Instituto Juan March de Estudios e Investigaciones

36 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Flower Development

Organized by

E. Coen, Zs. Schwarz-Sommer and J. P. Beltrán

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N. Battey
J. P. Beltrán
G. Bernier
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INTRODUCTION

José Pío Beltrán

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The molecular and genetic analysis of floral homeotic mutants are providing many important insights into the flower developmental biology field. Progress accomplished using two experimental systems, Arabidopsis thaliana and Antirrhinum majus has led to the so called ABC model of floral organ identity specification. In this model there are three activities that are present in overlapping manner in an early floral primordium, such that four domains of activity are present. Thus, A function causes, in the floral periphery, the differentiation of sepals. Cells with both A and B functions present develop petals. Stamens result from combined B and C activity and carpels from C activity alone. Besides, A activity and C activity are mutually exclusive.

Genetic evidence for this model has been obtained. Genes associated with each activity A, B, C, have been isolated and characterized. Besides, two clases of upstream genes have been identified: the meristem identity genes which are positive regulators of the A B C genes and the cadastral genes which would act to present ABC genes from becoming active in unappropriate places.

This workshop has brought together twenty invited speakers and twenty eight participants from Europe, the U.S., Canada and Australia to discuss their recent progress in two related and challenging areas: the mechanisms governing the transition from vegetative to reproductive meristems and the genetic and molecular bases of pattern formation and differentiation during flower development. From presentations and discussion floral induction appears as a very complex process in which many environmental factors, such as daylength, vernalization and light quality participate. Experimental conditions that allow growth and flowering of Arabidopis plants under complete darkness were reported. Genetic analysis of dark morphogenesis could help to dissect and understand floral transition. The cloning of constants a gene that promote flowering in response to long days has allowed the molecular analysis of light mutant alleles, and suggest the presence of a new family of transcription factors controlling floral induction. Impatiens appears as an interesting experimental system to test the determination of floral meristems. Some signals were suggested to participate in the floral transition, although a clear signal transduction pathway both in floral transition and flower development remains to be established. The biochemical patways leading to concrete mechanism governing cell differentiation under the control of MADS-box genes remain uncovered as a great challenge for the future. Major progress in the molecular characterization of the MADS-box gene family was reported. The isolation and characterization of MADS-box genes from other plants of agronomic interest such as Petunia, tomato, pea and maize among others, open the door to future biotecnological manipulations in these species.

The mixta gene, controls the development of specialized shape of petal epidermal cells. As it was reported its ectopic expression in tobacco influenced the shape of epidermal cells from other plant parts, besides petals, and, in some instances, induced trichome differentiation. Interestingly, mixta is a myb gene, like GL1 from Arabidopsis which also controls trichome differentiation. Plant myb genes, however, were first described because their involvement in the regulation of phenylpropanoid

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biosynthesis. In fact, as proposed in the workshop, this metabolic pathway might be a common aspect in the action of the plant myb gene family.

The genetic analysis of flower shape and floral simmetry reported promises new exciting times in flower development.

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1st Session:

Floral induction and early events

Chairperson: Elliot M. Meyerowitz

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Physiological and genetical analysis of the sucrose signal at the transition to flowering

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Available evidence indicates that the control of the transition to flowering is multigenic and multifactorial (Bernier *et al.*, 1993). One of the controlling factors or signals in many plants is apparently a better supply of sucrose to the shoot apex. Supporting evidence has been obtained in both the long-day plant (LDP) *Sinapis alba* induced by a single LD or by a single displaced short day (DSD) (Lejeune *et al.*, 1993) and the SDP *Xanthium strumarium* induced by a single long night (LN) (Houssa *et al.*, 1991). Remarkably, this increased sucrose supply is observed not only in inductive conditions involving an increase in photosynthetic CO_2 fixation (*Sinapis* exposed to one LD) but also in conditions where induction does not modify (*Sinapis* exposed to one DSD) or decreases photosynthetic CO_2 fixation (*Xanthium* exposed to one LN). This strongly suggests that the extra-sucrose arises in many cases not from increased photosynthesis but from mobilization of reserve carbohydrates, presumably starch, stored in various plant parts.

In *Sinapis* the effect of the extra-sucrose supply is manyfold. Upon arrival at the apex, sucrose causes an increase in acid invertase activity, mitochondrion number and presumably stimulates the energy metabolism of meristematic cells (Bernier, 1988). Sucrose also cooperates with cytokinins (CKs) in decreasing the duration of the cell cycle and its component phases in these cells (Bernier *et al.*, 1993). On the other hand, the experimental evidence strongly suggests that the extra-sucrose is the leaf-to-root signal causing an early increased export of CKs out of the root system. This extra-CK supply to the shoot, eventually reaching the apex, is another essential signal for the flowering transition in *Sinapis*. The sucrose signal - either alone or combined with the CK signal - is unable however to trigger the whole process of the floral transition in *Sinapis*, and thus other signals are at work in this species.

The conclusion of the physiological analysis is that sucrose is an essential but insufficient signal of the flowering transition.

Mutant and transgenic lines of *Arabidopsis thaliana* with a defect in their carbohydrate metabolism have been used to test this inference : two starch mutants of the Columbia wild type (WT) (a) the *phosphoglucomutase* (*pgm* TC 75) starchless mutant (Caspar *et al.*, 1985), and (b) the *starch overproducer* (*sop* TC 26) mutant which results from a deficiency in an unknown step of starch degradation (Caspar *et al.*, 1991); the transgenic line A41-49 produced from the C24 genotype expressing the yeast invertase gene *suc2* in the cell walls (von Schaewen *et al.*, 1990) (Expression of this gene in the walls

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should block any apoplastic movement of sucrose and thus reduce export of sucrose out of the mature leaves). All mutant and transgenic lines are facultative LDPs and facultative cold-requiring plants as are the Columbia WT and the C24 genotype, but all mutant and transgenic lines have a late-flowering phenotype compared to their respective WT or untransformed lines. The delayed flowering is progressively more detectable as daylength is decreased. In all lines seed vernalization suppresses the late-flowering character, so that the *pgm* and *sop* mutants now flower at the same time as the unvernalized Columbia WT and the transgenic line A41-19 almost at the same time as the vernalized C24 line.

These observations support the conclusion that carbohydrate metabolism, especially starch mobilization and sucrose transport, is an essential process in the control of the flowering transition.

Research supported by a grant from the University of Liège (PAI). L.C. is a fellow of I.R.S.I.A. The *pgm* and *sop* mutants were provided by Drs C. Somerville and C. Dean, and the transgenic line by Dr. L. Willmitzer.

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Genetic control of floral transition in *Arabidopsis thaliana*

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Flowering in Arabidopsis is promoted by long photoperiods and, in some ecotypes, by low temperature exposure (vernalization) (1). Mutations that produce a late flowering phenotype under floral inductive conditions have have been used to identify more than 10 loci, thought to be involved in the promotion of the flowering process in this species (1, 2, 3). Additionally, early flowering mutants, which likely identify loci involved in the repression of the flowering process have recently been identified (4, 5). Physiological and morphological characterization of the responses of late flowering mutants to daylength, vernalization and light quality has allowed their classification in different phenotypic groups that could represent, at least, two different floral promoting pathways. Mutations at loci like *FVE, FCA, FPA, FY* or *LD*, produce a delay in flowering time under any daylength and a reduction in stem elongation that can be rescued by vernalization (1, 3, 6, 7). On the other hand, mutations at loci like *CO, GI, FWA* or *FT* only produce a delay in flowering time under long days and are very similar to wild type under short days (1, 3). These mutants also show a very reduced response to vernalization (3, 6).

Based on morphological features, the development of Arabidopsis can be divided in four different developmental phases, early rosette, late rosette, early inflorescence and late inflorescence (8, 9). When this morphological analysis was performed in several late flowering mutants belonging to both phenotypic groups, we found that the delay in flowering time caused by these mutations under long days is the consequence of an elongation of all the developmental phases of the plants, from the early rosette phase to the specification of the floral meristems (9, 10). A similar effect in Arabidopsis development is caused by non inductive photoperiods (10). These results suggest a function for the corresponding loci either very early in plant development or all along the development of the plant (10). In this way, the analyses of double mutants carrying late flowering mutations and mutations in floral meristem identity genes, such as *LFY, AP1* and *AP2* (8, 11), indicate that the products of the wild type genes identified by late flowering mutations in both promotive pathways are directly or indirectly involved in the activation of the floral meristem identity genes. On the other hand, the analysis of double mutants carrying late flowering mutations and mutations at *TFL*, a locus which activity precludes the establishment of the floral program in coflorescences and inflorescence meristems (12, 13), suggest a differential interaction among *TFL* and genes belonging to different floral promotive pathways.

Based on previous reports of dark flowering in Arabidopsis (14, 15), we have identified experimental conditions that allow succesful growth and flowering of Arabidopsis plants under complete darkness. Under these conditions, the only restriction for morphogenesis seems to be the availability of carbon at the aerial part of the plant, what is provided by growing the plants on vertical Petri dishes. Dark morphogenesis taking place at the apical meristem gives rise to etiolated plants that flower after the production of a determined number of leaves. Late and early ecotypes, showing strong differences in

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flowering time and leaf number under long days, flower with the same number of leaves when grown under our conditions. Moreover, all the late flowering mutants tested, except those at loci *FWA* and *FT*, flower with the same number of leaves, suggesting that, under these conditions, only those two loci are involved in the dark morphogenic proccess.

All these results will be consider to discuss possible models on the genetic control of floral transition in Arabidopsis.

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***Insights into flowering from reversion in* Impatiens**

Nick Battey, Sylvie Pouteau, David Nicholls, Fiona Tooke (Plant Sciences, University of Reading, Reading RG6 2AS, UK) & Enrico Coen (John Innes Institute, Colney Lane, Norwich NR4 7UH, UK).

I.balsamina cv. Dwarf Bush Flowered forms a terminal flower when grown in inductive short days. Transfer to non-inductive long days during flower development causes reversion to leaf initiation. The greater the number of short days before transfer, the further developed the flower becomes before reversion, giving a range of reversion types classified as R0-R7 (Battey & Lyndon, 1984).

We have concentrated our analysis on plants showing the R4 reversion type, which, in place of the flower, develop organs intermediate between leaves and petals. The reverted meristem then initiates leaves in whorls. This arrangement is characteristic of floral organs, and together with the absence of axillary meristems, indicates that the leaves are not purely vegetative. This is confirmed when the reverted plants are returned to inductive conditions: the young 'leaf' primordia are able to switch to petal development; they gradually lose this developmental flexibility until, when they are approx 750 um long, they are fully committed as leaves (Battey & Lyndon, 1986; 1988). This is in marked contrast to leaf primordia on the true vegetative meristem which, in common with leaf primordia of other species, are committed to vegetative development at or very close to the time of their initiation (Battey & Lyndon, 1988). The inductive treatment therefore does not commit the meristem to flower development, but it does result in an alteration in the determination time of the leaf primordia subsequently initiated.

This pattern of flowering, reversion and re-flowering offers the opportunity for a number of insights into the control of flower development. First, it contrasts with the situation in many plants, in which floral commitment appears to occur very early, so that the meristem is considered committed well before the appearance of the first floral organs (Bernier *et al.*, 1981). However, reversion has been reported in a wide variety of cases, often in photoperiodically sensitive species in marginally inductive conditions (Battey & Lyndon, 1990). Reversion could therefore imply that commitment to flower is not a real event; alternatively, *Impatiens* (and other plants when they revert) could be demonstrating a weak or partial functioning of the normal commitment step.

The second point that *Impatiens* makes is that organ arrangement and identity are controlled independently. To study how this control is achieved during flowering and reversion, PCR fragments corresponding to the homologues of the homeotic genes *FLORICAULA* and *FIMBRIATA* have been isolated from *Impatiens*. *FLO* and *FIM* expression has been analysed by RNA *in situ* hybridisation with digoxigenin-labelled probes at different times after induction and compared to vegetative controls. An unexpected finding is expression of *FLO* and *FIM* in the vegetative meristem. The expression pattern of *FLO* does not show any clear difference in vegetative and floral terminal apices, indicating that its transcriptional control does not play a key role in flower induction. By contrast, the *FIM* pattern of expression changes during petal initiation, reflecting the involvement of *FIM* in the control of organ identity.

The implications of these results for understanding commitment and flower development will be discussed.

Acknowledgement
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Molecular genetic analysis of the control of flowering time in response to daylength in *Arabidopsis*

George Coupland, Joanna Putterill, Frances Robson, Karen Lee, Rüdiger Simon, Isabel Igeño, Katherine Dixon, Laurian Robert, Sudhansu Dash and Giovanni Murtas.

Arabidopsis is a facultative long-day plant, flowering earlier under long (16 hours light) than short (10 hours light) days. We have used molecular-genetics to study this phenotype. The approach that we used was to identify mutants that flower at approximately the same time under both long and short days (i.e. are day-neutral), and then to isolate the genes affected by the mutations. Broadly two types of mutants were identified: early day-neutral mutants that are early flowering under both long and short days, and late day-neutral mutants that are late flowering under both conditions.

Mutants in the late class were identified by Redei (1) and Koornneef and colleagues (2). Three late flowering mutants, *constans* (*co*), *gigantea* (*gi*) and *fha*, flower later than wild type under long days, and approximately the same time as wild type under short days. These three genes therefore probably promote flowering in response to long days. We are cloning genes in this class to determine their function. Their locations on the *Arabidopsis* genetic map were previously determined (2). We used this information as a starting point to clone *CO* by chromosome walking. The protein product contains two zinc fingers, suggesting that it binds DNA and is involved in gene regulation (3). The *CO* mRNA is present at higher abundance in long day grown than in short day grown seedlings, which is consistent with its proposed role of promoting flowering under long days. The mRNA is present at low level in leaves and stems, making it difficult to predict whether *CO* is involved in inductive processes in the leaves, or whether it is required at the shoot apex. Analysis of eight mutant alleles showed that 5 affect the zinc fingers, supporting the suggestion that these sequences are important for *CO* function. Two others affect neighbouring amino acids near the carboxy-terminus and one is a promoter mutation. Double mutant plants containing *co* as well as mutations affecting genes involved in light reception, gibberellic acid (GA) metabolism or floral morphology were made and analysed. These suggested that the *GA INSENSITIVE* (*GAI*) gene, that is involved in GA metabolism, can partially compensate for loss of *CO* function. Furthermore *co* greatly enhances the phenotype of *leafy*, suggesting that *CO* might directly or indirectly promote the activity of meristem identity genes.

The second class of flowering time mutants that we have studied are those in the early day-neutral class. By screening M2 populations derived from gamma irradiated seeds, we identified three early flowering mutants, and were provided with two others by Maarten Koornneef. We have studied in most detail a severe mutation called *early short days* 4, that causes mutants to flower very early under short days. We have demonstrated that in double mutant combinations *esd4* suppresses the late flowering phenotype of *co*. We have mapped *esd4* to a short interval on chromosome 4, and are making use of the available physical map of this region (Schmidt and Dean, unpublished) to isolate the gene.

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MOLECULAR AND GENETIC ANALYSIS OF EARLY FLOWER DEVELOPMENT IN *ANTIRRHINUM MAJUS*

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SUMMARY

Our main scientific interest concerns the molecular genetic mechanisms acting between the flower promoting signal(s) and the establishment of floral meristem identity.

Flower formation in our model species *Antirrhinum majus* is preceded by the conversion of a vegetative shoot apical meristem into an inflorescence apical meristem. Floral meristems will subsequently arise as secondary meristems on the flanks of this primary inflorescence apical meristem.

In *Antirrhinum* the genes *FLORICAULA* (*FLO*) and *SQUAMOSA* (*SQUA*), encoding putative transcription factors, are among the earliest genes known to become activated after floral induction. Transcription of these genes is highly up-regulated in the inflorescence secondary meristems, and is, as deduced from the respective mutant phenotypes, required to determine their floral identity (Coen et al. 1990; Huijser et al. 1992).

However, *FLO* and *SQUA* seems to act relatively late in the genetic hierarchy controlling the flowering process as a whole. In fact, their activation demarcates the start of what actually represents the final stage of the flowering process, i.e. the realization of a flower.

In recent years, several floral homeotic genes were isolated that could be under the control of the floral meristem identity genes. However, our knowledge of the mechanisms controlling these meristem identity genes themselves or even earlier stages of the floral transition, remain unsatisfactory.

During early steps, i.e. floral induction and evocation, the mechanisms controlling flowering can be strongly influenced by many aspects of plant growth. Therefore, it is to be expected that many mutations of genes involved in the general metabolism of the plant will affect flowering time, that is the appearance of the first flower and thus the activation of the floral meristem identity genes.

To avoid the possible isolation of 'flowering time genes' that are only secondarily involved in the floral transition, we started a search for genetic factors directly involved in the regulation of the floral meristem identity genes. Using an electrophoretic mobility shift assay (EMSA) we identified putative regulators of *SQUA* through their specific interaction with the *SQUA* promoter region. One particularly clear *in vitro* complex formation was found between a defined sequence motif and nuclear protein extracted from inflorescences. The same motif showed no specific interaction with nuclear protein extracted from non-flowering plants. Subsequent south-western screening of an inflorescence specific expression library using this specific sequence as a probe resulted in the isolation of two different genes named *SBP*1 and *SBP*2 (*SBP* for *SQUAMOSA PROMOTER BINDING PROTEIN*).

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The encoded proteins share a conserved domain necessary and sufficient for sequence specific DNA-binding and were found to represent a small family of at least four genes.

No sequences similar to the *SBP* genes and their products could be detected in the databases. Conversely, highly related genes could be isolated from *Arabidopsis* and seem to be present in other flowering plants as well. In addition, the protein products of the *SBP*1 and *SBP*2 releted genes in *Arabidopsis* recognize *in vitro* a similar DNA motif present in the promoter of *APETALA*-1, the presumed *SQUA* orthologue in this species (Mandel et al. 1992).

The *SBP*1 and *SBP2* genes, as well as their *Arabidopsis* counterparts, are developmentally regulated whereas other members of the family seem to be expressed more constitutively. Their importance for the floral transition is currently investigated in transgenic plants.

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2nd Session:

Mechanisms of pattern formation

Chairperson: Georges Bernier

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Between meristem and organ identity: establishment of organ number and whorl position in developing *Arabidopsis* flowers.

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In the development of flowers, organ primordia can arise in stereotyped positions and numbers, as if the cells of the floral meristem can measure and count. To find out how floral organ numbers and positions are established, we have collected a group of *Arabidopsis* mutants in which floral organ number is increased or decreased, and have proceeded to genetically analyze some of the mutations.

The first-studied set of mutations was an allelic series of lesions in the *CLAVATA1* gene, which maps toward the bottom of the first chromosome. Mutations in the gene were first identified and mapped by Koornneef and his coworkers (Koornneef et al., 1983), and were shown to affect apical meristem size, and to cause fasciation by Leyser and Furner (1992). We have looked at several alleles, and found that the stronger (greater departure from wild type) ones cause increases in the numbers of organs in all floral whorls, and also cause the formation of additional whorls internal to the fourth whorl (these have also been noted by Shannon and Meeks Wagner, 1993). The additional whorls develop into pistils, giving flowers with multiple nested ovaries. Laser scanning confocal microscopy has shown that the basis of all of the phenotypes is that there are many extra cells in all apical meristems, whether vegetative or floral (Clark et al., 1993). In strong alleles and in some environmental conditions such as low temperature, the cell divisions in apical meristems appear to grow completely out of control, and a huge overgrowth of the meristem results. This causes different types of stem fasciation or floral fasciation, and can lead to shoot apical meristems on *Arabidopsis* much larger than normal for *Arabidopsis*, and in fact larger than known for any plant, including such giants as oaks. The wild-type function of *CLV1* thus appears to be the limitation of cell division in apical meristems.

To better understand the processes of cell division control in meristems, we have now isolated mutations in additional genes. Allelic series of *CLAVATA2* (chromosome 1) and *CLAVATA3* (chromosome 3) have been obtained, and *clv3* mutations studied in detail (Clark and Meyerowitz, 1994; Clark, Running and Meyerowitz, submitted for publication). The phenotype of *clv3* mutations is similar to that of strong *clv1* mutations. Double mutants between the strongest *clv1* and *clv3* alleles show no phenotypic difference from either single homozygote. This implies that the genes might act in the same, and not in parallel, pathways. This notion is strengthened by the finding that *clv1*/+; *clv3*/+ transheterozygotes show nonallelic noncomplementation, that is, such transheterozygotes show a strong Clavata phenotype. It thus appears that a heterozygous lesion in one *CLAVATA* gene makes the plant much more sensitive than usual to a quantitative change in the amount of product of another *CLAVATA* gene. Additional, similar mutations have been found, for example mutations in a new gene named *MULTIPETALA* also cause increased cell and floral organ number (Running and Meyerowitz, in progress).

These studies lead to two different questions. One is, How are cell divisions controlled in meristems? To investigate this, we have looked at some new mutations that reduce floral meristem cell number. One group are alleles of *TSO1* (chromosome 3; Liu and Meyerowitz, in progress). The *tso1* mutant phenotype is cessation of cell division in floral meristems, with only minimal effects on vegetative development. Transmission electron microscope analysis of the mutants shows that cell division appears to be blocked in cytokinesis: one can find binucleate cells with partial cell walls, and with what appear to be unfused phragmoplast vesicles. In older flowers large cells with large nuclei are found; quantitation of the DNA content of these cells by microspectrophotometry shows that they are highly polyploid.

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The second question raised by the cell division mutations is the original question: How do cells count? We can see from the fact that extra cells (or large meristems) lead to extra floral organs that the organ primordia are somehow measuring cell number or distance, and not a fixed angle. But how is the number or distance measured? We have only one mutation that seems primarily to affect organ number without affecting cell number, and this is *PERIANTHIA* (chromosome 1; Running and Meyerowitz, in progress). Mutations in this gene cause the *Arabidopsis* flowers to have five-fold symmetry. The mutant flowers typically have five sepals, five petals, five stamens and two carpels, rather than the usual 4,4,6, and 2.

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FUNCTION OF PLANT MYB GENES IN THE REGULATION OF THE BIOSYNTHESIS OF PHENYLPROPANOIDS. INSIGHTS FROM BIOCHEMISTRY

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Phenylpropanoids are a class of metabolites, originating from phenylalanine, which serve several functions in plants: structural components of cell walls, protectants against UV ligth,pathogens and oxidative stress, attractants of polinators, signal molecules etc. The pathway leading to the synthesis of phenylpropanoids is a multibranched pathway, reflecting the chemical diversity of this class of compounds, despite their common precursor. Genetic and biochemical evidence have shown that myb genes are one of the classes of genes involved in the regulation of the phenylpropanoid pathway. This family includes a large number of members, at least 70, in each plant species, most of them encoding a highly related MYB DNA-binding domain, composed of two repeats (R2 and R3), suggesting that they migth share some target genes. However, at present, there are only three myb genes, the maize C1, Pl and P genes, which have been conclusively shown to regulate phenylpropanoid biosynthetic genes. To evaluate to what extent the remaining myb genes could also participate in the regulation of this metabolic pathway, we have chosen the myb.Ph3 gene from Petunia for biochemical analysis, since this is one of the plant myb genes most distantly related to the maize C1, Pl and P genes. Using a binding site selection protocol, the DNA sequences recognized by MYB.Ph3 were found to fall into two classes, resembling the two known types of MYB DNA-binding sites: consensus type I (MBSI), aaaAaaC(G/C)GTTA, and consensus type II (MBSII), aaaAGTTAGTTA. Optimal MBSI was strongly bound by c-MYB from animals and not by Am305 from Antirrhinum majus , whereas optimal MBSII was bound by Am305 and not by c-MYB protein. Different constraints on MYB.Ph3 binding to the two classes of sequences were demonstrated by DNA-binding studies with mutated MBSI and MBSII. These studies, as well as hydroxyl radical footprinting analysis, pointed to the R2 repeat as the most involved in determining the dual DNA binding specificity of MYB.Ph3 and supported the idea that binding to MBSI and MBSII does not involve alternative orientations of the two repeats of MYB.Ph3 despite the resemblance of MBSI and MBSII to (imperfect) inverted and direct repeats of the GTTA motif, respectively. A minimal promoter containing either MBSI or MBSII was specifically activated by MYB.Ph3 in yeast, indicating that both types of binding site can be functionally equivalent. In aggreement with this, MYB.Ph3 induced very similar distorsions on both MBSI and MBSII. MYB.Ph3 binding sites were found at the promoter of several phenylpropanoid (flavonoid) biosynthetic genes, specially *chs* genes, such as the *Petunia chsJ* gene, which was shown to be transcriptionally activated by MYB.Ph3 in tobacco protoplasts. Moreover, MYB.Ph3 was detected by *in situ* immunolocalization in the epidermal cell layer of petals, a cell type in which flavonoid biosynthetic genes, are actively expressed, strongly suggesting a role of this protein in the regulation of flavonoid biosynthesis, and, thus consistent with the notion that many myb genes regulate this or related pathways .

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"Floral organ initiation and development in wild-type *Arabidopsis* and in the organ identity mutants."

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The flowers of *Arabidopsis thaliana* (Brassicaceae) were examined for histological events during organ initiation and later development. An inflorescence plastochron of the main stem raceme was used as a basis for the timing and staging of developmental events. Sepals, petals, stamens, and carpels in wild-type landsberg *erecta Arabidopsis* are distinguishable as primordia in terms of cell division events associated with initiation, size, and component cell numbers. Flower organogenesis in the organ identity (homeotic) mutants, *pistillata*, *apetala2-1* and *agamous-1* was compared with that of the wild type. In the mutants, each whorl of floral organs initiates much like the wild type and only subsequently produces visibly altered organs with mosaic features. The flower organ identity mutants achieve their mature phenotypes by alterations in tissue differentiation that occur after initiation and early primordial development. An argument is made for the value of documenting the histology and timing of initiation and subsequent development of flower organs in order to understand the genetic mechanisms underlying these events. The data support a sequential model for control of floral organogenesis in *Arabidopsis*.

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Genetic and Molecular Studies of Floral Pattern Formation in *Arabidopsis thaliana*. E.M. Meyerowitz, Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125, USA.

We are trying to understand the mechanisms by which flower patterns develop. Our initial work was on the radial pattern of organ identity, and suggested the present ABC model of floral organ identity specification. In this model there are three activities that are present in overlapping fashion in an early floral primordium, such that four domains of activity are present. In the floral periphery A function is present, causing the organ primordia that develop there to differentiate to sepals. Internal to this there is a ring of cells with both A and B functions present, causing petals to be specified. Stamens result from combined B and C activity, and carpels from C activity alone. Furthermore, A activity and C activity are mutually exclusive, so that removing one enlarges the domain of the other. The early evidence for this model came from the phenotypes of loss of function mutations in individual genes necessary for A, B or C function. More recently, we have been using cloned A, B and C genes to see if ectopic expression causes phenotypes that fit the predictions of the model, and to see if any of the individual genes cloned (or combinations of them) are in fact sufficient to provide the activity for which they are necessary. Some ongoing work has used the two known B function genes. We have found that combined expression of *APETALA3* and *PISTILLATA* is sufficient to provide B function in flowers (but not outside of flowers). This indicates that, within developing flowers, the only spatially localized gene products necessary for B function are those coded by these genes. It also indicates that there is an additional factor that is generally present in flowers, but not in vegetative plant parts, that is necessary for B function.

The ABC model raises a series of questions. One concerns the way in which the three activities are spatially regulated in developing flowers, so as to provide the rings of activity that lead to the sepal-petal-stamen-carpel radial pattern of organ types. We have conceptually divided the upstream genes into two classes. One contains the meristem identity genes, which are positive regulators of the ABC genes (and which so far seem to act uniformly in floral primordia). The other contains the cadastral genes, which, formally at least, act to prevent ABC genes from becoming active in inappropriate places. Among these genes are *SUPERMAN*, which formally acts to maintain the inner boundary of B gene expression, and *CURLY LEAF* and *LEUNIG*, which participate along with A function genes in preventing activation of C function in outer whorls. *SUPERMAN* has been cloned, and its expression pattern indicates some new ways of thinking about cadastral activities. It has also become clear that ABC genes themselves, in addition to organ identity activities, have interactions with each other that are important for refinement of the initial pattern in which they are active. An example is provided by the B function genes, which positively interact at transcriptional and posttranscriptional levels so that B function exists only where both genes are activated. The two genes are initially transcribed in different but overlapping domains; the interactions between the genes result in B function being present only in the region of overlap.

Another problem raised by the ABC model is that of how the organ identity activities of the ABC genes come about. A number of these genes are now cloned, and all appear to be transcription factors. What genes do they activate and repress, and how? We have some information on the protein activities of four of the gene products, those of the A function gene *APETALA1*, the two B function genes mentioned above, and the C function gene *AGAMOUS*. This information indicates that the specificity of action of each protein is not conferred by different DNA binding regions, but by other parts of the proteins that may interact with as-yet unknown accessory proteins.

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In addition to the radial pattern of organ identity, there are other patterns in flowers that require molecular explanations. We have been studying genes responsible for setting proper organ number in *Arabidopsis* flowers, and have found two classes of them. One class, which includes *CLAVATA1* and *CLAVATA3*, is necessary for appropriate numbers of cell divisions in all above-ground meristems, including floral ones. The other class consists of one gene, *PERIANTHIA* which changes the normally tetramerous *Arabidopsis* flower to one with five-fold symmetry.

Some recent references from our laboratory:

Goto, K. and E.M. Meyerowitz (1994) Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*. Genes Devel. **8**, 1548-1560.

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3rd Session:

Transcription factors in the control of differentiation

Chairperson: Enrico Coen

Instituto Juan March (Madrid)

DIVERSE ROLES FOR MADS-BOX GENES IN *ARABIDOPSIS* DEVELOPMENT

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After floral induction, the first step in the development of flowers is the generation of floral meristems. Genetic studies show that *APETALA1* and *CAULIFLOWER* encode partially redundant functions in specifying floral meristem identity[1]. In addition to its role in meristem identity, *APETALA1* is required for the initiation and proper development of sepals and petals. Molecular characterization of *APETALA1* shows that it encodes a MADS-domain and thus likely functions as a transcription factor[2]. RNA tissue *in situ* hybridization analyses show that *APETALA1* RNA begins to accumulate as soon as floral meristems are visible on the flanks of the inflorescence meristem. Although *APETALA1* RNA accumulates uniformly throughout young flower primordia, later in development it is excluded from cells that will give rise to stamens and carpels. The lack of *APETALA1* RNA accumulation in stamens and carpels is due to the activity of the organ identity gene *AGAMOUS* [3].

When mutations in the *APETALA1* gene are combined with mutations in *CAULIFLOWER*, cells that would normally constitute a floral meristem instead behave as inflorescence meristems[1]. The result is a massive proliferation of meristems resulting in an inflorescence phenotype that is strikingly similar the familiar "cauliflower curd" of the dinner table. Isolation of the *CAULIFLOWER* gene reveals that it is closely related to *APETALA1*, sharing approximately 80% identical amino acid residues, and encodes a MADS-domain that is nearly identical to that of *APETALA1*[4]. Thus, *CAULIFLOWER* and *APETALA1* likely recognize the same target sequences, and thus likely regulate many of the same downstream genes. Like *APETALA1*, *CAULIFLOWER* RNA accumulates uniformly in young flower primordia. However, in contrast to *APETALA1*, whose RNA accumulates at high levels in sepals and petals throughout flower development, *CAULIFLOWER* RNA accumulates at very low levels in these organ primordia. This likely explains why *CAULIFLOWER* is able to substitute for *APETALA1* in specifying floral meristems, but is unable to substitute for *APETALA1* in specifying sepals and petals.

Studies in distantly related plant species, such as in *Arabidopsis* and *Antirrhinum*, suggest that the underlying mechanisms controlling flower development have been highly conserved[5]. Because the *cauliflower* phenotypes in *Arabidopsis* and *Brassica oleracea* var. *botrytis* are similar, it would not be surprising if the molecular basis for these phenotypes was related. We have characterized the *CAULIFLOWER* gene from *Brassica oleracea* var. *botrytis*, and molecular studies demonstrate that this gene is non-functional[4]. These studies suggest that the *cauliflower* phenotype in *Brassica oleracea* var. *botrytis* is due in part to a nonfunctional *CAULIFLOWER* gene.

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In addition to the MADS-box genes that determine floral meristem and organ identity, six functionally anonymous MADS-box genes have also been cloned[6]. To determine if this family of genes plays additional roles in flower development, and to see if MADS-box genes control other aspects of plant development, we have isolated and characterized fifteen new MADS-box genes from *Arabidopsis*. The onset of expression of two of these genes, *AGL4* and *AGL9*, is shortly after the onset of *APETALA1* and *CAULIFLOWER* gene expression. This suggests a possible role for these genes in mediating between the meristem and organ identity functions. The onset of *AGL5* expression is immediately after that of *AGAMOUS*, and *AGL5* requires AGAMOUS activity for its activation. These and other studies suggest that *AGL5* may be a target of AGAMOUS, and that it plays a role in carpel development[7]. Two other MADS-box genes, *AGL11* and *AGL13*, are initially expressed later in carpel development, with expression being limited to specific cell types of ovules[8]. Taken together, these and other studies suggest that MADS-box genes function throughout flower development. MADS-box genes likely play developmental roles outside of flower development as well. For example, *AGL15* is preferentially expressed during embryo development, with its expression initiating prior to the eight-cell stage of embryo formation[8]. Three genes, *AGL12*, *AGL14*, and *AGL17*, are preferentially expressed in roots, suggesting a possible developmental role for these genes in root formation[8]. *AGL8* represents a gene that is expressed in the inflorescence meristem, and is negatively regulated in floral meristems by *APETALA1*[9]. Taken together, it is likely that members of the MADS-box gene family play regulatory roles in diverse aspects of plant development.

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Interactions between plant MADS-box genes

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In recent years the study of floral developmental mutants has led to the isolation of a number of key regulatory genes. Many of these genes are members of the MADS-box gene family of presumed transcription factors. Several members of this gene family have been isolated from various different plant species with, for example, more than twenty-five different MADS-box genes existing in *Antirrhinum majus*. Each of these individual genes has a different, overlapping, temporal and spatial expression pattern, leading to the expression of various combinations of MADS-box genes in different floral organs and parts of those organs at different developmental stages. It is likely that the developmental fates of groups of cells within the flower are determined by the particular expression profiles of these and other genes.
The MADS-box is also found in well-characterised transcription factors from mammals (serum response factor (SRF) and the rSRFs (MEFs)) and yeast (MCM1). In both of these cases the proteins bind DNA as a dimer and also recruit other ternary factors by protein-protein interactions. In the case of one pair of the plant MADS-box proteins (DEFICIENS and GLOBOSA of *Antirrhinum* and their presumed *Arabidopsis* orthologues AP3 and PI) heterodimerisation has been demonstrated (Schwarz-Sommer, Hue et al. 1992; Goto and Meyerowitz 1994).
We have begun to study the network of potential interactions between the different MADS-box proteins of *Antirrhinum* using a yeast two-hybrid system (Chien, Bartel et al. 1991). To assess the feasibility of this approach we initially screened for proteins capable of interacting with the *Antirrhinum* protein PLENA (considered to correspond to AGAMOUS in *Arabidopsis* (Bradley, Carpenter et al. 1993)). PLENA was expressed in yeast as a fusion protein with the DNA-binding domain of GAL4. A directionally cloned *Antirrhinum* cDNA library was constructed such that any cDNA which was expressed in yeast would be expressed as a fusion with the GAL4 transcription activation domain. The library was introduced in to a strain of yeast which allowed the selection of proteins which interact with PLENA by activating two separate markers. Screening $2x10^6$ recombinants in the yeast expression library led to the isolation of ten cDNAs the protein products of which specifically interacted with PLENA. Nine of the cDNAs are MADS-box genes, corresponding to squamosa (1), and two other genes from our collection of *Antirrhinum* MADS-box genes, defh72 (2) and defh84 (6). A two-hybrid experiment designed to test whether PLENA could form homodimers was negative confirming our failure to detect DNA-binding with PLENA alone in band shift experiments.

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These results suggest that PLENA forms a range of different heterodimers and that it will be possible to build up a picture of the network of possible interactions between the various MADS-box genes by applying this strategy. It is also expected that this approach will allow us to isolate any factors which might interact with the MADS-box protein dimers or heterodimers and thus modulate their activity.

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Instituto Juan March (Madrid)

CELLULAR DIFFERENTIATION DURING FLOWER DEVELOPMENT

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The development of flowers requires the initiation of floral and inflorescence meristems, the development of organ primordia and their determination, and the development of the specialised cell types that go together to make up the floral organs. Petals serve a general role to protect the floral organs carrying the developing gametophytes early in floral development, and later to attract pollinators for successful reproduction. Many plants have developed highly specialised cells within the petals to serve this function. These cells are epidermal and contain large amounts of pigment (anthocyanins) to attract pollinators. The cells facing the pollinators are also specialised in shape to enhance pigmentation and to provide recognition signals for pollinators.

Anthocyanin biosynthesis is regulated by transcription factors which determine where and how much pigment is made by controlling the production of the anthocyanin biosynthetic genes. In flowers this pathway is regulated in a complex fashion by several interacting transcription factors. Regulation involves interaction between heterologous transcription factors to activate some genes, and also interactions between very similar factors to give fine tuning to the pathway and its products.

Similar regulatory genes are also involved in controlling the development of specialised cell shape. However, the production of anthocyanin and the development of specialised cellular morphology can occur independently, indicating that these two aspects of cellular differentiation are under separate control within a single cell. At present we are trying to relate the control of these processes to the activity of genes involved in organ determination to link mechanistically the early and later processes of floral morphogenesis.

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The Control of Ovule Morphogenesis in Arabidopsis thaliana: Biting the Hand That Feeds You.

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Ovules are specialized reproductive organs that develop on the carpels of higher plants. Recessive mutations in the Arabidopsis *BELL1* (*BEL1*) gene result in female sterility due to defects in ovule morphogenesis (Robinson-Beers et al., 1992; Modrusan et al., 1994a,b; Ray et al., 1994). In Bel1 ovules, the inner integument fails to form, the outer integument develops abnormally, and the embryo sac arrests at a late stage of megagametogenesis. During later stages of ovule development, cells of the outer integument of a Bel1 ovule sometimes develop into a carpel-like structure with stigmatic papillae and second order ovules. The frequency of carpel-like structures is highest when plants are grown under conditions that favour the induction of flowering and was correlated with ectopic ovule-expression of *AGAMOUS* (*AG*), an organ-identity gene required for carpel formation. Transgenic plants where *AG* is constitutively expressed under the control of the CaMV 35S promoter develop Bel1-like ovules (Ray et al., 1994). Finally, mutations in *APETALA2* (*AP2*), another negative regulator of *AG*, result in the replacement of ovules with carpel-like structures (Modrusan et al., 1994a). Taken together these results suggest that at least one and perhaps the primary role of *BEL1* is to negatively regulate *AG*.

We have cloned the *BEL1* gene using two T-DNA tagged alleles *bel1-2* and *bel1-3* (Reiser et al., unpublished results). The identity of the cloned gene was confirmed by complementation of the Bel1-3 mutation and analysis of different *bel1* alleles. Northern and *in situ* analyses have indicated that *BEL1* encodes a 2.4 kb transcript that is present at high levels in ovules and at lower levels in other tissues including leaves. The putative *BEL1* gene product shares amino acid sequence identity with the DNA-binding homeodomain and is most closely related to that of the Knotted gene family. The BEL1 amino acid sequence also predicts formation of a coiled-coil suggesting that *BEL1* function requires protein-protein interactions. We are currently using the yeast 2-hybrid system to identify proteins that may interact with BEL1.

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Instituto Juan March (Madrid)

MOLECULAR CONTROL OF REPRODUCTIVE ORGAN DEVELOPMENT IN PETUNIA HYBRIDA

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Flowers represent important plant organs involved in the vital function of reproduction via the attraction of pollinators and the formation of the male and female reproductive cells. Furthermore flowers are highly appreciated by their ornamental value and in the Netherlands the production and trade of cutflowers and potplants considers an important economic activity.

Despite the importance of flowers only a limited amount of knowledge is present about the molecular control of floral induction and the development of the different floral organs and suborgans. At CPRO-DLO we use *Petunia hybrida* as a model species and recently we have isolated key regulatory genes determining the development of the floral organs. These genes, designated as *fbp* genes, encode proteins belonging to the MADS box transcription factor family. Genetic modification of the expression of these genes by co-suppression or overexpression resulted in the generation of transgenic petunia plants with altered floral organ formation. In this presentation the action of the *fbp1* gene regulating petal and anther formation will be presented. In situ hybridization, GUS experiments and immunolocalisation experiments indicate that this gene encodes a transcription factor involved in the determination of corolla and stamen primordia. Co-suppression of this gene results in genetically modified transgenic plants with petals replaced bij sepals and stamen by carpels. Furthermore a new type of MADS box transcription factor was identified: *fbp11/fbp7*. Experiments indicating that these MADS box genes control the formation and development of ovules will be shown. We propose an extended ABC model describing how these genes might act as D-type homeotic genes in the control of ovule determination.

The use of genes encoding transcription factors regulating flower morphogenesis or genes involved in fertility combined with the availability of transformation methods will enable a true molecular flower breeding resulting in improved ornamental value or better breeding performance for the next century.

Instituto Juan March (Madrid)

Oral poster presentation and discussion - I

Chairperson: Cathie Martin

Instituto Juan March (Madrid)

THE PROMOTER OF A PHENYLALANINE-AMMONIA LYASE GENE IS ACTIVATED BY A FLOWER-SPECIFIC MYB-LIKE PROTEIN

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Synthesis of flavonoid pigments in flowers is a readily visible and non-essential trait which has been exploited extensively in genetical studies in *Antirrhinum* and *Petunia*. Precursors for flavonoid synthesis in flowers are provided by the phenylpropanoid biosynthetic pathway, which is also required in other parts of the plants, such as vascular tissue (for the production of lignin) and in responses to environmental insults (pathogen attack, UV-light). So far, genetical studies on flower pigmentation have only identified regulatory genes which control flavonoid biosynthetic genes but none controlling phenylpropanoid synthesis (1,2). This may be due to the effects that misregulation of phenylpropanoid synthesis could have outside flowers, or because the complex regulation of phenylpropanoid biosynthetic genes could involve redundant regulators. Therefore, studies on the regulation of these genes have relied mostly on a biochemical approach, starting with the identification of important cis-elements in the promoters and proceeding with the isolation of proteins likely to mediate the function of these elements.
The bean gPAL2 promoter (encoding phenylalanine-ammonia lyase, the first enzyme in the phenylpropanoid pathway) directs expression of the GUS reporter in flowers, vascular tissue, trichomes and root tips of transgenic tobacco, revealing conservation of PAL gene regulation between distantly related dicotyledons (3,4). We show that a G-box element and three AC-rich elements are important for gPAL2 expression in tobacco flowers and bind tobacco nuclear proteins. bZIP proteins binding to the gPAL2 G-box were cloned from tobacco and, like the G-box-binding nuclear protein, are expressed in a variety of organs, including flowers. The AC-rich elements are bound by a flower-specific protein related to Myb305 from *Antirrhinum* (5). Myb305 activates expression from the gPAL2 AC-rich elements in yeast and in tobacco protoplasts. Overexpression of Myb305 in tobacco leaves, using a plant viral vector, activates gPAL2 expression ectopically.
Tissue-specific expression of eucaryotic promoters is achieved in many cases through co-operation between ubiquitous and tissue-specific transcription factors. The data available suggest that gPAL2 activation in flowers may be achieved by co-operation between widespread G-box-binding factors and a flower-specific Myb factor and that gPAL2 expression in flowers mirrors the distribution of the Myb factor.
The gPAL2 AC-rich elements are conserved in many promoters of genes in the phenylpropanoid and flavonoid pathways in several plant species and are important not only for expression in flowers but also in vascular tissue and in responses to UV-light and pathogens. This, combined with the fact that plants contain large families of Myb proteins with varied tissue distributions, suggests that Myb factors may also play a role in gPAL2 expression outside flowers.

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Instituto Juan March (Madrid)

Molecular analysis of the *Arabidopsis thaliana* MADS-domain proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS.

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According to genetic analyses, the homeotic genes *APETALA1, APETALA3, PISTILLATA* and *AGAMOUS* act in a combinatorial mode to specify the identity of the *Arabidopsis* flower organs. Their gene products form part of a large family of related putative transcription factors. Is the combinatorial mode of action of the AP1, AP3, PI and AG proteins achieved by direct interactions between them, with the corresponding formation of different homo- and heterocomplexes? Immunoprecipitation experiments using *in vitro* translated AP1, AP3, PI and AG proteins indicate that all of these proteins are capable of interacting with each other. However, DNA binding experiments using several CArG-motifs as target sites have shown that only AP1 and AG homocomplexes and AP3/PI heterocomplexes can bind to DNA. These results indicate that these proteins present "partner-specificity" for the formation of a productive complex. In addition, the DNA binding experiments revealed that such complexes are dimers and that AP1, AG and AP3/PI are all capable of binding the same target sites (although with subtle differences in affinities), raising the question of how is their biological specificity achieved. On the basis of sequence comparisons, two different domains can be defined in these proteins: the so-called MADS-domain (DNA binding domain) and K-domain (with a presumed coiled-coil structure). The use of various mutant forms of the proteins in immunoprecipitation and DNA binding experiments has allowed us to delineate a functional map. The MADS-domain appears to be subdivided into two regions: the N-terminal half is the DNA binding domain but is (at least partially) dispensable for dimer formation; the C-terminal half, together with the region in between the MADS- and K- domains (that we call the L region) is essential for productive dimer formation. Determinants for "partner-specificity" reside in this L region. The K-domain and the C-terminal region of the AG protein are not essential for complex formation and DNA binding, although they also affect these activities. All these *in vitro* results correlate with *in vivo* experiments (ectopic expression in *Arabidopsis* of chimeric proteins formed through domain-swapping) that show that the biological specificity of the MADS-domain proteins does not reside in the DNA binding domain and suggest that interactions with additional cofactors (as yet unidentified) could modulate their ability to regulate the transcription of downstream genes.

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CURLY LEAF*: a gene regulating leaf and flower development in *Arabidopsis thaliana

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Genetic analysis of homeotic mutations has suggested a model for floral development. Three homeotic functions, **a**, **b** and **c**, act combinatorially to specify organ identity in the four floral whorls. The **a** function specifies sepal and petal identity in the outer two whorls and is mutually antagonistic with the **c** function, which specifies stamen and carpel identity in the inner two whorls. In *Arabidopsis thaliana*, the *APETALA2* (*AP2*) gene is required for the **a** function and the *AGAMOUS* (*AG*) gene for the **c** function. We have identified a novel gene, *CURLY LEAF* (*CLF*) which is also involved in the **a** function.

Plants carrying the recessive *clf*-2 mutation are early flowering and have leaves which curl inwards along the leaf margin. The flowers have small petals and occasionally display homeotic transformations of sepals into carpels and of petals into stamens. A similar phenotype has been observed in transgenic plants in which the *AG* gene is ectopically expressed. Consistent with this, Northern analysis indicates that *AG* is expressed in leaves of *clf*-2 mutants but not in those of wild type plants. In addition, genetic analysis shows that *ag* mutations are epistatic to *clf*-2, so that *ag clf*-2 double mutants have normal leaves, sepals and petals. Together, these results indicate that the *CLF* gene is not directly required to specify sepal and petal identity but is necessary for normal regulation of *AG* expression. The weak *ap*2-1 allele acts as a semi dominant enhancer of *clf*-2 in the flowers, suggesting that the *AP2* and *CLF* genes may act together to repress *AG* in sepals and petals. We are using in situ hybridization to define *AG* expression patterns in *clf*-2 flowers and in double mutants of *clf*-2 with floral homeotic genes regulating *AG*.

The *clf*-2 allele was obtained from a transposon mutagenesis experiment using transgenic lines that carried derivatives of the maize *Ac/Ds* transposons. Two observations indicate that this allele is transposon tagged: first, the *clf*-2 allele cosegregates with a single *Ds* transposon copy; second, excision of this transposon correlates with restoration of the CLF+ phenotype. We cloned the genomic region flanking the *Ds* insertion at *clf*-2 and are currently attempting to identify the *CLF* transcript. In addition, we are characterizing various novel *clf* alleles that were derived from *clf*-2 by imprecise transposon excision. The molecular cloning and characterization of *CLF* will help understand its interaction with other **a** function genes that regulate *AG*.

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Homeotic mutants in *Pisum sativum*

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The aim of our work is the study of a number of homeotic mutants of *Pisum sativum*. As a first approach, we are carrying out the phenotypic characterization of these mutations by scanning electron microscopy. ***deficiens-like*** presents a complete transformation of petals into sepals and stamens into carpels, being such phenotype similar to those found in other plants (i.e. *deficiens* or *globosa* in *A. majus*; *pistillata* or *apetala3*. in *Arabidopsis*); in ***stamina pistillodea*** flowers only two of the stamens are converted in carpels, and it is also detectable the presence of strips of sepalloid tissue in some petals; ***petalosus*** shows phenotypic variability strikingly dependent on the environmental and physiological conditions, from some extra number of petals or partial transformations of stamens into petals, to a complete homeotic transformation of the third whorl into petals and the full development of a new flower inside the carpel; flowers in ***large bractea*** mutants develop an extra bractea, and the sepals display leaf-like morphology, as well as partial fusions with petalloid tissue, and some petal-like expansions that arise from the anthers. We are also performing genetic studies with double mutants.

In order to correlate these mutations with some specific genes, we have focused on the isolation and characterization of the MADS box gene family of pea. We have performed a screening of a cDNA library using as heterologous probe the MADS box coding region of DefA from *A. majus*, and we are currently working on the study at the molecular level of four different clones, namely pM1, pM2, pM3 and pM4.

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Oral poster presentation and discussion - II

Chairperson: José Pío Beltrán

Instituto Juan March (Madrid)

A Role For MADS Box Genes In Controlling Flower Development In The Dioecious Species *Rumex acetosa* ?

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In the vast majority of plant species the male and female reproductive organs develop in the same hermaphrodite flower. A relatively small number of plant species exhibit monoecy, where separate male (staminate) and female (pistillate) flowers occur on the same individual (e.g.maize) or dioecy, where the male and female flowers are borne on separate individuals (male and female plants). Although dioecy may also be accompanied by a sex chromosome system in some plant species, for example, hop (*Humulus*) species, *Rumex* species and date palm (*Phoenix dactylifera*), this is the exception rather than the rule. As in animal systems, there are two types of sex determining system in plants with sex chromosomes: in the active-Y system, found in *Melandrium*, the Y acts as a maleness enhancer as well as suppressing the gynoecium. The alternative, X/autosome dosage system has been found in *Rumex* (Polygonaceae) and *Humulus* (Cannabidaceae). The *Rumex* system is by far the best characterised sex system in plants. *Rumex acetosa* has an aneuploid sex system with $2n=12+XX$ in females and $2n=12+XY_1Y_2$ in males.

In *R. acetosa*, the male flowers consist of a whorl of six stamens and two whorls of perianth segments. The female flower consists of three fused carpels with one ovule and two whorls of perianth segments, one of the whorls later encircling the fruit. SEM studies show that, during early development, both male and female flowers appear identical, with an initial lobe, and a second lobe which forms the leading sepal. In the male flower primordium, which develops the six stamens, the central dome may indicate incomplete suppression of the carpel; in the female flower primordium, the central carpels develop, with a complete absence of male organs. A possible mechanism for the differentiation of the male and female flowers involves the differential expression of homeotic genes. A switch between the carpel whorl and the stamen whorls is all that is required for the homeotic-like interconversion of male and female flowers. Although the male and female *Rumex* flowers differ from homeotic mutants in the respect that only one organ whorl is effectively transformed rather than a pair of adjacent whorls, we can propose that a *deficiens* -like gene product is responsible for the development of the androecium in males and hermaphrodites. This would require that either the gene is not active in females or that if active in both males and females, then it is somehow unable to induce stamen development in the female. Similar models can be proposed for the development of the female flower.

The initial research aim is to isolate genes which are known to be involved in the initiation of floral organ development and, initially, to focus on the MADS box genes and their expression patterns. Developing bud RNA from male and female flowers has been used in the construction of cDNA libraries which have been screened with probes for floral homeotic genes from *Antirrhinum*. The resultant clones were assessed by Northern analysis as to whether they represent flower specific genes. Seven different full-length *Rumex* MADS box clones for genes expressed in flowers, have been sequenced. Multiple alignment of the deduced amino acid sequences with MADS box protein sequences from other plants suggests that we have isolated the *Rumex* homologues of *plena/agamous, deficiens/apetala3 and squamosa* in addition to several previously uncharacterised genes. These genes are being used in *in situ* hybridisation to male and female developing bud sections to evaluate their patterns of expression. These data will be presented.

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ISOLATION AND CHARACTERIZATION OF cDNA CLONES FOR GENES PREFERENTIALLY EXPRESSED IN REPRODUCTIVE MERISTEMS OF CAULIFLOWER *Brassica oleracea* (*var. botrytis*)

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In order to better understand the mechanisms underlying the process of floral transition at the apical meristem· we have undertaken the isolation of cDNA clones corresponding to genes showing a preferential expression in reproductive meristems. With this purpose, we followed a subtractive hybridization approach employing a PCR-based method, where cauliflower heads and Brussels sprouts, two commercial varieties of *Brassica oleracea* (var. *botrytis* and var. *gemmifera*, respectively), were used as sources of reproductive and vegetative meristems. After six subtraction rounds, 35 cDNA clones were selected as corresponding to possible cauliflower meristem genes. A more detailed characterization of these clones, including Northern and Southern hybridization and nucleotide sequence analysis, allowed to confirm which of them corresponded to genes preferentially expressed in the cauliflower meristem, and to determine which were derived from the same genes. The results obtained indicate that 24 clones, corresponding to 4 different genes, hybridized to RNA from cauliflower meristems and not to RNA from Brussels sprouts meristems. *In situ* hybridization experiments are currently in progress to corroborate these results.

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MADS-box genes involved in reproductive organ development.

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Homeotic genes directing floral organ development in *Antirrhinum majus* are expressed in overlapping domains consisting of two floral whorls. Floral organ identity is proposed to be specified by the unique combination of expression of the organ identity genes. In *A. majus* three organ identity genes have been isolated: *Deficiens* and *Globosa*, which are expressed in whorls 2 and 3 (petals and stamens), and *Plena*, which is expressed in whorls 3 and 4 (stamens and carpels). All three genes contain a MADS-box domain, which is a DNA-binding domain conserved amongst the members of the MADS-box class of transcription factors. Screening an *A. majus* floral specific cDNA library with a probe consisting of the *Deficiens* MADS-box domain resulted in the isolation of a cDNA clone, *DefH1*. The expression of the *DefH1* gene is limited to the reproductive floral whorls: stamens and carpels, and so might be an organ identity gene of the same class as *Plena*. Screening a *Nicotiana tobaccum* floral specific cDNA library with probes for both the *Antirrhinum Plena* and *DefH1* genes resulted in the isolation of the putative tobacco homologues for the two genes. Both genes were expressed in transgenic tobacco in both sense and antisense orientations under the control of the 35S-promoter. The phenotypes resulting from both ectopic expression and inhibition of expression reveal that both genes have a role in specifying reproductive organ development in tobacco. Ectopic expression of both genes caused coversion of the sepals into carpelloid organs, and petals towards stamenoid organs, whereas the 3rd whorl stamens and 4th whorl carpels developed normally. Progeny of the cross between the two parental lines expressing both the tobacco *Plena* and *DefH1* genes did not have a more severe phenotype. Antisense inhibition of expression resulted in the conversion of the 3rd whorl stamens into petalloid organs and a distortion of the 4th whorl carpels towards sepalloid/petalloid organs. Analysis of the effect of the transgenes on the endogenous MADS-box gene expression has provided information on the regulatory relationships between the MADS-box genes.

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THE ARABIDOPSIS HOMEOTIC GENE *AG* REGULATES FLORAL INDUCTION AND SEED DEVELOPMENT

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Genetic studies indicate that the Arabidopsis floral homeotic gene *AG* controls the identity of reproductive organs and the determinacy of the floral meristem. We have generated transgenic plants carrying an 35S-*AG* fusion in the sense orientation, and have previously reported the floral organ identity conversions observed in these plants. After further analysis of these transgenic plants, we have recently learned that the alteration of *AG* expression also affects the timing of flowering. We have analyzed flowering time of the transgenic plants, and *ag* mutant plants, under both long day and short day conditions. Our results strongly suggest that *AG* plays a role in regulating floral induction. We are currently further characterizing this newly discovered aspect of *AG* function using genetic and molecular approaches, to uncover possible interactions of *AG* with other genes controlling floral induction. In addition to *AG*'s function in floral induction, in floral meristem determinacy and organ identity, we found that *AG* is also involved in regulating the development of ovules and seeds. We observed that the *AG* transgenic plants behave in a way similar to *ap2* mutants. Both exhibit abnormal ovule development, abnormal seed development, and vivipary. We have performed a number of experiments to characterize the ovule and seed development in these plants. Our studies on *AG* function have revealed that *AG* plays several roles during flower development. In addition to its function in floral meristem determinacy and reproductive organ identity, as suggested by the mutant phenotypes, we have found that *AG* can also affect floral induction, and it regulates ovule and seed development. These and possible additional results will be presented.

Instituto Juan March (Madrid)

4th Session:

Transcription factors in the control of differentiation (Continuation)

Chairperson: Zsuzsanna Schwarz-Sommer

Instituto Juan March (Madrid)

Flower Development in *Antirrhinum majus*

Rosemary Carpenter, Da Luo, Desmond Bradley, Pilar Cubas, Mark Chadwick, Lucy Copsey, Coral Vincent and Enrico Coen.

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Wild type *Antirrhinum majus* plants consist of a vegetative region which develops an indeterminate inflorescence consisting of large asymmetrical (zygomorphic) flowers exhibiting a single plane of symmetry. However a number of mutations exist in which the symmetry of the flowers have been altered to radially symmetrical (actinomorphic). We have been studying several of these mutations and have isolated two of the key genes involved:- *cycloidea* (*cyc*) and *dichotoma* (*dich*). Extensive genetic and molecular analysis is underway to characterise these genes, study their expression patterns and their combinatorial effects, in an attempt to understand some of the interactions involved in the control of flower symmetry. In addition these genes are being studied in other species which have both symmetrical and asymmetrical flowers.

The *centroradialis* (*cen*) mutation deviates from wild type in two aspects:- the inflorescence becomes determinate and is "crowned" by a flower with altered symmetry. This is usually radially symmetrical, similar to that seen in the *cyc* series of mutations. This mutation is also known in other species , notably that of terminal flower in *Arabdopsis* and also in *Digitalis* (foxglove). The *cen* gene has recently been isolated from *Antirrhinum* and is being analysed both molecularly and genetically in parallel with the *cyc* and *dich* genes.

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Genetic control of flower shape in *Antirrhinum majus.*

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The shape of the *Antirrhinum majus* flower can be described in terms of differences between regional phenotypes within whorls along a dorso-ventral axis. For example, from the most dorsal to the most ventral positions, the flower corolla can be divided in 5 regions with the phenotypes D, DL, L, LV and V. To investigate how these differences are genetically determined we have analysed 3 mutations that share the common property of causing some regions defined by their positions along the dorso-ventral axis to develop the phenotypes proper to other regions in wild-type. These mutations fall in 2 classes. The first class includes 2 recessive mutations with ventralizing effects (*cycloidea* and *dichotoma*) which confer either a DL, LV, V, V, V phenotype (*cyc*) or a DL, DL, L, LV, V phenotype (*dich*). Being recessive with ventralizing effects, these mutations indicate that the wild-type *cyc* and *dich* alleles control dorsal functions.

The second class includes *divaricata*, a semi-dominant mutation with dorsalizing effect conferring the phenotype D, DL, L, LV, LV. By analysing the effect of duplications of the *div* locus we show that the phenotype of the ventral region is sensitive to the dosage of the wild-type Div^+ allele and insensitive to the dosage of the mutant allele. Hence, the *div* phenotype results from loss of a ventral function rather than gain of a lateral or dorsal function.

To investigate how the functions defined by these mutations might interact, we have constructed all possible combinations of the 3 mutants. Based on the analysis of regional phenotypes conferred by the various genotypes we propose a model for how *cyc, dich* and *div* may control flower shape.

Instituto Juan March (Madrid)

THE *K*-LOCUS OF BARLEY: A CASE OF EPIPHYLLOUS FLOWER FORMATION

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In plants, the direction of growth is reflected in several attributes like plane of cell division, orientation of hairs, symmetry of cells and tissues. The existence in barley of Hooded and related mutants provides a basis to study the problem of polarity at the molecular level. The homeotic Hooded mutation (*Hordeum vulgare* var. *trifurcatum*) causes the appearance of an extra flower[1] which is arranged in inverse polarity as compared to the primary floret. This extra flower is formed on the lemma, one of the protective bracts of the barley floret[2]. The foliar nature of the lemma is obvious in the barley mutant leafy lemma (*lel*)[3] in which the lemma is converted to a leaf with sheath and blade separated by a ligule. In awned barley, the lemma proper would correspond to the leaf sheath, while part of the leaf blade is modified to form the awn[4]. The mutant phenotype is governed by the genetic locus *K* mapping on the short arm of chromosome 4[5]. The mutant allele is dominant over the awned *K* wildtype allele, and the development of the extra floret largely suppresses the formation of the awn, the distal appendage of the wildtype lemma[6]. We have shown[7] that the homeobox gene *Knox3* corresponds to the genetic locus *K*. Ectopic expression of this gene is caused by a 305 bp duplication in *Knox3* intron 4, and phenocopies of the mutation have been obtained in the heterologous tobacco system by *Knox3* overexpression. The implications of our findings are discussed in relation to polarity of growth and epiphylly in flower formation.

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Maize, MADS-boxes, and Mutants; What's Happening in a Grass Flower?

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We are interested in elucidating the underlying mechanisms controlling floral organ specification and development in *Zea mays*. A number of floral regulatory genes have been identified in *Arabidopsis* and *Antirrhinum* that encode proteins containing a highly conserved DNA binding domain known as the MADS domain (reviewed in Davies and Schwarz-Sommer 1994; Weigel and Meyerowitz 1994). As a first step towards this goal we have identified homologues in maize of cloned floral homeotic genes from *Arabidopsis* that contain this conserved domain. Specifically we have isolated the putative *Zea mays* homologues (*ZAG1* and *ZAP1*) of the *Arabidopsis* floral homeotic genes *AGAMOUS* and *APETALA1* as well as a number of related genes that show preferential or specific expression in flowers (Schmidt et al. 1993; Veit et al. 1993). The *ZAG1* and *ZAP1* sequences are, respectively, 65% and 69% identical to their Arabidopsis homologues. Consistent with their *Arabidopsis* counterparts, RNA blotting and in situ hybridization analyses show *ZAG1* expression to be restricted to the reproductive organs while *ZAP1* expression appears restricted to the nonreproductive portions of the flower. Transgenic *Arabidopsis* plants ectopically expressing *ZAG1* produce abnormal flowers that resemble *ap2* mutant flowers. This phenotype is consistent with *ZAG1* being the functional homologue of *AG*. Using low stringency hybridization with *ZAG1* and *ZAP1* on genomic southerns indicates that maize contains a large number of related genes. Following the segregation of these genes among recombinant inbreds has allowed us to place 38 members of this gene family onto the RFLP map. Members of this putative MADS-box gene family are scattered throughout the maize genome. RFLP mapping has established close linkage for a few of these genes with candidate mutant loci affecting floral development. Progress towards using a PCR-based approach to identify transposon-induced mutant alleles of maize MADS-box genes will be discussed.

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POSTERS

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THE ROLE OF PROTEIN DEGRADATION IN NUCLEAR DIVISION

Haya Friedman and Michael Snyder

Accurate chromosomes separation during mitosis is dependent on proper function of the spindle apparatus, as well as on the temporal regulation of events prior to and during mitosis. Dynamic changes in protein levels occur during nuclear division and the selective removal of either regulatory or structural proteins is essential for this process. Despite the importance of specific proteolysis in these events, our knowledge of the mechanism of specific protein degradation is very limited.

In the course of searching for chromosome disjunction defective mutants, we have isolated a yeast gene called *PRG1* (for Proteasome Related Gene), which is closely related to *RING10,* a human proteasome gene implicated in antigen processing (1). Proteasome are multicatalytic multiprotein complexes involved in noncompartemental protein degradation including proteolysis of ubiquitin-tagged proteins. *PRG1* is essential for cell growth which indicate that it fullfills a unique function. We created four temperature-sensitive allels of *PRG1* and the analysis of the mutant strains showed that PRG1 function is neccesary for proper nuclear division. (2).

Currently we are searching for potential proteasome substrates. This is done by screening a *LAC Z* fusion library and looking for proteins that are stabilized in $prg1^{ts}$ mutant strain. It is expected that these studies will identify proteasomes substrates and therefore, elucidated the role of selective protein degradation in nuclear division.

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CHARACTERIZATION OF THE *Deficiens* GENE (*St-Def*) IN *Solanum tuberosum*.

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The homeotic gene *Deficiens* (described for the first time in *Antirrhinum majus*) controls flower organ identity determining proper development of petals and stamens (Schwarz-Sommer et al., 1990). According to the ABC model (Bowman et al., 1991; Coen and Meyerowitz, 1991; Meyerowitz et al., 1991) this is a B class gene and its loss of function causes the transformation of second whorl petals into sepals, and of third whorl stamens into carpels.

We have isolated the homologous gene to *Deficiens* in *S. tuberosum* (*St-Deficiens* or *St-Def*)(García-Maroto et al. 1993). ST-DEF protein is 79% identical to that from *A. majus*. Gene structure (intron-exon distribution) is also very similar, with 6 introns interrupting the coding sequence.

The promoter region of *St-Def* is been studied in detail. We have performed GUS fusions with a 1,2 Kb fragment upstream the ATG and GUS activity was detected in petals and stamens (mainly associated to the vascular bundles) but it is also found at a high level in the ovary wall.

Main transcriptional start has been mapped by primer extension at position -93 relative to the ATG although minor initiations are also detected upstream this point. At the moment we are studying 5'-RACE products to get additional information.

A search for similarity between the *St-Def* promoter and other *Deficiens* promoters revealed an 81 bp sequence (between -50 and -131 in *St-Def*) wich is 73% identical to an equivalent region in the *A. majus Deficiens* promoter but located in this case around 1 Kb upstream the ATG. It has been described that this region contains the mutation site in the *Chlorantha* allele that inactivates this gene as well as an SRE motif with a possible autorregulatory function (Schwarz-Sommer et al. 1992). We are performing directed mutagenesis of this DRR (Def Regulatory Region) in order to verify the importance of this element.

We have isolated three different *St-Def* genes: pD10, pD12 and pD13, present in a tetraploid potato cultivar (cv. granola) of potato. Coding sequences are almost identical for the tree genes. Two of them, pD10 and pD13, also share very similar 5'-non coding regions, suggesting that they migth represent different alleles, while for pD12 homology keeps only till position -340 and no homology is found upstream this point. Promoters for the three genes seem to be active and keep the same expression pattern as revealed by GUS fusion experiments. However genes pD10/13 and pD12 does not seem to be allelic as suggested by the fact that the two kind of genes are present in four different diploid potato lines. This raises the possibility that these two genes might play different roles by having a distinct activation program.

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Poster Abstract: Development of chimeric shoots during the transition-to-flowering in *Arabidopsis*. Frederick D. Hempel, Department of Plant Biology, University of California, Berkeley, CA 94720-3102

The induction of flowering in *Arabidopsis* can occur within one leaf plastochron. During that time, the shoot apical meristem ceases producing leaves and starts producing flowers.

Within populations of wild-type plants, some individuals will produce a single chimeric flower-paraclade shoot at the lower boundary of flowering on the main axis. The frequencies of chimeric flower-paraclade shoots and the phenotypes of flower-paraclades vary between ecotypes and with changes in induction treatments.

We have data which indicates that chimeric shoots are derived from young leaf anlagan/primordia present during photoperiodic induction. These primordia are secondarily "floralized."

The chimeric flower-paraclade shoots have a rough bilateral symmetry; the abaxial side is flower-like. This symmetry suggests that the abaxial region of a developing leaf primordium may be affected *directly* by the influx of floral stimulus during the induction of flowering.

The production of chimeric flower-paraclade shoots indicates that at least some *Arabidopsis* primordia are developmentally plastic (not strictly determined at inception).

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Expression of a MADS-box gene (*rcd1*) from Gerbera hybrida (Compositae) is spatially correlated with translation of flavonoid biosynthetic and putative cell-wall protein genes during floral organ differentiation

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We are interested in analyzing floral organ differentiation in Compositae through studies of gene expression in gerbera. We have approached this on one hand by studying flavonoid pathway genetics and on the other by cloning and characterizing genes abundant during corolla development.

Using differential screening experiments we isolated a cDNA, rcd1, which has high similarity with MADS-box genes of other plants, being closest to *fbp2* of petunia. We have studied rcd1 expression, focusing particularly on the differentiating floral organs. Rcd1 is specific for floral organs. Its expression is first detected in what appear to be the sepalar ring primordia of early flower buds. In differentiating flowers *rcd1* is expressed in all four whorls. First it can be detected uniformly and later it localizes to epidermal and vascular tissues (of corolla) and to epidermal and transmitting tissues (of the style).

During corolla development the late pattern of rcd1 expression correlates spatially with the expression of both early and late flavonoid biosynthetic genes (*pal, chs, dfr*) as well some putative cell-wall protein genes, *gtt5* (PRP-like) and *gty2* (with a cysteine rich domain), suggesting the involvement of *rcd1* in their genetic regulation.

In future work we will study the putative regulatory role of rcd1 and its linkage to marker genes by taking advantage of the transformation technology we have developed for gerbera. For example, transgenics with constitutive 35S-antisense-*rcd1* constructs should block both the floral organ determination and differentiation related rcd1 expression. We have recently isolated promoter region of *gltp1* (codes for lipid transfer protein) and based on the northern blot experiments, the promoter should be active only during the differentiation period. Thus the *gltp1* promoter-antisense-*rcd1* construct should block *rcd1* expression related only to floral organ differentation.

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Isolation of the *AP1/SQUA* homologous genes (*SQE1* and *SQE2*) from *Eucalyptus*.

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Eucalyptus has a very different flower structure to Arabidopsis and Antirrhinum. In order to understand the molecular basis of flower development we have cloned several floral genes from Eucalyptus. The *SQUAMOSA (SQUA)* and *APETALA1 (AP1)* genes are required for correct development of sepals and petals in Arabidopsis and Antirrhinum. In Eucalyptus sepals and petals are fused and form a cap-like structure called operculum. Here we describe the isolation and characterisation of *AP1/SQUA* homologous genes from Eucalyptus.

A PCR-based strategy was taken to clone the gene from Eucalyptus. Primers to the MADS-box were used to amplify DNA from *Eucalyptus globulus*. By screening a young flower bud cDNA library with PCR amplified MADS-box fragment as a probe, we obtained two cDNAs (*SQE1* and *SQE2*). Both are single copy genes in Eucalyptus. *SQE1* contains an open reading frame encoding a 245 amino acid polypeptide. *SQE2* produces two different length mRNAs by alternative splicing of the last intron which encode 205 and 243 amino acid polypeptides. The deduced amino acid sequences of SQE1 and SQE2 have high amino acid identity to each other and to the SQUA and AP1 proteins. All the deduced amino acid sequences contain highly conserved MADS-box and K-box domains.

Northern blot analysis showed that *SQE1* is expressed at all stages of flower development. *SQE2* is expressed only in young flower buds. Neither *SQE1* nor *SQE2* is expressed at a significant level in leaves, stems or roots. More detailed expression patterns of the two genes will be determined by *in situ* hybridisation.

To examine the function of the two genes in flower development, *SQE1, SQE2* and *AP1* cDNAs were ectopically expressed in Arabidopsis plants under the control of a constitutive promoter (CaMV35S). Ectopic expression of AP1 caused a reduction of plant height and a homeotic change from inflorescence meristem to flower. These plants resembled plants homozygous for the previously described Arabidopsis mutation terminal flower (*tfl*). A similar *tfl*-like phenotype was also observed in Arabidopsis plants carring 35S driven SQE1 and SQE2. These results suggest 1) Over expression of *AP1* can produce a *tfl* phenotype, 2) Eucalyptus coding sequences are functional in Arabidopsis, and 3) SQE1 and SQE2 have AP1-like functions in flower development.

These results together with the similarity in DNA sequences strongly suggest that we have cloned *AP1/SQUA* homologous genes from Eucalyptus.

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Bracteomania as a Tool to Study Early Flower Development in *Antirrhinum majus.*

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In *Antirrhinum* two classes of floral homeotic genes have been recognized: meristem identity and organ identity genes. Organ identity genes can be classified into three classes, corresponding to three pathways (1). As only gain of function and no loss of function mutations are known in *Antirrhinum* which affect the identity of whorl 1 and 2, the pathway that determines the identity of whorl 1 and 2 was defined as the ground state or basic pathway. With respect to the basic pathway and the interaction between the meristem identity genes and the organ identity genes not much is known yet.
Upon mutation, the meristem identity genes *SQUAMOSA* (*SQUA*, (2)), *FLORICAULA* (*FLO*, (3)) and *SQUAMATA* (*SQUAM*, (4)) show a bracteomaniacal phenotype: instead of flowers they develop secondary shoots with bracteose leaves. We investigated the action of *SQUA*, *FLO* and *SQUAM* by growing several wt and mutant lines under different conditions and by measuring several physiological parameters. Inflorescences were studied by SEM and Northern analysis, using *FLO*, all available MADS-box and *SBP* (J. Klein et al., in prep.) genes as probes. Double mutants were constructed to study the interaction between *SQUA*, *FLO* and *SQUAM*, and between these genes and PLENA (PLE, (5)) and CENTRORADIALIS (CEN, (4)). For all the three bracteomaniacal mutants allelic differences were found, which partly could be explained by the different genetic backgrounds. The dependance of the phenotype of the *squa* and *squam* shoots on the environmental conditions and developmental age demonstrates the presence of a gradient of flower inducing activity.
Our analysis allows the interpretation of the switch from vegetative to generative growth as an imposition of the floral identity upon the vegetative identity, without an intermediate inflorescence program. We concluded that *SQUA*, *FLO* and *SQUAM* can be classified as both meristem identity and ground state pathway genes.

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ANALYSIS OF THE FUNCTIONAL HOMOLOGY OF THE MAIZE *ZAG1* GENE AND THE ARABIDOPSIS FLORAL IDENTITY GENE *AGAMOUS*.

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The maize *ZAG1* gene has been isolated by sequence homology with the *AGAMOUS* gene that is required for the determination of stamen and carpel identity in Arabidopsis (Yanofsky et al.,1990). Several observations have been presented that suggest that *ZAG1* represents the maize homolog of *AG* (Schmidt et al., 1993). The amino acid sequence encoded by *ZAG1* is highly similar to that of *AG* both within and outside the well-conserved MADS motif. The ZAG1 protein produced in vitro binds to putative AG binding sites. Similar to *AG, ZAG1* expression is restricted to developing stamen and carpel primordia suggesting that, like *AG*, *ZAG1* plays a role in specifying the development of reproductive organs within the flower.

We have investigated the possible functional homology of these two genes by phenotypic and molecular analyses of transgenic Arabidopsis plants in which *ZAG1* is expressed constitutively under the control of the cauliflower mosaic virus 35S promoter. If *ZAG1* like *AG* is able to suppress the activity of the A function gene AP2, its ectopic expression is predicted to deviate the normal floral morphology toward an *ap2*-like phenotype (Bowman et al.,1991; Mandel et al., 1992; Mizukami and Ma, 1992). Most of the transformants carrying the 35S-*ZAG1* fusion did indeed produce abnormal flowers that resemble *ap2* mutant flowers. In the most severe cases, the first whorl organs develop as modified sepals that exhibit carpelloid characteristics such as the development of stigmatic tissue at the tips and the lack of trichomes. The second whorl organs differentiate as staminoid petals with their basal portion shaped like stamen filaments and a blade that is shorter and narrower than in wild-type petals and that in most cases has yellow patches. RNA blot hybridization analysis indicates that the level of *ZAG1* expression in flowers correlates with the severity of the *ap2*-like phenotype presented by independent transgenic lines. By introducing the *ZAG1* transgene into a mutant *ag* background we are also examining if *ZAG1* can fully or partially direct the specification of stamens and carpel and the determinacy of the floral meristem and, therefore, to rescue the *ag* mutant phenotype. Two additional functions of *AG*, the negative regulation of *AP1* and the positive regulation of *AGL5* expression, are being also tested for *ZAG1*.

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THE ROLE OF *MYB* GENES FROM *Antirrhinum majus* IN THE REGULATION OF FLAVONOID BIOSYNTHESIS

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In *Antirrhinum majus* a series of *myb*-related transcription factors expressed in flowers have been identified (Jackson et al., 1991, Plant Cell; Noda et al. 1994, Nature). We have shown that one, a flower-specific Myb protein (*myb*-305), activates transcription of phenylalanine ammonia lyase (PAL), the first step in phenylpropanoid biosynthesis and also binds to the promoter of the *Antirrhinum majus* chalcone synthase (CHS) gene (Sablowski et al, 1994, EMBO J.). The binding site (H-box-like) is an element conserved in the promoters of others genes involved in flavonoid pigment synthesis from Antirrhinum. The regulation of flavonoid biosynthetic genes in *Antirrhinum* appears to be complex (Martin et al., 1991, Plant J., 1:37-49). To test if *myb*-305 and *myb*-340, two flower-specific transcription factors, activate transcription of these biosynthetic genes we have used an *in vivo* yeast system. It consists of two plasmids, an integrative plasmid pMH1, which contains a truncated *cyc-1* promoter fused to the *LacZ* reporter gene and an effector plasmid (pYES2) with contains the *Gal1* promoter for expression of the transcription factor gene. We have cloned about 200 bp from the promoters of the biosynthetic genes: CHS, CHI, F3H, DFR and *candi* in pMH1 and the coding sequence of *myb*-305 and *myb*-340 in pYES2. The transcriptional activation by these Myb proteins to these promoters in yeast was studied by measuring the β-galactosidase activity under induction conditions of the transcription factor. Both Myb proteins activated transcription from PAL, CHI and F3H promoter but not CHS, DFR and *candi*. *Myb*-340 activation was always stronger than *myb*-305 activation. However, by EMSA studies, we have shown that they bound to all these promoters, being the binding activity by *myb*-305 stronger than that by *myb*-340. Their temporal expression during floral development is very similar and to determine whether their spatial expression in flowers is also similar, some work by *in situ* hibridization is in progress. These results suggest these two myb transcription factors could be competing in the regulation of these promoters. To test that we are co-expressing both proteins in the yeast system which will determine their function in the control of flavonoid biosynthesis. We are also studing some tobacco transgenic plants over-expressing Myb 305 protein.

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REDOX MODULATION OF THE EXPRESSION OF BACTERIAL GENES ENCODING CYSTEINE-RICH PROTEINS IN PLANT PROTOPLASTS

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Activity of neomycin phosphotransferase II (NPTII; gene *neo*; five cysteines) in tobacco protoplasts transfected with fusions of the octopine TR2' or cauliflower mosaic virus 35S promoter and the *neo* gene, with or without a signal peptide, increased up to 8-fold in response to externally added dithiothreitol (DTT) at concentrations that did not affect protoplast viability (up to 2.5 mM). Activity of phosphinothricin acetyl transferase (PAT; gene *bar*; one cysteine) expressed under the control of TR1' or 35S promoter was not similarly affected, thus excluding a redox modulation of transcription as the mechanism of NPTII activation by DTT. Western blot analyses showed an increase in the amount of protein in response to DTT, whereas the steady-state level of NPTII mRNA nor the specefic activity of the enzyme was affected. The same type of modulation was observed for transiently expressed B-glucuronidase (nine cysteines) produced from a fusion with the 35S promoter, with or without a signal peptide. Limitation of cotranslational and/or early posttranslational steps by excessively oxidizing sulfhydryl/disulfide redox potentials is postulated to explain the low net accumulation of cysteine rich proteins of bacterial origin (i.e., NPTII and B-glucuronidase) when expressed in plant protoplasts, and the marked increase in such proteins in response to externally added DTT.

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Rhythmic changes in expression of specific mRNA species during flower induction

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Early events associated with flower induction were examined in the sensitive leaves of two classic photoperiodic model plants, *Pharbitis nil*, and *Xanthium strumarium*. cDNA libraries prepared from mRNA isolated from induced and non-induced leaves were differentially screened to select clones whose expression increased or decreased during induction. The hybridization of two induction-specific *Pharbitis* cDNA probes to *Xanthium* RNA was studied using Northern analysis. One of the probes showed a low degree of hybridization to a *Xanthium* species. Transcript abundance was measured by Northern analysis of RNA isolated from leaves and other tissues throughout the inductive cycle. Abundance of some clones changed in a cyclical pattern which was changed by photoperiodic manipulations known to change the response to the inductive signal.

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The identification of genes involved in anther histodifferentiation and anther-specific gene regulation

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Floral development is firmly established as a key model system for the study of morphogenesis in higher plants. Genetic and molecular genetic studies have provided an insight into the regulatory events governing the establishment of the floral apex and the position and identity of the floral organs. Similarly, concerted efforts to clone and characterise genes expressed within one of these organs, the stamen, have shown that anther differentiation and function require the highly regulated expression of numerous organ specific structural genes. Consequently, with respect to the anther we have the beginning and end of a molecular genetic account of organ differentiation. However, we remain profoundly ignorant of the regulatory events and processes that lie between the floral homeotic genes and the anther-specific structural genes and which must form the bulk of this story. For example, the target genes for the floral homeotic gene *AGAMOUS* have not been found, nor the transcriptional activators of any anther-specific genes. For this reason, we have been attempting to identify genes in *Arabidopsis thaliana* that are required for the activity of a set of tapetum-specific structural genes.
Our approach involves the mutagenesis of *Arabidopsis* lines carrying the promoter of the target gene coupled to the coding regions of various reporter genes. The target genes (A6, A9) were isolated from *Arabidopsis thaliana* and display an extraordinary level of developmental regulation. Promoter fusions to the ribonuclease barnase showed that both are tapetum-specific. The gene A6 is first expressed in microspore-release stage anthers and probably encodes a glucanase involved in this process. The gene A9 is first expressed in premeiotic stage anthers and represents the earliest tapetum-specific gene documented to date.
Genes encoding components within the transduction pathway of the target gene are then identified by screening for M_2 individuals which fail to express the reporter gene. Our expectation was that we would identify several classes of genes including genes involved in the **histodifferentiation** of the anther (eg affected in archesporial cell or tapetal cell differentiation) as well as the direct transcriptional activators of the target genes. In the first of such screens, involving A6-gus and A9-gus lines mutagenised by gamma irradiation, **4** A6 and **8** A9 gus-negative and **6** A6 and **17** A9 gus-positive mutants were identified. **1** of the A6 gus-negative mutants was fertile and **3** were sterile; **5** of the A9 gus-negative mutants were fertile and **3** were sterile.
The recovery of gus-negative mutants is highly significant since preliminary experiments in which the reporter genes were introduced in 3 male-sterile genotypes from the Nottingham University stock centre collection, which were selected because the lesions were thought to be early acting, had shown that gus activity was maintained in these mutants. This suggests that these MS genes do not interact with A6 or A9 and act relatively late in tapetum development. Accordingly the screen appears to have identified a novel set of MS genes. The identification of gus-negative male-fertile mutants is also highly significant since the wt. genes must lie very close to the target genes in the regulatory cascade.
We have begun the detailed analysis of these mutants. Crosses to wild-type have shown that for each of the gus-negative mutants tested so far the reporter gene is functional. Allelism tests and genetic mapping are also underway and this data will be presented.
Histological studies have been carried-out on 2 male-sterile gus-negative A9 mutants and these show that in both cases that the gross-morphology of the anther is unaffected, but that tapetum-differentiation does not occur. This preliminary data suggests that these mutants are defective in tapetum histodifferentiation (tapetum-less). Similar analysis is underway for the other mutants. Once mapping is complete, some of the genes will be selected for cloning either by chromosome walking or subtractive hybridisation techniques.

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STUDIES ON THE EXPRESSION AND FUNCTION OF *CO* THE GENE CONTROLLING FLOWERING TIME IN *Arabidopsis*

Rüdiger Simon

The flowering time of many plant species is under photoperiodic control. The constans (co) mutant of Arabidopsis flowers later than wildtype under long photoperiods. The CO gene encodes a putative transcription factor, harbouring two zinc-finger domains, and a basic domain at its N-terminus. We have Isolated a number of CO-related genes from Arabidopsis, rice and maize, indicating that CO is the founding member of a new class of transcription factors. Experiments are under way to investigate wether CO-like genes control other aspects of flower devlopment in Arabidopsis.

Flowering time seems to be sensitive to CO copy number, indicating that CO expression is a critical determinant of flowering time. When wildtype plants are grown under long photoperiods, expression of CO is detectable already at the cotyledon stage, long before the first floral buds are visible. Under short photoperiods, CO expression is delayed until the first leaf primordia are visible.

Mutations in the CO gene enhance the effects of mutations affecting floral meristem identity, and delay the onset of flowering of early flowering mutants affected in phytochrome function. We are now investigating in more detail the regulation of CO expression during plant development, how CO interacts with other genes controlling flowering time, and wether CO does act as a DNA binding gene to regulate the expression of downstream target genes.

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A GENETIC AND MOLECULAR ANALYSIS OF FLOWER INITIATION IN *ARABIDOPSIS*.

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At least 14 loci have been identified of which the mutant delay flowering in long days in *Arabidopsis thaliana* ecotype Landsberg erecta (Ler) "wild type". The epistatic relations among 10 of these genes, as well as their response to vernalization and daylength have been determined. The cloning of *fwa*, one of the late flowering genes, by means of "chromosome walking" is in progress. The latest results about the gene, cDNA and expression will be reported.
For the cloning of *fwa* by means of chromosome walking the close linkage between *fwa* and *abi1* was used allowing the localization of the *fwa* gene on a YAC isolated by Leung *et al.* (1994). Subsequently by using subclones of this YAC in cosmids and λ phages we located *fwa* on one phage. This phage was used to screen a cDNA library (λPRL-2, constructed by Thomas Newman and obtained from the ABRC in Columbus, Ohio, USA). This provided us with 3 different cDNA's in this area, one of which is believed to be *fwa*. Fine mapping of *fwa* will probably further limit the number of possible cDNA's. Since *fwa* is a partially dominant mutation and therefore impossible to fully complement, we constructed genomic libraries of both mutant alleles and screened for the genomic fragments containing the mutant gene. These constructs will be used to introduce by transforming the mutant alleles in wildtype Landsberg *erecta* plants. Furthermore, all three alleles (wildtype and mutants) will be sequenced and the expected differences between the wildtype and the mutant alleles will indicate that the gene we isolated is *fwa*.

J. Leung et al. (1994), Science 264; 1448

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The effect of pollination conditions on the flower biology of *Vicia faba*.

Mondragao-Rodrigues, F. and Suso, M.J.

The low ratio of flower to pod is considered by many breeders a major problem of faba bean crop. As in many pulses the number of flowers per node is variable but always exceeds the number of pod per node.

Field studies were conducted to obtain more information on the relative importance of pollination conditions on floral abortion of *Vicia faba*. Two different pollination conditions were used: self-pollination, cages were used in the field to exclude pollination insects, and open-pollination.

Twelve entries with a broad variation in reproductive characters were studied.

Our observations showed that the number of nodes with flower increased in self-pollinated plants. Results also suggest that at least in some genotypes an "excess" of flowers would be produced until an optimal number of pods has been set; thus, flower production could be adjusted by the plant upwards as well as downwards in order to optimized the number of pods.

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List of Invited Speakers

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Workshop on

FLOWER DEVELOPMENT

List of Invited Speakers

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