Instituto Juan March
de Estudios e Investigaciones

107 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Biochemistry and Molecular Biology of Gibberellins

Organized by

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Introduction

P. Hedden and J. L. García-Martínez

The gibberellins (GAs) constitute a large group of diterpenoid compounds, some of which function as regulators of growth and development in plants. They were discovered about 60 years ago in the fungus *Gibberella fujikuroi*, the agent of the "bakanae" rice disease, which is characterized by a very elongated phenotype. GAs were shown later to occur naturally in plants, where they control developmental processes throughout the life cycle, including seed germination, stem elongation, flower induction and development, and fruit growth. They have also been shown to mediate the effects of environmental stimuli, such as photoperiod or low temperatures, on developmental processes, particularly germination, bolting and flowering.

Experiments with mutants and inhibitors of GA biosynthesis have indicated that relatively few of the 125 currently known GA structures function as hormones, many of the others being precursors or catabolites of the active GAs. The complex biosynthetic pathway to the active compounds is well understood. It consists of three stages: in the first, geranylgeranyl diphosphate is converted in two steps to *ent*-kaurene by cyclases (copalyl diphosphate synthase, CPS, and *ent*-kaurene synthase, KS) located in plastids; in the second stage, *ent*-kaurene is oxidised on membranes by cytochrome P450 monooxygenases to GA_{12} and GA_{53}, which, in the third stage, are converted by soluble, 2-oxoglutarate-dependent dioxygenases to the active hormones, GA_4 and GA_1, respectively. Dioxygenases also catabolize the inactivation of GAs by 2β-hydroxylation.

Within the last five years, spectacular progress has been made in our understanding of GA biosynthesis and its regulation though the cloning of genes encoding the biosynthetic enzymes. More than half the genes of the pathway have now been cloned, including the cyclases CPS and KS, the cytochrome P-450 *ent*-kaurene oxidase, and the dioxygenases GA 20-oxidase, GA 3-oxidase and GA 2-oxidase. A GA 3-oxidase in pea was shown to be encoded by the *LE* gene, mutation of which causes dwarfism in pea; difference in stem height between tall (*LE*) and dwarf (*le*) peas was one of the seven genetic traits used by Mendel in his studies on the nature of inheritance. The availability of these clones has enabled progress in several areas. Expression of cDNAs in heterologous systems has provided sufficient amounts of enzymes for characterisation of their function and mechanism. It has also been possible to investigate the regulation of GA biosynthesis in relation to plant development, tissue localisation and environmental stimuli.

Although progress on the mode of action of GAs has not been as rapid as that on GA biosynthesis, impressive advances have also been made in this area, particularly from genetic approaches. GAs are known to modify the expression of genes in relation to several developmental processes. In particular, considerable progress has been made in understanding the regulation of α-amylase gene expression by GA in cereal aleurone cells. There is compelling evidence for the presence of GA receptors on the outside of the plasma membrane of cereal aleurone cells. The involvement of heterotrimeric G-proteins, Ca^{2+}, calmodulin, and a GAMyb transcription factor as signal transduction elements mediating the induction of α-amylase synthesis by GA in such aleurone cells has also been demonstrated. In the model plant species *Arabidopsis thaliana*, three genes (*GAI*, *SPY* and *RGA*) that act as negative regulators of the GA signal transduction pathway have been isolated and their interaction and function are being studied intensively. There have been reports of further genes that modify the GA response so that there should be exciting developments in this area in the next few years.

From a practical standpoint, several laboratories are manipulating GA content in transgenic plants by modifying the expression of GA biosynthesis genes (overexpressing and underexpressing using antisense and ribozyme technologies), or are altering the expression of GA response genes. These approaches are providing the means to modify phenotypic characteristics, such as shoot length or parthenocarpic fruit development, in species of agricultural interest.

The workshop assembled scientists working on molecular aspects of gibberellin biosynthesis and mode of action. It provided an opportunity to review the rapid and exciting progress that is being made in these areas, covering both fundamental and applied aspects of the work. Perhaps more importantly, it brought together workers from these two areas, biosynthesis and action, which have tended to be worked on separately by researchers with different interests and expertise. This was particularly timely since it is now realised that the two areas are closely linked, with GAs regulating the expression of GA-biosynthetic genes in feedback and feedforward regulation of biosynthesis.

Peter Hedden and José L. García-Martínez

Session 1: Regulation of developmental processes by gibberellins
Chair: Peter Hedden

Regulation of stem growth by gibberellin

Hans Kende

Michigan State University, East Lansing, MI 48824-1312, USA. E-mail: hkende@msu.edu

Deepwater rice (*Oryza sativa* L.) is a subsistence crop in areas of Southeast Asia that are flooded during the rainy season. Survival of this rice depends on its capacity to elongate rapidly when it becomes partially submerged and to keep part of its foliage above the rising waters. This growth response is, ultimately, induced by gibberellin (GA). Internodal elongation is based on increased production of new cells in the intercalary meristem and on enhanced elongation of these newly formed cells (Kende et al., 1998).

Identification of GA-regulated genes complements characterization of GA-response mutants as an approach to determine steps in the GA response pathway. Because of the magnitude of the GA response in deepwater rice and the involvement of both cell elongation and cell division, we expected that GA would regulate, directly or indirectly, the expression of growth-related genes. Such genes were identified by a targeted approach and by differential display of mRNA. Early GA-induced genes fell into three categories: (i) Genes whose products are involved in regulating the cell cycle, namely genes encoding two cyclins, a $p34^{cdc2/CDC28}$ protein kinase, histone H3, and replication protein A1 (RPA1) (Kende et al., 1998); (ii) genes encoding the cell-wall-loosening protein expansin (Cho and Kende, 1997); and (iii) genes with products of unknown function. The effect of GA on the expression of expansin genes and of genes with unknown function will be described.

We studied the expression of four α-expansin genes in rice (Cho and Kende, 1997). Submergence and treatment with GA induced accumulation of *Os-EXP4* mRNA within 30 min of treatment, i.e., before the rate of growth started to increase. This is the most rapid induction of growth-related gene expression that we have observed in deepwater rice. We transformed rice with the sense and antisense constructs of *Os-EXP4*. The sense transformants, which overexpressed *Os-EXP4*, segregated into two populations, one that was significantly taller than the control plants transformed with the vector alone and one that was significantly shorter. Reduced growth may have been due to co-suppression. Among the antisense transformants, a subset of the population was significantly shorter than were the control plants. Recently, we started to study the occurrence of β-expansins, which may be more active in monocots than in dicots (Cosgrove, 1998). We found in the growing region of rice internodes a rapid GA-induced expression of genes encoding β-expansins (Lee and Kende, unpublished).

Three genes--*Os-DD3, Os-TMK, and Qs-GRF1*--whose functions are not known and whose transcripts accumulated in response to GA, were identified. The expression of *Os-DD3* mRNA increased in the intercalary meristem of rice internodes within 3 to 4 h of treatment with GA (Van der Knaap and Kende, 1998). The PSORT program predicted that Os-DD3 is a type 1A plasma membrane receptor with a short cytoplasmic C-terminal tail. The putative intracellular domain contains a consensus protein kinase C phosphorylation site, and the extracellular domain an RGD motif. The latter has been identified as the amino acid sequence in fibronectin and other proteins that mediates cell adhesion through binding to a family of

cell surface receptors, the integrins. The possibility exists, therefore, that Os-DD3 is involved in mediating the interaction between the cell wall and the inside of the plant cell.

The transcript level of *Os-TMK*, a transmembrane leucine-rich repeat receptor-like protein kinase, increased in the internode within 2 h of GA treatment and was high in all tissues undergoing cell division and cell elongation (Van der Knaap et al., 1999). The kinase domain of Os-TMK is enzymatically active and autophosphorylates on serine and threonine residues. The kinase interaction domain of a kinase-associated protein phosphatase, Os-KAPP, was phosphorylated in vitro by the kinase domain of Os-TMK. Li and Chory (1997) identified a leucine-rich repeat receptor-like kinase, BRI1, which is involved in brassinosteroid signaling. Because GA and brassinosteroids are both terpenoids, their signal transduction pathways may share similarities.

Expression of *Os-GRF1* (*Oryza sativa-GROWTH-REGULATING FACTOR1*) is induced in the intercalary meristem of the internode within 2 h of GA treatment (Van der Knaap et al., 2000). Os-GRF1 has sequence similarities to a protein-binding domain of SWI2/SNF2 of yeast, which is a subunit of a 2-MDa chromatin-remodeling complex, and to transcription factors or activators. It possesses a functional nuclear localization signal and a putative zinc finger. The rice database contains an expressed sequence tag (EST) with similarity to *Os-GRF1*, and the Arabidopsis database contains nine listings with similar sequences. We propose that Os-GRF1 is the prototype of a family of proteins with regulatory functions in transcription. Currently, we are trying to isolate the Arabidopsis ortholog of *Os-GRF1* whose expression is regulated by GA in the growing region of the Arabidopsis stem. To determine the function of GRF1-like proteins, we are screening the Wisconsin collection of Arabidopsis T-DNA insertional mutants for plants with interrupted *GRF*-like genes. In addition, we are using DNA microarray technology to identify GA-regulated genes in Arabidopsis (Kim and Kende, unpublished).

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Gibberellins and fruit-set and growth

J.L. García-Martínez, I. Alvarez and T. Fichet

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Avda de los Naranjos s/n, 46022-Valencia, Spàin

Fruit-set and fruit growth following pollination and fertilization depend on gibberellins (GAs) in many species (1). This conclusion has been drawn mainly from application experiments with GAs and inhibitors of GA biosynthesis, and by quantification of GAs during the early stages of fruit growth. In the absence of pollination, or when the developing seeds are destroyed the fruit fails to grow. The effect of developing seeds can be mimicked by applied GAs, suggesting that the fertilized ovules are the source of GAs for fruit growth. GA_1 has been proposed as the active GA regulating fruit-set and growth in pea, though its biosynthetic pathway in the seeeds is unknown (2). Furthermore, the function of seeds as a source of GAs for fruit-set and growth is still not clear since the pericarp, at least in the case of pea, has also some capacity of GA biosynthesis (2, 3).

The role of seeds and pericarp in the GA economy of pea fruits during fruit-set and early growth has been investigated by analyzing: a) the transcript levels, in the pod and seeds, of genes coding for dioxygenases that catalyze the last steps of the GA biosynthetic and metabolic pathway: GA 20-oxidase, GA 3-oxidase and GA 2-oxidase (a cDNA clone of a new gene coding for a GA 2-oxidase has been isolated from youg seeds), and b) the effect of seed destruction (by pricking) on the response to applied GAs and transcript levels of genes of GA biosynthesis and metabolism.

The pea ovary can also develop parthenocarpically (in the absence of pollination) when the plants are decapitated. Interestingly, the effect of decapitation was negated when the ovules were destroyed, indicating that the unfertilized ovules have also a role in the biosynthesis or metabolism of active GAs. Plant decapitation induces parthenocarpic growth due to the removal of auxin transported basipetally from the apical shoot (4). On the other hand, we have found that the pea *gio* mutant has a reduced response to applied GAs due to its overproduction of auxin in the apex (5). This is in contrast with the effect of auxin-like compounds (e.g. 2,4-D) applied directly to the ovary where they stimulate parthenocarpic growth. Results of the effect of auxin applied at the decapitated stump, or directly to the ovary on the expression of genes coding for enzymes of GA biosynthesis and metabolism will be presented.

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Gibberellins and Floral Induction in *Arabidopsis*

Detlef Weigel, Miguel A. Blázquez, and Greg F. W. Gocal

Plant Biology Laboratory, Salk Institute, La Jolla, CA 92037, USA; email weigel@salk.edu; URL http://www.salk.edu/LABS/pbio-w/

Arabidopsis is a facultative long-day plant and flowers much faster under long than under short days. The nature of florigenic factors that transduce floral inductive signals within the plant remains elusive, but physiological studies have provided valuable information on the florigenic properties of several plant hormones, in particular gibberellins (GAs). The importance of GAs for flowering of *Arabidopsis* is evident from several results: first, GA deficient mutants or mutants with reduced GA signaling flower late under long and short days, with particularly severe mutations such as *ga1-3* abolishing flowering under short days; second, mutants with constitutively elevated GA signaling flower early; and third, applied GA_3 decreases flowering time.

GAs are involved in the regulation of many developmental processes in *Arabidopsis*, from seed germination to cell elongation, so one important question is whether GAs play merely a permissive role in flowering, or whether they also play an instructive role. To answer this and related questions, we have initiated forward and reverse genetic approaches to study the link between GAs and flowering in *Arabidopsis*. In a reverse approach, we have been studying how GAs regulate the expression of *LEAFY* (*LFY*), a floral meristem-identity gene. In a forward approach we have been studying mutations that suppress the non-flowering defect of short day-grown *ga1-3* mutants.

The expression of *LFY*, which encodes a transcription factor that activates downstream genes required for floral morphogenesis (Parcy et al., 1998; Busch et al., 1999), increases gradually during the vegetative phase of plants grown in short days, and its expression is dramatically enhanced under inductive long days (Blázquez et al., 1997). Expression in short days is GA-dependent, since *LFY* expression is strongly reduced in short-day grown *ga1-3* mutants, which correlates well with the inability of this mutant to flower under non-inductive conditions (Blázquez et al., 1998).

Deletion analysis revealed several redundantly acting elements in the 2.2 kb full-length *LFY* promoter, but we were able to create a synthetic 0.6 kb promoter that was sufficient for rescue of *lfy* mutants when fused to a *LFY* cDNA. In the context of this derivative promoter, GOF9, a small element of a few base pairs became essential for short-day expression, such that the GO9m reporter was still active under long days, but not under short days. We demonstrated that the GOF9m defect was specific by analyzing GOF9m activity in various genetic backgrounds. The CONSTANS (CO) transcription factor is both necessary and sufficient to activate the long-day dependent pathway of floral induction (Simon et al., 1996). While GOF9m was no longer active in long-day grown *co* plants, constitutive expression of CO in short days restored GOF9m activity. In contrast, constitutive expression of *FT*, which acts partially downstream of *CO*, but in parallel with *LFY* (Kardailsky et al., 1999), could not restore vegetative GOF9m activity. Furthermore, GOF9m did not respond to GA application, neither in short nor in long days.

We evaluated the biological significance of the GOF9m mutation by fusing the GOF9m promoter to a *LFY* cDNA and introducing this construct into *lfy* mutants. In contrast to GOF, GOF9m rescued the *lfy* mutant only in long, but not in short days.

The sequence element that has been mutated in GOF9m was bound in vitro by the AtMYB33 protein, an *Arabidopsis* homolog of the GAMYB transcription factor, which transduces GA signals in cereal aleurone cells (Gubler et al., 1995). However, we have not established which MYB factor (if any) interacts with the GOF9m site in vivo. Overexpression of *AtMYB33* conferred partial resistance to the GA biosynthesis inhibitor paclobutrazol during seed germination, but did not affect flowering.

While the reverse genetic approach has identified a cis-element in the *LFY* promoter that mediates GA effects, we have used forward genetics to identify elements in trans that mediate the regulation of *LFY* expression by GAs. We have isolated mutations that specifically suppress the flowering defect of *ga1-3* mutants, in an attempt to define a GA-signaling branch different from the ones that control other GA responses. We have isolated mutations at four different *FOG* (for Flowering Of GA-deficient mutants) loci, of which two, in *FOG1* and *FOG2*, primarily suppressed the flowering defect, while *fog3* and *fog4* mutations suppressed also other defects of *ga1* mutants. *FOG3* turned out to be allelic to *RGA*, whereas *FOG1* and *FOG2* identified two new loci on chromosome 5. The mutations in *FOG1* and *FOG2* dominantly reduced flowering time under short days not only in *ga1-3*, but also in a *GA1*$^+$ background. *FOG1* acted upstream of *LFY*, as shown by the restoration of *LFY* promoter activity in *fog1-1D ga1-3* mutants.

One concern was that *fog1-1D* and *fog2-1D* were bypass suppressors that suppressed the *ga1-3* flowering defect by activating in short days the GA-independent long-day pathway, which is mediated by *CO*. However, *CO* was not upregulated in *fog1-1D*, which is different from other mutants that flower early in short days, such as *elf3* (G. Coupland, pers. communication).

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Gibberellins control trichome differentiation in *Arabidopsis*

Michel Herzog, Jean-Marc Bonneville, Daniel Perazza, Gilles Vachon

Génétique Moléculaire des Plantes, UMR CNRS 5575, Université Joseph Fourier, BP 53, F-38041 Grenoble, France. E-mail: michel.herzog@ujf-grenoble.fr

Recently, gibberellins (GA) have been credited a new role in trichome development in *Arabidopsis*. GA-deficient *ga1-3* plants have almost completely glabrous leaves in the absence of exogenous GA (1,2,3). Trichome formation has been studied extensively and requires more than 25 genes (4), two of which, *GLABROUS1 (GL1)* and *TRANSPARENT TESTA GLABRA (TTG)*, are required for trichome initiation. The *GL1* gene encodes a protein that contains a Myb domain, suggesting that GL1 is likely to function as a transcription factor (6). The recently cloned *TTG1* gene encodes a WD40 repeat protein distantly related to the β subunit of heterotrimeric G proteins, suggesting a role for TTG1 in signal transduction to downstream transcription factors (7). Before the *TTG* gene was cloned, it had been shown that a *ttg* mutation in *Arabidopsis* could be complemented by the maize R gene, which encodes a Myc transcription factor functionally but not structurally related to TTG (8,9). Because trichome cells are readily visible and non essential, they provide a powerful new cellular model for dissecting the GA-signalling pathways.

GA up-regulates GL1 *and possibly* TTG *expression in trichome cells.*

Using the constitutive GA-response mutant *spindly (spy)* (5) and uniconazol (a GA-biosynthesis inhibitor), we showed that the activation of GA-signal transduction pathway correlates positively with both trichome number and trichome branch number. Interestingly, *spy-5* displays overbranched trichomes and shows a high degree of ploidy (about 64C DNA) compared to the wild type trichome nucleus (32C). We observed that *spy gl1* double mutants are glabrous, indicating that SPY acts upstream of GL1. GA, thus, appears to be involved in both initiation and development of trichomes, including the negative control of endoreduplication.

To test the hypothesis that *GL1* and/or *TTG* genes were GA-target genes, we introduced *35S-GL1* (6) and *35S-R* (8) genes in the *ga1-3* mutant background : overexpression of *GL1* alone or *R* alone only poorly rescue trichome formation in the GA-deficient background. However, when *35S-GL1* and *35S-R* genes are simultaneoulsy expressed in the *ga1-3* background, trichomes overdeveloped on leaves. In addition, the *GL1* transcript is not detectable in the *ga1-3* background and the expression of the GUS reporter gene under control of the promoter/enhancer of *GL1* is positively regulated by GA. Using various transgenic lines of wild type or GA-deficient backgrounds *(ga1-3)* we were able to demonstrate that *GL1* gene is transcriptionnaly activated by GA (3), and that constitutive expression of both GL1 and TTG is sufficient to restore trichome formation when GA is missing.

We are currently attempting to dissect the GA signal-transduction pathway leading to the activation of *GL1*. To identify putative *trans*-acting factors from *Arabidopsis* able to control *GL1* gene expression we performed a one-hybrid screen in Yeast. We have identified a protein of 35kDa (p35) that binds to the 3'enhancer of GL1 fused to Gal4 activation domain,

both *in vivo* (Yeast) and *in vitro*. The p35 protein of unknown function displays two NLS sequences and a putative Leucine zipper domain suggesting that this protein is a transcription factor.

At least five genes are involved in the negative control of endoreduplications in trichomes.

Because *spy-5* mutants produce overbranched trichomes with an additional endoreplication round (64C, as estimated by DAPI-stained image analysis), we focused our efforts on various *Arabidopsis* genotypes showing a similar phenotype. Tetraploid lines develop trichomes with 4 to 5 branches and 64C DNA content. However, tetraploidy does not affect the number of endoreduplication rounds within trichomes, which reaches four as in the wild-type.

We next looked for diploid EMS-mutants and isolated 6 new lines whose trichome cells had additional branches and a DNA content of at least 64C, demonstrating that they underwent a fifth endoreplication round in trichomes (10). These mutants represent three loci which were mapped using SSLP markers, and named : *POLYCHOME* (*PYM*), *KAKTUS* (*KAK*, 4 alleles), *RASTAFARI* (*RFI*). Endoploidy is not affected in internal leaf cells of the mutants indicating that the genes involved in negative regulation of endoreduplication are specific to trichome or epidermal cells. In addition to *SPINDLY* mentioned above, one other gene, *TRIPTYCHON*, described earlier (4), is also affected in lateral inhibition of trichome formation. Genetic analysis of various double mutants revealed additive effects of some mutations caracterized by a high trichome branch number ranging from 7 to 12, and a DNA of 128C or higher. Interestingly, strong additive effects are observed in *spy try* double mutants but not in *spy kak, spy pym* nor *spy rfi* suggesting that at least two different pathways control trichome development and endoreduplications, one being dependant on GA signaling. We have performed a screen for T-DNA insertion mutants affected in trichome development in order to clone the possible negative regulators of trichome endoreduplication.

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Patterns of acidification, gene expression and aleurone cell death are coordinated by gibberellic acid in germinating wheat grains

Fernando Domínguez and Francisco J. Cejudo
Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC
Avda Américo Vespucio s/n, 41092-Sevilla, Spain

Cereal aleurone responses to gibberellic acid include activation of synthesis of hydrolytic enzymes, acidification of the external medium and cell death. We have studied the coordination of these aleurone responses in vivo during wheat grain germination. De-embryonated half grains show the capacity for GA_3-activated medium acidification when incubation is carried out at pH 6.0-7.0 but not at lower pHs. In addition, the activating effect of GA_3 on the expression of carboxypeptidase III and thiol protease genes is more efficient when the hormone treatment is carried out at neutral pH. *In situ* pH staining showed that starchy endosperm acidification takes place upon imbibition and advances from the embryo to the distal part of the grain. *In situ* hybridization experiments show a similar pattern of expression of a carboxypeptidase III gene, which is upregulated by gibberellic acid in aleurone cells. However, aleurone gene expression precedes starchy endosperm acidification. These findings imply that *in vivo* gibberellin perception by the aleurone layer takes place at neutral pH and suggest that the acidification of the starchy endosperm is regulated by gibberellic acid in germinated wheat grains. Aleurone DNA fragmentation increased as germination proceeded, the activating effect of GA_3 on DNA fragmentation was also pH-dependent. The TUNEL assay shows that cell death is started in cells proximal to the embryo and advances to the distal part of the grain but it is delayed with respect to carboxypeptidase III expression and starchy endosperm acidification.

Session 2: Genes of gibberellin biosynthesis and their regulation
Chair: Richard Hooley

The dioxygenases of gibberellin biosynthesis and their regulation

P. Hedden, J.P. Coles, S.G. Thomas, D.A. Ward and A.L. Phillips

IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK
(E-mail: Peter.Hedden@bbsrc.ac.uk)

Biosynthesis of gibberellins (GAs) in higher plants requires the action of diterpene cyclases, cytochrome P-450 monooxygenases and 2-oxoglutarate-dependent dioxygenases (Hedden and Kamiya, 1997). Three major types of dioxygenase are involved in the synthesis and deactivation of the growth active GAs, GA_1 and GA_4: GA 20-oxidases (GA20ox) sequentially oxidise C-20 from a methyl to an aldehyde, and then eliminate this atom in the formation of the C_{19}-GA skeleton; GA 3β-hydroxylases (GA3ox) catalyse the final step in the formation of the biologically active hormones, while GA 2-oxidases (GA2ox) introduce a 2β-hydroxyl group into C_{19}-GAs rendering them biologically inactive. The GA 2-oxidases have also the capacity to convert 2β-hydroxyGAs to the 2-ketones, which give rise, by rearrangement, to the so-called 'GA catabolites' (Thomas *et al.*, 1999). This multifunctionality is a characteristic of many of the oxygenases of GA biosynthesis (Hedden, 1997) and, coupled with broad substrate specificities, results in the large range of different GAs found in many plants.

The GA dioxygenases play important roles in the regulation of GA concentration. They are encoded by multi-gene families, members of which show differential patterns of expression. Arabidopsis contains at least four *GA20ox* genes; current evidence from transcript distribution for three of these genes (Phillips *et al.*, 1995) and from specific suppression of their expression by the introduction of antisense DNA (Coles *et al.*, 1999), suggests they may be involved in early seedling development (*GA20ox3*), and in the growth of shoots (*GA20ox1*) and the inflorescence (*GA20ox2*), respectively. More detailed analysis of sites of expression of these and other GA dioxygenase genes is currently being conducted using reporter genes and *in situ* hybridisation. Arabidopsis contains at least two *GA3ox* (Yamaguchi *et al.*, 1998) and three *GA2ox* genes (Thomas et al., 1999). In contrast to the *GA20ox* genes, two of the *GA2ox* genes were found to have similar patterns of expression at the tissue level, as determined by Northern hybridisation; the genes are expressed most highly in actively growing tissues: upper stems, flowers and young siliques (Thomas et al., 1999). However, two *GA2ox* genes are differentially expressed in pea, with one (*PsGA2ox1*) expressed most highly in developing seed, particularly in the testa (Lester *et al.*, 1999; Martin *et al.*, 1999), and a second (*PsGA2ox2*) expressed more highly in the shoot (Lester et al., 1999).

As well as the developmental regulation discussed above, dioxygenase gene expression is controlled by the action of GAs in a homeostatic mechanism. Expression of *GA20ox* and some *GA3ox* genes is reduced by GA, whereas, in Arabidopsis, expression of *GA2ox* genes is up-regulated by GA treatment (Thomas *et al.*, 1999). For example, application of 10 μM GA_3 to wild-type (Columbia) Arabidopsis seedlings resulted in a 3-fold decrease in *GA20ox1* transcript numbers, as determined by quantitative RT-PCR, whereas seedlings treated with the GA-biosynthesis inhibitor paclobutrazol (10 μM) contained 3-fold higher amounts of transcript than the untreated controls. We are currently using transgenic plants containing a range of reporter genes to study feedback regulation of GA biosynthesis with the long-term aim of elucidating the signalling pathways involved in this process.

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Gibberellin biosynthesis during seed development and germination of pumpkin

Andrea Frisse & Theo Lange

Botanisches Institut der TU Braunschweig, Mendelssohnstr. 4,
D-38106 Braunschweig, Germany, tlange@tu-bs.de

Gibberellins (GAs) control many aspects of plant development, including growth of seeds and seedlings. The recent cloning of GA biosynthetic genes, together with biochemical approaches have provided tools for investigating the site of GA-biosynthesis in plants (1-3).

We have isolated cDNAs encoding four GA-dioxygenases from developing pumpkin seeds, a GA 7-oxidase, 20-oxidase, 2ß,3ß-hydroxylase, and, recently, a 3ß-hydroxylase. Each of the four recombinant enzymes has unique catalytic properties, which have not been found with corresponding enzymes from other plant species. E. g. both pumpkin 3ß-hydroxylases hydroxylate C_{20}-GAs (e.g. GA_{15} or GA_{25}) at least as efficient than C_{19}-GAs (e.g. GA_9), while 3ß-hydroxylases from other plant species prefer C_{19}-GAs as a substrate.

The four GA-dioxygenase genes are expressed at very high levels in developing pumpkin seeds as shown by quantitative RT-PCR. Gibberellin 7-oxidase and 20-oxidase are strongly expressed in both, endosperm and embryos. However 2ß,3ß-hydroxylase is exclusively expressed in endosperm and 3ß-hydoxylase is only expressed in embryos. Recently, we have studied these expression pattern in more detail by *in situ* hybridisation.
To study the site of GA-biosynthesis, we have looked for expression pattern of the four GA-dioxygenase genes in 5-d to 7-d old pumpkin seedlings. Only GA 7-oxidase transcripts were detected at considerable levels in the seedling by quantitative RT-PCR and were found mainly in the tip of the shoot and the tip of the root. Correspondingly, in cell-free systems prepared from such seedlings we found 7-oxidase enzyme activity mainly in the tip of the shoot and in the tip of the root. However, we also detected considerable 20-oxidase and 3ß-hydroxylase activity in the tip of the root, but not in other parts of the seedling. These results suggest, that, in pumpkin seedlings, the root tip is the site of GA-biosynthesis. Furthermore, GA 20-oxidases and 3ß-hydroxylases expressed in vegetative tissues are distinct from those expressed in developing seeds. Compared with the two 3ß-hydroxylases from developing pumpkin seeds, the 3ß-hydroxylase expressed in root tips has different catalytic properties: Only C_{19}-GA_9, but not C_{20}-GA_{15} was metabolised.

There have been many reports that abundance of GA-dioxygenase transcripts is regulated by GA-levels (3). In our studies we analysed GA-dioxygenase activities directly in cell-free systems prepared of different parts of pumpkin seedlings treated with LAB150978, an inhibitor of GA-biosynthesis, and bioactive GA_3 and GA_4. In cell-free systems prepared from root tips GA 7-oxidase activity did not alter after treatment. However, in shoot tips 7-oxidase activity increased after LAB 150978 treatment and decreased after GA_3 or GA_4 treatment. GA 20-oxidase activity was not affected by any of these treatments. However, GA 3ß-hydroxylase activity was strongly enhanced in root tips after LAB 150978 treatment, but was not detectable in seedlings treated with GA_3 or GA_4. Our results suggest a feedback-type of regulation of GA-biosynthesis in pumpkin seedlings, controlling the early 7-oxidation step in the shoot tip, and, more directly, the GA hormone activating 3-hydroxylation step in the root tip.

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Regulation of GA biosynthesis by phytochromes

Yuji Kamiya[1], Shinjiro Yamaguchi[3], Tomonobu Toyomasu[2], Tahar Ait-Ali[1], Francis Chanon[1], Tai-Ping Sun[3]

[1]Plant Functions Laboratory, RIKEN, [2]Yamagata University, [3]DCMB Group, Dept. Botany, Duke University

Phytochrome regulation of GA biosynthesis during seed germination.

Seed germination is complex and is regulated by many factors such as nutrients, temperature, water and light. The endogenous content of GA_1, the main biologically active GA in lettuce seeds, increases after red light (R) treatment. GA_1 is synthesized from GA_{53} by two different 2-oxoglutarate dependent enzymes, GA 20-oxidase and GA 3β-hydroxylase. Lettuce seeds contain high levels of GA_{20} (about one hundred times higher than GA_1). Two GA 20-oxidase genes (*Ls20ox1* and *Ls20ox2*) and one GA 3β-hydroxylase (*Ls3h1*) are expressed in germinating seeds. The expression of *Ls3h1* is induced by R treatment and this effect is canceled by far-red light (FR) treatment. Expression of *Ls20ox1* and *Ls20ox2* is induced by imbibition alone in the dark. The level of *Ls20ox2* mRNA decreases with R treatment, whereas that of *Ls20ox1* is unaffected by light. These results suggest that R promote GA_1 synthesis by inducing *Ls3h1* expression via phy action. It is well known that GA 20-oxidation / accumulation of GA 20-oxidase mRNA is negatively feedback regulated by active GAs. Therefore, the down-regulation of *Ls20ox2* expression could be the result of the increased GA_1 content in germinating seeds. Interestingly, although the exogenous application of high levels of GA_1 decreases the expression of *Ls3h1*, this gene was not affected by the increase of endogenous GA_1. Thus, there may be some mechanism to suppress the feedback regulation of *Ls3h1* during seed germination of lettuce.

Arabidopsis *ga4-1* is a GA deficient semi-dwarf mutant. The *GA4* gene was cloned by T-DNA tagging and it was shown to encode a GA 3β-hydroxylase. Severe alleles of the GA-deficient mutants *ga1*, *ga2* and *ga3* fail to germinate without exogenous application of GAs, whereas even the putative null allele, *ga4-2*, can germinate without GAs, suggesting the presence of another GA 3β-hydroxylase in germinating seeds. Recently, a *GA4* homolog (*GA4H*) was isolated and shown to encode a GA 3β-hydroxylase. The *GA4H* gene was predominantly expressed during seed germination. Both *GA4* and *GA4H* genes in imbibed seeds are induced by R treatment. At least five loci in Arabidopsis encode phys. Among these, *PHYB* encodes the phyB, which plays a major role in germinating seeds shortly after the start of imbibition, and it was suggested that absolute concentration of FR-absorbing form of phy is important. In the phyB-deficient *phyB-1* mutant, *GA4H* expression is not induced by R, although *GA4* expression still is, indicating that the R-induced *GA4* and *GA4H* expression is mediated by one or more other phys. In contrast to the *GA4* and the *Ls3h1* genes, *GA4H* is not regulated by a feedback inhibition mechanism in germinating seeds (Note: *Ls3h1* gene is down regulated by applied GA_1 but not by the elevated endogenous GA_1 level after R). Although the endogenous GA levels of germinating *ga4* and wild-type (WT) seeds have not yet been analyzed, R treatment is expected to increase the level of biologically active GAs. The two GA 3β-hydroxylases of Arabidopsis therefore seem to play different physiological roles during light-induction of seed germination.

Phytochrome regulation of GA biosynthesis during seedling growth

Light inhibits stem elongation during photomorphogenesis, and the role that GAs (change of GA sensitivity and/or metabolism) play in that process has been the subject of long controversy. Recently, work from two independent laboratories has shown a rapid (within 2 h) and reversible decrease of GA_1 content (down to trace level) in the apical shoot of etiolated pea seedlings upon light irradiation. The light, however, increases the transcript levels coding for GA 20-oxidase and GA 3β-hydroxylase in the apical shoot, indicating that they do not contribute to the decrease of GA_1 content induced by light. Work with *phyA*- and *phyB*-deficient pea mutants showed that both phyA and phyB regulate the expression of GA 20-oxidase. The increase in the transcript accumulation is probably the result of feedback inhibition due to the reduction of the GA_1 level because it does not occur when the seedlings are treated with GA_1 before irradiation. The concentration of GA_8, the inactive product of GA_1 metabolism, increases transiently in irradiated seedlings, suggesting that GA 2β-hydroxylation may be regulated during de-etiolation. The recent isolation of clones coding for 2β-hydroxylases of *Phaseolus coccineus* and Arabidopsis should help to clarify this issue.

Treatment with end-of-day (EOD)-FR irradiation enhances stem elongation, and in cowpea also decreases the $[^3H]GA_1$ inactivation and increases the GA_1 content in the elongating region of the epicotyl, but not in the leaves, an effect that can be reverted by subsequent R. This suggests that phy may control stem elongation by regulating GA 2β-hydroxylation in light-grown seedlings. Work with the Arabidopsis *ga1 phyB* double mutant has shown that the full *phyB* mutant phenotype is expressed only in the presence of a completely functional GA system. However, the role of phy in the regulation of GA biosynthesis in light-grown plants is not clear. The overexpression of oat *PHYA* in tobacco and hybrid aspen decreases the content of active GAs and results in a short phenotype, that can be reversed by GA application. However, the *phyB* mutants of pea (*lv*), cucumber (*lh*) and Arabidopsis, that have an elongated phenotype, show no consistent differences in GA content compared to WT. Though some of these apparently contradictory results could be explained by inappropriate plant materials used for GA measurement, it seems clear that phy regulates both GA biosynthesis and GA signaling

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Photoperiodic regulation of gibberellin biosynthesis in long-day rosette plants

Jan A.D. Zeevaart, Dong J. Lee, and Yun-Ling Xu

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824-1312, USA. E-mail: zeevaart@msu.edu

Long-day (LD) rosette plants grow as rosettes and remain vegetative under short-day (SD) conditions, whereas under LD both stem growth and flower formation take place. There is extensive evidence that stem growth in rosette plants is mediated by gibberellins (GAs). The objective of our work is to determine which step(s) in the GA biosynthetic pathway is (are) limiting for production of bioactive GAs under SD, and how LD stimulates GA production. We are using two qualitative LD rosette species, spinach (*Spinacia oleracea*) and *Nicotiana sylvestris* (*Ns*.) to address these questions. The former is useful for preparation of cell-free extracts that can metabolize GAs (Gilmour et al., 1986), whereas *Agrobacterium*-mediated transformation can be readily achieved in the latter species.

The major GAs of spinach belong to the early-13-hydroxylation pathway: GA_{53}, GA_{44}, GA_{19}, GA_{17}, GA_{20}, GA_1, GA_{29}, and GA_8 (Talon et al., 1991). Previous work has shown that of these GAs, only GA_1 is active *per se* (Zeevaart et al., 1993). Transfer of plants from SD to LD causes an increase in all GAs of the 13-hydroxylation pathway, with GA_{20}, GA_1, and GA_8 showing the largest increases. A GA 20-oxidase has been cloned from spinach (Wu et al., 1996) whose expression is upregulated in various organs of plants moved from SD to LD, but especially in young leaves and shoot tips. This high level of GA 20-oxidase transcripts is correlated with a high GA_{20} content.

In addition to the 13-hydroxylation pathway, spinach is also a rich source of 2β-hydroxy-GA_{53}, now called GA_{97}. Smaller amounts of 2β-hydroxy-GA_{44} (=GA_{98}) and 2β-hydroxy-GA_{19} (=GA_{99}) are also found in spinach (Mander et al., 1996). At least in spinach, GA_{53} is at an important branch point: under LD conditions it is mainly converted via the 13-hydroxylation pathway to GA_{20}, whereas in SD it is preferentially converted to GA_{97}.

To demonstrate that GA 20-oxidase is limiting for GA production under SD, a GA 20-oxidase (*GA5=AtGA20ox1*) from Arabidopsis under control of the CaMV 35S promoter was introduced into *Ns*. When grown under SD conditions, the transgenic plants overexpressing *GA5* had a phenotype similar to that of plants grown in LD, or of plants in SD treated with GA. The GA 20-oxidase from Arabidopsis encoded by the *GA5* gene has preference for the non-13-hydroxylated substrate GA_{12} (Phillips et al., 1995). Thus, the possibility was explored that in transgenic *Ns/GA5* plants GA_{12} is preferentially converted via the non-13-hydroxylation pathway. The levels of members of the 13-hydroxylation pathway were lower in transgenic plants than in comparable *Ns* plants. This was especially striking for the C_{20}-GAs, GA_{53}, GA_{44}, and GA_{19}. *Ns*. plants accumulated relatively small amounts of GA_9 and GA_4, but much larger amounts of these GAs were present in the transgenic plants. In the case of GA_4, there was a 10-fold increase. Thus, in transgenic plants overexpressing *GA5*, the precursor GA_{12} is at an important branch point, being the substrate for both GA 13-oxidase ($GA_{12}\rightarrow GA_{53}$) and GA 20-oxidase ($GA_{12}\rightarrow GA_{15}$). In *Ns*. plants the former conversion predominates, because 13-hydroxylated GAs are present in much larger quantities than non-13-hydroxylated GAs. But in

the transgenic plants an abundance of GA 20-oxidase (constitutively expressed *GA5*) converts the bulk of GA_{12} to GA_{15}, which is then further converted to GA_9 and bioactive GA_4. When applied to *Ns.* plants, GA_4 has about the same activity as GA_1 in causing stem elongation and flower formation under SD conditions. Thus, we conclude that the much higher level of GA_4 in transgenic plants causes the long-day phenotype under SD conditions.

The *GA4* (=*AtGA3ox1*) gene from Arabidopsis encoding 3ß-hydroxylase has also been introduced into *Ns.* under control of the 35S promoter, but - unlike plants overexpressing 20-oxidase - the transgenic plants have no obvious phenotype. This indicates that the $GA_{20} \rightarrow GA_1$ conversion is not limiting GA production. However, transgenic plants expressing both the *GA4* and *GA5* genes show much more stem elongation under SD conditions than plants expressing *GA5* alone. This suggests that when more GA_9 is available due to overexpression of GA 20-oxidase, this GA_9 is rapidly converted to GA_4 by ectopically expressed GA 3ß-hydroxylase.

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Molecular genetics of gibberellin biosynthesis in *Gibberella fujikuroi*

Bettina Tudzynski

Westfälische Wilhelms-Universität Münster, Institut für Botanik
Schlossgarten 3, 48149 Münster, Germany

Gibberella fujikuroi produces large amounts of gibberellins under conditions of nitrogen limitation. In order to isolate genes of the gibberellin pathway, a differential screening of a cDNA library was performed. One of the isolated clones contained the conserved heme-binding motif of cytochrome P450 monooxygenases (FXXGXXXCXG). By sequencing the corresponding hybridizing 6.7 kb genomic *SalI* fragment, a second cytochrome P450 monooxygenase gene was found to be closely linked to the first one. Gene replacement experiments clearly demonstrated that both genes are involved in gibberellin biosynthesis. Since at least four cytochrome P450 monooxygenase-catalyzed steps were expected to participate in the synthesis of gibberellins, chromosome walking was perfomed to find further genes of this family or other genes involved in the gibberellin pathway. Next to the two P450 monooxygenase genes (*P450-1, P450-2*), a putative geranylgeranyl diphosphate synthase gene (*ggs-2*), the copalyl diphosphate/kaurene synthase gene (*cps/ks*), which was cloned by PCR before (Tudzynski et al. 1998), and two further P450 monooxygenase genes (*P450-3, P450-4*) were identified. Transcription of the 6 genes is co-regulated. Only recently a seventh gene which is highly expressed under GA producing conditions was found on the left side of the gene *P450-4*. These results suggest that at least most of the genes involved in the biosynthesis of gibberellins are closely linked and organized in a **gene cluster** in *G. fujikuroi* (Tudzynski and Hölter 1998). The function of several genes of this cluster was identified by gene replacement and following GC-MS and HPLC analysis of the mutants combined with feeding experiments. Another approach to find out the specific function of the genes is their expression in *G. fujikuroi* GA-defective mutants which totally lost the GA gene cluster and having large deletions on chromosome 4 (Linnemannstöns et al. 1999). By this procedure we, in collaboration with Peter Hedden (LAS, UK) and Cecilia Rojas (Chile), have already identified the multifunctional character of *P450-1*.

Furthermore, because of ammonium and glucose regulation of the gibberellin pathway, *areA*, *nmr* and *creA* genes coding for general transcription regulators were cloned from the fungus. Gene replacement of *areA* led to a significant reduction of gibberellin formation. Complementation of the *areA*-defected mutant with the wild-type copy of this gene completely restored the ability to produce gibberellins suggesting a direct transcriptional control of the pathway by the positive acting regulatory protein AREA (Tudzynski et al 1999).

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Session 3: Regulation of gene expression by gibberellin
Chair: Neil Olszewski

Gibberellins and G protein signalling in aleurone and *Arabidopsis*

Richard HOOLEY, Alison LOVEGROVE, Huw D JONES, Sally J SMITH, Jane NOLAN, Daniel COUCH and Konstantin KANYUKA

IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol, BS41 9AF, UK

The mechanism of action of gibberellin (GA) has been studied by cell and molecular biology in aleurone and by molecular genetics in *Arabidopsis*. In aleurone there is good evidence that GAs are perceived by a receptor at the plasma membrane. Two plasma membrane GA-binding proteins have been identified by photoaffinity labelling in aleurone and Arabidopsis and these may form part of a GA receptor complex[1]. The mastoparan analogue Mas7 stimulates GDP/GTP exchange by heterotrimeric G proteins thus mimicing activated G protein coupled receptors (GPCRs). When wild oat aleurone protoplasts are incubated with Mas7 they produced and secreted α-amylase in a dose-dependent manner and an identical time course to GA. The effect is largely overcome by ABA. This raises the possibility that Mas7 activates a heterotrimeric G protein in the GA signalling pathway. Further evidence supporting this has come from studying the effects of hydrolysis-resistant guanine nucleotides on GA-induction of *α-Amy2/54:GUS* expression. The hydrolysis-resistant GTP-γ-S and GDP-β-S bind to G_α subunits and hold them in either the activated (GTP-γ-S-bound) or inactivated (GDP-β-S-bound) form. GDP-β-S prevented GA induction of *α-Amy2/54:GUS* expression, whereas GTP-γ-S stimulated expression slightly. Taken together these data suggest that a heterotrimeric G protein or proteins are involved at an early stage of the GA signalling pathway in aleurone. It predicts that aleurone cells should contain G protein subunits and this has been confirmed by PCR and Northern analysis for a partial G_α subunit cDNA and two related G_β cDNAs[2].

Embryo-less half seed of the *Dwarf 1* mutant of rice produce very little α-amylase when treated with GA, and *Dwarf 1* seedlings elongate only slightly in response to GA[3]. Taken together these observations are consistent with *Dwarf 1* being a GA sensitivity mutant[3], although we await measurement of the endogenous GAs in *Dwarf 1* to see if they are elevated, as would be expected for a GA sensitivity mutant. Mutations giving rise to *Dwarf 1* are now know to be in the G_α subunit GPA1[4,5]. Because these clearly affect GA sensitivity of rice aleurone these observations add further support to the theory that a G protein is involved in GA signalling in aleurone.

In *Arabidopsis* a putative GPCR (GCR1) has been identified that may signal through a heterotrimeric G protein. Current evidence supports a role for this receptor in cytokinin signal transduction[6]. The presentation will consider the role of heterotrimeric G proteins and GPCRs in GA signal transduction.

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Gibberellin controls processes late in petunia flower development

David Weiss

Department of Horticultural, Faculty of Agriculture, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel

The process of petal growth and flower opening can be divided into two phases: a first, slow growth rate phase and a second, rapid growth phase. The early slow growth is caused mainly by cell divisions while the later rapid growth is caused only by cell expansion. In most flowers, the rapid growth of the petals coincides with pigment accumulation. We found that the transition of *Petunia hybrida* flower buds from the phase of cell division to the second phase of their development is controlled by gibberllin (GA). GA promotes cell expansion and is essential for anthocyanin accumulation in attached and detached corolla (1). Our results indicate that GAs are produced in the developing young anthers, then transported to the corollas to induce growth and pigmentation (3). GA is also required for normal anther development: inhibition of GA biosynthesis blocks anther development following microsporogenesis. The effect of GA on corolla and anther development results from the activation of various genes including those from the anthocyanin biosynthetic pathway (2,4). Our results suggest that GA controls master transcription regulators acting up-stream of the specific regulators of the anthocyanin pathways and other pathways involved in cell expansion. The activation of these pathways is required to complete the entire process of flower growth and opening. The early events in GA-signal transduction were studied and the results indicate that calcium, calmodulin and protein dephosphorylation are required for GA-induced gene expression (5). We also cloned the petunia homologue of the Arabidopsis GA-signal repressor, *spy* and studied its expression in corollas and anthers. Overexpression of SPY in transgenic petunia had no effect on flower development, and currently we are testing the effect of its suppression in antisense-transformed plants.

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Gene expression during fruit-set induced by gibberellins

Juan Carbonell, Manuel Cercós, Miguel A. Pérez-Amador, María Jesús Marcote, Cristina Urbez, David Alabadí, Xavi Cubells, Cristina Martí, Antonio Granell

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-Consejo Superior de Investigaciones Científicas, Valencia, Spain

Changes in gene expression have been analyzed during fruit set and early development induced by gibberellins in unpollinated ovaries of pea and tomato. In pea, treatment of the ovaries with gibberellic acid by two days after anthesis inhibits a senescence process and induces a rapid growth which is characterized by enlargement of the mesocarp and differentiation of the endocarp cells. In contrast, early fruit development in tomato is associated to cell division followed by cell enlargement. Our study on changes in gene expression induced by gibberellic acid has been directed to examination of genes involved in proteolysis, polyamine biosynthesis, and signal transduction pathways. Also analysis of gene expression by mRNA differential display was used to search for new GA-regulated genes.

Expression of several genes encoding cysteine proteases was found to increase during senescence of unpollinated pea ovaries (1, 6) which is in agreement with an active role of proteolysis in tissue degradation. Accordingly, the expression of these genes was prevented by gibberellic acid indicating that gibberellins may have a key role on the fate of the ovary. On the other hand, analysis by mRNA differential display in pea ovaries, treated or not with gibberellic acid, has shown changes in the expression of several genes. Identification and isolation of a serine carboxypeptidase that is induced associated to early stages of fruit development indicates that proteolysis has a role not only in ovary senescence but in early fruit development.

Changes in polyamine content have been correlated to events occurring during late ovary/early fruit development both in pea and tomato (3). We have found that the expression of genes coding for polyamine biosynthesis enzymes were transiently induced by treatment with gibberellic acid at early stages of fruit development (2, 4, 5). Results from polyamine biosynthesis and arginase activity suggest a redirection of nitrogen metabolism in early stages of fruit development.

Cloning of MAP kinases from pea ovaries resulted in the identification of *PsMAPK3*, whose expression was rapid and transiently induced by gibberellic acid and benzyladenine, but not by 2,4-D, that also induces fruit set. However, expression of other MAP kinase genes did not follow the same expression pattern. These results suggest that a variety of signaling pathways is active on fruit set and that some of them are controlled by gibberellins.

cDNAs homologous of Arabidopsis *SPY* and *GAI* have been isolated from pea and tomato. Transformation of tomato with *Atgai* under the control of 2x35S is beeing analyzed.

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GAMYB and SLENDER: roles in GA-regulated gene expression in barley aleurone cells

Frank GUBLER[1], Peter CHANDLER[1], Annie MARION-POLL[1,2], Jake JACOBSEN[1]

[1]CSIRO Plant Industry, GPO Box1600, Canberra 2602, AUSTRALIA, [2]INRA, 78026 Versailles cedex, FRANCE

Considerable progress has been made in determining the molecular mechanisms of gibberellin (GA) action involved in transcriptional control of hydrolytic enzyme gene expression in aleurone cells. We have focussed on identifying signal transduction components and transcription factors mediating GA-control of α-amylase gene expression. We have previously reported on the isolation and characterisation of a GA-regulated MYB transcription factor, GAMYB that binds specifically to a GA response element, the TAACAAA box, in an α-amylase gene promoter. We have shown in transient expression experiments that GAMYB is a transcriptional activator of α-amylase gene expression. Recent evidence indicates that it also activates the expression of a number of other GA-regulated genes in aleurone cells (1).

Studies are underway to determine the mechanisms for GA regulation of GAMYB expression and function. Nuclear run-on transcription experiments show the GA-induced rise in GAMYB mRNA and protein is due in part to an increase in the rate of GAMYB transcription. The GAMYB gene promoter has been isolated and fused to the GUS reporter gene as transcriptional and translational fusions to analyse control of GAMYB gene expression. Little difference was observed in the response of both constructs to GA compared with constitutive promoters indicating that cis-acting elements conferring GA-responsiveness are absent in the 6.4 kb genomic fragment. The cis-acting elements remain to be identified. Current investigations are also aimed at clarifying whether GA regulates GAMYB protein function. The presence of GAMYB protein and the absence of α-amylase expression in non-GA-treated aleurone cells support the idea that GA may regulate GAMYB function.

In addition to GAMYB, we have also investigated the role of SLENDER (SLN1) in GA-signaling in barley aleurone cells. Analysis of barley *slender1* (*sln1*) mutants indicates that SLN1 functions as a negative regulator of GA signal transduction in both aleurone cells and vegetative tissues. We have cloned the SLN1 gene in barley and shown that it belongs to a family that includes GAI, RGA and RHT in *Arabidopsis* and wheat. To understand how SLN1 is acting as a negative regulator we are currently examining the expression of SLN1 in aleurone cells in response to GA. We are also investigating the expression of GAMYB in a dominant dwarf *sln1* mutant that shows reduced responsiveness to GA in aleurone cells and expanding leaf tissues to determine whether GAMYB expression is regulated by SLN1. This mutant contains a mutation in the N-terminal region of the protein that presumably results in a negative regulator whose activity has reduced sensitivity to GA.

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Light control of plant development. A functional analysis of the *Arabidopsis thaliana* ATH1 gene

Marcel Proveniers, Steve Croker, Peter Weisbeek, Peter Hedden and Sjef Smeekens

In recent years it has become clear that many of the photomorphogenic responses initiated by phytochromes involve changes in metabolism of and/or responsiveness to phytohormones of the GA class. The molecular details of the interaction between the two pathways are not fully understood. In order to identify regulatory intermediates in light signal transduction, we have been investigating the role of light-regulated transcription factors in photomorphogenesis. Here, we describe a functional analysis of ATH1, a light-regulated *Arabidopsis thaliana* homeobox transcription factor gene (Quardvlieg et al., 1995). The ATH1 gene is highly active in the shoot apical meristem (SAM) and leaf primordia during early stages of seedling development. Prior to the switch from vegetative to generative development, ATH1 expression in the SAM is gradually down regulated and a function of ATH1 in floral transition is proposed. This hypothesis was tested by constitutive expression of ATH1 cDNA sequences in transgenic *Arabidopsis* and tobacco plants in either sense or antisense orientation. *Arabidopsis* plants with deregulated ATH1 expression displayed a flowering phenotype and were specifically affected in Rc-induced deetiolation and shade avoidance responses. In addition to Rc-hypersensitive deetiolation, ectopic expression of ATH1 in tobacco caused morphogological changes, including (semi)dwarfism due to reduced internode elongation and reduced apical dominance. When grown under LD photoperiods, flowering of these plants was delayed by up to ten months. Most aspects of this phenotype could be corrected by foliar applications of GA3. Quantitative analysis of intermediates in the GA biosynthetic pathway revealed that in severe phenotype ATH1 overexpressor plants levels of the biologically active gibberellin GA1 were dramatically reduced, due to a metabolic block of the conversion of GA19 to GA20, a step catalyzed by GA 20-oxidases. In addition, a disproportional reduction in GA1 content indicates that conversion of GA20 to GA1, catalyzed by 3b-hydroxylases, might also be inhibited. We conclude that ATH1 represents a positive mediator of a phyB-specific signal transduction cascade and controls various aspects of plant development by regulating the final steps of GA biosynthesis. We, therefore, propose that ATH1 integrates phyB-mediated environmental signals and endogenous growth regulators of the gibberellin-class to regulate a number of aspects of plant growth and development.

Session 4: Gibberellin perception and signal-transduction pathway
Chair: José Luis García-Martínez

Fusion genetic screens for GA signalling components

Carsten Meier, Thomas Bouquin, Dora Raventos, Mads Eggert Nielsen, Ole Mattsson and John Mundy

Dept. of Plant Physiology, Molecular Biology Institute, Copenhagen University, Oester Farimagsgade 2A, 1353 Copenhagen K, Denmark tel 45 35322131, fax 45 35322128, email mundy@biobase.dk

Most of the *Arabidopsis* GA-response mutants have been identified in screens for vegetative dwarfism, or for altered germination frequency. As an alternative, we used fusion genetic screens of gamma-mutagenized, transgenic plants expressing the firefly LUC and *E. coli* GUS reporters under control of two promoters differentially regulated by GA.

In one screen, the reporters were placed under control of the GA-responsive GASA1 promoter. Initial analyses determined the spatial and temporal patterns of reporter expression, and showed that reporter induction by GA was antagonized by ABA. M2 progeny with altered reporter activities were identified by LUC bioimaging followed by GUS assays and northern hybridization of the endogenous GASA1 mRNA. Genetic analysis showed that three mutants, which overexpressed both reporters and endogenous GASA1, were caused by recessive (GOE1&2, for GASA over-expressed) and semidominant (GOE3) mutations at different loci. These mutants were altered in their sensitivity to GA and the GA biosynthetic inhibitor paclobutrazol, and in the expression of several GA signaling related genes.

In a second screen, the reporters were under control of the GA-repressible, *GA5* GA 20-oxidase promoter. This bioimaging screen allowed the isolation of some 40 LUC-super-expressing (*lue*) and 40 LUC-low-expressing (*loe*)plants. Altered LUC expression was confirmed in progeny, and northern blots used to assess alterations in the expression of the endogenous *GA5* gene. The mutants were also found to have altered levels of mRNAs of genes involved in GA biosynthesis and metabolism (*GA4* & GA 2-oxidase), of genes encoding GA-signalling pathway components (*RGA*, *GAI*), and genes known to be GA-regulated (*GASA1*, *BIB*, *GLAB1*). Moreover, some mutants displayed GA-related growth phenotypes (giant and late flowering, *lue1*, *lue2* and *lue3;* dwarves, *lue4*, *lue5*) which may be associated with deficiencies in GA-biosynthesis and metabolism or in GA-perception. Backcrosses and progeny analysis indicated that all five of the *lue* mutations were inherited as monogenic recessive traits. The *lue5* mutation was mapped and will be described.

Gibberellin signal transduction presents ...The spy who *O*-GlcNAc'd me

Tina THORNTON, Tong-Seung TSENG, Lynn HARTWECK and Neil OLSZEWSKI

Dept. of Plant Biology, University of Minnesota, St. Paul, MN 55108-1095, USA

Manipulation of endogenous gibberellin (GA) levels affects virtually every stage of plant growth and development. The SPINDLY (SPY) protein is believed to be a negative regulator of the GA response pathway. Recessive loss-of-function mutations affecting SPY suppress the phenotypes of GA deficiency. Based on sequence comparisons, SPY is predicted to encode an *O*-GlcNAc transferase (OGT), a cytosolic and nuclear localized enzyme that in animals transfers single GlcNAc sugars to specific serine and/or threonine residues of target proteins. It has been shown that in animals *O*-GlcNAc modification can affect the localization, stability, phosphorylation status and/or activity of target proteins. Consistent with the hypothesis that SPY is an OGT, the pattern of GlcNAc modification is altered in *spy* mutants. Moreover, SPY produced in insect cells has OGT activity toward gp40, a tobacco nuclear pore protein that is known to be *O*-GlcNAc modified, and RGA, a component of the GA signal transduction pathway. As additional components of the GA signal transduction pathway become available, the activity of insect cell expressed SPY toward these proteins is being assessed.

Cell fractionation experiments with cauliflower inflorescence and arabidopsis seedlings were performed to determine the subcellular localization of SPY. Antisera against SPY detects a protein the same size as SPY in both the subcellular fraction enriched for soluble cytosolic proteins and in the nuclear-enriched fraction, suggesting that, like animal OGTs, SPY is present in both the cytosol and the nucleus. These studies also found that, while SPY is present at very low abundance in all tissues, it is most abundant in the cauliflower inflorescence. While SPY from insect cells has a molecular mass consistent with it being a trimer, SPY from cauliflower and arabidopsis has a predicted molecular mass of 850 kDa suggesting that it is complexed with other proteins. Interestingly, highly purified OGT from animal cells is also a trimer, raising the possibility that the studies of this enzyme have not characterized the native enzyme. The predicted sizes of the nuclear and cytosolic SPY complexes are similar suggesting that the complexes in these compartments are very similar or identical.

Experiments have been initiated to characterize the activity and protein components of the SPY complex. Two-hybrid screens have identified several proteins that interact with SPY in yeast cells. Experiments to confirm that these proteins interact with SPY *in vitro* are ongoing and several of the proteins have been shown to interact *in vitro*.

Expressed sequence tags for a second possible arabidopsis OGT have been identified. Genomic clones of the gene for this second OGT have been isolated and sequenced. Lines expressing antisense RNA to this OGT have been generated and two line with a T-DNA inserted into the gene have been identified. The insertion and antisense lines are being characterized for phenotypes associated with defects in GA signal transduction as well as for alterations in the pattern of protein *O*-GlcNAc modification.

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RGA, a repressor of gibberellin signaling in *Arabidopsis*

Aron L. Silverstone[2], Alyssa Dill[1], Hou-Sung Jung[1] and Tai-ping Sun[1]

[1]Department of Botany, Duke University, Durham, NC 27708, USA [2]Current addresss: Novartis Agribusiness Biotechnology Research, Inc., NC 27709, USA

The recessive *rga* mutation is able to partially suppress phenotypic defects of the Arabidopsis gibberellin (GA) biosynthetic mutant *ga1-3*, which include stem elongation, flowering time, and leaf abaxial trichome initiation. This indicates that *RGA* is a negative regulator of the GA signal transduction pathway. The *RGA* belongs to the GRAS regulatory protein family, which includes the radial patterning gene *SCARECROW* and another GA signal transduction repressor, *GAI*. The presence of several structural features, including homopolymeric serine and threonine residues, a nuclear localization signal and leucine heptad repeats, indicates that the RGA protein may be a transcriptional regulator that represses the GA response. In support of this, we showed that a green fluorescent protein (GFP)-RGA fusion protein is nuclear localized in transgenic Arabidopsis plants, and this fusion protein can complement the *rga* mutation. Confocal microscopy and immunoblot analyses demonstrated that exogenous GA treatment resulted in a reduced level of the GFP-RGA protein. This suggests that modulating RGA protein level by the GA signal is one of the mechanisms that allows expression of genes repressed by RGA. We are now examining the effect of GA on the level of the endogenous RGA protein using RGA antibody.

Comparing the GRAS family proteins, we have identified 3 conserved domains (named after motifs of highly conserved amino acid residues): the N-terminal DELLA domain, the central VHIID domain, and the C-terminal RVER domain. The DELLA domain is unique to RGA, GAI and RGL (for RGA-like). Peng et al. (1997) found that the semi-dominant *gai* mutant contains a 17-amino acid in-frame deletion within the DELLA domain. They proposed that this small deletion turns the gai protein into a constitutively active repressor of GA signaling. Because the sequences that are deleted in gai are identical between GAI and RGA, we tested whether an identical deletion mutation (*rga-□17*) in the *RGA* gene would have the same effect as the *gai* allele. Our results showed that the *rga-□17* allele is semi-dominant and causes a GA-unresponsive dwarf phenotype in transgenic Arabidopsis plants. Therefore, the DELLA domain is likely to be important for the inactivation of both RGA and GAI by the GA signal.

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Hormonal regulation of cell death in barley aleurone

Paul Bethke, Angelika Fath, Jennifer Lonsdale and Russell Jones

Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102. E-mail: rjones@nature.berkeley.edu

Gibberellic acid (GA) initiates a series of events in the cereal aleurone that culminate in death of the cell (Kuo et al. 1996, Bethke et al. 1997). Abscisic acid (ABA) prevents the induction of many GA-stimulated responses in the aleurone cell, including changes in cytosolic calcium and calmodulin, the synthesis and secretion of hydrolases, and cell death (Bethke et al. 1997). We have undertaken a detailed study of GA-induced cell death in the aleurone of barley to establish how these cells die. Do aleurone cells follow the apoptotic route of death characterized by condensation of chromatin, the cleavage of DNA into internucleosomal fragments differing by ca. 180 bp, and the fragmentation of the cytoplasm into apoptotic bodies? Our evidence indicates that aleurone cells do not follow the apoptotic route of cell death. Electrophoresis of nuclear DNA from dying aleurone cells shows that internucleosomal cleavage of DNA does not occur in dying aleurone cells (Fath et al. 1999). Aleurone cells do not form apoptotic bodies when they die. Rather, cells undergo autophagy whereby organelles are lost and the volume of the vacuole increases to almost fill the entire cell volume. Cell death occurs only when the GA-treated cell has reached the highly vacuolated stage. ABA prevents changes in DNA and cell structure, and aleurone cells incubated in ABA remain viable for up to 6 months (Bethke et al. 1999). Highly vacuolate GA-treated aleurone cells die as a result of the rupture of the plasma membrane. Death is dramatically hastened if GA-treated aleurone cells are exposed to light. ABA-treated cells on the other hand are insensitive to light. The action spectrum for cell death shows maxima in the blue and UV, typical of the absorption spectrum of flavins. Cell death brought about by light is indistinguishable from death brought about by GA, and it is manifested by changes in membrane permeability, rupture of the plasma membrane and loss of turgor. Using fluorescent dyes that are sensitive to H_2O_2 we have shown that blue and UV light stimulate H_2O_2 production in aleurone cells and the rate at which H_2O_2 is produced is higher in GA-treated than ABA-treated cells. Addition of H_2O_2 also dramatically stimulates death of GA-treated cells. After exposure of GA-treated cells to 1% H_2O_2 all cells die within 40 min, however, ABA treated cells are insensitive to 1% H_2O_2. Treatment of GA-treated aleurone cells with antioxidants such as ascorbate or BHT also reduces the rate of cell death, including death that is brought about by light. Taken together, these data strongly implicate H_2O_2 in aleurone cell death in GA-treated, but not ABA-treated cells. Catalase and other enzymes such as superoxide dismutase (SOD) that catabolize reactive oxygen species (ROS) are key enzymes in the response of aleurone cell to GA and ABA. Cells incubated in GA show a dramatic reduction in the activities of catalase and SOD, whereas the amounts of these enzymes increase in ABA-treated cells. We hypothesize that ABA-treated aleurone cells rapidly metabolize ROS, but that GA-treated aleurone cells cannot effectively metabolize these molecules. As a result, the amounts of ROS rise in GA-treated aleurone cells bringing about the oxidation of membrane lipids leading eventually to rupture of the plasma membrane.

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The expression pattern of the *SHI* gene is consistent with a role of SHI in the GA signal transduction pathway

Ingela Fridborg, Thomas Moritz and Eva Sundberg

Dept. of Physiological Botany, Uppsala University, Villav. 6, S-752 36 Uppsala, Sweden

shi (*short internodes*) is a semidominant dwarfing mutation in Arabidopsis, caused by a transposon insertion leading to overexpression of the *SHI* gene (Fridborg et al., 1999, Plant Cell 11:6). The *shi* mutation confers a phenotype typical of mutants defective in the biosynthesis of gibberellin, GA. However, application of exogenous GA on *shi* does not correct the dwarf phenotype, which suggests that *shi* is defective in the perception of, or the response to, GA. In agreement with this, the level of active GAs is elevated in *shi*, which is an expected result of reduced feedback control of GA biosynthesis.

SHI is a member of a small gene family encoding putative transcription factors with a single zinc finger motif. The expression pattern of *SHI* was examined using RT-PCR analysis and promoter::GUS fusion constructs. *SHI* was shown to be expressed in meristematic and expanding tissue, e.g. root tips, emerging lateral roots, developing shoots, young expanding leaves and developing flowers. This expression pattern is very similar to that of the GA biosynthesis gene *GA1* (Silverstone et al., 1997, Plant Journal 12:1), which is consistent with a role of *SHI* in the GA signal transduction pathway.

As most of the dwarfed putative GA signal transduction mutants are isolated in the semidwarfed L*er* background, we introduced *shi* into L*er*+ plants (lacking the *erecta* mutation). In this wild-type ERECTA background the *shi* mutation did not show a strong dwarf phenotype, suggesting that the *erecta* mutation is necessary for the penetrance of the *shi* mutant phenotype.

Session 5: Genetic manipulation of gibberellin biosynthesis and action
Chair: Russell L. Jones

Modifying gibberellin dioxygenase gene expression in transgenic plants

Andrew L. Phillips[1], Jeremy P. Coles[1], Stephen G. Thomas[1], Stephen J. Croker[1], Ian S. Curtis[2], Michael R. Davey[2], and Peter Hedden[1]

[1]IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, U.K.; [2]Plant Science Division, School of Biological Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, U.K.

GAs are involved in the regulation of a number of agronomically-important developmental processes in plants, notably shoot elongation and fruit development. The manipulation of GA levels in plants is, therefore, widely practised in agriculture and horticulture. Increased GA levels are achieved through the application of bioactive GAs while reduced GA levels are produced by the use of growth regulators that act through the inhibition of enzymes of the GA biosynthetic pathway. The safety of some synthetic plant growth regulators in use today has been questioned: the genetic manipulation of GA biosynthesis offers an environmentally-friendly, alternative strategy to controlling crop development. The analysis of levels of GA intermediates and activities of the biosynthetic enzymes, and more recently the analysis of transcript levels for these enzymes has suggested that the dioxygenases that catalyze the later reactions in the biosynthetic pathway are affected by factors such as light and feedback by bioactive GAs. This suggests that these enzymes catalyse regulatory steps in GA biosynthesis and are ideal targets for genetic manipulation.

GA 20-oxidase catalyses multiple consecutive steps resulting in the formation of the C_{19}-GAs and in Arabidopsis is encoded by a small multigene family with tissue-specific expression patterns (Phillips *et al.*, 1995). Overexpression of any of the genes in transgenic Arabidopsis using the CaMV 35S promoter results in increases in bioactive GAs and a phenotype that includes reduced seed dormancy, increased hypocotyl length, early flowering and increased stem elongation (Huang *et al.*, 1998; Coles *et al.*, 1999). This suggests that 20-oxidase activity limits bioactive GA levels in a number of plant tissues. Suppression of 20-oxidase transcript accumulation in Arabidopsis by expression of antisense RNA results in GA-deficient plants whose phenotype reflects the expression pattern of the gene family member: reduction in *GA20ox1* (*GA5*) expression yields seedlings with short hypocotyls and reduced rates of stem elongation. Antisense expression of *GA20ox2* reveals a phenotype only in short days, with a reduction in floral internode lengths, while antisense expression of *GA20ox3* yielded one line with short hypocotyls, suggesting a hitherto unknown role for this gene in early seedling development (Coles et al., 1999).

A GA 20-oxidase expressed in developing endosperm and cotyledons of pumpkin is unusual in that it produces only a small amount of the C_{19}-GA precursors to the bioactive GAs, its principal products being the C-20-carboxylic acids (Lange *et al.*, 1994). As these are inactive and cannot be converted to active forms, overexpression of this enzyme should, in theory, result in diversion of intermediates in the pathway into inactive by-products. In *Solanum dulcamara*, overexpression of the pumpkin cotyledon 20-oxidase results in a marked reduction in the GA_1 content of leaves and stems and a huge increase in the levels of GA_{17}, and the plants have small, dark-green leaves and reduced stem extension (Curtis, Phillips, Davey and Hedden, unpublished). Levels of non-13-hydroxy-GAs are less affected or even increased, possibly due to a combination of preference of the pumpkin 20-oxidase for non-13-hydroxy

substrates and up-regulation of the endogenous GA 20-oxidases by the feedback mechanism. In contrast, overexpression of a pumpkin endosperm 20-oxidase in Arabidopsis has only small effects on stem elongation (Coles, Phillips & Hedden, unpublished; Xu *et al.*, 1999), suggesting that the effects of overexpressing this enzyme differ between species and may be unpredictable.

An alternative strategy of reducing bioactive GA levels is to increase GA turnover by overexpression of GA 2-oxidase, a multifunctional enzyme that inactivates GAs by hydroxylation at the 2β position and also carries out a further oxidation to yield the GA-catabolites (Thomas *et al.*, 1999). Overexpression of GA 2-oxidase in Arabidopsis using the CaMV 35S promoter results in a reduction in GA_4 levels and a very large increase in 2β-hydroxy-GAs and GA-catabolites (Thomas, Phillips & Hedden, unpublished). The level of expression of the transgene determines the severity of the resultant phenotype, which range from extreme dwarf rosettes to plants that have a wild-type growth habit but that have elevated expression of GA 20-oxidase and GA 3β-hydroxylase genes, due to the operation of the feedback mechanism. Using this approach we have also produced transgenic lines of other crop and ornamental species including wheat, sugar beet, *Petunia* and *Nicotiana* with potentially useful GA-deficient phenotypes.

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Control of tuberization by gibberellins

Virginia Amador, Jordi Bou, Esther Carrera, Elena Monte, Mariana Rodríguez and Salomé Prat

Dpto. de Genética Molecular. Centro de Investigación y Desarrollo- C.S.I.C. Jordi Girona, 18-26, 08034 Barcelona, SPAIN. Tel. 34 93 400 61 89. FAX 34 93 204 59 04. E-mail spmgms @cid.csic.es

Gibberellins (GAs) are cyclic diterpenoid hormones that regulate many plant growth and developmental processes, including germination, stem growth, flowering and fruit development (Swain and Olszewski, 1996; Lange, 1998). In potato (*Solanum tuberosum*) they have an inhibitory effect on tuberization. Gibberellin activity decreases under conditions that promote tuber formation such as short days and increases in plants subjected to conditions which inhibit tuberization. Decreased levels of GA_1 were also observed in stolon tips during the early stages of tuber induction (Xu et al. 1998) and in this model system, the application of inhibitors of GA-biosynthesis like paclobutrazol or ancymidol was able to promote tuber formation. Hence, these studies have allowed to establish a strong correlation between decreased levels of GA activity and tuber initiation.

We have recently isolated three cDNA clones (*StGA20ox1*, *StGA20ox2* and *StGA20ox3*) encoding potato GA 20-oxidase (Carrera et al., 1999), a key enxyme in the GA biosynthetic pathway (Hedden and Kamiya, 1997). Characterization of these cDNA clones showed that they encode functionally identical enzymes with different patterns of tissue-specific expression. Gene *StGA20ox1* is abundantly expressed in shoot tips and leaves, and exhibits lower levels of expression in stem, stolons and tubers. We have shown that expression of this gene is regulated by daylength, suggesting that it may be involved in photoperiodic regulation of GA-biosynthesis and thus play a role in tuber induction in response to short days (SD). To investigate this possibility, we have obtained transgenic potato plants expressing sense and antisense copies of the *StGA20ox1* cDNA. Over-expressing transformants were taller than control plants, had lighter-green leaves and required a longer duration of SD to tuberize. Moreover, tubers obtained from these plants had a decreased time of dormancy and developed sprouts with highly elongated internodes. Antisense transformants had, in opposite, shorter stems, a decreased length of the internodes and in SD tuberized earlier than control plants. Tuber yield of these plants was increased, with a higher number of tubers produced per plant and a slight increase in tuber size. Expression of the antisense construct had no visible effect on the time of dormancy of the tubers, but at end of dormancy tubers formed sprouts with reduced internode length. These results demonstrate the involvement of the GA 20-oxidase activity encoded by *StGA20ox1* in the control of stem elongation and in tuber induction and indicate that another member of the gene family could be responsible for regulation of tuber dormancy (Carrera et al., 2000).

We have obtained as well transgenic plants that expressed a sense construct for the potato 3β-hydroxylase clone *StGA3ox1* under control of a double 35S promoter (Bou et al., in preparation). Transformants over-expressing this gene were taller than the control plants but to our surprise they tuberized in SD earlier than control plants. A co-suppression effect was observed in few transformants. Co-suppresed plants were shorter than the controls and

tuberized later than these when transferred to SD. Although very preliminary, these results may be indicative of a regulatory activity of GA_{20} in the control of tuberization, with GA_1 being mainly active in control of plant stature.

Interestingly, manipulation of the levels of expression of the GA-biosynthetic enzymes results in faster or slower tuberization in SD but is not able to overcome the requirement of SD conditions for tuber formation. In a similar way, the dwarf *ga1* mutant of *Solanum tuberosum* ssp. *andigena*, which is blocked in the 13-hydroxylation step catalyzing conversion of GA_{12} into GA_{53} (Bamberg and Hanneman, 1991), forms readily tubers after 4 days in SD but in LD conditions requires more than 5 months to tuberize. A different tuberization behaviour is exhibited by the *andigena* transformants bearing an antisense construct for the phytochrome *phyB* gene (Jackson et al. 1996). These plants (α-phyB) are able to tuberize in LD, with tubers being already observed 1 month after the plants were transferred to soil. Paradoxically, these transformants show also a characteristic elongated stem as if they would overproduce GAs. Therefore, their phenotype can not be simply explained by a change in endogenous levels of GAs. A change in GA sensitivity or constitutive activation of a tuberization inductive signal appears to be responsible for the photoperiod independent tuber induction in these plants. To investigate whether a change in GA sensitivity would result in tuber formation in LD, we have obtained *andigena* plants that expressed the *Arabidopsis* dominant *gai* mutation under control of the 35S promoter. We will report on the phenotype of these transformants and of the double α-*phyB*/*gai* transformants. Data will also be presented on a cDNA clone designated as *PHOR1* (from photoperiod responsive), isolated by differential display of leaves of plants induced to tuberize (grown in SD) and non-induced to tuberize (SD+NB). Antisense plants for this gene tuberized earlier than control plants and showed a dwarf phenotype that could be partially reversed by exogenous application of GAs. Studies of subcellular localization of the protein by fusion to GFP-GUS showed a cytosolic location of the fusion protein in the presence of ancymidol but a rapid migration to the nucleus upon treatment with GA_3. These results would agree with a function of PHOR1 in regulation of GA sensitivity or in GA signal transduction.

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Genetic manipulation of gibberellin biosynthesis in trees

Thomas Moritz, Maria E. Eriksson, Maria Israelsson

Umea Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83 Umea, Sweden

Gibberellins have been shown to affect many physiological aspects of growth in woody species. Hormone application studies have shown that GAs have effect on seed germination, differentiation of xylem fibres, the length of secondary xylem fibres, and longitudinal and radial growth. By modifying GA levels by transforming trees with genes encoding GA biosynthetic enzymes, it will be possible to both study the role of GAs in controlling different aspects of growth and development in trees, but also evaluating the possibility of practical implications (assisting in the production of modified trees of interest to the pulp, paper and forest industries). We will present data from transgenic hybrid aspen (*Populus tremula x P. tremuloides*) expressing either the *PHYA* gene from oat, or the GA 20-oxidase from *Arabidopsis*, showing the importance of GAs in controlling shoot elongation and secondary growth in trees. Hybrid aspen expressing the oat phytochrome A has reduced internode length and reduced GA levels. By application of GA_4 it is possible to restore the height growth showing that the reduction of biological active GAs is at least partly the cause of the dwarf appearance. Transgenic hybrid aspen overexpressing GA 20-oxidase show the complete opposite phenotype, with increased height growth, and interestingly, a delay in short-day induced shoot growth cessation. The later confirming the importance of GAs in controlling short-day induced growth cessation. Furthermore, the transgenic trees also show that the increase in GA levels results in increased secondary growth, increased biomass production and longer xylem fibres. This shows that GAs are involved in many aspects of growth and development in trees, but also implies that by genetically modification of GA levels in trees improvements in valuable traits such as growth rate and biomass are obtained.

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GAI: modulator of gibberellin responses

Nicholas Harberd, Tahar Ait-ali, Barbara Fleck, Lewin Hynes, Kathryn King, Jinrong Peng and Donald Richards

Molecular Genetics Department, John Innes Centre, Colney Lane, Norwich, NR4 7UJ, UK

Recent decades have seen large increases in world wheat grain yields. This so-called 'Green Revolution' is in part due to the widespread adoption of varieties containing the semi-dwarfing *Rht* mutant alleles, and of associated changes in agricultural practise. These yield increases have enabled wheat productivity to keep pace with the demands of the rising world population. Like *Rht* mutant alleles in wheat, the *gai* mutant allele of *Arabidopsis* confers a semi-dominant dwarf phenotype, and a dramatic reduction in responsiveness to the plant growth hormone gibberellin (GA). The *Arabidopsis GAI* and *RGA* genes have been cloned. These genes encode closely related proteins (GAI and RGA) that are members of a class of putative transcriptional regulators defined by the *Arabidopsis* SCR product. Recently, we have found that the DNA sequences of *Rht* (and *D8*, the maize orthologue) are closely related to those of *GAI/RGA*. Analysis of mutant *D8* and *Rht* alleles suggests that the wild-type proteins (GAI, RGA, D8, Rht) are bi-partite in structure, with an N-terminal GA-specificity domain, and a C-terminal putative transcriptional regulator domain. These proteins appear to act as repressors of stem elongation, whose activity is opposed by GA. The mutant proteins are altered in ways that make growth repression relatively resistant to the effects of GA. The significance of these findings for future plant improvements will be explored.

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POSTERS

Cloning of a gene encoding an *ent*-copalyl diphosphate synthase in the hybrid citrange Carrizo and analysis of its parentals

C.P. Alapont [1,2], A.M. Vidal [1,2], M. Talón[1] and J.L. García- Martínez[2]

[1] Instituto Valenciano de Investigaciones Agrarias, Moncada , Valencia; [2] Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia, Valencia.

Two cDNA clones have been isolated by PCR using degenerated oligonucleotides from citrange Carrizo (a hybrid from *Citrus sinensis* x *Poncirus trifoliata* extensively used like a rootstock in citrus). The sequences of those clones suggest that they encode *ent*-copalyl diphosphate synthases (*CcCDS1* and *CcCDS2*). A full length cDNA clone (*CcCDS1* gene) has been isolated by RACE-PCR that encodes a protein of 821 amino acids, which is being characterised by the enzymatic activity of the expression products of the gene in *E. coli*.

Because of the low levels of CDSs transcripts, we are using QRT-PCR technique to quantify the levels of those transcripts in different tissues of citrange Carrizo, and to know the effects of GAs and GAs biosynthesis inhibitors on *CcCDS1* expression levels.

To know the parental source of *CcCDS1* gene in the hybrid we have amplified cDNA from *C. sinensis* and *P. trifoliata* with *CcCDS1* specific primers. We are currently sequencing the amplified bands, which will allow to know the source of *CcCDS1* and, additionally to clone another CDS gene from the other parental.

Gibberellins and extracellular proteins expressed during fruit set in pea

José Pío Beltrán, Alicia Pérez-García and Manuel Rodriguez-Concepción

Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC). Camino de vera s/n. 46022-Valencia. Spain. E-mail:jbeltran@ibmcp.upv.es

Induction of fruit set and development in pea is regulated by gibberellins after fertilization of the ovules. Pollination and fertilization of the ovules can be prevented by removing the anthers from the flowers two days before anthesis. Before they enter senescence, unpollinated carpels can be stimulated to develop by exogenous aplication of gibberellic acid, giving rise to parthenocarpic fruits with the same morphology as those arisen from fertilization. The transition from the carpel of the flower to a developing fruit is not a well characterized process despite its agricultural importance. We have analyzed early changes in gene expression in pea carpels induced to develop by gibberellins by differential screening of a pea carpel cDNA library enriched by subtraction for carpel specific messages. We have identified two genes, *GIC19* and *GIC4* which are expresed after induction of pea fruit set by gibberellins. Analysis of their sequences and localization of fusion proteins with GFP indicate that both GIC19 and GIC4 are extracellular proteins. While GIC19 is a small proline-rich protein with no overall homology to other reported proteins, gic4 belongs to a novel family of proteins. Our results reinforce a model of gibberellin mode of action during pea fruit set and development involving enhanced synthesis of extracellular proteins and secretory activity to provide materials and energy for cell growth.

The promotion of Flowering by Gibberellins in Arabidopsis

Miguel A. Blázquez and Detlef Weigel
Plant Biology Lab. The Salk Institute, La Jolla, CA.

Flowering in *Arabidopsis*, as in many other plants, is regulated by both environmental and endogenous factors. This is particularly evident for photoperiod, since *Arabidopsis* can flower under short days, but it flowers earlier under long days. On the other hand, the role of endogenous factors is revealed by the need that plants acquire certain competence before they initiate the formation of flowers even under inductive conditions. There are three reasons why gibberellins (GAs) are thought to be an important regulator of flowering time in *Arabidopsis*, particularly under non-inductive conditions: first, exogenous application of GA_3 decreases flowering time; second, mutants defective in GA biosynthesis flower late under long days, and particularly severe mutations like *ga1-3* abolish flowering under short days; and third, mutants with constitutively high GA signaling flower early.

GAs are involved in the regulation of many developmental processes in *Arabidopsis*, from seed germination to cell elongation, so one relevant question is how direct the connection between GAs and flowering is. For instance, the effect of GAs on flower initiation might be a secondary consequence of a more basic effect on cell division or metabolism; alternatively, GAs might promote flowering through a specific branch of GA signaling. To discriminate between these and other possibilities, we have embarked in the isolation and analysis of suppressors of the flowering defect caused by *ga1-3*, which might define a GA-signaling branch different to the ones that may control other GA responses. We have isolated mutations at 4 different loci named *FOG* (for Flowering Of GA-deficient mutants). Two of them, in *FOG1* and *FOG2*, seem to suppress primarily the flowering defect, while mutations in *FOG3* and *FOG4* also suppress the size defect and the lack of trichome initiation characteristic of *ga1* mutants, and the mutation in *FOG4* also suppresses the germination defect. *FOG3* has turned out to be allelic to *RGA*. Interestigly, mutations in *FOG1* and *FOG2* reduce flowering time under short days in a *GA1*$^+$ background. One concern is that FOG1 and FOG2 might be involved in the activation of a GA-independent pathway for the promotion of flowering, such as the one induced by long days and mediated by CONSTANS. This possibility can be tested genetically, but preliminary analysis of the *fog* mutants indicates that all of them show enhanced sensitivity to GAs, which would place them in the GA signaling pathway.

AFLP experiment designed to investigate the role of Gibberellins in *Arabidopsis* germination

J. G. De Diego[1], F. D. Rodríguez[1], E. Battaner[1], P. Grappin[2] and E.Cervantes[3].

1: Dep. Bioquímica & Biología Molecular. Edificio Departamental, lab 125 Campus Unamuno, Universidad de Salamanca,37007 Salamanca, Spain. Laboratoire de Biologie des semences. INRA-Versailles. France. IRNA-CSIC. Apartado 257. Salamanca. Spain.

An experiment based on the AFLP protocol was designed to isolate cDNA fragments induced during *Arabidopsis thaliana* seed germination. Poly A+ RNA from wild type Wassilejska seeds as well as mutant ABC33 (GAI1) , 24 hours after imbibition, was isolated and reverse transcribed. Sequences from both cDNAs were amplified according to the AFLP protocol described by Bachem *et al.* (1996), and the resulting radiolabelled PCR products were ran on acrylamide gels. Bands showing specific expression on wildtype or mutant seeds were colected and re-amplified, cloned and sequenced. The aminoacid sequences derived from the obtained nucleotide sequences showed among the cDNAs several putative transcription factors and other proteins that may be involved in the control of *Arabidopsis* seed germination. One of the transcription factors, belongs to the b-Zip family and corresponds to a gene already sequenced, thus making straightforward the analysis of its promoter region. Other isolated cDNAs encode proteins with diverse degrees of homology with ABA induced proteins, titin, cytochrome oxidase and others. Some data concerning the regulation of their mRNAs during germination will be presented.

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Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length.

Maria E. Eriksson[1], Maria Israelsson[1], Olof Olsson[2], Thomas Moritz[1]

[1]Department of Forest Genetics and Plant Physiology, The Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden [2]Department of Cell and Molecular Biology, Göteborg University, Box 462, SE-405 30 Göteborg, Sweden.

Important aims in all tree-breeding programs around the world are to produce plants with increased growth rate, shorter rotation time and increased stem volume. Such trees would yield more biomass per area unit. Here we show that when ectopically over-expressing a key-regulatory gene in the biosynthesis of the plant hormone gibberellin (GA) in hybrid aspen, (*Populus tremula x P. tremuloides),* these traits are obtained.
GA content in *AtGA20ox1* over-expressers was determined both in leaves and internodes of actively growing tissue. Transgenic plants showed high levels of the 13-hydroxylated C_{19}-GAs; GA_{20}, GA_1 and GA_8, and the non 13-hydroxylated C_{19}-GAs; GA_9, GA_4 and GA_{34}, in both stem and leaves. The increase was more pronounced in stem tissue than in leaf tissue. The levels of the biological active GA_1 and GA_4 in stem tissue of line 7 were 22- respectively 24-fold higher than in the control. Furthermore, all transgenic lines showed lower levels of the substrates for the GA 20-oxidase. For example GA_{12} and GA_{53} levels in stem tissue of line 7 were 17 % and 9 % of the contents in the control, respectively.
In addition, these trees also display xylem fibres with an increased length compared to unmodified wild type (wt) plants. We also show that GA has an antagonistic effect on root development, since the transgenic plants had a decreased root mass compared to wt at early stages of development. However, the negative effect on rooting efficiencies of regenerating plants in tissue culture and on the initial establishment of young plants in the green house, did not negatively affect growth at later stages.

GAI- a negative regulator of gibberellin mediated growth in *Arabidopsis thaliana*
B. Fleck, T. Ait-Ali, K. King, J. Peng, D. Richards, N. Harberd

The semidominant *gai* mutation in *Arabidopsis* confers a dwarf phenotype with reduced GA responses. Loss of GAI results in a tall wild type looking phenotype and plants homozygous for *GAI* null alleles are impaired in GA signal transduction.
GAI encodes a predicted 532 aa protein containing several domains which may be functionally important. Among others, sequence comparison identified two Nuclear Localisation Sequences and the VHIID domain that characterises the GRAS gene family of putative transcription factors in Arabidopsis. GAI shows sequence similarity to Signal Transducers and Activators of Transcriptions (STAT proteins) over a putative SH_2 domain and a conserved C-terminal tyrosine. STAT proteins have been identified in non-plant species, they elicit transcription by interacting with a receptor mediated signal.
We are interested in investigating the importance of these domains thereby understanding more about the role of GAI in GA mediated growth.
Sequence analysis of *GAI* null alleles identified a mutant that contains a small in frame deletion in the putative SH2 domain of GAI. We propose that this deletion is responsible for restoring the tall phenotype. To study nuclear localisation of the GAI protein, translational fusions of GAI with the Green Fluorescent Protein are being expressed *in planta*. To examine how the SH_2 domain, NLS and other consensus motifs in the protein affect cellular trafficking of GAI, site directed mutagenesis of GAI was carried out and analysis of the transgenic plants expressing GAI-GFP fusions is in progress.

Regulation of *GA1* gene expression during early seedling development in Arabidopsis

Christine Fleet, Chien-wei Chang, and Tai-ping Sun

Biosynthesis of gibberellin (GA) is regulated by both environmental and endogenous factors to produce appropriate hormone levels, regulating plant growth and development in processes including seed germination and stem elongation. In *Arabidopsis thaliana*, the enzyme for the first committed step of GA biosynthesis is encoded by the *GA1* gene. We are interested in identifying factors that regulate expression of *GA1* to better understand how plants control GA biosynthesis.

GA1 shows a tissue specific expression pattern, corresponding to rapidly growing tissues including root tip, shoot apex, flowers, seeds, and leaf vasculature (Silverstone *et al*, 1997. Plant J. 12: 9-19). To identify endogenous factors that may regulate *GA1* expression, we are conducting a screen for mutations in *trans* -acting regulators of *GA1*. We are using ethyl methane sulfonate-mutagenized transgenic plants containing two copies of a *GA1* promoter-beta glucuronidase (GUS) reporter gene construct to identify plants with a change in the GUS expression at the seedling stage. Putative mutants have been identified which have altered staining, producing over- or under-expression or tissue-specific changes in GUS reporter staining. Lines with decreased GUS expression show a GA-deficient phenotype and GA-responsiveness, while those with increased GUS expression have a near wild-type morphology. Expression of the endogenous *GA1* gene in selected lines has been measured by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), and these results are in agreement with the observed GUS activity levels. A mapping population for several mutant lines is being generated by crossing mutants in the *Ler* background to Colombia plants containing the *GA1-GUS* reporter.

Additional work involves over-expression of *GA1* in Arabidopsis to determine any phenotypic effect and associated changes in overall GA biosynthesis. Transgenic plants have been generated containing the *GA1* coding region under the control of the 35S-CaMV promoter (Sun and Kamiya. Plant Cell. 1994 6: 1509-1518) in either the *Ler* or *ga1-3* backgrounds. Although some lines express high levels of GA1 protein, they show a near-wild type morphology in either background. These lines are being further characterized for any subtle morphological differences, and for expression levels of downstream GA biosynthetic genes (*GA4* and *GA5*), as an indication of feedback regulation within the biosynthetic pathway.

FUNCTIONAL ANALYSIS OF *ENT*-KAURENE SYNTHASE FROM THE FUNGUS *PHAEOSPHAERIA* SP. L487

HIROSHI KAWAIDE*, TAKESHI SASSA†AND YUJI KAMIYA*
*RIKEN and †Department of Bioresources, Yamagata University.

ent-Kaurene is the first cyclic intermediate of gibberellin (GA) biosynthesis in both higher plants and GA-producing fungi. In higher plants, copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) are involved in the conversion of geranylgeranyl diphosphate (GGDP) to *ent*-kaurene by way of copalyl diphosphate (CDP). In contrast, fungal *ent*-kaurene synthases from *Phaeosphaeria* sp. L487 and *Gibberella fujikuroi* convert GGDP to *ent*-kaurene. To understand the functions and mechanism of the bifunctional enzymes in fungal *ent*-kaurene biosynthesis, we studied the kinetics of the reaction catalyzed by *Phaeosphaeria ent*-kaurene synthase (FCPS/KS) and identified two cyclase domains in the 106-kDa (946 amino acids) polypeptide.

Kinetic analysis of a recombinant FCPS/KS protein produced in *E. coli* indicated that the affinity of FCPS/KS for CDP is higher than that for GGDP. *ent*-Kaurene production from GGDP by FCPS/KS was enhanced by addition of a plant KS but not by plant CPS, suggesting that the rate of *ent*-kaurene production of FCPS/KS may be limited by the KS activity. Interestingly, the activity of a plant CPS was inhibited by high concentration of substrate GGDP, both substrates did not inhibited enzyme activities of FCPS/KS. Such inhibition has not been found in cloned GGDP cyclases involved in secondary metabolites in plants.

Functional analysis using CPS inhibitor suggested that FCPS/KS resides in separate domains for the two-step cyclization reaction. We assigned two active domains of FCPS/KS by site-directed mutagenesis and deletion experiments. Replacement of aspartate residues by alanine indicated that the ^{318}DVDD motif near the N-terminus is part of active site for CPS activity, and that the ^{656}DEFFE motif near the C-terminus is required for KS activity. The other aspartate-rich ^{132}DDVLD motif near the N-terminus is thought to be involved in both activities. Functional analysis of the N- and C-terminally truncated mutants revealed that a 63-kDa polypeptide from the N-terminus (Δ 531-946 amino acid) catalyzed the CPS reaction and a 66-kDa polypeptide near the C-terminus (Δ 1–249 amino acid) showed KS activity which was severely reduced. The mutant encoding a truncated protein lacking the first 43 amino acid residues of the N-terminus (Δ 1-43 amino acid) showed no CPS activity and reduced KS activity rather than that of the C-terminal 66-kDa polypeptide. These results indicate that there are two separate interacting cyclase domains in the single polypeptide chain of FCPS/KS. A third domain near the N-terminus, which contains conserved amino acid residues with other plant diterpene cyclases, may be important for the both CPS and KS reactions in the enzyme.

Modification of plant height in transgenic tobacco plants by genetic manipulation of GA biosynthesis with GA 20-oxidases.

I. López-Díaz1, A. M. Vidal1, C. Gisbert1, P. Hedden2, A.L. Phillips2, S. J. Croker2, M. Talón3, José.L. García-Martínez1

1 Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia; 2 Long Ashton Research Station, Bristol, 3 Instituto Valenciano de Investigaciones Agrarias, Montcada, Valencia.

Gibberellins (GAs) control several plant developmental processes and, in the case of stem elongation, there is a direct correlation between the content of active GAs and the final length of the shoot.

Consequently, we can expect to achieve a reduction or enlargement of plant stature by manipulating GA biosynthesis in genetically modified plants. We have used tobacco plants as a model system to study different strategies for modifying plant height. To alter the active GA content in these plants, we have focused in the final part of the GA biosynthetic pathway, after GA12, where two enzymes (GA 20-oxidases and GA 3-oxidases) catalyze most of the steps that generate the active GA1 and GA4. We have transformed tobacco plants with heterologous genes for GA 20-oxidases, from different sources, altering the height of the plants in different qualitative and quantitative ways.

Tobacco transgenic plants overexpressing a cDNA clone coding for a GA 20-oxidase from the citrus Carrizo citrange (CcGA20ox1) were taller (up to 100%) and had longer flower peduncles than those of control plants. The ectopic overexpression of CcGA20ox1 enhanced the non-hydroxylation pathway of GA biosynthesis leading to GA4 (whose concentration in the shoot increased 2-3 times), apparently at the expense of the parallel alternative early-13-hydroxylation pathway (whose products decreased or were not affected). We conclude that the tall phenotype of tobacco plants was due to their higher content of GA4, since this was shown to be more active inducing growth of hypocotyls in tobacco seedlings. Hypocotyls of 9-day-old transgenic seedlings, which were longer (up to 400%) than those of control, did not elongate further by GA3 application, suggesting that the GA response was saturated by the presence of the transgene.

We have attempted the reduction of plant height by the antisense inhibition of the endogenous GA 20-oxidases, transforming tobacco plants with the citrange gene Cc20ox1 in antisense orientation. This GA 20-oxidase gene is up to 77% identical in sequence to one of the endogenous genes of tobacco. The antisense expression of Cc20ox1 generated plants significantly shorter that control, with a reduction in RNAm levels for the most homologous tobacco gene, Nt12. The hypocotyl length however was not affected. A second approach that we have tested to reduce plant height is the transformation of tobacco plants with the Cm20ox1 gene of pumpkin, which code for an atypical GA 20-oxidase. This enzyme leads to the biosynthesis of inactive GAs (GA17 y GA25) and its overexpression in Arabidopsis causes a reduction of stem length by deviating the pathway toward inactive products.

However, transgenic tobacco plants expressing the Cm20ox1 gene are not reduced in height, but are 60% taller than control and their hypocotyls are also elongated. The hypocotyl length is not much different in plants with one (hemizygous)

or two (homozygous) loci although GA responses are not saturated as shown by the GA3 induction of hypocotyl elongation. The lack of gene dose effect will be discussed in terms of substrate availability, as suggested by experiments with the inhibitor paclobutrazol.

Characterization of *cif*, a mutation of *Arabidopsis thaliana* that particularly affects the development of the late inflorescence.

Francisco Madueño[1], Leonor Ruiz-García[2], Isabel López-Diaz[1], Julio Salinas[3] y José Miguel Martínez-Zapater[2]

[1]*Instituto de Biología Molecular y Celular de Plantas, UPV-CSIC, Valencia;* [2] *Centro Nacional de Biotecnología;, Madrid;* [3] *CIT-INIA, Madrid.*

The aerial part of the *Arabidopsis thaliana* plant is composed of four parts that correspond to four different developmental phases. Both the *juvenile* and the *adult rosettes* are formed by leaves originated in nodes among which no elongation takes place. In opposition to this, the internodes of the of the *early inflorescence* stem, as well as those of *the late inflorescence* stem, exhibit elongation.

The *compressed inflorescence* (*cif*) mutation, isolated from an EMS mutagenized Landsberg erecta population, is recessive, maps to a region at the top of chromosome 3 and has a pleiotropic effect on the architecture of the inflorescence. The *cif* mutation provokes a moderate delay in the flowering time (under both long and short day conditions) and a strong alteration of the morphology of the inflorescence stem. In this way, whereas the internodes of the lower part of the inflorescence stem of *cif*, which separate those nodes bearing secondary inflorescences (early inflorescence phase), exhibit an essentially normal degree of elongation, the internodes of the upper part of the inflorescence stem, that separate the flower-bearing nodes (late inflorescence), do not elongate and this originates a characteristic compressed inflorescence. This striking effect on the inflorescence stem architecture is only observed when the *cif* mutation is found in combination with the *er* mutation (present in the Landsber erecta ecotype). Even though the delay in flowering time is partially rescued when *cif* is combined with the *spy-5* mutation, the double mutant plants still exhibit a compressed inflorescence phenotype. Exogenous application of GA3 or vernalization treatments do not correct the compressed phenotype either.

When the *cif* mutation is combined with mutations which delay (*lfy* or *ap1*) or accelerate (*tfl1*) the initiation of flowers in the inflorescence stem, the internode compression is consistently delayed or accelerated. This suggests that the internode compression provoked by the *cif* mutation is associated to the late inflorescence phase of development and makes unlikely the possibility that this phenotype is due to some non-specific defect as, for instance, a deficiency in the acropetal transport of some substance through the inflorescence stem.

Our results indicate that the elongation of internodes of the inflorescence stem in *Arabidopsis* is governed by different mechanisms in the early and late inflorescence phases.

Acknowledgments. The *cif* mutant was originally isolated in the laboratory of Dr. Javier Paz-Ares (Centro Nacional de Biotecnología, Madrid). We are grateful to Juan Manuel Pedrosa for technical assistance.

EARLY STEPS IN THE PHYTOCHROME B SIGNAL TRANSDUCTION PATHWAY.

J. F. Martínez-García, E. Huq and P. H. Quail, Plant Gene Expression Center (USDA-ARS, UC-Berkeley), 800 buchanan Street, Albany, CA 94710, USA.

Plants have evolved several photoreceptors to perceive the light environment continuously and adapt their growth and development optimally to the light conditions. The best characterized are the phytochromes (phys), which in *Arabidopsis* are a small family of five members (phyA to phyE). Perception of the light conditions activates transduction pathways leading to changes in gene expression that drive the photomorphogenic responses that occur throughout the plant life cycle, such as seed germination, seedling de-etiolation, shade avoidance and flowering. The isolation of *Arabidopsis* mutants deficient in the function of one phy has provide evidence that phyA and phyB have a major role in the seedling de-etiolation under continuous far-red (FR) and red (R) light, respectively. Recently it was isolated and characterized in our laboratory a phy interacting factor, PIF3, and shown to be a truly primary factor of both phyA and phyB signaling pathways (1). It has been shown that "in vitro" PIF3 binds preferentially, if not exclusively, to the active form of phyB, PfrB (2). PIF3 is localized constitutively in the nucleus of the plant cells (1). The recent finding in other laboratories that both phyA and phyB translocate to the nucleus in a light dependent manner (3), enables the observed interaction between PIF3 and PfrB to happen "in vivo". PIF3 shows similarity to the basic-helix-loop-helix (bHLH) superfamily of transcription factors, but its ability to act as one has not been proven. We were interested in addressing whether PIF3 is able to bind specifically the DNA and, if so, whether the DNA-bound form of PIF3 can still interact with the active form of phyB. A random binding site selection (RBSS) strategy combined with electrophoretic mobility shift assay (EMSA) experiments were performed to answer these questions. The latest results will be described.

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Do gibberellins come from mevalonate pathway or non-mevalonate pathway?

Kazunori Okada, Hiroshi Kawaide, Bunshiro Endo, Tomohisa Kuzuyama†, Haruo Seto † and Yuji Kamiya
RIKEN, Wako-shi, Saitama, Japan, 351-0198, †Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan, 113-0032.

Biosynthesis of isopentenyl diphosphate (IDP), which is an essential precursor of geranylgeranyl diphosphate (GGDP), occurs using two different pathways, namely mavalonate (MVA) pathway in citosol and non-MVA pathway in plastid. GGDP is converted to *ent*-kaurene via copalyl diphosphate (CDP) by CDP synthase and *ent*-kaurene synthase in plastids. Therefore, the non-MVA pathway is thought to be a major pathway to supply the precursor for GA biosynthesis. However, we still don't know how the non-MVA pathway involves in the GA biosynthesis and other IDP dependent isoprenoids biosynthesis. To address this question, we have been focusing on the genes related to IDP metabolism, such as GGDP synthase genes and some other non-MVA pathway dependent genes, and examining about their functions, localization, expression patterns, and phenotypic effects to plants caused by silencing of these genes.

There is a small gene family for GGDP synthases consisting of 5 isozymes in Arabidopsis. Subcellular localization of these enzymes using synthetic green fluorescent protein (sGFP) has been shown that GGPS1-sGFP and GGPS3-sGFP proteins were translocated into the chloroplast, GGPS2-sGFP and GGPS4-sGFP proteins were localized in the ER, and GGPS6-sGFP protein was localized in the mitochondria. RNA blot and promoter-GUS analysis showed that these genes are organ-specifically expressed in Arabidopsis. *GGPS1* were ubiquitously expressed, while *GGPS2*, *GGPS3* and *GGPS4* were expressed specifically at flower, root and flower, respectively. These results suggest that two GGDP synthases are involved in the plastidial GA biosynthesis with non-MVA pathway at each specific organ, and other three enzymes are working at the cytosolic/ER or the mitochondria with MVA pathway.

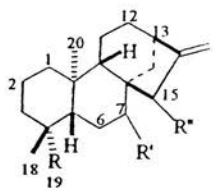
Recently, *Escherichia. coli* 2-*C*-methyl-D-erythritol-4-phosphate (MEP) cytidyltransferase that produces cytidil-MEP, a possible precursor of IDP in non-MVA pathway, has been reported. Therefore, we have cloned its Arabidopsis homolog (AtYACM) and purified His-tagged AtYACM protein. The protein showed the activity for production of cytidil-MEP from MEP and CTP like a bacterial enzyme. Now we are proceeding to antisense analysis and localization analysis of AtYACM protein. It can be expected that AtYACM protein be probably localized into chloroplast, and the plant lucks AtYACM activity could express pleiotropic effects including a dwarfism caused by GA deficiency. Expression patterns of AtYACM transcripts are also now in progress.

Stimulating Effect of Exogenous *ent*-Kaurenoic Acid on the Metabolism and/or the Excretion of other Kaurenoids in the SG138 Mutant of *Gibberella fujikuroi*

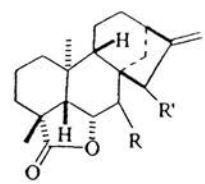
Alejandro F. Barrero‡, J. Enrique Oltra‡, José Justicia‡, Enrique Cerdá-Olmedo†, and Javier Ávalos†.

‡Departamento de Química Orgánica, Instituto de Biotecnología, Facultad de Ciencias, Universidad de Granada, Granada, Spain. †Departamento de Genética, Universidad de Sevilla, Sevilla, Spain. e-mail joltra@goliat.ugr.es

Feeding experiments with *ent*-kaurenoic acid (**1**), 15α-hydroxy-*ent*-kaurenoic acid (**2**), 15β-hydroxy-*ent*-kaurenoic acid (**3**) and mixtures of **1** plus **2** and **1** plus **3**, have been performed using the SG138 mutant of *Gibberella fujikuroi*. The results provide information about the substrate specificity of the enzymes involved in the *ent*-kaurenoic acid 7β-hydroxylation and subsequent ring B contraction to give the gibberellin skeleton. Biotransformation of **2** gave 7β,15α-dihydroxykaurenolide (**5**)[1] and the previously non described 7β,15α-dihydroxy-*ent*-kaurenoic acid (**4**). Biotransformation of **3** provided 7β,15β-dihydroxy-*ent*-kaurenoic acid (**6**)[2] and 7β,15β-dihydroxykaurenolide (**7**).[2] No biotransformation products with a gibberellin skeleton were detected in any of these experiments. The results confirm that an OH group at C-15 does not inhibit the 7β-hydroxylase activity[1] but completely prevents the ring B contraction reaction in our mutant, suggesting a relatively high substrate specificity for the enzymatic system involved. Unexpectedly, we found a strong stimulating effect of exogenous **1** on the excretion of *ent*-kaurene and the metabolism of **2** and **3**. A enzymatic cell model organized in two compartments connected by a bottle-neck channel, explaining the *ent*-kaurenoic acid effect, will be depicted in the poster.



1, R = CO_2H, R' = H, R" = H
2, R = CO_2H, R' = H, R" = αOH
3, R = CO_2H, R' = H, R" = βOH
4, R = CO_2H, R' = βOH, R" = αOH
6, R = CO_2H, R' = βOH, R" = βOH



5, R = βOH, R' = αOH
7, R = βOH, R' = βOH

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Decreasing the stature of *Arabidopsis thaliana* by expression of hammerhead ribozymes and antisense RNA against the *GA1* gene

José Pérez-Gómez and José Luis García-Martínez

Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC)
Universidad Politécnica de Valencia. Camino de Vera, 46022, Valencia, Spain.

Gibberellins (GAs) are plant hormones involved in many developmental processes such germination, stem elongation, flowering and fruit development. These processes can be modified by altering the endogenous GA content of the plant, therefore generating phenotypes of agronomic interest. The growing number of cloned genes involved in GA biosynthesis and metabolism makes feasible the genetic manipulation of their expression.

GA biosynthesis starts with the ciclation of GGPP to produce *ent*-copalyl diphosphate, a reaction catalized by the *ent*-copalyl diphosphate synthase (CPS). In *Arabidopsis* a gene coding this enzyme (*GA1*) is mainly expressed in young developing tissues, which makes CPS a good potential target for genetic manipulation.

Our aim is to silence the expression of the *GA1* gene in *Arabidopsis* using antisense RNA and hammerhead ribozymes (small RNAs with catalytic activity capables of cutting others RNAs in trans).

We have designed a hammerhead ribozyme against the *GA1* gene based on the secondary structure of its mRNA and the base composition of the hybridizing arms. A control ribozyme with a single base change that lacks catalitite acticity was also produced. The activity of these ribozymes was assessed *in vitro* and transgenic plants of *Arabidopsis thaliana* that express the ribozymes as well as antisense RNA against *GA1* were obtained. The phenotype of the transgenic plants, as well as the levels of GA1 transcripts quantitated by RT-PCR will be presented.

EBS: a new locus involved in the regulation of flowering time and germination in Arabidopsis.

Manuel Piñeiro(1), Concepción Gómez-Mena(1), José M. Martínez-Zapater(1) and G. Coupland(2).

(1) Centro Nacional de Biotecnología. Campus de la U. Autónoma de Madrid, Cantoblanco. 28049, Madrid. Spain.

(2) John Innes Centre. Colney Lane, NR4 7UH. Norwich. UK

Mutations in the *EBS* (*EARLY BOLTING IN SHORT DAYS*) gene cause a precocious transition from vegetative to reproductive development, especially in short day (SD) conditions. Specifically, *ebs* mutants show a reduction in the adult vegetative phase of development. In addition, *ebs* mutations result in altered floral morphology, semidwarf phenotype, reduced dormancy of seeds and increased resistance to the inhibition of germination caused by paclobutrazol. Since both flowering in SD and germination are developmental processes that require gibberelin (GA) biosynthesis, we tested whether the early flowering and the precocious germination of freshly harvested seeds observed in *ebs* mutants was dependent on GA biosynthesis or signal transduction. The analyses of double mutants constructed with *ebs* and *ga1-3*, *ga2-1* and *spy-5* show that both the early flowering and the reduced dormancy phenotypes of *ebs* require GA biosynthesis. Also, *spy-5* mutation acts additively with *ebs* in accelerating flowering and decreasing dormancy.

On the other hand, the genetic analyses of double mutants among *ebs* and late flowering mutants show that *FT* gene is also required for the early flowering time phenotype of *ebs*, both under long days (LD) and SD conditions, suggesting that *EBS* could be involved in the regulation of *FT* expression. To test this hypothesis we have analysed the accumulation of *FT* transcript in *ebs* mutant plants. Our results show that *FT* expression is upregulated in *ebs* mutant as compared to wild type, confirming that *EBS* participates in the regulation of the expression of *FT* gene.

We are currently analysing the possible involvement of *EBS* in the regulation of the expression of key genes in the control of the pathway of GA biosynthesis (*GA5* and *GA4*). The results of this study will be also presented.

The role of GAI in the gibberellin signal transduction pathway.
Donald E Richards, Jinrog Peng and Nicholas P Harberd. John Innes Centre, Colney Lane, Norwick UK.

Gibberellins are diterpenoid compunds that influence many plant developmental processes. *GAI* is a gene involved in the gibberellin signal transduction pathway that has been cloned in our laboratory. We have shown that its orthologs in wheat are the genes responsible for the "green revolution". We show here that these genes have sequence homology to the STATs (Signal Transducers and Activators of Transcription), a family of transcription factors present in metazoans. The STATs play a central role in the signal transduction pathway of cytokines and other extracelluar effectors. We discuss the evidence that the members of the recently discovered family of plant proteins, GRAS - whose members code for proteins that play diverse roles in development in plants-, also have homology to the STATs. In addition, we present the results of our recent experiments concerning the role that tyrosine phosphorylation plays in the gibberellin signal transduction pathway.

Gibberellins in Rumex acetosa L.

Tania Stokes

Developmental Botany, Dept. of Plant Sciences, Univ. of Cambridge
Downing Street, CB2 3EA Cambridge (U.K.)

Physiological changes during the development of a plant may be effected through changes in the levels of active plant growth regulators such as gibberellins. Quantitative changes in gibberellins have been shown to be important in the transition from the vegetative to the reproductive stages of development in rosette plants such as *Spinacia oleracea* and *Arabidopsis thaliana* [1]. The levels of gibberellins may be in turn be controlled though regulation of gibberellin biosynthesis pathways by exogenous and endogenous signals.

Therefore, as a first step to understanding the roles of gibberellins at various stages during the development of a plant, the identification of endogenous gibberellins and the gibberellin biosynthesis pathways operating in a plant is necessary. In this study GC-MS and combined HPLC/ELISA were employed to carry out quantitative and qualitative analytical studies on the gibberellins present in the dioecious long-day rosette plant *Rumex acetosa* (common sorrel).

In addition to a range of 13-hydroxylated gibberellins, the following gibberellins were identified in *R. acetosa* for the first time: GA17, GA8, GA29, GA18 and GA38. It is proposed that at least two biosynthesis pathways operate in the *R. acetosa* samples studied. Gibberellins were quantified in various tissues to identify those that would be suitable for further analysis. Young inflorescences of both male and female plants were found to be sites of high concentrations of gibberellins, with male inflorescences having significantly higher levels of GA18 ($p<0.05$) than females.

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Regulation and Genetic Manipulation of Gibberellin Catabolism

Steve Thomas, Andy Phillips, Steve Croker, Paul Gaskin and Peter Hedden

IACR-Long Ashton Research Station, Dept. of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK

Biologically active gibberellins (GAs) are inactivated by hydroxylation at the 2β position. We have employed a functional expression screening strategy to isolate a cDNA encoding a GA 2β-hydroxylase from late-developing seeds of *Phaseolus coccineus*, a rich source of this activity. A cDNA library was constructed using poly $(A)^+$ RNA isolated from *P. coccineus* seeds and a single clone was identified on the ability of the expressed protein to liberate ^3H_2O from [2,3-$^3H_2$]GA_9 and [1,2-$^3H_2$]GA_4. Functional assays, using ^{14}C labelled GA substrates, demonstrated that the protein product is a multifunctional GA 2-oxidase that converts GA_1, GA_4, GA_9, GA_{15} and GA_{20} to the respective 2β-hydroxylated products with GA_{51} and GA_{34} being metabolised further to their catabolites.

Sequence database searches identified three putative *Arabidopsis* homologues. Full-length cDNA clones were isolated from *Arabidopsis* and designated *AtGA2ox1*, *AtGA2ox2* and *AtGA2ox3*. Northern blot analysis demonstrated highest levels of expression of *AtGA2ox1* and *AtGA2ox2* in floral clusters, but expression of *AtGA2ox3* was undetectable. After expression in *E. coli*, *AtGA2ox2* and *At2GAox3* were shown to encode multifunctional GA 2-oxidases, whereas *AtGA2ox1* catalyzed only 2β-hydroxylation with no catabolite detected. As a consequence of feedback regulation, floral shoots of the *ga1-2* GA-deficient *Arabidopsis* mutant contain abnormally high transcript levels of the GA-biosynthetic enzymes, GA 20-oxidase (AtGA20ox1) and GA 3β-hydroxylase (*AtGA3ox1*); application of 10 μM GA_3 rapidly reduces these levels. However, transcript levels for AtGA2ox1 and AtGA2ox2 were increased by GA_3 application, indicating that GA inactivation is up-regulated by bioactive GAs.

We have investigated the potential utility of GA 2-oxidase genes for manipulating GA content in transgenic plants. Over-expression of the *P. coccineus* 2-oxidase, under the transcriptional control of the 35S promoter, has produced a severe dwarf phenotype in *Arabidopsis* and several other species. Transgenic *arabidopsis* lines, with high levels of expression of the 2-oxidase gene, possess a phenotype similar to that of the extreme GA-deficient mutant, *ga1-3*. When the transgenic plants were grown under long days they produced sterile flower buds but did not undergo stem elongation. Application of GA_3 to these plants rescued the wild-type phenotype. Feedback regulation of GA biosynthesis was apparent in the dwarf plants, with the transcript levels of *AtGA20ox1* and *AtGA3ox1* being elevated compared to wild-types. Conversely, transcript levels for *AtGA2ox1* were reduced in these plants. The metabolism of applied [^{14}C]GAs in the transgenic *Arabidopsis* plants was compared with that of wild-type (Columbia) plants. C_{19}-GAs were converted predominantly to their respective catabolites in the transgenic plants, but these products were not formed in the wild-type plants. Analysis of the GA content of the transgenic plants showed a significant increase in the levels of GA_{51}-catabolite compared to wild-type plants. A more detailed analysis of the endogenous GAs is currently in progress.

Regulation of Gibberellin Biosynthetic Genes in Germinating Arabidopsis Seeds

SHINJIRO YAMAGUCHI (1), Yuji Kamiya (2), Tai-ping Sun(1)

(1) Developmental, Cell and Molecular Biology Group, Dept. Botany, Duke University, USA

(2) Frontier Research Program, RIKEN, Japan

Gibberellin (GA) plays an essential role during germination of Arabidopsis seeds. We studied the regulation of two Arabidopsis genes, *GA4* and *GA4H* (for *GA4* homolog), both of which encode GA 3β-hydroxylases that catalyze the final biosynthetic step to produce bioactive GAs. Using RNA gel blot analysis, we showed that these genes are expressed during seed germination with distinct temporal patterns of mRNA accumulation. *In situ* hybridization showed that both *GA4* and *GA4H* transcripts are predominantly localized in cortical cells in embryo axis during seed germination. Interestingly, in contrast to the *GA4* gene, the *GA4H* gene was not down-regulated by GA activity. Therefore, the *GA4H* gene may play an important role in the maintenance of bioactive GA levels required for seed germination. The physiological importance of the *GA4H* gene during seed germination will be studied using a *ga4h* T-DNA insertion mutant that we have recently identified.

The Arabidopsis *GA1* gene encodes copalyl diphosphate synthase which catalyzes an early step of the GA biosynthetic pathway. We have previously studied developmental regulation of the *GA1* gene using *GA1* promoter-*GUS* reporter gene expression and quantitative RT-PCR. Unlike the *GA4* and *GA4H* genes, *GA1-GUS* expression is localized in shoot apex and provasculature during seed germination. Distinct localization patterns of these genes suggest intercellular translocation of a GA intermediate. To determine which intermediate could be transported, we are currently examining cellular localization of other GA biosynthetic genes in germinating seeds using *in situ* hybridization and promoter-*GUS* reporter gene expression.

LIST OF INVITED SPEAKERS

Juan Carbonell	Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia - CSIC. Camino de Vera s/n, 46022 Valencia (Spain). Tel.: 34 963 877 000. Fax: 34 963 877 859. E-mail: jcarbon@ibmcp.upv.es
José Luis García-Martínez	Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia - CSIC. Avda. de los Naranjos s/n, 46022 Valencia (Spain). Tel.: 34 96 387 78 65. Fax: 34 96 387 78 59. E-mail: jlgarcim@ibmcp.upv.es
Frank Gubler	CSIRO Plant Industry, GPO Box 1600, 2602 Canberra (Australia). Tel.: 61 2 624 65 269. Fax: 61 2 624 65 000. E-mail: f.gubler@pi.csiro.au
Nicholas Harberd	Molecular Genetics Department, John Innes Centre. Colney Lane, NR4 7UJ Norwich (UK). Fax: 44 1603 50 57 25. E-mail: nicholas.harberd@bbsrc.ac.uk
Peter Hedden	IACR-Long Ashton Research Station, Dept. of Agricultural Sciences, University of Bristol. Long Ashton, BS41 9AF Bristol (UK). Tel.: 44 1275 54 92 63. Fax: 44 1275 39 42 81. E-mail: Peter.Hedden@bbsrc.ac.uk
Michel Herzog	Génétique Moléculaire des Plantes, UMR CNRS 5575, Université Joseph Fourier, BP 53, F-38041 Grenoble (France). Tel.: 33 4 76 51 48 01. Fax: 33 4 76 51 43 36. E-mail: michel.herzog@ujf-grenoble.fr
Richard Hooley	IACR-Long Ashton Research Station, Dept. of Agricultural Sciences, University of Bristol. Long Ashton, BS41 9AF Bristol (UK). Tel.: 44 1275 39 21 81. Fax: 44 1275 392 1 81. E-mail: richard.hooley@bbsrc.ac.uk
Russell J. Jones	Dept. of Plant and Microbial Biology, University of California, Berkeley, CA.94720-3102 (USA). Fax: 1 510 642 4995. E-mail: rjones@nature.berkeley.edu
Yuji Kamiya	Plant Functions Laboratory, RIKEN. Hirosawa 2-1 Wako-shi, 351-01 Saitama (Japan). Tel.: 81 48 462 9375. Fax: 81 48 462 4691. E-mail: ykamiya@postman.riken.go.jp
Hans Kende	Michigan State University, East Lansing, MI.48824-1312 (USA). Tel.: 1 517 353 7865. Fax: 1 517 353 9168. E-mail: hkende@msu.edu
Theo Lange	Botanisches Institut der TU Braunschweig. Mendelssohnstr. 4, D-38106 Braunschweig (Germany). Tel.: 49 531 391 58 79. Fax: 49 531 391 81 80. E-mail: tlange@tu-bs.de

Thomas Moritz	Umeå Plant Science Centre, Dept. of Forest Genetics and Plant Physiology, Swedish Univ. of Agricultural Sciences, SE-901 83 Umeå (Sweden). Tel.: 46 90 786 7739. Fax: 46 90 786 5901. E-mail: Thomas.Moritz@genfys.slu.se
John Mundy	Dept. of Plant Physiology, Molecular Biology Institute, Copenhagen University. Oester Farimagsgade 2A, 1353 Copenhagen K (Denmark). Tel.: 45 353 22 131. Fax: 45 353 221 128. E-mail: mundy@biobase.dk
Neil Olszewski	Dept. of Plant Biology, University of Minnesota, 220 Biological Sciences Center, St. Paul, MN.55108-1095 (USA). Tel.: 1 612 625 3129. Fax: 1 612 625 1738. E-mail: neil@biosci.cbs.umn.edu
Andrew L. Phillips	IACR-Long Ashton Research Station, Dept. of Agricultural Sciences, Univ. of Bristol. Long Ashton, Bristol BS41 9AF (UK). Tel.: 44 1275 54 92 57. Fax: 44 1275 39 42 81. E-mail: andy.phillips@bbsrc.ac.uk
Salomé Prat	Dpto. de Genética Molecular. Centro de Investigación y Desarrollo-C.S.I.C. Jordi Girona, 18-26, 08034 Barcelona (Spain). Tel.: 34 93 400 61 89. Fax: 34 93 204 59 04. E-mail: spmgms@cid.csic.es
Tai-ping Sun	Dept. of Botany, Duke University, Durham,NC 27708 (USA). Tel.: 1 919 613 8166. Fax: 1 919 613 8177. E-mail: tps@acpub.duke.edu
Bettina Tudzynski	Wesfälische Wilhelms-Universität Münster, Institut für Botanik. Schlossgarten 3, 48149 Münster (Germany). Fax: 49 251 832 3823. E-mail: Bettina.Tudzynski@uni-muenster.de
Detlef Weigel	Plant Biology Laboratory, Salk Institute. 10010 North Torrey Pines Road, La Jolla, CA.92037 (USA). Tel.: 1 858 453 4100. Fax: 1 858 558 6379. E-mail: weigel@salk.edu
David Weiss	Dept. of Horticultural, Fac. of Agriculture, Food and Environmental Quality Sciences, The Hebrew Univ. of Jerusalem. P.O. Box 12, 76100 Rehovot (Israel)
Jan A. D. Zeevaart	MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI.48824-1312 (USA). Tel.: 1 517 353 3230. Fax: 1 517 353 9168. E-mail: zeevaart@msu.edu

LIST OF PARTICIPANTS

Manuel Acosta	Dpto. de Biología Vegetal, Fac. de Biología, Univ. de Murcia. Campus Univ. de Espinardo, 30100 Espinardo, Murcia (Spain). Tel.: 34 968 36 30 00. Fax: 34 968 36 39 63. E-mail: macosta@fcu.um.es
Carmen P. Alapont	Instituto de Biología Molecular y Celular de Plantas, Univ. Politécnica de Valencia. Camino de Vera s/n, 46022 Valencia (Spain). Tel.: 34 96 387 77 30. Fax: 34 96 387 78 59. E-mail: calapont@ibmcp.upv.es
José Pío Beltrán	Instituto Biología Molecular y Celular de Plantas, CSIC. Camino de Vera s/n, 46022 Valencia (Spain). Tel.: 34 96 387 77 30. Fax: 34 96 387 78 59. E-mail: jbeltran@ ibmcp.upv.es
Miguel A. Blázquez	Plant Biology Lab. The Salk Institute, La Jolla, CA.92037 (USA). Tel.: 1 858 453 4100. Fax: 1 858 558 6379. E-mail: blazquez@salk.edu
Pilar Carbonero	Lab. Bioquímica y Biología Molecular, Dpto. Biotecnología, UPM. ETSI Agrónomos, 28040 Madrid (Spain). Tel.: 34 91 336 5707. Fax: 34 91 336 5757. E-mail: pcarbonero@bit. etsia.upm.es
Francisco J. Cejudo	Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC. Avda. Américo Vespucio s/n, 41092 Sevilla (Spain). Tel.: 34 954 48 95 06. Fax: 34 954 46 00 65. E-mail: fjcejudo@cica.es
Emilio Cervantes	Instituto de Recursos Naturales y Agrobiología, CSIC. Apartado 257, 37080 Salamanca (Spain). Tel.: 34 923 21 96 06. Fax: 34 923 21 96 09. E-mail: ecervant@gugu.usal.es
Maria E. Eriksson	Dept. of Forest Genetics and Plant Physiology. The Swedish Univ. of Agricultural Sciences, SE-901 83 Umeå (Sweden). Tel.: 46 90 786 58 00. Fax: 46 90 786 59 01
Barbara Fleck	John Innes Centre, Molecular Genetics Dept. Colney Lane, NR4 7UH Norwich (UK). Tel.: 44 1603 452 571. Fax: 44 1603 505 725. E-mail: barbara.fleck@bbsrc.ac.uk
Christine Fleet	Depts. of Botany and Zoology, Duke University, Durham, NC.27708-1000 (USA). Tel.: 1 919 613 8167. Fax: 1 919 613 8177. E-mail: cmf7@duke.edu
Ingela Fridborg	Dept. of Physiological Botany, Uppsala University. Villav. 6, S-752 36 Uppsala (Sweden). Tel.: 46 18 471 28 08. Fax: 46 18 55 98 85. E-mail: Ingela.Fridborg@fysbot.uu.se

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Hiroshi Kawaide	RIKEN, Plant Functions Laboratory. Hirosawa 2-1, Wako, 351-0198 Saitama (Japan). Tel.: 81 48467 9552. Fax: 81 48462 4691. E-mail: hkawaide@postman.riken.go.jp
Isabel López-Díaz	Instituto de Biología Molecular y Celular de Plantas, Univ. Politécnica de Valencia, CSIC. Camino de Vera s/n, 46022 Valencia (Spain). Tel.: 34 96 387 78 66. Fax: 34 96 387 78 59. E-mail: ilopez@upvnet.upv.es
Francisco Madueño	Instituto de Biología Molecular y Celular de Plantas, UPV-CSIC. Camino de Vera s/n, 46022 Valencia (Spain). Tel.: 34 96 387 78 71. Fax: 34 96 387 78 59. E-mail: madueno@ ibmcp.upv.es
Jaime F. Martínez-García	Centre d'Investigació i Desenvolupament, CSIC. Jordi Girona 18-26, 08034 Barcelona (Spain). Fax: 34 93 204 59 04. E-mail: jmggms@cid.csic.es
José M. Martínez-Zapater	Centro Nacional de Biotecnología, Campus de la U. Autónoma de Madrid. Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 585 45 00. Fax: 34 91 585 45 06
Montaña Mena	Lab. Bioquímica y Biología Molecular. Dpto. Biotecnología. UPM. ETSI Agrónomos, 28040 Madrid (Spain). Tel.: 34 91 336 57 05. Fax: 34 91 336 57 57
Ove Nilsson	Dept. of Forest Genetics and Plant Physiology. Swedish Univ. of Agricultural Sciences, S-901 83 Umeå (Sweden). Tel.: 46 90 786 9082. Fax: 46 90 786 5901. E-mail: Ove. Nilsson@genfys.slu.se
Kazunori Okada	RIKEN. Wako-shi, 351-0198 Saitama (Japan). Tel.: 81 48467 9552. Fax: 81 48462 4691. E-mail: kokada@ postman.riken.go.jp
J. Enrique Oltra	Dpto. de Química Orgánica, Instituto de Biotecnología, Fac. de Ciencias, Univ. de Granada, 18071 Granada (Spain). Tel.: 34 958 24 33 21. Fax: 34 958 24 84 37. E-mail: joltra@ goliat.ugr.es
José Pérez-Gómez	Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Univ. Politécnica de Valencia. Camino de Vera s/n, 46022 Valencia (Spain). Tel.: 34 96 387 78 65. Fax: 34 96 387 78 59
Manuel Piñeiro	Centro Nacional de Biotecnología. Campus de la U. Autónoma. Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 585 46 88. Fax: 34 91 585 45 06. E-mail: mpineiro@ cnb.uam.es

Marcel Proveniers	Dept. of Molecular Plant Physiology. Fac. of Biology. Utrecht University. Padualaan 8, 3584 CH Utrecht (The Netherlands). Tel.: 31 30 253 31 32. Fax: 31 30 251 36 55. E-mail: M.C.G.Proveniers@bio.uu.nl
Donald E. Richards	John Innes Centre, Dept. of Molecular Genetics. Colney Lane, NR4 7UJ Norwich (UK). Fax: 44 1603 505 725. E-mail: donald.richards@bbsrc.ac.uk
Tania Stokes	Developmental Botany, Dept. of Plant Sciences, Univ. of Cambridge. Downing Street, CB2 3EA Cambridge (UK). Tel.: 44 1223 33 39 00. Fax: 44 1223 33 39 53. E-mail: tss25@cam.ac.uk
Eva Sundberg	Dept. Physiological Botany, Uppsala University. Villav. 6, S-752 36 Uppsala (Sweden). Tel.: 46 18 471 28 08. Fax: 46 18 55 98 85. E-mail: Eva.Sundberg@fysbot.uu.se
Manuel Talón	Instituto Valenciano de Investigaciones Agrarias. Dpto. Citricultura. Apartado Oficial, 46113 Moncada, Valencia (Spain). Tel.: 34 96 139 10 00. Fax: 34 96 139 02 40
Steve Thomas	IACR- Long Ashton Research Station, Dept. of Agricultural Sciences, Univ. of Bristol. Long Ashton, Bristol BS41 9AF (UK). Tel.: 44 1275 54 92 57. Fax: 44 1275 39 42 81. E-mail: steve.thomas@bbsrc.ac.uk
Shinjiro Yamaguchi	Developmental, Cell and Molecular Biology Group, Dept. Botany, Duke University, Durham, NC. (USA). Tel.: 1 919 613 8167. Fax: 1 919 613 8177. E-mail: shinjiro@acpub.duke.edu

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