Instituto Juan March
de Estudios e Investigaciones

13 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Approaches to Plant Hormone Action

Organized by

J. Carbonell and R. L. Jones

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A. B. Bleecker
J. Carbonell
R. Fischer
D. Grierson
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A. Jones
R. L. Jones
M. Koornneef
J. Mundy
M. Pagès
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A. Spena
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INTRODUCTION

J. Carbonell

Hormone action has been a topic of interest in Plant Biology since the thirties, when Kenneth Thimann's group studied the action of the first known plant hormone, the auxin indole-3-acetic acid. The classic book, *Phytohormones*, by Went and Thimann illustrates the first advances.

In the years that followed, advances were slow, while the study of hormonal action in animals progressed rapidly. In plants, studies of hormonal action followed the approaches used in animals. A new interest emerged in the seventies, when the search for changes in gene expression and for receptors was initiated. Other approaches including the action of plant hormones through signal transduction pathways, involving calcium and calmodulin, were introduced. The new methodologies developed during the eighties made possible the analysis of rapid changes in gene expression and the analysis of gene structure. Finally the molecular study of mutants with altered responses to plant hormones and the use of transgenic plants have introduced new and powerful tools that open very exciting possibilities.

The program of the meeting was designed to cover all the approaches that are currently used in the study of hormonal action in plants. However we cannot forget that we are studying different hormones, so the mechanisms of their action may be different. Progress in the search for receptors, hormone-regulated genes, hormone-response mutants, and signal transduction pathways vary for auxins, gibberellins, cytokinins, abscisic acid, and ethylene. All of these hormones are well represented in the program except cytokinins, which are not receiving much attention at present. About ten years ago, Leon Dure, at a meeting on Molecular Biology of Plant Hormone Action, suggested that all plant hormones exist in all cells and that different things happen in different cells because of different ratios of these hormone concentrations. However the study of the action of specific plant hormones, in the midst of this paucity of knowledge about the regulation of the level of physiologically active forms, and their interactions with other cellular compounds, is now possible at molecular level and we hope that in this meeting we will learn of recent advances that offer us a promising future.

SESSION I

Characterization of hormone response mutants

THE CHARACTERIZATION OF ABSCISIC ACID AND GIBBERELLIN INSENSITIVE MUTANTS IN ARABIDOPSIS AND THE GA HYPERSENSITIVE PROCERA MUTANT OF TOMATO.

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Plant hormone mutants can be affected in hormone metabolism or hormone action. Defects in the functioning of hormones can be expected to lead to insensitivity to exogenously applied hormones. Since an excess of some hormones, leads to growth inhibiting or even toxic effects, selection for resistance to high hormone concentrations allows the selection of hormone insensitive mutants as shown for abscisic acid insentive *abi* in Arabidopsis (1,3). Mutants at some loci were only affected in a subset of the ABA responses, indicating the interaction of the ABA transduction chain with developmental stage specific factors. Recently Giraudat and co-workers isolated the seed specific *ABI3* gene, which resembles the maize *Vp-1* gene, by a map based cloning strategy (2). Newly isolated extreme *abi3* alleles and genotypes that combine ABA deficiency and ABA insensitivity (*aba/abi3* double mutants) were used to investigate the physiological and developmental effects of ABA during seed development (5). In addition to a resistance phenotype, hormone insensitive mutants display characteristics of hormone deficient mutants. The resemblence of these two classes of mutants led to the identification of the gibberellin (GA) insenstive *gai* mutant in Arabidopsis (4, 6). In addition to hormone insensitive mutants, mutants that are hypersensitive to hormones have been found. The phenotype of such mutants resembles a wild type to which an excess of the hormone has been applied. With respect to GAs this is a mutant with an excessive elongation growth. The finding that several slender mutants have been associated with phytochrome defects made it possible to investigate the role of phytochrome in relation to GA action. The tomato *procera* (*pro*) mutant is an example of a GA hypersensitive mutant with normal photomorphogenesis and resembles the constitutive GA response mutants of barley and pea. An analysis of GA responsiveness for a number of traits with different sensitivity to GAs has been performed with this mutant, which shows how increased sensitivity and saturation of the response complicates the interpretation of the analysis of hormone hypersensitive mutants. The determination of GA levels in both GA insensitive (6) and GA hypersensitive mutants suggests that hormone action regulates hormone levels. By using GA deficient mutants it was found that GA levels affect hormone sensitivity at least in seed germination. It is expected that the cloning of the various genes, which especially in Arabidopsis is feasible nowadays, will help the elucidation of the hormone signal transduction pathways and their interaction with hormone levels and environmental factors.

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THE USE OF HORMONE-RESPONSE MUTANTS IN THE STUDY OF ETHYLENE-MEDIATED SIGNAL TRANSDUCTION PROCESSES IN ARABIDOPSIS

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The plant hormone ethylene influences a host of developmental processes and mediates responses to a variety of environmental stresses in higher plants. Single-gene mutations in Arabidopsis which confer insensitivity to ethylene are being used to clone and characterize genes that are relevant to early events in ethylene-mediated signal transduction. In addition, comparative studies of ethylene insensitive (etr) mutants with the isogenic wild-type line are providing insights into the specific roles that ethylene plays in complex biological processes such as pathogen defense responses, somatic tissue senescence, and the abscission of floral organs. These complex processes can be modeled by monitoring the ethylene dose-dependent activity of an ethylene-responsive reporter gene in transgenic Arabidopsis. Results from these experiments provide evidence for signal-transduction related phenomena such as signal adaptation and developmentally and environmentally altered changes in hormone sensitivity. Early biochemical events in the perception and transduction of the ethylene signal will be discussed in the context of the molecular characterization of the ETR1 locus.

A MOLECULAR APPROACH TOWARDS ETHYLENE BIOSYNTHESIS AND ACTION

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Two basic questions can be raised concerning ethylene biology: (1) Which are the molecular mechanisms controlling ethylene formation in plants? (2) How is this gaseous hormone perceived and its signal transduced resulting in the well characterized responses ? Our current interests are focussed on *Arabidopsis thaliana* as a model system to study these questions. The first step in the molecular analysis of ethylene biosynthesis, was the cloning of genes encoding 1-aminocyclopropane-1-carboxylic acid synthase (ACC synthase), the key regulatory enzyme in ethylene formation (1, 2). We have analyzed the temporal and spatial expression of one member of the *Arabidopsis* ACC synthase gene family (*ACS1*) using both RT-PCR and a promoter-GUS fusion. The expression of the *ACS1* gene is under developmental control both in shoot and root. Strong expression was observed in young developing tissues. In mature and senescent tissues expression was switched off. The ACC content and the *in vivo* activity of ACC oxidase were determined, in order to support the biological importance of the *ACS1* expression. ACC content correlated with *ACS1* gene activity. ACC oxidase activity was demonstrated in young *A.thaliana* seedlings, indicating that the ACC formed as a result of ACC synthase activity can be converted to ethylene.

The possible involvement of *ACS1* - and thus ethylene - in the determination of cell length/width ratio, which influences plant growth, is hypothesized.

The second question is addressed by the study of mutants with altered responses to ethylene and/or its precursor ACC. A population of M2 seedlings of *Arabidopsis thaliana* was screened for mutants in sensitivity to ACC. Several independent lines were obtained and proved insensitive to both ACC and ethylene. Two lines were identified as alleles of a single recessive mutation, designated *ain1*. Linkage analysis indicated that the *ain1* gene is located on chromosome 1 and genetically distinct from previously identified ethylene resistance loci.

An extensive comparison of morphological and physiological characteristics of wild type and mutants indicated certain interallelic variation in the mutants. Growth inhibition experiments showed that the *ain1* mutation does not confer resistance to other hormones. Thus, *ain1* most probably affects a step specific for the ethylene signal transduction pathway.

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SESSION II

Characterization of putative receptors and identification of signal transduction pathways

MULTIPLE RECEPTORS IN AUXIN ACTION

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Two rapid biochemical events can be induced by the addition of auxin to auxin-depleted cells: modulation of specific gene transcription and changes in cell wall viscoelastic properties. In the last few years, several auxin-binding proteins (ABP) have been identified and some or all of these are auxin receptors and may directly mediate these auxin-induced changes. I propose that auxin-induced growth involves multiple receptors and I focus on a nuclear-localized ABP and an ABP, designated ABP1, that is largely localized to the endoplasmic reticulum but moves to the cell wall space. The nuclear ABP was identified using anti-idiotypic antibodies and my lab is currently trying to purify and clone this putative receptor. The other ABP was identified by several laboratories using a variety of techniques. Recent evidence from other laboratories suggest that ABP1 has a site of action on the outer face of the plasma membrane or within the cell wall. Using immunocytochemical techniques, we find that some of ABP1 is found in the cell wall and at the plasma membrane consistent with an ABP1 site of action at these subcellular locations. We are investigating its trafficking and find that auxin modulates the steady-state levels of ABP1 in the cell wall space, thus its secretion and/or endocytosis is ligand mediated.

PROBES OF AUXIN RECEPTOR STRUCTURE AND FUNCTION

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Several entities designated as auxin binding proteins (ABPs) have been reported in recent years. However, only one of these - the ABP of maize microsomal membranes - is strictly an ABP in terms of binding auxins reversibly, with high affinity and appropriate specificity. It is also, so far, the only ABP for which evidence of receptor function has been obtained (1).

Maize ABP has been purified, cloned and sequenced in several laboratories (2-5). We have developed a range of monoclonal and polyclonal antibodies against maize ABP to probe aspects of its structure and function (6). The epitopes recognised by these antibodies have been identified through fragmentation studies and the use of an epitope mapping kit (7). The polyclonal sera recognise three principal epitopes, clustered around the N-glycosylation site. Two of these epitopes are conserved in other species. Two of the monoclonals recognise the endoplasmic reticulum retention sequence (-KDEL) at the C-terminus, which appears to be a conformationally active region, while another two recognise a region close to the N-terminus. Thus we have markers for specific parts of the auxin receptor, which can be used to identify structural features and functionally active parts of the protein. In addition, we have developed sera against a synthetic peptide, which was identified from the deduced ABP amino acid sequence as being likely to contribute to the auxin binding site. The antipeptide sera recognise all maize ABP isoforms on immunoblots as well as ABP homologs in other species. They also have potent auxin agonist activity on tobacco leaf mesophyll protoplasts, eliciting characteristic hyperpolarisation of the transmembrane potential (8). By contrast, antisera to native ABP act as auxin antagonists. This auxin agonist activity has been fully confirmed by patch clamp analysis of H^+ current, using maize protoplasts (9). We conclude that we have identified at least part of the auxin binding site and that the ABP is a genuine auxin receptor. The same experiments, and others with impermeant auxin-protein conjugates, show that the receptor is active on the outside of the plasma membrane. However, the bulk of the receptor protein is in the endoplasmic reticulum in accordance with its KDEL targeting sequence. This dual distribution will be discussed, as will experiments on the role of ABP in controlling gene expression.

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PERCEPTION AND TRANSDUCTION OF HORMONAL SIGNALS IN CEREAL ALEURONE

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The cereal aleurone responds to gibberellins (GAs) and abscisic acid (ABA) by modulating the synthesis and secretion of a variety of hydrolytic enzymes. We have sought to establish how the aleurone cell perceives and transduces these hormonal signals. Calcium and the calcium-dependent regulatory protein calmodulin (CaM) have both been implicated in the response of the aleurone cell to GA and ABA. Cytosolic calcium levels are maintained in the range of 50 to 100 nM in aleurone cells that have not been stimulated to produce secretory proteins by GA treatment. Following exposure to GA, however, cytosolic Ca^{2+} increases to about 300 nM. The increase in cytosolic Ca^{2+} precedes the GA-induced increase in enzyme synthesis and secretion in both barley and wheat aleurone tissue. ABA, which reverses the effects of GA on hydrolytic enzyme synthesis, reverses the GA-induced increase in cytosolic Ca^{2+}. The ABA-induced decrease in Ca^{2+} precedes ABA inhibition of enzyme production. GA also regulates the levels of CaM in aleurone cells. GA induces as much as a ten-fold increase in CaM levels after 24 hours of exposure to GA. The time course of the GA-induced increase in CaM closely parallels the increase in a-amylase synthesis by the aleurone layer. We have identified two proteins in the aleurone cell whose activities are regulated by Ca^{2+} and CaM, a Ca^{2+} ATPase located on the endoplasmic reticulum (ER) and an ion channel on the tonoplast of the protein body vacuole. The activity of the Ca^{2+} ATPase is markedly stimulated by GA, and our data indicate that Ca^{2+} and CaM may play a role in transducing the GA response. Calcium stimulates the activity of the ER Ca^{2+} ATPase by several-fold when Ca^{2+} concentrations are raised from 100 nM to 500 nM. Calmodulin is implicated in the regulation of the ER Ca^{2+} ATPase because addition of CaM activates ER Ca^{2+} ATPase activity *in vitro* and ER membranes isolated from GA-treated aleurone tissue have elevated levels of CaM-like protein associated with them. When ER membranes from GA-treated tissue are incubated with the CaM antagonist W7, the rate of Ca^{2+} transport is inhibited, indicating that the CaM associated with the ER plays a role in Ca^{2+} transport. A slow vacuolar ion channel located on the tonoplast of the protein body vacuole is also regulated by Ca^{2+} and CaM. W7 causes a marked reduction in the probability of channel opening and the effects of added W7 on channel activity can be reversed by addition of CaM. The opening of the channel is also sensitive to cytoplasmic Ca^{2+} concentration, the probability of opening increasing dramatically above 10 μM. We have tentatively localized the site of perception of GA and ABA to the plasma membrane of the aleurone cell by showing that cells microinjected with either GA or ABA do not respond to the hormone. By measuring α-amylase secretion from single aleurone protoplasts we have shown that when GA is injected into the protoplast, α-amylase is not secreted, whereas when the surface of the protoplast is exposed to GA, α-amylase is produced. Microinjected protoplasts secrete α-amylase if they are subsequently incubated in GA, showing that microinjection does not interfere with the response of the aleurone cell to hormones. ABA reverses the effects of GA on α-amylase secretion by protoplasts if ABA is presented to the outside of the aleurone cell. When ABA is injected into aleurone cells, it has no effect on α-amylase secretion. We conclude that the receptors for GA and ABA are located on the outer surface of barley aleurone protoplasts.

ABSCISIC ACID REGULATION OF ION CHANNELS DURING SIGNAL TRANSDUCTION IN GUARD CELLS

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The plant hormone abscisic acid (ABA) can induce stomatal closing in response to drought stress. Stomatal closing has been shown to be calcium-dependent and is mediated by efflux of K^+ ions and anions from guard cells (4). In early patch clamp studies, outward rectifying K^+ channels were identified as a major pathway allowing potassium efflux from guard cells during stomatal closing (2,8,9). We have identified anion channel currents in the plasma membrane of guard cells which provide a mechanism for strong depolarization which in turn drives K^+ efflux through K^+ channels (10). In recent studies two highly distinct types of depolarization activated anion currents, S-type (10,12) and R-type (3), and have been differentiated (12) in the plasma membrane of *Vicia faba* guard cells. S-type anion channels may provide a central driver for stomatal closing as these ion channels are capable of mediating long-term ion efflux (12) required for ABA-induced stomatal closing (4). Specific blockers for S-type anion channels have now been identified supporting the suggestion that S-type and R-type anion channels constitute two separate molecular entities. These anion channel blockers may be useful for discerning the contribution of each anion channel type to stomatal closing.

Initiation of ion efflux during stomatal closing requires anion channel activation. Data has shown that elevation in the cytosolic Ca^{2+} concentration enhances S-type anion channel currents in guard cells (10). Simultaneous application of patch clamp techniques and single cell Ca^{2+} measurements have shown that abscisic acid activates non-selective Ca^{2+}-permeable channels in the plasma membrane of guard cells as an early event in ABA-mediated signal transduction (5,11). Data suggest that intermediate events occur between ABA reception and Ca^{2+} channel opening. Whether putative ABA receptors are located on the extracellular or intracellular or both membrane sides of guard cells remains unknown. We have used a combination of several approaches to gain insight to the membrane side of ABA reception which controls stomatal movements.

In addition to induction of stomatal closing, ABA also inhibits opening of stomata which requires K^+ uptake. In earlier studies, inward-rectifying K^+ channels in guard cells have been identified as a major pathway for K^+ uptake into higher plant cells (8,9). Cytosolic Ca^{2+} elevation inhibits inward-rectifying K^+ channels (10) and K^+ uptake during stomatal opening. Recent evidence suggests that cytosolic Ca^{2+} may indirectly inhibit these K^+ channels. Clones of these K^+ channels have recently been functionally characterized (1,6,7) and should be useful for determining modulation sites of these K^+ channels. The precise sequence of events by which ABA induces stomatal closing remains unknown. A parallel processing model will be suggested in which ABA exerts control over nonselective Ca^{2+}-permeable channels, anion channels and outward and inward K^+ channels to control stomatal closing.

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SESSION III

Molecular cloning of hormone regulated genes

HORMONAL CONTROL OF EXPRESSION OF PROTEASES IN THE SENESCENCE AND FRUIT SET AND DEVELOPMENT OF PEA OVARIES

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Unpollinated pea ovaries enter naturally into a senescence process two days post anthesis (2 dpa). Alternatively, fruit set and development of pea ovaries can be induced by either pollination or treatment with plant growth substances by 2dpa, only gibberellic acid (GA) producing parthenocarpic fruits with identical shape and size as those produced by pollination (1, 2). One of the characteristics associated with the senescence of pea ovary is the presence of new proteolytic activities which are detected at neutral pH by the in vitro degradation of endogenous (ribulose-1,5-bisphosphate carboxylase/oxygenase) and exogenous (azocasein and gelatin) substrates (3, 4, 5). The former activity has been characterized as a thiolprotease and has been detected only in senescent ovaries and immunolocalized mainly in the exocarp (6, 7); the gelatin-degrading activity has been detected in the endocarp and ovules of senescent ovaries (5). All of these activities are not detected in unpollinated ovaries treated with GA. Screening of a cDNA library of senescent ovaries resulted in the isolation of a cDNA clone (tpp) with a high similarity to other plant and animal thiolproteases (8). Analysis of temporal and spatial expression of tpp showed that tpp-mRNA level is highest at the beginning of senescence and was localized mainly in the ovules and transiently in the endocarp of senescent ovaries. This pattern of expression did not occur when the ovaries were treated with GA, indicating that one of the actions of GA, in preventing senescence and inducing fruit set and development of pea ovaries, might be through the negative control of the expression of proteolytic activities.

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GENES CONTROLLED BY GIBBERELLINS DURING FRUIT SET IN *Pisum sativum.*

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There is evidence indicating that fruit set and development in pea are processes regulated by gibberellins (1).
Self pollination in the pea plant takes place 24-36 h before anthesis (day 0), and by the time of full blossom, fertilization has taken place and the zygote has started to divide. Non pollinated pea ovaries keep growing, although slowly, until two days after the day corresponding to anthesis (day +2) when both ovary growth and assimilate import by the ovary cease (2). Before this, fruit set and development of nonpollinated ovaries can be induced by application of GA_3. The pods of the parthenocarpic fruits developed by treatment with GA_3 to ovaries of emasculated pea flowers are similar to those which arise after self pollination of the ovaries (3). This experimental system, being inducible, allows the study of very early changes induced by GA_3 and their correlation with the growth responses associated with the development of the pea pod. Currently, we can envisage the mode of action of GA_3, in relation to assimilate unloading, covering a broad spectrum of mechanisms and processes: a) stimulation of the soluble neutral invertase activity, which can be associated with pod growth, in a process that requires protein synthesis (sensitive to cycloheximide); b) enhancement of the cell wall bound acid invertase activity (insensitive to cycloheximide), that can be associated with sink capacity and involves a promotion of processes of protein secretion; c) activation of glucose uptake and glucose metabolism (incorporation in the cell wall) coinciding in time with an increase in ovary growth rate (4, 5).
To study possible changes in gene expression in pea ovaries induced to develop by gibberellins we have prepared a cDNA library enriched by sustraction for messages specific of pea ovaries. We have isolated several clones corresponding to genes which expression is modulated by GA_3 by differential screening with probes +, derived from cDNAs of GA_3 treated ovaries and -, from non treated ones. Currently, we are characterizing one of them, which expression is constant in non stimulated ovaries but is repressed in ovaries induced to develop either by GA_3 or by pollination. *In situ* hybridization experiments show that in non stimulated ovaries the transcripts of this gene are localized in the endocarp and in cells of the mesocarp close to the endocarp. No signal is detected either in the exocarp or in the ovule tissues. When the ovary is pollinated, there is a general reduction of signal intensity, but in ovaries stimulated by GA_3 the levels of transcripts remain high in the endocarp and decerease in the mesocarp. Sequence analysis of the full size cDNA shows a significant homology with several plant lipoxygenases. We will discuss these results in the general contex of the role of gibberellins in the induction of fruit development and/or the repression on fruit senescence.

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ETHYLENE, FRUIT RIPENING, AND GENE EXPRESSION

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The pathway for ethylene biosynthesis has been elucidated with the first committed step being the conversion of S-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) by the ACC synthase enzyme. ACC is then oxidized to ethylene by the ACC oxidase enzyme. The E8 predicted polypeptide is a member of a family of dioxygenases found in plants and microorganisms, and is related to ACC oxidase, sharing 34% amino acid sequence identity over 295 residues. To determine the function of E8 we transformed tomato plants with an E8 antisense gene. Whereas it has been shown that reduction of ACC oxidase results in reduced levels of ethylene biosynthesis, we found that reduction of the related E8 protein produces the opposite effect, a 10-fold increase in ethylene evolution specifically during the ripening of detached fruit. Thus, E8 has a negative effect on ACC synthase enzyme activity and subsequent ethylene production in fruit. We have investigated the molecular mechanism responsible for the 10-fold increased ethylene production. Expression of the E8 antisense gene increased the level of ACC and ACC synthase activity 10- and 15-fold, respectively. There was no increase in the level of ACC oxidase activity or level of conjugated forms of ACC. RNA blot hybridization experiments showed that expression of the E8 antisense gene did not affect the concentration of either ACC synthase mRNA. Thus, action of the E8 protein, either directly or indirectly, acts at the posttranscriptional level to represses ACC synthase activity, the rate limiting enzyme for ethylene production during fruit ripening.

Transcription of the E4 gene is controlled by an increase in ethylene concentration during tomato fruit ripening. To investigate the molecular basis for ethylene regulation, we have examined the E4 promoter to identify *cis*-elements and *trans*-acting factors that are involved in E4 gene expression. In transgenic tomato plants a chimeric gene construct containing a 1.4 kilobase E4 promoter fused to a β-glucuronidase (GUS) reporter gene is rapidly induced by ethylene in ripening fruit. Deletion of E4 promoter sequences to 193 base pairs reduces the level of GUS activity, but does not affect ethylene induction. Transient expression of E4 promoter-luciferase chimeric gene constructs containing various deletions, introduced into tomato fruit pericarp by particle bombardment, indicates that a positive ethylene response element is localized between -161 and -85. DNase I footprint analysis shows that a nuclear factor in unripe fruit interacts specifically with sequences in this element, from -142 to -110, which are required for the ethylene response. The DNase I footprint of this factor is reduced in ethylene treated unripe fruit and undetectable in ripe fruit. Based on the correlation of a nuclear factor binding site with promoter sequences required for ethylene induction, we propose that this *in vitro* DNA-binding activity represents a factor that is involved in ethylene-regulated E4 gene expression.

SESSION IV

Hormone responsive elements

ABSCISIC ACID AND WATER STRESS RESPONSIVE GENES IN MAIZE

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Earlier studies on the regulation of gene expression during embryogenesis in *Zea mays* L. have shown that a group of stage-specific mRNAs and proteins which accumulate in late embryogenesis are induced precociously in immature embryos when abscisic acid (ABA) is supplied in the culture medium Gomez et al 1988). One gene *rab* 17 encoding a highly phosphorylated protein, has been previously sequenced and extensively characterized (Vilardell et al. 1990; Pla et al 1989).

Expression of maize *rab* 17 gene can be induced in tissues other than those of the embryo, and at other stages besides embryo development by ABA treatment or water-stress (Pla et al 1991). Several genes showing similar behaviour have been identified in different plant systems (Mundy et al. 1988, Bartels et al. 1990, Claes et al. 1990,). Transient expression studies have shown that the promoter region of rab genes contain ABA-responsive DNA motifs which confer ABA responsiveness on reporter genes in cereal protoplasts (Marcotte et al.1989, Mundy et al 1990,). Moreover a leucine zipper protein binding to one of the DNA motifs has been recently characterized (Guiltinan et al. 1990, Oeda et al 1991). Thus there is strong evidence for regulation of transcription of ABA-responsive genes, but control at the posttranscriptional level has also been suggested. Furthermore, differences in the level of expression of several ABA responsive genes have also been reported (Skriver et al. 1990). All these data suggest that more than one mechanism determines the level of expression of the various ABA-responsive genes.

Studies will be presented on the regulation of *rab* genes in transgenic Arabidopsis mutants and on the putative roles of two ABA-responsive proteins which may help to further characterize molecular mechanisms of ABA on gene expression during water stress.

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GA- & ABA-RESPONSIVE GENE EXPRESSION

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We are working on two different aspects of GA and ABA responsive gene expression. First, we are attempting to clone barley cDNAs encoding putative regulatory proteins which bind to a functionally defined GA-responsive DNA element (GARE)[1]. To date, a 2kB cDNA (Gab1) isolated by expression screening was shown by sequencing to encode a novel protein with two potential DNA-binding domains. Southern blotting indicates that GAB1 is encoded by a single gene. GAB1 fusion protein produced in *E. coli* binds specifically to the GARE probe used to isolate the cDNA. Northern blotting shows that the rare, 2kb GAB1 mRNA accumulates earlier than α-amylase mRNA in GA-treated aleurone layers. This pattern of expression is consistent with a model of α-amylase control involving an inducible regulatory protein.

Second, we are characterizing a novel rice gene (*rab27*) whose expression is responsive to osmotic stress and to ABA[2]. The 1.1kB mRNA, cloned by differential cDNA screening, accumulates rapidly following osmotic stress or ABA-treatment of embryonic and vegetative tissues. Hybrid selection/ translation confirmed the size of the mRNA and showed that rice contains at least three other related genes. Transcription/ translation confirmed the RAB 27 open reading frame determined by DNA sequencing. Northern blotting indicates that homologous genes are found in other cereals. The encoded protein (RAB27) shows sequence and secondary structural similarities to a mammalian Cl^-- channel. In collaboration with R. Hedrich (Hannover), we are testing whether RAB27 acts as a channel in *oocytes* by patch-clamp measurements[3]. While Cl^--channels may have several functions, the pattern of expression of RAB27 indicates that it is involved in osmotic adjustment.

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ABA-REGULATED GENE EXPRESSION : CIS-ACTING SEQUENCES AND TRANS-ACTING FACTORS Ralph S. Quatrano Dept. Biol., Univ. North Carolina, Chapel Hill, NC 27599-3280

The Em gene is normally expressed only in maturing embryos monocot grains and dicot seeds. ABA is required for the control of Em expression in cereal embryos and ABA can regulate the normal changes of Em gene products in embryos cultured in vitro. Expression of Em in embryos also requires the product of the viviparous (Vp1) locus. A mutation at Vp1 results in ABA insensitivity (no changes in ABA levels) which causes the precocious germination of immature embryos. Accumulation of Em transcripts occurs in vegetative tissue when 1-3 day old seedlings are treated with ABA or placed under water stress. Also, the Em gene is expressed in embryogenic rice suspension cultures following exposure of the cultures to various combinations of ABA and osmotic stress.

We have described the identification of ABA-responsive sequences in the 5' promoter of the Em gene using a rice protoplast transient assay and transgenic plants (Marcotte, et al. (1988), **Nature** 335:454-457; Marcotte, et al. (1989), **The Plant Cell** 1:969-976). A detailed analysis of the sequences involved in the transcriptional component of the regulation has shown that a 76 bp sequence is necessary and sufficient for greater than 10-fold induction of expression in the presence of ABA. In addition, a DNA binding protein of the basic-leucine zipper class (bZIP) has been shown to interact with a portion of this same sequence. The target sequence contains a core region, 5'-CACGTG-3', which appears to be conserved for several plant and non-plant DNA binding proteins. Mutation of 2 bp in the core sequence reduces or eliminates the ability of the 76 bp sequence to mediate ABA induction and to interact with this factor (Guiltinan, et al. (1990), **Science** 250:267-271). Further studies will be presented which have reduced the sequences necessary and sufficient for ABA-mediated induction to 20 bp (Em1a element) and which further characterize the bZIP protein (EmBp-1).

When the Vp1 protein is expressed from a constitutive promoter, transactivation of the Em gene can occur in protoplasts in the absence of exogenous ABA (McCarty, et al. (1991), **Cell** 66:895-905). In the presence of ABA, expression of the Vp1 product results in a synergistic effect on expression of Em. Analysis of the regions of the Em promoter required for the activation and enhancement effects of Vp1 define two regions; Em1a and a 129 bp region upstream from Em1a. Detailed analysis of the sequences in these regions responsible for these effects will be presented, as well as results that test whether a direct interaction occurs between EmBp-1 and Vp1.

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SESSION V

Expression and repression of hormone biosynthetic genes and analysis of hormone response in transgenic plants

Modifying Gene Expression, Leaf Senescence and Tomato Ripening by Inhibiting Production of the Ethylene Forming Enzyme Using Antisense Genes

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Ripening of fleshy fruits involves dramatic changes in physiology and biochemistry that alter their colour, flavour, texture, and aroma. These changes affect all cell compartments and require the expression of new genes encoding enzymes that catalyze reactions essential for development of quality attributes. In climacteric fruits, such as tomato, ethylene functions as a hormone to stimulate gene expression and ripening. Molecular cloning experiments have led to the isolation of cDNAs encoding many ripening proteins (Grierson *et. al.*, Proc. Roy. Soc. B 314, 399-40, 1986). Subsequent sequence analysis and experiments in transgenic plants have enabled the roles of several of these to be established (Gray *et, al.*, Plant Molec. Biol. 19, 69-87, 1992). This has led to the identification and manipulation of novel plant genes encoding enzymes involved in cell wall texture change (polygalacturonase, pectinesterase), carotenoid biosynthesis (phytoene synthase) and ethylene synthesis ('ethylene-forming' enzyme or ACC oxidase), and the identification of gene control regions involved in ripening-specific expression. Antisense gene techniques have been developed to generate transgenic plant lines in which specific genes can be permanently inactivated by molecular breeding, with implications for modifying ripening and other processes such as the senescence of leaves and flowers. The antisense approach has also been used to identify and assign functions to unknown genes. The effect of inhibiting ethylene synthesis on ripening of tomatoes and the senescence of leaves and flowers will be described. These fundamental studies have led to commercial evaluation of transgenic tomato lines with improved colour, texture, storage life, and processing characteristics. The future applications of this work, and possible further developments will be discussed.

Transgenic plants altered in phytohormone metabolism.

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Genes cloned from microbial plant pathogens have been expressed in transgenic plants to provide evidence that plant physiological and developmental processes can be altered by enzymes either able to synthesise auxin-conjugates (e.g. indolacetic-acid-lysine synthetase from *Pseudomonas savastanoi)* or to release free active cytokinins from their inactive conjugates (e.g. RolC ß-glucosidase of *Agrobacterium rhizogenes*). Alteration of phytohormone metabolism (i.e. auxin) results from the anther-specific expression of the *rolB* gene of *Agrobacterium rhizogenes*, which codes for a ß-glucosidase able to hydrolyse indoxyl-glucoside. Plants, which are mosaics for the expression of a cytokinin-synthesising enzyme (i.e. isopentenyltransferase of *Agrobacterium tumefaciens*) form adventitious buds on their leaves indicating that *in planta* cytokinin can trigger a novel and ectopic developmental plan. Epiphyllous buds are usually vegetative. However floral buds are observed too. Some epiphyllous floral buds are morphological abnormal. Abnormalities of flower development correlate with localised expression of the cytokinin-synthesising gene and a reduction in the steady state level of mRNAs corresponding to three homeotic genes involved in the control of floral organ identity. Lastly we report on the biological effects of the *rolA* gene of *Agrobacterium rhizogenes.* Altogether this line of research has contributed to show that plants altered in their hormone metabolism display alterations of form and growth, and in the case of the "cytokinin mosaics", an ectopic developmental program.

ANALYSIS OF AUXIN-RESPONSIVE PROMOTERS IN TRANSGENIC PLANTS.
Tom Guilfoyle, Gretchen Hagen, Yi Li, Zhanbin Liu, Tim Ulmasov, Tim Strabala and Xiang Yang Shi, Department of Biochemistry, University of Missouri, Columbia, MO 65211

We have cloned and sequenced a number of auxin-responsive cDNAs (i.e., GH3 and SAURs) and their corresponding genes from soybean and *Arabidopsis*. Each of these genes is transcriptionally regulated specifically by auxins within minutes after application of the hormone. While the GH3 and SAUR (Small Auxin Up RNA) mRNAs show a 25-50-fold increase in mRNA abundance over a broad range of auxin concentration (10^{-9} to 10^{-3} M), the mRNAs show distinct patterns of organ-specific and tissue-specific expression. GH3 mRNAs are most abundant in vascular tissues of roots and are transiently expressed during flower and seed development; however, external application of auxin leads to GH3 expression in most, if not all, organs and tissues in the plant. SAURs are most abundant in epidermal and cortical cells within elongation zones of hypocotyls, epicotyls, stems, and petioles, and application of auxin simply leads to increased levels of expression within these same organs and tissues. The SAURs are unstable, and one determinant of this instability resides in the open reading frame of the mRNA. The promoters of the GH3 and SAUR genes have been fused to the *E. coli uidA* gene which encodes β-glucuronidase (GUS) and transferred into tobacco and/or *Arabidopsis* via *Agrobacterium* T-DNA. These promoters or parts of these promoters have been used to follow the expression pattern and auxin-inducibility of these genes in transgenic plants. We are attempting to identify minimal auxin-responsive elements and gravity-responsive elements within these promoters. We have also fused the auxin-inducible promoters to cytokinin and auxin biosynthetic and conjugating genes to facilitate our studies on the role of auxin in gravitropic and other growth and developmental responses.

POSTERS

Effect of ethylene on gene expression during maturation of orange flavedo tissue

Alonso, J.M., Chamarro, J., and Granell, A.

The flavedo (outer coloured part of the rind about 1 mm thick) of orange fruits undergoes important changes during maturation. Some maturation-related changes can be stimulated by ethylene though oranges do not show the characteristics of a climateric fruit.

The more obvious physiological changes occuring during maturation of orange flavedo are chlorophyll degradation and carotenoid biosynthesis which can also be induced by ethylene. In a previous work, we presented evidence that some of the changes in the levels of specific proteins and mRNAs, as detected by this *in vitro* translation products, occurring during maturation could also be detected after exogenous application of ethylene to fully developed green fruits (mature green fruits).

The aim of this work is to better understand fruit maturation and the role of ethylene by studying genes whose expression appear to be regulated by ethylene. To do this, we constructed cDNA libraries using mRNA from ethylene-treated mature green fruits. Using differential screening we have isolated four cDNAs that showed increased levels after ethylene treatment. Two additional cDNAs appear to be down-regulated by ethylene.

Studies on the changes in the levels of specific mRNAs as a function of maturity and/or ethylene treatment are currently in progress. The nature of the gene products by homology searching in genebanks, together with the results from their pattern of induction will eventually shed some light on their role in the maturation process.

GENE EXPRESSION DURING WHEAT GRAIN GERMINATION

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During wheat grain germination the aleurone cells synthesize and secrete a number of hydrolytic enzymes to mobilize the reserve material in the endosperm. This process is under hormonal control: gibberellic acid (GA) activates transcription of genes coding for these enzymes while abscisic acid (ABA) counteracts this activating effect.

Some of these genes have been cloned and the expression of two of them, a carboxypeptidase III and a low-pI α-amylase (*Amy*1), during wheat grain germination has been studied using Northern analysis and *in situ* hybridization.

As previously reported, a high level of expression of both genes is observed in aleurone layers in response to exogenously added GA. Expression at a lower level is also detected in dissected embryos, however, this expression is not enhanced by GA.

In situ hybridization shows that one day after germination *Amy*1 mRNA is confined to the scutellar epithelium, no expression is detected in aleurone cells at this stage. After two days, however, expression in the epithelium declines while expression in the aleurone commences and progresses from the proximal to the distal end of the grain. The pattern of expression of carboxypeptidase III and *Amy*1 is very similar in aleurone cells, however, in the embryo no expression of carboxypeptidase III was detected in epithelial cells, the expression being restricted to the vascular tissue both in the scutellum and the coleoptile.

Expression in the scutellar epithelium and the aleurone cells is in agreement with a function of endosperm starch breakdown for *Amy*1 gene, however, a different role may be played by carboxypeptidase in the development of the vascular tissue. In addition, it is shown that effectors other than GA may control gene expression in the grain embryo.

EXPRESSION OF A THIOL PROTEASE GENE DURING GERMINATION OF CHICKPEA

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We are investigating the mechanisms that regulate synthesis and activity of degradative enzymes during chickpea germination. In contrast with the well known system of cereals, where both alpha-amylase and thiol proteases are regulated at the transcriptional level by the positive action of Gibberellic Acid and negative of ABA (Rogers et al,1985), in legumes, the regulatory mechanisms are less understood.

Alpha amylase transcript is already present at the onset of germination in chickpea, but the enzymatic activity increases notably in the course of germination, this suggesting that enzyme synthesis or activation during germination may be due to regulatory mechanisms acting at a posttranscriptional level.

To study the regulation of thiol proteases, we have amplified by RT-PCR a fragment of cDNA corresponding to a thiol protease gene expressed during the germination of chickpea. We have used this fragment as a probe to investigate the regulation of this gene during germination and the influence of hormonal and environmental factors. The corresponding mRNA is absent in embryogenic development and in the dry seed being first detected in the cotyledons 24 hours after imbibition. Possible mechanisms regulating the expression of this gene in cotyledons will be discussed.

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HISTOCHEMICAL ANALYSIS OF A MAIZE UBIQUITIN PROMOTER -β-GLUCURONIDASE GENE EXPRESSION IN TRANSGENIC RICE PLANTS

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Ubiquitin is a highly conserved eucaryotic protein involved in multiple cellular functions. The widespread occurrence of this protein, suggests that ubiquitin promoters are likely to be active in a variety of cell types. We studied the activity of the maize ubiquitin promoter and intron (UBI) in fertile transgenic rice plants (cv Taipei 309). Stable transformants were obtained from protoplasts co-transfected with UBI:GUS and UBI:BAR. Protoplasts were prepared from rice calli, bypassing the time-consuming establishment of suspension cultures. The distribution of GUS activity in transgenic rice reveals several features of UBI expression. This promoter is most active in rapidly dividing cells such as callus and meristematic zones. In rice plants, the strongest GUS expression was found in young roots. They appeared intensely stained throughout the meristematic, elongation and differentiation zones. However, in more mature roots, expression decreased drastically and was only associated with the central cylinder. GUS activity in rice leaves was located in the vascular system and also in the guard cells. At the time of flowering, GUS staining in leaves was almost undetectable. The somatic tissue of rice anthers did not show GUS activity, while a fraction of the pollen population showed intense GUS staining. Overall, the pattern of GUS expression from UBI in transgenic rice suggests that this promoter is expressed in cells with high metabolic activity and undergoes important changes during the development of transgenic rice plants.

HOW DOES ABA PREVENT GERMINATION?

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Maize and *Arabidopsis* mutants impaired in the synthesis or response to abscisic acid (ABA) germinate precociously, strongly suggesting that ABA is required to prevent germination of immature seeds. When ABA is applied exogenously to wild type mature seeds, it can also prevent their germination. The mechanism for this effect of ABA is not known. Several reports have shown that ABA inhibits the synthesis of α–amylase and stimulates the synthesis of an α- amylase inhibitor. This suggests that ABA might interfere with germination by impeding the use of reserve macromolecules, thus depriving the germinative process of the required building blocks and energy. We are addressing this possibility in *Arabidopsis*.

Germination of mature *Arabidopsis* seeds is not affected detectably by high concentrations of α- amanitine, a transcription inhibitor. This suggests that germination is not dependent on transcription. The inhibition of germination induced by ABA is also insensible to α- amanitine. These two results make unlikely that ABA blocks germination by repressing transcription of germination genes. ABA, at 10 μM, prevents germination completely when seeds are sown in mineral medium. However, if the medium is supplemented with a good carbon source (glucose, sucrose or peptone), seeds germinate and grow to become plantlets even in the presence of 30 μM ABA. Interestingly, under these conditions ABA directs the synthesis of the same conspicuous protein pattern it directs when no carbon source is added and the seeds are unable to germinate. So, it seems that the addition of a carbon source does not interfere with the normal regulatory effects of ABA. We have studied the degradation of the seed storage proteins. These proteins rapidly disappear during the first days of germination in mineral medium with or without a carbon source. If ABA is present, the storage proteins remain undegraded for many days; this holds true even when the seeds are allowed to become young plantlets by the inclusion in the media of a good carbon source.

Our results support the hypothesis that exogenous ABA prevents germination of mature seeds by restricting the availability of metabolites and oppose some hypothesis suggesting that ABA prevents germination by turning off the germinative genetic program. Stated in a different way: ABA inhibits the catabolic but not the anabolic processes of germination.

DIFFERENTIAL EXPRESSION OF SUCROSE SYNTHASE GENES IN BARLEY

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The enzyme sucrose synthase catalyzes the reaction: sucrose + UDP ⇌ fructose + UDP-glucose that is a key enzyme in starch metabolism. In maize and in wheat two types of non-allelic genes for sucrose synthase have been described (Werr et al. 1985; Maraña et al. 1990) and the pattern of expression under different conditions have been studied (Maraña et al. 1990)

In barley , we have isolated and sequenced two different clones pSS1 and pSS2 from a cDNA library from developing endosperm (Sánchez de la Hoz et al. 1992; Martinez de Ilarduya et al. 1992). By the way, from a genomic DNA library of Hordeum vulgare we have isolated a clon containing the complete gene for SS1 together with the corresponding promoter. We have sequenced the promoter, exons 1 and 2 and the first intron. Besides the location of different important motives in the control of gene expression, we have performed functional studies using transient expression in protoplasts (Díaz and Carbonero,1992). Chimeric genes produced by fusing promoter regions to the β-glucuronidase reporter gene have been used to transform protoplasts from different tissues.

Results of expression and its induction mediated by hormonal factors as well as by stress conditions are being studied.

Transgenic plants have also obtained with the constructs of the enzyme promoter and their expression have been analyzed.

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THE *le* MUTATION IS EXPRESSED IN YOUNG OVARIES OF *PISUM SATIVUM* BUT DOES NOT AFFECT THE GROWTH OF THE FRUIT

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There is clear evidence that fruit-set and development in the garden pea (*Pisum sativum* L.) are regulated by gibberellins (GAs). We have investigated whether the mutation *le*, which decreases the content of GA_1 (the purported active GA) in the shoot due to its partial blockage of GA_{20} 3β-hydroxylase, is also expressed in the reproductive organs. Fruit growth in the isogenic lines 205^+ (*LeLe*, tall) and 205^- (*lele*, dwarf) was similar. Pollinated ovaries from 205^- plants contained lower levels of GA_1 and GA_8 (a GA_1 metabolite) and higher of GA_{20} and GA_{29} (a GA_{20} metabolite) than those from 205^+ plants. These results, together with those from application experiments, indicate that the mutation is also expressed in the ovary. However, the content of GA_3, which is localised mainly in the young fertilised ovules, was similar in both lines. We hypothesize that whereas GA_1 controls stem elongation, GA_3 may be a native regulator for fruit growth.

Isolation and characterization of an ethylene-related gene from *Arabidopsis thaliana*

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Summary: The Ethylene Forming Enzyme is a key factor in ethylene biosynthesis. To better understand the regulation of ethylene biosynthesis in vegetative tissues, we set out to isolate and characterize a DNA complementary to the ethylene forming enzyme from *A. thaliana*. An *A. thaliana* cDNA library was screened with pTOM 13, a tomato cDNA coding for the Ethylene Forming Enzyme in that fruit. A cDNA clone (pEAT 1) was isolated. The cDNA is 1200 nucleotides in length and predicts a protein of Mr 36 663. The insert includes the complete open reading frame of 972 bp and shows strong homology with several reported sequences, both at the nucleic acid and amino acid level. In whole seedlings, expression of pEAT 1 was enhanced by wounding and ACC treatment. In contrast, heat shock had no effect on the expression.

ARE DNA METHYLATION PROCESSES RELATED WITH THE HORMONAL BALANCE IN WOODY PLANT CELL CULTURES?

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Plant DNA is highly modified by covalent attachment of methyl groups to certain bases. This moiety -the methyl group- appears coupled in different positions of adenine and cytosine. At least 30% of total cytosines are modified (Gruenbaum *et al.*, 1981). The sequences of bases normally methylated in plants are the dinucleotide CG and trinucleotide C, $^{A}/_{T}$ G.

The role of this methylation is still unclear, but in mammals could represent a gene regulation mechanism (Holliday, 1987). This idea is based on results that showed the inactivation of some genes are depending on methylation degree.

However, in plants there are evidences on its implication in different processes, such as development (Lo Schiavo *et al.*, 1989; Durante *et al.*, 1990), aging (Díaz-Sala *et al.*, 1989) or somaclonal variation (Phillips *et al.*, 1990) or transformation (Matzke *et al.*, 1991). Recently, many papers described the possibility of controlling plant gene expression by DNA methylation (Ngernprasirtsiri *et al.*, 1990; ; Zhu *et al.*, 1991).

On the other hand, Regeneration processes in tissue culture depends of several factors, such as explant age, **regulator balance** or physiological state. All of them are involved in the "competence" of tissues. Related to the **hormonal control**, it is a noteworthy that the auxin 2,4-D, widely used in cell culture, is associated with DNA hypermethylation (Lo Schiavo *et al.*, 1989). In the other hand, the cytokinins, usually implicated in regenerating steps in many species, show the reverse effect (Arnholdt-Schmitt *et al.*, 1991).

In view of this, **DNA methylation would be implicated in regeneration steps, as development and differentiation, by means of hormone action**.

The isolation and characterization of DNA methylases have been carried out in wheat (Theiss *et al.*, 1980), rice (Giordano *et al.*, 1991) and pea (Yesufu *et al.*, 1991). In this report we describe a method to detect DNA methyltranferase in woody plant cell suspensions, in order to establish **differences between hormonal treatments and DNA methyltransferase activity**. Simultaneously, the study of correlation between methylation patterns and regulators employed is carried out by restriction analysis and hplc determination.

GIBBERELLIN MODULATION OF GENE EXPRESSION IN THE PEA OVARY

Orzáez, D., Peñarrubia, L. & Granell A.

The ovary of a pea plant is a terminal organ, its fate being senescence and abscission unless a stimulus: pollination or hormone treatment, occurs. A single treatment with GA is as effective as pollination in promoting pericarp development and to induce a range of similar biochemical and physiological changes.

Although the transition of an ovary to a developed fruit is a very important process in which hormones play a decisive role, very little attention have been given to study genes involved in the process.

We are interested in defining genes which are involved or change as a consequence of the ovary entering one of these two alternative pathways of development: senescence (no stimulus) or growth (pollination or Ga treatment). The hormonal regulation of these genes is also of our interest. Different approaches have been used in our lab to characterize both pathways. One approach have been based on constructing cDNA libraries of both untreated and GA-induced ovaries conducting differential screening of the libraries to isolate messages that show levels induced or repressed by GA and /or differential expression in the above mentioned processes. Here we present evidence based on screening of ovary cDNA libraries and analysis of selected clones, that senescence and ovary growth are highly regulated pathways in which specific mRNAs are alternatively induced or repressed.

CHARACTERIZATION OF THE AUXIN RESPONSIVE SEQUENCE, GH1. Gretchen Hagen, Melissa Gee and Tom Guilfoyle. Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA.

We have been characterizing the soybean auxin-responsive sequence GH1. GH1 mRNA is moderately abundant in both mature and elongating regions of soybean seedlings, and the levels can be rapidly and specifically induced (several fold) by the external application of active auxins. In situ hybridization analysis has revealed that GH1 mRNA is expressed in all tissues and organs of soybean. The GH1 mRNA levels can be depleted by incubation of soybean organ regions in buffer lacking auxin; mRNA levels are subsequently induced in the same tissues by auxin application. A GH1 cDNA clone with an insert of 1.1 kb contains and ORF of 1kb, and detects a soybean transcript of about 1.7kb. A genomic fragment of about 8kbp corresponding to the cDNA has been sequenced. The gene contains 5 introns and 6 exons. A search of the database revealed no homologous nucleotide sequences. A search of the protein database, however, revealed that the GH1 deduced amino acid sequence was homologous to the soybean auxin-responsive polypeptides encoded by Aux 28 and Aux 22 (previously described by J.L.Key, Athens, GA). Several blocks of amino acids were highly conserved (80-100%) between the sequences. To begin a study of the function of the GH1 protein, we have raised polyclonal antibodies to the polypeptide encoded by GH1. To date, our studies show that the antibodies recognize several polypeptides from plant extracts, and these species are present in most soybean organs that were examined.

IDENTIFICATION OF NEW GENES INVOLVED IN THE MECHANISM OF ACTION OF GIBBERELLINS.

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Only a few gibberellin-regulated genes have been characterized so far: three of these are activated during germination (α-amylase, cystein proteinase, carboxypeptidase), the others (chalcone flavanone isomerase, chalcone synthase) being mainly expressed during flowering. Most of these genes, including the recently discovered GAST1 gene from tomato are activated by gibberellins and inhibited by abscissic acid. However, gibberellins appear to be involved in several other steps of plant growth and development and a number of functions controlled by the hormone as well as the signal transduction pathways are still unknown. GA-deficient mutants sensitive to exogenous GAs, as well as GA-insensitive mutants have been characterized mainly in *Arabidopsis* by M. Koornneef. Such mutants are particularly interesting to study genes involved in the biosynthesis of the hormone or in the hormonal response, respectively.

We have designed different strategies to identify new genes involved in the response to gibberellin signals in *Arabidopsis*. The approaches are primarily based on the use of growth retardants known to inhibit GA synthesis (GA inhibitors). Various GA inhibitors were tested for their ability to inhibit germination of *Arabidopsis* seeds, or to induce visible physiological or developmental changes in the plant. They provide a way of identifying, by differential screening of cDNA libraries, genes that are involved in either biosynthesis or transport of endogenous GA, or in the response to the hormone at different developmental stages. GA inhibitors also represent useful tools to identify resistant genotypes (potentially gibberellin-insensitive) by selectively screening collections of irradiated or T-DNA insertional mutants.

The potentiality of this strategy will be discussed and illustrated by preliminary data on new mutants and genes involved in the gibberellin response in *Arabidopsis*.

ISOLATION, CHARACTERIZATION AND REGULATION OF EXPRESSION OF TWO DIFFERENT EM-LIKE GENES OF ARABIDOPSIS THALIANA

Gillian Hull, Pascale Gaubier, Monique Raynal, Gordon Huestis, Françoise Grellet, César Arenas, Monserrat Pages and Michel Delseny. Laboratoire de Physiologie et Biologie Moléculaire, URA CNRS 565, Université de Perpignan, 66860 Perpignan cédex, France.

During the late maturation stage of the plant seed, at the onset of desiccation, a particular set of mRNA's accumulate in the embryo. The pattern of expression of the corresponding genes suggests that the proteins which they encode, called Late Embryogenesis Abundant (LEA) proteins, play a role in seed maturation and desiccation, and/or germination. The early methionine (Em) protein is a major LEA protein of wheat the expression of which has been shown to be induced by abscisic acid (ABA) and osmotic stress/saline stress. It has been suggested that Em may play a role in maintaining seed viability during desiccation.

We have isolated and characterised two genomic clones of *Arabidopsis* encoding two different proteins which are homologous to the Em protein of wheat. We have studied the pattern of expression of these genes, and their responsiveness to ABA and saline stress in seeds and young seedlings of *Arabidopsis* . The results of northern blot and *in situ* hybridization experiments will be presented.

Complimentary experiments have been carried out with transgenic *Arabidopsis* and tobacco plants transformed with constructs consisting of various regions of the promoter of one of the Em-like genes linked to the GUS reporter gene. The results of this functional analysis of the promoter will be presented.

INVOLVEMENT OF ABA IN THE REGULATION OF FIVE LOW- TEMPERATURE- AND DROUGHT-INDUCED GENES IN *ARABIDOPSIS THALIANA*

Viola Lång, Kerstin Nordin, Tiina Vahala, Björn Welin, Åke Olson, Einar Mäntylä, Marcela Baudo and E. Tapio Palva

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Freezing stress is proposed to have similar effects on plant cells as desiccation: extracellular freezing leads to dehydration of the cytoplasm when water is removed from the cell to the extracellular ice. Many plant species, including *Arabidopsis thaliana*, can tolerate such freeze-induced dehydration stress after exposure to low but non-freezing temperatures. This adaptive process, known as cold acclimation, is correlated to numerous physiological changes, including alterations in the level of endogenous abscisic acid (ABA) and in protein and mRNA profiles. Freezing tolerance can also be increased by exposure to exogenous ABA. It has been suggested that ABA may function as a trigger in both freezing and desiccation tolerance.

Our aim was to isolate genes that might be involved in freezing and desiccation tolerance and study the signal pathways regulating the expression of these genes. For this reason we have cloned a) three *rab / dehydrin*-related genes (*r*esponsive to *AB*A / *dehyd*ration *in*duced) and b) two novel low-temperature-induced genes (*lti* = *l*ow *t*emperature *i*nduced) from *A. thaliana*. The mRNAs corresponding to these genes accumulate in plants exposed to low temperature, water-stress or exogenous ABA but the level and timing of the accumulation is different between the genes. The studies with ABA-synthesis and ABA-response mutants of *A. thaliana* and with wild-type plants treated with the ABA synthesis inhibitor, fluridone, revealed that the genes could be divided into three different categories according to their requirement of ABA in the stress-induced expression:
Class I: low-temperature- and drought induction is independent of ABA.
Class II: low-temperature- and drought induction is ABA mediated.
Class III: low-temperature- but not drought induction is independent of ABA.

The alignment of *rab* and *lti* promoter sequences revealed putative ABRE- and "cold"-boxes and we are currently investigating these elements by deletion analyses. The role of these *rab* and *lti* genes in the cold acclimation process and desiccation tolerance is studied by overexpression / antisense techniques.

Modification of the Genetic Expression in *Saccharum officinarum* during Somatic Embryogenesis.

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Saccharum officinarum, sugarcane, is considered a model system for the study of the somatic embryogenesis in Gramineae as under optimal conditions the regeneration percentage is as great as 100%.

We chose for this work four stages during the somatic embryo development. The first stage include those calli which were in darkness for 30 days in a Murashige-Skoog (MS) medium supplemented with the auxin 2,4 D.

Calli from the first stage were subcultivated on fresh MS medium plus 3 mg/l 2,4 D during 10 days and maintained in darkness. The resulting calli looked highly embryonic and constituted the second stage calli.

These calli were transferred to an auxin free medium and cultivated in the light. After five days we considered the calli to be in the 3rd. stage. These calli showed some violet regions.

The last stage arose when a few little green shoots were clearly observed.

We analysed and compared the protein patterns obtained in a polyacrylamid gel bidimensional electrophoresis in the four types of calli mentioned above.

Via PCR technology, we have isolated an analogue sequence of the Em gene described in wheat (J. C. Litts et al. Nuc. Acid Res. 1987 15,3607-3617) whose expresion and regulation are being studied.

FRUIT SET IS MEDIATED BY GAs IN PISUM SATIVUM : The effect of constitutive GA-response mutations on reproductive development.

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Pea ovaries are induced to enter fruit development by pollination or hormone applications (like gibberellins, GAs). In the absence of these stimuli, ovaries stop growing and enter an alternative pathway of senescence that leads to their degeneration. It h as been proposed that GAs may play a role in fruit-set and development.

We have studied the reproductive development of mutants with GA-independent stem elongation (genotype *lacry*), which are considered to be constitutive for GA responses. We have found that *lacry* mutants can develop parthenocarpic fruits from emasculated flowers, as opposed to wild-type lines, where unpollinated ovaries degenerate instead. The pollination independent phenotype of different *lacry* mutants correlate quantitatively with their GA-independent phenotype and support a role for GAs in fruit-set. The molecular patterns (according to 2-dimensional gel electrophoresis) of proteins and mRNAs of fruits induced by GA application are similar to those of fruits induced by pollination but quite different to the molecular patterns of unstimulated ovaries which enter senescence. In the *lacry* mutants, however, unstimulated ovaries show the typical molecular patterns of stimulated fruits. We therefore conclude that pollination act through gibberellins in fruit set and that this GA response is constitutive in *lacry* ovaries.

We are currently investigating other developmental processes which are altered in this phenotype. and searching for molecular differences between mutants and wild type lines under different conditions

Isolation of genes involved in gibberellin control of cereal stem growth

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Gibberellin control of gene expression in germinating cereal seeds has been studied extensively, and the analysis of GA responsive genes in the aleurone layer is progressing rapidly. However, gibberellin is also involved in controlling the length and strength of grass straws, and inhibitors of gibberellin synthesis are widely used as growth regulators in agriculture. GA affects both the size and the number of cells in grass internodes, but genes involved in this control have not been characterised.
We have initiated a research project to identify and characterise genes involved in the GA response in vegetative parts of young barley seedlings. A number of screening strategies are currently being employed to identify genes differentially expressed in plants either blocked in GA synthesis or stimulated with GA. The results of this work will be presented.

ABSCISIC ACID PROMOTES NOVEL DNA-BINDING ACTIVITY IN DESICCATION TOLERANT CALLUS OF CRATEROSTIGMA PLANTAGINEUM

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DNA binding proteins have been detected in callus of the desiccation tolerant species *Craterostigma plantagineum* which bind specific elements in the promoter region of the ABA and desiccation inducible *det*27-45 gene. The level of one binding activity increases following ABA treatment and is correlated with mRNA accumulation, protein accumulation, and acquisition of desiccation tolerance. This activity binds to sequence elements unlike other abscisic acid response elements and is represented by a complex of four bands as detected by shift assay. Two of these bands are detected in untreated samples although at low levels and the accumulation of the other two after ABA treatment is prevented by protein synthesis inhibitors. As only a very limited region of DNA is protected by these binding activities as revealed by DNAseI footprinting, the complex potentially contains protein-binding proteins in addition to DNA binding proteins.

A second binding activity was also detected which binds an oligonucleotide containing the sequence element ACGT, the so called G-box, indicating it may be related to the common plant response factors (CPRFs), currently thought to be general transcription factors. The level of binding activity is similar in both untreated and ABA treated cells, supporting the idea that this second activity represents constitutive transcriptional factors.

Progress on the purification of the ABA-inducible DNA binding proteins by affinity column chromotography will be reported as well as results of studies to demonstrate that these proteins and the sequence elements are relevant to transcriptional control.

Induction of arginine decarboxylase activity in unpollinated pea ovaries treated with gibberellic acid

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In unpollinated pea ovaries the total amount of putrescine, spermidine, and spermine increases during fruit set induced by gibberellic acid (GA) indicating a stimulation of their biosynthesis (1). In plant tissues polyamine synthesis seems to be regulated via arginine or ornithine decarboxylase activity. We have determined both activities and arginase, which catalyzes the synthesis of ornithine from arginine, with the aim to explain the effect of GA on polyamine biosynthesis.

Incubation of extracts from GA-treated unpollinated ovaries produced an increase in putrescine level that was not observed in extracts from non-treated ovaries. The production of putrescine was inhibited by difluoromethylarginine, an inhibitor of arginine decarboxylase activity, and was not affected by difluoromethylornithine, an inhibitor of ornithine decarboxylase activity, suggesting that polyamines are synthesized in pea ovaries via arginine decarboxylase pathway. Extracts from pea ovaries showed arginine decarboxylase activity but ornithine decarboxylase and arginase could not be detected. Measurement of changes in arginine decarboxylase activity during different developmental stages of the pea ovary showed that arginine decarboxylase activity decreases during the last step of unpollinated ovary development, with a minimum level of activity at day two post-anthesis, just when the ovaries enter in a senescent processs; however, the activity increased shortly and markedly after induction of fruit set with gibberellic acid. Pollination and other plant growth substances (benzyladenine and 2,4-dichlorophenoxyacetic acid) that also induce fruit set and development of unpollinated pea ovaries, promoted increases of arginine decarboxylase activity. All these results suggest that arginine decarboxylase activity is associated with the development of pea ovaries induced by plant growth substances.

(1) Carbonell J, Navarro JL (1989) Planta 178:482-487

Accumulation of salt induced protein TAS 14 in organs, tissues and cells: evidence for a nuclear localization.

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Salt stress-induced changes in gene expression hav been reported in different biological systems. Some of these changes are also induced by osmotic and cold stress. Abscisic acid (ABA) has been extensively related to these kinds of environmental stress; in fact, correlations are found between changes in gene expression induced by ABA and those related to salt, water and cold stress. Endogenous levels of ABA increase dramatically in plants under these stresses; furthermore, the exogenous application of ABA accelerates the acclimation of culture plant cells to water and salt stress. From these data, a crucial role of ABA in mediating the response of plants to drought, salinity and low temperatures can be inferred.

TAS14 cDNA clone of tomato corresponds to a mRNA which accumulates in tomato seedlings upon treatment with NaCl, ABA or mannitol. It is also induced in roots, stems and leaves of hydroponically-grown tomato plants treated with NaCl or ABA. TAS14 clone codes for a highly hydrophilic and glycin-rich protein of 14 kDa molecular weight which is phosphorylated in vivo. TAS14 protein shows structural domains similar to other proteins of several species, which are expressed in late stages of embryogenesis (LEA proteins).

Pattern expression of TAS14 protein, tissue and subcellular localizations have been analyzed by using antibodies against β-galactosidase-TAS14 fusion protein.

TAS14 protein is expressed in radicles of tomato seedlings upon treatment with mannitol, salt and ABA. TAS14 protein appears as different polypeptides of m.w. between 16 and 21 kDa, which correspond to phosphorylated forms of the protein. TAS14 polypeptides were detected after 6 h of salt treatment. Every NaCl concentration analyzed (5g/L to 12.5 g/L) induced the protein, but

concentrations higher than 10 g/L turned to be toxic for seedlings. In untreated hydroponically-grown tomato plants, TAS14 was absent from roots, stems and leaves. However it was accumulated in every organ upon addition of salt to the hydroponic solution. The highest levels of the protein were detected in stems and leaves and it increased with time; in roots, a faint signal was hardly detected after 24 h of treatment, the attempts to detect it at any other times were unsuccessful. Pulse-chase experiments with ^{32}Phosphate suggested that TAS14 protein and its phosphorylated forms were stable as long as salt conditions were present. Amino acid sequence of TAS14 protein contains putative casein kinase 2 (CK-II) phosphorylable sites. Purified TAS14 used as substrate in presence of [γ-32P]ATP, Mg+2 and rat brain CK-II, became highly phosphorylated.

TAS14 protein was preferentially localized in the adventitious primordia, in the procambium of the apical meristem and leaf primordia and it was associated with the vascular system in leaves.

Immunoelectron localization with specific TAS14 antibodies and labelling with protein A-gold revealed the presence of TAS14 protein into the nucleus and the cytoplasm. Gold particles were associated to the nuclear and nucleolar chromatin, preferentially to euchromatin, where the number of gold particles were 13 times higher than in heterochomatin. Immunolabelling of cytoplasm was not associated to any particular organelle, but was mainly localized in the cytosol; the cell wall and cytoplasmic vacuoles of every cell examined were devoid of gold particles. The quantitative analysis showed that the relative number of gold particles into the nucleus were higher (2.5 times) than into the cytosol. Adventitious meristem cells of untreated tomato plants and preimmune controls were essentially free of labelling and background in every region of every cells.

SEQUENCE ANALYSIS OF 2-OXOGLUTARATE-DEPENDENT DIOXYGENASES

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The group of enzymes known as the 2-oxoglutarate-dependent dioxygenases are involved in the catalysis of a wide variety of reactions in plants, animals and microorganisms including the synthesis of penicillins/cephalosporins; gibberellins; ethylene; flavonoids and anthocyanins; hydroxyproline residues in collagen and plant glycoproteins; alkaloids and carnitine. Thus they perform key roles in both primary and secondary metabolism.

Suprisingly, there was little progress in the cloning of any member of this group until 1987. Since then, seven 2-oxoglutarate-dependent dioxygenases from plants, animals and microorganisms have been sequenced. The two plant enzymes, hyoscyamine 6ß-hydroxylase (H6H) and flavanone 3-hydroxylase (F3H) show strong similarity with the two 2-oxoglutarate-dependent dioxygenases cloned from the cephalosporium/penicillin biosynthetic pathway of *Streptomyces clavuligerus* (DAOCS and DACS). The three enzymes cloned from mammalian tissue, prolyl 4-hydroxylase, lysyl hydroxylase and aspartyl ß-hydroxylase show little similarity with each other or with the other sequences available.

Two enzymes which are not 2-oxoglutarate-dependent show strong homology to the F3H/H6H/DAOCS/DACS sequences. One of these, isopenicillin N synthase is also involved in penicillin/cephalosporin synthesis. The other is 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase), which catalyses the final reaction in ethylene synthesis in ripening fruit.

The sequences of the 7 2-oxoglutarate-dependent enzymes, ACC oxidase and isopenicillin N synthase are compared with each other and with other sequences available from the databases. Conserved amino acids and motifs are discussed in the light of the biochemical data available. The evolutionary relationships between 2-oxoglutarate-dependent dioxygenases and other oxygenases are considered.

MOLECULAR CHARACTERIZATION OF THE RESPONSE TO ABSCISIC ACID AND JASMONIC ACID OF A CELL SUSPENSION CULTURE FROM COMMON BEAN (*Phaseolus vulgaris* L.).

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The plant hormones abscisic acid (ABA) and jasmonic acid (JA) have been reported to mediate the conversion of various environmental stresses into changes in plant gene expression.

We have initiated the study of the response to ABA and JA of a bean, *Phaseolus vulgaris,* cell suspension culture , through the analysis of the proteins induced by these hormones. The synthesis of four proteins of 22, 36, 60, and 70 kD is clearly increased when 10^{-4}M ABA is added to the culture. One of this proteins, 22kD (P22), has been purified, and antibodies against it were raised in mice. The antibodies will be utilized to study the pattern of appearance of P22 under different growth conditions. P22 is also induced by 10^{-5}M JA.

In addition to that the 60 Kd protein, P60, which is also a glycoprotein, is being purified. This protein has novel properties in comparison with other proteins of bean ,induced by ABA or water deficit, identified until now.

Peroxidase activity is also induced by ABA treatment in this culture, we are trying to identify the protein responsible of this activity.

CLONING OF A PEA OVARY cDNA WHICH IS REPRESSED DURING DEVELOPMENT INDUCED BY GIBBERELLINS OR POLLINATION

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There is evidence that gibberellins (GAs) regulate fruit-set and pod development in pea. Application of gibberellic acid (GA_3) to pea ovaries of flowers previously emasculated to avoid pollination, induces the development of parthenocarpic fruits that are identical to those arisen by fertilisation.

The mode of action of GAs in this process is poorly known. Our interest consists in isolate and characterize genes that are modulated by GAs during pea ovary development. We have constructed a cDNA library enriched for GA_3-modulated sequences. The substracted library was differentially screened with cDNA probes from ovaries treated or not with GA_3. One clone was isolated and tested in Northern experiments, showing that it was constantly expressed in non-stimulated ovaries while it was repressed in GA_3-treated or pollinated ovaries.

We have also carried out *in situ* hybridization experiments in order to localize the transcripts in ovary tissue sections. The gene is expressed in the pod, mainly in the endocarp and in the close mesocarp; it is not expressed at all either in the exocarp or in the ovule tissues. In ovaries treated with GA_3 there is a general decrease of the signal in the mesocarp, while the levels of transcripts keep high in the endocarp.

The sequence analysis revealed that this clone has a significant similarity to some oxydizing enzymes which may be involved in the production of free radicals and cell wounding. The full-length cDNA is currently being analyzed. We discuss the molecular data and propose a model for a physiological role of this gene.

ROLE OF GIBBERELLINS IN THE REPRODUCTIVE AND VEGETATIVE DEVELOPMENT OF *Citrus*

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Research in our laboratories has been focused on the role of plant hormones in the regulation of the reproductive and vegetative development of *Citrus*. In a comparative study on two seedless mandarins differing in their potential to set fruits, we have recently shown (*Plant Physiol.* 99: 1575-1581, 1992) that the parthenocarpic development of the fruit is associated with high levels of 13-hydroxy-GAs. The effects of different inhibitors of GAs biosynthesis (paclobutrazol, inhibitor of ent-kaurene oxidation, and BX-112, which blocks 3-ß hydroxylation) on different processes of fruit development are currently under study. Analysis of the endogenous content of Abscisic Acid (ABA) suggests a negative relationship between ABA and GAs in the control of Citrus fruit development. Changes in polypeptides during fruit development induced by these treatments will be presented and discussed.

On the other hand, inhibition of GA biosynthesis reduced shoot development of Citrus seedlings, and this effect was counteracted by different GAs. The potential of these treatments in the control of Citrus dwarfism will be also evaluated and discussed.

SUMMARY AND PERSPECTIVES

R. L. Jones

Advances in molecular biology have had a profound impact on the field of plant hormone biology. In this workshop speakers emphasized the progress that has been realized over the past few years using molecular cloning methods, mutational analysis and transgenic plants, including the expression of antisense genes in higher plants.

Molecular cloning has identified genes that respond to hormones, and analysis of the promoters of hormone-regulated genes has led to the identification of cis-acting elements that control the expression of these genes. Progress is now being made in identifying specific trans-acting factors that interact with promotors, and in the case of the gibberellic acid response element (GARE) of the α-amylase gene in barley, a gene for a GARE-binding (GAB) protein has been cloned. Interestingly, the expression of GAB is also hormonally regulated. Transgenic plants have also proved valuable for the study of hormone biosynthesis and action. The introduction of hormone biosynthesis genes from bacteria into various angiosperms has helped clarify the relationship between the levels of auxins and cytokinin and the physiological and developmental responses that they elicit. It is clear from these types of experiments that the levels of hormones in plants have a profound effect on development.

A genetic approach, especially when coupled with molecular methods for gene identification, has also lead to significant advances in several areas of plant hormone biology. For example, the pathway of gibberellin (GA) biosynthesis is now well understood from work with dwarf phenotypes of *Zea mays* and *Pisum sativum*, and more recently *Arabidopsis thaliana*. Gene tagging methods are being used to isolate the genes in the GA biosynthetic pathway in maize, pea and *Arabidopsis*. Mutant analysis, particularly in *Arabidopsis*, is also leading to the identification of the genes involved in responses to ethylene and abscisic acid (ABA). Recent work reported at this workshop with ethylene-insensitive *Arabidopsis* mutants has led to the identification of a gene that encodes a protein kinase that may be part of the ethylene signal transduction pathway.

Several reports at this meeting described progress in the identification of plant hormone receptors. There appear to be numerous receptors for auxins located in various cellular compartments, although the low molecular weight (20-22 kD) auxin binding protein found in the endoplasmic reticulum (ER) has received the most attention because of the unique properties of the protein. In addition to possessing a C-terminal ER retention signal (the amino acid sequence KDEL), this protein is apparently secreted to the cell surface. The possibility that the ER auxin-binding protein interacts with the outside of the plasma membrane to effect auxin action is intriguing. Reports that the ABA and GA receptors might be also located at the surface of responsive cells were also presented.

Since a consensus is emerging that plant hormones have receptors at the plasma membrane, there is a considerable interest in the signal transduction pathways that link the receptor to the developmental response of the cell. There is growing acceptance of the view that calcium and the regulatory protein calmodulin are involved in responses to hormones in various cell types including stomatal guard cell and cereal aleurone cells. It is clear, however, that

the signal transduction pathways for plant hormones will involve complex interconnecting webs of signal transduction chains.

The utility of fruit development and ripening in pea and tomato, respectively, as models for the study of plant hormone action was also demonstrated at the workshop. The unpollinated pea ovary has been used to examine the effects of GAs on development and senescence, whereas the ripening tomato fruit continues to provide unique insights into the role of ethylene in development. The use of antisense genes has proven to be a particularly powerful tool for identifying the functions of unknown genes in tomato development.

The Workshop on Approaches to Plant Hormone Action was an unqualified success, and we learned that even more exciting developments in this field can be expected in the next decade. Biophysical approaches, such as the use of the patch clamp and microelectrode techniques are likely to rapidly advance our knowledge of receptors and signal transduction, whereas the use of mutants in well-defined genetic systems, such as corn and *Arabidopsis*, will lead to the identification of genes that participate in hormone action.

List of Invited Speakers

Workshop on

APPROACHES TO PLANT HORMONE ACTION

List of Invited Speakers

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List of Participants

Workshop on

APPROACHES TO PLANT HORMONE ACTION

List of Participants

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concerning workshops and courses organized within the
Plan for International Meetings on Biology (1989-1991)

246 **Workshop on Tolerance: Mechanisms and implications.**
Organized by P. Marrack and C. Martínez-A. Lectures by H. von Boehmer, J. W. Kappler, C. Martínez-A., H. Waldmann, N. Le Douarin, J. Sprent, P. Matzinger, R. H. Schwartz, M. Weigert, A. Coutinho, C. C. Goodnow, A. L. DeFranco and P. Marrack.

247 **Workshop on Pathogenesis-related Proteins in Plants.**
Organized by V. Conejero and L. C. Van Loon. Lectures by L. C. Van Loon, R. Fraser, J. F. Antoniw, M. Legrand, Y. Ohashi, F. Meins, T. Boller, V. Conejero, C. A. Ryan, D. F. Klessig, J. F. Bol, A. Leyva and F. García-Olmedo.

248 Beato, M.:
Course on DNA - Protein Interaction.

249 **Workshop on Molecular Diagnosis of Cancer.**
Organized by M. Perucho and P. García Barreno. Lectures by F. McCormick, A. Pellicer, J. L. Bos, M. Perucho, R. A. Weinberg, E. Harlow, E. R. Fearon, M. Schwab, F. W. Alt, R. Dalla Favera, P. E. Reddy, E. M. de Villiers, D. Slamon, I. B. Roninson, J. Groffen and M. Barbacid.

251 **Lecture Course on Approaches to Plant Development.**
Organized by P. Puigdoménech and T. Nelson. Lectures by I. Sussex, R. S. Poethig, M. Delseny, M. Freeling, S. C. de Vries, J. H. Rothman, J. Modolell, F. Salamini, M. A. Estelle, J. M. Martínez Zapater, A. Spena, P. J. J. Hooykaas, T. Nelson, P. Puigdoménech and M. Pagès.

252 **Curso Experimental de Electroforesis Bidimensional de Alta Resolución.**
Organizado por Juan F. Santarén. Seminarios por Julio E. Celis, James I. Garrels, Joël Vandekerckhove, Juan F. Santarén y Rosa Assiego.

253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
Organized by F. García-Arenal and P. Palukaitis. Lectures by D. Baulcome, R. N. Beachy, G. Boccardo, J. Bol, G. Bruening, J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. García-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

254 **Advanced Course on Biochemistry and Genetics of Yeast.**
Organized by C. Gancedo, J. M. Gancedo, M. A. Delgado and I. L Calderón.

255 **Workshop on The Reference Points in Evolution.**
Organized by P. Alberch and G. A. Dover. Lectures by P. Alberch, P. Bateson, R. J. Britten, B. C. Clarke, S. Conway Morris, G. A. Dover, G. M. Edelman, R. Flavell, A. Fontdevila, A. García-Bellido, G. L. G. Miklos, C. Milstein, A. Moya, G. B. Müller, G. Oster, M. De Renzi, A. Seilacher, S. Stearns, E. S. Vrba, G. P Wagner, D. B. Wake and A. Wilson.

256 **Workshop on Chromatin Structure and Gene Expression.**
Organized by F. Azorin, M. Beato and A. A. Travers. Lectures by F. Azorin, M. Beato, H. Cedar, R. Chalkley, M. E. A. Churchill, D. Clark, C. Crane-Robinson, J. A. Dabán, S. C. R. Elgin, M. Grunstein, G. L. Hager, W. Hörz, T. Koller, U. K. Laemmli, E. Di Mauro, D. Rhodes, T. J. Richmond, A. Ruiz-Carrillo, R. T. Simpson, A. E. Sippel, J. M. Sogo, F. Thoma, A. A. Travers, J. Workman, O. Wrange and C. Wu.

257 **Lecture Course on Polyamines as modulators of Plant Development.**
Organized by A. W. Galston and A. F. Tiburcio. Lectures by N. Bagni, J. A. Creus, E. B. Dumbroff, H. E. Flores, A. W. Galston, J. Martin-Tanguy, D. Serafini-Fracassini, R. D. Slocum, T. A. Smith and A. F. Tiburcio.

258 **Workshop on Flower Development.**
Organized by H. Saedler, J. P. Beltrán and J. Paz Ares. Lectures by P. Albersheim, J. P. Beltrán, E. Coen, G. W. Haughn, J. Leemans, E. Lifschitz, C. Martin, J. M. Martínez-Zapater, E. M. Meyerowitz, J. Paz-Ares, H. Saedler, C. P. Scutt, H. Sommer, R. D. Thompson and K. Tran Thahn Van.

259 **Workshop on Transcription and Replication of Negative Strand RNA Viruses.**
Organized by D. Kolakofsky and J. Ortín. Lectures by A. K. Banerjee, M. A. Billeter, P. Collins, M. T. Franze-Fernández, A. J. Hay, A. Ishihama, D. Kolakofsky, R. M. Krug, J. A. Melero, S. A. Moyer, J. Ortín, P. Palese, R. G. Paterson, A. Portela, M. Schubert, D. F. Summers, N. Tordo and G. W. Wertz.

260 **Lecture Course Molecular Biology of the Rhizobium-Legume Symbiosis.**
Organized by T. Ruiz-Argüeso. Lectures by T. Bisseling, P. Boistard, J. A. Downie, D. W. Emerich, J. Kijne, J. Olivares, T. Ruiz-Argüeso, F. Sánchez and H. P. Spaink.

261 **Workshop The Regulation of Translation in Animal Virus-Infected Cells.**
Organized by N. Sonenberg and L. Carrasco. Lectures by V. Agol, R. Bablanian, L. Carrasco , M. J. Clemens, E. Ehrenfeld, D. Etchison, R. F. Garry, J. W. B. Hershey, A. G. Hovanessian, R. J. Jackson, M. G. Katze, M. B. Mathews, W. C. Merrick, D. J. Rowlands, P. Sarnow, R. J. Schneider, A. J. Shatkin, N. Sonenberg, H. O. Voorma and E. Wimmer.

263 **Lecture Course on the Polymerase Chain Reaction.**
Organized by M. Perucho and E. Martínez-Salas. Lectures by D. Gelfand, K. Hayashi, H. H. Kazazian, E. Martínez-Salas, M. Mc Clelland, K. B. Mullis, C. Oste, M. Perucho and J. Sninsky.

264 **Workshop on Yeast Transport and Energetics.**
Organized by A. Rodríguez-Navarro and R. Lagunas. Lectures by M. R. Chevallier, A. A. Eddy, Y. Eilam, G. F. Fuhrmann, A. Goffeau, M. Höfer, A. Kotyk, D. Kuschmitz, R. Lagunas, C. Leão, L. A. Okorokov, A. Peña, J. Ramos, A. Rodríguez-Navarro, W. A. Scheffers and J. M. Thevelein

265 **Workshop on Adhesion Receptors in the Immune System.**
Organized by T. A. Springer and F. Sánchez-Madrid. Lectures by S. J. Burakoff, A. L. Corbi-López, C. Figdor, B. Furie, J. C. Gutiérrez-Ramos, A. Hamann, N. Hogg, L. Lasky, R. R. Lobb, J. A. López de Castro, B. Malissen, P. Moingeon, K. Okumura, J. C. Paulson, F. Sánchez-Madrid, S. Shaw, T. A. Springer, T. F. Tedder and A. F. Williams.

266 **Workshop on Innovations on Proteases and their Inhibitors: Fundamental and Applied Aspects.**
Organized by F. X. Avilés. Lectures by T. L. Blundell, W. Bode, P. Carbonero, R. W.Carrell, C. S. Craik, T. E. Creighton, E. W. Davie, L. D. Fricker, H. Fritz, R. Huber, J. Kenny, H. Neurath, A. Puigserver, C. A. Ryan, J. J. Sánchez-Serrano, S. Shaltiel, R. L. Stevens, K. Suzuki, V. Turk, J. Vendrell and K. Wüthrich.

267 **Workshop on Role of Glycosyl-Phosphatidylinositol in Cell Signalling.**
Organized by J. M. Mato and J. Larner. Lectures by M. V. Chao, R. V. Farese, J. E. Felíu, G. N. Gaulton, H. U. Häring, C. Jacquemin, J. Larner, M. G. Low, M. Martín Lomas, J. M. Mato, E. Rodríguez-Boulan, G. Romero, G. Rougon, A. R. Saltiel, P. Strålfors and I. Varela-Nieto.

268 **Workshop on Salt Tolerance in Microorganisms and Plants: Physiological and Molecular Aspects.**
Organized by R. Serrano and J. A. Pintor-

Toro. Lectures by L. Adler, E. Blumwald, V. Conejero, W. Epstein, R. F. Gaber, P. M. Hasegawa, C. F. Higgins, C. J. Lamb, A. Läuchli, U. Lüttge, E. Padan, M. Pagès, U. Pick, J. A. Pintor-Toro, R. S. Quatrano, L. Reinhold, A. Rodríguez-Navarro, R. Serrano and R. G. Wyn Jones.

269 **Workshop on Neural Control of Movement in Vertebrates.**
Organized by R. Baker and J. M. Delgado-García. Lectures by C. Acuña, R. Baker, A. H. Bass, A. Berthoz, A. L. Bianchi, J. R. Bloedel, W. Buño, R. E. Burke, R. Caminiti, G. Cheron, J. M. Delgado-García, E. E. Fetz, R. Gallego, S. Grillner, D. Guitton, S. M. Highstein, F. Mora, F. J. Rubia Vila, Y. Shinoda, M. Steriade and P. L. Strick.

Texts published by the CENTRE FOR INTERNATIONAL MEETINGS ON BIOLOGY

1 **Workshop on What do Nociceptors Tell the Brain?**
Organized by C. Belmonte and F. Cerveró. Lectures by C. Belmonte, G. J. Bennet, J. N. Campbell, F. Cerveró, A. W. Duggan, J. Gallar, H. O. Handwerker, M. Koltzenburg, R. H. LaMotte, R. A. Meyer, J. Ochoa, E. R. Perl, H. P. Rang, P. W. Reeh, H. G. Schaible, R. F. Schmidt, J. Szolcsányi, E. Torebjörk and W. D. Willis Jr.

2 **Workshop on DNA Structure and Protein Recognition.**
Organized by A. Klug and J. A. Subirana. Lectures by F. Azorín, D. M. Crothers, R. E. Dickerson, M. D. Frank-Kamenetskii, C. W. Hilbers, R. Kaptein, D. Moras, D. Rhodes, W. Saenger, M. Salas, P. B. Sigler, L. Kohlstaedt, J. A. Subirana, D. Suck, A. Travers and J. C. Wang.

3 **Lecture Course on Palaeobiology: Preparing for the Twenty-First Century.**
Organized by F. Álvarez and S. Conway Morris. Lectures by F. Álvarez, S. Conway Morris, B. Runnegar, A. Seilacher and R. A. Spicer.

4 **Workshop on The Past and the Future of Zea Mays.**
Organized by B. Burr, L. Herrera-Estrella and P. Puigdoménech. Lectures by P. Arruda, J. L. Bennetzen, S. P. Briggs, B. Burr, J. Doebley, H. K. Dooner, M. Fromm, G. Gavazzi, C. Gigot, S. Hake, L. Herrera-Estrella, D. A. Hoisington, J. Kermicle, M. Motto, T. Nelson, G. Neuhaus, P. Puigdoménech, H. Saedler, V. Szabo and A. Viotti.

5 **Workshop on Structure of the Major Histocompatibility complex.**
Organized by A. Arnaiz-Villena and P. Parham. Lectures by A. Arnaiz-Villena, R. E. Bontrop, F. M. Brodsky, R. D. Campbell, E. J. Collins, P. Cresswell, M. Edidin, H. Erlich, L. Flaherty, F. Garrido, R. Germain, T. H. Hansen, G. J. Hämmerling, J. Klein, J. A. López de Castro, A. McMichael, P. Parham, P. Stastny, P. Travers and J. Trowsdale.

6 **Workshop on Behavioural Mechanisms in Evolutionary Perspective.**
Organized by P. Bateson and M. Gomendio. Lectures by J. R. Alberts, G. W. Barlow, P. Bateson, T. R. Birkhead, J. Carranza, C. ten Cate, F. Colmenares, N. B. Davies, R. I. M. Dunbar, J. A. Endler, M. Gomendio, T. Guilford, F. Huntingford, A. Kacelnik, J. Krebs, J. Maynard Smith, A. P. Møller, J. Moreno, G. A. Parker, T. Redondo, D. I. Rubenstein, M. J. Ryan, F. Trillmich and J. C. Wingfield.

7 **Workshop on Transcription Initiation in Prokaryotes.**
Organized by M. Salas and L. B. Rothman-Denes. Lectures by S. Adhya, H. Bujard, S. Busby, M. J. Chamberlin, R. H. Ebright, M. Espinosa, E. P. Geiduschek, R. L. Gourse, C. A. Gross, S. Kustu, J. Roberts, L. B. Rothman-Denes, M. Salas, R. Schleif, P. Stragier and W. C. Suh.

8 **Workshop on the Diversity of the Immunoglobulin Superfamily.**
Organized by A. N. Barclay and J. Vives. Lectures by A. N. Barclay, H. G. Boman, I. D. Campbell, C. Chothia, F. Díaz de Espada, I. Faye, L. García Alonso, P. R. Kolatkar, B. Malissen, C. Milstein, R. Paolini, P. Parham, R. J. Poljak, J. V. Ravetch, J. Salzer, N. E. Simister, J. Trinick, J. Vives, B. Westermark and W. Zimmermann.

9 **Workshop on Control of Gene Expression in Yeast.**
Organized by C. Gancedo and J. M. Gancedo. Lectures by T. G. Cooper, T. F. Donahue, K-D. Entian, J. M. Gancedo, C. P. Hollenberg, S. Holmberg, W. Hörz, M. Johnston, J. Mellor, F. Messenguy, F. Moreno, B. Piña, A. Sentenac, K. Struhl, G. Thireos and R. S. Zitomer.

10 **Workshop on Engineering Plants Against Pests and Pathogens.**
Organized by G. Bruening, F. García-Olmedo and F. Ponz. Lectures by R. N. Beachy, J. F. Bol, T. Boller, G. Bruening, P. Carbonero, J. Dangl, R. de Feyter, F. García-Olmedo, L. Herrera-Estrella, V. A. Hilder, J. M. Jaynes, F. Meins, F. Ponz, J. Ryals, J. Schell, J. van Rie, P. Zabel and M. Zaitlin.

11 **Lecture Course on Conservation and Use of Genetic Resources.**
Organized by N. Jouve and M. Pérez de la Vega. Lectures by R. P. Adams, R. W. Allard, T. Benítez, J. I. Cubero, J. T. Esquinas-Alcázar, G. Fedak, B. V. Ford-Lloyd, C. Gómez-Campo, V. H. Heywood, T. Hodgkin, L. Navarro, F. Orozco, M. Pérez de la Vega, C. O. Qualset, J. W. Snape and D. Zohary.

12 **Workshop on Reverse Genetics of Negative Stranded RNA Viruses.**
Organized by G. W. Wertz and J. A. Melero. Lectures by G. M. Air, L. A. Ball, G. G. Brownlee, R. Cattaneo, P. Collins, R. W. Compans, R. M. Elliott, H.-D. Klenk, D. Kolakofsky, J. A. Melero, J. Ortín, P. Palese, R. F. Pettersson, A. Portela, C. R. Pringle, J. K. Rose and G. W. Wertz.

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