Abstracts of papers presented
at the 1991 meeting on

MOLECULAR BIOLOGY OF SIGNAL TRANSDUCTION IN PLANTS

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Arranged by
Frederick Ausubel, *Harvard Medical School, Massachusetts General Hospital*
Nam-Hai Chua, *Rockefeller University*
Gerald Fink, *Whitehead Institute*
Venkatesan Sundaresan, *Cold Spring Harbor Laboratory*

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Front cover: An *hy3* mutant of *Arabidopsis thaliana* (*right*) with a wild-type plant (*left*) shown for comparison. *hy3* mutants were originally isolated by Maarten Koornneef (Wageningen) in a screen for mutants that exhibit lack of hypocotyl inhibition in bright white light. The phenotype includes the long hypocotyl, elongated petioles, bolts and leaves, and slight paleness. Recent work indicates that *hy3* mutants are deficient in the type-B phytochrome. Photography by Marc Lieberman, Salk Institute. (See Abstract by Chory et al.)

PROGRAM

WEDNESDAY, October 2—7:30 PM

SESSION 1 RECEPTION OF ENVIRONMENTAL SIGNALS

Chairman: **J. Chory,** The Salk Institute

THURSDAY, October 3—9:00 AM

SESSION 2 RECEPTION OF HORMONAL SIGNALS

Chairman: **J. Ecker,** University of Pennsylvania

THURSDAY, October 3—2:00 PM

SESSION 3 POSTERS I

THURSDAY, October 3—4:30 PM

Wine and Cheese Party

THURSDAY, October 3—7:30 PM

SESSION 4 CELL–CELL RECOGNITION

Chairman: **J.B. Nasrallah,** Cornell University

FRIDAY, October 4—9:00 AM

SESSION 5 G PROTEINS AND PROTEIN KINASES

Chairman: **H. Ma,** Cold Spring Harbor Laboratory

FRIDAY, October 4—2:00 PM

SESSION 6 POSTERS II

FRIDAY, OCTOBER 4—7:30 PM

SESSION 7 TARGETS OF SIGNALS I

Chairman: **E. Coen,** John Innes Institute

SATURDAY, October 5—9:00 AM

SESSION 8 TARGETS OF SIGNALS II

Chairman: **S. Hake,** University of California, Berkeley

SATURDAY, OCTOBER 5—2:00 PM

SESSION 9 MUTANTS IN SIGNALING PATHWAYS

Chairman: **Y. Shimura,** Kyoto University, Japan

SATURDAY, October 5

BANQUET

Cocktails 6:00 PM Dinner 6:45 PM

SUNDAY, OCTOBER 6—9:00 AM

SESSION 10 TRANSGENIC APPROACHES AND TECHNOLOGIES

Chairman: R. Horsch, Monsanto Company

AUTHOR INDEX

BIOCHEMICAL DISSECTION OF PHYTOCHROME FUNCTION

J. Clark Lagarias, Department of Biochemistry and Biophysics, University of California, Davis CA 95616

Phytochrome is a biliprotein photoreceptor which enables plants to adapt to fluctuations of light intensity, direction and spectral quality which occur in the natural environment. The long term objective of the research in my laboratory is to define the components of a phytochrome-mediated signal transduction pathway in the higher plant *Avena sativa* L., where a family of phytochrome photoreceptors regulates many growth and developmental responses throughout the plant life cycle, and the unicellular green alga *Mesotaenium caldariorum*, where phytochrome functions to regulate chloroplast orientation. Biochemical characterization of the photoreceptors from these two diverse organisms has been undertaken in order to define structural features of the phytochrome molecule which are involved in the transmission of the light signal. Experiments which elucidate light-dependent structural changes within phytochrome will be discussed. *In vitro* assembly of spectrally active recombinant holophytochrome has enabled us to introduce non-natural chromophores and site-specific protein mutations at putative functionally important regions of the photoreceptor. Parallel investigations have sought to develop an *in vitro* biochemical assay for phytochrome function based upon the hypotheses that phytochrome is a protein kinase and that phytochrome regulates cytosolic calcium homeostasis. In conjunction with *in vitro* phytochrome assembly, such an assay system will facilitate experimental dissection of the molecular basis for phytochrome action as well as biochemical characterization of the components of a phytochrome-mediated signal transduction pathway.

A PROPOSED PATHWAY FOR LIGHT-REGULATED SEEDLING DEVELOPMENT AND GENE EXPRESSION IN *ARABIDOPSIS*.

J. Chory[1], P. Nagpal[1], A. Pepper[1], J. Reed[1], T. Delaney[1] A. Nagatani[2] and M. Furuya,[2] [1]Plant Biology Laboratory, The Salk Institute, La Jolla, CA; [2]Frontier Research Program, RIKEN, Wako, Japan.

The photoconversion of photoreceptors by light induces the diverse morphogenic responses which result in greening. Several regulatory photoreceptors are involved in the perception of light signals, but little is known of the transduction pathways that mediate light-regulated development. We are taking a combined genetic and molecular biological approach to identify potential components of the light signal transduction pathways. We have identified a class of *Arabidopsis thaliana* mutations, defining at least three genes, that uncouple the light signal from the downstream light-regulated responses. The mutants have the phenotype of light-grown plants, even when grown in total darkness. Thus, we call them *de-etiolated* (gene designation, *det*), because they do not have the developmentally blocked phenotype of dark-grown wild-type (etiolated) seedlings. Because *det* mutations are recessive and affect a number of downstream light-regulated processes (leaf and chloroplast development, light-regulated gene expression, anthocyanin production), we have proposed that the wild-type DET gene products function as negative regulators in light signal transduction. A second class of mutants have a long *hypocotyl* (*hy*) when grown in the light. Sixty-five mutant alleles have been analyzed by M. Koornneef and by our lab. These efforts have identified 7 complementation groups; 3 of these (*hy*1, *hy*2, and *hy*6) show deficiencies in photoreversible phytochrome activity, and are presumably signal perception mutants. Biochemical analysis of *hy*1, *hy*2, and *hy*6 mutants suggests that these genes may encode enzymes in the chromophore biosynthetic pathway. Mutations in a fourth *HY* gene, *HY*3, result in deficiencies of the type B phytochrome. We have constructed double mutants between the phytochrome-deficient *hy* mutants and the *det*1 and *det*2 mutants. The phenotype of the *hy*1-*det*1, *hy*2-*det*1, or *hy*6-*det*1 double mutant is *det*1, indicating that *det*1 is epistatic to *hy*1, *hy*2, and *hy*6. *det*2 is epistatic to *hy*1, *hy*2, *hy*3, and *hy*6. These results are consistent with a model where formation of the active form of phytochrome results in a decrease in activity of DET1 or DET2, which in turn leads to the de-etiolation response. The phenotype of *det*1-*det*2 double mutants is additive. This additive effect suggests that the DET1 and DET2 gene products do not interact, and thus implies that DET1 and DET2 function either in distinct pathways or in separate branches of a common pathway that affect downstream light-regulated genes. Further, these pathways are not utilized solely during early seedling development, but must also be required to regulate different aspects of the light developmental program during later stages of vegetative growth. Clearly, it will be important to know the mechanisms by which *DET*1 and *DET*2 act. We are attempting to clone these loci by chromosome walking and complementation of the mutant phenotypes with the wild-type copy of the gene.

PHYTOCHROME CONTROL OF DEVELOPMENT OF THE PHOTOSYNTHETIC APPARATUS IN WILD TYPE AND MUTANTS OF *ARABIDOPSIS* AND TOMATO

Benjamin A. Horwitz, Sarit Lifschitz, Yael Pauncz, Shifra Ken-Dror and Shimon Gepstein; Department of Biology, Technion - Israel Institute of Technology, Haifa 32000, Israel.

A brief pulse of red light (R) given to dark-grown seedlings results in the acceleration of greening upon their transfer to continuous white light. The R pulse thus accelerates the accumulation of chlorophyll and of polypeptides belonging to the photosynthetic apparatus. Mutants are a valuable adjunct in the study of the transduction steps between photoconversion and control of the levels of mature thylakoid proteins. A R pulse accelerated chlorophyll accumulation to an extent similar to that observed with wild type seedlings, in several long hypocotyl photomutants of *Arabidopsis*: *hy 1,2,3,4* and *5*. A far-red (FR) pulse had almost no effect on greening of etiolated *hy 1* and *hy 2* seedlings, consistent with their low phytochrome levels. FR, though, caused only partial reversal of the effect of R in mutants *hy 1* and *hy 2*. In an *aurea* mutant of tomato, the greening rate was slower than in the wild type, and as in the low phytochrome *hy 1* and *hy 2* mutants of *Arabidopisis*, FR or R in the very low fluence range were ineffective. In both plants, accumulation of LHC(II) polypeptides was much slower in low-phytochrome mutants (*hy 2* and *aurea*)than in wild type, and the acceleration by R was reduced. These results are consistent with a model in which Pfr controls accumulation of the LHC(II) by independently modulating chlorophyll and LHC(II) mRNA level. In addition to the characterization of available mutants, we are isolating new mutants. The criterion for selection is low chlorophyll fluorescence *in vivo*.

GT-2 A PROTEIN THAT BINDS IN A HIGHLY SEQUENCE SPECIFIC MANNER TO FUNCTIONAL GT MOTIFS IN THE RICE *PHYA* PROMOTER

K. Dehesh, W. Bruce, H. Hung, J. Teppermann and P. Quail, University of California, Berkeley / U.S. Department of Agriculture, Plant Gene Expression Center, Albany, CA 94710.

It is well established that phytochrome regulates gene expression in response to light , yet the molecular mechanism underlying this regulation is unclear. To approach this question we have chosen to analyse one of the phytochrome genes, *phyA*, in monocots, where negative autoregulation of expression at the transcriptional level is well documented. Our focus is to identify cis-elements and transcriptional factors involved in the expression of this gene. Cis-elements necessary to drive a high level of *phyA* transcription have been identified using microprojectile mediated gene transfer into the intact rice seedlings. These studies show the presence of several functional elements including GT motifs. There are three direct repeats of very similar but not identical GT motifs, designated GT-1, GT-2 and GT-3 boxes in the rice *phyA* promoter. Deletion and linker scan mutagenesis of either GT-1 or GT-2 motifs leads to a drop in *phyA* transcriptional activity to the basal level . These results indicate that both GT-1 and GT-2 motifs are functional positive elements required to drive a high level of *phyA* transcription. Previous screening of a rice expression library with oligonucleotides representing GT-1 and GT-2 motifs as a probe, resulted in isolation of a partial cDNA clone, designated GT-2, that binds in a highly sequence specific manner to the GT-2 motif . Recently isolation of overlapping cDNA and genomic clones has enabled us to obtain a sequence that encodes a full length GT-2 polypeptide as indicated by several criteria. A combination of Western blot analysis, using antibodies raised against the GT-2 polypeptide, and Southwestern analysis, with a GT-2 binding site probe, show that the GT-2 *in vitro* transcription-translation product comigrates with a single reactive band in nuclear extracts. Furthermore DNAase protection assays carried out either with *E.coli* extracts containing overexpressed GT-2 protein, or with plant nuclear extracts resulted in identical footprint patterns, showing protection of all three GT motifs in the rice *phyA* promoter. These results confirm that the new GT-2 clone encodes a full length polypeptide with identical binding properties to those of the protein in nuclear extracts. Moreover these data raise the possibility of involvement of the GT-3 motif as an additional functional element necessary for the transcriptional regulation of the rice *phyA* gene. This question is currently under investigation.

MOLECULAR LIGHT SWITCHES FOR PLANT GENES

Nam-Hai Chua, Philip Gilmartin[1], Kazuyuki Hiratsuka, and Xiao-Dong Wu, Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, New York 10021-6399, U.S.A., [1]present address: The University of Leeds, Department of Biochemistry and Molecular Biology, UK LS2 9JT

Many light responsive genes contain conserved sequences, such as boxes II and III that serve as binding sites for the nuclear factor GT-1. Analysis of site-specific mutants of the pea *rbcS-3A* promoter demonstrated that GT-1 binding *in vitro* is correlated with light-responsive expression of the promoter in transgenic plants. Indeed, a synthetic tetramer of box II (-152 TGTGTGGTTAATATG-138) inserted upstream of the -90 CaMV 35S promoter is sufficient to confer light-responsive transcription in chloroplast-containing cells of transgenic plants. The expression is correlated with the ability of box II to bind GT-1 *in vitro*. Our data shows that the binding site for GT-1 is likely to be a part of the molecular light-switch for *rbcS* activation. By screening cDNA expression libraries, we have isolated cDNA clones encoding tobacco and *Arabidopsis* GT-1. Comparative gel shift experiments using box II and its mutant derivatives as discriminating probes, demonstrated that the nuclear GT-1 and the recombinant GT-1 have the same binding specificity. Nucleotide sequence analysis showed that the encoded protein contains a basic region abutting a presumptive helix-turn-helix motif. A truncated protein containing only these two domains was competent in box II binding. The mRNA encoding GT-1 is present in light-grown, as well as dark-adapted leaf tissue. This is consistent with the finding that GT-1 activity is detected in both tissues. Binding of GT-1 to box II is sensitive to phosphatase-treatment, suggesting that GT-1 phosphorylation precedes DNA-binding and is a potential regulatory step.

A LIGHT-INDUCIBLE DNA-BINDING PROTEIN RECOGNIZING A PLANT PROMOTER ELEMENT WITH FUNCTIONAL RELEVANCE IN LIGHT RESPONSIVENESS

Bernd Weißhaar, Gregory A. Armstrong, Michael Feldbrügge, Kazufumi Yazaki, Annette Block, and Klaus Hahlbrock; MPI für Züchtungsforschung, Abteilung Biochemie, Köln, Germany.

To investigate the mechanism(s) of plant responses to short-wavelength light, the expression and regulation of chalcone synthase (CHS) has been analyzed. In parsley (*Petroselinum crispum*) CHS is encoded by one gene whose expression is light-dependent and mainly regulated at the transcriptional level. Therefore, the structure of the parsley CHS promoter was studied extensively.

We have recently analyzed three partial cDNAs encoding sequence-specific DNA-binding proteins from parsley (EMBO-J. **10**, (1991)). The three proteins bind specifically to Box II, one of two *cis*-acting elements present in a 52-bp CHS promoter region (Unit 1) which was shown to be sufficient for light responsiveness. All of them contain basic and leucine-zipper domains characteristic of transcription factors of the bZIP class. These putative transcription factors were designated CPRF-1, -2, and -3. The recognized nucleotide sequence contains an ACGT motif which is present in similar sequence context in many *cis*-acting elements including the G-box, the Motif I/Em1a element defined in abscisic acid responsive promoters, and the as-1 element from the CaMV 35S promoter. In cultured parsley cells, CPRF-1 mRNA accumulates to high levels upon irradiation. The timing of the transient increase in CPRF-1 mRNA and the characteristics of CPRF-1 binding to DNA are consistent with the hypothesis that this factor participates in light-mediated CHS-gene transcription.

The full-length CPRF-1 cDNA encodes a protein with a calculated molecular mass of 44 kD and a potential proline-rich activation domain N-terminal to the bZIP region. Data on the co-expression of CPRF-1 with reporter constructs containing hybrid promoters driving the GUS gene will be presented along with data on the genomic organisation of the parsley CPRF-1 gene.

IS SUGAR TOXIC TO PLANTS ?

J. Sheen, Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts

Using freshly isolated maize mesophyll protoplasts and a transient expression assay, I show that the transcriptional activity of seven maize photosynthetic gene promoters is specifically and coordinately repressed by the photosynthetic end products sucrose and glucose, and by the exogenous carbon source acetate. Analyses of deleted, mutated, and hybrid promoters show that sugars and acetate inhibit the activity of distinct positive upstream regulatory elements without a common consensus. The metabolic repression of photosynthetic genes overrides other forms of regulation, e.g., by light, tissue type, and developmental stage. Repression by sugars and repression by acetate are mediated by different mechanisms. Recent studies indicate that both calcium and phosphate can cause derepression of photosynthetic gene promoters in the presence of glucose. The role of protein phosphorylation and dephosphorylation by protein kinase and phosphatase in plant anabolic repression will be discussed.

ARABIDOPSIS β-1,3-GLUCANASE GENES: USEFUL MARKERS FOR STUDYING PLANT RESPONSES TO PATHOGEN INVASION AND TO HORMONAL AND ENVIRONMENTAL SIGNALS

X. Dong and F. Ausubel, Department of Genetics, Harvard Medical School, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114

The β-1,3-glucanase genes of plants are often considered defense-related genes for two reasons. First, the substrate of the gene products, glucan, is a major cell wall component of microbial phytopathogens and the lytic activity of β-1,3-glucanase may inhibit the pathogen growth *in planta*. Second, glucan fragments generated as a consequence of the interaction between pathogens and plants may then act as signals in activating genes involved in plant defense. We isolated three β-1,3-glucanase genes from *Arabidopsis thaliana* (BG1, BG2 and BG3). The expression of all three genes are induced 24 hours after infecting *Arabidopsis* with the pathogenic bacterial strain *Pseudomonas syringae* pathovar *maculicola* 4326 (*P.s.m.* 4326). Histochemical analysis of transgenic *Arabidopsis* lines carrying a BG2-GUS fusion showed that the BG2 gene was expressed in the marginal region of the lesion caused by the infecting bacteria. This implies that β-1,3-glucanase may play a role in preventing the spreading of the pathogen. We also found that in the wild-type *Arabidopsis*, the expression of BG2 and BG3 mRNAs were induced by the treatment with the "stress hormone", ethylene. However, in the well characterized ethylene insensitive mutant *Arabidposis* plants, *etr-1* and *ein2*, the induction of BG3 gene by ethylene was completely abolished. The induction of BG2 gene by ethylene treatment in these mutants were not affected. Since *etr-1* and *ein2* are mutants of different loci, the effect of the mutations on the BG gene expression is probably not an effect of a specific allele. If the *etr-1* and *ein2* loci encode the receptor for ethylene, our result implies that there is probably more than one ethylene receptor, one of which leads to the activation of BG2, in *Arabidopsis*. Since the induction of all three BG genes by the pathogenic bacterial strain *P.s.m.*4326 in *etr-1* were not affected by the mutation, it is likely that different pathways were involved in the induction the BG genes by ethylene or by bacteria and different promoter elements must be used in the responses to these signals.

LIPOXYGENASE EXPRESSION IN RESPONSE TO WATER DEFICIT AND WOUNDING

Erin Bell and John Mullet, Department of Biochemistry and Biophysics, Texas A & M University, College Station, TX.

Lipoxygenases (LOXs) are non-heme iron-containing enzymes which catalyze the incorporation of molecular oxygen into unsaturated fatty acids and can also catalyze carotenoid co-oxidation. Based upon these activities, roles for LOX in the biosynthesis of jasmonic acid, traumatic acid, and abscisic acid (ABA) have been proposed. In addition, increases in LOX activity in response to wound and pathogen stresses have been reported. In order to examine potential roles for LOX in response to stress conditions, we have isolated three different cDNA clones encoding LOX genes expressed in growing tissues, and examined levels of the corresponding mRNAs in tissues which have been wounded or exposed to water deficit. Two patterns of LOX mRNA accumulation are seen. A slow (>8 hr.) accumulation is seen in soybean plants in response to wounding, while a more rapid (<3 hr.) increase in LOX mRNA levels is seen in pea plants or soybean seedlings which are exposed to water deficit. The time frame of this rapid response is similar to that seen for ABA accumulation in these tissues in response to water deficit. We have also shown that certain LOX inhibitors reduce the magnitude of ABA accumulation in osmotically-stressed soybean cell cultures, suggesting that LOX or a similar enzyme may be involved in the stress-induced accumulation of ABA.

DEFINING THE ETHYLENE-MEDIATED SIGNAL TRANSDUCTION PATHWAY FOR FRUIT RIPENING USING REVERSE GENETICS*

Paul W. Oeller, Lu Min-Wong, Loverine P. Taylor†, Deborah A. Pike‡, Athanasios Theologis, Plant Gene Expression Center, University of California, Berkeley/U.S. Department of Agriculture, 800 Buchanan Street, Albany, CA, 94710.

Ethylene controls fruit ripening. Expression of antisense RNA to the rate-limiting enzyme in the biosynthetic pathway of ethylene, 1-aminocyclopropane-1-carboxylate (ACC) synthase, inhibits fruit ripening in tomato plants. Administration of exogenous ethylene or propylene reverses the inhibitory effect. This result demonstrates that ethylene is the trigger, and not the byproduct, of ripening, and raises the prospect of extending the life span of plant tissues, thereby preventing spoilage.

*Supported by grants from NSF (DCB-8645952, -8819129, -8916286), NIH (GM-35447), and USDA (5835-23410-D002) to A.T.

Current addresses: †Dept. of Horticulture, Washington State University, Pullman, WA 94164; ‡Cetus Corp., 1400 53rd St., Emeryville, CA 94608.

MOLECULAR GENETIC DISSECTION OF HORMONE SIGNAL TRANSDUCTION IN *ARABIDOPSIS.*

J. R. Ecker, J. Kieber, G. Roman, M. Rothenberg and A. Wollenberg, Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

We have utilized a simple response of etiolated seedlings to ethylene, the "triple response", to identify several new types of hormone mutants in *Arabidopsis thaliana*. These include mutations that affect either ethylene or auxin: production (*eto1, eto2, hls3*), perception or signal transduction (*ctr1, ctr2, ein1, ein2, ein3, eir1, hls1, hls2, ran1*) pathways.

Genetic, molecular, biochemical and physiological studies to define more precisely the roles of these genes in ethylene and auxin action pathways will be presented. Several alternative approaches toward the molecular cloning of these genes will also be described.

CHARACTERIZATION OF THE SAUR-AC1 GENE: EXPRESSION IN AUXIN-RESPONSIVE AND GRAVITY-RESPONSIVE MUTANTS OF *ARABIDOPSIS*

Pedro Gil[1], Candace Timpte[2], Yang Liu[1], Vladimir Orbovic[1], Ken Poff[1], Mark Estelle[2], and Pamela J. Green[1], [1]MSU-DOE Plant Research Laboratory, Michigan State University, E. Lansing, MI 48824-1312; [2]Indiana University, Biology Department, Bloomington, IN 47405.

The small auxin up RNA (SAUR) genes were originally characterized from soybean where they comprise a small gene family (McClure et. al., Plant Cell, 1:229-239). During gravitropism, there is a rapid redistribution of the SAUR transcripts in soybean hypocotyls, which is presumably mediated, at least in part, by auxin (McClure and Guilfoyle, Nature, 243:91-93). We have isolated and characterized an *Arabidopsis* SAUR gene, designated SAUR-AC1, that is highly homologous to the soybean SAUR genes over most of the coding region. In addition, SAUR-AC1 contains a sequence with similarity to the putative regulatory element DST, found in the 3' flanking regions of soybean SAUR genes. In the SAUR-AC1 mRNA, the DST sequence has the potential to form a stem-loop structure adjacent to the polyadenlyation site. The regulatory contributions of the sequences upstream and downstream of the transcription initiation site are presently being investigated in cultured cells and transgenic plants. We are also studying the expression of SAUR-AC1 in mutants of *Arabidopsis* that exhibit altered physiological responses to auxin and/or gravity. Our results show that the SAUR-AC1 transcript is highly inducible by 50μM 2,4-D in etiolated seedlings of wild-type *Arabidopsis*. However, in the *AXR2* mutant, which is resistant to auxin and exhibits defects in gravitropism (Wilson et al. MGG, 222:377-383), the SAUR-AC1 transcript not detectable in etiolated seedlings, with or without 2,4-D treatment. Wild-type rosette leaves accumulate low levels of the SAUR-AC1 transcript and the levels increase in response to exogenous auxin. Interestingly, AXR2 appears to express the SAUR gene constitutively in rosette leaves. The implications of altered SAUR-AC1 expression in AXR2 and other mutants of *Arabidopsis* will be discussed.

REGULATION OF GENES CODING FOR AUXIN-BINDING PROTEINS

E. Schwob[1], S.-Y. Choi[1], K. Palme[2] and D. Söll[1] [1]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511, USA; [2]Max-Planck-Institut für Züchtungsforschung, W-5000 Köln, Germany.

Plant hormones are the major determinants of growth and developmental responses in plants. Auxins, a major class of plant hormones, are involved in cellular differentiation, cell elongation and the initiation of the gravitropic growth response. Auxin's mode of action is mainly accounted for by the acid-growth and gene expression theories that imply plasma membrane or intracellular receptors, respectively. The characterization of these receptors and the elucidation of the signal transduction cascade leading to the hormone-specific biological response are a major challenge in plant molecular biology.

The *axr1* gene from corn codes for such a putative receptor (1), but the recent characterization of several other auxin-binding proteins (ABPs) from corn (24, 60 kD), zucchini (42 kD) and *Hyoscyamus* (31 kD) having different cellular localization (plasma membrane, cytosol, endoplasmic reticulum) raises the question of how complex the molecular mechanism of auxin action really is. In order to try to understand the biological role of these various ABPs, we have decided to study how their genes are regulated.

The *axr1* gene encodes the major 22 kD auxin-binding protein from corn coleoptile membranes whereas the *axr4* cDNA codes for a protein which is 85% identical to the mature *axr1* protein. Both proteins contain the C-terminal KDEL sequence which specifies their retention in the lumen of the endoplasmic reticulum; they differ significantly only in their signal peptide. We have now isolated and sequenced the genomic *axr1* and *axr4* genes from maize, as well as the corresponding AXR1 gene from *Arabidopsis*. We will describe their molecular structure and discuss their evolutionary origin. The tissue-specific and developmentally regulated expression of these genes, studied in transient expression assays using transformed BMS protoplasts or microprojectile-bombarded corn seedlings, as well as transgenic *Arabidopsis*, will be discussed.

Reference:

1. Hesse, T. Balshüsemann, D., Bauw, G., Vandekerckove, J., Puype, M., Löbler, M., Klämbt, D., Schell, J. and Palme, K. (1989) EMBO J. **8**, 2453-2461.

A MOLECULAR APPROACH TO STUDY THE PERCEPTION OF THE AUXIN SIGNAL

K. Palme, B. Brzobohaty, Feldwisch, J., Garbers, C., Hesse,F., Hesse, T., I. Moore,R. Walden, Zettl, R., and J.Schell
Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-5000 Köln 30, F.R.G.

Plant development is influenced by phytohormones, which elicit a remarkably wide variety of responses. Of particular interest are auxins which modify elongation growth, selective transcription of genes, control of cell division and vascular tissue differentiation. Receptor like proteins have been assumed to recognize and transmit the auxin signal. The search for such proteins led to the identification of auxin binding sites to the endoplasmic reticulum, the tonoplast and the plasma membrane. We have cloned from maize and A. thaliana members a gene family encoding auxin binding proteins located to the endoplasmic reticulum. We have expressed these genes in transgenic plants and will present the analysis. Using photoactivatable probes we were successful in isolating from Zea mays different auxin binding proteins which are located either in the cytosol or in the plasma membrane. We will present recent results on identification and characterization of a NPA binding protein that is located in the plasma membrane. We assume that this protein is a component of the auxin efflux carrier.

PROGRESS TOWARDS CLONING AND CHARACTERIZATION OF THE ABI1 AND ABI3 LOCI IN ARABIDOPSIS THALIANA

J. Leung,[1] B. Hauge,[2] H.M. Goodman[2] and J. Giraudat,[1] [1]Institut des Sciences Végétales, CNRS, Gif-sur-Yvette, France; [2]Department of Molecular Biology, Massachusetts General Hospital, Boston, USA.

Abscisic acid, or ABA, is known to play a crucial role in normal seed development and germination, and adaptive responses to a diversity of environmental stresses. However, the repertoire of molecular components involved in mediating ABA perception, as well as those in transducing the appropriate physiological signals, are unknown.

Koornneef has identified three distinct abscisic acid-insensitive mutants in *Arabidopsis thaliana* (*abi*1, *abi*2, *abi*3) that can germinate in the presence of toxic levels of ABA. All three response mutants show reduced seed dormancy. Additional biochemical and physiological analyses have shown that *abi*3 is essential for multiple events during seed matuation; whereas, both *abi*1 and 2 are required in regulating water relations, principally in vegetative tissues. It is worth emphasizing that none of the abnormal phenotypes of these mutants can be restored by an exogenous supply of ABA. Also, since the effects of these mutations are clearly pleiotropic, it is likely that they impair functions in the early steps of the signal transduction cascade, perhaps close to site of hormone reception.

Our group has undertaken the cloning and characterization of the *abi*1 and *abi*3 genes. The combined lines of evidence obtained from studies of the mutants above suggest that both genes are required for normal seed dormancy, but subsequently, effect distinct molecular cascades of ABA action. One of our aims is thus to compare features of these genes, and their products, that serve to impart their characteristic functions in triggering their corresponding response pathways. Our strategy for cloning the genes is based on "fine-structural" RFLP mapping in conjunction with chromosome walking. The "fine structural maps" constructed with recombinant plants in the regions of the *abi*1 and *abi*3 loci have already allowed us to (1) properly integrate the genetic positions of the two mutations onto the existing RFLP maps, (2) align closely linked RLFP markers relative to the target loci more precisely, and (3) assign new RFLP markers from cloned segments of DNA from successive steps to monitor the distance and direction of the chromosome walk. In the case of *abi*3, the mutation has been delimited to within a cosmid in an existing contig by RFLP mapping. We are in the process of identifying the gene corresponding to *abi*3 by functional complementation using a series of overlapping DNA segments from the cosmid to transform the mutant plant. In the case of *abi*1, we have initiated a walk using flanking RFLP's as departure points and genomic banks propagated as yeast artificial chromosomes (kindly provided by Drs. C. Somerville and E. Ward) to progressively home in on the locus. Current status of these projects will be presented.

SALICYLIC ACID AND SIGNAL TRANSDUCTION IN PLANT DISEASE RESISTANCE RESPONSES. Zhixiang Chen, Jocelyn Malamy, Jacek Hennig & Daniel F. Klessig, Waksman Institute, Rutgers, The State University of New Jersey, P.O. Box 759, Piscataway, New Jersey 08855.

It has been recently demonstrated that endogenous levels of salicylic acid (SA), a known exogenous inducer of plant disease resistance, increased dramatically in resistant, but not susceptible, tobacco cultivars after tobacco mosaic virus (TMV) infection (*Science* 250:1002, 1990). This observation strongly indicate that SA is a likely endogenous signal molecule in the resistance response of tobacco to TMV infection. Here, we report that when the resistance response of tobacco plant, including synthesis of pathogenesis-related proteins, is blocked at a high temperature (32°C), there is no rise in SA levels. However, when plants are then moved to a low temperature (22°C), SA levels rapidly increase at least 100-fold and resistance response resume soon after. These results are consistent with a model in which SA functions downstream of the temperature-sensitive step in the defense response signalling pathway. In a search for possible cellular factors which directly interact with SA, we have detected and partially characterized a SA-binding activity in tobacco leaves. The SA-binding activity is both SDS- and proteinase-sensitive and behaves as a soluble protein with an apparent molecular weight of 370 *K*Da. The protein has an apparent K_d of 14 μM for SA, which is consistent with the range of physiological concentrations of SA observed for the induction of plant disease responses. Furthermore, the ability of SA analogues to compete with SA for binding to this soluble protein is strictly correlated with their biological activity to induce the expression of genes associated with disease resistance. These results collectively indicate that this SA-binding protein may play a role in perceiving and transducing the SA signal to appropriate response elements, which ultimately activate one or more of the plant disease resistance responses.

ABSCISIC ACID IS INVOLVED IN THE IRON-INDUCED SYNTHESIS OF MAIZE FERRITIN

Jean-François Briat and Stéphane Lobreaux, Laboratoire de Biologie Moléculaire Végétale, Centre National de la Recherche Scientifique et Université Joseph Fourier B.P 53X. F-38041 GRENOBLE Cédex (FRANCE)

Iron is an essential element involved in important life processes such as respiration, photosynthesis, nitrogen fixation and cell division. However, in presence of oxygen, cells have to face both iron insolubility and toxicity through hydroxyl radicals formation. Part of these two problems are handled by ferritins, a class of multimeric proteins present in animals, plants and microorganisms. Their important role in cellular iron homeostasis is due to their ability to store up to 4500 iron atoms in their central cavity in a soluble, bioavailable, non toxic form (1).

Plant and animal ferritins are closely related proteins arising from a common ancestor but they differ by their cytological location and by the level of control of their synthesis in response to iron overload : plant ferritins are plastid located and transcriptionally regulated while animal ferritins are found in the cytoplasm and are translationally regulated (2). In order to investigate further the mechanisms by which ferritin gene expression is regulated in plants, ferritin cDNAs from maize have been cloned and a system allowing to induce a rapid and transient accumulation of ferritin protein and mRNA in roots and leaves of maize in response to iron has been developed. It is shown that in addition to ferritin, two different known maize RAB transcripts also accumulate in this system and that application of exogenous ABA is sufficient to induce ferritin mRNA accumulation in vegetative organs of maize. In fact, ferritin synthesis in response to iron overload is strongly decreased by application of exogenous gibberellic acid, indicating that ABA is involved in the pathway which transduces the iron signal in plants.

(1) Theil, E.C. (1987) *Ann. Rev. Biochem.*, **56**, 289-315.

(2) Lescure, A.M., Proudhon, D., Pesey, H., Ragland, M., Theil, E.C. and Briat, J.F. (1991) *Proc. Natl. Acad. Sci. USA* (in press).

TOMATO *Cf* (*CLADOSPORIUM FULVUM*) RESISTANCE GENE DEPENDENT INDUCTION OF DEFENCE GENE PROMOTER - GUS FUSIONS.

T. Ashfield, K. Harrison, K.E. Hammond-Kosack, J. Jones. The Sainsbury Laboratory, John Innes Centre for Plant Science Research, Colney Lane, Norwich, NR4 7UH, UK.

In numerous studies on the induction of defence genes, such as studies on genes in bean suspension cultures induced by elicitors, or on the PR genes of tobacco, relatively little data has accumulated on race-specific defence gene induction, and on whether different resistance genes vary in the manner and degree in which they induce different defence genes.

The genetically well defined interaction between tomato (*Lycopersicon esculentum*) and the leaf mould (*Cladosporium fulvum*), in which dominant resistance (*Cf*) genes are matched by dominant avirulence (*Avr*) genes in the pathogen, is an excellent system for investigating defence gene induction by the action of single major resistance genes. Isogenic lines containing different *Cf* genes respond differently to injection of race specific elicitors and vary in the degree to which the pathogen growth is contained.

The aim of this project is to characterise the spatial and temporal patterns of induction for a range of defence related genes in compatible and incompatible interactions between tomato and *C.fulvum*. Interactions involving *Cf*- 0, 2, 4, 5, and 9 will be investigated. This study will identify whether the patterns of gene induction triggered by the presence of the different *Cf* genes are identical or distinct. The data may also highlight the important components of a successful defence response.

Gene fusions have been made between the promoters of a range of defence related genes and the *E.coli* β-glucuronidase (GUS) gene. The promoters being used originate from the following genes:- (1) glucanase (extracellular and vacuolar members)(tobacco), (2) chitinase (tomato), (3) PR1a (tobacco), (4) HMGcoA reductase (tomato), (5) PAL2 and PAL3 (bean), and (6) proteinase inhibitor II (potato). GUS fusions will allow both quantitative analysis of gene induction and histochemical localization to the individual responding cells. Tomato plants containing no *Cf* genes (Cf0 plants) have been transformed with each of the GUS fusions. These primary transformants are being crossed to near isogenic lines homozygous for either *Cf*-2, 4, 5, or 9. Thus the response of the GUS fusion on each introduced T-DNA can be easily be compared in each resistance gene-containing genetic background. All the GUS fusions currently envisaged to be used in this study have now been constructed in-house or obtained through collaborations. At least one Cf0 primary transformant has been generated for each of the fusions and crosses to introduce the T-DNAs into the different Cf backgrounds are now in progress.

Preliminary results have been obtained on the induction of the extracellular glucanase/GUS fusion. Initial quantitative measurements after challenge with race 0 *C.fulvum* suggest that this fusion is induced in both compatible and incompatible interactions. However, in incompatible interactions the magnitude of induction is greater. Microscopic examination of infected leaves stained sequentially to reveal both GUS activity and the fungal hyphae has revealed markedly different patterns of induction at the cellular level in the different interactions. In incompatible interactions the induction is associated with sites of attempted fungal penetration of the plant tissue. In compatible interactions induction occurs behind the hyphal front and appears to be coincident with conidiophore formation.

The induction of this extracellular glucanase/GUS fusion in Cf0 and Cf9 backgrounds by the injection of intercellular fluid (IF) containing the *Avr* 9 race specific elicitor has also been studied. The IF induced the fusion in the Cf9 (approx. 10 fold over water control), but not the Cf0, genetic background.

Further progress will be reported.

ANALYSIS OF PLANT HORMONE-REGULATED PROMOTER EXPRESSION IN TRANSGENIC TOBACCO

M. Båga, W.H.R. Langridge and A.A. Szalay, Plant Molecular Genetics, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

We have previously shown that the dual *mas* promoters from the T_R region of the *Agrobacterium tumefaciens* Ti-plasmid are differentially regulated throughout development of transgenic tobacco plants and controlled by endogenous auxin/cytokinin levels in the plant tissue. Our results show that *mas* promoter expression is inhibited in the region of the apical meristem and in the immature inflorescence. A detailed functional analysis of the *mas* promoter region has been undertaken by fusing a series of *mas* promoter deletion constructs to the bacterial luciferase reporter fusion gene. Promoter activity was monitored by transient expression in tobacco protoplasts and in transgenic tobacco plants *in vivo*. From these studies we have identified several upstream activating domains for both promoters. Hormone regulation of the 1' promoter is localized to a 65 bp region and a non-overlapping 38 bp DNA sequence is essential for hormone-activation of the 2' promoter. Gel retardation assays using the regulatory DNA sequences as probe have enabled us to identify specific DNA-binding complexes formed *in vitro*. Nuclear extracts prepared from selected tobacco tissues were used to identify tissue-specific trans-acting factors.

SIGNAL TRANSDUCTION IN PLANTS: IS THERE A ROLE FOR THE *ras* PROTO-ONCOGENE?

K.M. Barnett and M.M. Stayton, University of Wyoming, Laramie, Wyoming.

Research efforts in our laboratory focus on understanding the process of signal transduction in plants. One candidate signal transduction molecule currently being examined is the Ras protein, a monomeric GTP-binding protein which functions in this capacity in other organisms. In *S. cerevisiae* and *D. discoideum*, for example, Ras mediates nutritional and developmental signaling. In higher organisms Ras seems to transduce mitogenic signals, and thus plays a role in the control of cell growth and proliferation.

Preliminary data presented here suggests that sequences similar to the *ras* proto-oncogene can be amplified by PCR from *Arabidopsis thaliana* genomic DNA using primers based on the *S. cerevisiae rasl* sequence. These amplification products are of the predicted size, and data produced by sequencing now underway will allow confirmation of their identity. Selected cloned PCR products will then be used to screen *Arabidopsis* cDNA and genomic libraries to identify positive clones, which will be analyzed by restriction mapping, DNA sequencing, and RFLP mapping. We may discover that the cloned genes correspond to already identified loci which affect a plant's response to its environment. Further experiments are directed towards determining *ras* gene function in plants.

PLANT DEFENSE ELICITOR PROTEIN PRODUCED BY *ERWINIA CHRYSANTHEMI.*

M. BOCCARA, R. VEDEL and M. DUFRESNES
Laboratoire de pathologie végétale, INA P/G, 16 rue Claude Bernard, 75006 PARIS, FRANCE.

Erwinia chrysanthemi 3937 produces an hypersensitive response when inoculated to tobacco cv. Xanthi leaves. Elicitor molecules are proteins secreted in the culture medium. Using a set of characterized mutants we have shown that the elicitor protein was not one of a major pectinases nor endoglucanases produced by *E. chrysanthemi* 3937.

Elicitation was monitored by symptom of local necrosis on tobacco leaves or measure of the phytoalexin, capsidiol, produced either by elicited leaves or suspension tobacco cells. Maximum elicitation was observed 16 hours post stimulation and rapidly decreased.

Purification of the elicitor protein was undertaken from the culture medium of an *E. chrysanthemi* strain producing solely the acidic pectate lyase (unable to macerate potato tuber). The purified protein exhibited polygalacturonate lyase activity, with a pi>9 and a MW of 32000.

EXPRESSION OF AUXIN-REGULATED GENES FROM TOBACCO IN CELL CULTURE SYSTEMS AND IN PLANTA

Cees J.M. Boot[1], Frans N.J. Droog[2], Albert M. Mennes[1], Paul J.J. Hooykaas[2], Kees R. Libbenga[1] and Bert J. van der Zaal[2], Leiden University, Department of Plant Molecular Biology, [1]Botanical Laboratory, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands; [2]Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands.

After treatment of auxin-starved tobacco cells with auxins several mRNAs are rapidly induced prior to cell division (Van der Zaal et al. (1987) Planta 172, 514-519; Plant Mol. Biol. 10, 145-157). Four different mRNAs are inducible independently of protein synthesis. The levels of these mRNAs were studied during the growth cycle of cell cultures in the presence or absence of 2,4-D. Subculturing of cells into 2,4-D-containing medium ($0.22\ 10^{-6}$M) leads to a rapid and transient elevation of the mRNA levels, being maximal around 6 hr after transfer and nearly back to the normal low level after 11 hr when the cells reach the S-phase. For three of the mRNAs the accumulation is at least partly due to enhanced transcription rates. The mRNAs hybridizing with cDNA clone pCNT103 were most extensively studied. CaMV 35S promoter-driven expression of 103 antisense RNA nearly completely blocked 103 mRNA accumulation but did not hamper cell division. Analysis of two promoters of 103-like genes, fused with the gene for GUS, indicated that a 100 bp region around -300 prior to ATG is responsible for auxin-regulated and root-tip specific expression. We are currently trying to elucidate the signal perception and transduction pathway responsible for the expression of 103-like genes.

ISOLATION OF A GENE ENCODING A PUTATIVE SEVEN TRANSMEMBRANE DOMAIN RECEPTOR FROM *ARABIDOPSIS THALIANA*

K. Brady, P. Albersheim,[1] A. Darvill,[1] and M.G. Hahn,[2] University of Georgia, Complex Carbohydrate Research Center and Depts. of Biochemistry[1] and Botany[2], 220 Riverbend Rd., Athens, GA 30602

Perception of a number of extracellular signals occurs at the cell surface via binding to specific receptors that are coupled to specific guanine nucleotide binding proteins (G proteins). The activated G proteins in turn initiate intracellular signaling events. Examples of signals transduced via such G protein-coupled receptors include neurotransmitters, peptide hormones, light, odorants, and mating pheromones. These G protein-coupled cell surface receptors have evolutionarily conserved domains consisting of seven hydrophobic transmembrane segments, and have been found in eukaryotic organisms ranging from yeast to humans. To date, no direct evidence for the existence in plants of G protein-coupled receptors has been obtained, although G protein homologs have been found.

We are attempting to isolate genes for similar seven transmembrane domain receptors from plants, specifically *Arabidopsis thaliana*. Degenerate oligonucleotide primers were designed to amino acid regions of the evolutionarily highly conserved transmembrane domains II, VI, and VII of several mammalian G protein-coupled receptors. These oligonucleotide primers were utilized to amplify homologous sequences in Arabidopsis genomic DNA using the polymerase chain reaction (PCR). High stringency primer annealing yielded a small number of discrete PCR products that were then used as templates in a second round of PCR amplification. These second round PCR products were recovered into a phagemid vector. The sequence of one of these PCR products (ca. 1500 bp) has been determined and shows significant homology to a mammalian muscarinic acetylcholine receptor. The predicted amino acid sequence of this clone displays two terminal regions very similar to the amino acid sequences upon which the oligonucleotide primers were designed, and also internal hydrophobic regions typical of transmembrane spanning segments. We are currently isolating a full-length cDNA copy of this gene and also determining the tissue localization of the expression of this gene.

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AUXIN-MODULATED GROWTH AND TRANSCRIPTION IN RICE COLEOPTILES

D. Breviario[1], S. Gianì[2] and I. Coraggio, [1]Istituto Biosintesi Vegetali, Milan, Italy; [2]Istituto Tecnologie Biomediche Avanzate, Milan, Italy.

IAA (indoleacetic acid) stimulates elongation of a large variety of stem sections. Results obtained in different laboratories indicate that the first phase of auxin-stimulated elongation may be controlled at transcriptional level by the enhancement of specific mRNAs coding for proteins affecting cell wall loosening. However,recent findings show that these enhancements are not specific for area undergoing cell extension.
We have undertaken a similar study using apical segments of rice coleoptiles. Within the first two hours of treatment, IAA-stimulated elongation of rice coleoptile segments is solely dependent on auxin. Afterward, a contribution to elongation due to ethylene produced in response to IAA can be observed. Rice coleoptile cell elongation stimulated by auxin is repressed by inhibitors of RNA and protein biosynthesis. Two-dimensional gel analysis of the in vitro translation products obtained from polyA+ RNA extracted after 1 hour of IAA treatment shows moderate enhancements in three different mRNA species and the disappereance of a 38 Kd translation product. Additional repression of another mRNA coding for a 28 Kd product begins to show by this time but becomes more evident after 4 hours of IAA treatment. At this time, 4 additional IAA specific mRNA enhancements can be observed.

In agreement with recent reports, these data suggest that IAA may also stimulates elongation by reducing the amount of mRNAs coding for proteins possibly involved in the maintainance of cell wall rigidity.

DEVELOPMENTAL EXPRESSION AND ALLELIC DIVERSITY OF THE MAIZE *Vp1* GENE.

Christian B. Carson and Donald R. McCarty
Vegetable Crops Dept. Univ. of Florida, Gainesville 32611

Maturation in developing maize kernels requires both *Vp1* expression and ABA accumulation. Mutant kernels with low ABA content (e.g. *vp5*) are viviparous. *vp1* null mutants are viviparous in the presence of normal levels of ABA. *Vp1* expression is required for anthocyanin pigmentation of aleurone and scutellar tissues during maturation. *vp5* mutant kernels do produce anthocyanins and *Vp1* mRNA is expressed. Developmental aspects of *Vp1* expression in whole embryo and endosperm tissue will be presented. Maturation related gene expression (i.e. *Glob1* and E_m homologous transcript) is dependent on *Vp1* expression, as is the expression of *C1* (anthocyanin regulatory gene). While *C1* is expressed in *vp5* mutants, the E_m related message is not. The dormant, colorless alleles, *vp1*-Mc and *vp1*-C, each encoding truncated gene products, express maturation, but do not promote *C1* expression. Structural analysis of this mutant suggests a distinct domain in the C-terminus region of the protein that is not essential for maturation expression in the kernel. Two other dormant alleles are currently being analyzed for comparison. Two leaky phenotype alleles, *vp1*-mum3 and *vp1*-mum5, are variably pigmented and viviparous, but may become dormant by suppression. Both are interrupted in the promoter region with a Mutator transposable element. *vp1*-mum3 encodes a full-sized protein and can become fully suppressed. The ramifications of insertions into regulatory regions of the gene will be discussed.

UNCOUPLING OF *CHLAMYDOMONAS* FLAGELLAR EXCISION, GENE EXPRESSION, AND OUTGROWTH BY MANIPULATION OF EXTRACELLULAR CA^{2+}

J.L. Cheshire and L.R. Keller, Department of Biological Sciences, Florida State University, Tallahassee, Florida.

Chlamydomonas cells respond to certain environmental stimuli by shedding their flagella, inducing a rapid, transient increase in expression of a specific set of genes encoding flagellar proteins, and initiating assembly of a new flagellar pair. While these three responses are normally tightly coupled during flagellar regeneration, our results demonstrate that these processes can be uncoupled by manipulating Ca^{2+} levels or calmodulin activity. In our experiments, cultures of wild-type cells were deflagellated by mechanical shearing, and at times after deflagellation, flagellar lengths were measured and flagellar mRNA abundance changes were determined by S1 nuclease protection analysis. When extracellular Ca^{2+} was lowered by addition of EGTA to cultures before excision, flagellar mRNA abundance changes and flagellar outgrowth were temporally uncoupled from flagellar excision. When extracellular Ca^{2+} was lowered immediately after excision or when calmodulin activity was inhibited with W-7, flagellar outgrowth was uncoupled from flagellar excision and flagellar mRNA abundance changes. Whenever events in the process of flagellar regeneration were temporally uncoupled, the magnitude of the flagellar mRNA abundance change was reduced. Our results suggest that flagellar gene expression may be regulated by multiple signals generated from these events, and implicate Ca^{2+} as a factor in the mechanisms controlling flagellar regeneration. Genetic and pharmacological approaches will enable us to examine the source(s) of Ca^{2+} involved in flagellar gene regulation.

CYCLING OF AUXIN-BINDING PROTEIN CAN ACCOUNT FOR MULTIPLE SITES OF AUXIN ACTION.

John W. Cross, EPL Bio-Analytic Systems, Box 1708, Decatur, IL 62525

Defining the cellular site for auxin hormone reception has been a major problem in plant cell biology. A major difficulty has been the inconsistency of independent experiments that point to different cellular sites. For example, although the best-known auxin-binding site (ABP) isolated from monocot shoots is a soluble protein and has been localized to the lumen of the ER and the Golgi, experimental addition of this isolated protein to protoplasts caused changes in the electrical potential at the plasma membrane. A new hypothesis was required to account for these results. I have proposed that the auxin receptors cycle between the endomembrane system of the cell and the cell surface in response to the presence of the auxin signal. The cycling of auxin receptors would also represent a mechanism for regulating the auxin-dependent secretion of cell wall precursors. This hypothesis makes several testable predictions, for example, that ABP will be secreted into the isolation medium by protoplasts, and should be recoverable from the endomembrane system when incubated with protoplasts.

ISOLATION AND CHARACTERISATION OF A CDNA FROM WHEAT ENDOSPERM ENCODING A DNA-BINDING PROTEIN WITH AN HMG DOMAIN

D. de Froidmont(1,2), C. Lejour(1), J.-M. Jacquemin(1).
(1) Station d'Amélioration des Plantes, Gembloux, Belgium;
(2) Faculté des Sciences Agronomiques, Gembloux, Belgium.

The prolamin genes, encoding cereal storage proteins, are transcribed exclusively in the endosperm during seed maturation. The control of their specific expression is essentially transcriptional. Homology search comparing prolamin gene promoters pointed out a conserved sequence: the endosperm box, which is a 30-base pair sequence lying in hordein, gliadin and low molecular weight glutenin gene promoters. The 5' part of this box is also present in α-zein and high molecular weight glutenin gene promoters. Wheat nuclear protein extracts from immature endosperm have been tested in mobility shift DNA binding assay. Specific binding activities were observed using a probe corresponding to the endosperm box. With this same probe we have screened a λgt11 expression library derived from wheat endosperm mRNA. We have isolated two clones. Their sequences revealed that they encode the same 161-amino acid protein. This protein is homologous to the high mobility group (HMG) and nonhistone chromosomal proteins. It contains an HMG domain in its central region and a highly acidic carboxy-terminal region. Northern blot analysis demonstrated that the mRNA of this wheat HMG protein is present in endosperm and in leaves. Southern blot analysis displayed several copies of this gene in the wheat genome.

HISTO-DEVELOPMENTAL CONTROL OF VEGETATIVE LECTIN mRNA DURING MERISTEM DEVELOPMENT

M. S. Dobres, D. Maiti, Jhang Ho Pak, Laura Belmont and A. List Jr.

We have examined the histo-developmental regulation of a previously characterized pea vegetative-lectin encoding transcript in pea (*Blec*, MS Dobres and WF Thompson, Plant Physiol.,1989, 89: 833-838). *Blec* RNA displays a novel pattern of accumulation that serves as a molecular marker for meristem development. During vegetative growth it is absent from the undifferentiated outer layer of the vegetative meristem and accumulates first in epidermal tissue adjacent to the first leaf primordia. During flowering it accumulates during development of the floral meristem into the floral bud. In addition,*Blec* RNA acts as a histological and molecular marker for lateral bud development: *Blec* RNA accumulates throughout the meristematic ground tissue of dormant lateral buds and becomes more peripheral in its accumulation upon lateral bud activation.

The physiological significance of *Blec* RNA expression in both vegetative and floral meristems is unknown. The *Blec* deduced amino acid sequence indicates that it encodes a protein similar to legume seed lectins such as concanavalin A, phytohaemaglutinin and the pea seed lectin . Numerous roles have been proposed for plant lectins including plant defense, seed dormancy factors and cell-adhesion (Sharon and Lis, FASEB J. 4:3298-3208, 1990). It is possible that the *Blec* protein plays a structural role in vegetative and floral differentiation, perhaps by altering the nature of cell-cell interaction during organ formation.

IDENTIFICATION OF REGULATORY GENES INVOLVED IN PLANT DEVELOPMENT

Jan Dockx, N. Quaedvlieg, P. Weisbeek and S. Smeekens
Department of Molecular Cell Biology,
University of Utrecht, The Netherlands

Light plays a critical role in the plants life cycle. It is essential for normal plant growth and development, not only as a source of energy but also as a stimulus that regulates numerous developmental and metabolic processes. Studies of several light-regulated genes from different species demonstrate that DNA elements responsible for light-responsive expression are usually located within 5' upstream sequences. An increase in the transcript levels of these genes occurs in response to light. The best known photoreceptor is phytochrome and it has been shown that this photoreceptor can mediate gene expression. Such observations suggest that many developmental processes in plants are controlled by interaction of regulatory proteins with specific DNA sequences.

Transcription factors can be divided into classes on the basis of their mode of interaction with the target promoter sequence. Different protein domains responsible for DNA recognition have been identified (e.g. zinc finger, helix-turn-helix, homeobox, leucine zipper etc.). We are interested in genes which control light-dependent plant development. Our hypothesis is that in many cases these genes will be transcription factors. More precisely we want to work out the chain of events by which light, through photoreceptors, gives signals to these developmental processes. The strategy we follow to isolate transcription factor genes involved in these processes is described as follows. A transcription factors library has been generated by screening a genomic lambda zap library with probes that encode DNA-binding motifs identified in plants, yeast, and animals. Out of this library we want to isolate transcription factor genes which are differentially expressed during light-dependent development. Interesting clones will then be further characterised.

CHARACTERIZATON OF HOMOLOGS OF *MYB* TRANSCRIPTION FACTORS FROM *A. THALIANA*.

Peter W. Doerner, Yoshihiro Ozeki, Diane M. Ruezinsky, Susan A. Hedrick and Christopher J. Lamb. Plant Biology Lab, The Salk Institute, 10010 North Torrey Pines, San Diego CA 92037

Homologs of the *myb* proto-oncogene have been cloned from many species including mammals, flies, plants and yeast. These genes are involved in the regulation of cellular differentiation, development and possibly also in housekeeping functions. The gene products have been shown to function as transcriptional activators and to bind DNA with a highly conserved DNA binding domain. The maize c1 gene, which is involved in the regulation of anthocyanin synthesis, was the first plant *myb* homolog to be identified. The *Arabidopsis* gl1 gene is a *myb* homolog regulating trichome development. Additional homologs have also been cloned from maize, barley, *Antirrhinum*, petunia and *Arabidopsis*.

We describe here the isolation of novel *myb* homologs from *Arabidopsis*. Five genes were isolated from a genomic library using PCR generated probes. Two additional clones were isolated using the DNA binding domain of the maize c1 gene as a probe. The deduced amino acid sequence of the DNA binding domain of these genes shares between 51-64% homology to the corresponding domain of the maize c1 gene. Each of these *myb* homologs are single copy genes, however they are part of a large gene family in the *Arabidopsis* genome, which consists of more than 16 genes. The genetic loci of two of these clones were mapped by RFLP based methods. None of these map positions coincides with loci identified previously.

RNA slot blot analysis shows that two genes are highly expressed in floral tissues, one gene is induced by high intensity light, whereas all others are expressed at levels too low to be detected. Results from analysis of expression at higher resolution using promoter GUS fusions will be shown.

We are currently focussing on the question of how to identify and analyze the function of these genes. We are introducing into *Arabidopsis* a series of constructs that we expect will generate phenocopies of mutants. Data on these transgenic plants will be presented.

SMALL G-PROTEINS IN THE GREEN ALGAE *VOLVOX CARTERI* AND *CHLAMYDOMONAS REINHARDTII*

S. Fabry[1], N. Naß[2], K. Palme[3], L. Jaenicke[2], and R. Schmitt[1]

[1]Universität Regensburg, Lehrstuhl für Genetik, D-8400 Regensburg; [2]Universität Köln, Institut für Biochemie, D-5000 Köln; [3]Max-Planck-Institut für Züchtungsforschung, D-5000 Köln-Vogelsang; Federal Republic of Germany

An increasing number of small G-proteins belonging to the so-called *ras*-superfamily has been documented in vertebrates, invertebrates, and certain fungi, whereas information on such G-proteins is scarce in green organisms. We have now identified several genes classified as members of the *ypt*-subgroup of small G-proteins in gene libraries of the colonial green alga *Volvox carteri* and its unicellular relative *Chlamydomonas reinhardtii*. This is the first report of such genes in simple algae. The *ypt*-genes, originally described in *Saccharomyces cerevisiae*, thus constitute a family of genes found in all eucaryotes examined so far. These genes encode highly conserved small GTP-binding proteins thought to be involved in intracellular vesicel transport and signal transduction.

Here, we present a comparative analysis of six algal *ypt* genes and the derived protein structures. Properties of *ypt* 1 from *Volvox carteri*, the best examined member of this group, will be reported. *The ypt* 1 gene belongs to the low expression class in *V. carteri*. Immunocytological studies together with the finding of strictly conserved cysteine residues at the C-terminus (suggesting posttranslational modifications such as palmitylation or isoprenylation) indicate a membrane anchoring of the *Ypt* 1 protein. We are currently investigating life-cycle dependent gene expression and the precise cellular localization of the various *Ypt* proteins in *Volvox* and *Chlamydomonas*. Possibilities of gene disruption and complementation analyses for defining the role of the *ypt* genes will be discussed.

MOLECULAR CLONING AND CHARACTERIZATION OF A CDNA ENCODING A NOVEL MEMBER OF SOYBEAN PROTEIN KINASE FAMILY

X.-H. Feng, Y. Zhao, P. Bottino and S.D. Kung, Center for Agricultural Biotechnology and Department of Botany, University of Maryland, College Park, MD 20742.

Protein kinases control diverse cellular processes by phosphorylating specific proteins in eukaryotic cells, probably as components of signal transducing systems. We have identified six members of a protein kinase family from soybean (*Glycine max* L.) by using PCR with two fully degenerate oligonucleotide primers corresponding two conserved motifs (DLKPENV and GTHEYLAPE) in the catalytic domains of eukaryotic protein serine/threonine kinases. By screening a soybean lambda gt11 cDNA library with the PCR products, we identified 8 cDNA clones that hybridize to one PCR DNA product (*GmPK6*). The composite nucleotide sequence of the *GmPK6* clones revealed an uninterrupted open reading frame of 1389 nucleotides capable of encoding a polypeptide (designated GmPK6) consisting of 462 amino acids with a calculated molecular mass of 52,561 daltons. The deduced primary structure of GmPK6 shows that it is a structural mosaic resembling both the protein serine/threonine and tyrosine kinases in the catalytic domains. It also contains a N-terminal putative regulatory domain similar to the central region of *Xenopus* U1 snRNP 70K protein where putative nuclear localization signals can be found. GmPK6 was produced and specifically labeled in *Escherichia coli*. Hybridization of the *GmPK6* coding region DNA sequence with genomic DNA from soybean reveals the presence of a single copy of *GmPK6* and at least one copy (copies) of *GmPK6*-related gene(s). Northern blot analysis reveals two transcripts of *GmPK6*, 2.5 kb and 1.9 kb, which are differentially expressed in soybean.

IDENTIFICATION OF NUCLEOTIDE DIPHOSPHATE KINASE FROM PEA MICROSOMAL MEMBRANES.

P.M. Finan, I.R. White, J.B.C. Findlay and P.A. Millner, Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, LS2 9JT, U.K.

Chromatography of detergent solubilised pea microsomal membrane proteins on GTP-agarose yielded a subset of tightly binding proteins of 95,35,31 and 17kDa Mr. These proteins could be eluted with 20mM GTP or 0.6M NaCl. N-terminal amino acid sequence data (17 residues) was obtained from one of these polypeptides, of Mr. 17 kDa, by protein microsequencing. The sequence displays a high degree of similarity to the nucleoside diphosphate kinase (NDP kinase) family of proteins and nm23, a protein whose expression is greatly reduced in tumour cells of high metastatic potential. NDP kinases are involved in maintaining the functional levels of non-adenine containing nucleoside triphosphates including GTP. Several members of this family have been recently isolated and cloned from a various sources including *Drosophila* and *Myxococcus xanthus* but their function is not fully understood. However, they would appear to be essential in processes requiring GTP, such as microtubule polymerisation, protein synthesis and signal transduction. In addition it has been suggested that a membrane localised form of NDP kinase may exist in a complex with the alpha subunit of the G-protein G_s in rat liver membranes.

The N-terminal sequence data obtained for the 17kDa polypeptide has been utilised to design degenerate oligonucleotide primers, which were used with oligo dT to amplify pea cDNA via the polymerase chain reaction (PCR). A PCR product of the expected size (~0.75 kilobases) was produced which hybridises to human nm23 cDNA which was also amplified by PCR. The pea cDNA product has been used for cloning and sequencing studies and the results of these studies will be presented. Finally, anti-peptide antibodies raised to the N-terminus of the 17 kDa protein crossreact specifically with 17 kDa and 35 kDa proteins isolated using GTP-agarose and should facilitate purification of these proteins.

PLANT CALMODULIN-RELATED PROTEINS AND CALMODULIN-BINDING PROTEINS

H. Fromm, E. Carlenor and N.-H Chua. Laboratory of Plant Molecular Biology. The Rockefeller University, New York, NY 10021.

We have found that a DNA-binding protein in petunia petal extracts can be phosphorylated by a calmodulin-dependent kinase. To learn more about this protein modification and its potential role in the regulation of gene expression we have used an *Arabidopsis* calmodulin cDNA as a probe to screen a petunia petal cDNA library. Clones encoding three types of calmodulin-related proteins were isolated. One clone (CAM81) has one amino acid residue different from previously reported spinach and barley calmodulins. Another clone (CAM72) is different from CAM81 at 14 residues. It contains two tyrosine residues in the same positions as vertebrate calmodulins. The third clone (CAM53) encodes a protein that is identical to CAM81 up to its penultimate residue but contains an extra domain of 35 amino acids. This domain is very basic (42% arginine and lysine) and has, roughly, a two fold symmetry regarding its charge distribution. All three recombinat calmodulins, bind to phenyl-sepharose in the presence of calcium, indicating that CAM53 as well as CAM72 and CAM81 are calcium-modulated proteins. Their mRNAs are not confined to petals and therefore most likely have a function which is not restricted to flower development. We are exploring the possibility that CAM53 has a specific function not shared by other calmodulin-related proteins.

The high expression level of the recombinant CAM81 protein in *E. coli* enables preparation of substantial amounts of ^{35}S-methionine-labeled protein. The purified protein can be used as a probe to screen cDNA expression libraries for isolating calmodulin-binding proteins. Results describing the isolation of a calcium-dependent calmodulin-binding protein from a root cDNA library will be presented.

BIOCHEMICAL EVIDENCE FOR G PROTEINS IN YOUNG TOMATO FRUIT. G. Gillaspy, D Schmitt, A. Loraine, and W. Gruissem, Department of Plant Biology, University of California, Berkeley, CA 94720

Signal transducing G proteins are a group of small GTP-binding proteins which have been implicated in growth control of a variety of eukaryotic cells. They function as single polypeptides such as the ras protein, or as heterotrimeric complexes with alpha, beta and gamma subunits. In an attempt to isolate molecules involved in signalling pathways of growth control in plant cells, we have searched for evidence of G proteins in young tomato fruit. This tissue was chosen because it is composed of rapidly dividing cells, and growth controlling molecules should be abundant. A group of antibodies which recognize human ras proteins was used in western blots against either young fruit extracts or crude membrane preparations. Of these, one polyclonal sera reacted specifically against at least two young fruit proteins in the 18 to 30 kd size range, which were present in membrane but not cytosolic extracts. The ability of proteins within these extracts to bind alpha-P^{-32} labelled GTP was investigated. Proteins of similar sizes as detected by the ras antibody were labelled with GTP, indicating that they may be G-proteins. An assay for ADP-ribosylation by either cholera or pertussis toxin was next used to detect whether these proteins or other substrates for this reaction were present in the abstracts. Higher molecular weight species were labelled by $NADP^{-32}$ when cholera toxin was used. To determine if these same proteins are targets of isoprenylation, H^{3}- mevalonic acid and the drug mevinolin are being injected into young fruit to label any isoprenylated species.

To isolate the genes which encode these proteins several approaches are being taken. Both the ras antibody and GTP^{-32} binding are being used to screen a young tomato fruit expression library. Also, DNA oligonucleotides specific for ras and G gamma sequences are being used in a PCR approach to clone homologues from young tomato fruit.

We are also attempting to purify a prenyltransferase activity from young fruit extracts with an <u>in vitro</u> assay using peptides containing target sequences for either farnesyl or geranylgeranyl transferases.

INDUCTION BY PHOSPHATE STARVATION OF EXTRA- AND INTRACELLULAR RIBONUCLEASES IN CULTURED TOMATO CELLS

K. Glund, M. Köck, K. Theierl, W. Jost, A. Löffler, T. Nürnberger and S. Abel; Universität Halle, Institut für Biochemie, 4050 Halle, Germany

We have previously identified in cultured tomato cells one extracellular and four intracellular (three vacuolar, one extravacuolar) ribonucleases which are induced upon depriving the cells of inorganic phosphate (Pi). Characterization of these enzymes with respect to catalytic and structural properties revealed close similarities from which we conclude that they might be encoded by a gene family. Induction of synthesis of these proteins is believed to be initiated by, at least, two mechanisms: (i) Synthesis is rapidly induced in the absence of extracellular, but presence of high intracellular Pi without any detectable changes in the cytoplasmic Pi pool (measured by *in vivo* ^{31}P-NMR). Synthesis of extracellular RNase ceases when extracellular Pi is supplied. This suggests a transduction through the plasma membrane. (ii) In the presence of extracellular Pi, ribonucleases were induced transiently by adding phosphorylatable metabolites (glycerol, mannose, deoxy-D-glucose, galactose) to the cells. This argues for transient changes of the cytoplasmic Pi pool as a signal. Furthermore, we have totally sequenced the extracellular enzyme, isolated a cDNA clone coding for this protein and used it to study the induction process in more detail.

LUPIN L-ASPARAGINASE GENE REGULATION IN TRANSGENIC TOBACCO.

Murray R. Grant and Michael W. Bevan, Molecular Genetics Department, Cambridge Laboratory, John Innes Centre for Plant Science Research, Colney Lane, Norwich, UK.

Legume seed development is highly regulated, with the synthesis of seed storage proteins subject to developmental, organ specific and environmental controls. The developing seed pod acts as a sink for nitrogen, which is transported in plants predominately as amides.

In lupin over 80% of the fixed nitrogen is transported as asparagine and about 80% of that entering the developing pod is metabolised (Atkins et al. 1975). Asparaginase (ASNase) catalysed deamidation is probably the major route in asparagine nitrogen remobilization in developing seeds. The ASNase gene from Lupinus angustifolius has been cloned (Lough et al. 1991), and a construct containing a translational fusion of -1163 to +82 of the ASNase gene with the reporter gene B-glucuronidase (GUS) has been used to study the induction and histochemical localisation of GUS during the development of tobacco seeds.

The results indicated that GUS activation was temporally and spatially controlled in a complex manner during the seed maturation process and this induction preceeded tobacco seed storage protein deposition (Chen et al. 1988).

The specific induction of asparaginase activity in localised endosperm cells in the vicinity of the funicle begs the question whether transcriptional control of the ASNase gene is mediated via metabolite responsive elements. To endeavour to answer this question deletion analysis of the ASNase gene upstream region is currently being undertaken to determine regions important in ASNase induction during seed maturation. Concomitant with this, we are footprinting with developing seed extracts from both homologous and heterologous systems in order to identify putative regulatory sequences.

REGULATION OF FLAVONOID BIOSYNTHESIS BY THE MAIZE P GENE, A TRANSCRIPTIONAL ACTIVATOR HOMOLOGOUS TO MYB

E. Grotewold[1], B. Bowen[2], B. Drummond[2], B. Roth[2], & T. Peterson[1], [1]Cold Spring Harbor Laboratory, New York; [2]Pioneer Hi-Bred International Inc., Johnston, Iowa.

Biochemical and genetic studies have suggested a regulatory role for the maize P gene in the biosynthesis of the red phlobaphene pigments found in certain floral organs (Styles and Ceska, 1977; *Can. J. Genet. Cytol.* **19**:289-302). We have shown that a functional P gene is required for the accumulation in the pericarp of transcripts of three genes (C2, CHI and A1) encoding structural enzymes required for the formation of flavan-4-ol, the precursor of the phlobaphene pigment. However, P is not required for anthocyanin synthesis. Anthocyanin formation requires expression of C2, CHI, A1, A2, Bz1 and Bz2 under the simultaneous control of two regulatory genes, Pl or C1 (myb homologues) and R or B (which carry a basic helix-loop-helix domain).

We have characterized two P-encoded mRNAs of 1802 and 945 bp from maize pericarps which arise by alternative splicing of their third exons. The 1802 nt transcript encodes a 43.7 kD protein with an amino terminal domain of high homology to the DNA binding domain of the myb family of oncoproteins. The carboxy terminus contains a negatively-charged amphipathic α-helical region resembling the activating domain of several other transcriptional activators (Grotewold *et al.* 1991, *PNAS* **88**:4587-4591).

We have used particle bombardment to test whether P cDNAs can transactivate a luciferase reporter gene driven by the promoter of the maize A1 gene, which is regulated by P *in vivo*. The 1802 nt P cDNA is sufficient to transactivate the A1/luciferase reporter gene approximately 100 fold; this level is similar to that obtained using a combination of R and C1. The luciferase gene under the control of the Bz1 gene promoter (not P regulated) was activated only 5 fold by the 1802 nt P cDNA. The 945 nt P cDNA had no activating or inhibiting effect in this assay.

The proteins encoded by P and C1 are over 70% homologous in the region corresponding to the DNA binding domain, and the P and C1 transcripts share some similarities in exon/intron structure. Furthermore, P and C1 regulate some of the same genes required for flavonoid biosynthesis. However, our results show that P, unlike C1, does not require the R or B gene product for its transcriptional activatory function. Possible mechanisms of transcriptional activation by P will be discussed.

CHARACTERIZATION AND EXPRESSION ANALYSIS OF A TOBACCO FLOWER-SPECIFIC THIONIN cDNA. Q. GU, H-M.WU, AND A.Y. CHEUNG. Department of Biology, Yale University.

Thionins are a class of low molecular weight cysteine rich basic polypeptides. A number of thionins have been shown to be toxic to bacteria, fungi, animal and plant cells. Thionins are found in the seeds of many monocotyledonous plants such as wheat (purothionins) and barley (hordothionins), as well as in some dicotyledonous plants such as in the stems and leaves of mistletoe (viscotoxins). The biological roles of these toxic molecules in plants have not yet been elucidated. Recently, etiolated leaf-specific thionins have been found and these thionins have anti-pathogen activities and their expression in light-grown leaves can be induced by pathogen treatment suggesting a role in plant defense systems for these leaf thionins (Ref. 1 and 2).

We have isolated a cDNA (NtFS) encoding a novel thionin specific to flowers in *Nicotiana tobacum*. NtFS encodes a polypeptide of 105 amino acids. It has a hydrophobic amino terminal signal peptide, a central thionin domain of 47 amino acids which is basic and has 8 cysteine residues, and a slightly acidic carboxyl terminal domain, the significance of which remains to be elucidated. This structure is similar to a number of thionin cDNA characterized. Mature thionins have only the central thionin domain. The thionin domain encoded by NtFS is highly homologous to three other thionins isolated from wheat. The most striking homology is in the perfect alignment of the 8 cysteine groups between these molecules. It is known that these cysteine residues form 4 disulfide bridges and thus are important to the secondary structure of thionins.

Expression of NtFS has been analysed by Northern blots and *in situ* hybridization. Expression is highly specific to flowers but drops precipitously at anthesis. Expression is restricted to petals, stamens and carpels. *In situ* hybridization also did not detect any expression of NtFS in the sepals and revealed that NtFS transcripts are constitutive to petal cells, highly concentrated in the style but are absent from the stigmatic surface. Expression in the stamen is enriched in the filament but is limited in the anthers. The biological significance of the differential expression pattern of this thionin in tobacco flowers and its functional role are being explored.

Ref. 1. F. Garcia-Olmedo et al. 1989. In Osford Surveys of Plant Molecular and Cell Biology *6*: 31.

2. H. Bohlman and K. Apel. 1991. Ann. Rev. Plant Physiol. and Mol. Biol. *42*: 227.

CHANGES IN PHOSPHOPROTEIN PATTERNS IN POTATO (SOLANUM TUBEROSUM) AND ARABIDOPSIS THALIANA DURING WATER STRESS

P. Heino[1]; B. Welin[1]; M. Baudo[1]; C. Barassi[2] and E.T. Palva[1],[1]Departement of Molecular Genetics, Swedish University of Agricultural Sciences, Box 7003, S-750 07 Uppsala, Sweden [2]Universitad Nacional de Mar del Plata, INTA Balcarce, c.c. 276, 7620 Balcarce, Argentina

Solanum tuberosum and Arabidopsis thaliana plantlets were exposed to water stress either by adding polyethylene glycol (PEG) 6000 to the liquid growth medium or by air drying the plants. The analysis of the pattern of in vitro phosphorylated polypeptides during the stress revealed that there are significant changes in phosphoprotein patterns in both plant species during water stress. Part of the observed changes were found to be strictly Ca^{2+}-dependent, suggesting that a Ca^{2+} -dependent protein kinase is involved. The concentration of the growth regulator abscisic acid (ABA) is known to increase in plants subjected to water stress. Therefore, the effect of exogenously applied ABA on phosphoprotein patterns was studied. The application of ABA to the growth medium of plants resulted in similar changes in phosphoprotein patterns as compared with those produced by water stress, suggesting that the observed changes may be mediated by ABA during water stress.

RHIZOBIUM MELILOTI NODULATION SIGNALS

G. Truchet[1], P. Roche[2], F. Debellé[1], P. Lerouge[2], C. Rosenberg[1], M.Y. Ardourel[1], N. Demont[2], J. Vasse[1], J.C. Promé[2] and J. Dénarié[1], [1]Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, CNRS-INRA, BP27, 31326 Castanet-Tolosan Cedex; [2]Centre de Recherches de Biochimie et de Génétique Cellulaire, CNRS-UPS, 118, route de Narbonne, 31062 Toulouse Cedex, France.

R. meliloti is a symbiotic procaryote which elicits the organogenesis of nitrogen-fixing nodules in the roots of specific leguminous hosts such as alfalfa. The first visible responses of the host to infection are root hair deformation and curling, infection thread formation, and mitotic activity in the root cortex leading to the formation of a nodule. The bacterial nodulation genes which control these processes, as well as host specificity, are classified into common *nod* genes present in all rhizobia studied so far and species-specific *nod* genes which control the host-range.

The common *nodABC* genes are required for the production of extracellular Nod factors which are sulphated lipo-oligosaccharides. They are tetra- or pentasaccharides of N-acetyl-D-glucosamine, N-acylated and O-acetylated on the terminal non-reducing sugar residue and sulphated on the reducing sugar (1, 2). On alfalfa, these purified factors elicit root hair deformation at nanomolar concentrations and cortical cell divisions and nodule formation in the micromolar-nanomolar range (1, 3). Structure-function relationship studies have shown that the sulphate group, the reducing function of the terminal sugar and the double bonds of the acyl chain are all important in eliciting nodule organogenesis on alfalfa (3).

The host-range genes *nodH* and *nodPQ* determine the sulphation of the Nod factors which is responsible for the alfalfa specificity. The role of other *nod* genes in the synthesis of Nod factors will be described.

Lipo-oligosaccharidic Nod factors elicit the transcription of some early symbiotic plant genes which are expressed in root hairs and cortical cells (4). Such plant genes could provide molecular markers for the developmental switch which occurs in the root cortex. Signal molecules amenable to chemical modifications and molecular markers should facilitate the analysis of how a signal is perceived, transduced and ultimately specifically induces a plant organogenesis.

(1) Lerouge et al. (1990) Nature, 344, 781.
(2) Roche et al. (1991) J. Biol. Chem. in press.
(3) Truchet et al. (1991) Nature, 351, 670.
(4) Nap JP & Bisseling T (1990) Science, 250, 948.

NOD FACTORS AND NODULIN GENE INDUCTION

H.J. Franssen,[1] B. Horvath,[1] H. Spaink,[2] C. van de Wiel,[1] B. Scheres[1] and T. Bisseling,[1] [1]Department of Molecular Biology, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands; [2]Department of Plant Science, University of Leiden, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands.

The interaction between roots of leguminous plants and (Brady) *Rhizobium* bacteria leads to the formation of a unique plant organ, the root nodule, in which the differentiated bacteria fix nitrogen. The development of the nodule proceeds through several distinct steps in a specific order; root hair deformation, the formation of the infection thread through which the bacteria enter the root, and concomittantly induction of cell divisions leading to the formation of a meristem from which the nodule tissues will generate. During these different steps the expression of specific plant genes, the nodulin genes is induced. Five different (early) nodulin genes have been identified that mark different early stages in the pea root nodule developmental process, among them two genes that are related to the infection process. Since the formation of a root nodule is induced by *Rhizobium*, signal molecule(s) secreted by the bacteria will induce this developmental process. Recently the bacterial produced compounds called the Nod-factors have been purified and the structures have been elucidated (H. Spaink). These Nod-factors are capable of inducing root hair deformation, cell division and nodulin gene expression related to infection. Hence, besides the Nod-factors no other bacterial signal molecule are essential in the induction of the initial steps that leads to nodule formation. Clues about the possible way of action of the Nod-factors came from experiments in which it was shown that legumes treated with auxin transport inhibitors (ATI) nodule-like structure were generated in which the expression of early nodulin genes could be detected. This observation indicates that the Nod-factors most likely influence the hormonal balance in the root and this will lead to nodule formation.

Identification and Molecular Mapping of a Disease Resistance Gene in Arabidopsis thaliana and Characterization of Putative Mutants Affecting its Action

Jeffery L. Dangl, Thomas Debener, Hiltrud Lehnackers, Claudia Ritter and Corinna Clemens; Max-Delbrück-Laboratorium, Carl-von-Linné-Weg 10, D-5000 Köln 30, Germany

We have developed a model pathosystem using phytopathogenic Pseudomonas syringae pv. maculicola (Psm) isolates and Arabidopsis thaliana. Our goals are to identify and isolate plant genes necessary for generation of a successful disease resistance response. These genes can full into at least two classes: signal perception (R-genes) and signal transduction.

We showed that there is phenotypic variability determined by both plant and bacterial genotypes in our system. In one interaction set Arabidopsis ecotypes Col-0 and Oy-0 are resistant and ecotype Nd-0 susceptible to infection with Psyringae pv. maculicola isolate m2, while all three are susceptible to isolate m4. Resistance against isolate m2, defined as the ability to rapidly generate a hypersensitive response (HR), segregates as a single dominant trait in the crosses Col-0xNd-0 and Oy-0xNd-0. We have mapped this locus (RPM1), via RFLP linkage to a small interval (~10cM) on chromosome 3 in both crosses. Chromosome walking is in progress. We also isolated a bacterial avirulence (avr) gene from the m2 isolate. This gene converts the normally compatible interaction between Psm isolate m4 and ecotype Col-0 to an incompatible one. Resistance directed against avrRpm1 co-segregates with resistance to the Psm isolate m2 from which it was isolated. One Arabidopsis mutant (from ~2000 screened) no longer responds to the avrRmp1 gene.

We also have isolated several candidate "lesion mimic" mutants from a T-DNA insertion mutagenized population (kindly provided by Ken Feldmann). We are characterizing these for constitutive induction of defense related transcripts and for enhanced resistance. Our progress in these areas will be detailed.

A VIRUS-HOST INTERACTION: ALTERED PLASMODESMATAL FUNCTION

C. Deom[1], P. Moore[2], C. Fenczik[1], and R. Beachy[1]
[1]Department of Biology, Washington University, St. Louis, Missouri. [2]Department of Agronomy, University of Kentucky, Lexington, Kentucky

The primary route for cell-to-cell spread of plant viruses is by way of plasmodesmata, plasma membrane lined, cytoplasmic channels which extend through cell walls to interconnect plant cells. A role of the 30-kilodalton movement protein (MP) of tobacco mosaic virus is to facilitate cell-to-cell movement of viral progeny in an infected plant. One mechanism by which the MP functions is to modify the molecular exclusion limit of plasmodesmata. Immunocytochemistry was used to examine the structure of plasmodesmata between leaf mesophyll cells in transgenic tobacco plants expressing the TMV MP. A change in structure was observed in plasmodesmata as leaves develop, both in control and MP expressing (MP+) plants. However, plasmodesmata of older leaf cells in MP(+) plants exhibit a fibrous material in the central cavity. The presence of the fibers is correlated with the ability to label plasmodesmata with anti-MP antibodies. The developmental stage of MP(+) leaf tissue, having plasmodesmata which exhibit the fibrous material, is similar to that of MP(+) leaf tissue in which an increase in the molecular size exclusion limit is detected and MP(+) leaf tissue in which a MP deficient strain of TMV is complemented. Cell fractionation and aqueous phase partitioning was used to identify the plasma membrane and the cell wall as the cellular compartments with which the MP stably associates. The nature of the interaction between the MP and the plasma membrane was investigated using sodium carbonate washes and Triton X-100 extraction. The data suggests that the MP behaves as an intregal membrane protein.

MOLECULAR CLONING OF A RECEPTOR PROTEIN KINASE GENE ENCODED AT THE SELF-INCOMPATIBILITY LOCUS OF *BRASSICA OLERACEA*

J.C. Stein, B. Howlett, D.C. Boyes, M.E. Nasrallah, J.B. Nasrallah, Division of Biological Sciences, Section of Plant Biology, Cornell University, Ithaca, New York.

We are interested in describing the molecular recognition and subsequent signal transduction events that lead to pollen rejection in the self-incompatibility (SI) response of *Brassica oleracea*. Our approach has been to clone and characterize genes derived from the highly polymorphic S locus, which determines "self" versus "non-self" recognition in the pollen-stigma interaction. One of these genes, *SLG*, encodes the secreted S-locus glycoprotein, and possesses several characteristics that make it a strong candidate for functioning as a recognition determinant. These include its genetic location at the S locus, its high degree of sequence polymorphism between alleles of different S-locus genotypes, and its exclusive expression within reproductive tissues. However, the amino acid sequence of *SLG* has yielded few leads on how *SLG* functions.

We have now discovered a second gene, S-Receptor Kinase (*SRK*), that is very closely related to *SLG*, but whose sequence provides powerful clues to its function. *SRK* encodes a receptor-like protein (predicted MW 98 kd) that is analogous in structure to the receptor tyrosine kinases in animals. Its predicted features include a 405 amino acid (aa) extracellular domain that is homologous to *SLG*, and could possibly function in ligand binding. This region is connected via a single-pass transmembrane domain to a 300 aa protein kinase catalytic center. Unlike most receptor kinases, which display ligand-activated phosphorylation on tyrosine residues, *SRK* possesses the consensus sequences typical of serine/threonine kinases, and thus joins an emerging class of genes that may encode receptor serine/threonine kinases. RFLP analysis of F2 populations segregating for different S-locus genotypes shows that *SRK* is tightly linked to *SLG* and to the S locus, thus raising the possibility that different alleles of *SRK* could impart the recognition specificity of SI. In support of this, we have isolated *SRK* genomic clones from two different S-locus genotypes, and have shown them to be highly diverged (67% aa identity over their *SLG*-homologous domains and 71% aa identity over their kinase-catalytic domains). *SLG* clones corresponding to the same two S-locus genotypes have also been isolated, and their sequences show a similar level of divergence. Interestingly, much greater conservation (90%) was observed between *SLG* and the *SLG*-homologous domain of *SRK* within each S-locus genotype, suggesting concerted evolution between the two genes and a functional interaction of their products. RNA blot analysis revealed a 3.0 kb transcript, consistent with the size predicted by cDNA clones of *SRK*. Moreover, expression was restricted to stigmatic and anther tissues and was not observed in vegetative tissues. Two additional *SRK* transcripts provide evidence of alternative splicing that may lead to the production of both C-terminally truncated and N-terminally truncated protein forms. These data provide foundation to a model for SI in which an S locus-specific receptor couples initial molecular recognition events (ie ligand binding) to the signal transduction chain via a phosphorylation cascade, leading to pollen acceptance or rejection.

CHARACTERIZATION OF A RECEPTOR-PROTEIN KINASE GENE FAMILY IN *ARABIDOPSIS*

R.E. Pruitt and S.E. Ploense, Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108

The S-locus of *Brassica oleracea* is involved in the mediation of self-recognition during fertilization. It encodes a small glycoprotein which is abundantly expressed in the stigmatic papillae where it is secreted to the surface of the cells. The SLR1 gene of *Brassica* encodes a similar product and has a similar pattern of expression but does not have a known function. Recently, Walker and Zhang (1990) have recovered a cDNA clone from maize which is composed of an extracellular domain similar to the *Brassica* S-locus linked to a cytoplasmic protein kinase domain. This gene is expressed in young shoots and roots as well as reproductive structures.

We have isolated a series of genes from *Arabidopsis* based on their strong homology to both the S-locus and to SLR1. Of the five genes which we have isolated one appears to encode a product similar to the S–locus which is primarily confined to floral tissues and one appears to be a pseudogene based on structural analysis. The remaining three each encode at least two different RNA species, one of which appears to correspond to an S-locus-type product and the other a membrane bound protein kinase bearing an extracellular S-locus-like domain similar to that described by Walker. These three genes are expressed at very low levels in the plants in a wide variety of tissues. Within a tissue each gene appears to be expressed in a limited subset of cells, as determined by reporter gene fusion experiments. The functions of all the *Arabidopsis* genes are unknown but the conservation of their sequences relative to the S-locus make it seem likely that they are involved in some type of cellular recognition process and the subsequent transduction of signals resulting from that process into the cell.

J.C. Walker and R. Zhang (1990) Nature 345: 743-746.

CALCIUM-SIGNAL RECEPTORS: CHARACTERISTICS OF PLANT ANNEXINS

H.D. Blackbourn and N.H. Battey. Department of Horticulture, School of Plant Sciences, University of Reading, Berks., RG6 2AS. U.K.

Proteins with annexin-like characteristics have recently been identified in plants (1,2). The term 'annexin' refers to a class of animal proteins which bind calcium and have the capacity to: aggregate and fuse secretory vesicles; bind to actin and inhibit phospholipase A_2 activity (3). In maize extracts, three proteins of relative molecular mass (M_r) 35,000, 33,000 and 23,000 have annexin-like properties (2). The evidence that these proteins are related to animal annexins is that they share a Ca^{2+}-dependent capacity to bind to liposomes composed of acidic phospholipids (2). Furthermore, antibodies specifically raised to the proteins of M_r 33-35,000 from maize also recognise annexin VI from animal tissues (2). Sequence data to be presented confirms that there is a high degree of homology between the proteins from maize and animal annexins.

We are studying the function of plant annexins in the tip-growing pollen tube as there is evidence that pollen tube growth is dependent on the availability of free Ca^{2+} (4) and that elevated levels of free Ca^{2+} are present at the tube tip (5). Western blotting reveals that immunoreactive polypeptides of M_r 33-35,000 are present in pollen tubes of *Lilium longiflorum*. Immunolocalising at the LM level suggests that annexin-like proteins are confined to the tube tip. The detection of annexins in the vesicle-rich tip of pollen tubes has encouraged us to examine the capacity of plant annexins to promote vesicle fusion and hence vesicle-mediated secretion and growth. Data to be presented will include the biochemical and functional characterisation of these plant annexins.

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STRUCTURAL AND FUNCTIONAL PROPERTIES OF THE CALCIUM CHANNEL BLOCKER BINDING PROTEIN FROM CARROT CELLS

R. Ranjeva[1], A. Graziana[1], C. Mazars[1], P. Thuleau[1,2] and J. Schroeder[2], [1]Centre de Physiologie Végétale,Université P. Sabatier et CNRS, Toulouse, France; [2]Department of Biological Sciences, University of California, San Diego, California.

Calcium channel blockers and compounds of microbiological origin control the entry of calcium into carrot protoplasts.
The first step involves the recognition of the effector by "receptor-like" structures mainly located at the plasma membrane. A binding protein has been identified by photoaffinity labeling with a radioactive azido derivative of calcium channel blocker that labels specifically a 75 kDa polypeptide.
The solubilized protein retains its ability to bind the calcium channel blocker and has been shown to contain transmembrane domains.
Incorporation of partially purified proteins into giant liposomes and single-channel patch-clamp studies on these proteoliposomes show calcium channel activities which are not found in control experiments. The currents are inhibited by the calcium-channel blocker, bepridil.
The occupancy of the binding sites results in the inhibition of calcium uptake by protoplasts. The efficiency of the inhibitor is proportional to its affinity for the "receptor-like" structure. Measurements with Fluo-3, a specific fluorescent probe, show significant decreases in cytosolic calcium. The channels may be activated by the phytotoxin zinniol which partially competes with calcium-channel blokers for common sites. Cell lines that are resistant to the toxin do not respond in this manner and remain insensitive to zinniol.
These data establish that calcium-channels exist at the plasma membrane of plant cells. They are functional and control both the entry of calcium and cytosolic calcium. Since their activities may be modified by signal compounds such as phytotoxins, plant calcium channel may be involved in the perception and transduction of external stimuli.

INHIBITION OF TOMATO HMG CoA REDUCTASE ACTIVITY ARRESTS FRUIT GROWTH, AND INTERFERES WITH SIGNAL TRANSDUCTION BY ARACHIDONIC ACID

J. O. Narita, S. M. Dean, and W. Gruissem, Department of Plant Biology, University of California, Berkeley, CA 94720

In animal and yeast, control of cell division, cell growth and signal transduction events are coupled to the isoprenoid pathway via the prenylation of ras and Gγ signal transduction proteins. Inhibition of mevalonic acid (MVA) synthesis results in arrest of cell division and cell growth, and disrupts e.g., the signal transduction pathway for the mating response in yeast. Thus, the modification of ras and Gγ proteins by farnesyl and geranylgeranyl transferases is required for their biological activity, and establishes the connection between the sterol biosynthesis pathway and coordination of cellular responses.

We are investigating the role of MVA in control of plant cell growth and signal transduction. In contrast to animals and yeast, tomato has at least four genes for HMG CoA reductase (*hmg*) which are differentially expressed during development. The plant enzymes lack the seven membrane-spanning N-terminal domain, which is characteristic of the animal and yeast proteins. The *hmg1* gene is expressed in growing tomato fruit, and we have previously shown that inhibition of MVA synthesis results in arrest of fruit growth, but not cell death (Narita and Gruissem, *Plant Cell* 1: 181-190, 1989). The arrested fruit phenotype is consistent with a block in cell division and/or cell growth, and can be rescued by mevalonic acid. Formation of seeds in arrested fruit is greatly reduced or absent.

Upon wounding and treatment of young tomato fruit with arachidonic acid (AA), *hmg1* expression is rapidly inactivated, and *hmg2* expression is induced to high levels. The *hmg2* gene is not expressed in young control fruit, and is normally activated only during ripening and lycopene synthesis. The inactivation of *hmg1* and induction of *hmg2* by AA is suppressed in fruit in which MVA synthesis was blocked 24 hrs prior to AA treatment. These results are consistent with the possibility that in plants signal transduction events are coupled to MVA synthesis, presumably via the prenylation of proteins that control signal transduction pathways and cell growth. We will discuss experiments and results to identify the molecular mechanisms involved in these processes.

ISOLATION AND CHARACTERISATION OF GUANINE NUCLEOTIDE BINDING PROTEINS FROM HIGHER PLANTS.

P.A Millner, J.Clarkson, A.Wise and I.R White.
Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, UK.

Specific, high affinity binding of the guanine nucleotide analogue GTPγS has been shown in the highly purified plasmalemma fractions from several species including the dicots *Nicotiana tabacum* and *Pisum sativum* and the monocots *Triticum aestivum* and *Zea mays*. In the latter species, binding was stimulated by sub-CMC concentrations of the detergent Lubrol and was optimum at pH 7.4. In the microsomal membrane fractions and purified plasmalemma from the above species, the presence of polypeptides which crossreacted with the antisera raised against synthetic peptides corresponding to animal Gα sequences or to the C-terminus of the *Arabidopsis* Gα-homologue (GPA1) was also shown. In particular, antisera SG1 directed against a C-terminal peptide of $G_i\alpha$ revealed a 37kDa crossreacting protein in *Pisum* and *Arabidopsis* membranes whilst antisera raised against the GPA1 C-terminus crossreacted with 43kDa and 37kDa polypeptides in *Arabidopsis* membranes. Subsequent work has indicated that the 37kDa, SG1-detectable polypeptide can be released from the *Arabidopsis* membranes and partially resolved by chromatography on Cibacron Blue agarose. Release of the 37kDa polypeptide from this affinity matrix was brought about by 20mM GTP or ATP, but not 2M NaCl. Finally, work aimed at the identification of G-protein linked receptors has been carried out, using affinity resins which contain peptides corresponding in sequence to the C-terminus of GPA1. In animal systems this region of the Gα subunit normally interacts with the third cytoplasmic loop of rhodopsin-like (7-helix) receptors. Preliminary data, indicating the existence of a receptor of this type in maize microsomal membranes, will be presented.

AT LEAST 10 DISTINCT G - PROTEIN α (INHIBITORY) SUBUNIT GENES ARE EXPRESSED IN THE TOMATO LEAF

K.E. Hammond-Kosack, M.J.Dickinson and J.D.G.Jones. The Sainsbury Laboratory, John Innes Centre for Plant Science Research, Colney Lane, Norwich, NR4 7UH, UK.

Many eukaryotic signalling systems involve plasma membrane spanning receptors that activate intracellular effectors via GTP binding proteins (G proteins). The G protein component is located on the cytoplasmic side of the plasma membrane and is a trimer, consisting of an α-subunit associated with a tight complex of β and γ subunits. The α-subunits are a key element of this signalling machinery and can interact with several effector target molecules including adenylate cyclase, phospholipase C, cGMP phosphodiesterase and K^+ and Ca^{++} ion channels. The cycle for α-subunit activation and inactivation is crucial for the amplification of the original external signal.

In yeast, certain mutants are constitutively "on" for the pheromone response signal transduction pathway. Some of these mutations correspond to recessive mutations that abolish the function of the α subunit. We hypothesized that some plant necrotic mutations, that appear to be constitutively "on" for the hypersensitive defence response, might correspond to mutations in such α subunit G-protein genes. We therefore set out to isolate α subunit G protein genes from tomato and localize them on the tomato RFLP map, to see if any were linked to known necrotic loci.

Comparison of α-subunit amino acid and DNA sequences from a range of species has revealed highly conserved domains throughout their primary structure. These conserved regions primarily participate in guanine nucleotide interactions. Two pairs of degenerate oligonucleotides were made to conserved domains in the mammalian primary nucleotide sequence. These were used firstly to prime 1st strand cDNA synthesis from a leaf poly A^+ mRNA template and then to amplify by PCR the intervening sequences.

A strong PCR band was observed of the expected size (180bp), and products were cloned and sequenced. The sequence data confirmed each insert encoded the invariant amino-acid consensus sequence characteristic of Gαi (inhibitory) subunits. 10 of the 14 clones sequenced are distinct; one clone was represented three times and two others twice. The degree of homology they exhibit to each other varies, at the nucleotide sequence level between 69-96% and at the deduced amino-acid sequence level between 85-97%. No gene homologous to *Arabidopsis* GPA1 was detected.

Several 180 bp PCR product clones were used as a probe to screen a λ gt 11 cDNA library. Numerous positively hybridising clones were obtained (56/60000).

Three independent clones were characterised further. These have been mapped on the tomato genome. SLJ 603 resides on chromosome 5 , and SLJ's 602 and 6041 are located near together on chromosome 12. Northern analyses with each cloned sequence has revealed different patterns of transcript abundance in the various plant organs. Sequencing of the three cDNA clones is in progress.

The diversity and multiplicity of these genes strongly suggests an important role for G-protein linked (heptahelical) receptors in plant signal transduction mechanisms.

ISOLATION AND MOLECULAR ANALYSIS OF SMALL GTP-BINDING PROTEINS OF ARABIDOPSIS THALIANA

M. Matsui[1], T. Anai[1,4], K. Hasegawa[2], Y. Watanabe[3], H. Uchimiya[4] and R. Ishizaki[1]

[1] Molecular Biology Laboratory, Nippon Medical School, Kawasaki, 211, [2] College of Liberal-arts, Kagoshima University, Kagoshima, 890 [3] Department of Bioscience, Teikyo University, Utsunomiya, 320, [4]Institute of Applied Microbiology, University of Tokyo, Tokyo, 113 (Japan)

Many small GTP-binding proteins (small G proteins) have been identified in various species. These molecules are thought to be involved in signal transduction pathways in eucaryotic cells. We previously isolated a member of this small G protein gene from the genome of an A. thaliana and named it ara (Matsui et al., gene,1989). Small G protein genes make up a supergene family in mammalian cells, and they are localized in different intra-cellular membranes. The amino acid sequences of their products are conserved in four regions, which are now known to be important for binding and hydrolysis of the GTP moiety. Small G proteins can be classified into three sub-family, ras, rho and YPT by differences around amino acid residue 35 which is thought to be important for the binding of proteins regulating GTPase activity and transducing this activity to subsequent unidentified molecules. Mainly from studies on S. cerevisiae, the YPT-family are known to be localized in the surface of traffic vesicles segregated from the Golgi apparatus, and to determine the fate of the vesicles. YPT is also important for cell viability and in S. cerevisiae lack of this gene is lethal. The structure of ara is most similar to that of YPT of S.cerevisiae.Three YPT-like genes were cloned from S. pombe and found to constitute a gene family, the members of which are thought to have slightly different functions. To examined whether there is also an ara gene family that functions in signal transduction pathways, we cloned ara-related sequences from a cDNA library of A. thaliana. The products of these genes, ara-2, ara-3, ara-4 and ara-5 showed conservation of amino acid residues in four regions. These products were especially highly homologous with those of the YPT genes of S. cerevisiae and S. pombe in the regions around amino acid residue 35 . The products of these four genes showed characteristic features at their C termini and end with Cys-Cys-X-X. Another characteristic of this family is serine in place of glycine in the first conserved region. The GTP-binding and GTPase activities were examined using T7 over-expression system in E. coli. Their functions were also examined in yeast cells using expression vector which expression was regulated by nutrient, because their products seemed to be toxic to both E. coli and yeast cells. Although there were no complementations of ypt1 and ypt3 gene, it was suggested that ara proteins have some regulatory role about the growth of cells. When ara genes were expressed, all transformed cells (with or without YPT mutation) stopped growing, but did not stop growing when expressed in an antisense orientation. I would also show the recent results about the functions of these proteins.

CLONING AND CHARACTERIZATION OF A $p34^{cdc2}$ HOMOLOG FROM *Arabidopsis thaliana*

J. L. Celenza and G. R. Fink, Whitehead Institute for Biomedical Research, Cambridge, MA.

We are interested in how plants regulate their growth, and as a first step we have cloned a $p34^{cdc2}$ homolog from the dicotyledonous plant *Arabidopsis thaliana*. Degenerate oligonucleotides spanning conserved regions of the $p34^{cdc2}$ family of protein kinases were used in a polymerase chain reaction in order to isolate homologous sequences from Arabidopsis genomic DNA. Clones that were similar to the $p34^{cdc2}$ family were used to probe an Arabidopsis cDNA library and a complete cDNA was identified that could encode a 34 kd protein that is 65% identical to the CDC28 protein of *S. cerevisiae*. The high level of shared sequence identity is conserved throughout the entire sequence, and the Arabidopsis protein (CDC2A) contains the VPSTAIR sequence unique to the $p34^{cdc2}$ family of protein kinases. We have also found that when CDC2A is expressed in yeast it can complement a temperature-sensitive *cdc28* mutation as well as an insertion mutation.

Ideally we would like to obtain plants that carry a mutation in CDC2A and determine whether these mutant plants have altered responses to growth regulators such as auxin. Since gene replacement techniques are not presently available an alternative is to express dominant mutations in plants. We are using a multi-pronged approach in which we are constructing transgenic plants that express: 1) novel dominant mutations identified by expression in yeast, 2) *in vitro* construction of known dominant mutations found in other $p34^{cdc2}$ homologues, and 3) anti-sense constructions driven by the CaMV 35S promoter. In order to detect the Arabidopsis protein we have epitope tagged CDC2A with an influenza hemagglutinin antigen and are currently making transgenic Arabidopsis that will carry the epitope-tagged protein.

ANALYSIS OF P34^{CDC2} PROTEIN KINASE FROM HIGHER PLANTS

J. Colasanti and V. Sundaresan, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

The p34^{cdc2} kinase is essential for the regulation of cell division in all eukaryotes. This protein is a serine/threonine kinase which is the central catalytic component of a complex that drives the cell cycle by phosphorylation of key substrates. Substrates include those involved in mitosis-specific events such as chromosome condensation, breakdown of the nuclear envelope and cytoskeletal reorganization. Plant cells differ from animal and yeast cells in many significant respects; for example, by the absence of distinct centrosomes or spindle pole bodies, by the appearance of an organized microtubular superstructure (the preprophase band) that predicts the plane of cell division, and by the formation of a new cell wall plate by the phragmoplast. As there is no cellular migration during plant development, precise orchestration of spatial and temporal signals specifying these cell divisions is essential in the formation of the plant. We have isolated a cDNA clone for a functional p34^{cdc2} homologue from maize and have found that expression of the mRNA is correlated with the cell division activity of the tissue. The functionality of the maize *cdc2* clone was demonstrated by its ability to complement a *S. cerevisiae cdc28* mutant defective in the G2/M phase of the cell cycle. However, complementation of *cdc28* mutants defective in G1/S is inefficient, suggesting that the cloned kinase may be G2/M-specific and further raises the possibility that G1/S-specific p34 kinases may exist. Southern blot analysis, and the isolation of a second *cdc2* cDNA, suggest that maize has multiple *cdc2*-like genes. In addition, expression of the maize *cdc2* clone is lethal to *S. pombe* and seems to result in the uncoupling of the coordination between nuclear division and cytokinesis. We have raised antibodies specific to the deduced maize p34^{cdc2} protein, based on the cDNA sequence, and show that levels of the protein are also correlated with cell proliferation. These antibodies are being used to localize the p34^{cdc2} protein during the plant cell cycle.

A PROTEIN KINASE CASCADE IN PLANTS AND THE CLONING OF PLANT PROTEIN PHOSPHATASES 1 AND 2A.

Robert W. MacKintosh, Stephen P. Davies and D. Grahame Hardie
Protein Phosphorylation Group, Department of Biochemistry, University of Dundee, Scotland DD1 4HN.

It is now apparent that, as in animal cells, *reversible* phosphorylation of proteins is central in transduction of extracellular signals into intracellular biochemical changes in plants. Recently three of the major protein phosphatases (PP), namely PP1, PP2A, PP2C, present in animal cells, were identified in higher plants and their catalytic properties were found to be virtually indistinguishable from the mammalian counterparts. The molecular explanation for these properties was revealed when we isolated *Brassica napus* cDNAs encoding proteins with remarkable similarity (70–80% identity) to rabbit PP1 and PP2A[1].

Since these protein phosphatases are so highly conserved, we became intrigued to know whether plants contain homologues of any of the mammalian protein kinase cascades whose activities are regulated by phosphorylation/dephosphorylation systems using PP1, PP2A or PP2C. In particular, several clues suggested to us that plants might contain a homologue of the mammalian AMP-activated protein kinase which inactivates *in vivo* three key regulatory enzymes of mammalian lipid metabolism, (ie, HMG-CoA reductase, cholesterol/isoprenoid biosynthesis; acetyl-CoA carboxylase, fatty acid synthesis; and hormone-sensitive lipase, triglyceride and cholesterol ester breakdown). For example, serine-872 which is the regulatory site on rat HMG-CoA reductase that is phosphorylated by the AMP-activated protein kinase is conserved in the sequence of HMG-CoA reductase from several plant species, and plant HMG-CoA activity is responsive to external stimuli.

Indeed, we have found a calcium-independent protein kinase in both monocotyledons (wheat) and dicotyledons (avocado fruit, pea leaf, carrot cells, oilseed rape seeds, and potato tubers) that shares many of the catalytic and structural properties of the mammalian AMP-activated protein kinase. For example, the plant kinase is itself regulated by phosphorylation being activated by a separate kinase kinase and inactivated by PP2A or PP2C. This represents the first example of a protein kinase cascade to be discovered in higher plants. The only difference so far detected between the plant and animal enzymes is that the plant kinase is <u>not</u> activated by AMP. The plant kinase cascade can regulate mammalian enzymes *in vitro* and in plants appears to be involved in regulating microsomal HMG-CoA reductase and hence isoprenoid biosynthesis which includes such plant secondary metabolites as phytoalexins and other terpenoids important in plant growth and division.

1. MacKintosh, R.W. *et al*, (1990) FEBS Lett. 276, 156-160

CALMODULIN GENE EXPRESSION AND CALCIUM/ CALMODULIN-DEPENDENT PROTEIN KINASE II IN PLANTS

A.S.N. Reddy[1], Z.Q. Wang[1], Y.J. Choi[2], G. An[2], A.J. Czernik[3] & B.W. Poovaiah[1]. [1]Lab. of Plant Mol. Biol. and Physiol, Dept. of Horticulture, [2]Institute of Biological Chemistry, Washington State University, Pullman, WA 99164; [3]Lab. of Mol. And Cell Neurosci, The Rockefeller University, NY 10021

Calmodulin, a ubiquitous calcium-binding protein, plays a central role in signal transduction. In our laboratory we are studying calmodulin gene structure and regulation of its expression. We have cloned and sequenced a potato calmodulin cDNA (pPCM-1) and shown signal-induced changes in the expression of calmodulin (Jena, Reddy and Poovaiah, Proc. Natl. Acad. Sci., Vol. 86, pp. 3644-3648, 1989). To study the consequences of altered levels of calmodulin, we obtained several independent transgenic plants containing calmodulin in sense orientation driven by CaMV 35S promoter. The data on the expression of calmodulin in these transgenic plants will be presented. Using pPCM-1 as a probe, we have isolated several genomic clones from rice and potato. Our studies indicate that there are multiple genes of calmodulin in plants. To study the activity of calmodulin promoter, we fused the 5' region of one of the rice genomic clones to a reporter gene and transformed tobacco. Studies on the expression of the reporter gene expression in transgenic plants indicate that the activity of calmodulin promoter is developmentally regulated. We have also studied the presence of multifunctional calcium/calmodulin-dependent protein kinase (CaM KII) in plants using affinity purified anti-peptide antibodies produced against α-subunit of rat brain CaM KII and specific substrates for CaM KII. The CaM KII antibodies recognized a 56 kDa protein in soluble proteins from different plant tissues. Two other anti-peptide antibodies raised against CaM KII also recognized the same protein. The molecular weight of the cross-reacting protein is similar to the mammalian CaM KII. *In vitro* phosphorylation and immunoprecipitation studies indicate that the protein recognized by the antibody is phosphorylated in a calcium/calmodulin-dependent manner. Soluble extract from corn roots phosphorylated synapsin I, a physiological substrate for mammalian CaM KII and BB40, and this phosphorylation was stimulated by calcium and calmodulin. One dimensional phosphopeptide mapping revealed that the site of enhanced phosphorylation of synapsin I was located with in the 30 kDa fragment that is the known site of phosphorylation by CaM KII. Our results suggest that plants contain a protein kinase with properties similar to multifunctional CaM KII.

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ISOLATION OF A PROTEIN TYROSINE KINASE (APK1) cDNA FROM *ARABIDOPSIS THALIANA*.

Takashi Hirayama and Atsuhiro Oka, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan

We have previously shown that *Arabidopsis thaliana* contains a *CDC2* gene family. One of the member genes, *CDC2a*, is both structurally and functionally similar to the *S. pombe cdc2* gene. *Arabidopsis* p34^{CDC2a} seems to be modulated by the level of its own phosphorylation like *S. pombe* p34^{cdc2} because three out of the four major phosphorylation sites of *S. pombe* and chicken p34^{cdc2} are conserved in *Arabidopsis* p34^{CDC2a}. Despite extensive efforts to search protein kinases which phosphorylate p34^{cdc2}, no candidates have been found; moreover no protein tyrosine kinase has been identified from yeasts and plants except for *wee1* and *YPK1* protein serine/tyrosine kinases, a novel type of kinases. To find plant tyrosine kinases which may relate to modification of the p34^{CDC2a} activity, polymerase-chain-reaction was done on an *Arabidopsis* cDNA mixture using a set of primers each corresponding to an animal tyrosine kinase-specific amino acid sequence motif. One of the PCR fragment products could code for a polypeptide which has amino acid sequences characteristic of both tyrosine and serine/threonine kinase. Thus the translation product of the corresponding gene may belong to the above novel type of protein kinases. To confirm this, an *Arabidopsis* cDNA library was screened with this PCR fragment probe, and a nearly full length cDNA (1.4 kb) was obtained. This sequence contained an open reading frame for a 45-kDa polypeptide (APK1), in which the kinase domain was flanked by non-kinase regions. This kinase domain is similar largely to serine/threonine kinases, but limitedly also to tyrosine kinases, and the NH2-terminal sequence implies myristylation of APK1. No proteins highly homologous to APK1 have been found in databases. To see if APK1 has actually the activity of protein tyrosine kinase, cDNA corresponding to the APK1 kinase domain was expressed in *E. coli* cells with the *tac* promoter and total proteins isolated from IPTG-induced those cells were subjected to Western blotting analysis using anti-phospho tyrosine antibody. The results showed that some *E. coli* proteins were Tyr-phosphorylated by APK1. Thus, APK1 belongs to the novel class of kinases, which phosphorylates tyrosine residues but largely resembles protein serine/threonine kinases. Since another cDNA highly homologous to the above APK1-coding cDNA was also obtained, Arabidopsis appears to have multiple APK1 genes.

CHARACTERIZATION OF A PUTATIVE cAMP DEPENDENT PROTEIN KINASE ASSOCIATED WITH THE ANAEROBIC STRESS RESPONSE OF MAIZE

P. M. Kelley, E. B. Simpson, S. Lal and J. Hyslop; School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, Nebraska 68588-0118

Flooded maize roots experience an energy crisis and must switch from efficient aerobic production of energy to the much less efficient anaerobic fermentation. The key enzyme in this switch is pyruvate decarboxylase (PDC). Initially during anaerobic treatment, roots metabolize pyruvate to lactic acid. As the pH in the cell drops , the enzyme PDC is activated and the cell switches to a mixed fermentation where ethanol is the major product. These changes allow the plant to survive for significant periods (72 hours) of anaerobiosis, avoiding toxic acidosis. Several glycolytic enzymes including glucose phosphate isomerase, aldolase, glyceraldehyde 3-phosphate dehydrogenase, enolase, lactate dehydrogenase, pyruvate decarboxylase, and alcohol dehydrogenase are known to be induced by anaerobiosis.

A putative cAMP dependent protein kinase cDNA has been purified from a cDNA library synthesized from mRNA isolated from maize roots treated 24 hours anaerobically. The clone size is about 1.7 kb. Northern analysis indicates a size of about 1.8 kb and suggest that levels of this mRNA corresponding to this cDNA may increase three-fold during anaerobic treatment. The sequence of the cDNA encodes a protein of approximately 36,500 daltons. The sequence contains all eleven conserved domains found protein kinases. It is very similar in sequence to the yeast TPK2 cAMP dependent protein kinase catalytic subunit. We believe this protein kinase may play an important regulatory function in adapting plant root cells in the transition from an aerobic to an anaerobic state.

GENETIC AND MOLECULAR ANALYSIS OF CONSTITUTIVE ETHYLENE RESPONSE MUTANTS IN *ARABIDOPSIS*

J. Kieber, M. Rothenberg, and J. R. Ecker, Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, PA 19104

We are studying the action of the plant hormone ethylene utilizing the "triple response" of *Arabidopsis thaliana*. The "triple response" in *Arabidopsis* consists of three distinct morphological changes in dark-grown seedlings upon exposure to ethylene: inhibition of hypocotyl and root elongation, radial swelling of the stem and exaggeration of the apical hook. We have isolated a collection of mutants which display the triple response phenotype in the absence of exogenously added ethylene. One class of mutants (Eto) no longer shows the triple response in the presence of inhibitors of ethylene biosynthesis or binding. *eto1* is recessive, and produces at least sixty-fold more ethylene than wild type seedlings. Several independent EMS, x-ray and DEB alleles of *eto1* have been isolated. A dominant Eto mutant, *eto2*, has been identified which also produces elevated levels of ethylene. A second class of constitutive mutants (Ctr) still display the triple response in the presence of ethylene biosynthetic inhibitors, and so are most likely affected at, or downstream of the receptor. *ctr1* mutants are recessive, do not produce elevated levels of ethylene and have a dramatically altered adult morphology. The adult phenotype of *ctr1* can be phenocopied by growth of wild type plants in the presence of 1 ppm ethylene. We have determined the interaction of these genes with other ethylene-related mutants (see also poster by Roman and Ecker).

In order to understand the effects of the *eto1 and ctr1* mutations at the molecular level, we are analyzing the expression of ethylene-regulated cDNA clones in the various constitutive triple response mutants. The transcripts of several ethylene-induced cDNAs are elevated in *ctr1* and *eto1* seedlings and adult plants relative to levels in their wild-type counterparts. We plan to test the effects of inhibitors of ethylene biosynthesis and action on the expression of these cDNAs in mutant and wild-type plants.

BIOACTIVE SUBSTANCES AND THEIR ENVIRONMENTAL REGULATION IN *ARABIDOPSIS THALIANA*

Hai-Hang Li, Koji Hasegawa, Mimako Urashima, Midori Amano, Junya Miztani & Nobuharu Goto*. Mizutani Pl. Ecochemi. Project Res. Devel. Corp. of Japan, 3-1-1, Meguminokita, Eniwa 061-13 Japan. *Bol. Dept, Miyagi Educat. Univ., Sendai 980, Japan.

5 weeks old *Arabidopsis thaliana* (Columbia) seedlings (21g) were extracted with 80% methanol and then extracted with water (water ext. 63.6 mg). The 80% methanol extracts were fractionated into hexane fraction (dr.wt.83.8 mg), EtOAc fraction (dr. wt.68.6 mg), n-butanol fraction (dr.wt.63.6 mg) and water fraction (dr.wt. 452mg). The activities of these fractions on the growth of plants and microorganisms were examined. The water frac. showed strong gowth promotion on etiolated seedlings of all the plant species tested. It increased the elongation of hypocotyls of lettuce, green amaranthu and cucumber by 85-120% and the elongation of coleoptiles of barnyard grass, timothy, barley,oat by 30-50% at the concentrations of 500 or 1000 ppm. Interestingly, the preliminary experiment showed that this fraction promoted the elongation of the floral axis (scapus) of heading *A. thaliana* at 500 ppm concentration. These results suggested that the active substance(s) might involve in the heading after induction of photoperiod in *A.thaliana*.Isolation and identification of the active substances and the researches of their physiological roles are undergoing. EtOAc frac.showed strong growth inhibitions on seed germination, seedling growth of lettuce, green amaranthu, timothy and barnyard grass at 500 or 1000 ppm. EtOAc frac.also showed strong antimicrobiol activities. It inhibited tne growth of *Cladosporium herbarum* at 1 μg on TLC bioassay and inhibited the growth of *E. coli* and *Staphylococus aureus* at 500 μg in paper disk bioassay. At least five antimicrobial spots were found in this fraction on TLC bioassay. The hexane frac. showed inducible activities of antimicrobiols by the treatments of $CuCl_2$ and injure.The activities of antimicrobiols in both hexane and EtOAc fractions were different according to the growth stages of A. thaliana. Separation of these substances, their regulation of synthesis and the molecular mechanisms of plant responses to environmental signals or stress (plant pathogens, heavy irons, injure et al.) are under studying.

AGL3, A MADS-BOX GENE WITH NON-FLOWER-SPECIFIC EXPRESSION

Hong Ma and Hai Huang, Cold Spring Harbor Lab., Cold Spring Harbor, NY 11724.

The *Arabidopsis* floral homeotic *AG* gene was previously found to be a member of a gene family, whose protein products share a sequence motif with known transcription factors SRF and MCM1 from humans and yeast, respectively. This motif, which is also been found in the *Antirrhinum* floral homeotic gene *Deficiens A*, has been called the MADS box. We have previously identified six additional members of *Arabidopsis* MADS-box gene family, *AGL1-AGL6*. Among these, all but *AGL3* are expressed preferentially in flowers. A partial *AGL3* cDNA was isolated, and RNA blot using this cDNA as probe indicated that *AGL3* is expressed not only in flowers, but also in stems and leaves. We have now isolated and analyzed full length cDNA as well as genomic sequences of *AGL3*. The sequence information confirmed our prediction that *AGL3* share the same general structure with the other members of the *Arabidopsis* MADS-box gene family. Furthermore, the *AGL3* sequence has some additional features that are much less pronounced in other plant MADS-box genes. The wider expression range suggests that *AGL3* may have a more general function than its floral counterparts. The understanding of the role of *AGL3* awaits future experiments.

ISOLATION AND ANALYSIS OF A TOMATO G PROTEIN α SUBUNIT GENE

Hong Ma[1], Martin F. Yanofsky[2], and Hai Huang,[1] [1]Cold Spring Harbor Lab., Cold Spring Harbor, NY 11724; [2]Department of Biology, University of California at San Diego, La Jolla, CA 92093.

G proteins are important signal transducers in eukaryotes. To study G protein function in plant signal transduction processes, we have taken the approach of isolating and analyzing G protein genes from plants. We have previously isolated a Gα gene (*GPA1*) from *Arabidopsis*. In addition to similarity to G protein concensus regions, the predicted *GPA1* gene product (GPα1) also has some differences from other known G proteins in the consensus regions. To determine whether these differences are shared by other plant G proteins, and therefore may suggest functional differences, we have attempted to isolate G protein genes from other plants. Using an *Arabidopsis GPA1* cDNA as a DNA probe, We have isolated cDNA's for a Gα gene (*TGA1*) from tomato (*Lycopersicon esculentum*, cv VF36). Hybridizations of tomato genomic DNA with a *TGA1* cDNA indicate that it is a single-copy gene. The sequences of the cDNA's indicate that the deduced amino acid sequence of the gene product (TGα1) has 384 amino acid residues (44 906 Da). The predicted TGα1 protein is highly homologous to the *Arabidopsis* GPα1 protein (85% identity), and exhibits similarity to all known G protein α subunits, with about 34% identical and 59% similar (identical and conservative replacements) amino acid to mammalian transducins. Furthermore, it has all of the consensus regions for a GTP-binding protein. Finally, some of the variations of the consensus sequences in the GPα1 protein are also found in the TGα1 sequence.

PROTEIN PHOSPHATASES IN PLANT CELL SIGNALLING

Carol MacKintosh, MRC Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Scotland, U.K.

About two years ago I became intrigued to know whether plant responses to external stimuli are regulated by protein phosphatases similar to those we study in the context of hormonal regulation of mammalian metabolism. Upon investigation, surprisingly high levels of protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein phosphatase 2C (PP2C) were found in a variety of extracts from both mono- and dicotyledonous plants. Moreover their properties (substrate specificity, sensitivity to inhibitors) were remarkably similar to those of the corresponding enzymes in mammalian cells.

These observations led me to study their physiological roles in plants. For example, we have now shown that PP2A is responsible for the light-activation (dephosphorylation) of sucrose-phosphate synthase in spinach leaves [1] and inactivation of quinate dehydrogenase in carrot cells [2]. However, chloroplast phosphoproteins, such as the thylakoid light-harvesting proteins, appear to be regulated by enzymes with different characteristics to PP1, PP2A and PP2C [2].

Although these results demonstrate roles for PP2A in the regulation of cytosolic metabolism, it is likely that, as in animal cells, PP1 and/or PP2A will turn out to be involved in regulating many other plant functions such as gene expression and cell cycle. More recently, we have found that the potent inhibitors of PP1 and PP2A, okadaic acid, acanthafolicin, tautomycin and microcystins [eg. 3] (but not their non-inhibitory analogues) can induce the characteristic defense responses of soybean plants to fungal attack; namely induction of phenylalanine ammonia-lyase and production of phytoalexic isoflavanoids (C.M., Gary Lyon and Robert MacKintosh, unpublished). In the course of these experiments we have also shown that radiolabelled microcystin binds exclusively to soybean proteins of 37kDa (the molecular weight expected for PP1 and PP2A catalytic subunits) on SDS-PAGE. The roles of PP1 and PP2A in the induction of isoflavanoid biosynthesis are being investigated at the level of gene expression and control of enzyme activity.

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LIGHT-MEDIATED TRANSCRIPTIONAL REPRESSION OF ASPARAGINE SYNTHETASE GENES IN PLANTS.

Susan J. Martino-Catt, Fong-Ying Tsai and Gloria M. Coruzzi, The Rockefeller University, New York, New York 10021-6399.

The studies in our laboratory are aimed at defining the environmental and developmental factors that regulate the expression of genes encoding amino acid biosynthetic enzymes in plants. Asparagine synthetase (AS) gene expression has been shown to be negatively regulated by light in both pea and transgenic tobacco plants. Light represses the transcription of AS fairly rapidly (within 20 min of exposure to light). The negative effect of light on AS gene expression is a phytochrome-mediated response. Unlike most light-regulated genes, AS expression is also dramatically negatively regulated by light in non-photosynthetic organs, such as roots. Experiments designed to identify the factors which are necessary for light-repression of AS transcription are currently being performed using 5' deletion analysis of the AS promoter in transgenic tobacco. In contrast to AS expression, another gene involved in nitrogen assimilation, glutamine synthetase (GS), is positively regulated by light. Thus, the levels of GS and AS expression are coordinately regulated by light via phytochrome in opposite fashions. One possible model for this differential regulation by light invokes a transcriptional activator/repressor protein which activates GS expression and represses AS expression in response to light. Our characterization of the trans-acting protein factors that interact with cis-acting DNA elements of the AS and GS promoters will allow us to test our proposed activator/repressor protein model. These studies will ultimately uncover the underlying mechanisms that regulate the expression of genes involved in nitrogen assimilation into amino acids in plants.

EVIDENCE FOR C-DI-GMP DEPENDENT CELLULOSE SYNTHASE AND DI-GUANYLATE CYCLASE IN PLANTS

R. Mayer[1], Y. Amor[2], G. Volman[1], L. Rivas[1], H. Weinhouse[1], D. Amikam[1], D. Delmer[2], and M. Benziman[1], Departments of Biological Chemistry[1] and Botany[2], Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel.

An efficient demonstration of plant cellulose synthase (CS) activity in vitro from plants has not yet been realized. We have taken therefore, a different approach towards identifying polypeptides involved in this process, employing as a model the well characterized Acetobacter xylinum cellulose synthesizing system. Of the four genes recently discovered to constitute the operon for bacterial cellulose synthesis (bcs) in A. xylinum, bcsB encodes the catalytic subunit of CS which directly interacts with its highly specific activator, cyclic di-guanylic acid (c-di-GMP). Polypeptides immunologically related to the bcsB gene product were detected in mung bean, barley, pea and cotton extracts using antibodies against the bcsB-gene product. The cross reacting polypeptides from cotton (83 and 48 kD) specifically bind ^{32}P-c-di-GMP with high affinity, and binding is correlated with the developmentally dependent rate of cellulose synthesis in vivo. The N-terminal sequence of the 48 kD polypeptide also indicates relatedness to the bacterial CS. The homology observed at the polypeptide level between bacterial and plant CS's, has been extended to the genetic level by high stringency hybridization of genomic DNA and mRNA from cotton, pea and barley with a DNA fragment derived from the bcsB gene. A polypeptide (58kD) immunologically related to the bacterial diguanylate cyclase was detected in mung-bean extracts using antibodies raised against the purified enzyme. Our results suggest a high degree of homology between the CS's of diverse organisms and raises the possibility that a common mechanism for cellulose biogenesis and regulation is employed throughout nature.

IMAGING THE ABA INDUCED INCREASE IN FREE CYTOSOLIC CALCIUM IN STOMATAL GUARD CELLS.

M R McAinsh[1], C Brownlee[2] & A M Hetherington[1], [1] Division of Biology, Lancaster University, Lancaster, LA1 4YQ, UK. [2]Marine Biological Association, Plymouth, PL1 2PB, UK.

Stomatal guard cells constitute an attractive system for the study of the early events in signal transduction. They respond in a readily quantifiable manner to a range of both environmental (light & CO_2) and hormonal (ABA, auxins & cytokinins) signals. Alterations in the aperture of the stomatal pore are achieved through changes in guard cell turgor which are largely driven by fluxes of K^+ salt. We have suggested that one of the primary events in the ABA induced closure response is an increase in the concentration of free cytosolic calcium ions ($[Ca^{2+}]_c$) (see Mansfield, Hetherington & Atkinson 1990, Annu Rev Pl Physiol Pl Molec Biol 41:55) which then triggers the machinery responsible for loss of turgor (Schroeder & Thuleau 1991 The Plant Cell 3:555; Hetherington & Quatrano 1991 New Phytologist, in press).

Using the dual excitation fluorescent calcium indicator Fura-2 we demonstrated that ABA induces increases in $[Ca^{2+}]_c$ in single guard cells (McAinsh, Brownlee & Hetherington 1990 Nature 343:186). Patch clamp and Fura-2 photometry studies by Schroeder & Hagiwara 1990 (PNAS 87:9305) also demonstrated that ABA induces increases in $[Ca^{2+}]_c$ and suggested that this increase may result from activation of a non selective Ca permeable channel. A recent report (Gilroy et al 1991, The Plant Cell 3:333) describes ABA induced increase in 40% of guard cells using photometry but failed to image this increase. Here we report the results of experiments using the dual emission indicator Indo-1. Our data shows variable ABA induced increases in $[Ca^{2+}]_c$ using ratio photometry and with Joyce-Loebl image analysis soft and hard we have captured images whch demonstrate ABA induced increases in guard cell $[Ca2+]_c$. These recent results will be discussed.

COLD ACCLIMATION-SPECIFIC (CAS) PHOSPHORYLATION OF PROTEINS REQUIRED FOR ACCUMULATION OF CAS TRANSCRIPTS, AND DEVELOPMENT OF FREEZING TOLERANCE IN ALFALFA

A. F. Monroy[1], F. Sarhan[2] and R. S. Dhindsa[1], [1]Department of Biology, McGill University, Montreal, Québec H3A 1B1, [2]Dept. de Sciences Biologiques, Université du Québec à Montréal, Montréal, Québec, Canada.

Changes in patterns of *in vivo* phosphorylation status of proteins during cold acclimation and subsequent deacclimation of suspension cultures of the freezing tolerant cultivar of alfalfa (*Medicago falcata*, cv *Anik*) were examined. Non-acclimated cells, cells cold-acclimated at 4° C, and deacclimated cells were pulse-labeled with ^{32}P-orthophosphate. Their proteins were extracted and analyzed by 2-D PAGE and autoradiography. Results indicated that during 24 h of cold acclimation, phosphorylation of a large number of proteins decreased while that of about 15 polypeptides increased. Three or 4 proteins were specifically phosphorylated within 30 min of cold acclimation and before CAS transcripts were detectable. All these changes were rapidly reversed on deacclimation (return to room temperature).

CAS phosphorylation was independent of protein synthesis. Several proteins specifically phosphorylated on cold acclimation were soluble. Based on eletrotransfer of proteins from gels to nitrocellulose membrane through NP-40 (a non-ionic detergent) containing g l indicated that one of the proteins specifically phosphorylated on cold acclimation, and having a relative mass of 63 kD, is likely to be a membrane protein. Treatment of cells with various kinase inhibitors and calmodulin antagonists during cold acclimation prevented CAS phosphorylation, and the treated cells did not accumulate CAS transcripts as determined by northern analysis using CAS cDNAs as probes. Significantly, these cells also lost their ability to cold acclimate.

It is, therefore, concluded that low temperature-induced phosphorylation of some pre-existing proteins is essential for triggering CAS gene expression and development of freezing tolerance in alfalfa.

POLYPHOSPHOINOSITIDES IN CARNATION FLOWER PETALS

T. Munnik[1], A. Musgrave[2], W.M.F. Jongen[1], and T. de Vrije[1]
[1]ATO-DLO Agrotechnology, P.O. Box 17, 6700 AA Wageningen, The Netherlands.[2]Department of Molecular Cell Biology, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

The phytohormone ethylene plays a key role in carnation petal senescence. Stress conditions or exogenously applied ethylene stimulate the autocatalytic ethylene production and induce wilting, while inhibitors of the ethylene-biosynthesis or -action are capable of delaying senescence. The molecular mechanism - how the ethylene signal is transduced and converted into responses leading to senescence is still unknown. We are investigating whether the $IP_3\backslash Ca^{2+}$ second messenger system is involved in this ethylene induced senescence. Our first step in this study was to identify the lipid precursors PIP and PIP_2 in carnation flower petals.

Carnation petals (*Dianthus caryophyllus* cv. White Sim) were radiolabeled with [^{32}P] orthophosphate and the lipids extracted and analysed by thin layer chromatography (TLC). Phospholipids were identified by comigration and mixing the extract with known standards, using two different TLC solvents. Preliminary results showed that, when labeled overnight (17h), most [^{32}P] label was incorporated into the structural phospholipids PC, PE, PG, and PI. A little was incorporated into PIP but PIP_2 could not be detected. In contrast, when labeling was followed upto 120 min, relatively high levels of [^{32}P] were quickly incorporated into PIP and PIP_2. Incorporation into the structural lipids was relatively slow and continued for many hours. These results imply that the polyphosphoinositides are turning over rapidly, as one would expect of signal precursors. The specific labeling of PIP and PIP_2 will enable us to study their turnover and their role in mediating ethylene induced senescence.

UV-B INDUCED CHANGES IN ARABIDOPSIS GENE EXPRESSION AND METABOLISM. Tsai-Mei Ou-Lee, Jiayang Li, Suzanne Fellows, Richard M. Raba, Robert G. Amundson and Robert L. Last. Boyce Thompson Institute for Plant Research, Ithaca, NY 14853-1801

We are pursuing a combined molecular biological, biochemical and genetic approach to understand how plants respond to ultraviolet-B (UV-B: 280-320 nm) radiation. Changes in the composition of the stratosphere are projected to cause significant increases in the amount of UV-B radiation reaching the biosphere. Plants are thought to synthesize light absorbing pigments, including flavonoids, as an adaptive mechanism to protect against UV-B damage to macromolecules. We are using the plant *Arabidopsis thaliana* to test the efficacy of flavonoid pathway pigments in protecting plants from UV-B damage and to understand the genetic regulation of the biosynthesis of these pigments in response to UV-B radiation. Preliminary results show that mRNA levels for the flavonoid biosynthetic enzymes phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) increased several-fold within hours following a shift of wild-type plants to different levels of UV-B. The growth rate and leaf morphology of plants were dramatically altered under these UV-B conditions. Data will be presented documenting physiological changes and flavonoid pathway pigment accumulation in Arabidopsis plants grown under UV-B light. We will also compare the UV-B response of wild-type and flavonoid-deficient *tt* (transparent testa) mutant plants.

GENETIC AND PHYSIOLOGIC ANALYSIS OF HORMONAL SIGNALS IN THE *Arabidopsis thaliana* TRIPLE RESPONSE

G. Roman, A. Wollenberg, and J.R. Ecker, Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, PA 19104

We are investigating the development of the *Arabidopsis thaliana* triple response through mutant analysis. In the presence of ethylene, etiolated *Arabidopsis* seedlings undergo drastic morphological changes. This seedling phenotype consists of an exaggerated apical hook, radial swelling of the hypocotyl, and an inhibition of root and hypocotyl elongation. There are two classes of mutants examined which are deficient in aspects of the triple response. The first class of mutants fail to form aspects of the triple response in the presence of ethylene. Two ethylene insensitive (*ein*) loci have been identified which do not form any of the triple response features. We have also identified a tissue specific mutation which has an elongated root in the presence of ethylene. This mutant, designated *eir* (ethylene insensitive root), is recessive and genetically distinguishable from the *ein* and *aux1* mutations. Another class of mutants, designated hookless (*hls*), are deficient in the formation of the apical hook. This deficiency can be phenocopied by NPA, an inhibitor of auxin transport or by high concentrations of 2,4-D.

To investigate the role of these genes during the development of the triple response, genetic interactions were sought with other genes affecting the ethylene seedling response. The *hls1* loci is epistatic to *ein2*, and is co-expressed with *eir1*. Both *ein* loci are epistatic to the ethylene overproducer, *eto1*. A mutation which confers a constitutive triple response (*ctr*) is epistatic to mutations at both *ein1* and *ein2* loci.

FUNCTIONAL ANALYSIS OF VP1.

Leonard Rosenkrans, Vimla Vasil, Indra K. Vasil and Donald R. McCarty. Vegetable Crops Dept. University of Florida, Gainesville, FL. 32611.

The VP1 protein controls expression of a number of genes which are required for normal processes involved with kernel maturation as well as anthocyanin accumulation in the aleurone. Previous work in this laboratory has demonstrated that VP1 regulates expression of two genes which represent examples of the two processes just mentioned: *C1*, which is itself a transcriptional regulator of structural genes in the anthocyanin biosynthetic pathway, and Em, a member of the LEA (late embryogenesis abundant) class of seed proteins. VP1 been shown to exhibit the characteristics of a transcriptional activator, although no sequence similarities to known transcription factors have been found. To address the functional significance of VP1 protein sequences, a series of terminal and internal deletion mutants were made in the VP1 protein and tested for ability to activate Em and *C1* GUS reporter constructs in maize protoplasts. Functional domains which have been identified include an acidic region near the amino terminus that is required for activation of both reporter constructs, and a C-terminal sequence that is essential for activation of *C1*. A mutant lacking 40% of the C-terminal sequence remained a weak activator of Em.

CHARACTERIZATION OF A SHOOT SPECIFIC, GA_3- AND ABA-REGULATED GENE FROM TOMATO

L. Shi, R. Gast, M. Gopalraj, and N. Olszewski, Department of Plant Biology and Plant Molecular Genetics Institute, University of Minnesota, St. Paul, MN 55108.

We have characterized a tomato gene (GAST1) that encodes a RNA whose abundance increases >20-fold in shoots of the GA-deficient *gib1* mutant following spraying with GA_3. An increase in GAST1 RNA levels is detectable 2 hrs after treatment and levels continue to increase for at least an additional 10 hrs. Between 12 and 24 hrs following treatment, the amount of the GAST1 RNA begins to decline and at 48 hrs the level is nearly equivalent to that of water treated control plants. Nuclear runoff analysis indicates that 8 hrs after treatment with GA_3, transcription of the GAST1 gene has increased only 3 fold, suggesting that GA acts both transcriptionally and post-transcriptionally. ABA partially inhibits the GA-mediated increase in GAST1 RNA abundance while ethephon, kinetin, and 2,4-D have little effect. GAST1 RNA is detectable in untreated leaves, stems, petioles and flowers, but not in roots. The GAST1 gene encodes a 0.7 kb transcript. The sequence of GAST1 cDNA and genomic clones indicates that the gene is interrupted by three introns and potentially encodes a 112 amino acid protein of unknown function.

TRANSPOSON TAGGING OF THE *TEOSINTE BRANCHED* LOCUS IN MAIZE

Patricia S. Springer and Jeffrey L. Bennetzen, Department of Biological Sciences, Purdue University, W. Lafayette, IN.

The *teosinte branched* (*Tb*) locus of maize specifies a switch between vegetative and gametic meristem development. Mutations at *tb* convert ear shoots into vegetative branches that terminate in tassels. We have begun a transposon mutagenesis program for *tb*, as a first step toward the eventual cloning of this homeotic locus. As an aid to our tagging experiments, we have initiated studies to precisely place *Tb* on the maize genetic map. We have obtained a number of recombinants between *tb* and *Adh1* which indicate that the *Tb* locus is approximately 4.5 cM proximal to *Adh1*. These recombinants have been used to generate a fine structure map containing RFLP markers in the chromosomal region surrounding *Tb*.

Two independent *teosinte branched* mutations, one a null and one that appears to be a partial inactivation, have been identified in testcross progeny of a *Mutator* transposable element stock. To date, we have not been able to identify a *Mu* element that is present at the *tb* locus, although a *Mu7* element was found to be closely linked to one of the mutations. The fine structure mapping stocks were used to determine that a *Mu7* element that co-segregated with the *tb* locus in 94 out of 94 testcross progeny was, in fact, approximately 2 cM distal to *tb*. We are also screening for *tb* mutations in a line containing a *Ds2* transposable element that is linked to *Tb*. We plan to study the segregation of additional *Mu* element subfamilies in our two *Mutator*-associated mutations at *tb*, and hope to perform analogous studies on any mutations that may be identified in our *Ds2* stocks. We will then clone any element that shows very tight co-segregation with the *tb* phenotype and use the existence of the other mutant alleles at *Tb*, and any germinal instability in the *Ds2* lines, to confirm that we have a *tb* clone.

CIS-ACTING ELEMENTS CONFERING TRANSCRIPTIONAL ACTIVATION DURING THE DEVELOPMENT OF LATE BLIGHT DISEASE IN POTATO

G. Strittmatter, N. Martini, C. Koch, B. Hunkirchen and M. Egen; Max-Planck-Institut für Züchtungsforschung, Carl von Linné Weg 10, D-5000 Köln 30, FRG.

Infection of potato with Phytophthora infestans (causal agent of late blight disease) results in cultivar/race-specific interactions (compatible or incompatible) in which resistant cultivars reveal a very rapid hypersensitive response. We have isolated genes which are transcriptionally activated at early time points after inoculation of leaves with fungal spores; two of them show a differential expression pattern in compatible and incompatible interactions. The transcription of a third gene (prp1) encoding a heat shock protein like product is induced in both types of interaction; most of the abiotic stresses tested, so far, did not lead to an increase of the prp1-mRNA level. Cis-acting elements confering rapid transcriptional activation of this gene in compatible host/pathogen-interactions were identified by in vitro methods (gel retardation, DNase I footprinting) and functional analysis in transgenic potatoes. Our results indicate that negative elements are involved in the transcriptional regulation of this gene. None of the elements corresponds to promoter sequences known from defense related genes of other plant species.
These signal elements can be used to drive the infection-dependent synthesis of products that interfere with processes necessary for the establishment of compatible host/pathogen-interactions and thereby prevent the development of disease symptoms.

MOLECULER MARKERS OF ORGANOGENIC DIFFERENTIATION IN TOBACCO THIN CELL LAYER

K.Tran Thanh Van, L.Richard and C.Gendy, Laboratoire de Physiologie Vegetale, Centre National de la Recherche Scientifique, Gif sur Yvette, France.

Tobacco Thin Cell Layer (TCL) systems respond to hormonal signals by differentiating specific organogenic patterns. Flowers, vegetative buds or roots are differentiated directly, without intermediate callus formation, from the subepidermal layer of the 3 to 6 cell layers of the TCL system upon reception of kinetin or dihydrozeatin for flower differentiation, of zeatin for vegetative bud differentiation or of indolyl butyric acid for root differentiation.Thaumatin-like (Thl) proteins of 46, 41 and 27 kD were exclusively detected in floral TCL. They were localized in the cytoplasm and were different from the ones synthesized under stress conditions and accumulated in the vacuole in tobacco cell suspension or root. Similar Thl proteins of 42 and 27 kD were detected in floral meristems in vivo. The purification of these Thl proteins and the identification of the corresponding genes will allow the study of their possible function in floral differentiation. Another significant molecular marker of organogenic differentiation is cytokinin oxydase based on data related to cytokinin catabolism of TCL under specific organogenic induction. These data will be discussed.

RHIZOBIUM MELILOTI EXTRACELLULAR SYMBIOTIC SIGNALS: STRUCTURE AND ACTIVITY

G. Truchet, P. Roche*, P. Lerouge*, F. Debellé, C. Faucher, J. Vasse, S. Camut, F. Maillet, F. de Billy, P. Gamas, M. Cock, J. Cullimore, J.C. Promé* and J. Denarié.

Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, CNRS-INRA, 31326 Castanet-Tolosan Cedex, France; *Centre de Recherche de Biochimie et de Génétique Cellulaire, CNRS LP8201, 31062 Toulouse Cedex, France.

Rhizobium meliloti is a symbiotic bacterium which elicits the morphogenesis of specific organs, the nitrogen fixing nodules, on the roots of specific legumes (eg *Medicago sativa* and *M. truncatula*). This process requires a series of *Rhizobium* nodulation (*nod*) genes: some (*nod A,B,C*) are common to all *Rhizobia* whereas others, such as *nodP*, *nodQ*, *nodH* are host-specific.

The early steps in the establishment of the symbiosis involve root hair deformations and initiation of cortical cell divisions leading to formation of nodule primordia. Both events can be elicited in *M. sativa* and *M. truncatula* by molecules, called NodRM factors, purified from the culture supernatant of *R. meliloti* genetically engineered to overproduce these factors. Moreover genuine nodules can also be elicited by NodRM factors in *M. sativa* at concentrations in the micro-nanomolar range.

NodRm factors have been identified as acylated N-acetyl-D-glucosamine oligosaccharides. Their production is strictly dependent upon *nodABC* genes. Structural analysis of Nod factors synthesised by *nodH*, *nodP* and *nodQ* mutants of *R. meliloti* has shown that these genes are responsible for sulphation of lipo-oligosaccharide precursors. An absolute correlation is found between the production of sulphated or non-sulphated Nod factors and the ability to infect two different host plants (alfalfa and vetch respectively). Chemical modification such as reduction of the terminal sugar or hydrogenation of the acyl chain result in a strong decrease in the morphogenic activity of Nod factors.

CALCIUM PERTURBATION AND HEAT SHOCK STIMULATED SYNTHESIS OF SPECIFIC PROTEINS IN BRASSICA NAPUS

F-S. Wu[1] and T-Y. Feng[2], [1]Department of Biology, Virginia Commonwealth University, Richmond, Virginia; [2]Institute of Botany, Academia Sinica, Taipei, Taiwan ROC.

We investigated the stimulated synthesis of several specific proteins in Brassica cells treated with the calcium chelator (EGTA), or the calcium ionophore A23187 which perturbs the intracellular calcium balance. EGTA treatment resulted in an increased synthesis if 88, 61, 55, and 45 kD proteins, and A23187 stimulated synthesis of 88, 55, 45 kD proteins in excised hypocotyl sections. However, only synthesis of the 45 kD protein was enhanced in young, etiolated seedlings. The potassium ionophore, valinomycin, did not stimulate synthesis of these proteins. 2-D PAGE demonstrated the enhanced synthesis of six proteins, including the 88, 55, and 65 kD proteins, in A23187-treated hypocotyl sections.

Heat shock at 38.5°C stimulated synthesis of 105, 84, 70 and a group of 16-28 kD proteins regardless of whether or not cells were pretreated with EGTA, A23187, or valinomycin. The 2-D PAGE revealed that in A23187 treated cekks at keast seven abd nine proteins were present in greater amounts at 25°C and 38.5°C, respectively. Heat shock did not prevent normal cellular protein synthesis, but it did nullify the stimulating effects of EGTA and A23187 on the synthesis of specific proteins. In addition, the overall protein synthesis of EGTA treated cells was more sensitive to inhibition under heat shock condition than control cells. Both EGTA and EDTA at 5 mM inhibited protein synthesis in hypocotyl sections at 25°C and 38.5°C, suggesting that the cytosolic divalent cations are essential for protein synthesis. High concentrations of either A23187 or EGTA also inhibit protein synthesis, and they act synergistically to enhance the inhibition. It can be concluded that specific proteins can be induced when plant cells are subjected to calcium perturbation, and that synthesis of heat shock- and A23187-stimulated proteins is controlled by separate mechanisms, both of which are sensitive to the balance of intracellular calcium ions.

SYSTEMIC INDUCTION OF PHOSPHORYLATION OF TWO PROTEINS BY LOCALIZED INFECTION OF TOBACCO MOSAIC VIRUS IN TOBACCO

X. S. Ye, S. Avdiushko, S. Q. Pan, and J. Kuc. Department of Plant Pathology, University of Kentucky, Lexington, Ky 40546.

Inoculation of 3 lower leaves of 2.5 month-old tobacco plants carrying the *N*-gene for the hypersensitive reaction to TMV with TMV or stem injection with *Peronospora tabacina* causes localized necrosis at the inoculation site and induces systemic resistance and a systemic accumulation of defense-related proteins. The systemic responses to a localized infection implicate the presence of a signal(s) which is produced at the site of infection, translocated systemically and amplified through signal transduction. A systemic transmissible signal(s) has been demonstrated in plants stem-injected with *P. tabacina* by phloem girdling and grafting experiments. *In vitro* phosphorylation of two proteins (designated as pp29 and pp51 according to their molecular weight) in uninfected leaves of plants induced with TMV was associated with induced systemic resistance. Plasma membranes (PM) were isolated from uninfected leaves of control and TMV-induced plants by aqueous two phase partitioning. Incorporation of ^{32}P from ATP into PM proteins was about 50 times higher than into soluble proteins. Protein kinase activity in PM of the TMV-induced plants was significantly higher than in PM of the control plants. Calcium markedly enhanced PM protein kinase activities. The soluble protein (pp51) is cytoplasmic and pp29 is a PM protein. PM protein phosphorylation was mostly calcium but not calmodulin dependent. Phosphorylation of pp60 was completely inhibited , while pp58 was highly phosphorylated in the presence of free Ca^{2+}. Spermidine strongly and spermine weakly enhanced phosphorylation of two PM proteins, pp34 and pp60. Putrescine and cadaverine had little effect on protein phosphorylation in general. The promotion by Ca^{2+} and spermidine of protein phosphorylation was additive, except for pp60. Inositol trisphosphate reduced this effect of Ca^{2+} and spermidine on protein phosphorylation. Phorbol esters and various phospholipids had no effect on protein phosphorylation, but a protein kinase C inhibitor [1-(-5-isoquinolinylsulfonyl)-2-methylpiperrazine] strongly inhibited the phosphorylation of pp60 and pp106 at micromolar concentrations. The results of this study indicate a fine regulation of protein kinase activities in plasma membranes by protein kinase effectors. The role of protein kinases, inducible phosphorylation of proteins and second messengers in transmembrane signaling in induced systemic resistance will be discussed.

ATP-DEPENDENT AND CELL-CONTACT DEPENDENT REGULATION OF FLAGELLAR ADENYLYL CYCLASE IN GAMETES OF THE GREEN ALGA, CHLAMYDOMONAS.

Y.H. Zhang and W.J. Snell, Department of Cell Biology and Neuroscience University of Texas, Southwestern Medical School, Dallas, TX 75235.

During fertilization in the unicellular, biflagellated, green alga *Chlamydomonas reinhardtii*, mt^+ and mt^- gametes adhere to each other via adhesion molecules (agglutinins) on the surfaces of their flagella. The interaction between agglutinins leads to an immediate, 10-fold increase in intracellular cyclic AMP (Pijst et al.,1984,FEBS Let.,174:132;Pasquale & Goodenough,1987,JCB 105:2279), inducing several events associated with fertilization including secretion of a serine protease responsible for wall degradation, and movement of agglutinins from the cell body to the flagella. To identify the mechanism of this cell-contact mediated signal transduction our laboratory has studied regulation of the flagellar adenylyl cyclase (AC). The AC is activated 3-fold when isolated mt^+ and mt^- flagella adhere to each other via their agglutinins *in vitro*. We and others have found no evidence for regulation of the AC by G proteins. Rather our evidence indicates that the AC is regulated by phosphorylation. The flagellar AC was inhibited by prior incubation at or below 30°C. The inhibition was ATP-dependent, did not occur with the ATP analog 5-adenylylimidodiphosphate (App(NH)p), and was blocked by addition of the protein kinase inhibitor staurosporine (2 μM). Incubation of the flagella at 45°C in the absence of ATP or incubation at lower temperatures in the presence of App(NH)p or staurosporine prevented the inhibition and caused an increase in adenylyl cyclase activity. In a 10 min assay, flagella that had been pretreated at 45°C for 12 min showed 11-fold greater activity than non-pretreated flagella. The heat-induced activation of adenylyl cyclase was prevented when the pretreatment was carried out in the presence of ATP plus an ATP-regenerating system. If the ATP-treatment was carried out in the absence of an ATP-regenerating system, adenylyl cyclase activity was initially inhibited, but ultimately recovered. The data suggest that the flagellar adenylyl cyclase in *Chlamydomonas* gametes is inhibited by phosphorylation and stimulated by dephosphorylation. This mechanism for regulating adenylyl cyclase may underlie the rapid increase in cyclic AMP that is induced by flagellar adhesion during fertilization in *Chlamydomonas*.

HOMEOTIC GENES CONTROLLING FLOWER DEVELOPMENT IN *ANTIRRHINUM*

Enrico Coen, Sandra Doyle, Robert Elliott, José Romero, Desmond Bradley, Sabine Hantke, Rüdiger Simon, Da Luo and Rosemary Carpenter. John Innes Institute, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.

Three classes of homeotic floral mutations have been defined in *Antirrhinum*, depending on whether they affect (1) the overall fate of the floral meristem (2) the identity and sometimes also the number of whorls in the flower (3) the assymetry of the flower. We have carried out a molecular and genetic analysis of these mutants in order to understand how genes interact to control the fate of meristems. An early-acting class(1) gene, *floricaula* (*flo*), is required for the initiation of the floral programme: in the *flo* mutant indeterminate shoots grow in place of flowers. The *flo* transcript is absent in vegetative apices and is detected 2 days following floral induction. The *flo* gene is transiently expressed in bract, sepal, petal and carpel primordia but no expression is detected in stamen primordia. Several class(2) genes have also been isolated from *Antirrhinum*. We will discuss models for how class(1) and class(2) genes interact based on studies of the timing and spatial patterns of gene expression together with phenotypic analysis of single and double mutants.

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FUNCTIONAL DOMAINS AND EXPRESSION OF *DEFICIENS*, A HOMEOTIC GENE WHICH CONTROLS PETAL AND STAMEN ORGANOGENESIS IN *ANTIRRHINUM MAJUS*

Zs. Schwarz-Sommer, P. Huijser, W.-E. Lönnig, H. Saedler and H. Sommer; Max-Planck-Institut für Züchtungsforschung, 5000 Köln 30, FRG

Floral organogenesis in *Antirrhinum majus* seems to be controlled by a set of homeotic genes. We have isolated some of these genes and characterized their structures and expression patterns. Mutations in *deficiens* (*def*) and *globosa* (*glo*) lead to homeotic transformation of petals to sepals and stamens to carpels. Accordingly, *def* and *glo* expression in the wild type is elevated in these organs. Analysis of expression patterns *in situ* revealed common features but also subtile differences in temporal and spatial expression patterns of *def* and *glo*.

The proteins encoded by these genes are putative transcription factors. They have a conserved domain, the MADS-box, at the N-terminus in common that displays extensive homology to two known transcription factors: SRF of mammals and MCM1 of yeast. The MADS-box was also found in twelve additional genes the majority of which is expressed only in floral organs, in a specific manner. We speculate that specific combinations of these MADS-box genes, expressed in the respective organs, specify organ identity in the process of organogenesis.

We utilized morphologically distinct alleles of *def* to identify functionally important domains of the DEF A protein. The study of such morphoalleles provides the proof that integrity of the MADS-box is absolutely necessary for *def* function and also indicates the presence of further functional domains. Correlation of the altered gene structure with its altered expression allows deeper insight into the molecular mechanisms which control *def*, and by which *def* controls floral organogenesis. In particular, we address the question of interaction between *def* and *glo* in the control of petal and stamen organogenesis.

THREONINE PHOSPHORYLATION OF DEFICIENS IS REQUIRED FOR BINDING TO ITS TARGET DNA

Anil Kush[1,2], Tim Conner[1], Hiroshi Takatsuji[1], Jorg Stockhaus[1] and Nam-Hai Chua[1], [1]Laboratory of Plant Molecular Biology, Rockefeller University, New York, USA and [2]Institute of Molecular and Cell Biology, National University of Singapore, Republic of Singapore.

The product (DEF) of the homeotic gene, deficiens, regulates organ identity in flower development. We have cloned the deficiens gene from Petunia hybrida based on its homology with the deficiens gene of Antirrhinum in the region encoding the putative DNA-binding domain. We found that the deficiens gene product P-DEF1 can bind to specific sites in the 5' upstream region of chalcone synthase and chalcone isomerase genes. However, the binding is dependent upon phosphorylation of threonine-20 in P-DEF1. The DEF-kinase is present in petal extract, but its activity is undetectable in nuclear extracts prepared from sepals or leaves. The enzyme uses ATP and requires calcium and calmodulin for its activity. Our results suggest that the activity of DEF can be reversibly modified by phosphorylation.

MOLECULAR GENETIC ANALYSIS OF THE INTERACTION OF AGAMOUS MADS DOMAIN OF *A. THALIANA* WITH DNA

Hideaki Shiraishi[1] and Yoshiro Shimura,[1,2] [1]National Institute for Basic Biology, Okazaki, Japan; [2]Department of Biophysics, Faculty of Science, Kyoto University, Kyoto, Japan.

Mutations of the *agamous* gene of *Arabidopsis thaliana* confer double-flowering phenotype to the plant. Structural analysis of the gene suggested that it encodes a putative DNA-binding protein shearing homology to yeast MCM1/PRTF and mammalian serum response factor (SRF) which are known to bind specific DNA sequences. Also, *def A*, anothor cloned gene affecting floral morphogenesis, has a homologous region to these proteins. Recently, it has been proposed that the homologous region of these proteins is to be called a "MADS box" for MCM1, AGAMOUS, DEF A and SRF. However, whether the AGAMOUS and DEF A proteins actually bind to specific DNA sequences remain to be shown experimentally.

We have employed an *in vitro* genetic approach to examine the DNA-binding activity of the AGAMOUS MADS domain. 79mer oligonucleotides having random sequences of 40 bp in the central region were incubated with the AGAMOUS MADS domain overproduced in *Escherichia coli*. After the incubation, the MADS domain was immunoprecipitated with an antibody specific to the protein. If the MADS domain actually has DNA-binding activity, some portion of the random oligonucleotides should bind to the domain and they should be coprecipitated with the protein. The precipitated DNA was amplified by PCR and the amplified product was used for further rounds of selection. After three rounds of such selection cycles, electrophoretic mobility shift assay was carried out using the selected DNA as a probe. A DNA-protein complex specific to the AGAMOUS MADS domain was detected. The DNA in the complex was inserted into a plasmid vector after PCR-amplification and the nucleotide sequences of randomly selected clones were determined. Comparison of the sequences has revealed a sequence motif which is common to the selected oligonucleotides. DNase I-footprinting and methylation interference experiments have shown that the AGAMOUS MADS domain actually binds to the motif in the selected oligonucleotides.

EXPRESSION OF THE FLORAL HOMEOTIC GENE *AG* IN AN *ag* MUTANT

Hong Ma[1] and Martin F. Yanofsky,[2] [1]Cold Spring Harbor Lab., Cold Spring Harbor, NY 11724; [2]Department of Biology, University of California at San Diego, La Jolla, CA 92093.

The *Arabidopsis AG* gene is a homeotic gene, mutations in which lead to replacement of one type of floral organs (stamens) by another (petals). The *AG* gene was recently cloned, and its sequence shows similarity to transcription factors MCM1 and SRF, suggesting the AG protein is a transcription regulator necessary for proper flower development. In the wild type, *AG* is expressed in the organs affected by the mutations: stamens and carpels, as well as earlier cells which develop into stamens and carpel. Based on genetic results, it has been proposed that AG and another floral homeotic gene product, AP2, regulate each other's expression or activity. This is further supported by the finding that *AG* expression is altered in *ap2* mutants. We are interested in determining whether AG regulates its own expression. Northern analysis indicates that *AG* mRNA is present at near wildtype level in the *ag-1* mutant, which has the same phenotype as an insertion mutant. Because most of the tissues expressing *AG* (stamens and carpels) are absent in the *ag-1* mutant, *AG* expression must be altered in the *ag-1* mutant. We are in the process of studying *AG* expression in *ag-1* mutant in more detail, using in situ RNA hybridizations. The pattern of *AG* expression in the *ag-1* mutant should promise to be very informative, and may provide additional clues about AG's role in flower development.

ECTOPIC EXPRESSION OF CARPEL PROPERTIES IN *ARABIDOPSIS* MUTANT *FIDDLEHEAD*. S.J. LOLLE, J.M. SUSSEX*, AND A. Y. CHEUNG. Department of Biology, Yale University. *Current address: Department of Plant Biology, University of California, Berkeley.

Under most circumstances plant epidermal cells do not respond to surface contact with adjacent plant parts, ensuring organ autonomy and unrestricted growth of the plant body. Thus developmentally, epidermal cells differ from cells in internal tissue layers in their inability to fuse with other contacting cells through dedifferentiation and redifferentiation. However, under special conditions or during limited developmental windows, eg. in the postgenital fusions between the emerging carpels of the gynoecium, epidermal cells can de- and re-differentiate and fuse.

In wildtype *Arabidopsis*, postgenital fusion is limited to the formation of the ovary septum and the transmitting tissues of the gynoecium. We have isolated an EMS induced mutant in *Arabidopsis* which displays postgenital fusion in most of its epidermis on the aerial part of the plant. We named this mutant *fiddlehead* because fused floral buds cause apical curling of inflorescences resulting in structures reminescent of fern fiddleheads. As in wildtype, *fiddlehead* floral organs are initiated as discrete primordia. Upon enclosure of the flower bud, sepals fuse to each other as well as to sepals of neighboring floral buds. Newly initiated floral buds become trapped within the inflorescence by surrounding older buds while internode and pedicel elongation continue giving rise to the elaborate *fiddlehead* structure. Fusion is most prominent in the inflorescence and also occurs frequently between leaves. Analysis of contacting tissues reveals that fusion in *fiddlehead* involves only epidermal cells and occurs without cytoplasmic union, both also being criteria for postgenital fusions. Furthermore, cell morphology characteristic for epidermal cells associated with a given organ is altered at the fusion suture suggesting involvement of morphogenic substances in the fusion process. Morphogens have been implicated in signalling epidermal fusions in the classical example of postgenital fusion in the gynoecium of *Catharanthus roseus* (ref. 1).

The promiscuous fusions in *fiddlehead* suggest ectopic expression of a normally carpel-specific program throughout the plant. Current efforts center on identifying other carpel-specific properties which have become ectopically expressed in *fiddlehead* plants. Elucidation of the genetic and cellular bases of the *fiddlehead* mutation should lead to considerable understanding of mechanisms signalling the control of epidermal determination.

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PHOSPHORYLATION OF THE NUCLEAR FACTOR GBF-1 BY CASEIN KINASE II FROM BROCCOLI.

Leszek J. Klimczak, Ulrike Schindler, and Anthony R. Cashmore. Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, PA 19104-6018

To study the phosphorylation of one of the G-box binding factors from *Arabidopsis thaliana*, GBF-1, we have obtained large amounts of this protein by expression in *Escherichia coli*. Bacterial GBF-1 was shown to be phosphorylated very efficiently by nuclear extracts from broccoli (*Brassica oleracea* var. *italica*). The phosphorylating activity was purified by chromatography on heparin-Sepharose and DEAE-cellulose, and was characterized. It showed the essential features of casein kinase II activity: utilization of GTP in addition to ATP as a phosphate donor, strong inhibition by heparin, preference for acidic protein substrates, salt-induced binding to phosphocellulose, and salt-dependent deaggregation. The very low K_m value for GBF-1 (220 nM compared to ca. 10 μM for casein) was in the range observed for identified physiological substrates of casein kinase II. Phosphorylation of GBF-1 resulted in stimulation of the G-box binding activity and formation of a slower-migrating protein–DNA complex. The DNA binding activity of the endogenous plant GBF was shown to be reduced by treatment with calf alkaline phosphatase; this reduction was diminished by addition of fluoride and phosphate or incubation in the presence of casein kinase II and ATP. Current experiments focus on the determination of the phosphorylation sites and attempt to address the possible biological significance of this phosphorylation.

ACCURATE *IN VITRO* TRANSCRIPTION FROM THE CHS15 PROMOTER REQUIRES SEQUENCES UPSTREAM OF THE TATA BOX.

J.A. Arias and C.J. Lamb, The Salk Institute for Biological Studies, Plant Biology Laboratory, La Jolla, CA 92037

Chalcone synthase (CHS) catalyzes the first committed step in isoflavonoid biosynthesis and is regulated transcriptionally in response to light, wounding, and infection. To better understand the terminal steps in the signal pathway leading to CHS gene induction we prepared cell-free extracts for transcription analysis. Chromatin extracts from suspension cells of french bean (*P. vulgaris*) failed to accurately transcribe templates containing the CHS15 promoter. Although reactions containing nuclear extracts from HeLa cells were able to transcribe an Adenovirus "minimal" promoter, they required plant chromatin extract to transcribe the CHS 15 template. Primer extension analysis of *in vitro* transcripts and those generated *in vivo* from suspension cells of french bean revealed a common RNA start site. CHS 15 was transcribed in the heterologous extract system by RNA polymerase II. Using this system we identified regions of the CHS15 promoter required for accurate transcription *in vitro*. We observed that DNA template lacking CHS15 sequences 5′ to the TATA box (-35/CHS15) was poorly transcribed. Moreover, CHS15 promoter activity was selectively competed by a DNA fragment containing sequences -80 to -42 bp of the CHS15 promoter. Collectively, these data suggest that upstream *cis*-elements proximal to the TATA box are required *in vitro* for transcription from the CHS15 promoter, and that one or more factors in the plant extracts are necessary for this activity. This heterologous extract system should prove useful for studies aimed at isolating and characterizing plant transcription factors that regulate expression of CHS and other plant genes.

HOMEOBOX GENES IN PLANT DEVELOPMENT

S. Hake[1,2], B. Greene[2], R. Kerstetter[2], B. Lowe[1], L. Smith[2], N. Sinha[2], B. Veit[2] and E. Vollbrecht[1] [1]USDA/ARS Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710; [2]Dept. of Plant Biology, University of California, Berkeley 94720 USA.

The *Knotted* (*Kn1*) locus, defined by dominant mutations that affect leaf development, was cloned by transposon tagging. The mutation is characterized by alterations to the lateral veins of the leaf blade that include pockets of continued division, or knots, and displaced ligule, a tissue that is normally found at the base of the leaf blade. Clonal analysis has revealed the site of mutant gene action. The inner layer that contains the vascular layer and the bundle sheath cells require the dominant *Kn1* mutation in order for the mutant phenotype. At least nine mutations result from insertions into introns, while one mutation is a tandem duplication that results in a rearrangement in the 5' non-coding region. Sequence analysis demonstrates that *Kn1* contains a homeodomain, a DNA-binding motif encoded by a number of *Drosophila* developmental genes. While the dominant, gain-of-function mutations specifically affect the leaf, initial results demonstrate that in normal plants, the protein is abundant in meristems and not detectable in developing leaves. In mutant leaf primordia, the protein is present in developing lateral veins. These are the very cells predicted by clonal analysis to require the mutant genotype for the knots and ligule displacement. We have expressed the gene in tobacco and find a phenotype, suggesting the tobacco cells recognize the maize product. A number of other maize homeobox genes have been cloned and partially characterized. Mapping data suggest that at least two of them map to known morphological mutations.

ARABIDOPSIS ENCODES A LARGE NUMBER OF HOMEODOMAIN PROTEINS INCLUDING A PUTATIVE LEUCINE ZIPPER-CONTAINING FAMILY

M. Schena and R.W. Davis, Department of Biochemistry, Beckman Center, Stanford University Medical School, Stanford, California.

The homeodomain is a conserved 61 amino acid DNA binding motif found in transcriptional regulator proteins from all eukaryotes examined thus far including yeast and mammals. Given the fact that homeobox-containing genes encode a major class of developmentally important regulators in animal systems, we sought to determine the extent to which this motif is utilized by higher plants.

Using three pools of degenerate oligonucleotides complementary to sequences encoding helix three of the homeodomain, we screened a genomic library from *Arabidopsis thaliana*. Under conditions of high stringency, approximately 1% of the genomic clones scored positive, indicating that *Arabidopsis* probably contains upwards of 50 homeobox-containing genes. Using this same approach, 48 clones were isolated from an *Arabidopsis* complementary DNA (cDNA) library and named *HAT*2-49 (homeoboxes from *Arabidopsis thaliana*). Preliminary sequence analysis indicates that nearly all of the cDNAs were derived from unique messenger RNAs. Detailed analysis of a subset of the *HAT* clones indicates that each contain significant homology to the homeodomains encoded by known homeodomain-containing genes; interestingly, *HAT*4, 10 and 22 also encode a series of evenly spaced leucine residues reminiscent of the leucine zipper motif originally described for C/EBP. The putative leucine zipper domains of *HAT*4 and *HAT*22 are nearly identical at the amino acid level but highly divergent at the DNA level, suggesting that these regions have been functionally conserved, despite apparent selective pressures to alter the base composition of these sequences. We propose that these putative leucine zippers may facilitate homodimer formation of homeodomain proteins; moreover, given that *HAT*4 and *HAT*22 are differentially expressed during *Arabidopsis* development, it is tempting to speculate that heterodimers between *HAT*4 and *HAT*22 may also form to provide novel regulatory complexes. *HAT*24, unlike members of the putative leucine zipper-containing family, lacks a repeated series of leucine residues but contains unique residues in the helix 3 region.

While this work was in progress, reports by Vollbrecht *et al.* (*Nature* 350, 1991) and Ruberti *et al.* (*J. EMBO* 10, 1991) provided the first published reports of homeobox genes in Maize and *Arabidopsis*, respectively. Our findings confirm and extend these observations and indicate that *Arabidopsis* encodes a large and diverse family of homeodomain proteins. Functional analysis of these proteins should provide a wealth of information on the molecular mechanisms that govern higher plant development.

The CaMV as-1 Element is a Salicylate-Responsive Element.
Loreto Holuigue and Nam-Hai Chua, Laboratory of Plant Molecular Biology, The Rockefeller University, New York, New York 10021-6399.

Salicylic acid (SA), widely used as a chemical elicitor for the induction of pathogen-related proteins, has recently been identified as an endogenous signal in pathogenic defense response. The mechanism by which SA activates the expression of these genes has not yet been elucidated. Here we report that activation sequence (as)-1, a 21-bp element, located between -85 to -65 within domain A of the Cauliflower Mosaic Virus (CaMV), 35S promoter is responsive to SA. Previous evidence indicates that the as-1 element binds specifically to the transcriptional factor ASF-1 and confers preferential expression in roots with little activity in leaves. Treatment of transgenic tobacco plants containing the CaMV A domain linked to β-glucuronidase coding sequence (-90 35S/GUS), results in an increase of GUS activity in mesophyll cells and vascular elements of leaves and at the apical meristem and axillary buds of stems. The 21-bp as-1 element is also able to mediate SA response when inserted upstream of a heterologous promoter (pea rbcS-3A). In both cases, point mutations in the TGACG motifs of as-1 drastically reduce ASF-1 binding in vitro ando abolish the SA inductive effect in vivo. Similar results were obtained with the ocs element of the octopine synthase promoter, which also binds to ASF-1 and is related in sequence to as-1. These results strongly suggest that as-1 and related sequences can function as SA-responsive elements.
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FUNCTIONAL ANALYSIS OF THE MAIZE TRANSCRIPTION FACTOR OPAQUE-2

Stefan Lohmer, Massimo Maddaloni, Francesco Salamini and Richard D. Thompson, Max Planck Institut für Züchtungsforschung, Köln, Germany.

The Opaque-2 (O2) locus of maize controls the synthesis in the developing endosperm of a major storage protein family, the 22kD zeins, and a number of non-zein polypeptides including a 32kD albumin, b32. The genetically suggested cis-trans interaction between *O2* (1) and *b-32* (2) was confirmed in our lab on the molecular level. Our studies demonstrated that *O2* is a sequence specific DNA binding protein, which interacts with the promoter region of *b-32* and is sufficient for the transactivation of *b-32* in tobacco protoplasts (3). We took advantage of this specific interaction for the further characterization of *O2* and addressed the following questions : A. Is there an influence of the 3 short open reading frames (sORFs) located in the 5' 'untranslated' leader on the expression of *O2* ? B. Where is the transcriptional activation domain located in *O2*?

A.From Western blot analysis it is obvious, that the fourth ATG is used as the start codon, because constructs with or without the 3 sORFs produce a protein with the same molecular weight. Therefore frameshift and readthrough of the sORFs STOP codons can be ruled out. In order to prove if the sORFs are involved in the regulation of *O2* expression, we have carried out site-directed mutagenesis to change the START codons to missense codons. The different mutagenized *O2* constructs were cotransfected with the reporter construct b32-GUS via PEG into tobacco protoplasts. Comparision of the relative GUS-values of the different constructs obtained from the transient expression show, that the presence of the sORFs reduces the transactivation at least five-fold. The largest effect was attributable to ORF-2, which would give rise to a 21 Aa peptide.

B. For the determination of the transcriptional activation domain of *O2* a series of deletion mutants were created, covering N-terminal, C-terminal and internal deletions. The mutants were tested for their transactivation potential in the above-described transient expression assay using tobacco protoplasts. The results obtained indicate, that *O2* harbours two separated activation domains located in the N-terminal region. Both belong to the acidic class of activation domains and share also with these the computer-predicted ability to form α-helices. Interestingly, a C-terminal deletion which removes around 40% of the coding sequence results in a protein with a higher transactivating potential than the wildtype protein (wt = 100% and mutant = 250%).

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CHARACTERIZATION OF CIS- AND TRANS-ACTING ELEMENTS INVOLVED IN ACTIVATION OF TRANSCRIPTION IN RESPONSE TO DESSICATION AND ABSCISIC ACID

O. Perisic and E. Lam, AgBiotech Center, Box 759, Waksman Institute, Rutgers State University, Piscataway, NJ 08854.

It has been found recently that tetramer of a 21 base-pairs element, designated as *hex-3*, acts as an Abscisic Acid Responsive Element (*abre*) in transgenic tobacco. The sequence of *hex-3*, TTCGGCCACGCGTCCAATCCG, differs from that of another element, *hex-1* by only 3 base pairs. The *hex-1* element has been shown previously to bind tobacco nuclear factors ASF-1, HSBF and TAF-1. *Hex-3*, on the other hand, does not interact significantly with these tobacco factors. When fused upstream of a minimal promoter (-46 to +8 of the CaMV 35S promoter), the tetramer of *hex-3* can confer high expression in mature tobacco seeds. Upon germination, the activity of the *hex-3* element decreases by more than 10 fold but can be reactivated by dessication, 0.25 M NaCl or 0.1 mM ABA. The maximal activity induced by these treatments can be observed within a few hours. Under the same conditions and in mature seeds, a tetramer of the *hex-1* element is inactive. Our results suggest that there is at least one class of trans-acting factors which can interact with the *hex-3* element and mediate its activation by treatments that increase intracellular ABA concentrations.

By screening an expression library of tobacco cDNA, we have isolated two classes of cDNAs which encode *hex-3* binding proteins. One class of these DNA-binding proteins bind to *hex-3* but not to *hex-1*. The other class of cDNAs encode proteins which bind to *hex-3* as well as *hex-1*. The characterization of these cDNA clones will be presented.

THE ROLE OF THE *VIVIPAROUS-1* GENE IN REGULATION OF SEED MATURATION IN MAIZE. Donald R. McCarty, Leonard Rosenkrans, Tsukaho Hattori, Vimla Vasil, Christian B. Carson and Indra K. Vasil. Vegetable Crops Dept. University of Florida, Gainesville, FL. 32611.

The viviparous mutants of maize include several mutants that are blocked in abscisic acid biosynthesis and *viviparous-1* which reduces the hormone sensitivity of seed tissues. In addition to causing vivipary, *vp1* also blocks anthocyanin synthesis. This feature is not characteristic of the ABA deficient mutants. Certain *vp1* alleles prevent anthocyanin biosynthesis, but produce dormant seed. We have shown that VP1 regulates the anthocyanin pathway by controlling the *C1* regulatory gene. VP1 has properties of a transcriptional activator. Over-expression of VP1 protein in maize protoplasts causes trans-activation of several downstream genes including *C1*. Cis-analysis of the *C1* promoter suggests that ABA regulation and VP1 trans-activation functions are at least partially separable. Studies of VP1 regulation of the wheat Em gene suggest that maturation genes may interact with VP1 differently than *C1*. This idea is further supported by analysis of dormant/colorless mutants of *vp1* and directed mutagenesis of the VP1 protein. Our results suggest that VP1 action involves interaction with multiple transcription factors and that some of these interactions map to separate regions of the VP1 protein. VP1 may function to integrate the ABA response with other intrinsic developmental signals.

ANALYSIS OF ENDOGENOUS AND HETEROLOGOUS SIGNAL TRANSDUCTION PATHWAYS IN MAIZE BY TRANSIENT AND STABLE TRANSFORMATION

B. Roth, P. Solan, S. Maddock and B. Bowen, Pioneer Hi-Bred International, Inc., 7300 NW 62nd Ave Johnston, IA

We have utilized gene transfer by microprojectile bombardment and transient assays to investigate the feasibility of altering endogenous signal transduction pathways into maize. *R* and *C1* are genes which encode transcription factors that regulate the expression of the structural genes of the anthocyanin pigment pathway, including *Bronze1* (*Bz1*), to produce pigment in a variety of maize tissues. *In vitro*-synthesized dominant inhibitor mutations of the maize *R* and *C1* genes were constructed. These mutant transcription factors, denoted *R-I* and *C-I*, respectively, were co-bombarded into maize cells with CaMV 35S::*R* and 35S::*C1*. Inhibition of expression was assayed by either a reduction in the number of red cells (anthocyanin expression), or by measuring luciferase activity expressed from a *Bz1* promoter. These inhibitors were 5- to 20-fold more effective in reducing expression than analogous *R* and *C1* antisense constructs.

Additionally, we are interested in studying heterologous signal transduction pathways in maize. Expression results will be presented from *GAL4* fusion and chemically-regulated signal transduction pathways analyzed transiently and in stably transformed maize cells.

APPROACHES TO THE MOLECULAR CLONING OF SBF-1

M.J. Harrison[1], C.J. Lamb[2] and R.A. Dixon[1], [1]The Samuel Roberts Noble Foundation, Plant Biology Division, P.O. Box 2180, Ardmore, OK 73402; [2]The Salk Institute, P.O. Box 85800, San Diego, CA 92138.

SBF-1 (silencer box factor 1) is a DNA binding protein which recognizes three binding sites in the -170 to -326 region of the bean chalcone synthase promoter (1). The consensus sequence common to all three sites resembles the binding site sequence for the GT-1 factor originally noted in the upstream region of the pea rbcS-3A gene (2). Cross competition experiments suggest that the binding activity recognizing the CHS cis-acting elements is similar to the binding activity found in pea nuclear extracts known as GT-1 (1). We have purified SBF-1 from bean cell suspension nuclei (3). SBF-1 has a subunit molecular weight of approximately 95,000 and its binding activity can be modulated in vitro by de-phosphorylation. We have raised both polyclonal and monoclonal antibodies to partially purified preparations of SBF-1 which show cross reaction to SBF-1 as assessed by gel retardation and western blot analysis. We here report initial results on the use of these antibodies to obtain cDNA clones for this factor.

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TARGETS AND SIGNALS FOR CHLOROPLAST GENE EXPRESSION: DNA AND RNA BINDING PROTEINS INSIDE AND OUTSIDE THE ORGANELLE

K. Tiller, C. Fiebig, J. Nickelsen, A. Eisermann and G. Link,
Univ. of Bochum, Plant Cell Physiol. & Mol. Biol., Bochum, F.R.G.

Chloroplast gene expression involves a complex network of *cis/trans* interactions between DNA (RNA) sequence elements and their cognate protein factors, both inside and outside the organelle. We have looked at functional determinants that play a role in the transcription of genes for chloroplast constituents and the processing of their RNA precursors. In mustard (*Sinapis alba*), we find that the chloroplast *psbA* gene gives transcript levels that are much higher in the light than in darkness. This is reflected in part by *in vitro* experiments with extracts from chloroplasts and etioplasts, which were shown to differ in their promoter binding and transcriptional properties. We have purified three transcription factors which resemble bacterial sigma factors. These factors, which are present in either plastid type, differ in their sequence requirements and are subject to protein modification, suggesting the possibility of transcriptional regulation via signal transduction.

Much of the control of *psbA* gene expression, however, appears to reside at the post-transcriptional level, as is e.g. indicated by the result that a fraction of *psbA* sequences is cotranscribed with sequences of the preceding *trnK* gene. RNA-binding factors that interact with specific 3' sequence elements of the *trnK* precursor were purified and characterized.

In a search for nuclear transcription factors that are relevant for chloroplast biogenesis, we have studied 5' upstream regions of members of the *rbcS* gene family from *Brassica napus*. These genes fall into at least three different classes with regard to their mode of expression during seedling development. A class III gene (showing high *in vivo* transcript levels in the light and low levels in the dark) was shown to contain a 5' region that interacts more efficiently with 'light' than with 'dark' extracts. A critical sequence element defined by deletion analysis shows a modular architecture and appears related to the G-box motif of other plant genes. The focus on these and other factors could help define regulatory 'cascades' of protein-nucleic acid interactions inside and outside the chloroplast that are fundamental to its biogenesis and function.

MUTATIONAL ANALYSIS OF SIGNAL TRANSDUCTION PATHWAYS IN INFLORESCENCES AND ROOTS OF *ARABIDOPSIS THALIANA*.

K.OKADA[1], K.AKAMA[1], T.ITO[1], M.K.KOMAKI[1], H.OKAMOTO[1], K.SAKAMOTO[1], H.SHIRAISHI[1], A.YANO[1], Y.SHIMURA[1,2]. [1]National Institute for Basic Bilogy, Okazaki, Japan, [2]Kyoto University, Kyoto, Japan

We have been working on mutants of *Arabidopsis thaliana* defective in signalling pathways related with flower development and root growth responses toward physical stimuli. Process of flower development is based on successive interactions of inter- and/or intracellular signal transduction systems. Studies with an *Arabidopsis* mutant, *pin-formed*, indicate that the auxin polar-transport system is required for the initial step of floral bud formation. Decreased activity of the transport system caused by mutation or by treatment with inhibitors results in no floral bud formation at the top of inflorescence axis. Roots of wild type *Arabidopsis* seedlings show normal gravitropic and phototropic responses. When roots encounter obstacles, they alter their growth direction to evade the obstacles. We devised simple agar-plate procedures to test these responses, and isolated several mutants. Some of the obstacle-escaping mutants showed abnormal gravitropism and/or phototropism. Roots of wild type seedlings showed similar aberrant responses toward these stimuli when grown on agar plates containing auxin polar transport inhibitors. These results indicate that root responses toward different physical stimuli share, at least in part, a common regulatory mechanism, possibly in a process of signal transduction, and that the auxin polar transport system play an important role in the process.

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GENETIC CHARACTERIZATION OF THE *AXR1* GENE IN *ARABIDOPSIS THALIANA*

C. Lincoln, O. Leyser, J. Turner and M. Estelle, Indiana University, Biology Dept., Bloomington, IN 47405

The isolation and characterization of mutants of *Arabidopsis* which are altered in hormone response should provide a novel and effective way of studying plant hormone action. By screening for mutants which are resistant to exogenously applied auxin, we have identified several new loci which are important for normal plant development. Recessive mutations of one of these loci, *AXR1*, confer resistance to both auxin and cytokinin. The *axr1* mutants also exhibit an unusual morphological phenotype including decreases in apical dominance, root gravitropism, hypocotyl elongation and fertility. One mutant line, *axr1-3*, is less resistant to auxin and cytokinin than other mutant lines and has less severe morphological abnormalities. This correlation suggests that the morphological defects are a consequence of a defect in hormone action. Isolation of the *AXR1* gene will enable us to begin to determine the function of the *AXR1* gene product. The map position of *AXR1* lies between RFLP 488 (1) and RFLP 6811 (2) on chromosome 1. An overlapping series of cosmids spanning the region between these two RFLPs has been identified. We are currently using these cosmids to transform *axr1* plants in order to identify a cosmid which rescues the mutant phenotype.

We are also interested in identifying and characterizing genes which code for proteins that interact directly with the *AXR1* gene product. One means of identifying such genes is to isolate second site suppressors of the *axr1* mutant phenotype. Seed homozygous for an *axr1* mutation was mutagenized with EMS and the resulting M2 population was screened for mutant seedlings which displayed reversion to normal 2,4-D sensitivity. A number of mutant lines were isolated which exhibit a loss of auxin-resistance plus a unique morphology which differs from both *axr1* and wild-type plants. Preliminary genetic analysis indicates that in at least one of these mutant lines reversion to auxin sensitivity is due to a recessive mutation unlinked from the *AXR1* locus. Because this suppressor confers a novel morphological phenotype, we should be able to identify a plant which is homozygous for the suppressor but does not carry an *axr1* allele.

1. Chang et al 1988 *P.N.A.S.* 85:6856-60 2. Nam et al 1989 *Plant Cell* 1:699-705

THE ISOLATION OF PHYTOCHROME SIGNAL TRANSDUCTION MUTANTS IN *ARABIDOPSIS*

J. Brusslan[1], G. Karlin-Neumann[2] and E. Tobin[1] [1]Department of Biology, UCLA, Los Angeles, CA. [2]Dept.of Biochemistry, Stanford University, Palo Alto, CA.

Phytochrome is a photoreceptor involved in many aspects of photomorphogenesis. In etiolated seedlings phytochrome induces *cab* mRNA transcription (Tobin and Silverthorne, 1985), and we are interested in isolating mutants defective in this phytochrome-controlled response. We are currently using two selection schemes to isolate such mutants in *Arabidopsis*. In the first, the promoter of the *cab*1 gene of *Arabidopsis*, which is phytochrome regulated (Karlin-Neumann *et al.*, 1988), has been fused to the *tms*2 gene of *Agrobacterium*. The *tms*2 gene product converts IAM to IAA, which is toxic at high concentrations. The *cab*:*tms*2 gene has been shown to be under phytochrome control in transformed seedlings, and thus a screen can be done for seedlings that are not producing the *tms*2 gene product under inductive red light conditions (Karlin-Neumann *et al.*, 1991). So far we have isolated one interesting mutant in which *cab*1 mRNA levels are greatly reduced. Although the *cab*1 mRNA level can be increased by red light, the induction level is very low. This mutant has no obvious visible phenotypes.

Our other selection is based on phytochrome control of germination. We are screening M2 populations for seeds which do not germinate in response to red light, and rescuing such seeds with giberellic acid (GA), which overcomes the light requirement for germination. We have isolated alleles of *hy*1 and *hy*2 which require GA to germinate, and we believe these alleles have lower amounts of phytochrome than previously isolated germinating alleles of *hy*1 and *hy*2. The isolation of phytochrome-deficient mutants suggests that the germination screen should be successful for the isolation of signal transduction mutants.

Tobin, E.M. and Silverthorne, J. (1985) Ann. Rev. Plant Phys. 36:569-93

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Karlin-Neumann, G.A., Brusslan, J. and Tobin E.M. (1991) The Plant Cell 3:573-82

LIGHT SIGNAL TRANSDUCTION MUTANTS OF *ARABIDOPSIS THALIANA* WITH INCREASED *CAB* PROMOTER ACTIVITY IN THE DARK

L. Altschmied and J. Chory, The Salk Institute, Plant Biology Laboratory, 10010 N. Torrey Pines Road, La Jolla, CA 92037, USA

To identify light signal transduction mutants of *Arabidopsis thaliana* we constructed a transgenic line (107#2-1). It contains the T-DNA segment of the plasmid pOCA107 at one locus in the genome (chromosome 2, 3.0±0.4 cM from *hy*1) in a homozygous state. In this T-DNA the light regulated *cab*3 promoter (chlorophyll a/b binding protein; Leutwiler et al. 1986, Nucl. Acids Res. **14**, 4051-64) is used twice to direct expression of the *hph* (hygromycin phosphotransferase; Waldron et al. 1985, Plant Mol. Biol. **5**, 103-8; van den Elzen et al. 1985, ibid., 299-302) and the *gus*A gene (ß-glucuronidase; Jefferson 1989, Nature **342**, 837-8). The *cab*3 promoter is transcriptionally active in green tissue in the light, but inactive in the dark and in roots (Mitra et al. 1989, Plant Mol. Biol. **12**, 169-79). Therefore the 107#2-1 line allows us to identify mutants with increased *cab*3 promoter activity in the dark through their increased hygromycin resistance and to check for trans-acting mutations by analyzing expression of the *cab*3-*gus*A fusion.

50,000 seeds of the 107#2-1 line were mutagenized with EMS, sown to soil, and M2 seeds collected from 200 families. 100,000 etiolated M2 seedlings were screened for mutants with increased hygromycin resistance in the dark. As judged by an *A. thaliana* line with constitutive hygromycin resistance (Ohl et al. 1990, The Plant Cell **2**, 837-48), such mutants are revealed by their increased hypocotyl length when compared to the 107#2-1 line. 600 putative mutants were identified and 96 of them yielded M3 seeds. Etiolated M3 seedlings were screened for elevated GUS activity in darkness. 31 M3 plants from 28 M2 families with at least a twofold increase in GUS activity over the 107#2-1 line and more than three standard deviations away from the mean for the 107#2-1 line (19 samples) were found. In 8 of the mutants (8 M2 families) the increase was at least threefold and in one it was sixfold. So far the F2 generation from backcrosses to a Col-0 WT line showed that three mutations segregate as single genes, one being dominant and the other two recessive. Further genetic and biochemical characterization of the mutants is in progress and results will be presented.

IDENTIFICATION OF NEW DEFENSE GENES IN *ARABIDIPSIS* AND DEVELOPMENT OF A RAPID METHOD FOR SCREENING *AVR* INSENSITIVE *ARABIDOPSIS* MUTANTS

Guo-Liang Yu, Michael Mindrinos and Frederick Ausubel, Department of Molecular Biology, Massechusetts General Hospital, Boston, MA 02114

Upon infection of different bacterial pathogens, two types of defense responses are induced in *Arabidopsis*: the pathogenicity response which causes disease symptoms in infected tissue and the hypersensitive response that is associated with rapid plant cell death in the lesion and activation of defense genes. The hypersensitive response is attributed to the avirulence (*avr*) genes of the bacterium. Such an *avr* gene was previously cloned from *Pseudomonas syringae pv. tomato* 1065 (Dong et al., 1991, *Plant Cell*, 3, 61). We report here the identification of four new defense genes from *Arabidopsis* by differential screening of a cDNA library. The amino acid sequences deduced from two of the cDNA clones are highly homologous to calmodulin genes and one has homology to glutathione S-transferase. These plant genes were strongly induced by bacterial pathogens and were induced even more by the bacteria harboring the *avr* gene. Cloning of these genes suggest that calcium and glutathione may play roles in the defense response in plants. To understand the signal transduction pathway leading to the activation of the defense genes, we have developed a plate screening assay to isolate *Arabidopsis* mutants that fail to response to an *avr* gene in the bacteria. *Arabidopsis* seedlings were killed after vacuum infiltrated with a bacterial pathogen carrying an *avr* gene while the majority of plants were still alive after vacuum infiltration with the same bacteria that do not carrying the *avr* gene. This method allows screening of a large number of mutagenized seeds and will facilitate the identification of genes in the signal transduction pathway.

DEVELOPMENT OF A GENETIC APPROACH TO STUDYING THE *TCH* GENE STIMULATORY PATHWAY IN *ARABIDOPSIS*

G. Karlin-Neumann and R.W. Davis, Biochemistry Dept., Stanford University, Stanford, California.

Plants have recently been shown to respond to touch (mechanical) stimulation not only by morphogenetic changes, but also by rapid and transient changes in the expression of certain genes (Braam and Davis, 1990). Five such genes -- TCH1, TCH2, TCH3, TCH4 and TCH5 -- were originally shown to be induced in 2-4 wk-old *Arabidopsis* plants grown in soil. In order to more effectively apply genetic methods to the study of the stimulatory pathway involved, we are examining this behavior in young seedlings grown sterilely in petri dishes. Seedlings as young as 7d-old have been found to exhibit similar touch-inducibility of these genes as do older seedlings. In preparation for future screening for unresponsive and hypersensitive mutants, we are exploring both ways to administer and the effects of periodic mechanical stimulation on such sterilely-grown seedlings. Our method of choice for isolating such mutants will be through the creation of transgenic plants in which the *cis* element(s) conferring responsiveness to mechanical stimulation are fused to appropriate selectable markers (see *e.g.* Karlin-Neumann *et al* 1991). As a potentially useful tool in this type of approach, we have introduced the mammalian glucocorticoid receptor gene into *Arabidopsis* plants by the *in planta* transformation procedure of Chang *et al.* (1990). We are currently investigating these transgenic plants for the presence of functional receptor.

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ARABIDOPSIS THALIANA DNA HYPOMETHYLATION MUTANTS

Eric J. Richards, Rob Martienssen and Aurawan Vongs
Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724-2212

A large number of studies have shown a correlation between changes in DNA methylation patterns and activity of various processes in the eukaryotic cell, including gene expression and transposition. In order to directly assess the role that DNA methylation plays in control and patterning of these, and other, processes, we have isolated mutants of *Arabidopsis thaliana* that contain altered levels of cytosine methylation.

At present, three independent hypomethylation mutants have been recovered from M2 populations of EMS mutagenized seeds by screening for plants which have satellite arrays susceptible to digestion by methylation sensitive endonucleases. The three mutations are allelic and have been designated *dcm1-1*, *dcm1-2* and *dcm1-3*. Both satellite arrays and rDNA are hypomethylated in the *dcm1* mutants, but methylation at some single-copy sequences is unaffected. Global methyl-cytosine content is reduced 80% in *dcm1-2/dcm1-2* plants, based on the alteration of the 5mC content at the sequence TCGA.

Despite the severe reduction in methyl-cytosine content, no morphological phenotype in the sporophyte has been associated with the mutations. Progress in characterization of these mutants will be reported.

A MOLECULAR GENETIC APPROACH TO UNDERSTANDING ROOT DEVELOPMENT IN *ARABIDOPSIS*

Philip N. Benfey[1], Keith Roberts[2], John Schiefelbein[3], Davina Tang and Roger A. Aeschbacher;[1] Laboratory of Plant Molecular Biology, Rockefeller University, N.Y., N.Y. 10021; [2] John Innes Institute, Norwich, U.K.; [3] Biology Dept., University of Michigan, Ann Arbor, MI 48109.

Roots provide a useful model for understanding the repetitive post-embryonic organ elaboration that is characteristic of plant development. We have undertaken a molecular genetic approach to understanding root development by isolating and characterizing mutants in Arabidopsis with aberrant root formation. We have divided these mutants into three classes:
1) Initiation: plants that fail to form a primary root or form abnormal lateral roots.
2) Morphogenesis: plants that form abnormal root structures.
3) Differentiation: Plants that produce abnormal differentiated cells (e.g. root cap cells).
Here we present a preliminary characterization of three root morphogenesis mutants and describe a means of obtaining mutants in root cap differentiation. The morphogenesis mutants, *cobra*, *sabre* and *short-root* elaborate root structures that appear to be defective in different cell-types. We are using transgenic β-Glucuronidase (GUS) gene marker lines with tissue-specific expression patterns to characterize these mutants. Two of the mutants, *sabre* and *short-root* were generated by T-DNA insertion (by Dr. Ken Feldmann) and we have begun the process of identifying the affected genes.

EXPRESSION OF LUCIFERASE IN TRANSGENIC TOBACCO MEDIATED BY A PROMOTER ELEMENT FROM DESICCATION TOLERANT *CRATEROSTIGMA PLANTAGINEUM*

M. Båga[1], P.L. Dale[1], A.A. Szalay[1], D. Piatkowski[2], F. Salamini[2] and D. Bartels[2], [1]Plant Molecular Genetics, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7; [2]Max-Planck-Institut fur Zuchtungsforschung, Carl-von-Linne-Weg 10, D-5000 Koln 30, Germany

The phytohormone abscisic acid (ABA) is known to play a key role in drought tolerance of plants. In the desiccation-tolerant *Craterostigma plantagineum*, specific genes are transcriptionally activated in response to desiccation or application of ABA. Five of these genes have been identified and isolated (Piatkowski, D *et al* Plant Physiol 94:1682-1988, 1990). Two kb DNA from the upstream sequences of one of these ABA-induced genes and its truncated derivatives have been fused to the bacterial luciferase reporter fusion gene (*luxF*) resulting in a set of ABA-promoter *luxF* transcriptional fusions. These chimeric genes were used in separate experiments to transform leaf disks of SR1 tobacco plants. In regenerated transgenic tobacco plants, the luciferase activity was monitored *in vivo* during plant development. Light emission was found only in the maturing seeds and not in the vegetative portions of the analyzed plants regardless of the constructs used. In contrast, leaf mesophyll protoplasts from transgenic plants exhibited light emission only in the presence of high osmoticum (13% mannitol) or upon treatment with 10^{-4}M ABA. 5' truncations revealed the location of essential sequences required for seed specific activation as well as for promoter activation in the presence of osmoticum and ABA. The detailed functional analysis of the upstream sequences will help in elucidation of gene activation during dehydration of plants.

USE OF SYNTHETIC PEPTIDES IN THE ANALYSIS OF PLANT CELL SIGNALLING PATHWAYS.

I.R.White, Z.Ishak, and P.A.Millner.
Department of Biochemistry and Molecular Biology, University of Leeds, U.K.

Synthetic peptide technology has provided a valuable alternative to the classical approaches hitherto employed in the study of signal transduction processes. Antibodies raised against synthetic antigens corresponding to conserved domains of known transduction components have been used to identify and characterise novel signalling polypeptides. Peptides corresponding to predicted external loops have been employed to probe protein domains involved at the interfaces of transduction chain elements. Additionally, peptides have been utilised to identify and characterise effector enzymes *e.g.* protein kinases, and their substrates, and as immobilised affinity ligands to purify transduction chain components.

Previously we have characterised the substrate requirements of thylakoid LHCII kinase and are currently using peptides to elucidate the molecular regulation of this redox controlled enzyme. In addition, we have identified a number of putative plant cell guanine nucleotide binding proteins (G-proteins) using anti-peptide immunological probes and have isolated a candidate plasmalemma receptor using a G-protein derived peptide ligand (See poster by P.A.Millner *et al.*,)

Currently, our major interests focus on the polypeptides responsible for mediating events initiated by specific binding of auxins at the cell membrane of higher plants. Cytoplasmic loops of the auxin binding protein of *Zea mays* were identified by secondary structural prediction methods and have been used to define synthetic peptide sequences. The ability of these peptides (11-16 residues) to affect plasmalemma associated guanine nucleotide binding in *Zea mays* and *Pisum sativum* has been investigated.

Ac/Ds TRANSPOSON TAGGING SYSTEM IN *ARABIDOPSIS*

Mary A. Honma, Candace S. Waddell and B. Baker.
U.C. Berkeley-USDA Plant Gene Expression Center, Albany, CA 94710

We are developing a transposon mutagenesis/tag system in *Arabidopsis* based on the well-characterized maize transposon family *Activator/Dissociation (Ac/Ds)*. Our system has two components: a stable *trans*-activator Ac^{st} that supplies transposase and a responsive element *Ds*. Both the Ac^{st} and *Ds* are, separately, non-mobile elements. *Ds* transposes to new sites when *trans*-activated by Ac^{st} but these new *Ds* insertions become fixed when Ac^{st} segregates away from *Ds*. Consequently, stable *Ds* insertions are created that act as both genetic and physical markers in the genome.

All components of the system are genetically marked to facilitate assessment of transposition. Our *Ds*, *Ds(ALS)*, has an internal deletion of the transposase coding region and carries a mutated *Arabidopsis ALS* gene that confers resistance to the the sulfonylurea herbicide, chlorsulfuron. *Ds(ALS)* resides in the 5' untranslated region of a chimeric 1'NPTII gene such that *Ds* excision results in the expression of kanamycin resistance. The *Ds(ALS)* T-DNA also carries hygromycin (selectable) and nopaline (screenable) markers. Stable Ac^{st} *trans*-activators carry neither a functional 5' nor 3' terminus. The Ac^{st} constructs were made by deleting the *Ac* promoter and fusing one of three strong heterologous promoters to the transposase coding region. The promoters used to drive transposase expression are derived from the CaMV 35S gene, and the *Arabidopsis rbcS* and *chs* genes. The Ac^{st} T-DNA carries a DHFR gene which confers resistance to methotrexate (Mtx) and GUS. The use of selectable and screenable markers allow us to genetically monitor both the presence of Ac^{st} and *Ds(ALS)* -bearing T-DNAs, as well as the *Ds* excision and reintegration events. All Ac^{st} and *Ds* constructs were introduced into *Arabidopsis* using methotrexate, chlorsulfuron, or hygromycin for selection. Segregation analysis of selfed progeny using these same selective agents showed that most transformants contain T-DNA insertions at a single locus.

Ds and Ac^{st} -containing plants were crossed to activate transposition of *Ds(ALS)*. *Trans*-activation of *Ds(ALS)* by Ac^{st} leads to excision of *Ds(ALS)* from the 1'NPTII gene resulting in kanamycin resistance. Several Ac^{st} lines (Mtx^r) were used as pollen donors in crosses with two independent *Ds(ALS)* lines. F1 progeny containing both Ac^{st} and *Ds(ALS)* were identified and F2 seed was collected. F2 seed will be plated on kanamycin to assess transposition of *Ds(ALS)*. Results from this analysis will be presented.

REMOVAL OF SELECTION GENES FROM TRANSGENIC PLANTS.
Emily C. Dale and David W. Ow, Plant Gene Expression Center, USDA/ARS/UC Berkeley, Albany, CA 94710.

To explore novel approaches for the engineering of plants, we have investigated the use of a site-specific recombination system for the rearrangement of plant DNA. The bacteriophage P1 Cre/*lox* recombination system consists of the 38 kDa product of the *cre* gene (Cre), and the asymmetric 34 bp *lox* sites (locus of x-over). We have previously reported that the Cre/*lox* system is functional in plant cells (*Gene* **95**:79-85).

One application of site-specific recombination is in the engineering of transgenic plants. Current transformation technology relies on the use of genes that confer resistance to agents such as antibiotics as a means for selecting plant tissues that have incorporated genes of interest into the genome. Here we demonstrate the use of the Cre/*lox* site-specific recombination system to remove such selection genes from the genome of transformed plants. We constructed an *Agrobacterium* binary vector with the hygromycin phosphotransferase (*hpt*) gene flanked by *lox* sites of the same orientation. The luciferase (*luc*) cDNA, exemplifying a gene desired in transgenic plants, was inserted into this vector, and the resulting construct used to transform tobacco. Hygromycin-resistant/luciferase-producing plants so obtained were then re-transformed with a *cre*-expressing gene or were crossed to plants harboring the *cre*-expressing construct. Expression of the Cre recombinase resulted in the excision of the *lox*-flanked *hpt* marker but not the *luc* gene. As determined by sequence analysis of PCR products, the *hpt* gene was deleted by a site-specific recombination event between the *lox* sites and the recombination event was conservative. Subsequently, the *cre* locus was segregated away to produce progeny transformed with only the *luc* gene.

Other potential applications of the Cre/*lox* system include (i) clustering of transgenes into a designated genetic locus by site-specific integration, (ii) regulating gene expression by excision and inversion of DNA fragments, and (iii) rearranging the plant genome via translocation, deletion or inversion of chromosomal fragments. Our current efforts towards the use of the Cre/*lox* recombination system for gene targeting and chromosomal deletions will also be discussed.

ANALYSIS OF REGULATION OF TRANSCRIPTION FACTORS IN VIVO

G.Neuhaus[1], C.Neuhaus-Url[1], J.Potrykus[1], P. Katagiri[2], K.Seipel[2], N.-H.Chua[2], [1]Institut for Plant Sciences,ETH-Switzerland, [2]Laboratory of Plant Molecular Biology, The Rockefeller University, New York, USA.

So far microinjection technique was only used for transformation experiments. In our laboratory we have extended the range of applications by using the unique advantages of this system: 1.Injection can be performed into defined single cells, even in multicellular structures.2.Targeting of the injected substance to different cell compartments (nucleus, cytoplasm). 3. Injection of different kinds of molecules (DNA, RNA, proteins, chemicals) 4. Injection of defined quantity (titration of molecules). Using these possibilities we analysed the regulation of the transcrition factor TGA1a, a tobacco transcription activator that binds specifically to as-1 element (-83 to -63) of the CaMV 35S promoter in vivo. Results on the possible role of action of TGA 1a will be presented, as well as titration of transcription factor and plasmids was studied and will be shown.

MAIZE TRANSFORMATION

S. Brown, C. Armstrong, A. Barnason, D. Dean, R. Deaton, B. Dennehey, M. Fromm, B. Hairston, F. Morrish, M. Pajeau, W. Petersen, P. Sanders, C. Santino, S. Sims, F. Tamayo. Monsanto Company, St. Louis, MO.

Tremendous progress has been made recently in the development of systems to genetically engineer cereal crops. In the past year, several groups, including our own, have reported the production of fertile transgenic maize plants using the particle gun. We have utilized model systems such as transient gene expression assays and stable transformation of non-regenerable maize cells to develop the tools required for successful gene transfer, selection, and gene expression. Transient gene expression assay systems have been utilized as a simple and rapid means of testing new vectors and optimizing gene transfer parameters. The non-regenerable maize BMS (Black Mexican Sweetcorn) cell line has been used as a model stable transformation system. Selection schemes for BMS transformation have been developed using genes to confer resistance to both antibiotics (kanamycin/G418 and hygromycin) and herbicides (glyphosate, chlorsulfuron, and phosphinothricin). Stable transformation of BMS has demonstrated efficient co-transformation and co-expression of genes on separate plasmids.

Regenerable embryogenic suspension culture cells have been one of the most successful target tissues in the development of transformation systems to produce transgenic maize plants. Most of the selectable marker systems developed for BMS transformation have been used to produce transformed embryogenic cell lines, although the frequency is still quite low. The transgenic embryogenic cell lines can then be regenerated into fertile plants. Limited data on the transmission of the introduced genes to progeny has been obtained. We have observed the expected Mendelian segregation ratios as well as cases of reduced transmission.

One of our first applications of maize transformation is production of corn plants resistant to the European corn borer (ECB). Resistance to second brood ECB in commercial maize germplasm is inadequate and chemical control is not economical. The crystal protein endotoxins from the bacterium *Bacillus thuringensis* (B.t.) have been shown to have insecticidal properties. We have introduced B.t. genes into maize and demonstrated transmission to progeny. Leaf sections from the progeny killed nearly all the ECB larvae in laboratory feeding studies, while control sections support excellent growth. Preliminary field tests are being conducted this summer.

REPLICATING GENE TARGETING VECTORS AND BIOLISTICS FOR ARABIDOPSIS THALIANA TRANSFORMATION

Hans-Joachim Schalk and Gerald R. Fink, Whitehead Institute, Cambridge, MA

We are investigating the efficiency of homologous recombination using replicating targeting vectors that persist in the nucleus of plant cells during development. Vectors were derived from the genomes of DNA plant viruses of the caulimo- and geminivirus group. Highly efficient introduction of targeting vectors into Arabidopsis has been achieved by biolistic transformation. DNA is delivered into 10^5 cells of various tissues and is introduced into internally located cells. Two weeks after biolistic transformation of germinating seeds with GUS DNA, GUS enzyme activity can be detected in the cells of whole leaves. Using GUS as a marker we elaborated a visual test system to determine the recombination efficiency of different tissues *in planta* and to find optimal conditions for recombination between replicating targeting vectors and the chromosome. Homologous integration at multicopy sequences is being studied using vectors harboring rDNA sequences. We have constructed vectors to select for recombination in reproductive cells. These replicating targeting vectors contain a fragment of the gene for acetolactate synthase and harbor a dominant mutation conferring resistance against herbicide chlorsulfuron.

CLONING THE *ARABIDOPSIS GA-1* LOCUS BY GENOMIC SUBTRACTION

T.-P. Sun, H. Goodman and F.M. Ausubel, Department of Genetics, Harvard Medical School and Department of Molecular Biology, Masschusetts General Hospital, Boston MA 02114.

The *Arabidopsis thaliana ga-1* locus is involved in the conversion of geranylgeranyl pyrophosphate to *ent*-kaurene, an intermediate in the biosynthesis of gibberellic acid. Previously published genetic fine structure analysis by M. Koornneef (Koornneef *et al.*, Genet. Res., Camb. 41:57-68, 1983) suggested that one particular *ga-1* allele, 31.89, generated by fast neutron mutagenesis, is an intragenic deletion. We recently described a technique called genomic subtraction (Straus and Ausubel, Proc. Natl. Acad. Sci. USA 87:1889-1893, 1990) that identifies DNA sequences that are missing in a homozygous deletion mutant. The published procedure made use of a yeast model system. Using genomic subtraction, we now report the cloning of a DNA sequence that corresponds to a 5.0 kb deletion in the *ga-1* allele 31.89. Several lines of evidence indicate that the 5.0 kb deletion corresponds to the *ga-1* locus. First, RFLP mapping showed that sequences corresponding to the 5.0 kb deletion map to the *ga-1* locus. Second, several different cosmid clones containing approximately 20-25 kb inserts of wild-type DNA that span the deletion in 31.89 were used to transform mutant 31.89. The T2 generation of the transgenic plants carrying these clones did not require exogenous GAs for normal growth. Third, we identified molecular lesions in four additional *ga-1* alleles within the 5.0 kb region deleted in allele 31.89. One of these additional lesions is a 3.4 kb or larger insertion located within the most distal intron encoded by the *ga-1* locus. The three other lesions are all single base changes located within the two most distal exons. The *ga-1* locus encodes a 2.8 kb mRNA. We determined a recombination rate of 10^{-5} cM per nucleotide for the *ga-1* region of the *A. thaliana* genome.

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