DOE/ER/20021--1

DE92 002818

**Interdisciplinary Research and Training Program
in the Plant Sciences**

DOE Contract #DE-FG02-91ER20021

DOE Report #DOE/ER/20021-1

Technical Progress thru January, 1991

**MSU-DOE Plant Research Laboratory
Michigan State University**

**C. Peter Wolk, Director and
Principal Investigator**

MASTER

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

yes Received by OSTI

NOV 14 1991

Table of Contents

Investigator	Task Title	Page
de Bruijn, F.	Molecular Basis of Symbiotic Plant-Microbe Interactions	1
Delmer, D.P.	Enzymatic Mechanisms and Regulation of Plant Cell Wall Biosynthesis	3
Green, P.J.	Molecular Mechanisms that Regulate the Expression of Genes in Plants	7
Hanson, A.D.	Resistance of Plants to Environmental Stress	10
Kende, H.	Studies on Hormone Biosynthesis and Action	12
Lamport, D.T.A.	Plant Cell Wall Proteins	16
McIntosh, L.	Interaction of Nuclear and Organelle Genomes	17
Poff, K.	Sensory Transduction in Plants	20
Raikhel, N.	Molecular Mechanisms of Trafficking in the Plant Cell	23
Somerville, C.	Regulation of Lipid Metabolism	24
Somerville, S.	Molecular Bases of Plant Disease Resistance Mechanisms	29
Walton, J.D.	Biochemical and Molecular Aspects of Plant Pathogenesis	32
Wolk, C.P.	Developmental Biology of Nitrogen-fixing Cyanobacteria	35
Zeevaart, J.A.D.	Environmental Control of Plant Development and its Relation to Plant Hormones	38

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

Frans J. de Bruijn¹ "Molecular Basis of Symbiotic Plant-Microbe Interactions"

Azorhizobium caulinodans *nif/fix* gene regulation

We previously showed that N- and O₂ control of *Azorhizobium nif/fix* gene expression is mediated primarily via the promoter of the *nif*-specific positive regulatory gene *nifA* (6,11). We identified, in the *nifA* 5' region, specific DNA motifs which have been postulated to be responsible for regulating its expression (11): a nitrogen regulation (*ntr*) box (-12 to -24; GG-N10-GC; see 6), found in genes requiring the alternative sigma-factor NtrA (RpoN) for their expression under N-starvation conditions and the fumarate nitrate respiration (*fnr*) box (TTGAT-N4-ATCAA; see 12), found in the promoter region of genes anaerobically induced via the transcriptional activator Fnr. In order to examine the importance of these sequences, site-specific point mutations were constructed. Mutations in both the *ntr*- and *fnr* motifs were found to affect the expression of the *nifA* promoter drastically.

A novel regulatory locus (*ntrYX*; 4, 6, 10) of ORS571 was identified making the regulatory circuits for N- and O₂ control in this organism quite complex (see 6, 10). In addition, *Azorhizobium ntrA* and *fnr* genes have been cloned and transposon Tn5 insertion mutants in these loci created by gene-replacement. The *ntrA*::Tn5 mutants we isolated were found to be deficient in free-living and symbiotic nitrogen-fixation.

The *Enod2* gene is induced by cytokinin

The *S. rostrata* *Enod2* gene is expressed around 8 days after infection and encodes a proline-rich protein, consisting predominantly of two repeating oligopeptides (XEKPP(P) and XY(X)PP(P)) and a putative signal peptide (C. Dehio and F.J. de Bruijn, manuscript submitted). The *Enod2* gene has been shown to be specifically expressed in the nodule parenchyma cells (15) and the *Enod2* product has been proposed to be a cell-wall protein, which may play a role in the creation of an oxygen diffusion barrier in the nodule (see 9). We have started to examine the regulation of the *S. rostrata* *Enod2* gene using northern hybridizations and by analysing the expression of chimeric *Enod2*- β -glucuronidase (*uidA*, *gus*) genes in transgenic *Lotus corniculatus* plants (see 5). We have been able to show that exogenous application of physiologically significant concentrations of cytokinins (e.g. 10nM zeatin) induces *Enod2* gene expression in *S. rostrata* roots within 2 h. This response is cytokinin-specific and is not found in auxin-, gibberellin- or ethylene-treated plantlets, nor in plantlets stressed by heat, flooding, desiccation, wounding or heavy metals (C. Dehio and F.J. de Bruijn, manuscript submitted). This gene is therefore

¹The principal investigator of this task joined the P.R.L. in June of 1990, while remaining coordinator of the research activities of a group of collaborators at the Max Planck Institute in Cologne, F.R.G. (6-12/1990). Therefore, the work presented was carried out at both institutes.

not only a good candidate to study nodule-specific plant gene expression, but also serves as a unique model to study plant gene induction by cytokinin.

Cis-acting elements involved in nodule-specific expression of leghemoglobin genes

The leghemoglobin (*lb*) genes are maximally induced about 4 days after the Enod2 genes. A functional analysis of the *S. rostrata* leghemoglobin *g/b3* gene promoter region in transgenic *Lotus* and tobacco plants has revealed the presence of several positive regulatory regions and an ATG-proximal, tissue-specific element involved in nodule-specific gene expression (5, 13). Cytological Gus staining experiments have shown that the *g/b3* promoter is only expressed in the infected cells of the nodule (13). Moreover, the *g/b3* promoter has been found to be expressed in tobacco, primarily in the roots, which is interesting in light of the fact that the hemoglobin gene of the non-nodulated plant *Trema tomentosa* also shows a root-specific expression pattern in tobacco (1, 13).

Trans-acting factors interact with *lb* gene 5' regions

We previously reported the specific binding of a nodule factor to an A/T-rich binding site, repeated several fold in the *S. rostrata* *g/b3* and soybean *lbc3* 5' regions (3, 7, 8; BS2*). We have found that an oligonucleotide corresponding to this A/T binding site (BS2*) is capable of enhancing gene expression in transgenic plants, when fused *in cis* to a minimal plant promoter (P. Welters, K. Szczyglowski, L. Szabados and F.J. de Bruijn, unpublished results), suggesting that they play a role *in vivo*. However, this enhanced expression is not nodule-specific, confirming the requirement for other *cis*-acting elements (see section on *cis*-acting elements, above). Gel-retardation assays have also revealed a distinct G/C rich binding site, approximately 620 bp upstream of the transcriptional start-site (bacterial factor binding site; BBS1), which interacts specifically with a heat stable, proteinaceous factor from nodule extracts (BBF1). Surprisingly, this factor is produced by the infecting *Azorhizobium* (P. Welters and F.J. de Bruijn, unpublished results). An 11- kD protein that co-purified with the binding activity was partially sequenced and found to share a high degree of homology with histones and bacterial HU proteins (P. Welters, K. Palme and F.J. de Bruijn, unpublished results). Bacterial HU proteins are involved in gene transcription, possibly by bending DNA (see 14). The role of this peculiar protein-DNA interaction in *lb* gene expression remains to be elucidated.

Thus, multiple *cis*-acting elements and *trans*-acting factors play a role in *lb* gene regulation, and the architecture of the *lb* 5'upstream regions is correspondingly complex (see 2, 5).

BIBLIOGRAPHY

1. Bogusz, D., D. J. Llewellyn, S. Craig, E. Dennis, C. Appleby and W.J. Peacock. *The Plant Cell* 2:633 (1990).

2. de Bruijn, F.J. and J. Schell. In: *Control of Plant Gene Expression* (Verma, D.P.S., ed.) The Telford Press, Caldwell, N.J., USA, in press (1991).
3. de Bruijn, F.J., E.O. Jensen, B.A. Metz, H.-J. Hoffmann, P. Welters, K.A. Marcker and J. Schell. In: *Molecular Genetics of Plant-Microbe Interactions 1988* (Palacios, R. and D.P.S. Verma, eds.), A.P.S. Press, The American Phytopathological Society, St. Paul, Minn., U.S.A., pp. 333-339 (1988).
4. de Bruijn, F.J., K. Pawlowski, P. Ratet, U. Hilgert, C.H. Wong, M. Schneider, H. Meyer z.A. and J. Schell. In: *Nitrogen Fixation: Hundred Years After* (Bothe, H., F.J. de Bruijn and W.E. Newton, eds.) Gustav Fischer Verlag Stuttgart, New York, pp. 351-356 (1988).
5. de Bruijn, F.J., L. Szabados and J. Schell. *Dev. Genet.* 11:182 (1990).
6. de Bruijn, F.J., U. Hilgert, J. Stigter, M. Schneider, H. Meyer z.A., U. Klosse and K. Pawlowski. In: *Nitrogen Fixation: Achievements and Objectives*, (P. Greshoff, E. Roth, G. Stacey and W.E. Newton, eds.), Chapman and Hall, New York pp. 33-44 (1990).
7. Jensen, E.O., K. Marcker, J. Schell and F.J. de Bruijn. *EMBO J.* 7:1265 (1988).
8. Metz, B. A., P. Welters, H.-J. Hoffmann, E.O. Jensen, J. Schell and F.J. de Bruijn. *Mol. Gen. Genet.* 214:181 (1988).
9. Nap, J-P. and T. Bisseling. *Science* 250:948 (1990).
10. Pawlowski, K., U. Klosse and F.J. de Bruijn. *Mol. Gen. Genet.* , in press (1991).
11. Ratet, P., K. Pawlowski, J. Schell and F.J. de Bruijn. *Mol. Microbiol.* 3: 825 (1989).
12. Spiro, S. and J. R. Guest. *FEMS Microbiol. Rev.* 75:399 (1990).
13. Szabados, L., P. Ratet, B. Grunenberg and F.J. de Bruijn. *The Plant Cell* 2:973 (1990).
14. Schmidt, M.B. *Cell* 63:451 (1990).
15. Van de Wiel, C., B. Scheres, H. Franssen, M-J. van Lierop, A. van Lammeren, A. van Kammen and T. Bisseling. *EMBO J.* 9:1 (1990).

Deborah P. Delmer "Enzymatic Mechanisms and Regulation of Plant Cell Wall Biosynthesis"

Evidence for a c-di-GMP-dependent cellulose synthase in plants

Although c-di-GMP is a very specific activator of the bacterial cellulose synthase, no evidence has been available concerning its possible role in plants. The uniqueness of its structure and function makes it an attractive probe to search for any polypeptides in plants which may bear similarity to the bacterial cellulose synthase. Therefore, in collaboration with R. Mayer and M. Benziman of The Hebrew University, we have synthesized ³²P-c-di-GMP and used it for direct photo-labeling of cotton fiber membrane polypeptides. Two c-di-GMP-binding polypeptides, of 83 and 48 kD were identified with this probe. The ability to detect these c-di-GMP polypeptides is strongly correlated with the increase in the rate of cellulose synthesis *in vivo*. Neither polypeptide can be

detected by photo-labeling of membrane proteins derived from fibers engaged in low rates of cellulose synthesis during primary wall deposition; both are first detected at the transition to secondary wall deposition, and the ability to label the 48-kD species further increases markedly in fibers actively engaged in secondary wall cellulose synthesis where the rate of synthesis *in vivo* is >100-fold higher than that found in young fibers (6). Competition experiments with related nucleotides indicates that these polypeptides bind c-di-GMP with high affinity and specificity. Further strengthening the argument that these polypeptides are components of a cellulose synthase is our observation that both show cross-reaction during Western blotting with a polyclonal antibody prepared against a subunit of the bacterial synthase. Since both the 83- and 48-kD species cross-react, we tentatively conclude that the 48-kD is a proteolytic fragment of the 83-kD polypeptide, a situation analogous to that found in *A. xylinum* where a 91-kD primary gene product is cleaved to a 67-kD c-di-GMP-binding fragment (5). In sum, these observations lead us to the conclusion that higher plants very likely contain a c-di-GMP-dependent cellulose synthase which bears remarkable similarity to the bacterial enzyme. These findings offer hope that the genes coding for the synthase can soon be identified using sequence information derived from clones of the bacterial genes and/or the cotton c-di-GMP-binding polypeptides.

In search of the catalytic subunit of cotton fiber cellulose synthase

The question now arises as to whether the c-di-GMP-binding polypeptides described above also contain a site for binding of the synthase substrate UDP-glc. In previous work (3), we showed that an 84-kD cotton fiber membrane polypeptide was one of several which can be photo-labeled with ³²P-UDP-glc. This polypeptide migrates nearly, but not exactly, coincident with the 83-kD c-di-GMP-binding species during SDS-PAGE, and, in most, but not all, experiments, labeling with UDP-glc is markedly enhanced by the addition of unlabeled c-di-GMP, suggesting that it may indeed be the catalytic subunit of the synthase. We now have further evidence that the UDP-glc- and c-di-GMP-binding polypeptides are distinct species which do not exactly co-migrate during gradient centrifugation. Comparing our data with cotton fibers with recent published work on *A. xylinum*, we are now considering a model wherein the cellulose synthase is comprised of two types of non-identical subunits, one of which is an 84-kD catalytic subunit binding UDP-glc, and the other of which is a c-di-GMP-binding regulatory subunit of 83-kD (cleaving to 48 kD). Analyzing the recent work on *A. xylinum*, one can also propose a similar model for the bacterial enzyme wherein the enzyme is comprised of an 83-kD catalytic subunit (7) and a 91-kD regulatory subunit (cleaving to 67 kD, see refs. 5,10).

The effect of Ca²⁺ on localization and function of plasma membrane proteins in the cotton fiber

In our continuing studies on callose synthesis in cotton fibers, we have observed that activity for this enzyme is very high when extracts are prepared in the presence of 25 mM EDTA and much lower when low levels of Ca²⁺ are present during membrane isolation.

Subsequent washing of the latter type of membranes with EDTA restores callose synthase activity to high levels and also releases a characteristic set of proteins to the supernatant solution. The most notable of the polypeptides released are:

1. A very abundant 34-kD polypeptide that has the following properties. a) It can be photolabeled with α - ^{32}P -GTP; b) it can also be labeled with an analog of UDP-glc, UDP- ^3H -pyridoxal; c) it can be precipitated in almost pure form by addition of Ca^{2+} ; due to this latter characteristic, it is also found in abundance in pellets during product entrapment of callose synthase; and d) in many, but not all, experiments re-addition of the 34-kD species to preparations of gradient-purified callose synthase results in marked inhibition of enzyme activity. In terms of its size and Ca^{2+} -dependent interaction with the plasma membrane, this polypeptide resembles mammalian proteins called annexins which are thought to play a variety of regulatory roles including facilitating interaction of the cytoskeleton with the plasma membrane, secretion, and inhibition of phospholipase A_2 activity (1). An annexin-like protein has recently been characterized in tomato (9).

2. We have also observed to our surprise that the 48-kD c-di-GMP-binding protein described above is released to the supernatant solution when membranes are prepared in EDTA. This event, as well as that of its proposed origin by proteolytic cleavage from an 83-kD precursor, may offer some clues as to why cellulose synthase activity has not been detected previously.

These findings suggest that changes in intracellular Ca^{2+} levels may regulate glucan synthesis in very complex ways in addition to the well-known Ca^{2+} activation of callose synthase (2). This year we have also observed that the size of a soluble polypeptide which interacts with DCB (8) is markedly different when extracts are prepared in the presence of EDTA (42 kD) or divalent cations (18 kD). A further intriguing observation is that a soluble Ca^{2+} -dependent protein kinase activity is only detected in extracts prepared in the presence of EDTA and is absent in extracts prepared in the presence of Ca^{2+} .

A search for mutants of *Arabidopsis* impaired in glucan synthesis

Callose is not a normal wall constituent except in specialized cells such as pollen tubes or fibers derived from epidermal hairs of seeds. It is transiently deposited at the cell plate, and synthesized rapidly and in large amounts at sites of wounding or infection (2). Thus, the interesting question arises as to whether a plant impaired in callose synthesis could survive and what its phenotype would be. This year we devised a rapid screening procedure used for attempts to select such mutants in *Arabidopsis*. Pieces of leaves of young M_2 *Arabidopsis* seedlings are incubated with substrate and effectors of callose synthase, permeabilized with DMSO, and callose produced by trichomes is detected by fluorescence microscopy following staining with Sirofluor. After screening 400 such seedlings, one plant deficient in its ability to synthesize callose was tentatively identified,

seed collected, and is now being further analyzed. A search for more such mutants is in progress.

The structure of cell walls of DCB-adapted suspension-cultured tomato cells

The mechanism of adaptation of tomato cells to growth on the cellulose-synthesis inhibitor DCB is somewhat surprising. Instead of selecting cells with a pathway of cellulose synthesis resistant to DCB, we found that our adapted cells tolerated DCB because they had "learned" to grow with cell walls containing almost no cellulose (8). This year we learned that these cells still possess the capacity for cellulose synthesis and proceed to do so at normal, or even higher than normal, rates immediately after removal of DCB. We also now know that this type of adaptation is not unique to tomato, since we now have an adapted cell line of tobacco which has a similarly altered wall composition when growing on DCB.

Our early studies on the nature of the residual wall of tomato cells growing on DCB revealed that these cells also had reduced levels of hyp-rich protein, elevated levels of uronic acid-rich polymers, and very reduced levels of xyloglucan which, lacking cellulose with which to interact, was excreted to the medium in large amounts. This year, we have found that treatment of intact, adapted cells with the chelator CDTA causes the walls to break apart with substantial solubilization of uronic-acid-rich polymers. From this finding, we now conclude that the interaction of these polymers with Ca^{2+} provides one of the major load-bearing network in these walls. Nevertheless, a substantial part of the wall still remains insoluble after CDTA treatment, and we have devoted further effort toward determining the linkages between polymers in this fraction as well. Recently we have found that borohydride reduction at 4 C and pH 7.0, a treatment designed to reduce methyl esters of pectins (4), releases a fraction rich in extensin. We are now further examining the possibility that tomato cells may contain a cross-linked extensin network which is linked to other polymers in the wall via ester linkages, and that the level of this network may be relatively higher in the walls of adapted cells.

BIBLIOGRAPHY

1. Crompton, M.R., S.E. Moss and M.J. Crumpton. *Cell* 55:1 (1988).
2. Delmer, D.P. *Ann. Rev. Plant Physiol.* 38:259 (1987).
3. Delmer, D.P., M. Solomon and S.M. Read. *Plant Physiol.*, in press, (1991).
4. Maness, N.O., J.D. Ryan and A.J. Mort. *Analyt. Biochem.* 185:346 (1990).
5. Mayer, R., P. Ross, H. Weinhouse, D. Amikam, G. Volman, P. Ohana, R. Calhoon, H.C. Wong and M. Benziman. *Proc. Natl. Acad. Sci. USA*, in press.
6. Meinert, M. and D.P. Delmer. *Plant Physiol.* 59:1088 (1977).
7. Saxena, I.M., F.C. Lin and R.M. Brown, Jr. *Plant Mol. Biol.* 15:673 (1990).
8. Shedletzky, E., M. Shmuel, D.P. Delmer and D.T.A. Lampert. *Plant Physiol.* 94: 980 (1990).
9. Smallwood, M., J.N. Keen and D.J. Bowles. *Biochem. J.* 270:157 (1990).

10. Wong, H.C., A.L. Fear, R.D. Calhoon, G.H. Eichinger, R. Mayer, D. Amikam, M. Benziman, D.H. Gelfand, J.H. Meade, A.W. Emerick, R. Bruner, A. Ben-Bassat and R. Tal. *Proc. Natl. Acad. Sci. USA* 87:8130 (1990).

Pamela Green "Molecular Mechanisms that Regulate the Expression of Genes in Plants"

Development of methods to measure rates of mRNA degradation in suspension cultures

To date no system has been reported for the measurement of the degradation rates of specific transcripts in stably transformed plant cells. The development of such systems will allow the stabilities of a variety of engineered transcripts to be directly compared. We have sought to establish methods to measure mRNA degradation rates in a cultured line of *Nicotiana tabacum* cells (NT-cells) (10) based on methods used by others for cultured mammalian cells (e.g., 6,12). NT-cells provide an attractive model system for such an endeavor because they are homogeneous, grow rapidly and are very easy to manipulate. Moreover, transformation of NT-cells via *Agrobacterium tumefaciens* is particularly expedient (1). Our initial efforts have focused on the use of inhibitors of transcription to allow degradation of the mRNA to be studied in the absence of synthesis. Very few experimental data are available on the effectiveness of potential transcriptional inhibitors in plant cells. For this reason we tested several inhibitors for their ability to reduce the incorporation of ³[H]-uridine into polyadenylated mRNA in NT-cells. These experiments showed that Actinomycin D (ActD), which is the most commonly used transcriptional inhibitor for the study of mRNA stability in mammalian cells, effectively inhibits mRNA synthesis in NT-cells. α -amanitin, which is known to inhibit transcription in isolated plant and animal nuclei, had no effect on mRNA synthesis in intact cells. The lack of effect of α -amanitin, and another potential inhibitor, cordycepin, may indicate that these compounds do not enter intact NT-cells efficiently. Using several probes specific for endogenous genes or foreign genes stably introduced into NT-cells, we have identified candidates for inherently unstable and inherently stable mRNAs. The measurement of mRNA decay rates for the unstable mRNAs following ActD treatment is in progress. Our data indicate that the kinetics of mRNA degradation are first order over the course of our experiments. (Supported, in part, by the USDA grant # 9001167.)

Reporter genes for the identification of sequences that control mRNA stability

An effective way to identify sequence elements that control gene expression is to test putative regulatory elements for function within the context of a foreign reporter ("TEST") gene. We have constructed a TEST gene consisting of the strong 35S promoter from the Cauliflower Mosaic Virus, fused to the coding region of the bacterial β -glucuronidase gene (GUS), and polyadenylation signals from the *rbcS-E9* gene (E9). To facilitate the insertion of putative regulatory elements, the promoter, coding region, and 3' end of the 35S-GUS-

E9 TEST gene are separated with unique restriction sites. The sites flanking the GUS segment are located in the 5' and 3' noncoding regions of the transcription unit where sequences that affect mRNA stability are known to reside. In mammalian cells, an AU-rich sequence from an unstable lymphokine transcript was shown to destabilize the β -globin transcript when inserted into the 3' noncoding region of the mRNA (13). This AU-rich element and several others have been incorporated into the 35S-GUS-E9 TEST gene in either the 3' or 5' noncoding region. For another set of constructs, the TEST gene was modified by the insertion of the DST element, a sequence that is conserved among a set of unstable small auxin-up RNA transcripts. Each of the modified TEST genes has been introduced into NT-cells using a transformation vector containing a 35S-CAT-3C REFERENCE gene that will serve as an internal standard for all constructs. Because our experiments indicate that mRNA decay is first order, position effects that alter the rate of transcription should not alter the rate of decay. Nevertheless, our reporter gene configuration is very similar to a test/reference gene system used previously to normalize for position effects in transcriptional studies (2). Our initial experiments have shown that the CAT and GUS transcripts decay with similar half-lives in NT cells. Preliminary data indicate that insertion of two tandem copies of the DST sequence into the 3' end of the GUS TEST transcript markedly destabilizes the GUS mRNA.

A second set of reporter genes has been constructed to test whether sequence elements that regulate mRNA stability in NT-cells play the same roles in transgenic plants. In plants, we will use a regulated promoter from a chlorophyll a/b binding protein gene (Cab-1) to control transcription rather than the 35S promoter used for NT-cells. The Cab-1 promoter is regulated by light and a circadian clock such that it directs transcription transiently each morning in transgenic tobacco (11). Therefore it should be possible to follow the decay of transcripts driven by the Cab promoter by isolating RNA from leaf samples during the afternoon and evening after transcription has ceased. Control Cab-GUS-E9 and Cab-CAT-3C genes have been introduced into tobacco, and transgenic plants have been regenerated. Preliminary experiments have shown that the CAT and GUS transcripts disappear in the afternoon and evening as expected. F1 seedlings from the transgenic plants are being used to determine more precisely how rapidly these transcripts decay. While in theory, any promoter that can be induced transiently (e.g., a heat-shock promoter) could be used in this manner, the Cab-1 promoter offers the unique advantage of not requiring any alteration of normal growth conditions while stability is examined. (Supported, in part, by USDA grant #9001167.)

Characterization of SAUR-AC1, a small auxin-up RNA gene of *Arabidopsis*

The SAUR genes were originally characterized from soybean where they comprise a small gene family (9). During gravitropism, there is a rapid redistribution of the SAUR transcripts in soybean hypocotyls, which is presumably mediated at least in part by auxin. The kinetics of redistribution indicate that the SAUR transcripts are very unstable (7). We have cloned an *Arabidopsis* SAUR gene, designated SAUR-AC1, using PCR primers corresponding to the most conserved region of the soybean genes. The region amplified

by PCR was used as a hybridization probe to isolate the genomic clone. Like its soybean homologs, SAUR-AC1 is inducible by auxin in elongating seedlings. From amino acid 27 to 76 this clone is 80% homologous at the amino acid level with the prototype SAUR 15A gene from soybean and 74% at the nucleotide level. Sequences upstream and downstream of the coding region have also been determined. Interestingly, a sequence with similarity to the conserved DST element of the soybean SAUR transcripts exists in the 3' flanking region of SAUR-AC1. This sequence may be involved in the expression of SAUR-AC1 because other studies indicate that the DST element can act as an mRNA instability determinant as mentioned above. This implication will be tested by creating deletion and substitution mutations in SAUR-AC1, and monitoring the stability of the resulting transcripts.

Identification of major ribonucleases (RNases) from *Arabidopsis*

We have used a substrate-based gel assay to characterize the profile of RNases in *Arabidopsis*, as a first step towards identifying the RNases that play a role in mRNA stability. Using this approach, we have identified 16 RNases from *Arabidopsis* leaves and stems (Yen and Green, in press). These RNases vary in size from 9 to 41 kD. Most of the RNases exhibit a pH optimum of about 6.5; however, an activity at 22.6 kD is enhanced at low pH. A number of RNases in the 30- to 41-kD range are sensitive to EDTA and their activities are enhanced by the presence of a low concentration of zinc during renaturation. At least one RNase appears to co-migrate with a major DNase activity. The differential accumulation of several RNases in stems versus leaves indicates that some RNases are controlled in an organ-specific manner in *Arabidopsis*. Based on the biochemical properties of the individual activities (Yen and Green, submitted), *Arabidopsis* appears to contain representatives of at least three of the four major classes of RNA-degrading enzymes of higher plants (3,15). (Supported, in part, by a grant from the McKnight foundation.)

Identification of putative RNase genes from *Arabidopsis*

The only RNase genes that have been cloned from higher plants are those expressed in species known to exhibit self-incompatibility, such as the S-genes of *Nicotiana glauca* (8). We have investigated whether this particular type of RNase gene is restricted to self-incompatible species, or if similar genes are expressed in other plants. Using a polymerase chain reaction we have identified a set of three putative RNase genes in *Arabidopsis* (14), which is a self-compatible plant. These *Arabidopsis* genes, designated RNS1, RNS2, and RNS3, do not cross-hybridize to each other, and their products are homologous to both the S-gene RNases (28) and a set of fungal enzymes (4,5). The RNS1, RNS2 and RNS3 genes encode transcripts of 1.1, 1.3, and 1.1 kb, respectively; of the three genes, RNS2 is the most highly expressed in whole plants. Recent data indicate that the RNS2 gene is primarily expressed in flowers, similar to the S-RNases of self-incompatible species. The identification of the RNS genes in a self-compatible species

suggests that this class of RNases is of general significance in RNA catabolism in higher plants (14). (Supported in part by a grant from the McKnight Foundation.)

BIBLIOGRAPHY

1. An, G. *Plant Physiol.* 79:568 (1985).
2. Fang, R-X., F. Nagy, S. Sivasubramaniam and N-H. Chua. *The Plant Cell* 1:141 (1989).
3. Farkas, G.L. Encyclopedia of Plant Physiology, Vol. 14B (B. Parthier and D. Buolter, eds.) Springer-Verlag, Berlin pp.224-262, (1982).
4. Horiuchi, H., K. Yanai, M. Takagi, K. Yano, E. Wakabayashi E., A. Sanda, S. Mine, K. Ohgi and M. Irie. *J. Biochem.* (Tokyo) 103:408 (1988).
5. Irie, M., Watanabe, H., Ohgi, K., and Harada, M. *J. Biochem.* (Tokyo) 99:627-633.
6. Lindsten, T., C.H. June, J.A. Ledbetter, G. Stella and C.B. Thompson. *Science* 244:339 (1989).
7. McClure, B.A. and T.J. Guilfoyle. *Science* 243:91 (1989).
8. McClure, B.A., V. Haring, P.R. Ebert, M.A. Anderson and A.E. Clarke. *Nature* 342:955 (1989).
9. McClure, B.A., G. Hagen, C.S. Brown, M.A. Gee and T.J. Guilfoyle. *The Plant Cell* 1:229 (1989).
10. Nagata, T., K. Okada, I. Takebe and C. Matsui. *Mol. Gen. Genet.* 184:161 (1981).
11. Nagy, F., S.A. Kay and N.-H. Chua. *Genes Dev.* 2:376 (1988).
12. Schuler, G.D. and M.D. Cole. *Cell* 55:1115 (1988).
13. Shaw, G. and R. Kamen. *Cell* 46:659 (1986).
14. Taylor C.B. and P.J. Green. *Plant Physiol.* In press (1991).
15. Wilson, C.M. In: Isozymes: Current Topics in Biological and Medical Research 6:33-54, Alan R. Liss, Inc., New York, NY. (1982).

Andrew Hanson "Resistance of Plants to Environmental Stress"

Glycine betaine accumulation

Expression of betaine aldehyde dehydrogenase from sugar beet. In sugar beet, the activity of BADH was found to increase two- to four-fold in both leaves and roots as NaCl level was raised from 0 to 500 mM. The increase in enzyme activity was paralleled by an increase in the level of translatable BADH mRNA. To further investigate this stress induction, several cDNAs encoding BADH were cloned from a lambda gt10 library prepared from leaves of salinized sugar beet plants, by hybridization with a spinach BADH cDNA (1). Three nearly full-length BADH cDNA clones were obtained, one of which was used as a probe in RNA gel blot analyses of total RNA. These analyses showed that salinization to 500 mM NaCl increased BADH mRNA levels four-fold in leaves and three-fold in the taproot. (Supported in part, by grants from USDA-CRGO, and the BSDF.)

Novel osmoprotectant compounds

Determination of choline-O-sulfate by fast atom bombardment mass spectrometry.

A sensitive and specific method was developed for determining choline-O-sulfate by fast atom bombardment mass spectrometry, using a deuterium labeled (d_5) choline-O-sulfate internal standard. This method was used to survey thirty native halophytic species from fourteen families. Twenty three species had very little choline-O-sulfate ($<2 \mu\text{mol g}^{-1}$ dry wt) but *Avicennia germinans* contained $8 \mu\text{mol g}^{-1}$ dry wt, and all six members of the family Plumbaginaceae tested (*Limonium* and *Armeria* species) accumulated $>100 \mu\text{mol g}^{-1}$ dry wt. The identity of choline-O-sulfate was confirmed by high resolution and tandem mass spectrometry, by ^1H NMR, and by analysis of hydrolysis products.

Synthesis and accumulation of quaternary ammonium compounds in *Limonium* spp. in response to salt stress.

Fast atom bombardment mass spectrometry was used to determine QACs in 22 species from the widely distributed halophytic genus *Limonium* (Plumbaginaceae). Under saline conditions, all species accumulated choline-O-sulfate to high levels ($40\text{--}150 \mu\text{mol g}^{-1}$ dry wt). Leaves of 3 species accumulated glycine betaine ($>100 \mu\text{mol g}^{-1}$ dry wt) but contained no detectable β -alanine betaine; the other species accumulated β -alanine betaine ($40\text{--}150 \mu\text{mol g}^{-1}$ dry wt) but had little or no glycine betaine. Leaf disks of both β -alanine betaine- and glycine betaine-accumulating species metabolized [^{14}C]choline to choline-O-sulfate, but only the latter formed [^{14}C]glycine betaine. Conversely, only β -alanine betaine-accumulators converted β -[^{14}C]alanine to β -alanine betaine. When β -alanine betaine- and glycine betaine-accumulators were salinized with various levels of NaCl, the levels of their respective betaines, plus that of choline-O-sulfate, were closely correlated with leaf solute potential. Sulfate salinization resulted in an increase in choline-O-sulfate level and a matching decrease in glycine betaine level. These data establish that β -alanine betaine replaces glycine betaine, the common angiosperm betaine, in some but not all members of the genus *Limonium*, and indicate that both compounds as well as choline-O-sulfate may serve as compatible cytoplasmic osmolytes in this taxon. The data further suggest that the synthesis of choline-O-sulfate and glycine betaine may compete for choline. Such competition could have favored the evolution of the novel biosynthetic pathway for β -alanine betaine.

Osmoprotection of *Escherichia coli* and *Salmonella typhimurium* by choline-O-sulfate and β -alanine betaine.

Although choline-O-sulfate and β -alanine betaine can on physico-chemical grounds be reasonably assumed to be compatible solutes (2), there is little experimental evidence for this. The osmoprotective effects of these compounds were therefore evaluated in *E. coli* and *S. typhimurium*. When added at 1 mM to medium containing 0.6 M NaCl, β -alanine betaine and choline-O-sulfate acted as osmoprotectants comparable in effectiveness to glycine betaine. All three QACs decreased the doubling time to approximately 1.5 hours, although the lag phases for β -alanine betaine and choline-O-sulfate were consistently longer than for glycine betaine.

Lactate glycolysis

Lactate production and secretion by roots of *Limonium sinuatum*. Experimental measurements with mesophytes subjected to anoxia indicate that the flux to lactate is generally far less than that to ethanol. Because the roots of many halophytes penetrate completely anoxic soil horizons, the role of lactate fermentation was investigated in the salt marsh species *Limonium sinuatum*. Roots were incubated for 8 h under anoxic conditions in the presence of [U-¹⁴C]glucose, and labeled metabolites were analyzed by distillation, ion exchange chromatography and TLC. With roots previously acclimated to hypoxia, lactate was always a significant product; the ratio of labeled lactate/ethanol ranged from approximately 0.3 to 2.0 in various experiments. In all cases, most of the labeled lactate was found in the medium. (Supported in part by the Michigan Agricultural Experiment Station.)

BIBLIOGRAPHY

1. Weretilnyk, E.A. and A.D. Hanson. *Proc. Natl. Acad. Sci. USA*. 87:2745 (1990).
2. Wyn Jones, R.G. *Recent Adv. Phytochem.* 18:55 (1984).

Hans Kende "Studies on Hormone Biosynthesis and Action"

Ethylene biosynthesis

Recently, Van Der Straeten et al. (4) published the first full-length sequence of a cDNA encoding ACC synthase from tomatoes (subsequently referred to as ACC synthase 1). Partial amino acid sequences obtained by us from three CNBr cleavage peptides of ACC synthase were all contained in the cDNA-derived amino acid sequence of ACC synthase 1. We prepared oligonucleotide primers corresponding to two of our known amino acid sequences and amplified a 288-bp fragment from tomato-fruit cDNA using the polymerase chain reaction (PCR). This PCR product was cloned into pUC19 and sequenced. It turned out to have 85% homology to the corresponding portion of the clone of Van Der Straeten et al. (19) and was used to screen a cDNA library prepared from poly(A)⁺ RNA of ripening tomato fruits. Three cDNA clones were isolated which were homologous to each other but which differed in their nucleotide sequence from the cDNA clone encoding ACC synthase 1. This cDNA encoded, therefore, a new isoform of ACC synthase (subsequently referred to as ACC synthase 2). The overall DNA sequence similarity between the coding regions of ACC synthase 1 and 2 is 75%; the deduced amino acid sequences are 68% homologous at optimal alignment. Since the 3' ends of the two clones are dissimilar, gene-specific probes were amplified by PCR in order to compare the expression of the two genes. Poly(A)⁺ RNA was prepared from tomato pericarp tissue at four different stages of ripening, either immediately after isolation of the tissue or 4 h after wounding. RNA blot analysis showed that the level of mRNA encoding ACC synthase 1 increased

during ripening and as a result of wounding. The level of ACC synthase 2 mRNA increased during ripening but was not affected by wound stress. Further analysis using RNA from pink fruits wounded for up to 8 h showed that the level of mRNA for ACC synthase 1 greatly increased within 40 min, while that for ACC synthase 2 remained constant even after 8 h of wounding. Gene-specific probes hybridized to DNA fragments of different sizes on Southern blots, confirming that the two ACC synthase isoforms are encoded by different genes.

Mutants defective in ethylene formation would be very useful for studying ethylene biosynthesis and action. We have been screening M_2 populations of *Arabidopsis* with the aim of finding mutants deficient either in the activity of ACC synthase or of EFE. To date, approximately 125,000 M_2 plants have been examined. The ethylene-producing capacity of individual plants was assessed according to the following procedure. A mature leaf was removed from the plant and enclosed in a vial containing some water. After 2 and 5 h, the level of ethylene in the head space of each vial was determined by gas chromatography. In order to increase the number of putative mutants among the population of plants tested for ethylene-synthesizing capacity, a pre-selection scheme was employed. Since ethylene mediates closure of the plumular hook in etiolated dicotyledonous seedlings, a plant that produces less ethylene or is insensitive to it will have at least a partially open hook. Etiolated *Arabidopsis* seedlings displaying partly to fully opened hooks were considered putative ethylene biosynthetic mutants and were allowed to grow to maturity. To select mutants specifically impaired in EFE, ACC was included in the germination medium. Under these conditions, mutants blocked at or prior to the ACC synthase step would maintain a closed hook. Seedlings that are grown on ACC and still have open hooks would include ethylene-insensitive mutants as well as mutants blocked in EFE. Fully grown plants that had an open apical hook as seedlings were tested for ethylene-forming capacity as described above. Mutants that consistently produced low amounts of ethylene have now been tested through the M_4 or M_5 generation, and backcrossing to the wild-type parent has been started to remove unlinked mutations. When we began the search for ethylene biosynthetic mutants, we anticipated finding null mutants in ACC synthase and possibly in EFE. However, it has become clear from recent work that ACC synthase has multiple forms (see above), which appears to be the case in *Arabidopsis* as well (Theologis, personal communication). Since null mutations in ACC synthase activity (and possibly in EFE activity as well) are not likely to exist, pairwise crosses between the mutants have been started to define complementation groups and to construct double mutants that may lack ethylene-forming capacity. (Supported, in part, by USDA Grant No. 89-37261-4461.)

Studies on ethylene action in deepwater rice

Earlier results (3) indicated that ethylene stimulates growth of deepwater rice by increasing the plant's responsiveness to GA. Since treatment with ethylene reduces the level of abscisic acid (ABA) in some plants (5) and since ABA is a growth inhibitor counteracting the effect of GA, we examined the possibility that increased responsiveness

to GA is due to reduced levels of ABA in the tissue. In other words, enhanced growth may be the consequence of a shift in the ratio between a growth inhibitor and a growth promoter. We tested our hypothesis by examining the effect of applied ABA and GA on growth and by measuring the level of ABA in submerged and ethylene-treated plants. Applied ABA inhibited submergence-induced internodal growth of deepwater rice, and added gibberellic acid (GA_3) fully overcame this inhibition. In terms of applied growth regulators, the balance of ABA and GA did, therefore, determine the growth rate of deepwater-rice plants. Next, we examined the effect of submergence and ethylene treatment on ABA content of internodes. Growth was induced by submerging whole plants in 300-liter tanks or by enclosing them in plastic tubes through which air containing 3-5 μ l/l ethylene was circulated. Air-grown plants served as control. ABA was extracted from the growing zone of the uppermost internode, and its concentration was determined using an immunoassay. Both, submergence and treatment with ethylene, reduced ABA content in internodal tissue within 3 h to about one-fourth of its original level. Therefore, submergence and ethylene may indeed promote growth, at least in part, by reducing the concentration of ABA in the elongating region of the internode.

Rapid growth in deepwater rice very likely requires suppression of processes that restrict elongation and promotion of processes that support it. We examined lignification as one of the cellular events that limit growth irreversibly, and changes in the hemicellulosic composition of the cell wall that appear to be required for sustained cell elongation. We showed previously that the activities of two enzymes of the lignin biosynthetic pathway, phenylalanine ammonia-lyase and coniferyl alcohol dehydrogenase, were much lower in the newly formed internodal tissue of submerged or GA_3 -treated plants than in control internodes (Sauter and Kende, unpublished). These reduced enzyme activities were reflected in the lignification of the respective tissues. The lignin content per dry weight of cell wall material was five- to sixfold lower in the elongating part of submerged internodes than in the corresponding region of air-grown control internodes. These results demonstrate that suppression of lignification is one component of the growth process in deepwater rice.

β -Glucan, an unbranched homopolymer consisting of (1-3)- β -D- and (1-4)- β -D-glucosyl units, is a unique component of the cell wall of graminaceous monocots. It may comprise up to 30% of the hemicellulose fraction which, in turn, is at least 50% of the total cell wall material of this group of plants. Since the synthesis of hemicelluloses has been implicated in the control of cell growth (2), we set out to determine the pattern of β -glucan distribution in rapidly growing and in control rice internodes. We found that induction of cell elongation is correlated with a marked increase in the level of β -glucan in the elongation zone. Therefore, the increase in β -glucan levels is being studied as a process that is positively linked to growth. (Supported, in part, by NSF Grant No. DCB-8718873.)

Regulation of NR by cytokinins

We are studying the regulation of NR by cytokinins and nitrate in embryos of *Agrostemma*. While cytokinins enhance NR activity in several plants in the presence of nitrate, only in *Agrostemma* have cytokinins been shown to do so independently of nitrate. Therefore, *Agrostemma* may be a suitable plant to study the regulation of gene expression by cytokinins.

First, we optimized the experimental conditions for the induction of NR with regard to incubation conditions and the concentrations of cytokinins and nitrate. We used mostly benzyladenine (BA), a synthetic cytokinin, but the natural cytokinin zeatin elicited the same response. Since light had been shown to enhance the induction of NR by nitrate and BA in several plants, we examined the effect of illumination on the development of NR in embryos of *Agrostemma*. We did not find any interaction between light and either nitrate or BA on the induction of NR. Once the experimental conditions had been defined, we set out to determine whether or not BA and nitrate regulate the same or different genes encoding NR. We used a partial cDNA clone for NR from tobacco (BMC102010; ref. 1) to probe genomic DNA of *Agrostemma* for related or homologous sequences. Southern blot analysis showed three EcoRI and three HindIII bands ranging from 6.2 to 16 kb to which BMC102010 hybridized. The NR genes cloned thus far range from 5.3 to 6.1 kb. Therefore, each of the cross-hybridizing bands could contain a NR gene. This indicates, but does not prove, that *Agrostemma* may have two or three NR genes.

Poly(A)⁺ RNA was isolated from embryos induced with nitrate, BA, and nitrate plus BA. Blot analysis of this RNA from induced embryos showed a 3.5-kb band cross-hybridizing to BMC102010. No NR mRNA was detected in the RNA preparation from control embryos. We determined the relative amounts of NR mRNA present under each of the inductive conditions by densitometry of the autoradiographs and found that the effects of BA and nitrate at optimal concentrations were strictly additive, just as had been observed for the induction of enzyme activity. These results indicate that the regulation of NR by nitrate and BA may be at the transcriptional level.

BIBLIOGRAPHY

1. Calza, R., E. Huttner, M. Vincentz, P. Rouze, F. Galangau, H. Vaucheret, I. Cherel, C. Meyer, J. Kronenberger, M. Caboche. *Mol. Gen. Genet.* 209:552 (1987)
2. Carpita, N.C. In: *Physiology of Cell Expansion during Plant Growth* (D.J. Cosgrove and D.P. Knievel, eds.), pp. 28-45, Am. Soc. Plant Physiol., Rockville (1987)
3. Raskin, I. and H. Kende. *Plant Physiol.* 76:947 (1984)
4. Van Der Straeten, D., L. Van Wiemeersch, H.M. Goodman and M. Van Montagu. *Proc. Natl. Acad. Sci. USA* 87:4859 (1990)
5. Zeevaart, J.A.D. *Plant Physiol.* 71:477 (1983)

Derek T.A. Lamport "Plant Cell Wall Proteins"

Our most recent advances can be summarized as follows:

(a) Identification of the first gymnosperm extensin as a glycoprotein homologous both with dicot extensins *per se*, and the recently described "repetitive proline-rich proteins" [RPRPs] of soybean in particular (3): preprint attached. This work has generated an important rule for predicting extensin sequence types, based only on hydroxyproline arabinoside profiles, which will be described in more detail in next year's report.

(b) Identification of the first non-graminaceous monocot extensins and demonstration that they form a "bridge" between our earlier described graminaceous monocot [maize THRGP] extensin and P3 type extensin of dicots like bean and tomato (4).

(c) Isolation of extensins from both primitive and advanced gymnosperms and the highly advanced monocot family Orchidaceae [orchids represent 40% of **all** monocot species].

(d) Purification of the pl 4.6 crosslinking extensin peroxidase to the status of "major component" with $RZ > 1$ (5); this enzyme is specific for P1-type extensins [e.g., it does not crosslink maize THRGP or soybean RPRP]. Furthermore we have confirmed that anhydrous HF does not depolymerise the in vitro-generated crosslinked product, implying that the crosslink itself is stable to HF. This crucial experiment opens the way to the direct isolation of intermolecularly linked peptides following HF-deglycosylation of the crosslinked product followed by trypsinolysis and rapid sizing of the peptides. From the canonical 10-mer and 16-mer repetitive peptide motifs of tomato extensin P1, we predict that trypsinolysis should generate crosslinked peptides which are homo- or hetero-oligomers of the 10- and 16-mers.

(e) Elucidation of the new "twisted hairy rope" model for the gum arabic glycoprotein [GAGP] and corroboration by electron microscopy (6). Our demonstration of an essentially linear rather than proposed spheroidal ("wattle blossom" model) macromolecule (7), firstly means that the shape of all arabinogalactan proteins must be re-evaluated; second it solves the longstanding problem of explaining how the cell can secrete such large molecules through primary cell walls of relatively low porosity [MWCO ~68 kDa], namely by reptation - endwise migration of rodlike molecules (6).

(f) HF-deglycosylation of carcinoma-associated mucins for cloning and immunodiagnosis. We have continued this work with several different groups (1).

BIBLIOGRAPHY

1. Ceriani, R.L., J.A. Peterson, D.T.A. Lamport and R. Amiya. *Breast Cancer Research & Treatment* (submitted 1991).
2. Fincher, G.B., B.A. Stone and A.E. Clarke. *Ann. Rev. Plant Physiol.* 34:47 (1983).

3. Kieliszewski, M.J., R. de Zacks, J. Leykam and D.T.A. Lamport. *Plant Physiol.* submitted (1991).
4. Kieliszewski, M.J., A. Kamyab and D.T.A. Lamport. MS in preparation (1991).
5. Upham, B., H. Alizadeh and D.T.A. Lamport. MS in preparation (1991).
6. Qi, W., C. Fong and D.T.A. Lamport. *Plant Physiol.* in press (1991).

LEE MCINTOSH "Interaction of Nuclear and Organelle Genomes"

Photosynthesis

Photosystem II. A series of amino acid mutations in both D1 and D2 (the reaction center "core" of photosystem II) have been made in the areas surrounding and including the redox-active tyrosines, Y_0^+ and Y_2^+ (3,4,5). The time required for recovery of mutants in D1 has been lessened by the construction of a strain of *Synechocystis* 6803 which lacks all three resident *psbA* genes. Two types of mutations have been incorporated into this strain: structural mutations and mutations made to potential Mn amino acid ligands. All mutants were screened initially for the ability to evolve oxygen. Four classes of mutants have been isolated: those evolving oxygen at wild-type rates, those which do not evolve oxygen, and those which evolve oxygen at approximately 1/3 and 2/3 of wild-type rates. Further analysis of these mutants has begun and includes experiments measuring the effect of light intensity on oxygen evolution and fluorescence induction kinetics. Both of these approaches have been undertaken to study the quantum efficiency of cells with altered photosystems.

Photosystem I. The ability to grow *Synechocystis* 6803 heterotrophically has now allowed us to begin a molecular analysis of the reaction center of photosystem I in this organism. The reaction center genes *psaC*, *psaA* and *psaB* have been sequenced and their expression studied in *Synechocystis* 6803 (1,15). In *Synechocystis* 6803, *psaA* and *psaB* are arranged in an operon as they are in higher plants. The genes are transcribed as a 5.0-kb transcript which is rapidly turned over in *Synechocystis* 6803 (15).

The gene products of *psaA* and *psaB*, *PSA-A* and *PSA-B*, have eleven proposed membrane-spanning α -helices similar to those predicted for higher plants (7,8). Four cysteines are thought to bind the [4Fe-4S] center F_x with two of these residues residing in *PSA-A* and two in *PSA-B*. F_x is thus proposed to be shared by two separate polypeptides. Analysis of *PSA-A* and *PSA-B* sequences from a number of organisms has also shown that a series of leucine residues adjacent to the proposed iron-sulfur cysteine ligands are present as a possible "leucine zipper" shared between these two membrane proteins (11). The functional significance of these leucine residues is not known, although it is possible that they may play a role in the biogenesis of the photosystem I reaction center.

As a first step in a molecular/genetic analysis of photosystem I in *Synechocystis* 6803, we have genetically deleted the photosystem I core and recovered viable mutants grown under our light-activated heterotrophic growth conditions (2). The *psaA* gene was interrupted with a bacterial gene encoding kanamycin resistance. A mutant was selected under heterotrophic growth conditions which revealed complete segregation of a mutated *psaA* gene (14). This strain apparently lacks the chlorophyll molecules associated with photosystem I and thereby appears blue in color. Photosystem II activity is present in approximately wild-type amounts and the growth rate of this mutant strain is indistinguishable from wild type grown under heterotrophic conditions. The selection of this photosystem I mutation opens the door for further analysis of the possible redox-active amino acid residues within the photosystem reaction center, as well as those residues involved in its structural integrity and biogenesis.

Molecular bases of respiration

A "classical" material for studying plant wounding and respiration has been potato tubers. We have found that upon wounding of potato tuber tissue for 24 h the relative percent capacity of the alternative pathway increases while the relative percent capacity of the cytochrome pathway decreases (9). These results basically agree with previous work (10), with the exception that the increase in alternative pathway activity appears to correlate with *de novo* synthesis of the alternative oxidase. Partial purification of inner mitochondrial membrane protein complexes of potato, followed by probing with alternative oxidase antibodies, indicates that, in part, the potato alternative oxidase is synthesized *de novo* over at least the 24-h aging period and is composed of, at least in part, a 36-kDa membrane polypeptide (9).

We are also investigating the alternative-pathway respiration in *Nicotiana tabacum* NT1 suspension cell cultures. Preliminary results indicate that the alternative pathway capacity of these cells is approximately 10% of the total respiratory capacity and may fluctuate during the growth period. Monoclonal antibodies to the *S. guttatum* alternative oxidase recognize the oxidase from this cell line and it appears as a single 35-kDa polypeptide. This cell line is transformable and grows non-photosynthetically. In cell cultures of this type the amount of "carbon in" and "carbon out" (carbon balance) may also be measured (13). These features make this a model system for future studies on the genetic alteration of plant respiratory capacity.

Analyses of "non-plant" alternative oxidases. A collaboration with Dr. A. Clarkson (NYU Medical Center) was begun to study the alternative oxidase of the African trypanosome, *Trypanosoma brucei brucei* (the causative agent of African sleeping sickness). Our initial experiments indicate that the *T. brucei* alternative oxidase is composed of a single 36-kDa polypeptide which reacts with a monoclonal antibody produced to the *S. guttatum* oxidase. Ultimately, for comparative purposes, we will clone and sequence the alternative oxidase gene(s) from this organism.

Genes for the higher plant alternative oxidase. We have recently employed the antibodies to the *S. guttatum* alternative oxidase to clone the corresponding gene from a *S. guttatum* cDNA library (12). This gene, *aox1*, has been sequenced and the protein sequence deduced from the DNA sequence. *aox1* is encoded by nuclear DNA and codes for a 42 kDa polypeptide with an apparent transit peptide approximately 6 kDa in length. Upon computer analysis, the mature protein is predicted to have a minimum of three alpha-helical regions. Preliminary experiments employing this cDNA as a probe show that upon induction of increased alternative oxidase capacity in the *S. guttatum* inflorescence with salicylic acid, the mRNA pool for this gene increases. We had previously (6) shown that salicylic acid induces the appearance of the alternative oxidase proteins.

The *S. guttatum* gene has been employed as a probe to obtain cDNA clones from potato and rice. A partial clone from rice has been obtained (Nickels and McIntosh, unpublished) and a full-length clone from potato has been isolated and sequenced (Hiser and McIntosh, unpublished). Comparison of the deduced amino acid sequence with that of the *S. guttatum* sequence shows that the most highly conserved areas are those predicted to form α -helical regions (at least two of which are predicted to be membrane helices). The potato sequence starts with a transit sequence that is very dissimilar to that from *S. guttatum aox1*. Southern blotting with potato genomic DNA indicates that the potato *aox1* gene is present in low copy number.

BIBLIOGRAPHY

1. Anderson, S.L. and L. McIntosh. *Plant. Mol. Biol.* 16:487 (1991a).
2. Anderson, S.L. and L. McIntosh. *J. Bact.* 173:2761 (1991b).
3. Babcock, G.T., B.A. Barry, R.J. Debus, C.W. Hoganson, M. Atamian, L. McIntosh, I. Sithole and C.F. Yocum. *Biochemistry* 28:9557 (1989).
4. Debus, R.J., B.A. Barry, G.T. Babcock and L. McIntosh. *Proc. Natl. Acad. Sci. (USA)* 85:427 (1988a).
5. Debus, R.J., B.A. Barry, I. Sithole, G.T. Babcock and L. McIntosh. *Biochemistry* 27:9071 (1988b).
6. Elthon, T.E., R.L. Nickels and L. McIntosh. *Planta* 180:82 (1989b).
7. Golbeck, J.H. *Biochim. Biophys. Acta Rev. Bioenerg.* 895:167 (1989).
8. Golbeck, J.H. and D.A. Bryant. *Curr. Topics Bioenerg.* 16:83 (1991).
9. Hiser, C. and L. McIntosh. *Plant Physiol.* 93:312 (1990).
10. Laties, G.G. *Annu. Rev. Plant Physiol.* 33:519 (1982).
11. Webber, A.N. and R. Malkin. *FEBS. Lett.* 264:1 (1990).
12. Rhoads, D.R. and L. McIntosh. *Proc. Natl. Acad. Sci. U.S.A.* 88:2122 (1991).
13. Schnapp, S.R., R.A. Bressan and P.M. Hasegawa. *Plant Physiol.* 93:384 (1990).
14. Smart, L.B., S.L. Anderson and L. McIntosh. *EMBO J.* 10:3289 (1991).
15. Smart, L.B. and L. McIntosh. *Plant Mol. Biol.* in press (1991).

Kenneth L. Poff "Sensory Transduction in Plants"

Enhancement of phototropism in *Arabidopsis thaliana* is controlled by phytochrome

We have shown that the capacity of etiolated *Arabidopsis thaliana* seedlings to respond phototropically is altered by a previous exposure to light (5). This alteration, which we refer to as adaptation, consists of several successive components: a desensitization, a recovery, and an increased responsiveness (or enhancement). Within seconds of an exposure to a saturating pulse of blue light, the seedlings are desensitized. That is, the seedlings show no response to a pulse of unilateral blue light which would have induced normal phototropism in the absence of the blue pre-irradiation. The capacity of the seedlings to respond phototropically recovers in about 10-20 min. Following recovery, the phototropic responsiveness is enhanced over about 120 min. In contrast with desensitization, which is induced only by blue light, enhancement can be induced by both blue and red pre-irradiation.

We have measured an action spectrum for enhancement, derived from fluence response relationships measured at 15 wavelengths from 405 nm to 760 nm. This spectrum is virtually identical to the absorption spectrum of the red absorbing form of phytochrome.

The time threshold required for second positive phototropism is altered by a red light pre-irradiation

We have shown (4) that first positive phototropism and second positive phototropism exhibit the same fluence threshold. In addition, second positive phototropism exhibits a time threshold. If the time threshold is measured for etiolated *A. thaliana* seedlings which had not been pre-irradiated, curvature is seen at times of unilateral blue irradiation in excess of about 10-12 min. However, if seedlings are first pre-irradiated with red (669-nm) light at $10 \mu\text{mol m}^{-2}$ 2 h before the inductive unilateral blue irradiation, curvature is seen at times of unilateral blue irradiation exceeding about 4 min. We believe that this dependence of the time threshold for second positive curvature on the pre-irradiation of the seedlings with red light may account for the variable second positive time thresholds reported in the phototropism literature. Phytochrome absorbs blue light in addition to red light. Thus, a blue light irradiation is absorbed in part by phytochrome, which modulates the subsequent phototropic response regulated by the blue light photoreceptor pigment.

Kinetics for first positive phototropism by etiolated seedlings of *Arabidopsis thaliana*

An infra-red (IR)-sensitive imaging system was used to monitor the time course of first positive phototropism under radiation to which the organism does not respond. Phototropism was induced with blue light (BL) at $0.34 \mu\text{mol m}^{-2}$, and the etiolated seedlings were either placed on a stationary stage or mounted on a clinostat.

The time course for curvature by a seedling shows a lag time following the BL pulse. During this lag phase, the seedling shows no measurable curvature. Following the lag period, curvature toward the light source increases with time to a maximum and then decreases in a straightening phase. An average time course for phototropic curvature by a number of stationary seedlings shows a lag phase of about 10-20 min, and an average maximum curvature of 16.4° 80 min after photostimulation. Following maximum curvature, straightening accounts for an average loss of 6.4° of curvature by 180 min after the BL pulse. Control seedlings, treated in the same manner as the experimental seedlings but not irradiated with BL, showed no significant curvature.

In an earth-based experiment, it is impossible to examine any process in the absence of gravi-stimulation. However, a clinostat can be used to ensure that any gravi-stimulation is non-directional. Although we had previously shown that phototropic curvature was not significantly affected by gravitropism, the discovery of the straightening response meant that gravitropism had to be eliminated as a significant factor in a phototropism experiment. To accomplish this, seedlings were rotated on a clinostat immediately following photostimulation. The time course for phototropic curvature of such seedlings is similar to the time course for the stationary seedlings. The lag phase of 15 min, average maximum curvature of 17.7° and the average final curvature of 10.1° for the seedlings on the clinostat are essentially the same as those for stationary seedlings. However, the average length of time to reach maximum curvature was slightly greater (90 min) for the seedlings on the clinostat than for the stationary seedlings. In addition, the straightening phase is initiated more rapidly by the stationary seedlings than by those on the clinostat.

The effect of red light on the curvature and straightening was examined by measuring the time course for phototropic curvature of seedlings which had been pre-irradiated with 60 min of red light beginning 120 min before the BL pulse. The results show a curve shape which does not appear to be significantly different from that for seedlings not pre-irradiated with red light. The lag phase, time to maximum curvature, and straightening are similar. However, the amplitude of the curvature is greater for the red light pre-irradiated seedlings than for the non-pre-irradiated controls.

Based on these results, we conclude that gravity is not the primary cause for the straightening, although there is a small effect of gravity on the straightening. A red light pre-irradiation affects only the amplitude of the subsequent curvature to blue light and does not affect either the kinetics for the development of curvature or straightening.

Physiological and genetic characterization of gravitropism mutants in *Arabidopsis thaliana*

Four gravitropism mutant phenotypes in *Arabidopsis thaliana* have been identified (1). One of these, the "random" or agravitropic phenotype, is of particular interest since it contains the best candidates for perception or early transduction, mutants. Based on data from reciprocal crosses, four "random" gravitropism strains carry single recessive

nuclear mutations with respect to both the gravitropism and starch phenotypes. This is of particular interest because of the controversy concerning the role of starch in gravitropism (2, 6). Based on our data, there is no correlation between the starch and gravitropism phenotypes. For example, MG-32 and MG-65 are agravitropic lines which contain normal levels of starch. However, the agravitropic mutant MG-212 contains only

5% of wild-type starch levels. Therefore, the role of starch in gravitropism remains ill-defined.

Dose-response relationships for the wild-type parent and each of the four mutant strains have been determined in etiolated seedlings. The wild-type parent exhibits two thresholds. The first occurs between 10 and 30 s of stimulation at 1 g. After a peak response of 10° curvature at 5.5 min of stimulation, curvature decreases to nearly zero until doses exceed the second threshold of 1.6 to 2.7 h at 1 g. Doses above 2.7 h in wild-type lead to steadily increasing curvatures. All four mutants examined appear to lack the first response entirely. Three of the mutants also exhibit a slight shift to higher doses in the second threshold. Such shifts in threshold may indicate an alteration in gravity perception.

In these four mutant strains, the gravitropism phenotype appears to be repaired when the seedlings are not etiolated. That is, seedlings grown in white light prior to gravistimulation in darkness respond in a wild-type manner. This relationship between light exposure and the response of the plant to gravity may help explain the wide variability present in the literature with regard to gravitropism thresholds.

Thermotropism in primary roots of maize

We have previously described thermotropism in primary roots of *Zea mays* L. (3). We have now further characterized thermotropism as a function of gradient strength, temperature of exposure, pre-treatment temperature, and gravitropic stimulation. The magnitude of the thermotropic response increases with increasing gradient strength. The temperature for maximum thermotropism is independent of gradient strength and pre-treatment temperature. However, the range of temperatures for positive and negative thermotropism varied with gradient strength. In general, temperatures lower than 25°C resulted in positive tropic responses while temperatures of 39°C or more resulted in negative tropic responses. In addition, thermotropism can modify or even reverse the normal gravitropic curvature of a horizontal root when an appropriate thermal gradient is applied opposite to the 1 g vector. We conclude that root thermotropism is a consequence of thermal sensing and not simply an effect of temperature on growth. Moreover, curvature of the primary root results from the interaction of the thermal and gravitational sensing systems. Because of funding constraints, this area of work will be discontinued unless funded from a non-DOE source.

BIBLIOGRAPHY

1. Bullen, B.L., T.R. Best, M.M. Gregg, S.E. Barsel and K.L. Poff. *Plant Physiol.* 93: 525 (1990).
2. Caspar, T. and B.G. Pickard. *Planta* 177:185 (1989).
3. Fortin, M.-C. and K. Poff. *Plant Physiol.* 94:367 (1990).
4. Janoudi, A. and K. Poff. *Plant Physiol.* 94:1605 (1990).
5. Janoudi, A. and K. Poff. *Plant Physiol.* In press, (1991).
6. Kiss, J.Z., R. Hertel and F.D. Sack. *Planta* 177:198 (1989).

Natasha Raikhel "Molecular Mechanisms of Trafficking in the Plant Cell"

Sorting mechanism of barley lectin to the vacuoles of plant cells

Barley lectin is synthesized as a preproprotein with a transiently associated glycosylated carboxyl-terminal propeptide which is removed prior to or concomitant with deposition of the mature protein in vacuoles. Expression of a cDNA clone for barley lectin in transformed tobacco plants results in the correct processing and accumulation of active mature lectin in vacuoles (2). The glycan of the propeptide is not essential for vacuolar sorting but may influence the rate of processing (682). To investigate the functional role of the carboxyl-terminal propeptide (CTPP) in processing, assembly and sorting of barley lectin to vacuoles, a mutant barley lectin cDNA clone lacking the 15 amino acid CTPP was prepared. The CTPP deletion mutant of barley lectin was expressed in tobacco protoplasts, suspension cultured cells and transgenic plants. In all three systems the mutant barley lectin was synthesized as an active lectin and was secreted to the medium while the wild-type barley lectin construct was sorted to vacuoles. Therefore, we concluded that the carboxyl-terminal domain of the barley lectin preprotein is necessary for efficient sorting of this protein to plant cell vacuoles (1). To test whether CTPP is sufficient to redirect a secreted reporter protein to the vacuoles, we have prepared chimeric genes containing the carboxyl-terminal propeptide of BL fused with a reporter protein and have transformed the constructs into plants. Analysis of the protein fusions are in progress.

Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*)

Hevein is a chitin-binding protein which is present in laticifers of the rubber tree (*Hevea brasiliensis*). A cDNA clone (HEV1) encoding hevein was isolated by polymerase chain reaction using mixed oligonucleotides corresponding to two regions of hevein as primers and a *Hevea* latex cDNA library as a template. HEV1 is 1018 bp long and includes an open reading frame of 204 amino acids. The deduced amino acid sequence contains a putative signal sequence of 17 amino acid residues followed by a 187 amino acid polypeptide. This polypeptide has two striking features. The amino-terminal region (43

amino acids) is identical to hevein and shows homology to several chitin-binding proteins and to the amino-termini of wound-inducible proteins in potato and poplar. The carboxyl-terminal portion of the polypeptide (144 amino acids) is 74-79% homologous to the carboxyl-terminal region of wound-inducible genes of potato. Wounding, as well as application of the plant hormones abscisic acid and ethylene, resulted in accumulation of hevein transcripts in leaves, stems and latex, but not in roots.

BIBLIOGRAPHY

1. Bednarek S Y, TA Wilkins, JE Dombrowski and NV Raikhel. *The Plant Cell* 2:1145 (1990).
2. Wilkins, T.A., S. Y. Bednarek and N.V. Raikhel. *The Plant Cell* in press (1990).

Christopher Somerville "Regulation of Lipid Metabolism"

Characterization of enzymes and genes involved in lipid biosynthesis

We purified the stearyl-ACP desaturase from avocado mesocarp, produced antibodies against the enzyme and used the antibodies to isolate cDNA clones for the enzyme from castor, cucumber and rapeseed (12). The castor cDNA was expressed in both yeast and *E. coli* and functional enzymes were produced in both cases. The very high level of enzyme production in *E. coli* permits the isolation of hundred-milligram quantities of enzyme for biochemical studies. We have used this system to obtain preliminary evidence that the enzyme is an iron-sulfur protein and have initiated a collaboration with Carl Branden and colleagues (Uppsala) to examine the possibility that crystals of the protein can be obtained for X-ray crystallographic studies. Small diffracting crystals have been obtained and we are hopeful that larger crystals will be obtained in the next few months.

In order to determine if cytochrome b_5 is involved in the microsomal desaturase reactions of higher plants we have purified a trypsin-solubilized cytochrome b_5 from cauliflower microsomal membranes and have prepared antibodies against the cytoplasmic domain. The antibody inhibited Δ^{12} desaturation of fatty acids in safflower microsomes (6) providing direct evidence that this desaturase utilizes cytochrome b_5 . This information may facilitate the eventual purification and reconstitution of activity of the microsomal desaturases and provides a point of potential regulation of fatty acid desaturation.

Recently we extended our search for mutants to the enzymes which control fatty acid composition of seeds (11). We have identified mutants deficient in the desaturation of 18:1 to 18:2, and other classes deficient in desaturation of 18:2 to 18:3 in seeds and roots. We have also identified several classes of mutants deficient in the elongation of 18:1 to 20:1 and 22:1. These mutations have been genetically mapped and are currently being used in the isolation of the corresponding genes by chromosome walking in the laboratories of several collaborators.

The first enzyme in lipid biosynthesis is glyceraldehyde 3-phosphate acyltransferase. The properties of this enzyme are implicated as the determinative factor in one of the current theories of chilling sensitivity ("the Murata hypothesis") and are thought to regulate the quality of cocoa butter and other high value lipids. Murata and colleagues have recently cloned a cDNA for this enzyme from squash. We have used their sequence to isolate corresponding cDNA clones from *Arabidopsis* (chilling resistant) and squash (chilling sensitive). These genes are currently being introduced into the *Arabidopsis* mutant (*act1*) which lacks activity for this enzyme in order to directly test the importance of variation in the kind or amount of this enzyme on chilling sensitivity. (Carrie Schneider was supported in part by an NSF Postdoctoral Fellowship; Ellen Kearns was supported in part by an MSU-Biotechnology Fellowship; supported in part by grants from the USDA/NSF/DOE Plant Science Center Program and a gift from Pioneer Hybrid International.)

Production of biodegradable thermoplastic in higher plants

Poly- β -hydroxybutyrate (PHB) is an aliphatic polyester produced as a carbon storage reserve in numerous species of bacteria such as *Alcaligenes eutrophus*. We are exploring the possibility that PHB can be produced in high yield and at low cost by transferring the genes involved in PHB synthesis in *A. eutrophus* to a suitable higher plant. PHB is synthesized in bacteria by the sequential action of three enzymes which catalyze the conversion of acetyl-CoA to acetoacetyl-CoA, then to hydroxybutyryl-CoA, and then to PHB. The genes encoding the three enzymes have been cloned from *A. eutrophus* (13) and have been cloned into derivatives of the binary Ti plasmid PBIN19. The genes have been placed distal to the constitutive 35S promoter from cauliflower mosaic virus and introduced into a non-oncogenic strain of *Agrobacterium tumefaciens*. This, in turn, has been used to initiate the production of transgenic plants by established methods. (Yves Poirier was supported in part by a fellowship from NSERC; the work was supported in part by grant #DMB-9014037 from the SGER program at NSF.)

The pathway of sulfolipid biosynthesis

One of the unique constituents of chloroplast membranes is sulfolipid. Although the structure of this lipid was determined more than 20 years ago, the biosynthetic pathway has not been determined and the functional significance of this membrane component has not been determined. The purple photosynthetic bacterium *Rhodobacter sphaeroides* also accumulates substantial amounts of sulfolipid. Mutants which do not contain sulfolipid were identified by screening a mutagenized population by thin layer chromatography of lipid extracts from several thousand independent colonies. These mutants retain the capability to grow photoautotrophically and several of these mutants accumulate putative precursors of sulfolipid. Determination of the structure of these compounds may permit inferences to be made about the steps in the pathway.

The genes which complement three of the mutants were isolated by complementing the mutants with a cosmid library. The cloned genes will permit the creation of mutants, by

gene disruption techniques, which are completely lacking in sulfolipid. Two of the genes are very closely linked and are probably cotranscribed. Thus, other genes of the pathway may also be in the same operons and it may be possible to create mutations in additional steps of the pathway by gene disruption methods. In a series of complementary experiments we have found that cell-free extracts of the bacterium are capable of synthesizing sulfolipid from exogenous phosphoadenosine phosphosulfate. This has not previously been possible with any other system and should greatly facilitate the testing of hypotheses concerning the reactions involved in sulfolipid biosynthesis. (Supported in part by a grant from the USDA/NSF/DOE Plant Science Center Program.)

What determines the amount of membrane?

Act1 mutants lack activity for the chloroplast enzyme glycerol-3-phosphate acyltransferase (7,8) and are, therefore, largely unable to synthesize lipids by the plastid pathway which normally provides approximately 50% of total leaf lipids. Surprisingly, this mutation had a relatively slight effect upon the amount of any of the chloroplast or cytoplasmic lipids because the microsomal pathway compensated for the effects of the mutation by a several-fold increase in the rate of microsomal lipid synthesis, and in the rate of transfer of lipids from the endoplasmic reticulum to the chloroplast. This raised the question as to how the microsomal pathway allocates lipids to a particular membrane. A popular theory is that lipid movement between the endoplasmic reticulum and chloroplast membranes is mediated by a small class of proteins designated as lipid transfer proteins (LTPs). We cloned the gene for an LTP from spinach leaf (2), then used this cDNA to clone the related gene from *Arabidopsis*. Sequencing of the gene permitted us to attribute a function to several previously cloned genes which had been tentatively considered, on the basis of indirect evidence, to be amylase or protease inhibitors (1). When transcribed and translated *in vitro* in the presence of dog pancreatic microsomes, the LTP was translocated into the endoplasmic reticulum with concomitant cleavage of the signal peptide (2). Since there are no known examples of soluble cytoplasmic proteins in which synthesis involves insertion into the endoplasmic reticulum, this observation suggested that the LTP is not a cytoplasmic protein but may be located either outside the cell or in the vacuole. The protein has subsequently been localized outside the cell by immunoelectron microscopy (S. Thoma, unpublished). The protein appears to be most abundant in the cell walls of vascular tissues. This location effectively excludes a role for the protein in lipid transport and raises new questions about the role of the protein. In order to examine the role of the protein we have produced a number of transgenic plants which express antisense copies of the gene. Preliminary results indicate that, although we have reduced the amount of the protein ten to twenty-fold, there is no effect on the growth of the plants. We are, therefore, currently at a loss as to the function of this very abundant protein. (Ursula Hecht was supported in part by a fellowship from the Humbolt Foundation; supported in part by grants from the USDA/NSF/DOE Plant Science Center Program and the National Science Foundation.)

Role of lipid desaturation

Mutants lacking activity for fatty acid desaturases have very minor differences from wild type in growth or appearance of plants at standard temperatures. However, both *in vivo* and *in vitro* evidence was obtained indicating that decreased unsaturation leads to enhanced thermal stability of the chloroplast membranes at elevated temperatures (4,9,10). In the case of the *fadB* and *fadC* mutations, which control the activity of a chloroplast ω 3 and an ω 6 desaturase, respectively, this was correlated with a significantly higher growth rate of the mutants than the wild type at elevated temperature. Thus, these results provide striking evidence that a decrease in lipid unsaturation is a component of the thermal acclimation response observed in many species of higher plants. Recent analysis of the effects of the *fadB* and *fadC* mutations on the growth and development of plants at low temperature revealed that tissue which develops at low temperature is chlorotic (unpublished). This provides the first direct evidence for a role of fatty acid unsaturation in low temperature fitness. The chlorosis is associated with impaired chloroplast development. We are currently examining the mechanistic basis for this effect. However, it is clear that a high degree of lipid unsaturation is required for normal growth at low temperature.

YAC-based methods of gene isolation

In order to exploit the YAC library (3) we have mapped several loci of interest (i.e., *fad2*, *fad3*, *fadD*, *fae1*) relative to the flanking RFLPs and have initiated the isolation of the YAC clones which carry the DNA between the flanking RFLPs as a prelude to identifying the genes by genetic complementation tests. In the course of characterizing the library, we and several collaborators (Caroline Dean and Howard Goodman) have mapped YACs comprising about 33% of the total genome. A collaborative paper describing this effort has been accepted for publication. (Supported in part by a grant from the National Science Foundation; Erwin Grill was supported by a fellowship from the Deutsche Forschungsgemeinschaft; Bertrand Lemieux was supported by an NSERC fellowship; Sue Gibson was supported by an NIH fellowship; Vincent Arondel was supported by an EMBO fellowship.)

Genetic dissection of the cell wall

We hypothesized that many of the polysaccharide structures present in the cell walls of higher plants are not required for growth in laboratory conditions. Since it would be very useful to have an extensive collection of mutants with altered cell wall composition, we devised a direct screening method based on gas chromatography of alditol acetates. Approximately 100 mutants with altered cell wall composition were recovered from approximately 5000 plants. We are completing the preliminary genetic analysis and backcrossing the lines to remove spurious mutations. I believe this work will create many new opportunities in the area of cell wall research.

Phosphate-deficient mutants

In order to establish a system to examine the factors which regulate mineral nutrient acquisition, we isolated mutants which are deficient in phosphate accumulation by screening leaf tissue from several thousand plants for phosphate content. A mutant which has a 20-fold reduction in leaf phosphate content was isolated (Poirier et al., in press). The defect is associated with a deficiency in ability to transfer Pi through the stele into the xylem, presumably due to a defect in a Pi-specific translocator.

Chilling sensitive mutants of *Arabidopsis*

We are interested in the possibility that chilling resistant species have gene products which are not required for growth in temperate conditions but which are required for survival at low temperature. In order to test this hypothesis we have isolated several mutants which transform the chilling resistant species *Arabidopsis thaliana* to an extremely chilling sensitive phenotype. Analysis of one line, designated PM11, indicated that chilling sensitivity in this line is due to a single recessive nuclear mutation at a locus we designate *chs1*. In addition to chilling-induced chlorosis, this line exhibits massive ion leakage following 24 to 48 h of chilling at 13°C. Detailed metabolic tracer studies of the mutant have revealed only one metabolic alteration - a change in the amount of label in steryl-esters (5). Analysis of the mutant suggests that either variation in steryl esters may cause chilling sensitivity, or that this aspect of metabolism is uniquely sensitive to changes in cell structure or function which precede the onset of visible signs of chilling injury.

In order to determine how many different kinds of mutations can give rise to chilling sensitivity, we have isolated a large number of additional mutants with this phenotype. Several of these mutants carry additional mutant alleles of the *chs1* locus indicating that the mutation in PM11 is not a rare event. Thus, it is possible that the *chs1* gene product is specifically required for growth at low temperature. We are currently placing the other mutants of this type into genetic complementation groups in order to obtain an insight into the biochemical complexity of the phenomenon. (Supported in part by a grant from the USDA-CRGO #USDA-87-CRCR-1-2507; Carrie Schneider was supported by an NSF Postdoctoral Fellowship.)

BIBLIOGRAPHY

1. Bernhard, W., C.R. Somerville. *Arch. Biochem. Biophys.* 269:695 (1989).
2. Bernhard, W.R., S. Thoma, J. Botella, C.R. Somerville. *Plant Physiol.* 95:164 (1991).
3. Grill, E., C.R. Somerville, *Molec. Gen. Genet.* in press.
4. Hugly, S., L. Kunst, J. Browse and C.R. Somerville. *Plant Physiol.* 90:1134 (1989).
5. Hugly, S., P. McCourt, J. Browse, G.W. Patterson, C.R. Somerville. *Plant Physiol.*
6. Kearns, E.V., S. Hugly, C.R. Somerville. *Arch. Biochem. Biophys.* 284:431 (1991).

7. Kunst, L., J. Browse and C R. Somerville. *Proc. Natl. Acad. Sci. USA* 85:4143 (1988).
8. Kunst, L., J. Browse and C.R. Somerville. *Plant Physiol.* 90:846 (1989a).
9. Kunst, L., J. Browse and C.R. Somerville, *Plant Physiol.* 90:943 (1989b).
10. Kunst, L., J. Browse and C.R. Somerville, *Plant Physiol.* 91:401 (1989c).
11. Lemieux, B. M. Miquel, C.R. Somerville, J. Browse. *Theor. Appl. Genet.* 80:234 (1990).
12. Shanklin, J., C.R. Somerville. *Proc. Natl. Acad. Sci. USA*, in press.
13. Slater, S.C., Voige, W.H., Dennis, D.E. *J. Bacteriol.* 170:4431 (1988).

Shauna Somerville "Molecular Bases of Plant Disease Resistance Mechanisms"

Barley mutants with altered disease reaction phenotypes

Host-pathogen interactions in the powdery mildew disease are characterized by a high degree of specificity that is typical of interactions described by the "gene-for-gene" hypothesis (2,11). We have utilized mutational analysis to study the race-cultivar specific interactions of the barley powdery mildew disease. Barley line CI-16137 (AlgR) carrying the *MI-a1* resistance allele (12), was treated either with sodium azide or by γ -irradiation. Approximately 118,000 M2 seedlings were screened for an altered phenotype when inoculated with race CR3 of *E. g. hordei*. Forty-one mutants were recovered; 23 of the mutants are fully susceptible to race CR3 and 18 mutants have intermediate levels of susceptibility. No fully susceptible mutants were recovered from populations of M2 seedlings derived from sodium azide mutagenesis. For each of the mutants, the rachilla type and the hordein protein pattern were determined in order to confirm that the line was a true mutant and not a contaminant.

Based on these preliminary results, we suggest that either the *MI-a* locus is genetically complex or that resistance is conditioned by more than one gene. If the latter explanation is true, then the gene products must act in series to mediate the extreme form of resistance observed in immune lines like AlgR, and these genes are closely linked. It is possible that γ -irradiation, but not sodium azide mutagenesis, is sufficiently damaging to the *MI-a* allele found in AlgR to fully disrupt expression of the *MI-a* locus resulting in highly susceptible mutants. We plan to test the latter hypothesis by complementation analyses among the 41 mutants.

Molecular markers associated with the *MI-a* encoded resistance

The congeneric barley lines AlgR and AlgS differ by the respective presence or absence of the dominant *MI-a1* resistance allele (12). We examined the protein profiles of AlgR and AlgS by 2-D PAGE (4,13). Two constitutive differences between the barley isolines were observed in healthy, uninoculated tissue. A set of polypeptides of 37-40 kD with an

apparent pI of 6.2 were observed in the protein profile of AlgS but were absent in the resistant isoline. In addition, a polypeptide of pI 6.7 was identified that was 101 kD in AlgR and 103 kD in AlgS. The difference in mobility of the latter polypeptide was verified by the presence of both the 101 kD and 103 kD polypeptides in the protein profile of the F_1 [AlgR x AlgS]. Since these barley lines are related by 18 generations of crossing, any polymorphisms that are found are likely to be at or closely linked to the *MI-a* locus.

In order to determine whether either of these polymorphic polypeptides is likely to be an *MI-a* gene product, the protein profiles of additional barley genotypes were examined. In a mutant of AlgR with increased susceptibility to *E. g. hordei*, the 101 kD polypeptide displayed a 63% reduction in the intensity of labelling with 35 S-methionine+cysteine when quantitated with the Bioimage 110 Image Analyzer. Additionally, we found that a polymorphism for this polypeptide (pI 6.8, 101 kD) existed in the susceptible cultivar Siri. The Siri form of the polypeptide was replaced by the AlgR form in the line S-01, which was derived by backcrossing the *MI-a1* resistance allele from AlgR into Siri (9). This correlation between resistance to race CR3 of *E. g. hordei* and the presence of the 101 kD, pI 6.7 polypeptide did not hold in two further results. A second partially susceptible mutant derived from AlgR, M332, displayed normal levels of the 101 kD polypeptide. Also, the barley line P-01, which was generated by backcrossing the *MI-a1* allele into Pallas (10), did not exhibit the AlgR form of the polymorphic polypeptide. Based on these results, we concluded that protein polymorphisms associated with the *MI-a1* allele could not be detected in 2D-PAGE profiles with the methods we were using and that the *MI-a* gene product is likely a minor protein that is not represented in the 500 most abundant coleoptile polypeptides. Thus, more sensitive methods are required in order to detect *MI-a* gene products.

Subtractive hybridization techniques of recovering mRNA species present in one genotype or in one tissue type have been used to recover species with a relative abundance of only 0.001% and, thus, may be useful in identifying *MI-a* cDNA clones (14). We are attempting to make a subtractive cDNA library of uninoculated AlgR coleoptile tissue (1). Assuming the two isolines are >99% similar (12) and barley expresses about 30,000 different mRNA species (3,16), then the number of mRNA species that are recovered in the subtractive cDNA library should be about 300. In fact, this number is expected to be substantially less than 300 mRNA species because few of the genes in the introgressed region of the genome are expected to exhibit substantial differences in amount or sequence between AlgR and AlgS. To date, the cDNA populations have been constructed and are being amplified via the polymerase chain reaction to levels sufficient to drive renaturation reactions for the subtractive part of the experiment. Once the subtracted cDNA library has been established, the cDNA's will be tested for co-segregation with the resistance allele in 300 F_3 [AlgR x AlgS] families. By this means, we hope to identify one or a limited number of cDNA's that are unique to AlgR and altered or missing in AlgS.

Peroxidases: biochemical markers of infection

Plant peroxidases increase during some infections, and as a result, have been implicated in a number of host defense responses (5,6,7,15). Notably, wall peroxidases are responsible for initiating the polymerization of hydroxy-cinnamyl alcohol into lignin-like structures (15). We wish to address the arguments that peroxidases play a role in host-pathogen interactions and possibly in host resistance mechanisms by determining the localization and timing of occurrence of specific peroxidases during the infection sequence in compatible and incompatible interactions between barley and *E. g. hordei*. A more detailed knowledge of this induction process will lead to a better understanding of the molecular events that occur in host-pathogen interactions and will help to distinguish infection-specific responses from general stress responses.

The intercellular wash fluid of barley leaves contains at least seven peroxidase isozymes (8). Two of these isozymes with pI's of 8.5/8.6 and 5.2 increase in response to infection by *E. g. hordei*. The relative specific activity of the pI 7.7 isozyme does not change in response to inoculation with *E. g. hordei*, and will be used as a control in future experiments. The pI 8.5/8.6 isozyme has been purified to homogeneity by chromatography on carboxy-methyl cellulose, isoelectric focusing in a Rotofor apparatus (BioRad), and gel filtration chromatography on a Sephadex G75 matrix. At present, methods for purifying the pI 5.2 and pI 7.7 peroxidase isozymes are being developed. Once the extracellular peroxidases have been characterized, the localization and timing of induction of the infection-related peroxidases will be compared to such cytological responses as papillae formation and the deposition of phenolic compounds in the cell wall adjacent to penetration sites.

Synthesis of a phytoalexin in *Arabidopsis*

Previously, leaves of *Arabidopsis* were found to accumulate a phytoalexin following inoculation with *P. s. syringae*. About 2.5 mg of the *Arabidopsis* phytoalexin were purified from 1.28 kg fresh weight of leaf tissue by a combination of reverse phase flash chromatography, TLC, and reverse phase HPLC. A molar extinction coefficient of $14,800 \text{ M}^{-1} \text{ cm}^{-1}$ was calculated for the purified phytoalexin using the Beer-Lambert law. This extinction coefficient was then used to quantitate the accumulation of the *Arabidopsis* phytoalexin. In leaves inoculated with *P. s. syringae*, the phytoalexin accumulated within 12 hours post-inoculation and reached a maximum level of approximately $8 \mu\text{g g}^{-1}$ fresh weight by 36 hours. The accumulation of the phytoalexin was negatively correlated with the growth *in planta* of *P. s. syringae*. In addition, the *in vitro* growth of both the fungus *Cladosporium cucumerinum* and the bacterium *P. s. syringae* was inhibited by the *Arabidopsis* phytoalexin.

BIBLIOGRAPHY

1. Akowitz, A. and L. Manuelidis. *Gene* 81:295 (1989).
2. Ellingboe, A.H. *Phytopathology* 62:401 (1972).
3. Goldberg, R. B., G. Hoschek and J.C. Kamalay. *Cell* 4:123 (1978).
4. Granier, F. *Electrophoresis* 9:712 (1988).
5. Hammerschmidt R. and J. Kuc. *Physiol. Plant Pathol.* 20:61 (1982).
6. Hammerschmidt, R., E.M. Nuckles and J. Kuc. *Physiol. Plant Pathol.* 20:73 (1982).
7. Holden, D.W. and R. Rohringer. *Plant Physiol.* 79:820 (1985).
8. Kerby, K. and S.C. Somerville. *Physiol. Mol. Plant Pathol.* 35:323 (1989).
Techniques" (I. Rubenstein, B. Gegenbach, R.L. Phillips, E. Green, eds.) University of Minnesota Press, Minneapolis, MN, pp. 1-7 (1980).
9. Kølster, P. and O. Stølen. *Plant Breed.* 98:79 (1987).
10. Kølster, P., L. Munk, O. Stølen and J. Løhde. *Crop Sci.* 26:903 (1986).
11. Moseman, J.G. *Ann. Rev. Phytopathol.* 4:269 (1966).
12. Mcseman, J.G. *Crop Sci.* 12:681 (1972).
13. O'Farrell, P.H. *J. Biol. Chem.* 250:4007 (1975).
14. Travis, G.H. and J.G. Sutcliffe. *Proc. Natl. Acad. Sci., USA* 85:1696 (1988).
15. Vance, C.P., T.K. Kirk and R.T. Sherwood. *Ann. Rev. Phytopathol.* 18:259 (1980).
16. Willing, R.P. and J.P. Mascarenhas. *Plant Physiol.* 75:865 (1984).

Jonathan D. Walton "Biochemical and Molecular Aspects of Plant Pathogenesis"

Cloning of the *Tox2* locus from *C. carbonum*

Host-selective toxins have been the critical factors in two major epidemics of crop plants in the U.S. in the past fifty years, including the Southern Corn Leaf Blight epidemic of 1970 (7). Host-selective toxins are also critical determinants of other diseases caused mainly by other species of *Cochliobolus* and by species of *Alternaria*. We have chosen the leaf spot disease of maize, caused by *Cochliobolus carbonum*, as a model toxin system. *C. carbonum* produces a host-selective toxin called HC-toxin, which is a cyclic tetrapeptide of structure cyclo(D-Pro-L-Ala-D-Ala-L-AOE), where AOE stands for 2-amino-8-oxo-9,10-epoxidecanoic acid. The toxin and the producing fungus affect only maize that is homozygous recessive at the nuclear *Hm1* locus. Production of HC-toxin is controlled by a single Mendelian gene in *C. carbonum* called *Tox2*. Interestingly, production of the chemically unrelated host-selective toxins, T-toxin and victorin, made by *C. heterostrophus* (*Helminthosporium maydis*) race T and *C. victoriae*, respectively, are also controlled by single, but different, genes. These other genes are called *Tox1* and *Tox3*, respectively. Our work is aimed at understanding, first, how single genetic loci can control production of complex secondary metabolites such as toxins, and second, the evolution of *Cochliobolus*, especially the apparently rapid appearance of new toxicogenic races and species (4).

We reported the identification, purification, and characterization of two enzymes involved in the biosynthesis of HC-toxin (6,8). These enzymes, HTS-1 and HTS-2, catalyze activation and epimerization of L-proline and L-alanine, respectively. Both activities are linked to the *Tox2* locus and are lacking in isolates of the fungus that do not make HC-toxin (6). Using antibodies and oligonucleotide probes based on the amino acid sequences of the enzyme we have identified the gene for HTS-1. The entire gene as well as flanking DNA totalling 22 kb is lacking in all tested isolates of *C. carbonum* that do not make HC-toxin. The presence of this 22-kb region of DNA genetically co-segregates with HC-toxin production. This result indicates that HC-toxin production did not evolve by a simple mutation or by a genetic re-arrangement. We hypothesize as the simplest explanation for the combined experimental data that the *TOX2* "gene" is a gene cluster containing all of the genes necessary to encode all of the enzymes involved in HC-toxin biosynthesis. This hypothesis is currently being tested.

Our analysis of the 22 kb of *Tox2*-unique DNA, done in 1990, has revealed that it is more complicated than originally expected. Most and perhaps all of it is present in two copies in the genome of *tox*⁺ isolates. At one end of the 22- kb unique region there are restriction fragment length polymorphisms (RFLP's) indicating that some of the 22 kb of DNA is present in at least four copies. At the other end of the 22 kb-unique region, where the gene for HTS-1 lies, there is also an RFLP, but only with the enzyme *Apal*. The presence of multiple, functional copies of the putative *Tox2* locus considerably complicated efforts to convert a *tox*⁺ isolate to a *tox*⁻ isolate by transformation-mediated gene disruption. By using two selectable markers we were finally successful in constructing a strain of *C. carbonum* in which both copies of the gene for HTS-1 were disrupted. The strain could not make HC-toxin and was no longer pathogenic on maize, thereby proving that the 22-kb region was indeed the *Tox2* locus.

Metabolism of HC-toxin by maize as the basis of host-selectivity

Resistance to *C. carbonum* race 1 (*tox*⁺) and to HC-toxin in maize is controlled by a single dominant Mendelian gene called *Hm1*. HC-toxin contains an epoxide group which is required for activity, and plants are known to contain epoxide hydrolases. We have been testing the possibility that *Hm1* encodes or controls expression of an enzyme that metabolizes HC-toxin to a non-toxic derivative, such as the hydrolyzed epoxide. Radiolabelled HC-toxin was prepared by feeding [³H]D-alanine to the fungus. Tritiated HC-toxin was fed to maize leaves and the resulting products analyzed by reverse phase HPLC and an in-line radioactivity detector.

Maize leaves were able to metabolize HC-toxin very effectively to a single product. Contrary to our expectations, this was not the diol formed by hydrolysis of the 9,10-epoxide. In 1990 we showed by several chemical methods that the structure of the HC-toxin metabolite was HC-toxin in which the 8-ketone of the side chain of AEO was reduced to the 8-alcohol. This compound is non-toxic (3).

When tritiated HC-toxin was fed to leaves through the transpiration stream, there were no significant differences in rate or extent of HC-toxin metabolism by resistant and sensitive plants. This originally suggested that metabolism of HC-toxin is not the basis of its host-selectivity. Nonetheless, in order to study an apparently novel enzyme and to see if the enzymes from resistant and susceptible maize differed in any way (e.g., in K_m or V_{max}), we have developed an *in vitro* assay for HC-toxin reduction. "HC-toxin reductase" (HCTR) requires NADPH and is inhibited by p-hydroxy-mercuribenzoate. Although the leaf-feeding studies indicated that both susceptible and resistant plants could metabolize HC-toxin equally well, despite numerous attempts in 1990 we were able to find the enzyme only in resistant maize (inbred Pr1) and not in susceptible maize (inbreds Pr and K61). We have developed a method to assay both disease reaction and HCTR activity in single maize seedlings. We have found that resistance to *C. carbonum* race 1 and ability to detoxify HC-toxin are genetically linked. In fifty seedlings of a population that was segregating 1:1 for disease reaction, no recombinants were detected. In collaboration with Steve Briggs at Pioneer Hi-Bred International, we have been assaying HCTR in a variety of experimental maize lines that have transposon-induced mutations to sensitivity at the *Hm1* locus. In every case, there is a perfect correlation between susceptibility to the fungus and inability to detoxify HC-toxin. Our studies in combination with Briggs' work on the cloning of the *Hm1* locus have, for the first time, given us a complete genetic, molecular, and biochemical understanding of specificity in a plant disease interaction.

Analysis of the role of polygalacturonase and other cell wall-degrading enzymes in plant disease

Endo-polygalacturonase (endoPG) is an enzyme made by all cellular plant pathogens. It has been the most extensively studied of all cell-wall degrading enzymes (2). In addition to its role in degrading the pectinaceous middle lamella, this enzyme can also trigger plant defense responses (1). In order to evaluate definitively the role of endoPG in disease, in 1990 we cloned the gene by PCR using oligonucleotides based on N-terminal and tryptic peptide amino acid sequences. To the best of our knowledge this is the first endoPG gene to be cloned from a plant pathogenic fungus (5). In 1990 we constructed a specific endoPG-minus strain of the fungus using transformation-mediated gene disruption. This is the first time that the technique of gene disruption has been used to evaluate the role of a putative fungal pathogenicity factor. The resulting mutant lacked detectable endoPG activity but was still pathogenic, demonstrating that at least in this interaction endoPG was not required (5). The mutant still made exoPG and could still grow on pectin as sole carbon source. Whether exoPG is important in pathogenesis is under investigation.

BIBLIOGRAPHY

1. Cervone, F., M.G. Hahn, G. De Lorenzo, A. Darvill and P. Albersheim. *Plant Physiol.* 90:542 (1989).
2. Collmer, A. and N.T. Keen. *Annu. Rev. Phytopathol.* 24:383 (1986).

3. Kim, S.-D., H.W. Knoche and L.D. Dunkle. *Physiol. Mol. Plant Pathol.* 30:433 (1987).
4. Leonard, K.J. *Phytopathology* 63:112 (1973).
5. Scott-Craig, J.S., D.G. Panaccione, F. Cervone and J.D. Walton. *The Plant Cell* 2:1191 (1990).
6. Walton, J.D. *Proc. Natl. Acad. Sci. USA* 84:8444 (1987).
7. Walton, J.D. In: *Molecular Industrial Mycology*. (R. Berka and S. Leong, eds.), pp. 225-249, M. Dekker, New York (1991).
8. Walton, J.D. and F.R. Holden. *Mol. Plant-Microbe Interact.* 1:128 (1988).

Peter Wolk "Developmental Biology of Nitrogen-fixing Cyanobacteria"

Use of transposons, and luciferase as a promoter probe, to identify environmentally responsive genes in the heterocyst-forming cyanobacterium, *Anabaena* sp. strain PCC 7120

Nitrogen-deprived *Anabaena* initiates the differentiation of heterocysts at semiregular intervals along its filaments. Models of the mechanism of this pattern formation depend on the nature of the spatial relationship, if any, between the cells that differentiate and the cells that first sense N-deprivation (8). We therefore wish to identify the latter cells. Because it is possible to localize transcription of specific genes to individual cells of a living filament (4), we sought to identify *Anabaena* genes that are activated rapidly in response to removal of fixed nitrogen.

Genes that are activated upon a change in environmental conditions can be mutagenized under conditions in which the genes are inessential, and then identified by their response to alteration of those conditions. Seeking to identify such genes, we have used as a mutagen Tn5-1063, a derivative of Tn5 that incorporates the *Vibrio fischeri luxAB* genes, encoding luciferase, as a transcriptional reporter. Tn5 transposes in *Anabaena* (2). We monitored the luminescence of libraries of transposition-derived colonies by use of a Hamamatsu Photonic System model C1966-20 (Photonic Microscopy, Inc., Oak Brook, IL) coupled to a Nikon 35-mm f 2.0 macro-lens. Sources of differential luminescence identified by comparing images of luminescence made under different environmental conditions were localized to specific colonies by further comparison with corresponding bright-field images.

Mutants responding rapidly to N-deprivation by increase or decrease of luminescence were identified upon transfer of mating filters bearing transposition-derived colonies from agar plates containing nitrate to ones lacking fixed N. Two purified mutants, TLN2 and TLN6, showed increased luminescence within 1 or 4 hr, respectively. Luminescence increased equivalently if NaNO₃ and KNO₃ were omitted or replaced with equimolar NaCl and KCl. Sequences of DNA contiguous with the one copy of the transposon in the genome were recovered by digestion of total DNA with ClaI or EcoRV, self-ligation (the

transposon contains an origin of replication functional in *E. coli*), and transfer to *E. coli* by electroporation. The transposon in TLN2 and TLN6 was localized within fragments SalB and SalJ on the map of Bancroft et al. (6). tn2 is at a position of 3.5 Mb on that map, and is transcribed clockwise. Strains TLN2 and TLN6 differentiate normally. We are trying to identify, by transposon mutagenesis, regulatory genes whose products control the activation of the genes mutated in those strains. Such regulatory genes may also control differentiation.

We have isolated, similarly, mutants in which transcription of luxAB is activated by transfer from phosphate-sufficient to phosphate-deficient conditions, and mutants (including TLT2) in which luminescence is much (ca. 100-fold) greater after 24 hr at 20°C than after incubation at 30°. Mutant TLT2 diminished in luminescent intensity with $t_{1/2} \approx 2.6$ hr when returned to 30°. As with proteins that are induced in *Escherichia coli* by cold shock (7), expression of TLT2 can be induced at 30° by certain antibiotics that target the prokaryotic ribosome, namely chloramphenicol, erythromycin, spectinomycin, spiramycin and tetracycline. (Supported in part by NSF grant DCB-8702368.)

Regulation and localization of expression of hetA

The hetA gene (9) was replaced by a transcriptional fusion of the hetA promoter with luxAB. The resulting strain was subjected to transposon mutagenesis with Tn5-1058, the lux-less progenitor of Tn5-1063. Whereas luminescence of the parent strain was induced by nitrogen-deprivation, mutagenized derivatives were identified in which luminescence was not thereby induced, and others in which luminescence was expressed constitutively in the presence of nitrate. A majority of the mutants of the latter group were found to be deficient in uptake or utilization of nitrate. However, three mutants that expressed hetA in the presence of nitrate did not form heterocysts in the presence of nitrate but did in the absence of nitrate. One of these mutants had a transposon insertion between the hetA promoter and the luciferase reporter; the transposon in the remaining two mutants was at least 0.5 kb from the hetA gene. The implication of these observations is that at least one gene (hetA) involved in stability of the heterocyst envelope may be subject to both positive and negative regulation.

Whereas P_{rbc} luxAB and P_{nif} luxAB show strong luminescence at the single cell level (4), single-cell luminescence from P_{hetA} luxAB is feeble. The signal, amplified ca. 15-fold by use of a combination of (i) a fusion (P_{hetA} rpoT7) of the promoter of hetA to the structural gene for T7 RNA polymerase and (ii) plasmid pRL1050, bearing a fusion of a promoter recognized by T7 polymerase, to luxAB, appeared to localize luminescence to proheterocysts. In order to test whether the localization of luminescence seen from the *Anabaena* derivative carrying P_{hetA} rpoT7 is due to inactivity of T7 polymerase in vegetative cells, derivatives of *Anabaena* PCC7120 [pRL1050] carrying independent insertions of Tn5-1065 (with rpoT7 as reporter gene) were selected for their ability to express luciferase well when grown on nitrate. Three strains chosen for more detailed study were shifted to a nitrate-free medium. After 11 h of starvation for nitrogen, filaments were examined

under the microscope. In all three cases, luminescence was visible from vegetative cells. T7 polymerase evidently is able to couple promoter activity to luciferase expression in nondeveloping as well as in developing cells. Luminescence amplified by means of the T7 polymerase-luciferase couple appears then to reflect, at least qualitatively, the spatial activity of regulated promoters and may be used to detect the sites of weak, regulated transcription. (Supported in part by NSF grant DCB-8702368.)

Screening for mutants incapable of aerobic fixation of N_2

Ca. 1 to 2% of transposon-mutated colonies, unable to fix N_2 aerobically, become and remain yellow after a filter bearing small colonies is transferred from +N to -N conditions. Such mutants were purified, and examined by microscopy 7 days after transfer to AA medium containing 10 μ g Sm/ml. All but a small proportion of the mutants make heterocysts, although the heterocysts in many mutants are morphologically aberrant. Notable mutants include two which lack, apparently specifically, the envelope glycolipids of heterocysts, and eight (including α -41 and α -63; see below) which lack obvious differentiation.

Insertion sequences

Anabaena sp. str. PCC 7120 has (3) at least 6 distinct insertion sequences, so that upon selecting for a transposon, resistance might be due to the transposon but a phenotype due to transposition of an insertion sequence. The transposon and sequences contiguous to it were, therefore, transferred from α -41 and α -63 to the wild type strain, thereupon showing that the phenotypes of α -41 and α -63 are indeed due to the transposon.

IS892, one of a family of at least 9 related insertion sequences in PCC 7120, not all of which are identical, was sequenced. IS892 is 1675 base pairs in size with 24-bp near-perfect inverted terminal repeats, generates an 8-bp directly repeated target duplication upon insertion into its target site, and has two open reading frames (ORFs) that code for proteins of 233 and 137 amino acids, respectively. It has been suggested that the *E. coli* integration host factor (IHF), a histone-like DNA binding protein, plays a strong stimulatory role in many types of site-specific recombination, including transposition of transposable elements. An extended 48 bp-long consensus sequence of the binding site for IHF has been proposed based on a compilation of published studies. Computer search revealed two possible IHF-binding sites in the sequence of IS892 that closely match the consensus sequence.

Further efforts to facilitate gene transfer to strains of *Anabaena*

Plasmid pRL528, which methylates *Ava*I and *Sau*96I (including *Ava*II) sites, has proven very helpful for research with *Anabaena* sp. PCC 7120 (2,4,6), which would otherwise restrict conjugally transferred DNA bearing those sites. Our preliminary evidence indicates

that the presence of unmethylated *Ava*III sites also leads to restriction. Plasmid pEC22 (10), which contains genes for restriction and methylation of sites for *Eco*T22I, an isoschizomer of *Ava*III, was therefore mapped, and the methylase gene subcloned as a 1.2-kb *Hpa*II fragment, in preparation for its insertion into pRL528.

Extracts of *Anabaena variabilis* ATCC 29413, a heterotrophic strain, have very high activity of a non-specific nuclease that degrades RNA and single- or double-stranded DNA extensively. Much weaker activity of a corresponding nuclease is observed in extracts of *Anabaena* sp. PCC 7120. In collaboration with A. Muro-Pastor and E. Flores of U. Sevilla, Spain, we have cloned the gene for the nuclease from *Anabaena* 7120, and used it to inactivate, site-specifically, the corresponding nucleases from the two strains of *Anabaena*. It will be very interesting to determine whether, as is true of a somewhat similar nuclease from *Streptomyces glaucescens* (1), this nuclease serves a restriction function in *Anabaena* 29413.

BIBLIOGRAPHY

1. Aparicio, J.F., C.G. de los Reyes-Gavilán, C. Barbes, C. Hardisson and J. Sanchez. *J. Gen. Microbiol.* 134:2345 (1988).
2. Borthakur, D. and R. Haselkorn. *J. Bacteriol.* 171:5759 (1989).
3. Cai, Y. and C.P. Wolk. *J. Bacteriol.* 172:3138 (1990).
4. Elhai, J. and C.P. Wolk. *EMBO J.* 9:3379 (1990).
5. Golden, J.W. and D.R. Wiest. *Science* 242:1421 (1988).
6. Thomas, J., J.C. Meeks, C.P. Wolk, P.W. Shaffer, J.M. Austin and W.-S. Chien. *J. Bacteriol.* 129:1545 (1977).
7. VanBogelen, R.A. and F.C. Neidhardt. *Proc. Natl. Acad. Sci. USA* 87:5589 (1990).
8. Wolk, C.P. *Plant Syst. Evol.* 164:27 (1989).
9. Wolk, C.P., Y. Cai, L. Cardemil, E. Flores, B. Hohn, M. Murry, G. Schmetterer, B. Schrautemeier and R. Wilson. *J. Bacteriol.* 170:1239 (1988).
10. Yoshida, Y. and K. Mise. *J. Bacteriol.* 165:357 (1986).

Jan A.D. Zeevaart "Environmental Control of Plant Development and its Relation to Plant Hormones"

Only 3 β -hydroxylated gibberellins are active per se in stem growth of spinach

Spinach is a long-day (LD) rosette plant in which stem elongation under LD conditions (8 h high-intensity light, 16 h low-intensity light) is mediated by GAs. In previous work (8) it has been shown that the early-13-hydroxylation pathway (GA_{33} , GA_{44} , GA_{19} , GA_{20} , GA_1 , GA_3) is the major biosynthetic pathway in spinach. The 3 β -hydroxylated GAs, GA_4 and GA_7 , have also been identified in spinach, but their levels are always very low. In the experiments reported here the growth retardants AMO-1618 (which inhibits kaurene synthetase A activity) and prohexadione or BX-112 (which inhibits dioxygenases that

require 2-oxoglutarate as co-substrate) (4) were used to determine which GA is active per se in spinach. Both AMO-1618 and BX-112 suppressed stem growth in plants under LD. In the case of AMO-1618, applied GA_{20} and GA_1 were equally effective in overcoming the growth inhibition, but with BX-112 only GA_1 , not GA_{20} , could restore normal growth. GA_1 applied to plants treated with BX-112 was just as effective as GA_1 in causing stem growth. Analyses of endogenous GAs in plants treated with BX-112 showed that the levels of GA_1 and GA_3 were much reduced, while GA_{20} and GA_{19} accumulated. These findings indicate that the main effect of BX-112 in spinach is inhibition of the 3 β -hydroxylation step from GA_{20} to GA_1 . It also establishes unequivocally that 3 β -hydroxylation is required for GA activity in spinach. (Supported, in part, by a fellowship from the Spanish Ministry of Agriculture to Manuel Talon, and by USDA grant #88-37261-3434.)

Fluctuations in gibberellin levels under various light-dark conditions

With the use of deuterated GAs as internal standards, accurate and reproducible determinations of GAs can be made in relatively small tissue samples. Such measurements have been carried out with plants grown under SD conditions, or after transfer from SD to LD. In SD, the GA levels fluctuated, increasing during the light, and decreasing during the dark period. After transfer from SD to LD, GA_{33} and GA_{19} decreased significantly during the first supplementary light period, indicating continued activity of GA_{33} - and GA_{19} -oxidases, whereas GA_{20} increased. During the following high-intensity light period the GA_{20} level decreased and GA_1 increased. These fluctuations in GA levels continued in LD, resulting in an increase in absolute levels of GA_{20} and GA_1 which in turn resulted in stem growth. These observations are compatible with the idea that the flow through the GA biosynthetic pathway is much enhanced in LD, particularly during the period of high-intensity light, as compared to SD and darkness. (Supported, in part, by a fellowship from the Spanish Ministry of Agriculture to Manuel Talon, and by USDA grant #88-37261-3434.)

Cellular changes induced by exogenous and endogenous gibberellins in the shoot tips of *Silene armeria*

In the present investigation with the long-day rosette plant, *Silene armeria*, strain S 1.2, (7) microscopic observations on early changes in the shoot apical meristem following LD induction or GA treatment in SD (6), were combined with GA analyses of sections located at various distances below the shoot apex. Within 8 days after the beginning of LD induction or after GA application to plants in SD, the cells in the subapical meristem were of similar size and were predominantly oriented in long files, indicating that many transverse cell divisions had taken place. In contrast, in shoot tips of plants in SD the cells in the subapical region varied in size and were not oriented in any particular direction. Staining longitudinal sections of induced plants with calcofluor white showed that cellulose deposition occurred mostly in the longitudinal walls, whereas in the control plants in SD both longitudinal and transverse walls were stained. This indicates that in induced plants the cells elongated mainly in the longitudinal direction, giving rise to an elongated stem.

Material for GA analyses was obtained by cutting thin sections at various distances below the shoot apex. Endogenous GAs increased in all sections of the subapical meristem from plants in LD as compared to those from SD. For example, in the zone 500 - 1500 μm below the apex, the GA content increased from 4 ng/g dry wt. in sections from SD to 136 ng/g dry wt. in material harvested after 8 LD. Thus, an increase in GA content either by application in SD or following LD induction stimulated cell division in the subapical meristem which resulted in stem elongation. (Work done in collaboration with Dr. F.R. Tadeo, INRA, C. R. Bordeaux, Station de Recherches d'Arbiculture Fruitières, 33883 Villenave d'Ornon Cedex, France. Supported, in part, by a fellowship from the Spanish Ministry of Agriculture to Manuel Talon, and by USDA grant #88-37261-3434.)

Characterization of GA-responsive, dwarf mutants of *Arabidopsis thaliana*

There are five non-allelic dwarf mutants in *Arabidopsis* which are GA biosynthetic mutants (4). Experiments directed towards determining the biosynthetic step at which each of these mutants is blocked are in progress. By analysis of the endogenous GAs and quantitation of their levels the ga4 and ga5 mutants were found to be blocked late in the GA biosynthetic pathway. A similar analysis of endogenous GAs in the ga1, ga2, and ga3 mutants indicated that all three are blocked at steps prior to GA₁₂ synthesis. Furthermore, application of ent-kaurene and all precursors later in the GA pathway causes a growth response in ga1 and ga2 plants, whereas ga3 plants respond to applied ent-kaurenoic acid and all later GA precursors. These results suggest that the ga1 and ga2 mutants are blocked at steps prior to ent-kaurene, presumably at kaurene synthetase A and B, while ga3 is blocked in the oxidation of ent-kaurenal to ent-kaurenoic acid.

For further characterization of these mutants, the ability to convert geranylgeranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP) and CPP to ent-kaurene, was measured in cell-free extracts of immature siliques from normal and mutant plants. Based on tentative identifications of the products CPP and ent-kaurene by HPLC (1), it was found that the ga3 mutant was deficient in kaurene synthetase A activity, whereas the ga2 mutant lacked B activity. In the case of the ga1 mutant, both A and B activities were comparable to those measured in extracts from normal plants.

It is clear that the observations made with applications of precursors to mutant plants and measurements of kaurene synthetase A and B activities in extracts from immature siliques are not in agreement with respect to the blocks in the ga1 and ga3 mutants. Additional experiments are required to precisely locate the biosynthetic lesions in these mutants.

Absciscic acid biosynthesis in the *flacca* and *sitiens* mutants of tomato

We have further investigated the shunt pathway from ABA-aldehyde $\rightarrow$ ABA-alcohol $\rightarrow$ ABA by feeding [²H₆]ABA-alcohol to wild type and *flacca* mutant leaves in an atmosphere with 20% ¹⁸O₂. Both wild type and mutant tissues converted [²H₆]ABA-alcohol to [²H₆¹⁸O]ABA with similar efficiency which indicates that the shunt pathway is not exploited

by the mutants to maximize ABA synthesis. The conversion of [$^2\text{H}_6$]ABA-alcohol to [$^2\text{H}_6$]ABA in leaves of the *sitiens* mutant in the presence of carbon monoxide was inhibited 80%, whereas in an N_2 atmosphere this conversion was completely inhibited. These findings provide evidence for the involvement of a P-450 type monooxygenase which catalyzes the conversion of ABA-alcohol to ABA with the incorporation of molecular oxygen (5). (Supported, in part, by NSF grant #DMB-8703847.)

Characterization of the *aba* mutant of *Arabidopsis thaliana*

Three alleles of the *ABA* locus of *A. thaliana* are deficient in ABA (2). We have used $^{18}\text{O}_2$ to label ABA in water-stressed leaves of mutant and wild-type *Arabidopsis*. Analysis by selected ion monitoring and tandem mass spectrometry of [^{18}O]ABA and its catabolites showed that the mutant alleles are impaired in ABA biosynthesis and that they have an abnormally small precursor pool containing the ring oxygens of ABA, presumably oxygenated carotenoids (xanthophylls). Quantitation of the carotenoids from mutant and wild type leaves showed that leaves of the mutants are deficient in the epoxy-carotenoids, violaxanthin and neoxanthin, and accumulate the biosynthetic precursor zeaxanthin. These results provide strong evidence that ABA is a breakdown product of xanthophylls. Interestingly, the different alleles of the *aba* mutant are less impaired in biosynthesis of trans-ABA than of ABA. Feeding studies with xanthoxin isomers suggest that trans-xanthoxin may be a precursor to ABA in *Arabidopsis*. (Supported, in part, by NSF grant #DMB-8703847.)

Relationship between ^{18}O incorporation in carotenoids and abscisic acid

In earlier labeling studies of ABA with $^{18}\text{O}_2$ it was established that in water-stressed leaves and unripe fruits ^{18}O enrichment is mainly in the carboxyl group, while only small amounts of ^{18}O are incorporated in the ring oxygens. However, in ripening fruits there is considerable ^{18}O enrichment of the oxygens on the ring (9). These results suggest that xanthophylls, as presumed ABA precursors, must become ^{18}O labeled to a different extent in the various tissues analyzed. The present experiments were undertaken to determine whether the ^{18}O -labeling pattern of ABA is related to that of the xanthophylls, violaxanthin and neoxanthin.

In ABA from apple tissue, ^{18}O enrichment of the ring oxygens was higher than that of the carboxyl group. In the same tissue, approximately 50% of the violaxanthin and 25% of the neoxanthin molecules had incorporated four ^{18}O atoms. Thus, the labeling pattern of the xanthophylls closely paralleled that of ABA. The observation that the percentage of labeled violaxanthin was greater than that of neoxanthin is consistent with the former being the precursor of the latter.

In unripe avocado fruit most of the ABA was labeled with a single ^{18}O in the carboxyl group, while in the same tissue the xanthophylls had incorporated little ^{18}O . In ripe avocado fruit, ^{18}O incorporation in to ABA was low, with molecules containing 2 or 3 atoms

of ^{18}O being more abundant than singly labeled ones. This corresponded to a high incorporation of ^{18}O into the xanthophylls. ABA accumulated during ripening of avocado fruit, while the xanthophyll pool decreased by 90%. In their entirety, these results establish a close correlation between ^{18}O labeling of the oxygens on the ring of ABA on the one hand and ^{18}O incorporation by violaxanthin and neoxanthin on the other. They provide, therefore, further evidence that these xanthophylls are precursors to ABA. (Work done in cooperation with D.A. Gage, MSU/NIH Mass Spectrometry Facility. Supported, in part, by NSF grant #DMB-8703847.)

BIBLIOGRAPHY

1. Bensen, R.J. and J.A.D. Zeevaart. *J. Plant Growth Regul.* 9:237 (1990).
2. Koornneef, M., M.L. Jorna, D.L.C. Brinkhorst-van der Swan and C.M. Karssen. *Theor. Appl. Genet.* 61:385 (1982).
3. Koornneef, M. and J.H. van der Veen. *Theor. Appl. Genet.* 58:257 (1980).
4. Nakayama, I., Y. Kamiya, M. Kobayashi, H. Abe and A. Sakurai. *Plant Cell Physiol.* 31:11483 (1990).
5. Rock, C.D., T.G. Heath, D.A. Gage and J.A.D. Zeevaart. *Plant Physiol.* In press (1991).
6. Sachs, R.M. *Adv. Chem. Ser.* 28:49 (1961).
7. Talon, M. and J.A.D. Zeevaart. *Plant Physiol.* 92:1094 (1990).
8. Talon, M., J.A.D. Zeevaart and D.A. Gage. *Plant Physiol.* Submitted (1991).
9. Zeevaart, J.A.D., T.J. Heath and D.A. Gage. *Plant Physiol.* 91:1594 (1989).

END

**DATE
FILMED**

12/11/191