. It tests for protein interaction under physiological conditions that are expected to be much more like those of the original organism than any bacterial or in vitro system. A host of eukaryotic factors are present such as chaperonins which increases the likelihood of potential interactors being in a native conformation and therefore competent to interact. The yeast 2-hybrid assay utilises selection for auxotrophy as an initial screen for potential interactors enabling a large number of clones to be screened rapidly. It leads directly to identification of the genes encoding a partner protein, from which the amino acid sequence of the product can be deduced.

The yeast two hybrid assay was used in a screen for proteins that interact with the product of the PHAN gene. The PHAN prey vector construct, pY2HPHANa (PHAN-bait), had previously been made by Andrew Hudson using in vitro mutagenesis to introduce an Eco RI restriction site into a PHAN cDNA clone, so that ligation into pGBT9 would create an open reading frame between the GAL4-DB sequence of the bait vector and the second codon of PHAN (Fig. 12). The junction between GAL4-DB and PHAN in pY2HPHANa had been sequenced to confirm that the insert was in frame with the GAL4-DB.

An Antirrhinum whole plant cDNA prey library was provided by Brendan Davies, Leeds, UK. This was thought to be appropriate for use with PHAN baits because PHAN mRNA is expressed in all lateral organs derived from vegetative and inflorescence meristems of Antirrhinum. To take full advantage of the library it was necessary to attain a high level of transformation efficiency so that as many as possible of its estimated seven million primary clones could be screened for interaction.

The highest efficiency of co-transformation with bait and prey is obtained by sequential transformation, that is transformation first with the bait plasmid, followed by maintainance of the strain by selection during culture before carrying out a second transformation with the prey library. This method ensures that the yield of co-
transformants is maximised. Hf7c harbouring PHAN bait was found to grow slowly and slow growth often led to flockulation which made it difficult to determine the cell density of the culture. Since a specific cell density was an important requirement for high efficiency transformation, liquid culture conditions which avoided flockulation were devised.

Growth was found to be best in medium consisting of 50% YPDA: 50% YMM without tryptophan. Although these culture methods probably led to a reduction in the total number of co-transformants, over 30 million were produced in the three screens. This should have been adequate to represent the library.

Before the screen could be commenced two possibilities had to be eliminated: (1) that the bait fusion protein could activate the reporter genes alone or by recruitment of an endogenous yeast factor and (2) that PHAN and the GAL4 activation domain did not interact. To test these possibilities the reporter strain, HF7c, was co-transformed with PHAN-bait, which encodes full length PHAN, and pGAD424 which encodes the GAL4 activation domain. pGAD424 was used in place of the prey library vector pACT2, which was not available at the time, since the GAL4 activation domain encoded by the two vectors is identical. The full length PHAN fusion protein encoded by PHAN-bait showed no significant transcriptional activation of the HIS3 or β-galactosidase reporter genes, either in the presence or absence of pGAD424, enabling the yeast two hybrid assay to be carried out.

4.2 YEAST TWO HYBRID SCREEN RESULTS

Because the stoichiometry of interaction is not revealed by the yeast 2-hybrid assay, terminology appropriate to the lowest order of oligomerization possible (that is 1:1 or dimerization) will be used throughout this work.

Three screens were done with PHAN-bait. The transformation efficiency (T.E.) and total number of co-transformants obtained in each is shown below.
Table 6. Co-transformation efficiencies, estimated number of clones screened and fate of candidate clones in three yeast two hybrid screens.

All six colonies from the first two transformations were tested for β-galactosidase reporter gene activity using the X-gal overlay assay (see Materials and Methods). Due to doubts about the sensitivity of the X-gal overlay assay and problems of bacterial contamination caused by the necessity to pour the overlay in a fume hood, 119 histidine prototrophic colonies from screen 3 were screened for β-galactosidase reporter gene activity by the more sensitive filter lift method. One candidate interactor was obtained from screen 1 and five from screen 2. These were tested further and recovered by streaking on -LWH medium. Four candidates failed to grow which left two candidates that were designated S1-1 and S2-16.

The yeast 2-hybrid vectors were all yeast-E. coli shuttle vectors which enabled plasmids in yeast to be recovered by transformation of E. coli with crude yeast DNA extracts. However both bait and prey vectors encode ampicillin resistance for selection in E. coli and the majority of ampicillin resistant colonies resulting from transformation with DNA from yeast were found to carry the bait plasmid. This was possibly due to the bait vector being more abundant than the prey vector in sequentially transformed yeast.

Since no counter-selection against the bait vectors was possible in yeast with pACT2 and pGBT9, attempts were made to obtain a strain carrying only the prey plasmid by culturing the yeast strain without selection for tryptophan auxotrophy until the bait plasmid had been lost randomly during cell division. Colonies that had lost the bait might then be identified by replica plating. Even after repeated culture and dilution of the S1-1 strain in -L+W media for 5 days followed by replica plating from -L to -W agar, only one trp' colony was obtained from approximately 150 colonies carrying

<table>
<thead>
<tr>
<th>No.</th>
<th>T.E.</th>
<th>TOTAL</th>
<th>TEST MEDIA</th>
<th>CANDIDATES</th>
<th>β-GAL. ASSAY</th>
<th>POSITIVES (after testing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.09x10^5</td>
<td>5.56 million</td>
<td>-LWH</td>
<td>6</td>
<td>All</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2.58x10^5</td>
<td>13.1 million</td>
<td>-LWH</td>
<td>All</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.39x10^5</td>
<td>12.18 million</td>
<td>-LWH</td>
<td>119</td>
<td>119</td>
<td>NONE</td>
</tr>
</tbody>
</table>
the prey plasmid. Therefore, loss of bait from cells in culture is likely to be very low. Subsequently the *E. coli* strain HB101, which carries a mutation in the bacterial B6 leucine biosynthesis pathway was used to enable isolation of prey vectors by selecting transformants for complementation of the *leu* mutation on M9 plates with ampicillin.

To confirm the interaction between PHAN-bait and the proteins encoded by S1-1 or S2-16 the prey vectors were reintroduced into yeast harbouring PHAN-bait, pGBT9 or neither plasmid. This was done to demonstrate that reporter gene activation was prey dependent and not due to other possibilities such as wild yeast contamination, genomic recombination or autoactivation by the preys. Additionally, since more than one prey plasmid might have been present, it would also determine which was responsible for an interaction. Both S2-16 and S1-11 were recovered and shown to be capable of activating reporter gene expression only in the presence of the PHAN bait.

**4.3 INTERACTION BETWEEN S2-16 AND PHAN IN THE YEAST TWO HYBRID ASSAY**

Sequencing of S2-16 revealed a 130 bp insert which contained two stop codons in the same open reading frame (ORF) as the GAL4-AD. This was followed by two additional ORFs (both in frame 2) of approximately 300 and 620 bp separated by 130 bp of ambiguous sequence. The sequences of the ORFs corresponded to an *Arabidopsis* expressed sequence tag (accession F19794) but neither showed homology to any gene with a known function or any domain recognisable at the time. Recently three homologues with very high amino acid sequence similarity from *Arabidopsis* and Rice have appeared in the databases as predicted WD40 domain proteins.

S2-16 required the C-terminal domain of PHAN to activate the yeast reporter genes strongly, despite causing high levels of *HIS3* expression, activation of the β-galactosidase reporter gene was undetectable (Table 7.). The lack of a continuous open reading frame with GAL4-AD and slight activation of the *HIS3* reporter gene in presence of the GAL4-DB alone, weighed against S2-16 being a genuine PHAN interactor and it was rejected as a candidate. The possibility remained however that one of the S2-16 open reading frames was capable of producing a protein that could
bind PHAN and activate transcription in yeast independently of the GAL4-AD or as an intermediary between PHAN and a transcriptional activator. Another argument in favour of S2-16 being relevant was that the expression level of the β-galactosidase reporter gene is always relatively low in HF7c where it is under control of the GAL4 UASG17-met(x3) synthetic activator which binds GAL4 less strongly than the native UAS sequence controlling the HIS3 reporter.

Table 7. Growth on selective media and reporter gene activation in HF7c transformed with prey and bait vectors or prey and empty GAL4-DB vector alone (pGBT9). - denotes no reporter gene activation, ± low activation and + relatively strong activation.

<table>
<thead>
<tr>
<th>media</th>
<th>prey only</th>
<th>prey + pY2HPHANa</th>
<th>prey + pGBT9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1-1</td>
<td>S2-16</td>
<td>S1-1</td>
</tr>
<tr>
<td>-W</td>
<td>-</td>
<td>-</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-LW</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>β-gal</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

4.4 INTERACTION BETWEEN S1-1 AND PHAN DETECTED WITH THE YEAST TWO HYBRID ASSAY

Sequencing of the second prey plasmid, S1-1, revealed an open reading frame of 128 amino acids fused in frame with the GAL4-AD. S1-1 caused activation of the HIS3 reporter gene in the presence of [5 mM] 3-amino-triazole (3AT a histidine anti-metabolite) (Fig. 10C.). This suggested that the reporter gene was strongly activated due to the affinity of interaction between PHAN and S1-1 reconstituting the GAL4 transcriptional activator.

S1-1-dependent activation of the β-galactosidase reporter gene was similarly strong, producing blue colonies in 35 minutes comparable to the the native GAL4 transcriptional activator which produced the same intensity of staining in ~20 minutes. This provided further support for bait-dependent activation and supported the hypothesis that S1-1 activates the reporter genes by protein-protein interaction with the bait rather than by binding directly to the GAL4 UAS.

To determine whether the interaction with S1-1 was specific, S1-1 prey was tested against pLAM5'-1 which encodes a human lamin bait in pGBT9. The lack of interaction with this bait served to demonstrate that S1-1 was not susceptible to
non-specific interactions. Similarly, evidence that PHAN was not susceptible to non-specific interactions was provided by the low frequency of positive clones obtained from the library screens.

It was also necessary to demonstrate that S1-1 interacted with the PHAN-encoded part of the bait and not with the GAL4-DNA binding domain. Therefore HF7c was co-tranformed with S1-1 and pGBT9 which encodes the GAL4-DB domain alone. This combination failed to activate the HIS3 reporter, indicating that PHAN sequences are necessary for interaction (Fig. 10C, D.).

Further confirmation was provided by a vector swap experiment in which the insert of S1-1 was fused to the GAL4 DNA binding domain and PHAN was fused to the GAL4-AD. This combination of fusion proteins activated both the histidine and β-galactosidase reporter genes suggesting that interaction was specific to PHAN and the protein product of S1-1 (Fig. 11.). The vector swap experiment also eliminated a further possibility which could not be eliminated by co-transformation of S1-1 with the GAL4-DB domain alone: that interaction of S1-1 with the bait requires the motif created between the PHAN and GAL4-DB domains of the bait. It also eliminated the possibility that PHAN interacted with the boundary between the S1-1 region and the GAL4-AD domain of the prey.

To map the region of PHAN necessary for interaction with S1-1, two further bait constructs were made. These encoded fusion proteins corresponding to either the MYB domain of PHAN (pY2HPHANb) or its carboxy terminal region (pY2HPHANc; Fig. 12.). Neither of these constructs caused auto-activation of reporter genes. Yeast co-transformed with S1-1 and pY2HPHANc showed reporter gene activation, whereas yeast carrying S1-1 and PHAN-bait did not. This suggested that S1-1 interacts specifically with the C-terminal domain of PHAN (Fig. 10.).
Fig. 10. Interaction between PHAN and PULP in yeast. In Figures 10, 11, 19 and 25 baits are in rows and preys are in columns. (A) -LW media - selection for bait and prey plasmid; (B) -LWH media selection for expression of the HIS3 reporter gene dependent on interaction by baits and preys; (C) -LWH with [10mM] 3AT for repression of weak interactors; (D) β-galactosidase assays. The β-galactosidase is typically activated at 1/10th of the level or the HIS assay. PHAN or PHAN-CT interaction with S1-1 is strong in each of panel B, C and D.
Fig. 11. Interaction between PHAN and S1-1 or PULP in directionally opposite GAL4-domain-fusion configuration compared to Figure 10. Full length PULP bait interacts weakly with PHAN in this assay but cannot be detected in Fig. 10 see panel B; PHAN-CT interacts with PULP in both configurations panel B but is weaker in Fig 10.
4.5 FURTHER SCREENS WITH DOMAINS OF THE PHAN PROTEIN

A single copy of one strong activator, S1-1, had been isolated from over 30 million bait and prey library co-transformants. Such a low yield of candidate clones raised the possibility that potential interactors were being lost due to properties of PHAN. For example, steric effects within the PHAN protein itself might have prevented interactions being detected. Therefore yeast two hybrid screens were repeated using baits consisting either of the MYB or C-terminal domains of PHAN separately, (Fig. 12.).

![Bait constructs used in yeast 2-hybrid assays. Domains are colour coded; GAL4 DNA binding domain, Myb domain, PHAN C-terminal domain, PHAN 3' untranslated region, Alcohol dehydrogenase transcriptional terminator. (A) pY2HPHAna (PHAN-bait). The arrow indicates the position of an Nco I site used to make the deletion derivatives: (B) pY2HPHAnb (PHAN-Myb2-147); (C) pY2HPHAnc (PHAN-CT147-351).](image)

Two screens with the MYB domain were carried out. The first produced a total of 97,500 co-transformants whilst the second produced approximately the same number, although accurate quantification of this transformation failed. Of these transformants, 60 colonies were picked from -LWH plates and streaked on -LWH to isolate them from contamination. Fifty-four colonies were then restreaked on -LWH with 5 mM 3AT to counteract a problem of irregular growth. The 16 colonies which grew strongly in the presence of 3AT were then tested for β-galactosidase activity: three tested strongly positive, whilst eight showed lower β-galactosidase activity. Prey plasmids from one strong activator and two weak activators were recovered in HB101. The sequence of only one weak activator could be obtained. This revealed a 280 bp insert that added 6 novel amino acid residues to the GAL4-AD after the Eco
RI cloning site, and were partly encoded by the linker used in cDNA cloning (Section 3.19). No long ORF was apparent in the remainder of the clone. The most likely explanation for the weak interaction observed for this strain was that a second prey plasmid, which was not recovered, was responsible for the interaction. Using the C-terminal domain of PHAN as bait, 2.85 million bait-prey co-transformants were obtained in two screens. 60 colonies were recovered from -LWH plates. Four tested positive for β-galactosidase activation and three grew on -LWH with 10 mM 3AT. The prey plasmid could be obtained from only one of these by transformation of HB101. This was sequenced and found to be a second isolate of the S1-1 clone.

Further screens with the two domains were abandoned due to a declining supply of library DNA and problems with bait strain culture and transformation efficiency. The interaction between PHAN and S1-1 appeared strong and bait-dependent and had been detected in two different library screens. Analysis of this candidate was therefore pursued.

4.6 FURTHER CHARACTERISATION OF S1-1 (PULP)

S1-1 had the potential to encode a predicted 128 residue peptide that interacted strongly with the C-terminal domain of PHAN in the yeast two hybrid assay. It was likely that S1-1 represented part of an expressed gene because the prey library was constructed from cDNA. In order to confirm this and to eliminate the possibility that the S1-1 insert was contaminating genomic DNA, Antirrhinum cDNAs including the S1-1 sequence were isolated.

To obtain cDNA sequences, an Antirrhinum cDNA library was screened using the S1-1 insert as a probe. The cDNA library, constructed with RNA isolated from whole plants, had been made in λGT10 by Rüdiger Simon, John Innes Centre, Norwich. The template for a 32P labelled probe was made by PCR using primers PACTF2 and PACTR1, which flank the insert in the prey plasmid pACT2. Inserts were recovered from positive phages by PCR from purified λ phage DNA followed by cloning into Eco RV-digested pBluescript II. All the S1-1 cDNAs obtained appeared to be incomplete because they lacked poly(A) tails and had different 5' termini within the ORF. A second Antirrhinum
cDNA library became available from Hans Sommer in Cologne, Germany. This library had been constructed in λ Zap Express (Stratagene) which provided in vivo excision of pBKCMV phagmid containing cDNA clones. Screening of this library followed by successful recovery of phagemids by in vivo excision yielded two clones of 2.8 kb and five clones truncated at the 5' end at various points. The 2.8 kb clones showed characteristics of plant protein-coding genes having apparent 5' and 3' untranslated regions, a significant open reading frame and 3' polyadenylation (Gallie, 1996). These characteristics suggested that they were near full length and that S1-I was part of an expressed protein-coding gene in Antirrhinum.

All the clones obtained had identical DNA sequences except that their polyadenylation sites differed in location by 110bp. The S1-I clone corresponded to a region 384 bp long starting 1556 bp from the first methionine codon in the open reading frame (Fig. 13.). Comparison of this cDNA sequence with PCR products from Antirrhinum genomic DNA revealed that that the gene has no introns within the region contained in the cDNA clones.

A search of sequence databases with the nucleotide sequence of S1-I using the BLAST program (Altschul et al., 1997) initially revealed significant similarity to the product of an Arabidopsis EST, accession number T20403. The structure of the S1-I gene and the T20403 are discussed further in section 9.1.

With a considerable level of scepticism the S1-I gene was provisionally named PULP from PHANTASTICA's Unlikely Partner and the presence of the name of a prominent actor in the film Pulp Fiction (KEITEL) in the deduced amino acid sequence of its protein product. It was hoped that a mutant, either classical or novel, would be obtained later to enable a name to be given in line with traditional nomenclature.
Fig. 13. Alignment of PULP (upper rows) and T20403 deduced amino acid sequence. The region over-scored by a red line corresponds to S1-1. Conserved heptad leucine repeats are present throughout the sequence but within S1-1 are marked with arrows. Amino acid residue colouring is as follows: hydrophobic; aromatic; positively charged polar groups; negatively charged polar groups; uncharged polar groups; other.
INTERACTIONS OF PHAN AND PULP IN THE YEAST TWO HYBRID ASSAY

The C-terminal domain of PHAN had been found to interact with the S1-1 fragment of PULP. The question arose whether other parts of the two proteins could interact and whether interaction of the whole gene products was different. This was of particular interest because the MYB domain of the maize CI protein had been found to interact with the N-terminal domain of a bHLH transcription factor encoded by the B gene (Goff et al., 1992). This interaction is required for CI-mediated transcriptional activation and it was therefore possible that similar processes may occur with PHAN. To test this, PULP cDNA8, which contained the full-length ORF, was cloned into pGAD424 to make a PULP prey fusion protein. It was then tested for interaction with the MYB domain of PHAN, its C-terminal domain and full length PHAN baits in yeast (Fig. 10.). This revealed that only the C-terminal domain of PHAN interacted strongly with full length PULP. Lack of interaction between PULP and the MYB domain was again observed, suggesting that interaction involved the C-terminal region of PHAN. However, interaction involving the MYB domain in vivo could not be ruled out because there was no experimental evidence that the MYB domain bait was stably expressed in yeast. Western blotting of yeast extracts with antibodies to the GAL4 DNA binding domain was not sensitive enough to detect the low levels of protein expression characteristic of this vector in HF7c. Transcriptional activation by fusion proteins in the 2-hybrid assay is known to be susceptible to three main factors: these are the affinity of the binding partners, whether the assay is carried out with a given binding partner fused to the DNA binding or activation domain (directionality) and the reporter gene activation level. Estojak et al. (1995) found in most cases, that affinity of baits for preys in the 2-hybrid assay is the predominant determinant of the level of reporter gene transcription. Furthermore, they concluded that it is possible to discriminate between high, intermediate and low affinity interactions on the basis of relative ability to activate reporter gene transcription. Reporter gene expression, however, does not correspond linearly to dimerisation affinity reported in vitro. Consequently, affinities can be compared but not quantified with yeast 2-hybrid assays.

The second significant factor, directionality of the assay, has been seen to influence transcription level of numerous protein pairs in 2-hybrid systems (Finley and Brent
unpublished). Analysis of the interaction between Myc and Max baits and preys using the lexA 2-hybrid system (an alternative to the GAL4 system) provides a specific example. Whilst a LexA-Max bait interacted strongly with AD-Myc, LexA-Myc interacted only weakly with AD-Max (Estojak, et al., 1995).

The effect fused moieties have on one another provides a possible explanation for directionality: whether a given binding partner is fused to the DNA binding or activation domain can affect its binding affinity (Estojak et al., 1995). Fusion of moieties to the DNA binding domain, in some systems, can also affect its affinity for DNA and therefore the transcriptional activity of the reporter gene (Golemis and Brent, 1992). Additionally, oligomerisation of baits or preys could obscure residues necessary for interaction or interfere with conformation of the protein in such a way as to render it unable to interact.

Third, it is necessary to use reporter genes with different promoters to help detect false positives that activate transcription by binding directly to promoter sequences. This can result in differences in the transcriptional activation level of the reporter genes. Estojak et al. (1995) found that some reporters recognise only moderate to high affinity interactions ($K_d$ for dimerisation < 1μM) whilst others could also recognise weak interactions. In Hf7c, transcriptional activation of the β-galactosidase reporter gene is approximately 1/10th of the HIS3 reporter gene and can detect weak or transient interactions only when highly sensitive β-galactosidase assays are used, such as a liquid culture assay with a chemiluminescent substrate. Because of the differences in reporter gene sensitivity, the effect on transcription caused by diverse fusion proteins and directionality, interaction affinities are best ranked based on the activation levels of more than one reporter gene.

The yeast-2-hybrid data for PHAN and PULP interaction in directionally opposite assays are shown in Figs 10. and 11. These suggested that S1-1 interacts with the C-terminal domain of PHAN with high affinity as the transcription level of both reporter genes was similar to that induced by the native GAL4 transcriptional activator expressed from pCL1 (data not shown).

From these data, it was also possible to rank the affinity of interaction between parts of the PHAN and PULP proteins based on the relative levels of reporter gene activation. With PHAN as bait, equally strong interactions were observed between full-length PHAN protein or its C-terminal region and the S1-1 region of PULP.
Interaction between the C-terminal region of PHAN and full-length PULP was weaker, and interaction between full-length PHAN and full-length PULP indistinguishable from that between full-length PHAN and GAL4-AD (which was assumed to be zero). The same order of interaction strengths was observed when PULP was used as bait, except in this case it was possible to distinguish the strength of interaction between full-length PULP and PHAN from the interaction between the S1-1 region of PULP and the GAL4-AD (which was zero). Because the transcription levels could be ranked in order from high to zero (zero being equivalent to bait or prey with the GAL4 domain alone) it remained possible that the weak interactions observed in yeast were real but below the threshold detectable with the β-galactosidase assay and yeast strain used here. (the threshold of activation effect can be seen particularly in Fig. 19.C, D).

It was reasonable to assume that full-length PULP (particularly as bait) was stably expressed because it was able to make intermediate strength interactions with the C-terminal domain of PHAN (Fig. 11.B and C). Furthermore growth on -HIS medium due to the weak transcriptional activation of the HIS3 gene by full-length PULP interacting with full-length PHAN was significantly higher than background growth of bait with vector-only controls on the same agar plate.

The question of why full length PHAN and PULP appeared to interact less strongly than their sub-domains therefore remained. One possibility was that the size or geometry of fulllength PHAN and PULP fusion proteins affected transcriptional activation of the reporter genes. However, proteins of similar size and type have been observed to interact in GAL4 based yeast two hybrid assays although the strains and vectors used in these cases give higher levels of prey and bait protein expression (Tsukada et al., 1999).

A second possibility is that one of the full-length partners requires modification for full interaction to occur. An example of this is provided by the cyclic AMP response element binding domain protein which has a coiled-coil structure and requires phosphorylation by pKA before its KIX domain can interact with CREB binding protein (Richards et al., 1996). PULP has numerous predicted phosphorylation sites and its interactions might therefore be regulated by phosphorylation in a similar way.
4.8 CHOICE OF FUNCTIONAL TESTS

An *in vitro* binding assay is often employed to support protein-protein interaction detected with the yeast 2-hybrid assay. This typically involves expressing one protein as a fusion to Glutathione S transferase (GST) and using it to isolate a radio-labelled partner transcribed and translated in vitro. The *in vitro* binding assay eliminates the possibility that a yeast molecule is acting as an intermediary between the bait and prey fusion proteins enabling them to interact. However, there is no guarantee that the assay will be successful for proteins that interact *in vivo* because expression of functional proteins *in vitro* can be highly problematic. Only a positive result is useful because a negative result can be explained as a failure of the protein to adopt an active configuration. A demonstration of binding *in vitro* also cannot reveal the functional significance of an interaction. More relevant evidence can be obtained *in vivo*, for example, by genetic analysis. If mutation of a putative interactor results in a similar phenotype to that of a mutation in the bait gene, and the two mutations condition either an additive phenotype or the same phenotype when combined, the two genes might contribute to the same biological process or pathway. This would support the yeast 2-hybrid results and provide information about biological function. Additionally, for proteins to interact they must be present in the same cells at the same time. Therefore expression patterns of the candidate genes are expected to overlap although lack of overlapping expression patterns could be inconclusive as proteins can act non-cell autonomously. Regardless of this pitfall, the mRNA expression pattern of a gene can suggest biological function. Because resources for gene inactivation with transposons were available from the John Innes Centre, Norwich, and detection of mRNA by *in situ* hybridisation is well documented for *Antirrhinum*, mutant screens and detection of mRNA expression were pursued in preference to *in vitro* binding assays.
RESULTS. MUTAGENESIS OF PULP

5.1 SCREENING FOR MUTATIONS IN THE ANTIRRHINUM PULP GENE

To obtain a pulp mutant a PCR screening approach was taken using stocks of transposon mutagenised plants from the John Innes Centre (see Materials and Methods). Five gene-specific primers from different positions in the PULP gene were used with five transposon-specific primers in PCR with DNA from pools of mutagenised plants (Table 4.). The reaction products were fractionated in agarose gels (of which an example is shown in Fig. 14A.). The gels were blotted and probed with $^{32}\text{P}$ or DIG-labelled PULP sequences as shown in Fig. 14B. Lanes that had unique hybridising bands were selected as containing potential transposon-induced mutants and DNA from eight subpools corresponding to each positive superpool then screened separately. Although PCR products that hybridised to PULP probes were detected in superpools, results tended not to be reproducible. In addition, similar products were often not detected in the corresponding subpools although these contained the same DNA that had contributed to superpools. Some bands which appeared as strong PCR products but hybridised weakly with DIG labelled probes were isolated by band stabbing and reamplified. A number of these products were sequenced but none were found to correspond to the known sequence of PULP or to show significant homology with it, except in the primer sequences (Fig. 14C, 14D.). Hybridisation of the probe to these bands was probably presence of primer sequence within the probe which were also present in the PCR primers. Probe hybridised with positive control but not to the DNA markers demonstrating high sensitivity and specificity. Therefore failure to detect a transposon insertion was unlikely to result from lack of probe specificity. As no mutants were obtained using the plant pools available from the John Innes Centre mutagenesis program, a second source of mutants was utilised. This was a collection of 440 classical mutants that had arisen spontaneously, probably by transposon insertion, and had been maintained at Gatersleben, Germany. DNA of these was also available from the John Innes Centre as pools, each extracted from 20 or 22 plants. Four gene-specific primers and three transposon-specific primers were used in PCR screens of the Gatesleben pools, including PULP-189 which is directed 5' towards the promoter region. No transposon insertions in PULP were detectable in any of the classical mutants.
Fig. 14. (A) PCR products of Antirrhinum transposon mutagenesis superpools A to H using the primer combinations indicated above each set. Apparently unique bands are visible in the left two sets. (B) Autoradiograph of a filter obtained by Southern blotting from the gel in A when probed with digoxygenin labelled \textit{PULP} probe. Levels of signal were higher from some weak bands on the gel which suggested gene specific product amplification. (C) Reamplification of individual \textit{pulp2354} and \textit{W2052} products from superpools A to H. (D) Autoradiograph showing weak hybridisation between PCR product and \textit{PULP} probe but not with size markers. This was probably due to high sensitivity of the DIG Southern method detecting hybridisation between sequences in \textit{PULP} probe that were homologous to the \textit{PULP} primers. None of the products sequenced were homologous to \textit{PULP} except in the primers.
5.2 VIRAL CO-SUPPRESSION

The major advantages of a transposon-induced mutation are that it is heritable and often unstable, allowing excision of the transposon to be correlated with reversion of the mutation to wild-type. However, alternative methods of reducing gene activity and therefore of testing gene function are available. Use of antisense or double-stranded RNA expression are two common approaches but both involve generation of transgenic plants. Because genetic transformation of *Antirrhinum* was not yet routine and required considerable time, attempts were made to reduce *PULP* expression by viral co-suppression (Ratcliff *et al.*, 1999; MacFarlane and Popovich, 2000). This makes use of a modified RNA virus (in this case a tobravirus - tobacco rattle virus) The tobacco rattle virus genome consists of two RNA molecules that are packaged separately in rod shaped particles (Harrison and Robinson, 1986) RNA1 encodes the viral proteins which are essential for infection and inter-cellular movement. RNA2 encodes a coat protein and non essential genes but requires RNA1 for infectivity. The nonstructural genes of RNA2 have been shown to be necessary for transmission of tobraviruses by vector nematodes thus constructs in which these genes are deleted should be effectively contained in the plants under test. Plant sequences are inserted into a modified cDNA copy of RNA2, transcribed *in vitro* and used to infect susceptible tobacco to produce viable virus particles. Other plants that may be resistant can then be infected with active virus from the tobacco plants. The virus initiates infection but is then silenced as part of the resistance process, along with the introduced and endogenous copies of the target gene (Ratcliff *et al.*, 1999; MacFarlane and Popovich, 2000). While the phenotypes induced by this method are not heritable the technique can be applied directly to existing mutants to examine genetic interactions. The disadvantage of viral co-suppression is that any phenotype obtained may in fact be due to co-suppression of homologous sequences. For example, transfer of phytoene desaturase from tomato to tobacco and subsequent viral co-suppression of the tobacco homologue by the virus associated tomato sequence had demonstrated that the level of homology required for this process can be as low as 92% over a 395 bp segment (Kumagai *et al.*, 1995). As extensive PCR screening for a transposon induced *pulp* mutant had been unsuccessful, viral co-suppression was attempted in collaboration with Stuart MacFarlane, SCRI, Dundee, Scotland.
A construct for expression of viral RNA which included PULP sequence was made using a plasmid, pK20-GFPc, provided by Stuart MacFarlane (see section 3.19). The resulting clone encoded an RNA corresponding to nucleotides 1770-2120 of PULP. Whether this fragment was expressed as a peptide during the infective stage is unknown. The resulting plasmid, pAP9, was sent to Stuart MacFarlane at SCRI for in vitro transcription and co-infection of tobacco plants with RNA1. Innoculum was then obtained from these plants and tested for infectivity in tobacco (also at SCRI). The innoculum was then used to infect 8 plants each of *Antirrhinum* JI75, *phan-249* and *phan-250* on the four youngest leaves at the five node stage. Control plants were inoculated with another construct encoding GFPc, TRV-GFPc, to determine if TRV could infect *Antirrhinum* and enable expression from RNA2 from this construct to be monitored. Innoculated leaves and leaves that might be systemically infected from these plants were examined under UV light but no fluorescence could be distinguished from background chlorophyll fluorescence. Whole RNA was purified from inoculated and systemic leaves and sent to Stuart MacFarlane for RTPCR of RNA1 and RNA2. Transcript of the correct size was detected by this method for AP9 but not TRV-GFPc in inoculated leaves (Stuart MacFarlane, pers. comm.). However, no phenotypic difference could be seen between plants that had been inoculated with TRV-GFPc, AP9 RNA or plants mock inoculated with water and grown under the same conditions. To make sure that reduction in PULP expression was not aphenotypic, RNA gel blots of systemic leaf tissue from three inoculated plants was used to test if PULP expression level was altered but there was no apparent difference in PULP expression level in these plants compared to mock inoculated plants or un-inoculated plants using this technique (Fig. 16.). Because infection appeared to be weak in *Antirrhinum* it was thought that heavier infection at an earlier stage of growth might be effective (Stuart MacFarlane, personal observation.), therefore, JI98 plants were inoculated on all leaves at the four leaf stage (3 node). Four out of ten plants inoculated in this way had a phenotype that was not seen in mock inoculated plants, TRV-GFPc inoculated plants or plants inoculated with constructs including sequence of *Antirrhinum CURLY LEAF* homologues (Carol Wilson, pers. comm.). The plants had disrupted inflorescence phylotaxy which mainly resembled variably reduced internode length and increased numbers of leaves at some nodes. The most obvious feature was knots of cell
proliferation in the leaf mesophyll which were visible as transparent spots lacking chlorophyll under reverse lighting of leaves Fig. 15A. A median section through leaf tissue with this phenotype is shown in Fig. 15B, revealing changes in cell organization in all adaxial and some abaxial cell layers. Similar features were apparent in the stem which caused bulging and splitting of the epidermis possibly due to ectopic cell growth in the cortex. To determine whether PULP message levels had been changed by the viral co-suppression experiments described above, frozen tissue from inoculated leaves and leaves that may have been systemically infected on plants with or without phenotype was stored to enable RNA purification and northern analysis.
Fig. 15. Features of Antirrhinum J198 leaves showing phenotype induced by infection with tobacco rattle virus encoding PULP sequences. (A) leaves from equivalent nodes of unaffected (left) and affected (right) plants when lit from behind. (B) median section of a “knot” of cells from affected leaf showing loss of palisade cells, disruption of cell layers and emergence of cells through the adaxial surface of the leaf.
RESULTS. EXPRESSION ANALYSIS OF PHAN AND PULP

To demonstrate the potential of PHAN to interact with PULP in vivo and gain insight into the potential role of PULP, the expression pattern of PULP mRNA was investigated. This initially involved RT-PCR analysis. PCR from genomic DNA using primers designed to the PULP cDNA revealed that PULP has no intron within its open reading frame. This presented a problem for RT-PCR in that genomic DNA contamination might produce a signal that could not be distinguished from cDNA signal by size difference. Therefore a poly(A)-specific primer, QT, which had two nested primer sites in its 5' region was used for cDNA synthesis. PCR was then done using a gene-specific primer (PULP1839) and a QT-specific primer so that only polyadenylated cDNA could act as a template. The products were used in Southern blots and probed with a region of PULP that did not include the sequence of the gene-specific primer used in RT-PCR. However, this did not resolve gene-specific products because of a high level of background hybridisation to other PCR products. A similar level of background was seen using a second PULP-specific primer, PULP894. Regardless of adjustments to the PCR conditions, the background remained too strong to resolve a gene-specific product by Southern hybridisation. Although nested PCR could have been used to increase specificity of PCR, it was expected to provide a less quantitative result. Therefore Northern hybridisation, which can provide quantitative expression data but requires larger amounts of tissue, was carried out on total RNA from different tissues of wild-type and phan mutants. Hybridisation with a PULP antisense RNA probe detected a band in all tissues, although it was present at a lower level in floral organs and roots. Because PHAN is expressed in lateral organs, the PULP expression pattern demonstrated that PULP had the potential to interact with PHAN at least at the resolution of the tissue types from which mRNA was purified (Fig. 16.).

To further localise PULP RNA expression, in situ hybridisation was carried out (Jackson, 1991; Long, 1997). For this purpose, antisense digoxigenin-labelled RNA probes were made complementary to the 3' UTR of PULP, the full length cDNA excluding the poly(A) tract or a 460 bp fragment immediately 5' of the S1-1 region. These detected no message in wild-type tissues, although PHAN probes used as positive controls worked on each occasion. Finally the S1-1 region was subcloned into pBluescript making pAB1 and this enabled T7 RNA polymerase to be used to
make an antisense probe for this region of *PULP*. Probe generated from this
template produced signal in the L2 and L3 and single cells of the abaxial L1 of bracts
and sepals (Fig. 17A.) More diffuse staining was present in the cortex of internodes
but absent from the inner cells (Fig. 17B.). Re-examination of tissue previously
probed with other *PULP* probes revealed similar, but weaker, staining (not shown).
However, the sense probe corresponding to the same region of *PULP* produced a
similar staining pattern (Fig. 18.). Although hybridisation with sense probes is
widely used to reveal background staining, a sense probe is not an ideal control for
non-specific binding as antisense DIG labelled RNA can be produced by T7 RNA
polymerase as an artefact of sense strand synthesis. This was confirmed for the
*PULP* sense probe by using it against RNA gel blots of *Antirrhinum* RNA samples
(Fig. 16.). Although *PULP* sense RNA probes were expected to detect no
transcripts, they identified a band in RNA from young leaves in which *PULP* sense
RNA is expressed at a high level. Because the hybridising band was the same size as
detected with the *PULP* antisense probe, it seemed likely that the *PULP* “sense”
probe also contained antisense sequences.

A second possibility was that the sense probe detected an endogenous antisense
*PULP* RNA. However, examples of antisense transcripts in plants are few
(Klaff *et al*., 1996) and persistence of such molecules is likely to be brief since
known examples are associated with RNA degradation (Bourque, 1995).

Furthermore expression of vertebrate and fungal double stranded ribonuclease in
transgenic plants has no effect on their growth or development (Mitra *et al*., 1996;
Sano *et al*., 1997). The possibility therefore remained that the signal obtained from in
situ hybridisation reflected expression of *PULP*. 
(far left) lanes V1 to V3 are whole RNA of young leaves from viral co-suppression infected plants and young leaves (YL) from a mock inoculated plant demonstrating no change in PULP expression level when probed with antisense PULP RNA. (near left) young leaf and stamen (ST) RNA probed with sense in situ probe produced a band of the same size as bands in all other samples. This was not detectable in tissue where PULP was expressed at low level.

Fig. 16. Northern blots of whole RNA from various Antirrhinum tissues probed with PULP in situ probes. (Top) PULP expression appears to be almost ubiquitous when probed with anti-sense RNA probe. Tissue samples are (veg) vegetative tissue; (AP) apices; (YL) young leaves less than 0.5 cm; (OL) old leaves; (YF) young flowers; (INF) inflorescence; (NLS) needles; (HSL) heart-shaped leaves. The mutants tested were Hirzina (Hirz) which is a dominant misexpressor of AmSTM1; graminifolia (gram) which is a mutant with similar phenotype to phan having narrow leaves; handle bars (hb) was isolated by Richard Waites in a screen for temperature sensitive enhancers of phan.
Figure 17. The expression pattern of PULP in *Antirrhinum* inflorescence tissue as determined by insitu hybridisation. (A) single cell expression in L2 and abaxial L1 of bracts and sepals but absent from the adaxial L1. (B) expression in the inflorescence internode cortex, c; but absent from inner cell layers, i.
Figure 18. In situ hybridisation of PULP sense probe to *Antirrhinum* inflorescence tissue showing signal in the cortex of the inflorescence and in single cells of the bracts (arrows) but absent from the adaxial L1. Signal characteristic of background is visible in trichomes.
7 RESULTS. THE *ARABIDOPSIS* PULP HOMOLOGUE

7.1 T20403 GENE STRUCTURE AND COMPARISON WITH *PULP*

The *PULP* cDNA sequence was used to search databases using the BLAST program (Altschul *et al.*, 1997), which revealed a single *Arabidopsis* homologue, initially as a single EST (Genbank accession number T20403). The deduced protein product of this EST showed 39% amino acid identity and a further 17% similarity to the *Antirrhinum* PULP sequence over a 100 amino acid region. The EST clone, 78G3T7, was obtained from the *Arabidopsis* Biological Resource Centre at Ohio State University, USA and sequenced to reveal a 408 bp insert with a poly(A) tract at the 3' end. However, 78G3T7 terminated 3' of the region of the *Arabidopsis* *PULP* homologue which corresponded to *Antirrhinum* S1-1, suggesting that it was incomplete. Therefore 78G3T7 was used as a probe to screen an *Arabidopsis* cDNA library in λ Zap which had been made from wild-type inflorescence tissues and provided by Detlef Weigel, Salk Institute, La Jolla, California. This library screen yielded three partial cDNA clones from the same gene which were polyadenylated at their 3' ends and had the potential to encode proteins with considerable similarity to *Antirrhinum* PULP. None of the clones extended as far 5' as the *Antirrhinum* PULP gene, suggesting that they were also incomplete, probably as a consequence of using a probe to the 3' end of the gene. Shortly afterwards, the full genomic sequence of the *Arabidopsis* *PULP* locus was published as part of the European *Arabidopsis* sequencing project (Bevan *et al.*, 1998) The *Arabidopsis* *PULP* homologue appeared as a predicted gene, designated AtF10.170, which included sequences present in the cDNA clone T20403. This enabled the 5' region of the gene cDNA to be obtained by PCR with primers designed from the genomic sequence. An alignment of the deduced amino acid sequences of PULP and its *Arabidopsis* homologue, encoded by T20403, is shown in Fig. 13. Searches of the *Arabidopsis* sequence database revealed an EST, from *Arabidopsis* ecotype Columbia roots, that showed 97% identity at the nucleotide level to the 3' region of T20403. Because of this high degree of similarity, the differences between this sequence and T20403 probably reflected allelic polymorphism or sequencing errors. Subsequent analysis of the completed *Arabidopsis* genome sequence failed to reveal a second PULP-like gene, supporting this conclusion. The occurrence of the EST in a
library from root tissues also suggested that the *Arabidopsis* gene was expressed in roots. Comparisons of the genomic and cDNA sequences suggested that no introns were present within the *Arabidopsis PULP* coding region. This was confirmed by RT-PCR for the region of T20403 covered by cDNA sequences. The lack of introns was therefore a feature that T20403 has in common with the *Antirrhinum PULP* gene which lacks introns throughout its coding region and 3' UTR.

Searches of the non-redundant protein database at the EBI with MPsearch_pp, which compares sequences based on a prediction of their secondary structure (Collins and Sturrock, 2000; Smith and Waterman, 1981a, b) revealed that the protein with the highest similarity to both PULP and T20403 (apart from with each other) was the coiled-coil domain of the human centromeric protein E (CENP-E; Yen et al., 1992) between amino acids 1400 and 1980. Within this region, PULP showed 30% identity and 51% similarity over a 260 amino acid stretch, and T20403 showed 25% identity and 52% similarity over a 446 amino acid stretch. Both proteins also shared similarity with Restin (24% and 59%); myosin II non-muscle heavy chain (52% and 57%, for PULP and T20403 respectively to *Drosophila* myosin II). Like CENP-E, restin and myosin form coiled-coil structures in the region being compared. The Coils program (Lupas et al., 1991) also predicted with high probability that PULP and T20403 are likely to form coiled-coils in this region. Therefore although these similarities suggested that PULP and T20403 are likely to form coiled-coil structure, they appeared unlikely to suggest the biochemical or developmental functions of PULP and T20403. PULP and T20403 structure are discussed in relation to the predicted structure of PHAN homologues in section 9.1.

### 7.2 Interaction of AS1 and T20403 in the Yeast 2-Hybrid Assay

The yeast 2-hybrid assay had been successfully employed to isolate PULP and characterise its interaction with PHAN. Therefore it was employed again to test whether similar interactions occurred between the products of the orthologous genes from *Arabidopsis: AS1* (the orthologue of *PHAN*) and T20403 (the putative orthologue of *PULP*). Unlike *Arabidopsis*, the *Antirrhinum* genome contains at least one other *PHAN*-like gene, *PHANL-1*, therefore this was also tested for interaction with *PULP* and its *Arabidopsis* homologue. Initially full-length AS1 and T20403
bait and prey constructs were made but no interaction could be detected. Because interaction between full length PHAN and PULP had been found to be weaker than between their sub-domains it was possible that this phenomenon also affected interaction between AS1 and T20403. Therefore a prey construct was made which included the region from T20403 equivalent to that required for strong interaction between PHAN and PULP (S1-1). The T20403-S1-1 equivalent was made from T20403 using a PCR primer (ATPI198) to introduce an Eco RI site at the start of the region equivalent to S1-1 for fusion to the GAL4 domains. Once cloned into bait vectors the insert was subjected to exonuclease deletion by the method of Henikoff (1987). A clone corresponding to the S1-1 region was obtained by this method and designated T20403403-539. The un-deleted clones were also used for yeast 2-hybrid assays. Concurrently with sequencing of the T20403 deletions it was discovered that a single base pair insertion was present in the AS1 bait clone at nucleotide 835, (Fig. 33) resulting in a missense mutation and predicted substitution of 14 amino acid residues before premature termination of the peptide. The missense mutation had probably been introduced as a PCR error but turned out to be informative since it disrupted the equivalent region of AS1 to that required for PHAN to interact with PULP. Replacement bait and prey constructs were made using proof-reading *Pfu Turbo* DNA polymerase (Stratagene) in PCR reactions to reduce the risk of PCR errors being incorporated into the clones. The PCR products were then sequenced before subcloning into yeast 2-hybrid vectors. The results of yeast 2-hybrid assays using these constructs are shown in Fig. 19.

Fig. 19(B),(C) shows that the C-terminal end of T20403, encoded by T20403403-764, was weakly auto-activating as prey. A component of this appeared to be due to interaction with the GAL4-DB domain since weak HIS3 activation was seen when yeast was co-transformed with T20403403-764 and pGBT9. Autoactivation by T20403403-764 appeared to be below the threshold of detection for the β-galactosidase assay and was suppressed entirely on media containing 10mM 3AT. T20403403-764 bait appeared strongly auto-activating because the HIS3 reporter gene was expressed at high levels in yeast transformed with T20403403-764 bait and pGAD424 (Fig. 19B). This appeared not to be dependent on the presence of the GAL4-AD domain encoded by pGAD424 since T20403403-764 bait also caused autoactivation when present alone in yeast (see section 8.2).
Figure 19. Yeast 2-hybrid assays for T20403 and AS1. Baits are on the left and preys are along the top. Interaction between T20403\textsuperscript{403-764} and T20403\textsuperscript{403-539} was not included. (A) -LW media for selection of expression plasmids; (B) -LWH selection for interaction and activation to the HIS reporter gene; (C) increased selection for activation of the HIS reporter gene imposed by including [5mM] 3AT; (D) $\beta$-galactosidase assay. Interaction between AS1 bait and the C-terminus of T20403\textsuperscript{403-764} can be seen in B and C but the $\beta$-gal assay is not sensitive enough to reveal it. The opposite assay shows directionality similar to that seen with PHAN and PULP because T20403\textsuperscript{403-764} bait interacts more strongly with AS1 prey and is detectable with the $\beta$-gal assay in C. The strongest interaction was homodimerization of AS1. The C-terminus of T20403 also appeared to dimerize as suggested by the $\beta$-gal assay although a component of this was autoactivation which was independent of the GAL4 AD, see section 8.2.
T20403\textsuperscript{403-764} autoactivation was also susceptible to suppression of the histidine biosynthetic pathway with 10mM 3AT, enabling interaction between AS1-bait and T20403\textsuperscript{403-764} to be detected (Fig. 19C). The interaction was clearly weaker than that observed between PHAN and PULP and below the threshold detectable with the β-galactosidase assay.

Directionality, as found for the interaction between PHAN and PULP, was also observed in the interaction between AS1 and T20403. Full-length T20403 interacted more strongly with full length AS1 than vice versa (Fig. 19C). Importantly S1-1 bait did not interact with PHANL1 prey or with AS1 prey (Fig. 25C). The converse interaction between T20403\textsuperscript{403-764} (the nearest T20403 equivalent to S1-1 subjected to this test) and PHAN or PHANL1 was less clear because of autoactivation by PHANL1 bait. (Fig. 19C). Together, these results suggest that interactions of PHAN with PULP and AS1 with T20403 are specific and are conserved between \textit{Antirrhinum} and \textit{Arabidopsis}.

One caveat to this proposal is that no interaction was observed between AS1 bait and T20403\textsuperscript{403-539} (Fig. 19), the equivalent region to S1-1 in T20403 based on the overall protein alignment. This may be because T20403\textsuperscript{403-764}-derived preys were unstable, as suggested by patchy interaction with T20403-bait. Alternatively, it might have been because the interacting region was no longer entirely coded by this region in T20403.
7.3 EXPRESSION PATTERN OF T20403

To enable further comparisons between PULP and T20403, attempts were made to examine the mRNA expression pattern of T20403 by *in situ* hybridisation. No signal could be detected with probe made from the whole ORF of T20403, however, probes made from 5' and 3' deletion clones both produced signal. The staining obtained with T20403 was similar to that of *PULP* in *Antirrhinum* with signal appearing diffusely in the cortex of inflorescences but also detectable across the apical domain of both inflorescence and floral meristems (Fig. 20A). No signal was detected with sense probe however (Fig. 20B). Expression of *ASI* in *Arabidopsis* is equivalent to that of *PHAN* in *Antirrhinum* (Byrne *et al.*, 2000), and therefore overlaps with that suggested for T20403. Time was not available to optimise the *in situ* experiment and obtain either the vegetative expression pattern or improved signal in the inflorescence, both of which are required to enable an informed assessment of T20403 function in relation to AS1.
Fig. 20. In situ hybridisation of T20403 antisense RNA probe to *Arabidopsis* inflorescence tissue. (A) shoot apex; (B,C) axillary inflorescences and stems. Signal suggesting T20403 expression occurs in the cortex of the inflorescence, across the SAM and within organs (A and B) but no signal was obtained with sense probe (C). (c) cortex; (s) SAM; (f) floral organs; (fp) floral primordia.
7.4 MUTAGENESIS OF T20403

7.4.1 MUTAGENESIS BY TRANSPOSON TARGETING

The Sainsbury Laboratory *Arabidopsis thaliana* transposon (SLAT) mutagenesis program (Tissier et al., 1999) was used to screen for a transposon insertion in T20403 with the expectation that a mutant would provide insight to biological function. The SLAT collection consisted of 48,000 *Arabidopsis* lines, each of which carried a different insertion of a defective *Spm* transposon (dSpm). The transposon carried a gene conferring resistance to PPT (BASTA) herbicide and had initially been introduced within an *Agrobacterium* T-DNA. The T-DNA also included the *Spm* transposase gene and the cytochrome p450 gene from *Streptomyces griseolus* (*SUI*) as a counter selectable marker, that causes plants carrying the T-DNA to have a small, dark green phenotype and to be susceptible to the proherbicide R7402 (Dupont). DNA had been isolated from 48 pools of 50 plants and subjected to inverse display of insertions (IDI) which utilises iPCR to amplify sequences flanking the transposon insertions in the lines. The iPCR products had been spotted by superpool in an array on a filter which could be probed to rapidly identify plant pools with an insertion in, or near, a gene of interest. Probing of the filter with a 32P-labelled probe from T20403 revealed a potential insertion in subpool 14 of superpool 06. The DNA corresponding to this pool of 50 plants was obtained and PCR carried out with dSpm- and T20403-specific primers. Primers located at the ends of the T20403 gene and directed into the open reading frame of T20403 in combination with dSpm-specific primers gave no product. Use of primer ATP1988, which is located at the 3' end of the T20403 ORF pointing away from the gene, in combination with dSpm specific primers, yielded conflicting results because a product that hybridised with the T20403 probe was obtained with primers from both ends of the transposon. Sequencing of PCR products revealed that ATP1988 was priming spuriously 3' of the site of a dSpm insertion. Hybridisation with the T20403 probe was probably due to presence of the ATP1988 primers in the PCR product. However, the sequence clearly indicated an insertion close to T20403, so seeds from the subpool of 50 plants were sown. Approximately 280 seedlings were grown in seven trays (5 rows to a tray and approximately 8 plants to a row) and selected for PPT resistance. PCR was done on DNA pools from each tray of plants, and then
from candidate rows and individual plants. This enabled a candidate mutant to be isolated in tray 6, row C, plant 6 (C6). Using primers ATP1988 and DSPM11 or PASY1 with DNA from C6 or a sibling gave the results shown in Fig. 21. PASY1 was located 3' of the transposon insertion point relative to T20403 and could not prime amplification across dSpm. In the sibling lacking the insertion, the product of ATP1988 and ASY2 of 1166bp was visible. The converse was seen with primers ATP1988 and DSPM11 for which a 1088 bp PCR product was dependent on presence of the dSpm. The PCR results suggested that C6 was homozygous for the dSpm insertion but it was unclear if more than one tDNA insertion was present.

Fig. 21. PCR products obtained with transposon specific or gene specific primers flanking the dSpm insertion site near T20403. PCR product A-B was only obtained with gene and dSpm specific primers in C6 which harboured the insertion. This product was not obtained in a sibling plant (SIB). Conversely, PCR product A-A was only obtained in the sibling and absent from C6 due to the size of the intervening sequence caused by dSpm insertion. This suggested that C6 was homozygous for the insertion. During PCR screening product was obtained with ATP1988 and primers specific for both ends of the dSpm due to mispriming by ATP1988 at a 3' site (ATP1988 misprime).

Sequencing of the ATP1988-dSPM11 PCR product revealed that insertion of the dSpm had occurred 754 bp 3' of the ORF of T20403 and also beyond the poly(A) sites of the characterised cDNAs. It was also very close to the 5' end of the neighbouring gene, ASYNAPTIC2 (ASY2). This is a homologue of the *Arabidopsis* ASYNAPTIC1.
gene (ASY1) which is required for meiotic synaptonemal complex assembly (Caryl et al., 2000). The point of dSpm insertion was 56bp upstream of the transcriptional start point of ASY2 (Anthony Caryl pers. Comm.). In long days (16 hrs light), C6 plants could not be distinguished from wild-type, but when grown in short days (8 hrs light) they were smaller and greener than wild-type Columbia plants (Fig. 22). Although this phenotype might have resulted from disruption of the ASY2 gene, it also resembled the phenotype of plants carrying the SUI marker associated with the T-DNA from which dSpm had transposed. Although SLAT lines had been subjected to counter-selection against SUI, it was possible that they carried a T-DNA with a leaky suI mutation. However, because the insertion was unlikely to have affected T20403 function, this analysis was not pursued.

Fig. 22. Ecotype Columbia (left) and C6 plants (right) grown in short days showing reduced growth and dark green phenotype of C6 plants relative to wildtype. (22A,B) Mobilisation of the dSpm transposon located close to the 3' end of T20403. (22A) PCR bands produced from genomic DNA of candidate mutants A1, A3 and A8 with primers DSPM1 and ATP1 (which is located 3' of the ORF priming upstream) were barely visible in gels but hybridised strongly with T20403 probe when subjected to Southern analysis (22B). This suggested dSpm mobilisation had occurred and was present in T20403.
7.4.2 MOBILISATION OF THE dSpm TRANSPONSON NEAR ASY2

Although the dSpm insertion between T20403 and ASY2 yielded no useful phenotype it was known that plant transposons have a marked preference for insertion into genetically linked sites - a property that has been used to perform local mutagenesis in a region of interest (Das and Martienssen, 1995). Therefore an attempt was made to mobilise the dSpm located near ASY2 into T20403 by providing Spm transposase in trans. At that time the only plants expressing Spm transposase which were available were those carrying the T-DNA used in production of the SLAT lines. These carried Spm transposase under control of either the 35S promoter (line 8313#1) or Spm promoter (line 8337#9) together with the counterselectable marker SUI and a dSpm element encoding PPT resistance (Tissier et al., 1999). Both lines had been selected for a relatively high frequency of transposition to unlinked sites from the T-DNA "launchpad". The T-DNA in 8313#1 was located in chromosome 1, and was therefore unlinked to T20403 which was on chromosome 4. The location of 8337#9 in the genome was unknown. In the flow diagram below the procedure and expectation of the steps in this process are outlined on the left and the results of each cross are on the right (Fig. 23).

<table>
<thead>
<tr>
<th>PROCEDURE</th>
<th>EXPECTATION</th>
<th>8313#1 transposase controlled by 35S</th>
<th>8337#9 transposase controlled by Spm</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASY2 X T-DNA</td>
<td>ASY2 HOMOZYGOUS T-DNA HETEROZYGOUS</td>
<td>5 crosses</td>
<td>3 crosses</td>
</tr>
<tr>
<td>F1</td>
<td>F1</td>
<td>F1</td>
<td>F1</td>
</tr>
<tr>
<td>CHOOSE SUI AND ALLOW SELFING</td>
<td>ALL PLANTS WITH SUI CARRY ASY2 AND ARE HEMIZYGOUS FOR BOTH LOCI</td>
<td>choose 35</td>
<td>choose 3</td>
</tr>
<tr>
<td>APPLY DOUBLE SELECTION TO F2</td>
<td>1/8TH OF F2 ARE ASY2 HETS (AND NOT SUI), 1/16TH OF F2 ARE ASY2 HOMOZYGOUS (AND NOT SUI)</td>
<td>4 potential mutants</td>
<td>10 potential mutants</td>
</tr>
<tr>
<td>POOLS OF SURVIVORS SCHREENED BY PCR</td>
<td>NEW ALLELES FROM F1 BECOME HOMOZYGOUS</td>
<td>75000 seed sown of which 1600 pooled and screened by PCR</td>
<td>6500 seed sown of which 190 pooled and screened by PCR</td>
</tr>
<tr>
<td>ALLOW SELFING</td>
<td>F3</td>
<td>F3</td>
<td>F3</td>
</tr>
<tr>
<td>OBSERVE FOR PHENOTYPES</td>
<td>NEW ALLELES FROM F2 BECOME HOMOZYGOUS</td>
<td>10 flats of seed</td>
<td>4 flats of seed</td>
</tr>
</tbody>
</table>

As both C6 and transposase parents carried PPT resistance markers, 8313#1 and 8337#9 plants were crossed as male to C6 so that the SUI phenotype could be used
as a marker for F1 progeny that had inherited the transposase-containing T-DNA. C6 was homozygous for the insertion near ASY2, and therefore all its F1 progeny would be hemizygous for the dSpm insertion. F1 plants showing the SUI phenotype were retained because activation of dSpm by Spm transposase could potentially occur in these plants to generate T20403 loss-of-function mutations which would be transmitted to the F2 generation. These mutations were expected to be heterozygous in the F2 generation, and therefore detectable either by screening the genotypes of F2 plants, or by screening F3 progeny for recessive mutant phenotypes. It was assumed that the ASY2 and T-DNA loci would each segregate independently and each allele would recombine in the following manner AA, Aa, aA, aa thus producing a 1:2:1 ratio. Consequently when plants heterozygous for the insertion in ASY2 (and T-DNA) were selfed the ASY2 alleles (and a low proportion of transpositions to linked sites) would occur in the ratio ¼ homozygous, ½ heterozygous, ¼ wild-type. Considering the two classes where ASY2 alleles (or transposants) are present, assuming the T-DNA segregates ¼ (T-DNA --) and ¼ (++) and applying the product rule then 1/8th (¼ x ¼) would be expected to occur without the T-DNA and be heterozygous for an ASY2 allele (or transposition) and 1/16th would be expected to occur without the T-DNA and be homozygous for an ASY2 allele (or transposition). Double selection enabled this class to be obtained directly. From five separate crosses between 8313#1 and C6 plants, 35 F1 plants had the SUI phenotype and were allowed to self pollinate to produce an F2 generation of approximately 75,000 seedlings. Double selection for dSpm (PPT resistance) and against transposase (insensitivity to R7402) was applied. Four plants with abnormal morphology, which potentially carried dominant mutations were also selected at the seedling stage. Seeds from the double resistant (DR) F2 plants were pooled and sown to reveal recessive phenotypes in the F3 generation. DNA was purified from potential mutants and from 20 pools of approximately 80 phenotypically wild-type DR plants for PCR-based screens. From three separate crosses between 8337#9 and C6, three F1 plants had acquired the SUI marker. Approximately 6,500 of their F2 progeny were sown and 10 potential mutants selected at the seedling stage. Seeds from DR F2 plants were treated as above, but no mutant phenotypes were observed in the F3 generation. The 10 potential mutants and approximately 150 DR plants from the F2 were screened by PCR in pools of eight or 30 plants. PCR of the mutant and pooled DR
plant DNA using T20403 and dSpm-specific primers suggested that transposition had occurred because novel bands were observed. Some of these probably included T20403 sequences because they were detected strongly by T20403-specific probes in Southern hybridisation (Fig. 22). However, these bands could not be reproduced when smaller pools of F2 plants were screened, possibly because the bands represented somatic dSpm insertions that were not present in all tissues of any particular plant. Control reactions with transposon-specific primers also produced a range of bands unique to each pool. When sequenced, these were found to consist of T-DNA, even though the plants had been selected for absence of the SU1 marker present in the T-DNA. One explanation for this was that the dSpm present within the T-DNA had inserted into the SU1 gene to inactivate it, and that a copy of dSpm was also retained at its original site, so that amplification between dSpm elements could occur.

The frequency of transposition to sites unlinked from the T-DNA for line 8313#1 is now known to be 5.5x10^{-4} (Tissier et al., 1999) whilst the frequency for line 8337#9 has been reported as 6x10^{-5}. Since around 3/16 of plants generated from the crosses should have survived double selection (assuming that new insertions affect these numbers insignificantly), crosses with 8313#1 should have produced approximately five transposants and those with 8337#9 less than one. However, 6 and 14 potential mutants were detected at the seedling stage respectively. These would have to have carried either dominant mutations or recessive mutations that occurred early in development of their parents and were therefore made homozygous when their parents self pollinated. PCR screening for transposition of the ASY2 linked dSpm was handicapped by the presence of the original insertion. Also, in some cases it appeared that T-DNAs had escaped selection and may have caused new somatic insertion events.

Mutagenesis of T20403 by mobilisation of the ASY2-linked dSpm had proved difficult to achieve and the observed frequency of potential mutations appeared higher than predicted. This may have been a consequence of transposase activity from undetected T-DNAs or combined transposition from both ASY2 and the T-DNA. Alternatively the frequency of mutations may have been caused by the ASY2-linked dSpm which was not selected for unlinked transposition.
7.4.3 A GENETIC SCREEN FOR T20403 CANDIDATE MUTANTS

No insertions into T20403 were detectable by PCR, and the possibility remained that the T20403 locus was refractory to insertion or an essential gene. Based on the expectation that a T20403 mutant might alter the phenotype of as1-1 mutants a genetic screen of some candidates was attempted. One plant from a cross between 8313#1 and C6 plants was isolated on the basis of having a single spoon shaped cotyledon. Selection for presence of dSPM and counterselection against transposase had been applied and the plant did not have the small green phenotype characteristic of plants carrying the SU1 marker. When DNA from the plant, which was called A21a, was subjected to PCR with a T20403 and dSPM specific primers it consistently produced poorly resolved product detectable by Southern blotting with T20403 probe when other similarly treated plants did not. PPT resistant progeny from A21a crossed into wild type Columbia plants could be categorised into three phenotypes including wider short, sometimes serrate, leaves somewhat like as1-1; highly reduced plants with poor fertility and almost wild type plants with pointed rather than rounded rosette leaves. A genetic ratio for these phenotypes has yet to be established. It seemed reasonable that an insertion or deletion might have occurred within the region of T20403 that could not be detected by PCR and this might have been detectable using Southern hybridisation to genomic DNA digests. However, the presence of other candidates made it less labour intensive to cross A21a and the other candidates to as1-1 mutants and test for a phenotype. as1-1 mutants were crossed as female to A21a plants with a rounded leaf phenotype so that the progeny carrying A21a could be selected for with PPT. Self pollination of the PPT resistant progeny produced a variety of phenotypes. Occurring at low frequency (approximately 1/16 in a small sample) were plants with the phenotype shown in Fig. 24A. The cotyledons are relatively normal but leaves appear progressively abaxialized. Rossette leaves have patches only of adaxial “jigsaw” pattern cells on the adaxial surface of reduced blades. As leaves are produced they become progressively needle-like and after node 8 the primordia start to produce trichomes at an early stage Fig. 24B. When an inflorescence is produced the cauline leaves are very reduced and radially symmetrical. The putative T20403 as1-1 mutant has normal flowers except that the stamens become shrivelled and don’t reach the stage of dehiscense. One expectation of interaction between two proteins is that one might enhance the
Fig. 24. At low frequency crosses between as1-1 and A21a (a candidate mutant obtained from the dSpm mobilization experiment) produce progeny with the phenotype shown above. (A) A plant showing progressively severe leaf phenotype where adaxial tissue with characteristic jigsaw pattern shaped cells is reduced to patches such as that indicated by the arrow. Successively older leaves become reduced in width and start to acquire radial symmetry. (B) Later leaves are needle-like and have trichomes around their circumference suggesting that they are adaxialized or that they are abaxialized and have passed the developmental stage when Arabidopsis start to produce trichomes on the abaxial leaf surface (Telfer, et. al., 1997).
Fig. 24C. Compared to wild-type (left), A2la Arabidopsis mutants (bottom right) have wider or more heart shaped leaves which resemble those of as1-1 mutants (top right). One expectation of interaction between proteins is that one may enhance the function of the other, on this basis the phenotype of A2la is consistent with reduction of AS1 function. Scale bar length is 1cm.
function of the other. The rounded leaf shape of A21a resembles that of asl-1 which is a reduction of AS1 function (Fig 24C). When asl-1 and A21a are crossed a phenotype that has features of the phan phenotype is obtained. If the following factors are considered: first, T20403 was found to interact with AS1 in the yeast 2-hybrid assay; second, A21a arose from an attempt to mobilise a transposon located close to T20403 and transposons are known to move short distances (Das and Martienssen, 1995); third, the phenotype of the A21a asl-1 double is more like PHAN than asl-1 or A21a; forth, A21a was initially selected on the basis of having an abnormality (a spoon-shaped cotyledon) a phenotype that does not bear any relation to AS1 and therefore was not selected because it resembled asl; then it appears likely that (A) A21a contains some disruption of T20403 (B) AS1 and T20403 interact in vivo and (C) the interaction is biologically relevant to the function of AS1. The possibility that the A21a phenotype is due to mutagenesis at another site has a lower probability given these points, however both genetic and molecular methods can be used to determine whether this is the case or whether T20403 is affected.

7.4.4 RNA INTERFERENCE OF T20403 EXPRESSION

Chuang and Meyerowitz (2000) had shown that expression of RNA capable of duplex formation was an effective method of gene silencing in Arabidopsis. They used Agrobacterium transformation to introduce inverted repeats of transcribed regions driven by the viral 35S promoter. Four different genes required for development of Arabidopsis were efficiently silenced by this method and produced phenotypes similar to previously described loss-of-function mutants. Because it appeared that transposon mutagenesis might not provide a T20403 mutant an RNAi approach was also taken to attempt silencing of T20403. An interrupted inverted repeat of T20403 sequences was made using part of the T20403 gene as a linker between the repeats instead of the β-glucuronidase gene fragment used by Chuang and Meyerowitz. To circumvent the possibility that a T20403 mutant was lethal, the duplex construct was placed under the control of an ethanol inducible promoter derived from Aspergillus nidulans. The ethanol-inducible system had been generated by Caddick et al. (1997) and consisted of two components: the ethanol response gene (AlcR) and a promoter fusion including the A. nidulans alcohol dehydrogenase promoter (palcA) and the cauliflower mosaic virus 35S minimal promoter. The alcR
gene product activates transcription from the palcA fusion promoter only in the presence of ethanol. Two T20403 inverted repeat constructs, pAS3 and pAT5, were placed under control of the palcA promoter in the Agrobacterium binary vector, pGreen (Hellens et al., 2000) and a construct, pAU2, consisting of the T20403 ORF in sense orientation under control of the alcA promoter was also made (see Section 3.19). Inducible expression of T20403 sense or duplex RNA could then be achieved by crossing transformants with an Arabidopsis line harbouring a 35S:alcR construct in the kanamycin selectable T-DNA derived from pBIN19. Agrobacterium-mediated transformation of Col-0 and asl-1 mutant plants was then performed, and approximately 8,000 seeds from each transformation selected for PPT resistance encoded by the T-DNA from pGreen. Only one and two PPT resistant plants were identified in transformation of the asl-1 mutant with pAS3 and pAT5. Because none showed an asl mutant phenotype, they were likely to be PPT resistant contaminants. However, the mature plants differed from wild type Columbia in having smaller than normal rosette leaves and more branched inflorescences. In addition when 50 F1 seed from the putative asl-1 pAT5 plants were sown without PPT selection 1/4 had the asl mutant phenotype, indicating the primary transformants carried the asl mutation. However, an equal proportion of both asl mutants and their wild-type siblings were sensitive to PPT, demonstrating that the semi-wild-type phenotype was not caused by a T-DNA carrying PPT resistance (thus arguing against the possibility that leaky PULP suppression suppressed the asl-1 phenotype). The simplest explanation for the genotype of these plants was therefore that they had either (1) resulted from contamination of asl-1 parents with wild-type pollen carrying a PPT resistance gene unlinked to ASl or (2) were hemizygous asl contaminating seed that carried a PPT resistance gene.

Transformation of Col-O plants yielded one PPT resistant plant with pAU2 and six with pAH54. Whether these are genuine transformants remains to be determined. In the case of pAH54 and pAU2 this may be confirmed by PCR using M13 primers which are complementary to part of the T-DNA encoded by pGreen. PCR across the stem loop was found not to be an efficient process during clone construction but pAS3 and pAT5 transformants may be confirmed using an M13 primer and T20403 specific primer situated in the flank of the stem-loop construct.
8 RESULTS. OTHER ASPECTS OF INTERACTION BETWEEN PHAN HOMOLOGUES

8.1 DIMERISATION OF PHAN AND HOMOLOGUES

Previous yeast 2-hybrid assays using a full length PHAN bait and the C-terminal domain prey revealed the possibility that the C-terminal domain interacted with itself (Fig. 11C, D). This experiment was expanded by comparing full length PHANL1 and AS1 bait and prey to test whether the homodimerisation was conserved between PHAN-like proteins in Antirrhinum and Arabidopsis. Both the HIS3 and β-galactosidase assays suggested that PHAN, PHANL1 and AS1 formed dimers in a series of affinities: PHANL1 and AS1 homodimerisation appeared strongest and PHAN-PHANL1 heterodimerisation stronger than PHAN-PHAN homodimerisation (Fig. 25C and D). The strength of PHAN-PHANL1 interaction may be intermediate with PHAN and PHANL1 homotypic dimerisation or nearly equal to that of PHANL1 dimerisation. This was not completely clear because of PHANL1 autoactivation. Consequently the assay with PHAN as bait was probably more reliable because autoactivation by bait tethered to the promoter was minimised. PHANL1, bound to the promoter by interaction may contribute to the activation observed but this was probably considerably less than that caused by PHANL1 tethered to the promoter by GAL4-DB fusion. The highest activation level was observed in interactions envolving PHANL1 which, as described below, has strong transcriptional activation activity in yeast and therefore the relative strength of interaction cannot be fully resolved.

Interestingly growth on his media suggested that AS1 interacted with PHAN and PHANL1, and appeared to be approximately equivalent in strength to the same interactions made by PHAN with PHANL1 Fig. 25,C, column 2. This supports the hypothesis that AS1 is the orthologue of PHAN (Byrne et al., 2000).
Figure 25. Interaction of PHAN and PULP HOMOLOGUES. The baits are in rows and the preys are in columns; (A) -LW media enables selection for bait and prey plasmid; (B) -LWH enables selection for expression of HIS3 reporter gene dependently on interaction between bait and prey fusion proteins; (C) -LWH + [10mM] 3AT, increases selection to reveal strong interactors; (D) βgalactosidase assays. PHAN, PHANL1 and AS1 interaction can be seen in C row 1 where PHANL1 auto-activation is reduced to a minimum. AS1 interaction with self appears to be strongest (D row 3) and PHAN interaction with self appears to be weakest of the three. The S1-1 interaction is highly specific to PHAN but appears to interact with T2040340764.
Fig. 26. Transcriptional activity of PHAN-like genes and T20403 C-terminus baits when expressed alone in yeast. Yeast expression constructs are arranged in rows, selective media is arranged in columns. The left column selects for plasmid retention and all strains grow approximately equally. Media in the centre column selects for transcriptional activation of the histidine reporter gene and in the right column increased selection is imposed by including the histidine biosynthesis pathway inhibitor 3AT at a concentration of 10 mM. Because of the homology between PHAN, PHANL1 and AS1 the properties of each fusion protein are expected to be similar and they should therefore be comparable in yeast. Yeast expressing PHANL1 and T20403-403-764 grow under selection whilst those expressing AS1 and PHAN show reduced or zero growth respectively suggesting that the former are transcriptional activators and the latter are not.
8.2 TRANSCRIPTIONAL ACTIVATION BY PHAN AND PULP HOMOLOGUES IN YEAST

The yeast strain HF7c and yeast 2-hybrid expression vectors have previously been used to characterise transcriptional activation of plant factors such as APETALA1 (Cho et al., 1999). To confirm the results Cho et al., (1999) employed transcriptional activation assays by transient expression in mammalian COS-1 cells. This was not within the scope of this study so the yeast data alone was used to provide an indication of transcriptional activation potential with the expectation that further work could be done if a significant result was obtained. The yeast 2-hybrid assay controls for autoactivation initially revealed that PHANLI and the C-terminal domain of T20403 cause autoactivation in the presence of pGAD424. To determine whether the activation was autonomous or dependent on binding to the GAL4 activation domain PHANL1 and T20403403-764 were tested alone HIS3 activation on -WH media. Streaks of multiple colonies from a transformation were used for these assays. PHAN, AS1 and T20403403-764 bait were also tested. Each was streaked on -W, -WH and -WH [10mM] 3AT media to enable the relative strength of transcriptional activation to be seen. Fig. 26. suggests that both PHANL1 and T20403403-764 bait are transcriptional activators in these conditions whilst AS1 transactivation is low and PHAN has no detectable transcriptional activation. This result provides an indication of transcriptional activation by PHANL1 and T20403403-764 bait, but further testing in vivo is desirable. This might be achieved, in the absence of any known DNA binding activity for PHANL1 and T20403 by expressing the GAL4-DB fusion proteins used here so that they would activate a reporter gene in vivo under the control of the GAL4 UAS. This approach would require expression of a strong activator such as VP16 and the GAL4-DB domain alone in the same system as control experiments. A similar approach has been taken previously with Mybs in maize using co-delivery by microprojectiles of effector plasmids encoding deletions of the B gene; a plasmid encoding a reporter gene; and a transformation control plasmid (CAT) which enabled the relative level of expression to be assessed (Goff et al., 1992). However it might be better done by expressing the fusion proteins from the fiddlehead promotor (which is L1 specific) in Arabidopsis harbouring a yellow-flourescent-protein-tagged histone reporter gene under control of the GAL4 UAS which is easily visible under UV light (Gwyneth Ingram, pers. comm.)
Another aspect of T20403 interaction which was apparent from yeast 2-hybrid assays was that T20403 may have the potential to interact with itself (Fig. 19.). This was predicted by the homology PULP and T20403 share with Restin, myosin II and CENP-E all of which form dimers through coiled-coil interactions (Warrick and Spudich, 1987; Scheel, et al., 1999; Thrower et al., 1995).

The \textit{HIS3} and \(\beta\)-galactosidase assays shown in Fig. 19(C) and (D) both suggested that T20403\textsuperscript{403-764} bait and T20403\textsuperscript{403-764} were able to dimerise. The \textit{HIS3} reporter gene was activated at a higher level than the auto-activation by T20403\textsuperscript{403-764} bait. No conclusion was possible concerning interaction between T20403\textsuperscript{403-764} bait or T20403\textsuperscript{403-764} with full length T20403 because it could not be distinguished from background.

A synergistic effect of the autoactivation by T20403\textsuperscript{403-764} bait and T20403\textsuperscript{403-764} may have been responsible for the level of activation but it could equally have been caused by dimerisation. Similar parts of PULP were not compared for dimerisation or autoactivation. Another point of interest was that T20403\textsuperscript{403-764} also appeared to interact with S1-1 bait (Fig. 25C). The \(\beta\)-galactosidase assay however suggested that the level of activation was low (Fig. 25D).
DISCUSSION

9.1 THE INVOLVEMENT OF PHAN IN LATERAL ORGAN AXIS SPECIFICATION

The initial hypothesis that inspired this work was that \textit{PHAN} was required for aspects of lateral organ axis specification. This came from comparison of wild type \textit{Antirrhinum} leaves with those of \textit{phan} mutants and revealed loss of adaxial cell identity and the lamina outgrowth in the adaxial domain of the primordia which is thought to be contingent on interaction of adaxial and abaxial tissue (Waites and Hudson, 1995). Conversely \textit{PHAN} mRNA expression appeared throughout the lateral organ initials and early primordia suggesting that other spatially restricted factors might interact with \textit{PHAN} to modify its function and specify the difference between the adaxial and abaxial leaf domains. However, a number of factors now make it unclear to what extent \textit{PHAN} and its homologues are specifically involved in axis specification.

Firstly, The function of \textit{PHAN} in specifying lateral organ axes is unclear because characterisation of \textit{PHAN}-homologues in \textit{Arabidopsis} and maize reveal that they vary in their contribution to lateral organ axis specification. The differences may partly result from varying function of \textit{knox} genes that are repressed by \textit{PHAN}-homologues in each species, differences in the timing, position and level of the \textit{knox} genes misexpressed or by other factors which may mask the features of the \textit{phan} phenotype in other species.

Secondly, specification of abaxial and adaxial lateral organ cell layer identities have recently been found to be specified by the \textit{YABBY} and \textit{KANADI} or \textit{PHABULOSA} and \textit{PHAVOLUTA} genes respectively. \textit{PHB} and \textit{PHV} are genes of the class III homeodomain zipper family with an additional sterol lipid binding (START) domain, whilst \textit{YABBY} genes encode proteins with a zinc finger motif and an HMG domain and \textit{KANADIS} are GARP family putative transcription factors (Siegfried et al., 1999; Bowman, 2000; McConnell and Barton, 1998; Kerstetter \textit{et al.}, 2001). Both loss and gain of function mutations of \textit{KANADI} genes suggest they promote abaxial cell fate. In the loss-of-function case proper abaxial location of \textit{YABBY} gene expression fails whilst expression of \textit{KAN1}, \textit{KAN2} or \textit{KAN3} under control of the 35S promoter results in gain of abaxial tissues in adaxial positions, (Eshed \textit{et al.}, 2001).
Like \textit{PHAN} and \textit{ASI}, \textit{YABBYs}, \textit{KANADI}s, \textit{PHB} and \textit{PHV} are initially expressed throughout the primordia but the latter four are progressively localized to the abaxial and adaxial domains between the time of primordia emergence and P3 (Sawa \textit{et al.}, 1999; Siegfried \textit{et al.}, 1999; Eshed \textit{et al.}, 2001; McConnell \textit{et al.}, 2001).

Each of the known semi-dominant alleles of \textit{PHB} and \textit{PHV} have mutations in the putative sterol lipid binding domain (McConnell \textit{et al.}, 2001) and are initially expressed throughout lateral organ primordia but later fail to be localized to the adaxial domain. In \textit{phb-d} mutants transcript is detected in both adaxial and abaxial sides of organs at a higher level than wild type plants. This lead McConnell \textit{et al.}, (2001) to propose that PHB determines leaf polarity by being initially expressed at low levels throughout the unpolarized leaf primordia but then, in response to a polar ligand originating from the meristem, promotes adaxial leaf development and also positively regulates its own transcript (an alternative possibility is a negative acting ligand with highest expression in the abaxial domain). Auto-regulation via the START domain is probably the key to adaxial localization and mutation of it causes constitutive positive autoregulation throughout the primordia. In wild-type tissue autoregulation of PHB synthesis causes it to persist in the adaxial domain, which is consistent with the finding that organ primordia develop the adaxial-abaxial axis autonomously if isolated from the meristem after a certain stage (Sussex, 1955; Snow and Snow, 1959; Steeves, 1961). Eshed \textit{et al.}, (2001) suggested a model for interaction of the adaxial and abaxial specifying genes as shown in Fig. 27, below in which \textit{KANADI}s and \textit{YABBY} genes are regulated in an overlapping pathway to specify abaxial organ identity but are negatively regulated by \textit{PHB} family genes which positively regulate adaxial identity in response to a signal from the centre of the meristem.
Meristem derived signal (sterol) → anlagen factors

\[ \text{PHABULOSA} \rightarrow \text{KANADI} \rightarrow \text{YABBY} \]

adaxial ↔ abaxial

Blade outgrowth

axillary meristem formation

Fig. 27. A model for polarity establishment in lateral organs according to Eshed \textit{et al.}, (2001). A signal probably originating from the meristem is perceived by PHB, PHV or REV that promote adaxial cell fate. PHB, \textit{KANADI} and \textit{YABBY} genes are initially expressed throughout the primordia but become localised to mutually exclusive domains as the primordia become dorsiventral. abaxialized organs form as a default if signal is removed by isolation of the primordia from the meristem - probably because the abaxial identity genes \textit{KANADI}s and \textit{YABBY}s fail to be repressed by PHB PHV or REV. \textit{KANADI} activity may mediate between PHB and KAN activities. Interaction between adaxial and abaxial tissue promotes lamina outgrowth after the model of Waites and Hudson, (1995).

Although \textit{ASJ} is expressed earlier than \textit{PHB}, \textit{KANADIs} and \textit{YABBY}s in a similar pattern throughout the primordia it is unclear whether they are regulated by it (the same is true in \textit{Antirrhinum}). This appears unlikely in the case of \textit{PHB} firstly because \textit{as1-1} mutants have no known polarity defects like \textit{phan} and \textit{Rs2} and secondly \textit{PHB} is expressed (where \textit{ASJ} is not known to be expressed) in the meristem at low level.
and at higher level in radial lines from the primordia to the centre of the meristem (McConnell et al., 2001). However, the expression in the meristem is lower than in the primordia and \( AS1 \) may be required to increase \( PHB \) expression there. Also the radial expression of \( PHB \) is absent in \( phd-1d \) mutants suggesting that it is dependent on establishment of the polar organ axis (McConnell et al., 2001). The only evidence that a \( PHAN \)-homologue may regulate a \( PHB \) gene is that mRNA of a \( PHB \)-homologue cannot be detected in \( PHAN \) needle-like leaves by RT-PCR (John Golz pers. comm). Therefore, \( PHB \), \( PHV \), \( KANADI \)s and \( YABBY \)s may be part of a pathway that specifies organ axes downstream or independently of \( PHAN \)-homologues.

A third factor which makes in unclear to what extent \( PHAN \)-homologues are involved in axis specification is that they share a number of similarities with animal Mybs (some of which have been revealed by this work) which suggest that \( PHAN \)-homologues may function more like animal Mybs than homology would suggest. The most important similarities between \( PHAN \) and animal Mybs are found in;

1. similar domain structure and protein interactions
2. the kind of tissue in which \( phan \) and aMyb or cMyb are expressed,
3. similarity in the relationship between \( PHAN \)-homologues and \( knox \) genes with their homologues in animals - cMyb and TALE homeodomain proteins. There is no evidence that animal Mybs specify three dimensional axes, however, they are connected with an axis of differentiation. Examination of the literature reveals that cMyb is important for a pathway of simultaneous proliferation and differentiation of cells that are progressing from pleuripotent-stem to lineage-restricted identities.

### 9.1 COMPARISON OF PHAN AND Myb STRUCTURE

Outside of the Myb repeats there is little amino acid sequence homology between \( PHAN \) family gene products and animal Mybs. In addition neither \( \alpha \)-helix forming or breaking predictions nor hydropathy plots suggest much structural conservation (Fig. 8.). Furthermore, the presence of three repeat Mybs (Kranz, et al., 2000,) and the large expansion of the R2R3 Mybs in plants argues against \( PHAN \) being a strict
functional homologue of animal Mybs. Despite this it is possible that PHAN retains some functions of animal Mybs because of its role in tissue that is proliferating and changing differentiation state. This prediction is not refuted by failure of the yeast 2-hybrid assay to detect PHAN interactors homologous to heterologous Myb interactors because the screen was clearly incomplete. This was apparent because the PHAN and PHANL1 interaction was not detected by library screening despite it being strong and easily detectable in direct tests. Furthermore PHAN and AS1 need not engage in all the interactions of animal Mybs to retain some of their functions.

The principal structural similarities between cMyb and PHAN-homologues are that they form homodimers and also interact with heterologous proteins via a motif (which may be a coiled-coil) located C-terminally to the Myb repeats. The C-terminal region of PHAN and AS1, which includes the region that may form a coiled-coil structure, mediates dimerization as well as interaction with PULP homologues in yeast. This is similar to the C-terminal domain of cMyb, part of which has been postulated to form a leucine zipper (Biedenkapp et al., 1988; Kanei-Ishi et al., 1992). The cMyb C-terminal domain appears to act as a negative regulatory domain by dimerization at higher protein concentrations which prevents cMyb binding to DNA (Nomura, et al., 1993). In support of this deletion of the C-terminal domain increases transcriptional activation by cMyb (Sakura et al., 1989; Weston et al., 1989; Grasser et al., 1991; Dubendorf et al., 1992). C-terminal truncation also increases transcriptional activation by other animal Mybs (Facchinetti et al., 1997; Lane et al., 1997).

It is noted that the existence of the leucine zipper of cMyb has been challenged based on circular dichroism spectroscopy measurements of α-helical content (Ebneth et al., 1994) although the region which includes the putative leucine zipper is required for protein-protein interaction. Evidence for this is that substitution of the leucine residues to either proline or alanine abrogates the interaction with other proteins which can be isolated with cMyb by pull-down experiments from hematopoietic cell line extracts (Favier and Gonda, 1994; Boyle et al., 1984; Klempnaur et al., 1984).
Dimerization or oligomerization has not been previously reported for plant R2R3 MYBs but is known for single repeat telomeric MYB-related proteins which bind the direct repeats at chromosome ends. These have been reported to dimerize in plants and animals where they function in telomere maintenance (Bianchi et al., 1997; Yu et al., 2000). Oligomerization by one example Human Telomeric Binding Protein (hTRF1) is required to form stable complexes with DNA (Bianchi et al., 1997) and structural studies suggest that the way that hTRF1 binds DNA is slightly different from that of other MYB domains (Nishikawa et al., 1998). Therefore, oligomerization of single MYB domain proteins may be required to form an adequate DNA binding domain and accommodate the unusual direct repeat binding site found in telomeres. The DNA binding domain of R2R3 MYBs like PHAN however, is formed by both repeats therefore homodimerization seems unlikely to be needed for PHAN to bind DNA. Dimerization of cMyb is the only other example of Myb dimerization which has been recorded but the biological function of the dimerization remains unclear.

Whilst native cMyb is not transcriptionally active in animal cells it is in yeast cells. For this reason it has been suggested that transcriptional repression of cMyb in animals requires species specific factors (Chen and Lipsick, 1993; Seneca et al., 1993). In contrast AS1 and PHAN have low, or no, transcriptional activity in yeast. One explanation for this may be that PHAN-like proteins require specific protein modifications or transcriptional activators, which cMyb does not, and these were not present or detected during the yeast 2-hybrid screen. Alternatively a negative regulatory domain in PHAN and AS1 might be more autonomous than that of cMyb. This hypothesis could be tested, initially by making serial deletions of the PHAN-bait vector used in this study and testing them for transactivation alone in yeast.

9.2 COMPARISON OF TISSUE TYPES WHERE PHAN AND Myb ARE EXPRESSED

At first sight the function of aMyb and cMyb appears to have nothing in common with development of lateral organs of plants because vertebrate cMyb expression is most important and highest in hematopoietic cells of all lineages. It is also expressed
at low levels in epithelial cells, smooth muscle, skin, retina, intestinal crypt and hair follicles (Kastan et al., 1989; Sitzmann et al., 1995; Reilly et al., 1989; Queva et al., 1992; Gonda and Metcalf, 1984; Sheiness and Gardinier, 1984; Duprey and Bottinger, 1985; Ess et al., 1999). The importance of cMyb in hematopoiesis is demonstrated by laboratory mice with disrupted cMyb which die in early development due to failure of fetal hematopoiesis (Mucenski et al., 1991). aMyb is required for development of lymph, central nervous system and reproductive tissue (Mettus et al., 1994; Trauth et al., 1994; Toscani et al., 1994) but is predominantly expressed in adult male germ cells. In female mice a-Myb is expressed in breast duct epithelium during pregnancy induced ductal and alveolar development. In contrast bMyb is expressed ubiquitously from early development onwards (Nomura et al., 1988) consistent with a role in the cell cycle in a less tissue specific manner (Weston, 1998). PHAN expression is more similar to the tissue specific expression pattern of aMyb and cMyb than it is the ubiquitous expression of bMyb.

Previously, it was observed that Myb genes are associated with proliferating tissue (Weston, 1998; Graf, 1992). Comparison of PHAN with animal Mybs suggests that PHAN, aMyb and cMyb, are required for elaboration of tissues that are derived from a small population of stem cells and replenished throughout the life of the organism. This is more obvious in plants if perennials which produce leaves and flowers annually are considered.

Evidence exists to suggest that PHAN, like cMyb, is also required for cell proliferation. In plants cell proliferation is required for proper lateral organ development and the highest rate of cell division in the meristem occurs (in some plants) where PHAN and ASI are expressed in the lateral organ primordia (Laufs et al., 1998). Furthermore, at low temperature in Antirrhinum, PHAN is absolutely required for this process because organ growth stops in phan mutants whilst organ growth is not affected at the same temperatures in wild type plants (Waites and Hudson, 1998). The evidence that cMyb is important for cell proliferation is that when its function is removed by a dominant interfering MYB construct in cytotoxic T cells (Lyon and Watson, 1996) they arrest in G1 of the cell cycle suggesting that MYB proteins are required for this transition (Weston, 1998). The Drosophila Myb is
also required for cell cycle transition though at the G2/M boundary (Katzen et al., 1998). Also overexpression of Myb proteins can push cells to cycle abnormally (Sala et al., 1996; Lane et al., 1997). cMyb also regulates genes associated with cell proliferation or cycling such as topoisomerase IIa and cdc-2 (Brandt et al., 1997; Ku et al., 1993).

Transcriptional repression is a function also shared by PHAN, AS1 and cMyb although to date the kind of gene repressed is different. The best characterised function of both PHAN and AS1 is repression of knox genes (section 1.5). During expansion of myeloid progenitor cells cMyb functions as a transcriptional repressor of the G-CSF receptor gene c-fms (Reddy et al., 1994). Because C-terminal deletions fail to achieve the repression of native cMyb it is thought to function in the same way as the Drosophila even-skipped (eve) homeodomain protein represses Ultra bithorax (Ubx). The model for eve function entails eve binding to the Ubx proximal promoter and a 1500 bp distant high-affinity silencer element. This then serves to target more eve molecules to low affinity sites 3' of the transcriptional start site by looping of DNA and co-operative interaction which makes the transcriptional start site inaccessible (TenHarmsel et al., 1993). The repression function which is conserved between PHAN and AS1 could work in this way if dimerization occurs in vivo. A second mechanism of repression reported for cMyb - of the human epithelial growth factor receptor gene c-erbB-2 - is by binding DNA at a position overlapping the TATA box and competing with TFIID to block transcriptional initiation. This is demonstrated because only the Myb domain is required for repression (Mizuguchi et al., 1995). This mechanism of repression is also possible for PHAN and AS1 but would probably require involvement of the C-terminal domain which is most commonly disrupted in PHAN and AS1 mutants that misexpress knox genes (Fig. 33 and 34).
Barton, (2001) speculates whether the regulatory program involving knox genes and PHAN orthologues, which is conserved between Antirrhinum, Arabidopsis and maize, is present also in primitive plants such as ferns because of the presence there of knox genes. Because other regulatory systems appear to be very similar between the animal and plant kingdoms such as the COP9 signalosome (Wei and Deng, 1999) and polycomb genes or CURLY LEAF (Goodrich et al., 1997) this question could also be extended to animals.

In plants knox genes such as kn1, STM and HIRZINA are expressed in a region of the meristem which overlaps the stem cell population and based on loss-of-function mutations for Kn1 and STM are postulated to maintain cells in an undifferentiated state (Kerstetter et al., 1997; Barton and Poethig, 1993). PHAN family genes however act where knox genes are down regulated in lateral organ initials and primordia to promote processes that require cell proliferation and changes in fate. Based on these features a functional relationship which is remarkably similar to those of knox and PHAN in plants can be established in hematopoiesis between the TALE class of homeobox genes and cMyb.

In addition to roles in development exemplified by the antennapedia gene of Drosophila (Garber et al., 1983), HOX genes are involved in mammalian hematopoietic development (Lawrence et al., 1996) and are involved in the genetic interaction between TALE homeodomain proteins and Mybs. In metazoans class I HOX genes are organized in four clusters of 8 to 13 paralogues (Bocinelli et al., 1989) which are activated sequentially in a pattern that is colinear with the axes along which the genes function to specify body patterns (Mcginnis and Krumlauf, 1992). Of particular interest are the conventional HOX genes such as hoxb3 and hoxA9 which interact with TALE class homeodomain proteins and have functions in early stages of hematopoiesis.
Myeloid precursor cells (also called spleen colony forming units, CFU-S) are characterised by expression of the CD34 antigen. *HoxA9* is highly expressed in bone marrow myeloid precursor cells and down regulated as cells leave the CD34⁺ compartment. Other more 5' genes in the HOX cluster such as *HoxB3* are down regulated within the CD34⁺ compartment as cells progress to the committed progenitor stage (Giampaolo *et al.*, 1994; Sauvageau *et al.*, 1994). Lawrence *et al.*, (1997) found that *HOXA9* expression was highest in Human CD34+ cells and myeloid progenitors as well as being detectable in erythrocyte progenitors but very low or not expressed in CD34⁻ fractions. *HOXA9* was also found in developing lymphoid fractions. Mice with targeted *HoxA9* interruptions had reduced granulocytes and disruptions to certain subsets of developing lymphocytes. This was due to a reduced committed progenitor cell population demonstrating that *HOXA9* is active in normal primitive hematopoietic cells and various lymphoid populations (see Fig. 28. Adapted from Gilbert, 1999). In both mammalian and insect systems conventional HOX genes have been shown to collaborate with the TALE class of homeodomain proteins (Mann, 1995) which differ from other homeodomains by being located at unclustered sites in the genome and having a Three Amino acid Loop Extension between helix I and helix II of the homeodomain (Bertolino *et al.*, 1995). TALE homeodomain proteins, which can be divided into a number of subclasses, are found in plants, fungi and animals demonstrating relatedness via a common ancestor (Burglin, 1997).

In animals four different subclasses of TALE homeodomain proteins have been found including PBC, comprised of human *PBX* and Drosophila *EXTRADENTICLE* genes (Burglin and Ruvkun 1992); MEIS comprised of mouse and human *MEIS, PREP1* and Drosophila *HOMOTHORAX (HTH)* (Steelman *et al.*, 1997). Two other PBC subclasses are TGIF and the Drosophila IROQOIS (IRO) complex (Burglin, 1998).
Fig. 28. Differentiation pathways of hematopoietic lineages. Circles represent cell types and colouring denotes lineage: • pluripotent stem cells; 0 lymphoid lineage; 0 erythroid lineage; 0 myeloid lineage; dotted arrows indicate additional stages; growth factors are written in blue; CFU, BFU signifies colony and burst forming units respectively.
The TALE class of genes is represented in plants by *knox* and *BEL* homeobox genes (Bertolino *et al.*, 1995; Reiser *et al.*, 1995; Quadvlieg *et al.*, 1995). *knox* genes are most closely related to *MEIS* genes because of significant homology in a domain outside of the homeodomain called the MEINOX domain (Bharathan, 1997; Burglin, 1998). The conserved MEINOX domain is absent from other TALE proteins suggesting that MEINOX containing proteins may be more conserved with a common ancestor of plants and animals and that the other TALE proteins are divergent (Burglin, 1997).

*MEIS1* undergoes direct protein-protein interaction with paralogues 9-13 of the HOX clusters in vitro (Shen *et al.*, 1997). HOX paralogues can also dimerize with *PBX* TALE family homeodomain proteins (Shen *et al.*, 1997). *MEIS1* proteins can also bind both *HoxA9* and *PBX* in the absence of DNA and be isolated together from nuclear extracts of the myeloid precursor cell line KG1 (Chang *et al.*, 1997; Shen *et al.*, 1999).

Schnabel *et al.*, (2000) demonstrate that *HoxA9* transformed hematopoietic progenitors maintain a primitive phenotype in culture with growth factors and require a functional *MEIS1* interaction domain for this. In contrast cells transformed with a control vector differentiate under these conditions. *HoxA9* can also form trimers with Pbx2 as an intermediate with *MEIS1* on a modified target enhancer oligo in vitro and in leukemic cell line nuclear extracts. The trimer complex has 16 fold higher transcriptional activation above background level whilst *HoxA9* and *HoxA9*-Pbx2 has only 5-fold transcriptional activation. This suggests a role for *MEIS1* in transcriptional enhancement of *HoxA9* although the *HoxA9* C-terminal end was required for the transcriptional activation.

Further evidence of a role for MEIS genes in regulating development of myeloid precursor cells by interaction with HOX genes is suggested because they are frequently up-regulated with HOX genes in mouse BXH-2 leukemias. In 19 out of 20 BXH-2 mouse leukemias viral integration induced increases in expression level are found in both *MEIS* and *HoxA9* (Moskow *et al.*, 1995; Nakamura *et al.*, 1996a; Nakamura *et al.*, 1996b). Primary bone marrow cells retrovirally induced to
overexpress HoxA9, Pbx1a and Meis1a alone were not transforming which demonstrates dependence of HoxA9 on Meis1a in this process (Kroon et al., 1998). Whereas northern blots have failed to demonstrate expression of Meis1 in HoxA9 immortalised myeloid progenitors (Calvo et al., 2000) western blotting demonstrated that PBC and MEIS proteins are expressed in primary bone marrow cells with Pbx1, Meis1 and Meis1b (transcript splice variants) being prominent members (DiMartino and Cleary, unpublished).

This data suggests a role for MEIS genes during early stages of hematopoietic progenitor cell specification although it is noted that most of these genes also have roles during embryogenesis.

**9.3.1 THE ROLE OF cMYB IN PROLIFERATION AND DIFFERENTIATION OF CELL TYPES DERIVED FROM HEMATOPOIETIC PRECURSOR CELLS**

A characteristic of HoxA9, Meis1 or PBX transformed cells is that they frequently remain dependent for proliferation on growth factors such as granulocyte/macrophage colony stimulating factor (GM-CSF) (Schnabel et al., 2000; Thorsteinsdottir et al., 2001; Kamps and Wright, 1994; Kroon et al., 1998). This helps to establish cMyb as a key effector of proliferation and differentiation of cell types derived from the myeloid precursor cell (see Fig. 28.). The work of Largaespada et al., (1996) demonstrates that reduced GTPase activating protein (GAP) function in suppression of RAS signalling causes GM-CSF hypersensitivity resulting in a myeloproliferative symptoms in mice. In addition Bollag et al., (1996) have found a specific role for NFI GAP in negatively regulating GM-CSF signalling through RAS in hematopoietic cells. In lymphoid as well as erythroid cells cMyb is placed at the bottom of a RAS signal transduction pathway involving p100 and pim-1 kinase (Leverson et al., 1998).

During hematopoiesis pluripotent stem cells are present in very small numbers (Spangrude et al., 1998, Spangrude and Johnson, 1990) so early stages in differentiation are associated with mitotic expansion (Metcalf, 1980; 1989; White and Weston, 2000). cMyb expression is present in proliferating progenitor cells and
extinguished during terminal differentiation (Gonda and Metcalf, 1984). Forced expression of cMyb inhibits terminal differentiation of erythroid and lymphoid leukemia cell lines suggesting that cell cycle arrest is required for terminal differentiation (Bies et al., 1995; McMahon et al., 1988; Selvakumaran, 1992). Figure 29. shows a GM-CSF dependent signal transduction pathway leading to activation of cMyb and stem cell factor receptor cKit (White and Weston, 2000). Some other known cMyb targets are also shown such as mim-1, which is a marker for granulocyte differentiation (Ness et al., 1989) and is regulated by a combinatorial mechanism between cMyb and NF-M, the chick homologue of C/EBPβ (NF-IL6) (Ness et al., 1993; Burk et al., 1993). Other markers for differentiation regulated by cMyb are myeloperoxidase (MPO) which is restricted to late promyelocyte stages of granulocyte differentiation (Bies et al., 1995; Ford et al., 1996). In birds Myb is also known to activate expression of a nonclustered homeodomain gene (GBX2) which in turn activates a member of the IL6/G-CSF growth factor family called chicken myelomonocytic growth factor (cMGF) (Leutz et al., 1984, 1989). This has been shown to promote differentiation of myelomonocytes to macrophages and a monoblast phenotype which expresses monocyte marker molecules (Kowenz-Leutz et al., 1997). Although this same function has not been demonstrated in mammalian hematopoiesis GBX2 and cMyb are both highly expressed in a human progenitor cell line K562 (Kowenz-Leutz et al., 1997) and murine GBX2 expression level correlates inversely with nuclear localisation of a dominant interfering cMyb construct in FDCP-mix cells (White and Weston, 2000).

This example appears to place cMyb in a pathway of proliferation and differentiation of hematopoietic precursor cells towards one of the myeloid lineages (other examples within hematopoiesis may be possible as Myb is essential for growth of most lineages) which accounts for the role of Myb in simultaneous cell proliferation and differentiation (White and Weston, 2000; Graf, 1992).
Fig. 29. Putative signal transduction pathway linking GM-CSF to cMyb activation of stem cell factor receptor c-kit and homeodomain protein GBX2. GBX2 contributes to differentiation of hematopoietic precursor cells to one of the myeloid lineages by regulation of a myelomonocytic growth factor. Other targets of cMyb involved in differentiation are also shown. Components coloured black are mammalian; blue are avian.

9.4 THE MAIN FUNCTION OF PHAN MAY BE TO MEDIATE PROGRESSION OF CELLS TOWARDS DIFFERENTIATED LATERAL ORGAN FATE

During plant development cells originating in the meristem are marked by *knox* genes and during elaboration of lateral organs cells progress along a pathway of differentiation controlled by *PHAN*-homologues. The lateral organ initials and primordia are marked by *PHAN* expression and cell proliferation in a manner remarkably similar to the progression from myeloid precursor cells to lineage restricted myeloid cells mediated by cMyb. Therefore, it appears that MEIS1 and Myb as well as *knox* and PHAN, which are homologues in animal and plant have similar functions.
One good reason to suppose that this is correct is that plant cells are fixed in position by the cellulose cell wall and consequently isolated from each other to a greater extent than animal cells. The evolution of plasmodesmata (Graham et al., 2000) may have facilitated closer communication between related cells but not all cells are connected in this way continuously (Gisel et al., 1999; Rinnie and van der Schoot, 1998). Therefore interaction between plant cells at long or short range is particularly suited to interactions involving diffusible molecules. This is also a feature of hematopoiesis where isolated cells are induced to differentiate in response to inductive microenvironments specified by stromal cell cytokine production (Gilbert, 1999). The homology between TALE homeodomain proteins and the similarity in their function in plants and hematopoiesis, the similarity between some of the protein-protein interactions of Myb proteins in both these systems, and the relatively isolated nature of the tissues they function in all contribute to the idea that similar pathways of differentiation could be conserved in these tissues and that PHAN and animal Mybs have some functions in common.

A second piece of evidence to support the hypothesis that knox and PHAN have functions conserved with TALE homeodomains and Mybs in animals is that a second example of the relationship between a Myb and a non-TALE homeodomain exists in plants. GL1, a Myb, regulates trichome cell differentiation by transcriptional regulation of the homeodomain-zipper protein, GL2, in collaboration with TRANSPARENT TEST GLABROUS, (TTG) and GL3 (Walker et al., 2000; Payne et al., 2000; Szymanski et al., 1998). Trichome development can also be argued to involve a process similar to cell proliferation because in Arabidopsis the trichome undergoes endoreduplication during differentiation and mutation of the Arabidopsis SIAMESE gene results in formation of multicellular trichomes (Walker et al., 2000) similar to those found in other species such as Antirrhinum. Regulation of homeodomain proteins by Mybs could be simply a consequence of the evolutionary radiation of Mybs in plants but it could equally be part of a conserved mechanism of Myb function.
The phenotype and expression pattern of *PHAN* in *Antirrhinum* suggests a function both in specifying lateral organ identity as well as promoting the adaxial-abaxial axis or dorsal cell identity. The expectation was that *PHAN* might be modified in the adaxial leaf to specify the adaxial leaf domain. Alternatively a gene downstream of *PHAN* could be the target of adaxial modification.

The signal transduction pathway outlined in Fig. 29. could provide a model for *PHAN* dependent specification of the adaxial domain with an *Antirrhinum* non-TALE homeodomain adaxial specifier (such as *PHB*) in place of the *HOX* protein *GBX2*. This model could accommodate the possibility that *PHAN* or AS1 regulate expression of the adaxial specifier throughout the primordia - and its adaxial localisation is autonomous or that they contribute to its adaxial localisation directly in response to an adaxial signal.

Fig. 30. Models for *PHAN/AS1* role in specification of adaxial identity mediated by a non-TALE homeodomain protein (such as *PHB/PHV/REV*). (A) *PHAN/AS1* promotes *PHB* expression throughout the primordia and it localises adaxially in response to a positively acting adaxial signal or a negatively acting abaxial signal (after the model of Eshed *et al.*, 2001; see Fig 27 and Fig 29). (B) *PHAN/AS1* contribute specifically to *PHB* expression in the adaxial domain in response to an adaxial signal.
In *Arabidopsis* the evidence suggests that *PHB* and *PHV* are responsive to an adaxializing signal but in *Antirrhinum* a homologue of *PHB* which is normally detectable in leaves is not expressed in the abaxialized tissues of *phan* needles (John Golz pers. comm.). Whether *ASI* regulates *PHB, PHV* or *REV* is unknown partly because their loss-of-function mutations have not been described for comparison with the *asi* phenotype, consequently it is still unclear whether *PHAN* could regulate *PHB*.

No conclusion can be made about the potential role of *PHAN*-homologues to specify lateral organ axes based on the data obtained here because evidence for an adaxially or abaxially expressed interactor was not found, however, comparison of *knox* misexpression phenotypes in *Arabidopsis, Antirrhinum* and maize; the similarities between function of TALE homeodomain and Myb genes with *knox* and *PHAN*-homologues; the known function of *PHAN*-homologues in repression of *knox* genes; the common dimerization, similar domain structure and protein interaction of *PHAN* and cMyb all suggest that *PHAN* acts in an axis of cell differentiation similar to cMyb. If differentiation of tissues is important for development of lateral organ axes this has the potential to explain the variable contribution of *PHAN*-homologues to axis specification in *Arabidopsis, Antirrhinum* and maize as subtle differences in other pathways that contribute to differentiation are likely to be present in these species. Experimental approaches to obtain further evidence for these ideas will be discussed in section 13.
In the yeast 2-hybrid assay PHAN and PHANL1 were found to heterodimerize. Similarly both PHAN and PHANL1 also heterodimerized with AS1 suggesting that the interaction is conserved.

Heterodimerization of transcription factors has been postulated as a means to enhance their transcriptional regulation potential by increasing the number of different transactivating complexes that can be formed. The outcome of heterodimerization depends on the potential of factors to modify each other's functions. This can occur through alteration of the DNA binding properties and kinetics or stability of transcription complex formation (Jones, 1990). For example, members of the vertebrate βZip transcription factor family, cFos and cJun, heterodimerize and bind activator protein (AP1) cis-regulatory sites with much higher efficiency than homodimeric complexes of either protein. This occurs because stability of heterodimers in solution is much greater than that of homodimers. Heterodimerization by cJun with ATF/CREB which are also members of this same transcription factor family changes the DNA binding specificity of the heterodimer again to favour the cAMP response element over the AP1 site.

Heterodimerization can also act as a repressor of transcriptional activation. In Drosophila, negative regulation of peripheral nervous system development is achieved by Emc and Hairy which heterodimerize with corresponding AS-C and Daughterless proteins. All are basic helix-loop-helix (bHLH) proteins, but the former lack DNA binding domains so that they disrupt DNA binding of AS-C and Daughterless and act as dominant repressors (Garell and Modelell, 1990; Ellis et al., 1990). bHLH proteins such as HBP1 and EmBP1 have also been found in plants (Tabata, et al., 1989; Carlini et al., 1999). All appear to homodimerise although heterodimerization has yet to be demonstrated. Heterodimerization has, however, been observed between homeodomain zipper (HD-Zip) transcription factors which have so far been found only in plants. The homeodomain consists of a helix-turn-helix (HTH) motif and HD-zip proteins have been shown to heterodimerize with related proteins. For example in maize, members of HD-Zip families I and II heterodimerize extensively within families (Meijer et al., 2000).
Heterodimerization of transcription factors from the same family is therefore a common process in animals and plants with diverse regulatory functions. Whilst homeodomain and Myb proteins both consist of helix-turn-helix motifs homeodomain proteins have been observed to heterodimerize by several mechanisms (Sessa et al., 1993; Shen et al., 1999). This demonstrates that there is no gross inhibition of HTH protein dimerization and makes the lack of heterodimerization in Mybs all the more surprising particularly as MYBs are abundant in plants and have undergone duplication and divergence to regulate numerous plant specific functions (Kranz et al., 1998; Jin and Martin 1999). This is a process that might have been accompanied by novel protein-protein interactions. In the same way that HD-zip proteins appear to have evolved dimerization by acquisition of the bZip domain, PHAN homologues may have acquired dimerization through evolution of a novel C-terminal domain. Alternatively the PHAN homologues may have retained and modified dimerization from a common ancestor of the three-repeat MYBs found in animals. Whether dimerization occurs in other plant MYB families is not known.

Insitu suggest that the expression pattern of PHAN and PHANL1 overlap in Antirrhinum floral primordia (Richard Waites; John Golz, pers. Comm.). Although no information on protein levels is currently available mRNA levels for PHAN and PHANL1, based on cDNA library screening and insitu hybridization, appear to be relatively low. Where PHAN and PHANL1 expression has been detected by insitu hybridization to floral primordia tissue, PHANL1 mRNA levels appeared to be about \(1/10^6\) of the level of PHAN (Richard Waites, pers. comm.). In yeast, PHAN homodimer formation appeared weaker than heterodimer formation with PHANL1, whilst PHANL1 homodimer formation appeared to be the strongest interaction. Differences in expression levels in vivo could therefore, enable a different distribution of dimerization than predicted by the strength of interaction seen in yeast.

However, it is possible that the interaction between PHAN and PHANL1 could modify one or other of their functions. Experiments in yeast suggested that PHANL1 is a transcriptional activator and PHAN is not, leading to two possibilities: PHAN could recruit transcriptional activation potential by binding PHANL1, or be involved in repression of transactivation by PHANL1. There is 14% difference
between the amino acid sequences of PHAN and PHANL1 DNA binding domains and other differences in the protein overall. Although plant Myb domains appear to have relaxed DNA binding specificity (Jin and Martin., 1999) differences in the amino acid sequence proximal to the DNA binding domain could be just as important for DNA binding specificity due to interaction with other proteins at promoter sequences (Lamb and McKnight., 1991; Mann, 1995). These differences allow the possibility that they have different DNA binding specificity which could enable them to regulate an additional set of genes where they are both expressed.

In principle co-expression of PHAN and PHANL1 could enable further possibilities if repression and activation are also considered: as a heterodimer, binding to sites that were bound only by PHAN dimers could relax suppression and/or gain activation, binding to sites that would only be bound by PHANL1 could reduce or repress activation. This kind of interaction has been demonstrated for the Drosophila homeodomain proteins Aristaless-like4 (Alx4) and Goosecoid (Gsc). Alx4 and Gsc homodimers and heterodimers each have distinct binding properties, Alx4 activates transcription in a site specific manner and Gsc can antagonise Alx4 mediated activation only at promoter elements that support heterodimer binding. (Tucker and Wisdom, 1999).

The observation that a PHANL1 homologue does not exist in *Arabidopsis* tends to abrogate any hypothesis that a combinatorial mechanism is broadly conserved in plants. For example, a mechanism could be envisaged that relied on combinations of genes on opposing concentration gradients (or nuclear localisation gradients) to specify identity of epidermis, parenchyma and mesophyll cell layers in leaves or petals. This may appear partly supported by the phenotype of *phan* mutants because the adaxial-abaxial axis of the later leaves is disrupted and the leaf lacks a lamina, however *phan* abaxialized leaves still have differentiated layers of cells including epidermis, mesophyll and vasculature despite them all being characteristic of the abaxial domain (Waites and Hudson 1995). More importantly, a mechanism of this kind would have to postulate that AS1 is able to specify all these cell types independently when in fact, based on homology, mRNA expression pattern and behaviour in yeast, it resembles PHAN. In addition, based on their transcription
patterns there is currently no evidence that PHAN or PHANL1 proteins are expressed in adaxial or abaxial domains which may enable their function to be modified by interaction. They could however function synergistically in a common domain of expression in response to modification by spatially restricted factors. The possibility exists that PHANL1 acts redundantly with PHAN although the apparent differences in interactions with PULP and transactivation activity in yeast would suggest that the redundancy is incomplete. This could be another explanation for the differences between *phan* and *asl* phenotypes.

11 COMPARISON OF THE STRUCTURE OF PHAN AND PULP HOMOLOGUES

Based on yeast 2-hybrid data the interaction domains of PHAN homologues were localised to the C-terminus. However, whilst PHANL1 interacted with itself, PHAN and AS1 it was observed not to interact with PULP. This suggested a difference between PHANL1 and the other PHAN homologues which may shed light on the interaction domains and help localise them more precisely within the C-terminal domains. The interaction domain of PULP and T20403 were also known to be localised within the S1-1 region of PULP or the C-terminus of T20403. The homology shared by PULP homologues with coiled-coil proteins and interactions of cMyb, by a postulated coiled-coil interaction domain, suggested a similar mechanism in PHAN homologues.

Coiled-coils are a common interaction domain occurring in many classes of proteins. They are comprised of two, three or four right-handed α-helices which wrap around each other by means of a slight super helical twist. The sequence of all coiled-coils consists of a heptad repeat commonly denoted (abcdefg)ₙ (McLachlan and Stewart, 1975) where positions “a” and “d” are mainly occupied by hydrophobic residues, positions “e” and “g” by charged residues and positions b”, “c” and “f” by polar or charged residues. The hydrophobic residues in position a and d form a non-polar interface - burying of which drives association of the helices in aqueous solution. Interhelical ion pairs can form between complexed helices when oppositely charged residues occur at positions e and g (either side of the hydrophobic core). These may contribute stability and direct registry of the helices (see Tripet et al., 2000).
Fig. 31. Prediction of coiled-coil tertiary structure formation for AS1 (top), PHAN (centre) and PHANL1 (bottom) obtained using the Coils program, see page 137. Unweighted prediction is in blue, weighted in red. (x-axis) amino acid residue number; (y-axis) probability of coiled-coil formation.
Coils output for pulp

Coils output for T20403

Fig. 32. Prediction of coiled-coil tertiary structure formation for PULP (top) and T20403 (bottom) obtained with the coils program. (x-axis) - amino acid residue number; (y-axis) probability of coiled-coil formation.
The coils program (Lupas, 1991) was used to detect the probability of coiled-coil regions occurring in PHAN and PULP homologues. Because coiled-coil proteins are generally fibrous solvent exposed structures all but the internal a and d positions have a high likelihood of being occupied by hydrophilic residues. If the occurrence of all residues are weighted equally prediction can be biased towards hydrophilic proteins that have a high frequency of charged residues. The coils program enables weighting for residue frequencies which reveal these characteristics when scans are done with or without weighting. If the score for highly significant regions does not decrease more than 20-30% when prediction is done with and without weighting for residue frequencies this indicates a region with coiled-coil forming potential (Lupas, 1996).

Fig. 31. shows the weighted and unweighted predictions for PHAN homologues. Two significant peaks for predicted coiled-coil formation appeared in PHAN, PHANL1 and AS1 between residue positions 250 and 350. The most obvious feature was that in PHANL1 the peaks were separated whilst they were adjacent in PHAN and AS1. This may reflect the transcriptional activity or lack of interaction between PHANL1 and PULP observed in yeast. However these factors could also be entirely dependent on sequence and residues present in each protein as this could affect the specificity of coiled-coil interactions.

Fig. 32. shows the coils output for PULP and T20403. This predicts, with high probability, a similar complex discontinuous coiled-coil structure for both PULP and T20403. Examination of PHAN homologue sequences revealed heptad repeats with hydrophobic residues in positions a and d but in many cases these have hydrophobic residues only in position a (Fig. 33)

However, other residues are possible in positions a and d and have a thermodynamic stability order (Wagschal et al., 1999; Tripet et al., 2000). The type of hydrophobic residue in a or d positions as well as polar interactions of residues flanking the core can dictate the oligomeric state of the helices and their parallel or anti-parallel arrangement because of the “knobs in holes” packing arrangements of their side chains (Crick, 1953) and the geometry of the side chains (Lupas 1996). For example due to the packing effects of side chains in the hydrophobic core, isoleucines in a and leucines in d form dimers, the opposite form tetramers and either residue in both
positions tend to form trimers. These effects are not easy to extrapolate to other residues for example valine is very similar to isoleucine yet a core of leucines in a and valine in d yields trimers not tetramers (see Lupas 1996). Therefore, the oligomerisation state and residues involved in interaction between PHAN and PULP homologues is not easy to predict from sequence.

![Alignment of C-terminal domain of PHAN, AS1 and PHANL1 amino acid sequence showing one possible sequence of conserved discontinuous heptad repeats. Hydrophobic residues that fall in positions “a” of the heptad are boxed in red, those that fall in position “d” of the heptad repeat are boxed in blue. Positions of transposon insertions in PHAN 249 and 164 are shown as numbered arrows. The position of a point deletion in the faulty AS1 bait construct is also shown (see section 7.2). The heptad repeat is not so clear between residues 317 and 332 which are deleted in phan-249 and may account for the large phenotypic difference between phan-249 and phan-164 mutants.](image)

Fig. 33. Alignment of C-terminal domain of PHAN, AS1 and PHANL1 amino acid sequence showing one possible sequence of conserved discontinuous heptad repeats. Hydrophobic residues that fall in positions “a” of the heptad are boxed in red, those that fall in position “d” of the heptad repeat are boxed in blue. Positions of transposon insertions in PHAN 249 and 164 are shown as numbered arrows. The position of a point deletion in the faulty AS1 bait construct is also shown (see section 7.2). The heptad repeat is not so clear between residues 317 and 332 which are deleted in phan-249 and may account for the large phenotypic difference between phan-249 and phan-164 mutants.
An alignment of PULP and T20403 shows that conserved within the S1-1 region there are heptad repeats with hydrophobic residues in positions a and d as well as the majority of other positions having residues with the correct properties. Although, in both cases the heptad repeats are not extensive coiled-coils need not be very long to function in protein-protein interaction (Lupas, 1996). Proline which is considered to be a helix breaking residue is absent (Fig. 13).

Although the heptad repeat is discontinuous in both PULP and PHAN homologues this occurs in many coiled-coil proteins (Lupas et al., 1995) for example myosin which forms dimers which in turn form multimers has numerous discontinuities, these may function to introduce flexibility into the structure (Warrick and Spudich, 1987). Therefore an interaction between PHAN and PULP homologues may occur by a mechanism similar to coiled-coil interaction.

Another question of interest was whether the interaction region within PHAN and AS1 for dimerization was the same as that for interaction between PHAN and PULP or AS1 and T20403. If this was the case then a region of homology with PHAN may appear in the S1-1 region of PULP or in T20403. Results obtained with the “best fit” program in GCG confirmed only that the highest identity (28-37%) between PHAN and PULP homologues was localised to the regions in PHAN homologues predicted to form coiled-coils. A consistent region of homology between PHAN-homologues and PULP-homologues could not be defined that suggested the interaction region within PHAN-homologues for PULP is separate from that of PHAN-homologues. Therefore, PHAN may interact with itself by a different region within the C-terminal domain than is required to interact with PULP. Alternatively, interaction between PHAN homologues and PULP homologues may occur simultaneously by oligomerisation.

The *Antirrhinum* mutations *phan*-164 and *phan*-249 indicate that an important function resides within the C-terminal domain. The phenotypic difference between these two mutants is that *phan*-164 merely has narrow leaves whilst *phan*-249 has severely reduced and radially symmetrical organs characteristic of the null mutation *phan*-250 (Waites and Hudson, 1998). The genotypic difference is that 16 additional amino acids of the *PHAN* protein between residues 316 and 332 are disrupted (Fig. 31, 33). Coincidentally this is in a region of the sequence where the hydrophobic
residue distribution characteristic of a coiled-coil protein is less clear (Fig. 33). In both cases a number of missense amino acid residues are added to the PHAN protein which could affect its function, alternatively the 16 amino acid residues may encode the interaction region and interaction may occur by a mechanism other than that of a coiled-coil. Additionally this region may have a positive cis-regulatory effect on the sequences N-terminal of it (which may form a coiled-coil). Further implications of dimerization will be discussed in section 13.

12 HETEROLOGOUS INTERACTIONS OF PHAN HOMOLOGUES

Using the yeast 2-hybrid assay interaction was identified between the PHAN and PULP proteins of *Antirrhinum* and the homologous *Arabidopsis* proteins, AS1 and T20403. Whilst PHAN and AS1 are MYB DNA binding protein homologues, PULP and T20403 resemble coiled-coil proteins with potential for interaction by a coiled-coil mechanism. A potential coiled-coil domain was also detected in the C-terminal domain of PHAN and AS1 suggesting the interactions with PULP and T20403 occur by this mechanism. However, further work is required to confirm that the interactions between PHAN-homologues and PULP-homologues occur in vivo (see section 13). It is possible that interaction in yeast was mediated by a yeast endogenous protein and it was not shown in this work that interaction between PHAN and PULP is direct. However, PHAN-PULP interaction appeared to be highly specific because the S1-1 portion of PULP did not interact with the close homologues of PHAN, PHANL1 or AS1 (the equivalent part of T20403 did interact with *PHAN* suggesting that it was less specific; section 8.3) which would require an intermediary protein to also make highly specific interactions. These would necessarily be highly specific spurious interactions or interactions conserved between plants and yeast that are not conserved for PHANL1 and AS1. Since this is somewhat implausible there is no reason to reject the hypothesis that PHAN and PULP interact in vivo on the basis of having only obtained yeast 2-hybrid data. Further evidence that *PHAN* and PULP interaction is direct could be obtained using an in vitro interaction test such as GST pulldowns (Spillane, *et al.*, 2000) but due to constraints on time the potentially more productive approach of mutational analysis and characterisation of expression patterns was attempted (section 4.8).
12.1 THE EXPRESSION PATTERNS OF *PHAN* AND *PULP* HOMOLOGUES DEMONSTRATE POTENTIAL FOR THEM TO INTERACT IN VIVO

The mRNA expression pattern of PULP determined by RNA gel blots appeared to be widespread, occurring in lateral organs as well as in the internode and in roots. However, insitu suggested that it was expressed in single cells of the abaxial L1, the L2 or L3 of bracts and sepal primordia, in the base of floral meristems and diffusely in the cortex of internodes but not in the inflorescence meristem. Insitu also suggested that the expression pattern of T20403 was similar occurring diffusely in the cortex of inflorescences. Unfortunately, greater definition in *Arabidopsis* and *Antirrhinum*, or expression in vegetative tissue of either PULP or T20403 were not obtainable in the time available for this project because it required substantial further work to repeat the insitu experiment. This was partly due to the number of PULP antisense insitu probes that had to be tested before a recognisable signal was obtained. T20403, like PULP, may also be expressed in roots because an ecotype Columbia cDNA is present in the databases derived from this tissue but single cell expression could not be detected in sepals or cauline leaves of *Arabidopsis* and bracts are highly reduced in *Arabidopsis* (Smyth et al., 1990; Long and Barton, 2001) so they could not be used for comparison of T20403 expression with the potential single cell expression of PULP in *Antirrhinum* bracts. *PHAN* and ASI transcripts are detectable in the L1 and primordia of all organs so in both species PHAN or ASI and PULP or T20403 have potential for their protein expression pattern to overlap in vivo.

The results with PULP sense probe called into question the results obtained with antisense probe by producing a similar signal. This may have been caused by spurious antisense probe synthesis from the 3' end of the PCR generated template. This was suggested by positive signal obtained when the sense probe was used against gel blots of RNA from *Antirrhinum* leaves (Fig. 16) but this was a situation where only sense signal should be present. Spurious 3' probe synthesis can be reduced or eliminated by digestion of the template at a 3' site (with respect to the desired transcription) with a restriction enzyme that produces a 4 base 5' overhang,
this serves to prevent T7 polymerase from initiating transcription at the single base 3' overhang typically produced by Taq polymerase (Gwyneth Ingram pers. comm).

Attempts to reduce PULP and T20403 expression using RNAi both failed to produce concrete results. The attempt to reduce PULP expression by viral co-suppression was not repeatable and in plants where RTPCR had suggested local, but not systemic, infection of the PULP viral co-suppression construct northern analysis provided no evidence that PULP expression was reduced. Furthermore there is a possibility that the knots and splitting of stem epidermis in affected plants may have been due to gall forming thrip infection (Thomas Guebitz, pers. comm.), although this occurs predominantly on the abaxial leaf surface and the knots observed occurred on the adaxial leaf surface Fig. 15. Low infectivity of tobacco rattle virus in Antirrhinum and/or poor response to it by Antirrhinum probably caused failure of the viral co-suppression experiment. The alternative that Antirrhinum suppresses tobacco rattle virus vigorously is less likely because TRV-GFPc transcript was found at low levels in systemically infected leaves (Stuart Macfarlane, pers. comm.), however pursuit of these hypotheses was beyond the scope of this project. Additional time would have enabled a more systematic approach to assessing whether infection was initiated for the PULP viral co-suppression construct and enabled a better conclusion.

The RNAi approach to reduction of T20403 expression was also unsuccessful due to failure of the Arabidopsis transformation. Failure of the transformation which is normally a reliable process was most likely due to poor condition of the plants used for transformation which were persistently infested with scarid fly larvae. Condition and fecundity of the plants has been found to be a significant factor in transformation efficiency of Arabidopsis (Clough and Bent, 1998). Again sufficient time was not available to repeat the transformation.
Mutagenesis screens for transposon insertions in PULP and T20403 also failed initially to produce concrete results. Screens for insertion of endogenous *Antirrhinum* transposons in *PULP* were not successful using the transposon specific primers available at the time however recently a number of *Antirrhinum* CACTA family transposons have been isolated by Andrew Hudson which demonstrate a highly conserved terminal sequence. I used these to design a primer specific for CACTA transposons including those that may not be recognised by previous primers used (see Table 4.) and have successfully screened through the John Innes *Antirrhinum* mutagenesis DNA pools down to a single family of 15 plants. This work is ongoing with good prospects of obtaining a transposon insertion approximately 500 bp 3' of the *PULP* transcriptional start site. Since transposons are known to move short distances it is possible that, in the event that the insertion has no phenotype, transposition and reinsertion could be obtained for further mutagenesis of *PULP*.

Mobilisation of the dSpm located near *ASY2* into T20403 also failed to produce a clear result. Subsequently a screen of the Syngenta garlic database of *Arabidopsis* T-DNA insertion flanking sequences identified an insertion in T20403. When 44 seed of this insertion were sown 1/3 were PPT sensitive and 2/3 were PPT resistant (14/30) but PCR and Southern hybridisation to genomic DNA suggested that 12 of these were heterozygous. The genetic ratio of 3:1 suggested that 1/3 were absent raising the possibility that the insertion is embryo lethal. The alternative explanation that the mutation is gametophytically lethal is less likely because this would produce a ratio of 1:1 heterozygotes to wildtype. Further Southern can be done to confirm that ¾ of the plants are heterozygous for the insertion and that other T-DNAs are not present. The possibility that insertions in T20403 are lethal helps to explain failure of the transposon mobilization in *ASY2* to produce a T20403 mutant phenotype - as recessive mutants would not appear in the F3 (except in the case of A21a which has not been shown to segregate in a Mendelian fashion). In retrospect it also appears that insufficient plants were screened by PCR particularly in the case of line 8337#9 because 1/8 would be expected to survive double selection and the transposition ratio (to unlinked sites from the T-DNA) is published as being $6.5 \times 10^{-5}$/generation (Tissier *et al.*, 1999) when this is applied to the number of seed sown it predicts the number of new insertions to be less than one ($6500 \text{ seed} \times 1/8 \times 6.5 \times 10^{-5} = 0.05$).
12.2 THE FUNCTION OF PHAN AND PULP

It is difficult to formulate a function for PULP or T20403 with the information currently available. Further basic characterisation of their expression patterns or a mutant phenotype are required to suggest their function. The hypothesis that primed this experiment was that $PHAN$ function is modified in the adaxial (or abaxial) domain by protein-protein interaction which could promote a function in specifying development of lateral organ axes. However, the PULP and T20403 predicted structure are uninformative in this respect and neither of their expression patterns as determined by insitu hybridization are sufficiently characterised to make conclusions. The information obtained does not confirm that they are adaxially or abaxially expressed which would support the hypothesis. On the contrary the ubiquitous expression of PULP determined by northerns and appearance of T20403 in tissue from roots suggests a completely different role from the hypothesis. For example, because PULP expression is ubiquitous and PHAN is expressed in a fraction of PULP domain PHAN may modify PULP function.
The lack of information about PULP or T20403 derived from expression pattern, mutant phenotype or genetic interaction with PHAN and AS1 mutants constricts further testing of PULP-homologue function by interaction with PHAN or AS1 to (1) to obtain better expression data that could suggest what role interaction of PULP and T20403 with PHAN and AS1 may serve; (2) To demonstrate that PULP-homologues have a related function to PHAN or AS1; (3) further test the potential for PHAN and PULP to interact in vivo.

The preceding discussion concentrated on the possibility that PHAN-homologues have functions in common with cMyb and mediate the transition from pleuripotent-stem cell identity towards differentiated cell states. Whether the similarities between PHAN and cMyb are significant may become apparent as it is further characterised so experimental methods to continue this work could concentrate on two areas (4) further tests of the potential of PHAN-homologues to dimerize in vitro and in vivo which might suggest function explore the potential of PHAN to interact with PHANL1 or PULP and determine if these interactions compete or abrogate one another which might suggest function. (5) To test whether PHAN or AS1 regulate KNAT1, HIRZ or PHB-homologues.

(1) To obtain better expression data that could suggest what role interaction of PULP and T20403 with PHAN and AS1 may serve, the obvious approach is do further in situ hybridisation experiments using the probes already tested. These have the potential to demonstrate PULP and T20403 expression patterns. Better sense control probes with reduced potential to produce antisense probe can also be made as described in section 12.1. Sense probes are always used in conjunction with positive controls in the same batch so that no-signal cannot be confused with the experiment failing. Alternatively the promoter of T20403 can be obtained from the Arabidopsis genome sequence and used to control a GUS reporter gene and provide additional evidence for the T20403 expression domain.

(2) To determine if PULP-homologues have a related function to PHAN or AS1 a classical genetics approach to could be employed with the expectation that partial rescue or enhancement of PHAN or AS1 phenotypes might be observed in PULP or
T20403 mutants. Recent screening of the John Innes Centre *Antirrhinum* transposon mutagenesis lines using a primer specific for previously unknown CACTA transposons has detected a potential insertion in the PULP promoter region. This may eventually enable a mutant phenotype for PULP to be determined and compared to *phan* (see section 12.1). However the Syngenta *Arabidopsis* T-DNA insertion line described in section 7.4 suggests that mutation of T20403 is recessively lethal to the zygote. A homozygous recessive mutant may be obtainable using an ethanol inducible T20403 construct which has already been made in pGreen II. This could be used in conjunction with kanamycin selectable pBIN 19 carrying alcA and alcR::GUS in Col0 ecotype *Arabidopsis* to enable homozygous T20403 knockout seed to be produced. Withdrawal of ethanol could then enable a T20403 mutant phenotype to be observed and compared to *as1-1*. The Syngenta T20403 insertion line is also being crossed to as1 in columbia in the hope that one wild-type copy of T20403 might be insufficient in the presence of *as1*. Since the *Arabidopsis* genome sequence demonstrates that T20403 is a single copy gene the RNAi approach to reducing T20403 expression is still valid. Accordingly transformation of ecotype Columbia harbouring pBIN 19 alcA, alcR::GUS with ethanol inducible T20403 RNAi constructs in T-DNA of pGreenII is in progress.

(3) Further tests of the potential for PHAN or AS1 and PULP-homologues to interact in vivo. Co-immuno precipitation with antibodies specific for each protein can be difficult to achieve as was demonstrated by attempts to raise an antibody to AS1. An alternative method is to tag a target protein so that it can be recognised using a readily available antibody. This approach could be used with GST fusion proteins to test in vitro for interaction between PULP or T20403 and PHAN or AS1. One protein is expressed as a GST fusion protein and purified on glutathione-agarose beads, the second protein is tagged with an epitope such as Xpress by cloning in pRSET (Invitrogen) and expressed in *E. coli*. If the proteins interact the epitope tagged protein may be purified with the GST-fusion protein and observed by western blotting. Lysate from bacteria not expressing the GST-fusion protein should not purify the epitope tagged protein.
Co-localisation of PULP or T20403 and PHAN or AS1 in vivo could be observed by fusing them to yellow and cyan fluorescent proteins and expressing them in cowpea protoplasts by transient transfection and observing their localisation with confocal microscopy. Both these approaches have recently been used to support interaction of MEA and FIE proteins (Spillane et al., 2000).

(4) further tests of the potential of PHAN-homologues to dimerize in vitro and in vivo could be done which might suggest function particularly the potential of PHAN to interact with PHANL1 or PULP and to determine if these interactions compete or abrogate one another.

As mentioned in section 11. there is a large phenotypic difference between \textit{phan}-164 and \textit{phan}-249 mutants yet the deduced genotypic difference, shown in (Fig.34), is that 15 additional amino acids are deleted between residues 316 and 332 in \textit{phan}-250. Furthermore in \textit{phan}-249 thirty four missense residues are added before a stop codon in the sequence of the mutagenising transposon whilst in \textit{phan}-164 only eight missense residues are added (coloured red).

\begin{verbatim}
GLRREAEEVKEQKLAEQWAALK
GLRSTTTKNPYWDTEMCPKRIGLRREAEEVKEQKLAEQWAHY
HLRLTKFLEQTGYSRIAGELNGR*
DLLQLHYLLYLGTLAVSVA*
KKNHKLK*
PHAN
phan-249
phan-164

Fig. 34. The deduced amino acid sequence of \textit{phan} mutants 164 and 249 starting 43 residues from the terminus in the top set and terminating in the bottom set. \textit{Phan}-164 has a mild phenotype yet \textit{phan}-249 has a phenotype as severe as that of \textit{phan}-607 which is a null mutation (Waites et al., 1998).

Some possible interactions mediated by the C-terminus of PHAN are dimerization, interaction with PHANL1, interaction with PULP or an intramolecular interaction, but whether none of these is affected would also be useful to know because the interaction domain within PHAN has not been localised more precisely than to within the C-terminal domain. Since PHAN is known to act as a repressor the simplest interpretation of the allelic series caused by serial deletion of the C-terminus is that the C-terminus is important for its repression function. Therefore which of the possible interactions mentioned above might be affected by the deletion mutants and
may contribute to a repression function. This can be tested with the yeast 2-hybrid assay employing baits or preys made with *phan* mutants. The potential of PULP to disrupt the intramolecular interaction or dimerization of PHAN or PHANL1 could also be tested in this system by expressing PHAN, PHANL1, PULP or S1-1 without a GAL4 domain fused to it and observing the effect on PHAN dimerization (for example). The outcome of the yeast 2-hybrid assays would suggest further work that may clarify whether the interactions occur in vivo perhaps utilizing gene delivery by particle bombardment in *Antirrhinum* leaves as has been used to test transcriptional activation and interaction in maize (Goff *et al.*, 1992).

(5) To test whether PHAN or AS1 regulate *KNAT1, HIRZ* or *PHB*-homologues. The starting point of testing if AS1 regulates PHB in *Arabidopsis* would be to examine PHB expression in *as1-1* mutants. Other approaches might be to obtain PHB or *KNAT1* promotors by PCR using the sequence of the *Arabidopsis* genome. These could then be subjected to deletion analysis in yeast 1-hybrid assays to look for AS1 binding sites. Regulation of PHB by AS1 might be tested in vivo by transformation of *Arabidopsis* with fusion proteins including AS1 and the VP16 strong transcriptional activator or a class II ERF active repression domain (Ohta *et al.*, 2001) under control of the AS1 promoter then monitoring PHB expression by RT-PCR. The region of the AS1 promoter sufficient for expression in lateral organ primordia is known (Eshed *et al.*, 2001). Although this would help to ensure that timing and position of expression was normal the limitation of expressing fusion proteins might be that it could abrogate necessary processes such as signal transduction that may be necessary for function. Expression of the repressor domain fusion would be expected to give a *phan*-like leaf phenotype (abaxialized) because this might allow KANADIs and YABBYs to invade the adaxial domain.

Therefore there are numerous ways to substantiate the results obtained here and further test their significance.


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