Instituto Juan March
de Estudios e Investigaciones

60 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Abscisic Acid Signal Transduction in Plants

Organized by

R. S. Quatrano and M. Pagès

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The lectures summarized in this publication were presented by their authors at a workshop held on the 28th through the 30th of October 1996, at the Instituto Juan March.

Depósito legal: M. 44.561/1996
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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INTRODUCTION

R.S. Quatrano and M. Pagès

Introduction

The mechanism(s) by which phytohormones trigger physiological responses have long eluded plant physiologists. How these relatively small and simple molecules can elicit such major responses that differ from tissue to tissue during plant development has been a very difficult question to approach. However, through the use of molecular and genetic approaches, tremendous strides have been made within the last decade to elucidate the molecular basis of phytohormone action. These advances have been most notable with responses elicited by the phytohormones ethylene, auxin and abscisic acid. Overall progress in this important area of plant science research is represented in this publication that focuses on one of these phytohormones, abscisic acid (ABA). It is clear that the data accumulated from recent research has led to considerable progress in our understanding of the ABA response pathway; from perception of the ABA signal, through the expression and function of specific genes in a given physiological response. Future research, using the approaches outlined in this publication, will undoubtedly lead to the further clarification of the role of ABA in plant development and serve as paradigm by which the modes of action of other phytohormones and plant signals can be better understood.

The role of ABA in the typical life cycle of a higher plant is mostly confined to the development of the seed, and in response to environmental stresses in vegetative tissue. Levels of endogenous ABA increase during the development of the seed, and is part of a developmental pathway which promotes maturation of the seed and the acquisition of desiccation tolerance, as well as prevents precocious germination. Evidence from genetic and molecular data, as well as from embryo culture indicates that this pathway is an integral component of the developmental program within all higher plants. An early response of vegetative plant tissue to environmental stresses, such as osmotic and temperature extremes, involves increases in the endogenous levels of ABA and or in the sensitivity of cells to ABA, as an internal signal to trigger a set of responses to protect immobile plants from these perturbations. Although the signaling pathway from ABA to gene expression may be similar in seeds and in vegetative tissue, it is clear that different sets of genes are expressed in the different tissues. Hence, the importance of understanding the molecular and genetic basis of the ABA response pathway has enormous implications in agricultural practices and in engineering crops in the future with improved traits in seeds and in tolerance of environmental stresses.

Genetic approaches using the model plant *Arabidopsis* have identified mutants whose phenotypes are defective in ABA responses in both vegetative and reproductive tissues. Since one can easily map these mutant loci in *Arabidopsis*, which consists of an extremely small genome, positional cloning techniques are available to isolate the mutated genes. Also, insertional mutagenesis in *Arabidopsis*, as well as in maize, has also led to the further isolation and characterization of genes active in the ABA response pathway. Studies of two such ABA-insensitive mutants, *abi1* and *abi3*(*vp1*), have resulted in the isolation of genes that have been identified as a phosphatase and a transcriptional activator, respectively. The identification of other genes that interact with each of these gene products, as well as the substrates for their action, is the subject of intense genetic and biochemical research and study. More biochemical/cytological approaches have identified the ionic intermediates and the membrane channels responsible for these ionic fluxes that transduce the ABA signal (e.g. Ca) and drive various physiological responses to ABA (e.g. stomatal closure). Direct microinjection techniques have identified unique intermediates (e.g. cyclic ADP-ribose) that are responsible for specific gene expression at the level of ABA-responsive promoters. Furthermore, molecular approaches have detailed the cis-elements and several transacting factors (including VP1) that are critical for the transcription of ABA-responsive genes. Although the initial receptor of ABA has not been identified, new approaches are being targeted to this major unknown. Likewise, although the function of ABA-responsive genes in various physiological processes (e.g. desiccation tolerance) are not known in molecular terms, considerable progress has been made on the molecular structure and important domains of these proteins.

It is clear from these recent studies outlined in this publication, that our knowledge of the ABA signal transduction pathway has progressed greatly in the last several years, but major gaps still exist in our understanding. For example, what is the nature of the ABA receptor, what are the critical changes that occur in response to the ABA signal at the level of transcription resulting in ABA-dependent gene expression, and, what is the link between the proteins that appear in response to ABA and the tissue-specific physiological responses ?

Ralph S. Quatrano
Montserrat Pages

SESSION I. ABA AND GENE EXPRESSION

Chairperson: Ralph Quatrano

REGULATION OF EM GENE EXPRESSION: THE ROLE OF ABA AND REGULATORY FACTORS

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The Em gene is a member of a gene set which is expressed only in response to ABA during embryo maturation. This specific pattern of expression requires ABA and the product of the viviparous-1 (*vp1*) locus in maize and its homolog in *Arabidopsis ABA insensitive-3* (*abi 3*) (1). Overexpression of the ABI3 protein in vegetative tissue of *Arabidopsis* treated with ABA results in the ectopic expression of Em, indicating that tissue-specific expression is regulated by both ABA and ABI3 (2). VP1 and ABI3 proteins are over 85% identical in three regions representing over 35% of the protein (2). Vp1 has a transcriptional activation domain in its N-terminal region, suggesting a role in transcription (3).

Using a protoplast transient assay (4), we have identified a 76 bp ABA-response element (ABRE) within a 650 bp promoter of the Em gene from wheat. Overexpression of VP1 in cereal protoplasts transactivates the same Em promoter without exogenous ABA (3), possibly by making the protoplasts more sensitive to endogenous ABA. In the presence of excess ABA and VP1, a synergistic response is observed (3). A tetramer composed of a 20 bp sequence within the ABRE, which includes a G-box (5'-CACGTG-3'), can support ABA-induced expression, Vp1 transactivation and the synergy between ABA and VP1(5). Deletion of an 18 amino acid in a highly conserved region of VP1 (BR2) eliminates its ability to transactivate the Em promoter. A DNA binding protein (EmBP-1) of the basic-leucine zipper class (bZIP) can bind to the G-boxes in the ABRE(6). A 2 bp mutation in the G-box(es) reduces or eliminates the ability of the ABRE to bind EmBP-1(4,5), or to exhibit the responses to ABA and VP1 in the transient assay.

EmBP-1 and a partial VP1 without its activation domain were purified from *E. coli* as fusions with the maltose-binding protein. We have demonstrated that VP1 can greatly enhance the DNA-binding activity of EmBP-1 to the ABRE in a gel retardation assay (7). Deletion of the BR2 domain eliminates the enhancement while a 40 amino acid fragment containing BR2 can substitute for VP1. Attempts to demonstrate a physical interaction between VP1 and EmBP-1 have not yielded convincing results, but a weak interaction of BR2 with DNA can be demonstrated by UV-crosslinking. The capacity of VP1 to enhance DNA-binding activity has also been observed on transcription factors as diverse as opaque-2, max, NF-kB and Sp1. In view of a recently publication (8), it might be interesting to determine if the specificity of this activity is modulated by nucleosome positioning along the ABRE.

Previous work demonstrated that a 30 kDa protein (GF-14) is part of the transcription complex that binds to a G-box in the ADH gene from *Arabidopsis*. GF-14 has homology with a class of proteins known as the 14-3-3 proteins which participate in many signaling pathways. Using Em1a as a probe, we recently showed (9) that three different antibodies to different regions of the GF14 protein can interact with nuclear protein-DNA complexes formed with nuclear extracts prepared from untreated, ABA- and NaCl-treated embryogenic rice suspension cultures. No interactions were observed when an AT-rich region of the Em promoter was used as a probe. GF-14 antibodies recognize a doublet protein (28-30 kDa) in both cytoplasmic and nuclear extracts. A bacterially-expressed maltose-binding GF14 fusion protein (MBP-GF14) from rice, when added to nuclear extracts, can form a G -box binding complex when assayed by gel retardation. MBP-GF14 cannot bind to the G-box element alone. VP1 does not appear to be required to form G-box complexes containing GF14, since nuclear extracts prepared from maize wild and *vp1* mutant embryos show similar gel retardation patterns with and without GF14 antibodies. However, VP1 and GF-14 can interact in a yeast two-hybrid assay.

The possible role of these proteins in the regulation of Em expression will be discussed.

This research was supported in part by a grant from the NIH (GM44288) to RSQ and a training grant from the Spanish Ministry of Education and Science to Joaquin Medina.

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EXOGENOUS ABA DOES NOT MIMIC ENDOGENOUS ABA IN THE INDUCTION OF GENE EXPRESSION

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In the past ten years many genes have been identified that are responsive to ABA. A subset of these genes have been shown to require ABA (Bray, 1993). Several ABA-requiring genes from maize, *Arabidopsis* and tomato have been identified since they are not expressed in mutants of these species which are deficient in ABA biosynthesis. In tomato, four genes have been identified that are not expressed in the mutant *flacca* in response to water deficit. *Flacca* plants are blocked in the last step of the ABA biosynthetic pathway and accumulate approximately one third of the amount of ABA under well-watered situations and only 6% of the ABA of the wild type in response to a 6 h period of water deficit (Bray, 1988). Two of the four genes are *lea* genes; *le4* is a group 2 *lea*, which has also been referred to as *rab* and *dhn*, and *le25* is a group 4 *lea* and is similar to the cotton gene D113 (Cohen *et al.*, 1992). *le16* is a member of the group of genes called non-specific lipid transfer genes, although the name of these gene products does not necessarily reflect a function during stress (Plant *et al.*, 1991). *le20* is a stress-induced variant of H1 histone and is now called *H1-S*.

The ABA-deficient mutant was used to establish these genes as ABA-requiring genes; they are not expressed in the absence of ABA accumulation. Therefore, ABA is necessary for gene expression, however, it has become apparent that ABA alone is not sufficient to mimic the plant stress response with respect to expression of these genes. In transgenic plants (tomato and tobacco), in which an ABA-requiring promoter is fused to the reporter gene GUS, GUS activity in response to water deficit compared to ABA treatment alone is as much as 10-fold greater even though ABA content is similar (Imai *et al.*, 1995, and unpublished results). GUS activity is a convenient quantitative assay for gene expression, however, GUS activity can be a combination of transcriptional, post-transcriptional, translational and post-translation regulatory mechanisms. Therefore, quantitative measurements of RNA and ABA content of three of the ABA-requiring genes, *le4*, *le16* and *le25*, were made to determine if the difference can be explained at the transcriptional and post-transcriptional levels. Tomato leaves were detached and were given either different concentrations of ABA for 6 hours or were wilted to different water contents and maintained in plastic bags for a total of 6 hours. mRNA and ABA content was quantified. It was found that water deficit induced a greater mRNA content at a given ABA content. In each case there was a good correlation between ABA content and mRNA content, although in the leaves subjected to water deficit the ABA was more efficient in inducing gene expression. Therefore, there must be a transcriptional/posttranscriptional component to the different response to water deficit and ABA application. To further define these components run-on transcription experiments are currently being planned.

To determine if sensitivity to applied ABA can be altered by stress, ABA was applied to *flacca* leaves which were subsequently subjected to water deficit. mRNA and ABA content was determined. Since the ABA was applied to *flacca* leaves, only a small percentage of ABA was synthesized in response to the stress and the majority of the ABA in the leaf was ABA applied to the leaf. Water deficit increased the amount of mRNA measured compared to ABA treatment alone and actually decreased the content of total leaf ABA.

There are a number of possible explanations that might explain an increased sensitivity to ABA during periods of water deficit. These include a difference in cellular or tissue compartmentation of ABA when it is applied compared to endogenous synthesis, additional signals or signal transduction pathways may be induced by water deficit which interact with the ABA-induced signal transduction pathway(s), and/or one of the ABA metabolites may be responsible for ABA gene induction and ABA metabolism may be controlled by water deficit.

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Acclimation *vs* Shock response to water deficit: what is the role of ABA?

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While ABA has been widely demonstrated to play a fundamental role in controlling the rapid response of plants to physical changes in the environment, there is no clear evidence of its putative intervention in the ability of plant cells to maintain cellular homeostasis under persisiting stress conditions.

To compare rapid and long-term response to water stress, a potato cell population was treated abruptly with 20% PEG or acclimated by gradual transfer into a medium cointaining increasing concentration of this osmotic compound. Acclimation to water stress was accomplished by means of a set of metabolic and molecular cellular modifications, including proline accumulation, alteration in membrane lipid composition, synthesis of *de novo* proteins, changes in gene expression (Leone *et al.*, 1994 a, b; Leone *et al.*, 1996), which permitted active cellular growth at otherwise inhibiting conditions.

The two cellular systems were different in the cellular level of endogenous ABA, which did not vary during acclimation, but increased five-fold in shocked cells. Tolerance to otherwise non permitting cellular growth conditions was then acquired through the activation of ABA-indipendent genes. This was confirmed by the observation that several heterologous *lea* genes (D-11, *rab17* and *rab 28, Em*) were not up-regulated in acclimated cells.

Differential Display Reverse Transcription-PCR (DDRT-PCR) is being currently used as a strategy to clone genes specifically related to the ability of plant cells to increase tolerance through acclimation (Colonna-Romano *et al.*, 1996). Data on the first genes cloned and their role in the acclimation process will be presented and discussed.

This work was supported by a grant of the Italian Ministry of Agriculture and Forestry in the framework of the National Project "Biotecnologie Vegetali" to A.L.

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DEHYDRINS: EMERGENCE OF A BIOCHEMICAL ROLE OF A FAMILY OF PLANT DEHYDRATION PROTEINS

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Dehydrins (a.k.a. LEA D-11 family) are one of several immunologically distinct families of proteins that typically accumulate in plants in response to ABA, low non-freezing temperatures, or any environmental stimulus that has a dehydrative component, including drought, salinity, seed maturation drying, and freezing. About 100 complete dehydrin polypeptide sequences from angiosperms and gymnosperms are in databases, and there is immunological evidence of dehydrins in ferns, lycopods, mosses and liverworts, green and brown algae, cyanobacteria, and yeast (1). Their precise role is not known, but immunolocalization in plant cells (2, 3) and studies of purified dehydrins are consistent with stabilization of the basic cell architecture in the cytoplasm and nucleus. The available evidence is consistent with a hypothesis that stabilization occurs through a surfactant mechanism involving interaction of dehydrin amphipathic α-helical domains with the surface of stabilized molecules, and interaction of hydrophilic domains with the solution. Analogies can be drawn between the hydrophobic interaction component of this model and chaperone-assisted protein folding (4) or apolipoprotein-lipid interactions (5). The model also takes into consideration the possibility that stabilization is enhanced by exclusion of the hydrated hydrophilic domains of dehydrins from hydrated compatible solutes (6). Finally, the presence of a phosphorylatable serine tract (3) at one or both ends of the consensus blocks is taken into consideration as a molecular switch that may mediate dehydrin activity, with an analogy being made to the role of phosphorylation of serines modulating the binding of HSP27 to actin filaments (7). Since several families of plant stress proteins have similar primary amino acid characteristics (putative amphipathic α-helical domains and hydrophilic domains), the mode of action of dehydrins and these proteins may be fundamentally similar (8, 9).

Some dehydrin (*Dhn*) genes have been placed on linkage maps. Three barley chromosomes (4H, 5H, 6H) carry clusters of *Dhn* genes. The main seed dehydrin (*Dhn4*) and the main cold-induced dehydrin (*Dhn5*) map to 6H. The main freezing tolerance QTL in a doubled haploid mapping population from a cross of winter (Dicktoo) X spring (Morex) barley maps to 5H at a position that overlaps another cluster of *Dhn* loci that presumably encode low MW dehydrins (10, 11). This region of the Triticeae (wheat, barely, rye and relatives) genome is of particular interest since it has been shown in several crosses to be the location of genes controlling winter versus spring growth habit, vernalization responses, and freezing tolerance. Included in this list are barley *Sh2*, wheat *Vrn1*, *Vrn3*, *Vrn4, and Fr*, and rye *Sp1* (11). In addition, a determinant of drought-induced ABA accumulation in wheat has been mapped to this region (12).

We and others found in barley that the high MW (apparent MW 83 kD) *Dhn5*, which does not map to the major freeze-tolerance QTL, is expressed during mild cold acclimation conditions, both in controlled environments (13) and in the field (Riverside, CA), and under these conditions there is little or no expression of the low MW *Dhn* genes that are tightly linked to the main freezing tolerance QTL. In contrast, we found that several low MW *Dhn* genes are expressed under harsher field conditions (Saskatoon, Canada [with cooperation of Ping Fu and LV Gusta]), which evoke a larger difference in freezing tolerance between

Dicktoo and Morex. Consistent with a difference in freezing tolerance, expression of low MW *Dhn* genes is highest in Dicktoo (the more tolerant cultivar). Laboratory experiments conducted as a follow-up to these observations lead us to conclude that non-lethal freeze-thaw stimulates expression of chromosome 5H and other *Dhn* genes. Since similar size (low MW) dehydrins are also produced in drought-stressed seedlings and in developing embryos, one explanation of these observations is that drought stress or ABA is the primary inducer of low MW *Dhn* gene expression on chromosome 5H. If this is true, then the basis of the freeze-tolerance QTL on barley 5H (if it is indeed a manifestation of *Dhn* gene expression) may be an ABA response rather than a direct response to temperature. We are now developing fine structure maps of the *Dhn* clusters on chromosome 5H in Dicktoo, Morex and doubled haploids that represent recombination in this region to facilitate further tests of linkage between *Dhn* genes and freezing tolerance. This research was supported, in part, by NSF grant IBN-9205269 and USDA/CSREES grant 95-37100-1595.

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SESSION II. ABA AND GENE EXPRESSION

Chairperson: Montserrat Pagès

REGULATION OF ABA AND WATER STRESS RESPONSIVE GENES

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The maize ABA responsive genes are induced during late embryogenesis when ABA levels are high, and they are also ABA and water stress inducible in young embryo and vegetative tissues. Several genes homologous to *rab17* and *rab28* have been identified in different plant systems.
A genetic study on the role of ABA in the control of *rab* gene expression in ABA deficient and ABA insensitive mutants of maize and transgenic Arabidopsis showed a differential regulation of the *rab* genes in embryos and vegetative tissues. Moreover, gel retardation experiments have shown that distinct protein factors bind to the same ABRE element in embryo and leaves.
We performed *in vivo* footprinting and transient transformation of *rab17* in embryos and vegetative tissues to characterize the *cis*-elements involved in the regulation of the gene. Differences in the footprints indicated that distinct proteins interacted with these elements in the two tissues suggesting that the regulation of the promoter changed during development.
By transient transformation, the *cis*-acting sequences were divided in embryo-specific, ABA-specific and leaf-specific elements on the basis of protein binding and the ability to confer expression of *rab17*.
The general pattern of expression of Rab proteins indicate that they are synthesized during normal embryogenesis at the period where ABA increases, they accumulate in mature and dry embryos, disappearing during the first hours of germination. The Rab proteins are also synthesized in ABA-treated or drought-stressed vegetative tissues. Therefore these proteins are good candidates to be involved in the desiccation tolerance of the cells.
The rab-17 protein accumulates in maize embryos as a highly phosphorylated protein. Phosphorylation by casein kinase 2 in vitro is restricted to the serine cluster region of the rab-17 protein.
The subcellular localization of Rab17, and Rab28 proteins was performed by using optical and electron microscopy. We have localized the Rab17 protein to the nucleus and cytoplasm of embryogenic cells. Rab28 has been found in the nucleolus and is highly abundant in vascular cells. Transgenic plants overexpressing Rab proteins have been obtained and analyzed for subcellular targeting and possible role in salt and water stress tolerance. Preliminary data seeems to indicate that transgenic plants of Rab17 are more resistant to different concentrations of NaCl.

Novel plant Ca2+-binding proteins expressed during late embryogenesis and in response to abscisic acid and osmotic stress

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A cDNA corresponding to an mRNA which accumulates in germinating rice seeds in response to the phytohormone abscisic acid was isolated by differential hybridization (1). Northern blotting indicated that the mRNA also accumulates in vegetative tissues in response to treatment with abscisic acid and to osmotic stress. Sequencing identified a major open reading frame encoding a novel protein of 27.4kDa. The identity of the open reading frame was confirmed by comparing the translation products of cellular, hybrid selected and in vitro transcribed RNAs, and by immunoprecipitation.

Database searches indicated that two similar proteins are expressed in Arabidopsis (2). Sequence analysis indicated that the rice and Arabidopsis proteins may contain conserved features. First, they may contain a single Ca+2-binding EF-hand in the N-terminal region (3). This was confirmed by showing that a fusion protein purified from E. coli containing the EF-hand region of the rice protein bound Ca2+ in blot binding assays. Second, they may contain a single transmembrane helical domain (4). In line with this, western blotting of rice and Arabidopsis cellular extracts indicated that the protein is associated with microsomal or membrane fractions. Third, the proteins may contain a single tyrosine phosphorylation site and two casein kinase phosphorylation sites in their C-terminal regions (5). We are currently analyzing the phosphorylation patterns of the Arabidopsis proteins using antisera raised against them and against phosphotyrosine. We are also analyzing Arabidopsis lines transformed with sense and antisense EFA27 constructs, and aim to map the genes by RFLPs in recombinant inbreds and YAK hybridization.

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ROLE OF ABA IN GENE EXPRESSION AND SIGNAL TRANSDUCTION IN *ARABIDOPSIS* PLANTS UNDER WATER STRESS

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Plants respond to conditions of drought through various physiological and developmental changes to tolerate the stress. ABA is produced under water deficit conditions and plays important roles in tolerance to drought. Recently, a number of genes have been described that respond to drought at the transcriptional level. Most of the genens that have been studied to date are also induced by ABA. It appears that dehydration triggers the production of ABA, which, in turn, induces various genes. To understand the molecular process of signal transduction from initial water-stress signal to gene expression, we cloned 25 cDNAs (named RD or ERD) for genes that are induced by water stress in *Arabidopsis thaliana* by differential screening (1, 2). In the present study we report the expression of three genes, *rd29A*, *rd29B*, and *rd22* which are induced by ABA. The GUS reporter gene driven by these *rd* promoters was induced at significant level by dehydration and ABA in transgenic *Arabidopsis* and tobacco. We precisely analyzed the *rd29A* promoter and identified a novel *cis*-acting element containing 9 bp, TACCGACAT (DRE, Dehydration Responsive Element), that is involved in dehydration-responsive expression (3, 4). DRE is involved in the induction by low temperature or high salt conditions, but does not function in ABA-responsive slow expression of *rd29A*. Different *cis*-acting elements seem to function in the dehydration responsive expression in *rd29A* and *rd29B* .

The induction of an *Arabidopsis* drought-inducible genes, *rd22*, is mediated by ABA, but requires protein biosynthesis for its ABA-dependent expression (5). A 67-bp region of the *rd22* promoter is essential for its ABA-responsive expression, and contains several conserved motifs of DNA binding proteins, such as MYC and MYB, but no ABREs (6). Recently, a cDNA for a transcription factor MYC homologue, designated rd22BP1, was cloned by southwestern method using the 67-bp DNA as a probe. The expression of *rd22BP1* gene was rapidly induced by dehydration and treatment of ABA in Arabidopsis rosette plants and suspension-cultured cells, which suggests that drought-inducible MYC homologue may function in the ABA-inducible expression of *rd22* (Abe, Yamaguchi-Shinozaki and Shinozaki, unpublished data). On the other hand, a cDNA for a transcription factor MYB homologue, Atmyb2, by screening a cDNA library prepared

from dehydrated *Arabidopsis* plants (7). The *Atmyb2* gene is induced by dehydration, high salt and ABA treatment. The recombinant ATMYB2 protein was shown to bind to the myb recognition sequence found in the 67-bp region of the *rd22* promoter. These results indicate that drought- or ABA-inducible MYB and MYC may function in the ABA-responsive gene expression under drought stress, and that there are two independent transcription system for ABA-resposive gene expression, one is ABRE/bZIP system and the other may require de novo synthesis of ABA-inducible transcription factors. Moreover, there is an additional signal transduction pathway which may function only in drought response. Therefore, there are at least four independent signal transduction pathways between initial drought-stress and gene expression (8). The existence of complex signal transduction pathways in drought-stress responses of plants gives a molecular basis for the complex physiological responses of plants to drought stress.

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Regulation of the Arabidopsis Em genes during seed maturation and in response to ABA

The Em genes belong ot the class I Late Embryogenesis Abundant (LEA) protein genes which are abundantly expressed during the seed desiccation phase. There are two genes in *Arabidopsis* : AtEm1 located on chromosome 3 and AtEm6 located on chromosome 2. They differ by the presence in AtEm1 of 4 repeats of 20 aminoacids which are present only once in AtEm6. Both genes were cloned in Arabidopsis and their differential expression during development and in response to ABA was analysed using northern blots, *aba* and *abi* mutants, specific antibodies and transgenic plants.

Both genes are differentially expressed during development. They are completely dependant upon ABI 3 expression but some alleles of *abi3* can discriminate between the two genes. The two genes can be differentially expressed at a basal level in roots with a strong specificity of AtEm1 for provascular tissues. They are both ABA responsive in roots of young seedlings but cannot be induced in leaves unless ABI 3 protein in ectopically overexpressed.

AtEm1 promoter was dissected by deletion analysis and we could define several regions which are required for enhanced level of expression, response to ABA and to ABI3. Transgenic plants expressing the GUS reporter gene under the control of AtEm1 promoter were used in mutagenesis experiments and we selected several types of mutants. One class down regulates the reporter gene as well as the endogenous genes, while the other one up regulated the reporter gene.At least one of the down regulated mutants is not allelic with *abi 3* and its germination is sensitive to ABA as the wild type.

In an attempt to understand the function of the AtEm genes we regenerated several antisens lines. Preliminary data suggest that extinction of Em1 gene might alter dormancy.

MOLECULAR REGULATION OF *PKABA1*, A WHEAT PROTEIN KINASE mRNA RESPONSIVE TO ABA

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Elucidation of ABA signal transduction in plants requires identification of signalling components that are unique to plants. In wheat we have identified a novel protein kinase mRNA, *PKABA1* (protein kinase - ABA responsive) that accumulates in response to applied ABA, cold temperature treatment or dehydration. The deduced *PKABA1* kinase sequence has the conserved features of a serine-threonine protein kinase with a characteristic stretch of acidic amino acid residues beyond the catalytic domain. *PKABA1*-like protein kinases have now been identified in several types of plants, but no similar protein kinase sequence has been reported in animals or microorganisms. We report here the rapid down-regulation of the *PKABA1* transcript with recovery from stress, the identification and mapping of a second *PKABA1*-like protein kinase, and initial biochemical characterization of the expressed protein kinase. Within 1-2 h of dehydration of wheat seedlings, there is a 500-fold increase in ABA, and up-regulation of *PKABA1* mRNA levels. Seedlings subjected to cold temperature (2°C) exhibit an increase in ABA levels by 10 h and *PKABA1* mRNA levels are up-regulated by 12 h. The seedlings exhibit rapid recovery from stress. When dehydrated seedlings are resupplied water or cold-treated plants are returned to normal growing conditions, ABA levels decline rapidly and *PKABA1* mRNA levels decline to basal levels within 2 hours. The *PKABA1* kinase mRNA can be repeatedly induced by alternating cold and warmer temperatures. A second *PKABA1*-like protein kinase, called *TaPK3*, has been identified that accumulates in greening seedlings. Accumulation of *TaPK3* mRNA in greening tissue coincides with accumulation of light-responsive *cab1* mRNA. Use of *TaPK3*-specific gene probes shows that this gene is not ABA responsive. Both kinases map to the same locus on homoeologous group 2 chromosomes of wheat, suggesting that these two kinase genes are tandem or clustered. The PKABA1 protein kinase has been expressed in a bacterial system using a pET vector system, and demonstrated to have kinase activity including autophosphorylation and casein phosphorylation activities. Constructs of *PKABA1* and a mutant version of *PKABA1* are being produced for evaluation in a transient gene expression system. This characterization of the ABA-responsive *PKABA1* kinase and related kinases is serving as the basis to examine the role of ABA-responsive protein kinases and protein phosphorylation/dephosphorylation in ABA signal transduction.

SESSION III. ABA SIGNAL TRANSDUCTION: GENETIC AND MOLECULAR ANALYSIS

Chairperson: Maarten Koornneef

The Genetic Analysis of ABA biosynthesis and Action with special emphasis on Seed Dormancy.

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The plant hormone abscisic acid (ABA) regulates a number of physiological and developmental processes, such as stomatal closure, embryo dormancy and the adaptation to environmental stresses. ABA deficient mutants have been isolated in a number of plant species. In Arabidopsis mutants at the *aba1* locus were isolated as germinating revertants of gibberellin (GA) deficient mutants that normally require GA for germination (Koornneef et al. 1982). Mutants that do not require GA for germination can also be isolated by their ability to germinate in the presence of inhibitors of GA biosynthesis such as paclobutrazol and tetcyclacis. This selection procedure led to the isolation of new alleles at the *ABI3* locus (Nambara et al. 1992) and mutants at the loci *ABA2* and *ABA3* which affect ABA biosynthesis. The physiological characterization of these mutants indicated the lack of seed dormancy and enhanced water loss of excised leaves, which is characteristic for ABA biosynthesis mutants in all species investigated. All mutants at these loci are leaky as shown by their reduced but not absent ABA levels and by the more extreme deficiency phenotype of the *aba2,aba3* double mutant (Léon-Kloosterziel et al. 1996b). Biochemical analysis showed that these new loci *ABA2* and *ABA3* affect the last two steps of ABA biosynthesis, which are respectively the conversion of xanthoxin into ABA aldehyde and the conversion of ABA aldehyde into ABA (Schwartz et al. 1996).

Mutants of at least three additional loci exhibiting wiltyness and reduced seed dormancy are being characterized for their sensitivity to ABA, GA inhibitors, water relations and ABA levels. One of the mutants was identified in a population containing active En/I transposable elements (Pereira pers. comm) which may allow the cloning of this gene in the near future.

Since seed dormancy is affected in all ABA biosynthesis and ABA response mutants thus far, the involvement of this plant hormone in this process is well established. This target of ABA action can be separated from the effect of ABA on stomatal behaviour e.g. in *abi3* mutants (Koornneef et al. 1984). The latter predicts that mutants specifically affecting seed dormancy but otherwise responsive to ABA might be identified. Such mutants, isolated on the basis of their reduced dormancy led to the identification of the reduced dormancy 2 (*RDO2*) locus. This *rdo2* mutant has an enhanced resistance to GA biosynthesis inhibitors but with wild type ABA levels and wild type sensitivity to ABA. Another mutant (*rdo1*) has a reduced dormancy but does not show resistance to GA inhibitors indicating that dormancy might be controlled by a pathway controlled by ABA and GA and other factors not related to these two hormones (Léon-Kloosterziel et al. 1996a).

Research on seed dormancy in Arabidopsis, is hampered by the relatively low level of dormancy in freshly harvested seeds of the commonly used laboratory ecotypes L*er* and Col, which disappears rapidly upon dry storage, . However, other ecotypes such as Cvi show very strong dormancy. By using molecular markers and a Recombinant Inbred Lines (RIL) mapping population derived from the cross L*er* x Cvi, we were able to locate genes controlling this trait. The isolation of Ler genotypes introgressed with Cvi alleles at these dormancy loci is in progresss and the map based cloning of such genes is anticipated.

Seed dormancy being part of the seed maturation process, is in addition to ABA controlled by developmental factors involved in this process. Seed maturation in Arabidopsis is controlled by at least four major control genes; *ABI3, FUS3, LEC1 and LEC2*, identified on the basis of viviparous and desiccation sensitive mutants at these loci (Meinke et al.1994). The effect of these mutations on their germination behaviour during development has been analysed with respect to their GA requirement. The isolation of such mutants in T-DNA and transposon containing lines will facilitate the cloning of these genes.

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ABA SIGNALLING IN ARABIDOPSIS: THE *ABI1* AND *ABI3* GENES

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To begin dissecting the abscisic acid (ABA) signalling network, we have cloned and characterised the *ABI1* and *ABI3* genes, two of the known loci controlling ABA sensitivity in *Arabidopsis* [1].

The semi-dominant *abi1-1* mutation results in reduced seed dormancy and impairs a wide range of ABA responses in vegetative tissues. In particular, this mutant transpires excessively as a result of abnormal stomatal regulation. The *ABI1* gene has been cloned independently by us [2] and by Meyer et al. [3]. The C-terminal domain of the predicted ABI1 protein is related to the 2C class of serine-threonine phosphatases. A combination of in vitro assays and yeast mutant complementation studies confirmed that ABI1 is a functional protein phosphatase 2C [4]. The *abi1-1* mutation converts Gly_{180} to Asp, and in the above test systems, causes a partial loss of the ABI1 phosphatase activity. In transgenic *Nicotiana benthamiana* guard cells, the *abi1-1* gene reduces ABA sensitivity of both the outward- and the inward-rectifying potassium channels in the plasma membrane [5]. However, normal sensitivity of both potassium channels to, and stomatal closure in, ABA was recovered in the presence of protein kinase antagonists. These results suggest that ABI1 is part of a phosphatase/kinase pathway that modulates the sensitivity of guard-cell potassium channels to ABA. To identify other components of the ABI1-regulated pathway, we have screened for genetic suppressors of the *abi1-1* mutation, as well as for ABI1-interacting proteins using the yeast two-hybrid system. Current status of these approaches will be presented.

The *ABI3* locus encodes a putative seed-specific transcriptional activator homologous to the maize VP1 [6, 7]. The severe *abi3-4* mutation impairs the expression of many but not all members of the various gene expression programs associated with mid- and late-embryogenesis. Comparative analysis of the ABA-deficient *aba* mutant indicates that ABI3 participates in ABA-related and additional developmental pathways controlling gene expression in seed [7]. Interactions between *ABI3* and other *Arabidopsis* loci known to affect late embryogenesis are presently being characterised.

ABI1 and ABI3 have been proposed to act in separate ABA signalling pathways. However, ectopic expression of *ABI3* confers to transgenic plantlets the ability to accumulate the seed-specific *AtEm1* mRNA in response to ABA, and the *abi1* mutation inhibits this ABI3-dependent induction of *AtEm1* by ABA. Furthermore, ectopic expression of *ABI3* also influences diverse *ABI1*-dependent responses that pre-exist in wild-type vegetative tissues. These data demonstrate that *ABI1* and *ABI3* genetically interact in controlling various ABA responses in transgenic vegetative tissues, and suggest that the endogenous *ABI1* and *ABI3* genes may act in a common ABA signalling pathway in seed [8].

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Genetic analysis of ABA-mediated control of plant growth

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The long term goal of our research group is to elucidate the molecular events that mediate the regulation of plant growth and stomatal aperture by ABA. We are pursuing a genetic approach to accomplish this aim by identifying ABA response mutants of *Arabidopsis* and employing positional cloning strategies to isolate the affected genes.
A screening program comprising of more than 100,000 M2 seedlings of *Arabidopsis* resulted in the isolation of 22 mutants that are insensitive to ABA-mediated inhibition of growth. Subsequently, the mutants were subgrouped based on three ABA responses: seed dormancy, regulation of water status, and growth inhibition into 1) pleiotropic mutants insensitive to ABA in all tested responses, 2) mutants with 2 impaired responses, and 3) those that are solely affected in the ABA growth response[1]. Allelism analysis revealed that they represent 8 new loci, *gca1* to *gca8* (growth control via *A*BA), besides the well-characterized pleiotropic mutants *abi1* and *abi2*.

ABI1 locus has been cloned[2] and its gene product has been characterized as a Mg^{2+}-dependent protein serine/threonine phosphatase of type 2C that is highly affected in its activity by changes in pH and free Mg^{2+} concentrations[3]. Surprisingly, a unique amino acid exchange in the primary structure of the ABI1 protein results in both the ABA-insensitive dominant phenotype of the *abi1* mutant as well as in a strongly reduced phosphatase activity *in vitro*. This finding suggests that the *abi1* gene product is part of a protein complex which acts as a repressor of ABA responses. Thus, the mutant ABI1 protein phosphatase could incompletely deactivate a repressor upon ABA signaling.

In order to identify such interacting components of ABI1 we are engaged in cloning those loci also providing pleiotropic alterations of ABA sensitivity such as *abi2* and *gca2*. In addition, protein-protein interaction cloning strategies are employed to identify binding partners of ABI1. Currently, a candidate protein with unique features is characterized.

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ISOLATION AND CHARACTERIZATION OF *frs1*, A MUTANT OF ARABIDOPSIS AFFECTED IN ITS CAPABILITY TO TOLERATE FREEZING TEMPERATURES

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Many plants from temperate regions increase their freezing tolerance after being exposed to low-nonfreezing temperatures. This process is known as cold acclimation (1) and involves a number of biochemical and physiological changes including levels of sugars and proteins, plasma membrane composition, phytohormone levels and gene expression (2). Different lines of evidence suggest that ABA has an important role in the process of cold acclimation. First, low temperatures can increase the ABA levels in different plants (3). Second, exogenous application of ABA can induce freezing tolerance in a number of plants (4). Third, the ABA-deficient mutant *aba1* of Arabidopsis is less freezing tolerant (5). Fourth, several low temperature-induced genes are also responsive to exogenous ABA (2).

In an attempt to identify genes involved in freezing tolerance, we have used EMS mutagenized populations of Arabidopsis to select mutants showing an altered cold acclimation response. We have isolated a freezing sensitive mutant (*frs1*) that shows a reduced cold acclimation response. This reduction is caused by a single recessive mutation that we have mapped on chromosome 1. The *frs1* mutation causes a general decrease in freezing tolerance in both acclimated and nonacclimated plants, suggesting that the *FRS1* gene is involved in freezing tolerance. Phenotipycally, *frs1* mutant plants are smaller and darker than the L*er* wild type and show a wilty phenotype. Furthermore, dehydration experiments show that they lose water three fold faster than the wild type. Interestingly, exogenous treatments with ABA are able to partially rescue the wilty phenotype, the capability to tolerate dehydration as well as the capability to tolerate freezing tmperatures. These results suggest that the *frs1* mutant could have altered ABA levels. Experiments are underway to analyze the levels of endogenous ABA, and to characterize the expresion patterns of different Arabidopsis cold-regulated genes we have isolated. These data will be discussed to point out the relevance of ABA in maintaining plant hydric equilibrium and, as a consequence, freezing tolerance.

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COLD ACCLIMATION IN *ARABIDOPSIS*

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The ability to cold acclimate is a common adaptive response in plants native to temperate climates and results in enhanced tolerance to freezing stress, allowing plants to survive the severe cellular dehydration caused by extracellular freezing. We have shown that in *Arabidopsis* the acclimation response is triggered by low nonfreezing temperatures, drought as well as exogenous application of ABA. Using ABA-deficient and ABA-insensitive mutants we could demonstrate that ABA controlled processes appear essential for the development of freezing tolerance.

The acclimation process has been associated with altered gene expression and we have characterized a number of such low-temperature-induced (*lti*) genes from *Arabidopsis*. All of the genes isolated were shown to respond to ABA as well as to low temperature and drought, although different genes exhibited differential expression to these stimuli. With the help of mutant studies we have demonstrated the presence of distinct ABA-independent and ABA-mediated signal pathways that control the expression of *lti* genes. According to the requirement of ABA in their expression the genes isolated sofar seem to fall into different categories including ABA-dependent as well as ABA and low temperature responsive but ABA-independent genes. We are currently characterizing components of the ABA-pathway. Our recent studies have also indicated the involvement of Ca in the sensory process that leads to cold acclimation in *Arabidopsis*.

Regulation of zeaxanthin epoxidase gene expression in *Nicotiana plumbaginifolia* and *Arabidopsis thaliana*

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Abscisic acid is a plant hormone involved in seed development and germination and in plant tolerance to various stresses. In higher plants, ABA is synthesized from C40 carotenoid precursors, e.g. zeaxanthin, violaxanthin and neoxanthin.
Mutants deficient for ABA biosynthesis have been isolated in different plant species and have contributed to the clarification of the pathway. They show an early germinating phenotype and a strong tendency to wilt and can be restored to wild type phenotype by application of exogenous ABA.

An ABA-deficient mutant *aba2* of *Nicotiana plumbaginifoiia* was isolated by *Ac* transposon tagging (Marin *et al.*, 1996, EMBO J 15, 2331). It is impaired in the first step of ABA biosynthesis which is catalyzed by zeaxanthin epoxidase. *aba2* mutation was linked to *Ac* insertion and *Ac* flanking sequences were used to screen a *N. plumbaginifolia* cDNA library. The cloned cDNA has been shown to complement *N. plumbaginifolia aba2* mutation and *Arabidopsis aba1* mutation (Koornneef *et al.*, 1982, Theor Appl Genet 61, 385). It encodes a 72 kDa chloroplastic imported protein, sharing similarities with different monooxigenases and oxidases of bacterial origin. ABA2 protein, produced in *Escherichia coli*, catalyzes *in vitro* the conversion of zeaxanthin into antheraxanthin and violaxanthin.

An homologous cDNA of *Arabidopsis thaliana* has been isolated and zeaxanthin epoxidase gene expression is currently studied in both plant species by northern analysis. In leaves, mRNA accumulation is high at the beginning of the day, then decreases and is undetectable during the dark period. In constrast, mRNA accumulation is very low in roots and undetectable during seed development. During water stress, mRNA levels do not significantly vary for several days.

Transgenic plants of *N. plumbaginifolia* carrying sens and antisens constructs under control of 35S promoter have been obtained. Preliminary results show a good correlation between mRNA synthesis and zeaxanthin epoxidase activity. Consequences on stress tolerance and seed germination are now studied and will be presented.

SESSION IV: ABA SIGNAL TRANSDUCTION: GENETIC AND MOLECULAR ANALYSIS

Chairperson: Donald R. McCarty

Genetic control and integration of maturation and germination programs of seed development in maize.

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The viviparous mutants of maize identify genes that control the developmental regulation of the synthesis and perception of ABA in the developing seed. These genes illuminate the mechanism which integrate ABA signalling with other developmental processes. On the perception side we have focused on the *vp1* gene which encodes a seed specific transcriptional activator (1, 2,4,5) and repressor (3) that is required for expression of many ABA regulated genes. The biochemical mechanisms underlying the complex functions of VP1 have been elusive, but are now beginning to yield to in vitro analysis. Studies of the VP1 regulated C1 and Em genes have identified at least two distinct classes of cis-regulatory sequences, the Sph and G-box elements, that are capable of mediating VP1 transactivation (1, 2,5). Functional analyses of the VP1 protein indicate that the conserved B3 domain of VP1 is absolutely required for activation of Sph coupled genes, where as, a truncated protein lacking B3 is capable of at least partial activation of G-box coupled promoters. The B3 domain is found to have a cryptic DNA binding activity. Intact recombinant VP1 does not bind Sph or G-box sequences detectably in DNA binding assays: however, when expressed as an isolated peptide, the 140 amino acid B3 domain exhibits sequence specific binding to the Sph element. Non-functional mutants of the Sph element (1) and G-box sequences (5) bind nonspecifically to B3, but do not competitively inhibit Sph binding. DNAse I footprinting analyses show excellent an agreement between bases involved in B3 binding and the functionally defined Sph element. The DNA binding activity of the B3 domain shows a critical dependence on protein concentration. In the effective protein concentration range binding is highly cooperative with a Hill coefficient of 6, indicating that B3 may bind as hexamer. The cryptic nature of B3 DNA binding suggests that interactions of other domains in the VP1 protein inhibit or regulate the DNA binding activity. These salient features of the B3 DNA binding activity are discussed in terms of a model for signal integration in C1 promoter.

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Characterization of genes encoding putative regulatory molecules in the ABA signal transduction pathway in the resurrection plant *Craterostigma plantagineum*

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The role of ABA in mediating stress responses such as dehydration, high salinity or cold has been established by molecular studies identifying genes responsive to both stress and ABA. The resurrection plant *Craterostigma plantagineum* is being used as an experimental model system to study the role of ABA in regulation of gene expression triggered by dehydration. This plant can recover from severe desiccation within 24 hours of contact with water. In addition to whole plants *Craterostigma* callus is used to study the involvement of ABA in gene induction and desiccation tolerance, as callus is not normally desiccation tolerant and has a strict requirement for exogenously applied ABA to withstand drying. During drying or ABA treatment novel gene products accumulate rapidly in leaves and other tissues. Many of such response genes have been characterized including a set of genes homologous to late embryogenesis abundant (LEA) genes in seeds.

The following approaches are being used to isolate genes which regulate the expression of LEA genes:
- Characterization of proteins which interact with promoters of ABA responsive genes.
- Isolation of mutants in the signal transduction pathway using an activation tagging approach.
- Using the differential display technique to isolate transcription factors differentially expressed and active during the early steps of drought stress.

From these approaches several genes have been isolated which have a potential role in gene regulation and signal transduction during ABA mediated gene expression. The nature of the genes and therefore also their functions in the signal

transduction chain are very different. Two genes considered are probably transcriptional activators and are closely related to the *Arabidopsis* ABI3 gene and a leucine zipper protein, respectively. The other two genes under study are likely to function early in the signalling cascade downstream of ABA. The possible involvement of these genes in ABA mediated gene expression will be discussed.

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Modular nature of abscisic acid responsive promoter complexes: structural features and related signal transduction

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The promoters of two ABA/stress induced barley genes, *HvA1* and *HvA22*, have been analyzed following both the loss- and the gain-of-function approaches. The modular features of two minimal promoter units, ABA response complexes 1 and 3 (ABRC1 and ABRC3), which are necessary and sufficient for ABA induction have been defined. The ABRC1 consists of an ACGT-core containing box and a downstream distal coupling element (CE1), and the ABRC3 is made of an ACGT-core containing box linked to a upstream coupling element (CE3). Exchange experiments have been conducted to study the interaction among modular elements in these ABRCs. The two ACGT-boxes in these ABRCs are interchangeable, indicating that an ACGT-box can interact with either a distal or a proximal coupling element to confer ABA response. On the other hand, the two coupling elements are not fully exchangeable. Although CE3 can function either proximal or distal to the ACGT-box, CE1 is only functional at the distal position. The presence of both the distal and the proximal coupling elements has a synergistic effect on the absolute level of expression as well as on the ABA induction. These ABRCs function in both seed and vegetative tissues. In seeds, ABA induction of the ABRC containing the proximal CE3, but not the ABRC with the distal CE1, is enhanced in the presence of the transcription regulator, Viviparous-1 (VP1), indicating these two ABRCs are mediated by different ABA signal transduction pathways.

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A Bipartite Model of Developmental and ABA Regulation of the Carrot *Dc3* Gene

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Abscisic acid (ABA) mediates the response to a number of environmental stresses, including drought, cold, and salt, and it also plays a significant role in embryo development and seed maturation (reviewed in Thomas et al., 1996; Giraudat et al., 1994; Thomas, 1993). ABA levels peak in the developing seed prior to desiccation; this is accompanied or followed by the expression of a phylogenetically conserved group of genes known as late embryogenesis abundant (*lea*) genes. Expression of some *lea* genes is modulated by endogenous ABA in embryos. Furthermore, many *lea* genes can be induced in immature embryos and non-embryonic tissues by water stress or treatment with exogenous ABA (Thomas et al., 1996; Vivekananda et al., 1992).

A number of ABA response genes including cold-, and drought-inducible genes contain ABA responsive *cis*-regulatory elements with the consensus CACGTG. However, promoter deletion and mutation analysis of several ABA-responsive genes subsequently demonstrated that not all of these genes contain this consensus, and in some cases sequences containing the CACGTG consensus are either not functional or are insufficient for ABA response (reviewed in Thomas et al., 1996).

Dc3, a *lea* class gene, is normally expressed in developing seeds and in vegetative tissues in response to drought and exogenous ABA. To identify the *cis*-elements involved in ABA response, various 5' upstream deletions including internal deletions and site-directed mutations were constructed; the effect of these mutations on GUS reporter gene expression was studied in transgenic tobacco. These studies showed that the distal promoter region (-1500 to -117) confers ABA responsive expression in vegetative tissues and the proximal promoter region (PPR; -117 to +26) confers seed-specific expression and does not respond to ABA. A 100 bp fragment (-449 to -351) confers ABA-inducibility to the PPR in vegetative tissues but not to a minimal CaMV 35S promoter. Interestingly, there are no Em-like consensus sequences in this region; instead repeated TTTCGTGT motifs were found. These results are consistent with the PPR being necessary for ABA induction of *Dc3* in vegetative tissues even though the PPR itself does not respond directly to exogenous ABA. Furthermore, these results suggest that combinatorial interactions of *Dc3 cis* regulatory elements with their cognate *trans*-acting factors occur during the response to ABA.

We employed a modified version of the yeast one-hybrid system to clone factors that bind to the promoter region of *Dc3*. 25 million yeast transformants were screened in a single experiment; we isolated 9 independent cDNA clones which specifically bind to functional *cis*-regulatory elements in the *Dc3* promoter. These cDNAs represent three different mRNA species which encode two distinct basic leucine zipper (bZIP) proteins. The proteins, named DPBF-1 and 2 (Dc3 Promoter-Binding Factor-1 and 2), respectively, are nearly identical to each other and are similar to the *Arabidopsis* G-box binding factor GBF-4 in their basic regions. Outside the basic region, however, DPBF-1 and 2 diverge significantly from each other and from other known factors. Both factors have transcriptional activity in yeast and bind to DNA as homo- and heterodimers. Compared to other bZIP proteins, DPBF-1 and 2 recognize a broader range of DNA sequences that contain the consensus ACACNYG (Y=C or T). The role of DPBF-1 and 2 in the control of *Dc3* will be discussed.

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Regulation of Transcription by Abscisic Acid.

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During the late phase of seed development, the accumulation of characteristic sets of mRNAs and proteins called LEA are induced by ABA. A seed specific transcriptional activator VP1 is implicated in the regulation of LEA and other ABA-regulated genes by ABA.

To study the molecular mechanism of transcriptional regulation by ABA, we analyzed *cis*-elements in the promoter of *Osem* gene (a rice LEA gene) that are required for the regulation by VP1 and ABA using a rice protoplast transient assay system. The analyses revealed that a G-box like sequence designated motif A and an adjacent sequence designated region I are required for both ABA and VP1 regulation. Since G-box like sequences have been shown to be typical binding sites for a group of plant bZIP proteins, a possibility is suggested that VP1 might interact with a bZIP protein. We have cloned several bZIP proteins from rice embryo, one of which, *OSBZ8* was found to be induced by ABA. The induction of *OSBZ8* was not inhibited by cycloheximide and preceded the induction of other ABA-regulated genes such as *Osem* and *Rab16*, which were inhibited by cycloheximide. These results strongly suggest that is involved in the regulation of transcription by ABA. We have also isolated *OSBZ8* genomic clone and sequenced it. The *OSBZ8* gene consists of 12 exons. The basic and the leucine-zipper regions are separately encoded by the exons 10 and 11, respectively. The promoter region of *OSBZ8* contains several G-box like ABRE sequence, suggesting that the expression of *OSBZ8* is self-regulated.

We have been making an attempt to genetically identify the genes involved in ABA-responsive gene expression using transgenic Arabidposis. We have made transgenic Arabidposis carrying the luciferase gene fused to the promoter of *Cor15a*, which has been shown to be regulated by ABA and low-temperature. We have mutagenized the seeds of the transgenic Arabidopsis and been screening them for mutants in which luciferase is not induced by ABA and those in which the reporter gene is constitutively expressed or induced by ABA at a very low concentration by a non-destructive method using a liq. N_2-cooled slow-scan CCD camera.

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The Arabidopsis homeobox gene ATHB-7 is induced by water deficit and by abscisic acid

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The HD-Zip class of transcription factors are encoded by a class of homeobox genes with at least 15 members in Arabidopsis thaliana. We have found two genes of this class to have functions related to the plant response to water deficit. *ATHB-7* expression is detectable at low levels in all organs of the plant, but expression is strongly induced by drought as well as by abscisic acid, at concentrations as low as 10^{-8} M. Induction is fast, an increase detectable within 30 minutes after ABA treatment, and transient. The absence of drought-induction of the gene in an ABA-deficient indicates that the drought-induction of the gene is mediated by ABA. Further induction of *ATHB-7* is impaired in the *abi1* mutant, indicating that *ATHB-7* may act as a mediator of a drought response in a signal transduction pathway down-stream to *ABI1*. Data on the phenotypic effects of elevated expression levels of ATHB-7 in transgenic Arabidopsis are consistent with this notion. A second HD-Zip gene, *ATHB-6*, is also inducible by ABA, but induction appears independent of *ABI1*. ATHB-7 is preferentially expressed in cells associated with stomata.

Bzip transcription factors and control of gene expression in barley endosperm

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We have studied the control of gene expression in barley, focusing mainly in two genes *Itr1* and *Ss1*, encoding respectively trypsin inhibitor BTI-CMe and type I sucrose synthase. *Itr1* is endosperm specific and appears to be controlled "in trans" by the Lys 3a locus. *Ss1* is expressed not only in the seed but also in roots and leaves, where it is induced by anaerobiosis and cold temperatures.

Functional dissection of the promoters of both genes through transient expression in transfected barley protoplasts and, more recently, in barley tissues through the biolistic approach, have indicated the existence of cis-motives, putatively involved in interactions with Bzip transcription factors, and recognised by nuclear proteins, as ascertained by gel mobility shift assays and DNase I foot printing analyses.

We have cloned a Bzip (*Blz1*) from barley after screening cDNA and genomic libraries. This *Blz1* gene has been expressed in *E. coli* and the protein interacts with promoter fragments of endosperm specific *Itr1* but not with those derived from *Ss1*. Moreover, using as effector the *Blz1* gene under the control of the ubiquitin promoter and as reporter the β-glucuronidase (GUS) gene, under the control of the *Itr1* proximal promoter, in cotransfection experiments, the effector in sense, increases, and the effector in antisense orientation, decreases the GUS expression driven by the *Itr1* promoter. All this evidence supports the involvement of *Blz1* in the transcription control of endosperm specific *Itr1*.

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Apoptosis in barley aleurone and inhibition of apoptosis by abscisic acid

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Abstract

Apoptosis is a form of programmed cell death characterized by cytoplasmic boiling, chromatin condensation, and DNA fragmentation (Wyllie 1980), which is studied and considered to be very important in embryogenesis and to diverse stresses (Tomei and Cope, 1991, 1994; Hengartner and Horvitz, 1994). Recently, it has been demonstrated that in plant environmental stress or fungal infection could induce apoptosis as well (Ryerson and Heath,1996; Wang et al., 1996; Kuo et al., 1996). The plant hormone abscisic acid (ABA) is a crucial factor during seed development, being involved in storage protein synthesis, desiccation tolerance and prevention of precocious germination. ABA plays also important role in induction and maintenance of dormancy, and acts as an antagonist of another plant hormone gibberellin which is a germination stimulator.

During germination of barley grains, we observed apoptosis in barley aleurone. The appearance of DNA fragmentation was in a time dependent manner. Under osmotic stress, apoptosis could also be seen in aleurone. During protoplast isolation, a very strong DNA fragmentation occurred. ABA was able to inhibit apoptosis in barley aleurone under osmotic stress and during protoplast preparation. To study the role of phosphorylation in ABA signal transduction leading to control of apoptosis, the effects of phosphatase inhibitor phenylarisine oxide and okadaic acid on apoptosis were analyzed.

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SESSION V. ABA SIGNAL TRANSDUCTION: SIGNALLING MOLECULES

Chairperson: Nam-Hai Chua

SIGNAL TRANSDUCTION PATHWAYS FOR ACTIVATION OF ABA-RESPONSIVE GENES.
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Elucidation of the molecular mechanisms by which plants respond to osmotic stress elicited by desiccation, salt, and cold is a first step toward developing osmo-tolerant food crops. Physiological research has shown that plants subjected to osmotic stress undergo an acclimation process allowing the plants to modulate their osmoticum and ultimately withstand these adverse conditions. Certain acclimation processes can be correlated with an increase in endogenous concentrations of the plant hormone abscisic acid [ABA]. However, despite the wealth of knowledge concerning the physiological effects of ABA on plants, the components of ABA signal perception and transduction on a molecular level have yet to be identified.

We have taken two complementary approaches in an attempt to elucidate components of the signal transduction pathway for activation of ABA-responsive genes. In the first approach we have used microinjection techniques to examine the effects of a number of putative signal intermediates for their ability to activate transcription of ABA-responsive genes in single cells [Neuhaus et al., 1993; Bowler et al., 1994]. Promoter fragments of the Arabidopsis *rd29a* and *kin2* were fused to the β-glucuronidase [*GUS*] coding sequence and used as reporter constructs. We found that *rd29a-GUS* and *kin2-GUS* were inactive when injected into hypocotyl cells of the tomato mutant aurea. These two reporter genes, however, were activated upon incubation of the injected seedlings with 50 μM ABA for 2 days. ABA-dependent activation of the reporter genes can be blocked by 8-amino cyclin ADPribose [cADPR] and thio-NADP. In the absence of ABA, cADPR as well as NAADP can induce *GUS* expression of the two reporter constructs. Taken together, these results provide strong evidence that cADPR and NAADP may serve as second messengers for ABA signaling in higher plants.

Because both cADPR and NAADP are agonists for specific gated calcium channels in animal cells our results imply that similar channels exist in plant cells and that transient calcium increases in the cytoplasm may be an obligatory step in ABA signaling. Indeed, ABA-induction of *rd29a-GUS* and *kin2-GUS* can be inhibited by microinjection of EGTA; moreover, calcium alone can activate both these two genes in the absence of ABA. Finally, activation of *rd29a-GUS* and *kin2-GUS* by ABA, cADPR, NAADP, or calcium is sensitive to two kinase inhibitors, K252a and staurosporine, suggesting the involvement of a protein phosphorylation in this process.

In the second approach we have developed a novel screen to isolate mutants that aberrantly express ABA responsive genes. Several lines of Arabidopsis thaliana transgenic for an ABA-responsive reporter gene were generated. The *kin2*::luc reporter was constructed by cloning the 1416 bp *kin2* promoter fragment upstream from the TMV Ω 5' untranslated leader and protein coding region of the firefly luciferase gene. CCD mediated photon counting showed that the *kin2*::luc transgene is transiently expressed in response to exogenously added 100 μM ABA and that this effect is dose dependent, a manner similar to that observed for the endogenous *kin2* gene. EMS-mutagenized plants were selected that [1] responded to subthreshold ABA concentrations, and [2] failed to exhibit desensitization to the ABA response. Molecular characterization of these hypersensitive and desensitization mutants with respect to the expression pattern of ABA-, cold-, and desiccation-responsive Arabidopsis genes will be described.

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CELL BIOLOGICAL AND GENETIC IDENTIFICATION OF EARLY ABSCISIC ACID SIGNALING MECHANISMS IN GUARD CELLS

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By studying guard cell membrane signaling mechanisms, an "enzymatic cascade" of ion channels has been identified that is activated within the first 2 minutes of ABA-induced stomatal closing (1). These ion channels include Ca2+ channels, slow anion channels (2), and K+ channels (3). However, the upstream mechanisms and sequences of the initial intracellular ABA signaling pathways in higher plants remain largely unknown. The *abi1* locus encodes a protein phosphatase 2C (4,5) and effects guard cell K+ channels in transgenic tobacco (6). Slow anion channels in the plasma membrane of guard cells have been suggested to function as a central rate-limiting mechanism which controls ion efflux leading to stomatal closing (2). Detailed studies show that phosphorylation/ dephosphorylation events in *Vicia faba* guard cells can strongly up- and down-regulate slow anion channels and ABA signaling, thereby allowing anion channels to function as an "on-off" switch during stomatal regulation (7). Slow anion channels could provide an opportune system to genetically dissect upstream signaling pathways, because of their large degree of regulation (2,7). However, direct evidence for ABA regulation of slow anion channels is still lacking and furthermore patch clamp studies on *Arabidopsis* guard cells have been deemed unamenable because of the small cell size.

To allow quantitative analysis of genetic signaling mutants, patch clamp and confocal Ca^{2+} imaging techniques were developed and performed on *Arabidopsis* guard cells from wild-type, *abi1* and *abi2* mutants. Data will be presented that demonstrate that ABA directly and strongly activates slow anion channels in wild-type *Arabidopsis* guard cells. Interestingly, the ABA activation of slow anion channels was completely abolished in *abi1* and *abi2* guard cells. Further studies demonstrate that the impairment in ABA signaling in the *abi1* and *abi2* mutants can be functionally separated. Confocal imaging showed that ABA triggers cytoplasmic Ca^{2+} increases in both wild-type and *abi1* guard cells suggesting that initial cytoplasmic Ca^{2+} increases lie upstream or parallel to ABI1. The presented findings directly demonstrate that ABA activates slow anion channels. Furthermore, the *abi2* locus plays a direct role in early ABA signaling mechanisms and *abi2* and *abi1* differentially affect ABA signaling. A novel branched ABA signaling pathway can be derived from these findings and putative roles of phosphorylation events, Ca^{2+}, ABI2, and the ABI1 protein phosphatase will be discussed.

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REDUNDANCIES IN GUARD-CELL ION CHANNEL CONTROL

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Two, possibly three distinct signalling pathways and their messengers -- entailing changes in cytoplasmic-free Ca^{2+} ($[Ca^{2+}]_i$), cytoplasmic pH (pH_i) and protein phosphorylation -- appear to underpin K^+ and anion channel control during ABA-evoked stomatal movement. Such a degree of redundancy is probably not unique among plant cells, and is wholly consistent with the ability of the guard cells to integrate the wide range of environmental and hormonal stimuli that affect stomatal aperture. In principle, signal convergence enables a spectrum of graded responses extending beyond simple interference and allows one pathway to "gate" transmission via the next, so boosting or muting the final output. Current evidence supports such a role for the ABI1 protein phosphatase, and by inference, protein kinase elements in gating K^+ channel sensitivity to pH_i and ABA (Armstrong *et al.* 1995). In turn, changes in $[Ca^{2+}]_i$ are also subject to pH_i (Grabov and Blatt, 1997). Because these signal pathways affect discrete subsets of ion channels at the guard cell plasma membrane, their coupling may be seen to add a further layer of control necessary for coordinating the ensemble of channel response during stomatal movements.

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Calcium ions as second messengers in guard cell ABA stimulus response coupling

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Stomatal guard cells represent an excellent system for studying ABA signal transduction. They are tractable to a range of single cell *in vivo* techniques and the control of the ionic fluxes which underlie the changes in stomatal aperture are comparatively well understood. The major focus of the work at Lancaster has been the identification of early cellular responses to ABA in guard cells. In particular we have concentrated on the role of calcium ions as second messengers in ABA stimulus response coupling. On the basis of results from pharmacological investigations (McAinsh, Brownlee & Hetherington, 1991 Proc Roy Soc, (Lond) B 243, 195) and direct measurements of free calcium in stomatal guard cells it is possible to conclude that there is a calcium dependent ABA signalling pathway in stomatal guard cells (McAinsh, Brownlee, & Hetherington, 1990 Nature, 343, 186; 1992 Plant Cell, 4, 1113). These data integrate well into current models of the regulation of guard cell turgor by ABA (see for example Schwartz et al 1995 Pl Physiol109, 651). However, several key questions remain to be addressed. The recent data which point to the existence of calcium dependent and independent signalling pathways in guard cells (Allan et al 1994 Plant Cell 6, 1319) together with the possible existence of membrane and, or cytosolic receptors (Assmann 1994, Plant Cell 6, 1187) raises questions concerning where ABA signalling pathways divergence and or convergence. To compound this problem we must add our own recent data which show that guard cells are also competent to relay the ABA signal to the nucleus (Taylor et al 1995, Plant J 7, 129) and preliminary data suggesting a role for calcium in this signalling pathway. Another question which requires attention is mechanisms of encoding specificity into the guard cell calcium signalling system. It is now clear that calcium is used as a second messenger in both closure (extracellular calcium, McAinsh et al, 1995 Plant Cell 7, 1207; CO_2 ,Webb et al 1996 Plant J 9, 297 and roi's McAinsh et al 1996 Pl Physiol 111, 1031) and opening (auxins, Irving et al 1992 PNAS 89, 1790) induced signalling pathways. Possible solutions to this problem include the involvement of other second messengers and the generation of stimulus specific Ca signals - calcium signatures. The presentation will discuss these issues.

Transgenic tomato plants carrying the Arabidopsis *abi1-1* mutant allele are altered in their wound response.

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Wound damage of potato and tomato plants leads to a rapid accumulation of several proteinase inhibitors in the aerial parts of the plant. Accumulation of these inhibitors is not limited to the injured leaves but also occurs in tissues distal from the actual wound site. This systemic activation apparently is mediated by a hormone-like inducing factor that is transported out of the leaf via the vascular system. Several lines of evidence indicate that the phytohormones abscisic (ABA) and jasmonic acid (JA) are essential components in the systemic response to mechanical damage. Proteinase inhibitor induction does not occur in ABA deficient plants in response to wounding, though ABA and JA are able to induce high levels of transcript accumulation in these plants. Furthermore, wound-induced activation in wild-type plants can be inhibited by blocking JA synthesis. These results indicate that JA acts downstream of ABA in regulating the wound response, and that a rise in endogenous levels of ABA is required to trigger JA biosynthesis. Mutants impaired in their response to ABA have been isolated from several plants. From the different mutants characterised, only the Arabidopsis *abi1*, *abi2* and *abi3*, and the maize *vp1* mutants have been analysed to a substantial extent. While *abi3* and *vp1* are active exclusively in seeds, the *abi1* mutants show a wilty phenotype and are defective in numerous ABA-responses during vegetative growth. The *ABI1* locus has been cloned recently and found to encode a calcium-modulated protein phosphatase. We have taken advantage from the fact that the Arabidopsis *abi1-1* mutation has a dominant character, to obtain transgenic tomato plants carrying one or more copies of the mutant gene. Expression of *abi1-1* in tomato resulted in wilty plants that showed to be insensitive to ABA. Treatment with ABA does not induce stomata closure in these plants, neither the accumulation of TAS14 or other ABA induced transcripts. Preliminary results indicate that the *abi1-1* transgenic plants are as well impaired in their wound response, as they do not appear to accumulate proteinase inhibitor mRNAs upon wounding or ABA treatment. This result suggests that ABI1 would play a role in ABA transduction in the wound signalling cascade, by modifying the phosphorylation state of some as yet unknown target protein. In agreement with this observation, results that strongly implicate a protein phosphorylation/dephosphorylation step in the induction of plant defence responses have been reported by several groups.

8'-Methylene ABA - An Effective and Persistent Analogue of Abscisic Acid

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We report the synthesis and biological activity of a new persistent abscisic acid analogue, 8'-methylene ABA (8'-CH_2 ABA). This ABA analogue has one additional carbon atom, attached through a double bond to the 8'-carbon of the ABA molecule. The alteration in the ABA structure causes the analog to be metabolized more slowly than ABA, resulting in longer lasting and more effective biological activity relative to ABA in several biological assays. (+)-8'-Methylene ABA is more active than the natural hormone (+)-ABA in reducing transpiration in wheat seedlings and in inhibiting germination of cress seed and excised wheat embryos. (+)-8'-Methylene ABA is 10 times stronger than natural ABA in inhibiting germination of excised embryos of wheat cv Clark's cream. In reducing growth of cultured corn cells, (+)-8'-methylene ABA exhibits stronger growth inhibition than (+)-ABA at all tested concentrations. At 0.33 μM, ABA inhibited growth by 17% relative to the control whereas 8'-methylene ABA produced a 64% reduction. The (+)-8'-methylene analog is slightly weaker than (+)-ABA in increasing expression of ABA-inducible genes in transgenic tobacco but equally active in stimulating corn cell culture medium acidification. In metabolism studies in corn cells, both (+)-ABA and (+)-8'-CH_2 ABA are oxidized at the 8' position. ABA is oxidized to phaseic acid, and (+)-8'-CH_2 ABA is converted more slowly to two isomeric epoxides. Adding a methylene group to the 8'-carbon of ABA in 8'-CH_2 ABA provides two possible mechanisms to increase resistance to oxidation. First, increasing the steric bulk with additional carbon atoms on the 8'-carbon atom should hinder binding in the active site of the hydroxylase enzyme, reducing the rate of oxidation, thus prolonging the lifetime of the hormone analogue. Secondly, terminal olefins analogous to 8'-methylene ABA have been shown to inactivate cytochrome P-450 monooxygenases (Ortiz de Montellano, 1991).

The new analogue has potential as a new tool for maintaining ABA-induced gene expression and for investigating and manipulating physiological processes including seed dormancy and stress tolerance.

POSTERS

ISOLATION OF NEW ARABIDOPSIS MUTANTS ALTERED IN EXPRESSION OF THE ABA-REGULATED Em GENES USING A TRANSGENIC PLANT SCREENING APPROACH

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In order to identify new factors implicated in ABA transduction signal we chosed to study the regulation of the *Arabidopsis Em* genes (*AtEm1* and *AtEm6*) which belong respectively to the LeaA and Lea class. Our aim is to isolate new mutants distinct from *abi3*, *abi4* or *abi5* affected in *Em* expression in order to identify new elements potentially implicated in ABA transduction pathway.

A γ mutagenised transgenic population (approximatively 5 000 independent progenies harvested by bulk from 50 plants, containing the GUS reporter gene under the control of the *AtEm1* promoter) was screened by a non destructive GUS assay in order to select lines altered in GUS expression. For all the lines selected (399 lines) the level of GUS expression was checked in the progenies and 216 lines were confirmed and classified according to their level of GUS activity in dry seeds. We present here only the results obtain with the 28 lines which are characterised by a GUS activity lower than 25 % of the control (non mutagenised line).

First we verified in all the lines the integrity of the transgene by PCR then the alteration of the accumulation of the Em proteins in dry seeds was analysed by western blot method. Six lines (NEM lines) present an important diiinution in the two Em proteins accumulation. These lines were selected from the same bulk so it is possible that they contain the same mutation, we are analysing this possibility by allelism tests. We also started to characterise the phenotype of these NEM lines. Their germination is sensitive to ABA and therefore these lines are not altered in one of the already known *abi* genes. Some of the NEM lines have a reduced seed dormancy. We believe that the mutation responsible of the alteration of seed dormancy is independent than the one implicated in the alteration of the *Em* genes expression. At present we are testing if the altered dormancy lines are allelic to *aba* mutants. At the molecular level, we are analysing the expression of others Lea genes (northern blot approach) in the NEM lines. For the moment, our results suggest that we have isolated a new kind of mutant affected in the expression of the ABA regulated Em genes. Now we are mapping the mutation in order to be able to isolate the responsible gene.

Regulatory Elements *In vivo* in the Promoter of the Abscisic Acid Responsive Gene *rab17* from Maize

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The *rab17* gene from maize is transcribed in late embryonic development and is responsive to abscisic acid and water stress in embryo and vegetative tissues. We performed *in vivo* footprinting and transient transformation of *rab17* to characterize the *cis*-elements involved in development, abscisic acid and drought response. By *in vivo* footprinting protein binding was observed to nine elements in the promoter which correspond to five ABREs (abscisic acid responsive elements) and four other sequences. The same *cis*-elements were involved in protein-DNA interactions in embryo and in leaves. However, differences in the footprints indicated that different proteins were interacting with these elements in different tissues.
Transient transformation suggested that three positive elements, one ABRE and two other sequences, are necessary for high level induction of the *rab17* promoter. One positive, new element (GC-rich *rab* Activator) was identified with the sequence CACTGGCCG. This element was the most important for both basal and hormone induced transcription of the *rab17* promoter. The other non-ABRE element that stimulated transcription resembles previously described abscisic acid and drought inducible elements.
There were differences in protein binding and function of the five ABREs in the *rab17* promoter. Mutation showed that only three were responsive to abscisic acidand only one mutant had less than half of the wildtype activity.
Our data suggest that *rab17* gene regulation is achieved by multiple transcription factors that interact with positive and negative cis-regulatory elements and that the function of the ABREs is determined by a combination of sequence of the elements and their position in the promoter.

HORMONAL CONTROL OF pH CHANGES IN THE STARCHY ENDOSPERM OF GERMINATING WHEAT SEEDS

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INTRODUCTION

Following cereal seed germination a process of acidification occurs in the starchy endosperm. It has been shown that this acidification is produced by the aleurone layer, that secretes organic and phosforic acids, a process estimulated by gibberellic acid (GA) and abscisic acid (ABA) (Drozdowicz and Jones, 1995). In this communication we present data showing the pattern of acidification of the starchy endosperm of germinating wheat seeds as well as the effect of the external pH on the expression of several GA-responsive genes.

METHODS

The pH of the starchy endosperm of germinating wheat seeds was determined in longitudinally dissected seeds with the pH indicator Bromcresol purple. To test the effect of the external pH on acidification and mRNA accumulation, sterilized half-seeds were incubated on filter paper soaked with different buffers: 20 mM Na Acetate, pH 5.0 and 5.5, 20 mM Na Phosphate, pH 6.0, 6.5, and 7.0, and 20 mM MOPS-NaOH, pH 7.0. All buffers were supplemented with 10 mM $CaCl_2$. When required 5 μM GA, or 25 μM ABA were added. Northern-blot and *in situ* hybridization were performed as previously described (Cejudo et al, 1992).

RESULTS

A tissue stain with the pH indicator Bromcresol purple was carried out to study the variation of pH in the starchy endosperm of wheat seeds following germination. Early after imbibition the pH of the starchy endosperm was neutral. As germination proceeded it was observed a progressive acidification that avanced from the embryo to the distal part of the seed. The aleurone layer showed capacity to acidify the external medium, this acidification was pH-dependent and stimulated by GA_3. When de-embryonated half-seeds were incubated at an initial pH of 7.0 in the presence of GA_3, the external pH decreased to around 4.5 after 5 days of incubation. However, when the initial pH was acidic (5.0-5.5) no additional acidification was detected regardless of the presence of GA_3. Since the plant hormone GA stimulates the production of hydrolases (amylases and proteases) by the aleurone layer, the effect of the external pH on this stimulation was studied. A higher accumulation of transcripts in response to GA_3 was observed at neutral than at acidic pH. This effect was independent of the presence of phosphate in the incubation medium. In addition *in situ* hybridization experiments revealed a temporal pattern of expression of GA-regulated genes in the aleurone layer of wheat germinating seeds that paralleled the pattern of acidification of the starchy endosperm. Our results show a coordination between the pH of the starchy endosperm and the regulation of gene expression in the aleurone layer of wheat seeds, this coordination being achieved by GA.

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Differential expression of ABA-regulated genes in drought tolerant and drought sensitive sunflower lines

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A large number of drought- induced genes have been identified in a wide range of plant species, however there is little direct evidence for the implication of a given gene in drought adaptation. Correlating phenotypic adaptations with molecular responses should enable the potential role of drought- induced changes of gene expression during adaptation to be evaluated. Indeed, linking the expression of a gene to a high degree of tolerance suggests a possible role for this gene in adaptation. Abscisic acid (ABA) concentration increases in plant organs in response to drought, leading to many physiological changes. It has been postulated that ABA mediates general adaptative responses to drought and therefore that ABA-regulated gene products are involved in stress tolerance.

To identify genes potentially involved in drought tolerance, We have studied two lines of sunflower (*Helianthus annuus* L.) selected in the field as drought tolerant (R1 genotype) or drought sensitive (S1 genotype). When subjected to drought conditions, the R1 line was able to maintain high leaf water potential longer and wilted later, than the S1 line. Therefore, this indicates that R1 tolerance includes a leaf adaptive response. By subtractive hybridization, we have isolated six different cDNAs (designated *sdi* for sunflower drought induced) corresponding to transcripts accumulated in R1 leaves during adaptive response . Analysis of transcript accumulation in response to drought in both genotypes shows a preferential expression of three *sdi* genes in the tolerant line. Abscisic acid- mediated induction, analysed in R1 leaves, was observed for these *sdi* genes [1]. To determine in which extend ABA mediate the preferential expression of these *sdi* genes in the tolerant line we have compared *sdi* transcript accumulation in response to exogenous ABA in both lines. Furthermore, we have monitored steady state level of*sdi* transcripts, ABA concentration and plant hydric status upon progressive drought in both genotypes. Our results suggest that part of the R1 adaptive response to drought could be ABA-dependent.

1. O. Ouvrard, F. Cellier, K. Ferrare, D. Tousch, T. Lamaze, J-M. Dupuis and F. Casse-Delbart Identification and expression of water stress- and abscisic acid- regulated genes from a drought tolerant sunflower genotype. *Plant Mol. Biol* **31**: 819-829 (1996)

Trehalose accumulation in transgenic tobacco alters carbohydrate metabolism and leads to phenotypes with improved drought tolerance

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The yeast *trehalose-6-phosphate synthase* gene (*TPS1*) was engineered under the control of the cauliflower mosaic virus regulatory sequences (CaMV35S) for expression in plants. Using *Agrobacterium* mediated transfer, the gene was incorporated into the genomic DNA and constitutively expressed in *Nicotiana tabacum* plants. Trehalose was determined in the transformants, by anion exchange chromatography coupled to pulsed amperometric detection. The non-reducing disaccharide accumulate up to 0.15 mg / g fresh weight in leaf extracts of transgenic plants. Trehalose accumulating-plants exhibit multiple phenotypic alterations, including stunted growth, lancet-shaped leaves, reduced sucrose content and improved drought tolerance. These pleiotropic effects, and the fact that water loss from detached leaves is not significantly affected by trehalose accumulation, suggest that synthesis of this sugar, rather than acting by accumulation as osmoprotectant, has altered sugar metabolism and regulatory pathways affecting plant development and stress tolerance.

RAB-17 BINDS DOUBLE STRANDED DNA IN A NON-SEQUENCE-SPECIFIC MANNER

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Rab-17 is a gene from maize whose expression is specific for ABA-induced or water-stressed tissues. The Rab-17 protein has been found into the nucleus and the cytoplasm by immunolocalization, and several "in vitro" assays have shown the capacity of the phosphorylated forms of Rab-17 to bind specifically to NLS (nuclear localization sequences).

Since after expressing Rab-17 into Xenopus oocytes, the nucleus disappeared and due to the nuclear localization of Rab-17, we analyzed the capacity of Rab-17 to bind and degrade DNA. Several band-shift assays using Rab-17 protein purified from maize or overexpressed in bacteria indicated that Rab-17 binds dsDNA. The binding was competed by polidIdC-polidIdC and polidAdT-polidAdT, but could not be competed with ssDNA. In order to check if Rab-17 had any DNase activity we performed DNase-assays by band-shift and sequencing gels. This analysis indicated that Rab-17 does not have any capacity to degrade DNA.

As Rab-17 does not have DNase activity, binds to DNA in a non-sequence-specific manner and interacts specifically with dsDNA, we propose that Rab-17 can act during water stress as a DNA protector.

ETHYLENE RELEASE AND SUBSEQUENT LEAF ABSCISION IN WATER STRESSED SEEDLINGS OF CITRUS REQUIRE PREVIOUS ABA ACCUMULATION IN ROOTS.

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We have previously shown that ethylene controls leaf abscission in drought-stressed seedlings of citrus. During water stress, ACC largely accumulates in roots and when the plants are rehydrated ACC is soon afterward translocated to the aerial parts. This ACC transported from roots to shoots is rapidly converted to ethylene, which in turn causes leaf abscission (Plant Physiol. 1992, 100: 131-137). In the present work, we have studied the effect of norflurazon, an inhibitor of carotenoid biosynthesis and therefore indirectly of ABA, on ethylene and ABA levels as related to leaf abscission. In addition to ACC, ABA also increased in both water-stressed roots (20-fold) and xylem sap of rehydrated plants. Norflurazon treatments did not modify water potential, stomatal resistance and ABA levels in leaves of drought-stressed plants. However, this inhibitor reduced both ABA accumulation in roots (4-fold) and ABA transport through the xylem fluid of these plants. Furthermore, norflurazon inhibits the ethylene raise which normally occurred after rehydration in drought-stressed plants and consequently arrested also leaf abscission. The results show that water stress promotes both ABA and ACC accumulation in citrus seedlings roots. The data also indicate that the inhibition of the ABA accumulation in roots reduces the ethylene increase observed in leaves as well as final leaf abscission. Thus, The ACC accumulation which take place in roots in response to water stress is probably mediated by a previous increase in root ABA.

Expression of small heat shock protein genes during zygotic embryogenesis and their differential response to ABA and water-stress

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Earlier work in our lab described the concurrent expression of small heat shock protein (sHSP) and late embryogenesis abundant (lea) genes during zygotic embryogenesis, from maturation to desiccation. We suggested that genes from these two families might share regulatory elements, as indicated for example by their similar responses to exogenous ABA in young seedlings (1). We have recently obtained two different genomic clones that correspond to sunflower sHSP genes expressed in seeds at normal growth temperatures: Ha hsp17.7 G4 (2) and Ha hsp17.6 G1 (unpublished results). Whereas mRNAs from Ha hsp17.7 G4 accumulated during zygotic embryogenesis from late maturation, and in vegetative tissues in response to either heat-shock, abscisic acid (ABA), or mild water-stress treatments, the transcripts from Ha hsp17.6 G1 accumulated later in embryogenesis, but not in response to ABA or water-stress. These results demonstrate differential regulation of these two sHSP genes and, in the case of the seed-expression of hsp17.7 G4, suggest a possible involvement of regulation by ABA. We have used deletion analysis and site directed mutagenesis to define regulatory *cis*-elements of Ha hsp17.7 G4 in transgenic tobacco (2, and other unpublished results). Our aim is to determine the role, in the regulation of hsp17.7 G4 and Ha hsp17.6 G1, of heat shock factor binding sites (HSEs) and/or elements in the embryo-specific ABA signal transduction pathways (i.e., abi3); as suggested from preliminary evidence from our lab (2) and other groups (3,4). Although initial deletion analysis failed to separate seed regulation of Ha hsp17.4 from its heat shock response (2) more recent data suggest that this separation could be achieved. The involvement of ABA/abi3 in the seed regulation of Ha Hsp17.7 G4 would be consistent with this result and shall be additionally investigated by analyzing the expression of chimeric genes transgenic *Arabidopsis* wild type and mutant backgrounds (i.e. abi3, fus3).

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ABA and stress response of zmHyPRP and zmHRGP in maize immature embryos.

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We have studied during embryo development the regulation of two Proline-rich genes, zmHyPRP and zmHRGP by northern analysis and "in situ" hybridization.

zmHyPRP is a cortex embryo specific gene. zmHyPRP first is a marker of scutellar cells early in embryogenesis while later at stage III of embryo development expression is reduced to the cells placed below the epidermis of the embryo abaxial side and to cortex cells sorrounding developping vascular bundles in the embryo axis. Expression is arrested at the beginning of the maturation process by abscisic acid (ABA). zmHyPRP expression in mize ABA deficient mutants (vp2), the presence of an hormone responsive element in its promoter and the ability of exogenous ABA to repress its expression in excissed immature embryos support this idea.

zmHRGP expression is associated to meristematic tissues and to the defence response. zmHRGP is a marker of suspensor and coleoptile cells during early embryogenesis and of embryo vascular development in the differentiated axis at stage III of embryo development. zmHRGP expression is arrested at the beginning of the maturation process by ABA. zmHRGP expression is arrrested from maize immature excised embryos by exogenous ABA, while in maize ABA deficient mutants (vp2) its expression is never arrested. We show also that different stresses are able to increase zmHRGP expression by an ABA independent pathway as well as the normal cellular pattern of expression is modified.

ABA - REGULATED DEHYDRIN EXPRESSION IN WATER - STRESSED WHEAT SEEDLINGS

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Plants respond to water stress with a number of physiological and biochemical changes. Many of these responses are mediated by plant hormone abscisic acid (ABA). The expression of several dehydration-inducible genes has been shown to be ABA-regulated (1, 2). Among the products of such genes characterized up to date, dehydrins (LEA D-11 proteins) are very intriguing because of the various protective roles they have been proposed to play during plant cell dehydration (3, 4).

We investigated the expression of dehydrins in young wheat (Triticum aestivum L.) seedlings subjected to PEG-mediated water stress and their possible regulation by ABA. Using anti-dehydrin antibodies (produced to a synthetic peptide including the KIKEKLPG sequence, kindly provided by Prof. T. J. Close) we demonstrated that dehydrins of approximately 35kD,38kD and 93kD were induced in 3d-old wheat seedlings in response to PEG-mediated water stress. The expression of these dehydrins was found to be tissue specific. It was restricted to the stem of water-stressed 7d-old seedlings.

To determine whether 35kD, 38kD and 93kD dehydrin expression is ABA-responsive, intact wheat seedlings were transferred either to 5 micromolar ABA for 4d or 100 micromolar ABA for 48h. Western analysis revealed that only 93kD wheat dehydrin was expressed in response to exogenously applied ABA. These data suggest a differential regulation of dehydrin expression in water-stressed wheat seedlings - ABA-dependent and ABA-independent.

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Identification of Cyclic ADP-ribose in Plants and Its Involvement in ABA-mediated Signal Transduction

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Cyclic ADP-ribose (cADPR) has recently emerged as a signaling molecule involved in calcium-induced calcium release via its interaction with the ryanodine receptor (RyR). It has been detected in invertebrate, amphibian, and mammalian cells and is thought to be produced by an ADP-ribosyl cyclase which catalyzes the conversion of nicotinamide adenine dinucleotide (NAD^+) to cADPR. In plant single-cell microinjection experiments, this novel cyclic nucleotide can replace abscisic acid (ABA) or calcium as the injected compound which induces ABA-responsive genes (Wu Y., Foster R., and N-H. Chua, unpublished results). cADPR has also been shown to elicit calcium release from plant vacuolar membranes (Allen G. J., Muir S. R., and D. Saunders, Science 268:735 (1995)). This suggests that it is an important molecule in ABA-mediated signal transduction. In order to further support its role as a signaling molecule, we set out to detect cADPR in plants and quantify the amount of this molecule during ABA-mediated gene induction.

By using a variety of analytical techniques, the presence of cADPR in *Arabidopsis thaliana* and tobacco was confirmed. A series of experiments were conducted in which the expression of an ABA and cold induced gene, *kin2*, was correlated to cADPR levels after plants were exposed to ABA for various intervals. cADPR levels increased prior to *kin2* mRNA levels and reporter gene activity (*kin2*-luciferase). These results, in conjunction with the microinjection experiments, validate the importance of cADPR as a signaling intermediate in plant ABA responses.

TOWARDS CLONING OF AN ABSCISIC ACID RECEPTOR.

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Explicit messenger components downstream abscisic acid perception are confirmed to participate in the plant hormone signal transduction pathway of plant cells. Ca^{2+} is likely to be a second messenger in the signal transduction pathway of ABA in plant cells (Blatt and Thiel. 1993, Ward *et al.* 1995). However, little is conclusive about recognition of ABA by these cells. Published data demonstrating the spatial organization of reception are controversial and suggest the existence of several receptors either inside the cytosol or/and at the plasma membrane (Assmann 1994). By using a heterologous expression system, such as *Xenopus leavis* oocytes, ABA perception can be assayed and cloned in any of above-mentioned cases. In order to clone the ABA receptor, mRNA purified from *Nicotiana tabacum* leaves was injected in the *Xenopus leavis* oocytes and translated (2 days incubation at 20°C). A new cross-coupling was established of the plant ABA receptor and/or downstream proteins -of which transcripts are represented in this RNA sample- with an endogenous signal pathway of the egg cell. Therefore ABA response can be assayed via an increase of Cl^- currents in injected oocytes. These endogenous Cl^- currents are activated by elevating the cytoplasmic Ca^{2+} concentrations (Miledi, 1982).

In analogy with the method used by Julius *et al.* (1988), a sucrose gradient was used to fractionate the mRNA by size into 10 different portions. Only one fraction showed activity in response to ABA after translation in the oocytes. The transcripts of this fraction were used as template to synthesize cDNA and make an unidirectional library into the pSPORT expression vector. Complementary RNA was generated from a pool of 20.000 linearized plasmids of this library using the T7 RNA polymerase in the presence of the cap analogue GpppG. This cRNA was injected into *Xenopus leavis* oocytes, and cells were assayed for ABA induced Cl^- current. A small but significant signal was detected in the injected oocytes. The pool was subdivided into pools of 2000 colonies, and consequently the positive sample into pools of 200 colonies. The positive combination of 200 clones showed an ABA response increased over the signal evoked by the 20.000 or 2000 mixtures. Further sib-selection has yielded some clone(s) of interest.

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ALTERATION OF ANION CHANNEL KINETICS IN WILD-TYPE AND *abi1-1* TRANSGENIC *Nicotiana benthamiana* GUARD CELLS BY ABSCISIC ACID

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The influence of the plant water-stress hormone abscisic acid (ABA) on anion channel activity and its interaction with protein kinase and phosphatase antagonists was examined in stomatal guard cells of wild-type *Nicotiana benthamiana* L. and of transgenic plants expressing the dominant-negative *Arabidopsis* abi1-1 protein phosphatase. Intact guard cells were impaled with double-barrelled microelectrodes and membrane current was recorded under voltage clamp in the presence of 15 mM CsCl and 15 mM tetraethylammonium chloride (TEA-Cl) to eliminate K^+ channel currents. Under these conditions the free-running voltage was situated close to 0 mV (+9±6 mV, n=18) and the membrane under voltage clamp was dominated by anion channel current (I_{Cl}) as indicated from tail current reversal near the expected chloride equilibrium potential, current sensitivity to the anion channel blockers 9-anthracene carboxylic acid and niflumic acid, and by its voltage-dependent kinetics. Pronounced activation of I_{Cl} was recorded on stepping from a conditioning voltage of -250 mV to voltages between -30 and +50 mV, and the current deactivated with a voltage-dependent halftime at more negative voltages (τ=0.3 s at -150 mV). Challenge with 20 μM ABA increased the steady-state current conductance, g_{Cl}, near 0 mV by 1.2- to 2.8-fold with a time constant of 40±4 s and slowed I_{Cl} deactivation as much as 4-fold at voltages near -50 mV, introducing two additional voltage-sensitive kinetic components to these current relaxations. Neither the steady-state and kinetic characteristics of I_{Cl}, nor its sensitivity to ABA were influenced by H7 or Staurosporine, both broad-range protein kinase antagonists. However, the protein phosphatase 1/2A antagonist Calyculin A mimicked the effects of ABA on g_{Cl} and current relaxations on its own and exhibited a synergistic interaction with ABA, enhancing I_{Cl} sensitivity to ABA 3- to 4-fold. Quantitatively similar current characteristics were recorded from guard cells of *abi1-1* transgenic *N. benthamiana*, indicating that the abi1-1 protein phosphatase does not influence the anion current or its response to ABA directly. These results demonstrate that ABA stimulates I_{Cl} and modulates its voltage-sensitivity. Furthermore, they show that ABA promotes I_{Cl}, either by introducing additional long-lived states of the channel or by activating a second anion channel with similar permeation characteristics but with a very long dwell time in the open state. Overall, the data are broadly consistent with the view that ABA action engenders coordinate control of I_{Cl} together with guard cell K^+ channels to effect solute loss and stomatal closure.

The ABRE-like or coupling-like ACGCGTGG element of rice *Ltp2* promoter is necessary for ABA response

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Plant lipid transfer proteins (LTPs) have been identified by their ability to catalyze lipid transport from one membrane to another *in vitro*. A rice gene coding for a LTP type 2 has been cloned, sequenced and its expression pattern determined (1). *Ltp2* mRNA accumulates in mature seeds and during rice seed germination. In vegetative parts of the rice seedlings, mRNA was only detected upon treatment with NaCl or abscisic acid (ABA). The *Ltp* gene promoter region contains five ACTG core sequences of the so-called G-boxes identified as ABA-responsive-elements (ABREs) in the majority of ABA responsive genes (2); an ABRE-like sequence ACGCGTGG, named box I, very similar to the sequence known to be responsive to ABA in transgenic tobacco (3) and the sequence CGGCCGGGCTC, called box II, which is similar to the consensus sequence motif II of rice Rab genes (4). Box I is also similar to the coupling element defined by Shen and Ho (2) which is necessary for ABA responsiveness together with a typical ABRE element.

In order to ascertain which of these boxes are involved in the increase of LTP mRNA accumulation in response to ABA, a series of chimeric deletion constructs of *Ltp* promoter have been produced. The promoter deletions ending in 5' at -1114, -591 and -140 (being the A of the ATG start codon +1) were translationally fused to the ß-glucuronidase (GUS) gene coding region and delivered to maize immature embryos by particle bombardment. The level of GUS activity driven by the different promoter deletions was monitored in maize immature embryos of different days after pollination (DAP). The basal level of expression without ABA diminished accordingly to gradual shortening of *Ltp2* promoter. After ABA treatment all the 3 *Ltp* promoter constructs, but not the CaMV 35S promoter, showed a significative increment of the GUS activity. Interestingly, the higher level of induction was attained with -140 construct, ranging from around 14 to 25 fold ABA induction, compared to 3 to 8 times ABA induction, for example, for the full promoter construct.

The -140 construct still has two typical ABRE sequences, GTACGTCT and CCACGTGT, at 22 and 44 nucleotides upstream the TATA box, respectively. The -140 construct also has the ABRE-like box I (AC<u>GCGT</u>GG), 56 bp upstream of the TATA box. In the latter box, there is a G instead of A in the ABRE core sequence (ACGT). Mutations affecting to this box in the -140 construct have been analyzed using the particle bombardment-mediated transient expression system. When the core sequence ACGT was restored (AT<u>ACGT</u>GG) the level of induction upon ABA treatment dropped from 13-18 times for the wild *Ltp2* sequence to 6-14 times for that sequence, resembling to the rice *Rab* 16D ABRE box (GTACGTGG). An additional decline in the ABA responsiveness was reached (2 to 8 times) when the Hex-1 wheat histone box (3) was restored (TG<u>ACGT</u>GG). Remarkably, ABA responsiveness was completely abolished when the pair CG was substituted by GC (AG<u>CCGT</u>GG), suggesting that the alternation between purines and pyrimidines is important in box I to mediate ABA response.

These results indicate for the first time in a natural occurring promoter that the ABRE-like sequence ACGCGTGG of *Ltp2* promoter, is necessary for ABA responsiveness. Experiments under way will shed light on wether this sequence is or not sufficient for ABA response.

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UNCOVERING THE FLORAL C-FUNCTION ACTIVITY IN MAIZE

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The maize *ZAG1* gene (Schmidt et al., 1993, Mena et al., 1995) is the apparent orthologue of *AGAMOUS*, an Arabidopsis gene required for male and female reproductive organ development and for determinacy of the flower (Bowman et al., 1989; Yanofsky et al., 1990). We recently identified a transposon-induced mutant allele of *ZAG1*, designated *zag1-mum1*, and examined the functional homology between these dicots and monocots genes (Mena et al., 1996) . Here we present the most relevant results of this study.

ZAG1 mutants exhibit a loss of determinacy, but the identity of reproductive organs is largely unaffected. Unlike what has been observed in dicotyledons, loss of *ZAG1* expression impacts fourth whorl (carpel) development but leaves third whorl (stamen) development unaffected. That is, *ZAG1* mutant plants elaborate extranumerary carpels in the ear florets but have normal pollen-producing stamens in the tassel. This mutant phenotype results from a change of fate of the cells that are destined to become carpel primordia. This group of cells now acquire the ability to proliferate and reiterate a program of organ initiation, characteristics of an indeterminate pattern of growth.

This apparent redundancy in maize sex organ specification led us to the identification and cloning of a second *AGAMOUS* homolog, *ZMM2*, that has a pattern of expression distinct from that of *ZAG1*.These results suggest that in maize, unlike the situation in *Arabidopsis* and *Antirrhinum*, C-function activity is orchestrated by two closely related MADS-box genes that have evolved distinct, yet partially redundant roles in flower development. These genes appear to have partially specialized their activities for either male or female reproductive organ development.

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Isolation and characterization of a protein kinase regulated by ABA and calcium in dormant beechnuts.

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SUMMARY

Abscisic acid (ABA) has been involved in the induction and maintenance of dormancy in several seeds, including beechnuts (*Fagus sylvatica*). In this work we have isolated a cDNA clone (PK-F1) coding for a protein kinase, similar to serine/threonine kinases, which is up regulated by ABA in dormant seeds. Study of its expression during the stratification that leads to dormancy breakage shows that the transcripts corresponding to the PK-F1 cDNA clone disappear under the treatments that break dormancy, but increase when dormancy is maintained by adding ABA, which suggests that they may be involved in the action of ABA on dormancy. Additionally, exogenous application of Ca^{2+} increases the transcript levels while calcium chelants decrease them, and the expression is even higher when ABA and Ca^{2+} are added together, which indicates that Ca^{2+} is required for the expression of this ABA-induced clone, probably acting as a second messenger in this process.

CONCLUSION

In *Fagus sylvatica* dormant seeds, ABA induces the expression of a transcript encoding for a serine/threonine protein kinase (involved in phosphorylation processes leading to protein activation), which seems to be related to dormancy. This induction requires calcium, which suggests that Ca^{2+} may act as a second messenger in the synthesis of a protein kinase involved in the effects of ABA on the maintenance of seed dormancy.

List of Invited Speakers

Workshop on

ABSCISIC ACID SIGNAL TRANSDUCTION IN PLANTS

List of Invited Speakers

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Workshop on

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The lectures summarized in this publication were presented by their authors at a workshop held on the 28th through the 30th of October 1996, at the Instituto Juan March.

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