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DIRECTOR'S INTRODUCTION

Since the Plant Research Laboratory was organized in 1965, our personnel have developed a variety of interactions that enhance our research and educational efforts. 1991 saw two new integrative endeavors added: we had our first Retreat and we instituted a Plant Biochemistry Facility. The Retreat provided an opportunity both to emphasize an overall perspective on our areas of research and to enhance fruitful scientific interchange. The Plant Biochemistry Facility, which Tom Newman has helped to organize, provides both tools and instruction for a wide variety of techniques including HPLC purification of proteins, two-dimensional scanning of radioactive samples, and accession to databases.

The 1991 Annual Report of the Plant Research Laboratory summarizes the overall achievements of the PRL during the past year. The following paragraphs are intended to highlight a few of our accomplishments, in part to illustrate our approaches to research.

In an attempt to identify the basis of resistance of certain maize genotypes to HC-toxin (produced by the fungus, *Cochliobolus carbonum*), Jonathan Walton and his associates developed an in-vitro assay for chemical reduction of the toxin, and detected such an activity only in resistant maize. Genetic tests confirmed that resistance to HC-toxin is determined by a specific reductase that detoxifies the toxin. As reported in last year's Annual Report and in this volume, Walton's group identified and sequenced a 15.7-kb, intron-free, fungal open reading frame encoding a 570-kDa cyclic peptide synthetase that is responsible (at least in part) for production of the toxin. These combined biochemical and molecular genetic approaches have rapidly brought the maize/*C. carbonum* interaction from obscurity to the state of being arguably the best-characterized plant/pathogen interaction.

For over a decade, my group had sought to achieve the power of transposon mutagenesis of the cyanobacterium, *Anabaena*. Recently, we showed that luciferase could be used to assess transcription from single genes at the single-cell level, and thus provided a critical tool for studying the multicellular development of *Anabaena*. This year, by incorporating the genes for luciferase as a reporter in a variant of transposon Tn*5*, luciferase was used to identify genes controlled, as is heterocyst differentiation, by the availability of nitrogen. A rich harvest was obtained of mutants, and genes, that are important for differentiation and aerobic fixation of dinitrogen. We hope that analysis of these mutants will help to elucidate the origin of the pattern of spaced heterocysts in *Anabaena*. Already, three other groups at MSU, including Frans de Bruijn's in the PRL, are finding the *lux*-transposon/light-detection system fruitful for investigation of environmentally regulated genes.

Two other long-standing problems appear to be getting tantalizingly close to clarification. Having shown that an *aba* mutant of *Arabidopsis* interferes with the metabolism of zeaxanthin, Jan Zeevaart and coworkers are in position to determine the role of zeaxanthin derivatives in the protection of photosystem II against photoinhibition. A second such problem concerns cellulose, the most abundant polymer on earth. Efforts to obtain its synthesis in vitro have heretofore been unavailing, except for a biosynthetic system extracted from the bacterium, *Acetobacter xylinum*. This system requires cyclic di-GMP for activation. Debby Delmer has now identified two polypeptides from cotton fibers that bind c-di-GMP, resemble a subunit of *A. xylinum* cellulose synthase antigenically and in amino acid sequence, and increase in abundance markedly during the onset of secondary wall cellulose synthesis in cotton fibers. Delmer's work offers the hope that the elusive synthase from higher plants may be close to elucidation.

I mention last a project of great potential. The issue is whether agriculture may, via genetic engineering, be capable of producing wholly new products, including chemical feedstocks that could reduce our dependence on imports of petroleum and edible oils. In a first, highly significant step, Yves Poirier and coworkers in Chris Somerville's laboratory have engineered the production, by *Arabidopsis*, of the bacterial reserve material, poly-ß-hydroxybutyric acid (PHB). The significance of this achievement lies in the fact that PHB is a biodegradable thermoplastic. Will it soon be possible, by harnessing knowledge of plant metabolism, to replace normal reserve materials with PHB in the storage organs of crop plants? What products can be created from this material that are currently generated from petroleum-based plastics? No better example can be given of our effort to couple so-called basic research to matters of economic importance, nor of the practical need for deep insight into plant physiology, biochemistry and genetic regulation as bases for genetic engineering.

Derek Lamport, an outstanding researcher of plant cell wall proteins, has just retired as Professor Emeritus. I also note with regret the loss of Andrew Hanson from our University. We hope and anticipate that he will have continued success at his new position at the University of Montreal.

RESEARCH REPORTS

The following progress reports are not intended as publications and should not be cited without specific permission by the responsible investigator.

MOLECULAR BASIS OF SYMBIOTIC PLANT-MICROBE INTERACTIONS

Frans J. de Bruijn, Rujin Chen, Susan Fujimoto, Anurag Goel, Pyung Ok Lim, Ken Nadler, Silvia Rossbach, Uwe Rossbach, Johannes Stigter, Maria Schneider, Krzysztof Szczyglowski and Peter Welters (*Max-Planck-Institut für Züchtungsforschung, Köln, Germany)*

The induction of nitrogen-fixing root and stem nodules on leguminous plants by soil bacteria belonging to the *Rhizobiaceae* is a highly evolved process, requiring a fine-tuned interaction between the two symbiotic partners. Multiple (regulatory) signals go back and forth between the bacterium and the plant during the formation of an effective nodule (see Nap and Bisseling, 1990; de Bruijn and Downie, 1991). We are studying the molecular basis of the nodule-specific induction of both rhizobial and plant genes. Our goals include the characterization of the physiological signals and regulatory circuitry controlling free-living versus symbiotic expression of *Azorhizobium caulinodans* nitrogen-fixation genes, as well as the *cis*-acting elements and *trans*-acting factors responsible for nodule-specific expression of plant genes encoding early (Enod2) and late (leghemoglobin, Lb) nodulins. We are also studying environmental (stress) control of gene expression in rhizobia, and are investigating the role of nodule-specific opine-like compounds in competition, and the potential of using the corresponding synthesis and catabolism genes to create "biased rhizospheres".

Regulation of *Azorhizobium* nitrogen-fixation (*nif/fix*) genes

Biological nitrogen fixation is an extremely energy-intensive process. It is therefore not surprising that free-living nitrogen-fixing organisms only derepress their *nif/fix* genes when nitrogen-starved (N-regulation: de Bruijn et al., 1990a). Generally, oxygen (O_2) supports the production of ATP necessary for nitrogenase activity in symbiotically nitrogen-fixing bacteria, such as rhizobia, but O_2 is also capable of severely inhibiting nitrogenase (see Hill, 1988). Therefore, the intracellular O_2 concentration represents a second very important signal for rhizobia, which have evolved several mechanisms to regulate their *nif/fix* genes accordingly (O_2-regulation: see Hill, 1988; de Bruijn et al., 1990a).

We have been studying N- and O_2-regulation of the *nif/fix* genes of *Azorhizobium caulinodans* ORS571 (see de Bruijn et al., 1990a). Strain ORS571 was selected for this analysis since it has the unique capacity to fix N_2 in stem and root nodules induced on its host, the tropical legume *Sesbania rostrata*, as well as to grow at the expense of N_2 in the free-living state (see de Bruijn, 1989). N- and O_2-control of *Azorhizobium nif/fix* gene expression is mediated primarily via the promoter of the *nif*-specific positive regulatory gene *nifA* (Ratet et al., 1989). The NifA protein, in turn, acting in concert with the alternative sigma factor NtrA(RpoN), activates the other *nif/fix* promoters (e.g., p*nifHDK*; see de Bruijn et

al., 1990a). The *nifA* 5' upstream region contains a nitrogen-regulation (*ntr*) box (GG-N10-GC), found in the promoter of genes induced under N-starvation conditions (see de Bruijn et al., 1990a) and a fumarate nitrate respiration (*fnr*) box (TTGAT-N4-ATCAA), found in the promoter region of genes anaerobically induced via the transcriptional activator Fnr (see Spiro and Guest, 1990). Mutations in both the *ntr* and *fnr* boxes drastically affect the expression of the *nifA* promoter, suggesting that they constitute important *cis*-acting elements (J. Stigter and F.J. de Bruijn, unpublished results).

It has been demonstrated in enteric bacteria that the *ntr*-box is involved in the interaction with an alternative sigma factor (NtrA, RpoN) and the *fnr*-box with the Crp-like Fnr regulatory protein (Thöny and Hennecke, 1989; Spiro and Guest, 1990; de Bruijn et al., 1990a). Using heterologous DNA probes, we have cloned *ntrA* and *fnr*-like genes from *Azorhizobium* and have created insertion mutations in these genes. *NtrA*::Tn5 mutants are strictly deficient in free-living and symbiotic nitrogen fixation (Nif$^-$, Fix$^-$), and are unable to grow on nitrate as sole N-source—a phenotype commonly associated with rhizobial *ntrA* mutants (de Bruijn et al., 1990a). As expected, a *nifH-lacZ* reporter gene fusion is not expressed in the *ntrA*::Tn5 mutant strains, but although the *nifA* promoter is *ntr* controlled and contains a *ntr*-box, a *nifA*-lacZ fusion is expressed to a wild-type level in this *ntrA* mutant background. Surprisingly, the *fnr*::Tn5 mutant is nitrogen-fixation proficient in microaerobic cultures and only slightly impaired in symbiotic nitrogen fixation, while being impaired in dissimilatory nitrate reduction (Fnr$^-$ phenotype). These results suggest that other *ntrA* and *fnr*-like genes are responsible for the regulation of *nif(A)* gene expression in *Azorhizobium*, in response to N-starvation and microaerobiosis.

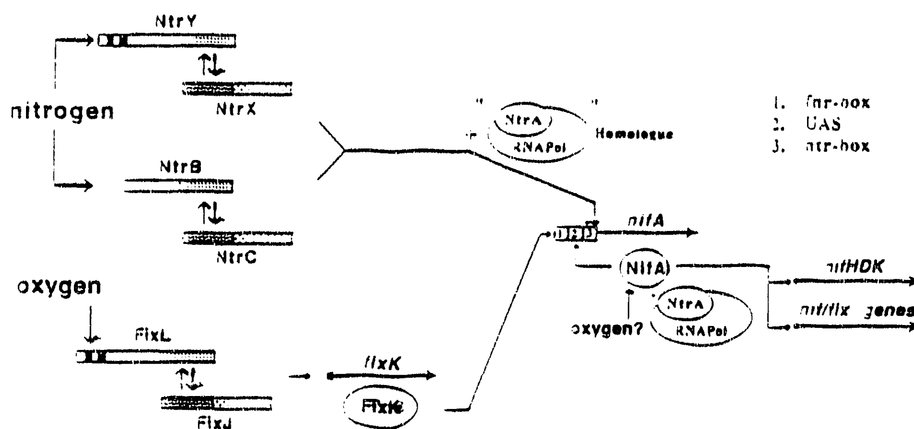

Figure 1. Regulatory circuit controlling expression of nitrogen fixation genes in *Azorhizobium*.

Recently, three ORS571 loci have been identified that may be responsible for O_2 control of *nifA* gene expression (*fixLJ*, *fixK*: Kaminski and Elmerich, 1991; Kaminski et al., 1991). Considering that the FixK protein shares considerable homology with Fnr proteins, it is reasonable to postulate that it, in fact, interacts with the *fnr* box in the *nifA* promoter. Moreover, a novel regulatory locus (*ntrYX*) of ORS571 has been identified that affects nitrogen fixation in culture, stem-nodulation, nitrogen fixation activity, and ability to grow on nitrate as N-source (de Bruijn et al., 1990a; Pawlowski et al., 1991). Mutations in the *ntrYX* locus significantly reduce, but do not abolish, *nifA* promoter activity, suggesting that both *ntrC* and *ntrYX* are important for N-regulation of *nifA* gene expression (Pawlowski et al., 1991). However, the nature of the NtrA sigma factor involved in *ntrC/ntrYX*-mediated regulation and directly interacting with the *ntr* box in the *nifA* promoter remains elusive.

Regulation of early and late nodulin genes in *S. rostrata*

Plant genes which are specifically induced during the symbiotic interaction between rhizobia and their host plant (nodulin genes: Van Kammen, 1984) are commonly designated "early" or "late" genes, reflecting the length of time from infection to first expression. Early nodulins are involved in the infection process and structural aspects of nodule ontogeny. Late nodulins are induced in the mature nodule, around the onset of nitrogen fixation, and participate in various aspects of nodule functioning, such as oxygen transport, nitrogen assimilation, and carbon metabolism (see Nap and Bisseling, 1990). Nodulin genes are interesting plant genes to study, since they are not only developmentally regulated in response to signals coming directly or indirectly from the infecting rhizobia, but are also expressed in a tissue- (cell-) specific manner (see Nap and Bisseling, 1990; de Bruijn et al., 1990b; de Bruijn and Downie, 1991).

The *S. rostrata* Enod2 gene is induced, by cytokinin and expressed in nodule parenchyma cells

The *S. rostrata* Enod2 gene (*SrEnod2*) is expressed around 8 days after infection and encodes a proline-rich protein, consisting predominantly of two repeating pentapeptides (PPEYQ, PPHEK) and a putative signal peptide (Dehio and de Bruijn, 1992). The Enod2 protein 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 Nap and Bisseling, 1990).

Since auxin transport inhibitors (ATI's) have been found to induce on alfalfa roots, nodule-like structures, in which the Enod2 gene is expressed (Hirsch et al., 1989), the effect of plant hormones and the ATI TIBA (2,3,5-triiodobenzoic acid) on *SrEnod2* expression has been investigated. We have been able to show that the *SrEnod2* gene is induced in the roots of *S. rostrata* seedlings treated with cytokinins (Dehio and de Bruijn, 1992). The cytokinin response appears to be

very specific, is observed with a variety of cytokinins, and is time- and concentration-dependent. This effect has also been observed when the intracellular cytokinin concentration in *S. rostrata* stems has been altered by other means, such as transformation of stem tissues with *Agrobacterium tumefaciens* (Dehio and de Bruijn, 1992).

Preliminary data on the expression of chimeric *SrEnod2-gus* (glucuronidase) reporter gene fusions in transgenic *Lotus corniculatus* plants reveal that the *SrEnod2* gene is expressed in the inner cortex of the nodule (nodule parenchyma), confirming the *in situ* RNA hybridization results of Van den Wiel et al. (1990). These results suggest that the *SrEnod2* promoter may not only confer symbiotic activation and cytokinin induction, but also cell- (nodule parenchyma-) specific expression. Whether rhizobial cytokinin production plays a direct role in *SrEnod2* gene induction and whether the *cis*-acting elements in the *SrEnod2* promoter region responsible for nodule (parenchyma)-specific expression correspond to those involved in cytokinin-induced gene expression are questions presently under study. (This work has been supported, in part, by a grant from NSF DCB 9105392.)

Cis-acting elements involved in nodule-specific expression of the _S. rostrata_ leghemoglobin (_glb3_) gene: delimitation of the NICE element

The leghemoglobin (*lb*) genes are maximally induced about 4 days after the Enod2 genes and encode oxygen-carrying proteins, which facilitate diffusion of oxygen to the actively respiring, nitrogen-fixing bacteroids within the infected zone of the nodule. Lbs operate at an intracellular O_2 concentration of 10 nM, which is below the concentration known to irreversibly inactivate the nitrogenase enzyme complex (Appleby, 1984). This constitutes the third mechanism which has evolved to deal with the O_2 problem in nitrogen-fixing nodules and may be particularly relevant to *S. rostrata* stem nodules, since they contain photosynthesizing (O_2-evolving) tissues in close proximity to the infected, nitrogen-fixing cells (see de Bruijn, 1989).

A functional analysis of the *S. rostrata* leghemoglobin *glb3* gene promoter region in transgenic *Lotus* plants has revealed the presence of two positive regulatory regions and an ATG-proximal element involved in nodule-specific gene expression (de Bruijn et al., 1990b; Szabados et al., 1990). The latter element has been delimited to an 80-bp region between positions -194 and -114 relative to the start codon of the *glb3* gene (K. Szczyglowski, S. Fujimoto, L. Szabados, P. Ratet and F.J. de Bruijn, manuscript in preparation). Cytological Gus staining experiments have revealed that the *glb3* promoter is expressed only in the infected cells of the nodule (Szabados et al., 1990).

Thus, an 80-bp DNA segment, 69 bp upstream of the transcriptional start site of the *S. rostrata glb3* leghemoglobin gene, appears to be essential for Nodule-

Infected-Cell-specific Expression (NICE element). In the NICE element, DNA sequences with a high degree of homology to motifs present in other late nodulin promoters (e.g. AAAGAT, TTGTCTCTT, CACCCT; Figure 2; de Bruijn and

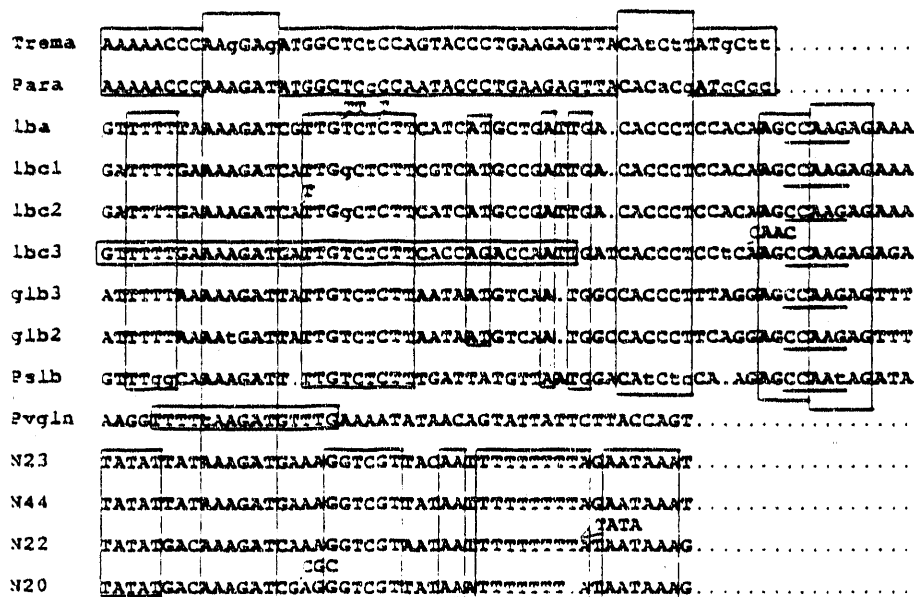

Figure 2. Conserved motifs in the 5' upstream regions of late nodulin genes

Schell, 1992) have been identified. The homology observed even extends to the promoters of hemoglobin genes (Fig. 2) of *Parasponia* (nodulated non-legume) and *Trema* (non-nodulated relative of *Parasponia*; Bogusz et al., 1990). In order to determine the significance of the conserved motifs in the NICE element, we have subjected them to saturation mutagenesis. Twenty-nine selected mutant oligonucleotides corresponding to the NICE region have been used to reconstruct 1.9-kb *glb*3 promoter fragments and have been fused to the *gus* reporter gene. The resulting chimeric genes have been introduced into *Lotus* plants, as described (Szabados et al., 1990; de Bruijn et al., 1990). Transgenic plants harboring these constructs are presently being analysed. (This work was supported, in part, by a grant from the USDA 90-37262-5781.)

Trans-acting factors interacting with DNA elements in the _S. rostrata_ g/_b_3 5' upstream region: the Bacterial Binding Factors (BBF1 and 2)

Previously we found a DNA sequence in the *glb*3 5' upstream region (ATTTTTAAATTATTAATTAAA; Binding Site 2* or BS2*), located immediately upstream of the NICE element, that interacts specifically with a proteinaceous

DNA binding factor from nodule extracts (Metz et al., 1988; de Bruijn et al., 1989). We have also identified a region 570-700 bp upstream of the *glb3* start codon that does not contain BS2*-like sequences, but does interact specifically with a proteinaceous DNA binding factor from nodule extracts (Fragment 202). The DNA binding activity of the nodule factor has been found to be heat-stable (resistant to boiling the extract) and Ca^{+}-dependent, and to be derived from the (infecting) *Azorhizobium caulinodans* bacteria (bacteroids), rather than the host plant (Welters, 1991; see de Bruijn and Schell, 1992). This intriguing observation suggests a putative involvement of bacterial (*Azorhizobium*) proteins (Bacterial Binding Factors or AcBBF's) in *lb* gene expression. The binding site for the first AcBBF has been delimited by footprinting analysis: CCTATACATACTTTATGTG-ATATCC (Bacterial Binding Site 1 or BBS1; Welters, 1991).

In order to gain further insight into the AcBBF1/BBS1 interaction, we have purified two proteins from free-living *Azorhizobium* cultures that bind BBS1 (Welters, 1991; P. Welters, K. Palme and F.J. de Bruijn, unpublished results). The highest BBS1 binding activity co-purifies with a protein of 9-10 kd (*A. caulinodans* Bacterial Binding Protein 1 or AcBBP1), which shares homology (46% identity or 68% similarity over a stretch of 23 amino acids) with the major DNA binding protein ICP8 (Gao et al., 1988) of herpes simplex virus (Welters, 1991; P. Welters, K. Palme and F.J. de Bruijn, unpublished results). We are presently cloning the *Azorhizobium* gene encoding AcBBP1, in order to examine its significance *in vivo*. We have also started to study the interactions of the NICE element with DNA-binding proteins from *S. rostrata* nodules, leaves, roots and free-living *Azorhizobium* cultures. The NICE element forms protein-DNA complexes of similar mobility when incubated with extracts of *S. rostrata* nodules and free-living *Azorhizobium* cultures (K. Szczyglowski, K. Nadler and F.J. de Bruijn, unpublished results). The BBF responsible for this interaction (AcBBF2) appears to be different from AcBBF1, since it is not heat stable and the binding to the NICE element is not Ca^{+}-dependent.

Environmental control of gene expression in *Rhizobium meliloti*: isolation of genes induced by N-, C- and O_2-stress

To understand the symbiotic interaction between rhizobia and plants, to improve its efficiency, or to extend it to presently non-nodulated crop plants, we must also develop an understanding of how rhizobia, especially modified or improved strains, compete with endogenous microbial populations in the rhizosphere and how they respond to a variety of metabolic stress conditions in the soil. To contribute to this goal we are characterizing rhizobial genes that respond to environmental stress conditions, and plan to monitor their expression pattern in the rhizosphere and their role in competition.

Using a Tn*5-lux* transposon constructed by Wolk et al. (1991), a collection of 5000 insertions in the *R. meliloti* 1021 genome has been generated. This collection has

been screened for genomic *lux* fusions induced by N- starvation, C-starvation and O_2-limitation. Twenty N-starvation-induced gene fusions have been characterized and 12 of them have been examined for induction/repression by a variety of N-sources, symbiotic properties and temporal expression patterns. The Tn*5-lux* mutated loci of these 12 isolates have been recloned from the *R. meliloti* genome and the Tn-target junctions subjected to DNA sequence analysis. Preliminary data indicate that at least one of the mutated loci shares significant homology with a known (N-regulated) gene, namely nitrite reductase (P.O. Lim, M. Renner and F. J. de Bruijn, unpublished observations). Strains carrying inducible *lux* gene fusions are being examined under controlled conditions for *lux*-reporter gene expression and competitive ability in the rhizosphere of alfalfa plants. (This work is supported by the NSF Center for Microbial Ecology at MSU.)

Rhizobial opine biosynthesis and catabolism genes and the creation of "biased rhizospheres"

The stable introduction of more efficient (genetically modified) microbes of a beneficial nature in the rhizosphere of (crop) plants has proven to be difficult because that they are usually outcompeted by the indigenous microflora. In order to ameliorate this problem, we are examining the possibility of creating "biased rhizospheres," using a specific nutritional mediator and it's corresponding synthesis and catabolism genes.

We have previously described a nodule-specific, opine-like compound from alfalfa nodules induced by *Rhizobium meliloti* strain L5-30 (Murphy et al., 1987). This compound was designated "opine-like" by analogy with *Agrobacterium*, since only the strain that induced its synthesis could catabolize it and use it as growth substrate. Unlike that in *Agrobacterium*, this rhizobial opine (rhizopine) has been found to be synthesized by the bacterium and the rhizopine synthesis (*mos*) and catabolism (*moc*) genes are closely linked on the symbiotic plasmid (Murphy et al., 1987). In fact, the mos genes are symbiotically induced (in the bacteroids) via the *nif*-regulatory protein NifA (see above), revealing a close linkage between the two processes (Murphy et al., 1988). It has been postulated that the *moc/mos* system may confer on its host bacterium a selective advantage, both in the nodule and in the rhizosphere (Murphy and Saint, 1991).

In order to examine the distribution of the *moc/mos* system, we have delimited the *moc* genes, using genetic and molecular means, and used a specific *moc* hybridization probe to screen a variety of soil bacteria and a collection of divergent *R. meliloti* isolates described by Eardley et al. (1990). We have found only one additional *R. meliloti* strain (102F51) that carries the *moc* genes and this strain is capable of catabolizing rhizopine (S. Rossbach and F.J. de Bruijn, unpublished results). Interestingly, strain 102F51 is used as a commercial inoculant in the US and appears to be highly competitive. For studies of catabolism, we normally isolate the rhizopine out of nodules induced by strain

L5-30 on alfalfa plants, but for large-scale screening the amount one can obtain is limiting. Therefore, we are presently synthesizing this compound. We have (re-)examined the rhizopine structure in collaboration with R. Hollingsworth of the MSU Department of Biochemistry, using mass spectroscopy and nuclear magnetic resonance spectroscopy. We have found two minor, but nevertheless crucial, deviations from the structure proposed by Murphy et al. (1987).

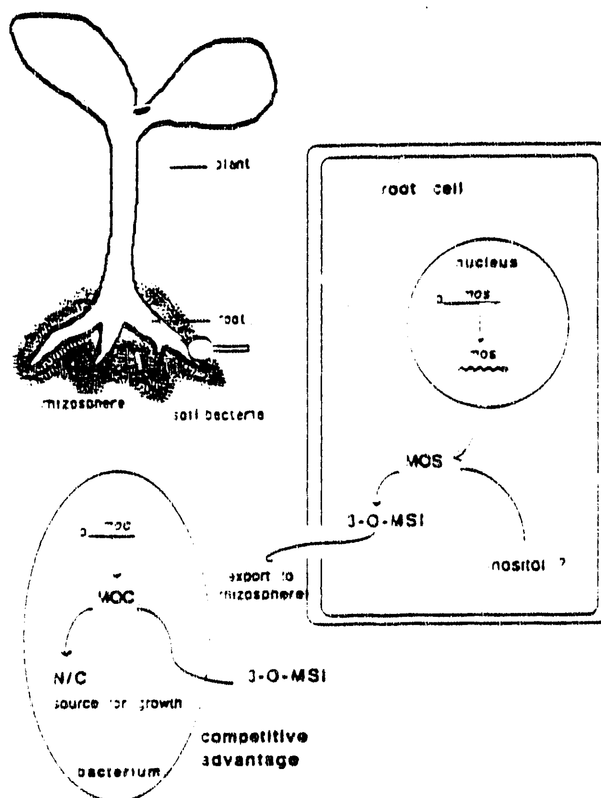

Figure 3. Schematic representation of a "biased rhizosphere"

We are planning to express the *mos* genes in transgenic plants and to examine the secretion of the rhizopine into the rhizosphere. We will also introduce the *moc*-cassette into different soil bacteria, in order to determine whether they will gain a selective advantage in the rhizosphere of rhizopine-producing plants (creation of "biased rhizospheres"; Figure 3). (This work is supported by the NSF Center for Microbial Ecology at MSU.)

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ENZYMATIC MECHANISMS AND REGULATION OF PLANT CELL WALL BIOSYNTHESIS

Deborah P. Delmer. *Yehudit Amor, Andrawis Andrawis, Liat Gonen, Esther Shedletzky, Miri Shmuel and Mazal Solomon (The Hebrew University)*

One of the major goals of this task continues to be to gain an understanding of the mechanism and regulation of cellulose synthesis in higher plants (see Delmer, 1991, for recent review). Although no group has yet succeeded in detecting an activity for a cellulose synthase in plants, for the bacterium *Acetobacter xylinum*, such an enzyme has been found, purified to homogeneity, and characterized (Ross et al., 1991). Furthermore, an operon of 4 genes coding for a catalytic subunit and regulatory subunit of the synthase, plus two other polypeptides of unknown function, has been cloned and sequenced (Wong et al., 1990; Saxena et al., 1990 and 1991). Last year, our group discovered two polypeptides in cotton fibers which bear remarkable similarity to the regulatory subunit of the bacterial cellulose synthase (Amor et al., 1991). This year, we have continued our characterization of these polypeptides. In addition, since our data strongly support the idea that the pathway for cellulose synthesis may be surprisingly conserved between bacteria and higher plants, we have designed strategies for cloning cellulose synthase genes from plants based upon such homology, and we report here our efforts in this direction.

We have also continued to characterize a set of polypeptides which interact with the plasma membrane of cotton fibers in a Ca^{2+}-dependent manner. We now believe that these polypeptides have key regulatory functions, some of which relate to the regulation of cellulose and callose synthesis. We report here further findings which support this concept.

Finally, we have continued our studies on plant cells adapted in culture to growth on the cellulose-synthesis inhibitor 2,6-dichlorobenzonitrile (DCB). We have already shown that such an adapted cell line of tomato possesses quite unique cell walls which virtually lack a cellulose-xyloglucan network (Shedletzky et al., 1990). This year we have extended our studies to compare the mechanism of adaptation of two dicot cell lines (tomato and tobacco) with that of a graminaceous monocot (barley endosperm cultures). We chose this approach because it is well-known that such monocots have primary cell walls with quite a different structure from that of the dicots (Bacic et al., 1988); we therefore suspected that the compensatory changes in cell wall structure used by the monocot to cope with reduced levels of cellulose in the wall may be quite different from those which occur in the dicots. The results presented here indicate that this is indeed so, and also shed interesting light on the nature of the various load-bearing networks in the primary walls of both dicots and monocot grasses.

Since the leader of this task (D.P.D.) is a Professor at The Hebrew University in Jerusalem and an Adjunct Professor at the PRL, research is conducted in both locations.

Further characterization of cotton fiber polypeptides which bind c-di-GMP; efforts to clone genes which code for polypeptides of the cellulose synthase of cotton fibers

Last year, we reported the discovery of two cotton fiber polypeptides, of 83 and 48 kD, which bind the bacterial cellulose synthase activator c-di-GMP with high affinity and specificity (Amor et al., 1991). This work also showed that both polypeptides possess antigenic determinants in common with the regulatory subunit of the bacterial synthase (product of the *bcsB* gene; Wong et al., 1990), and also show striking developmental regulation. Thus, we cannot detect the polypeptides by affinity labeling with ^{32}P-c-di-GMP using membranes prepared during the phase of cotton fiber primary wall synthesis when rates of cellulose synthesis are low; however, as fibers enter the transition to secondary wall synthesis and as rates of cellulose synthesis *in vivo* increase >100-fold, we see a marked increase in the level of these polypeptides.

Last year, we were still uncertain as to the relationship between the 83- and 48-kD polypeptides; because they shared antigenic determinants, we suspected that the 48-kD species was a proteolytic breakdown product of the 83-kD polypeptide. This conclusion is now much more strongly supported by N-terminal sequencing of the 48-kD polypeptide. (Figure 1 below).

A. xyl. 93 kD (from res. 479)	L	R	E	T	H	G	L	V	-	G	A
A. xyl. bcsB (from res. 457)	L	R	E	P	D	S	T	I	-	D	Q
Cotton fiber 48 kD (N-ter)	M	R	E	I	(D)	S	V	I	X	(D)	Q

Figure 1. Comparison of bacterial and cotton fiber peptide sequences. The N-terminal amino acid sequence of the cotton fiber 48-kD polypeptide is compared with that of an internal sequence of the product of the *A. xylinum bcsB* gene (Wong et al., 1990) and an analogous internal sequence of a 93-kD polypeptide deduced from cloning of the analogous gene in a different strain of *A. xylinum* by Saxena et al., (1991). Sequences in black boxes indicate perfect homology; those in white boxes indicate group homology. X indicates that no amino acid appeared in that cycle of sequencing.

By examining the amino acid sequence deduced from the *bcsB* gene cloned by Wong et al. (1990), we were able to find a region about halfway into the translated

region which shows at least 45% homology with the N-terminal sequence of the cotton fiber 48-kD polypeptide; this homology rises to 55% if the comparison also includes amino acids showing group homology, and is even higher if the two glutamic acid residues (D) which were only tentatively identified by us are also included in the comparison.

An additional comparison of the cotton fiber sequence with an analogous gene more recently cloned by Saxena et al. (1991) from a different strain of *A. xylinum* shows less homology, but this appears to be a region which is not highly conserved between the two strains. Thus, these results support the concept that the 48-kD polypeptide is a C-terminal cleavage product of the larger 83-kD c-di-GMP-binding polypeptide. It is interesting that a similar C-terminal cleavage fragment of the *bcsB* gene product which binds c-di-GMP has also been found in purified preparations of the *A. xylinum* cellulose synthase (Mayer et al., 1991), and it is not clear, either for the bacterial or the plant case, whether the proteolytic fragment is an artifact of *in vitro* isolation or part of a normal processing event.

Oligonucleotide probes based on the N-terminal sequence of the cotton fiber 48-kD polypeptide, as well as upon other selected sequences from the *bcsA* and *bcsB* genes of *A. xylinum*, were synthesized and used in PCR experiments in attempts to amplify portions of higher plant cellulose synthase genes using our cotton fiber cDNA library as the source of DNA. Several bands were amplified which had the predicted lengths; however, when subcloned and partially sequenced, the homology with the bacterial sequences was not sufficiently convincing to conclude that they were in fact derived from the correct genes. We therefore took a different approach. Since the clones of the bacterial genes were not available to us, we decided to create our own "clones" by using PCR to amplify portions of the *bcsA* and *bcsB* genes using *A. xylinum* DNA. For these experiments, we chose oligonucleotide probes which should have allowed us to amplify portions of these genes which were highly conserved between the two strains used by Wong et al. (1990) and Saxena et al. (1990, 1991). This approach was highly successful, as we succeeded in generating large quantities of specific fragments of the predicted length; results of sequencing indicated that we had amplified the desired regions of both genes. These amplified fragments are now being used as probes to screen our cotton fiber cDNA library. A number of putative clones have been identified which have continued to screen positive through several rounds of screening. Sequencing of these clones is in progress.

Characterization of regulatory proteins involved in ß-glucan synthesis in cotton fibers

Last year, we reported the discovery of a set of polypeptides which can be eluted from the membranes of cotton fibers by washing with high concentrations of EDTA or EGTA. The most abundant of these was a 34-kD polypeptide which, in

terms of its size and Ca^{2+}-dependent interaction with membranes, resembled a class of animal proteins called annexins (Crompton et al., 1988). Other polypeptides eluted included the 48-kD c-di-GMP-binding polypeptide, as well as some undefined protein kinase activity. Below, we summarize the results of our more recent studies with these polypeptides.

1. We now have sequence information on several N-terminal fragments derived by V-8 digestion of the 34-kD polypeptide (p34). One of these sequences shows striking homology with a similar fragment from a recently characterized annexin-like protein from tomato (Smallwood et al., 1990). A second fragment showed some homology with a conserved region from animal protein kinase C. Two-dimensional gel electrophoresis indicates that cotton fiber p34 is composed of two major, and several minor, 34 kD polypeptides which show antigenic relatedness, and therefore resemble the situation for animal annexins which exist as a family of at least 7 polypeptides. Using an affinity-purified polyclonal antibody against p34, we have screened our cotton fiber cDNA expression library, and succeeded in isolating 9 clones which continue to screen positive after 3 rounds of screening. These clones are currently being characterized with respect to insert size, overexpression of the expected antigen in Blue-Script plasmids, and nucleotide sequence.

2. One possible role for animal annexins is to serve as a membrane anchor for protein kinase C (Mochly-Rosen et al., 1991); in this regard, we note that protein kinase activity is present in the set of polypeptides released with p34. Addition of micromolar Ca^{2+} back to this fraction results in precipitation of the 34-kD polypeptide in almost pure form as judged by SDS-PAGE, but we have noted with interest that an 18-kD autophosphorylating protein kinase is also co-precipitated under these conditions. The kinase shows high activity but is present in concentrations too low to detect by staining of gels. We have also observed that p34 itself can be phosphorylated under some conditions. Furthermore, we have recently observed that a plasma-membrane localized 62-kD polypeptide, which is highly concentrated at sites of high callose synthase activity such as plasmodesmata, can be phosphorylated in a calcium-dependent manner; re-addition of EDTA-eluted polypeptides back to plasma membranes results in complete inhibition of the phosphorylation of this polypeptide.

3. Most exciting to us are results of recent experiments designed to probe the possible function of p34 in regulating glucan synthesis in cotton fibers. We have now clearly shown that re-addition of microgram quantities of proteins from the EDTA wash of membranes (highly enriched for p34) can stimulate callose synthase, but only under conditions where Ca^{2+} is limiting (Table 1).

Table 1. Effect of proteins washed from plasma membrane by EDTA on activity of partially purified detergent-solubilized cotton fiber callose synthase. Plasma membranes were prepared by two-phase extraction in the presence of 25 mM EDTA. Callose synthase was solubilized in 1% digitonin and partially purified by glycerol gradient centrifugation. Each assay contained 0.8 μg of the callose synthase preparation.

Proteins of the EDTA wash (μg)	Free Ca^{2+} (μM)	Callose Synthase Activity (nmol/min/mg protein)
—	1.2	39
0.04	1.2	68
0.13	1.2	57
1.32	1.2	192
13.20	1.2	399
—	5,000	411
13.20	5,000	408

Callose synthase an enzyme which requires micromolar levels of calcium for activity, and this information has been used to explain how callose synthesis is activated upon wounding and/or pathogenesis (Kauss, 1987). However, recent studies indicated that wounding or elicitation may result in only very transient increases in calcium in plants (Knight et al., 1991); thus, it remained a mystery how callose synthesis could continue for hours after calcium levels returned to their resting state. We now propose that p34 and the associated kinase are normally soluble proteins; when a transient rise in cytoplasmic calcium occurs, these proteins rapidly associate with the callose synthase on the plasma membrane and serve as activators of the enzyme even after calcium returns to its resting state. Possible changes in the state of phosphorylation of these or other proteins, such as the 62-kD polypeptide, may be involved in the later dissociation of these proteins from the membrane. Further support for this notion comes from our direct demonstration that p34 can associate directly with highly purified plasma membranes in a calcium-dependent manner, and that p34 is also found in high amounts in preparations of detergent-solubilized callose synthase partially purified by product entrapment.

In sum, we believe we are dealing with a very complex set of regulatory systems, at least some of which are involved in regulation of glucan synthesis. The fact that the 48-kD c-di-GMP-binding polypeptide also can be released from membranes by EDTA suggests to us that changes in calcium levels may regulate cellulose synthesis as well, perhaps by affecting the interaction of cellulose synthase subunits with some of these regulatory proteins present in the EDTA eluate. To make the story even more complex, we have also discovered a GTP-binding polypeptide in the EDTA eluate (specific for GTP; doesn't bind c-di-GMP). Its size, of about 45 kD, is quite similar to that of the alpha subunits of the well-known G-proteins which regulate many processes in animals.

DCB-adapted plant cells contain unique cell walls: a comparison between two dicots and a graminaceous monocot

Our previous work (Shedletzky et al., 1990) showed that suspension-cultured tomato cells adapted to growth on DCB have unique cell walls with markedly reduced levels of the cellulose-xyloglucan network. This year we have compared the wall compositions of two adapted dicot lines (tomato and tobacco) with that of a graminaceous monocot (barley endosperm). Such monocots are known to have a primary wall structure quite different from that of the dicots (Bacic et al., 1988), and this difference is also reflected in the very different types of wall modifications induced by growth on DCB. Both dicot lines have reduced levels of cellulose and xyloglucan, and they possess walls, the integrity of which is provided principally by Ca^{2+}-bridged pectates. Not surprisingly, we have found that the tensile strength of these walls is considerably less than of walls from their non-adapted counterparts. Remarkably, protoplasts can be prepared from adapted dicot cells simply by treatment with divalent cation-chelator CDTA and a purified endo-polygalacturonase (no protoplasts at all are released by a similar treatment of non-adapted cells). These protoplasts can then generate a new wall in the presence of DCB, a characteristic not shared by protoplasts of non-adapted cell lines.

Unlike the dicots, walls from both non-adapted and adapted barley cells contain limited amounts of pectic material. In contrast to the situation for xyloglucan in the dicots, adapted barley walls have normal levels of a major hemicellulosic polymer, glucuronoarabinoxylan, and elevated levels of the other hemicellulosic polymer, a mixed-linked 1,3-1,4-ß-glucan. These results indicate to us that the major two hemicellulosic polymers in these walls do not play a role similar to that of xyloglucan; that is, they are not polymers which normally interact with cellulose in the wall.

Surprisingly, the adapted barley walls have tensile strengths even higher than their non-adapted counterparts, even though cellulose levels are reduced by 70%. The following findings indicate that this additional strength is provided by altered patterns of phenolic cross-linking of hemicellulosic polymers: 1) in contrast to the

characteristic green autofluorescence (from ester-linked ferulic acid) in normal monocot walls, adapted barley walls show a yellow fluorescence indicative of altered phenolic composition and/or cross-linking; 2) analyses of phenolic materials released by mild alkali indicate the presence of much less ferulic and di-ferulic acid, more free coumaric acid, and more conjugated phenolics; 3) esterase treatment of non-adapted walls releases only about 0.4% of the non-cellulosic carbohydrates of the wall, whereas about 3.7% of these components from adapted walls is solubilized; 4) adapted walls contain an altered pattern of isozymes for basic peroxidases believed to be involved in phenolic cross-linking in walls. The ability to maintain high wall strength under conditions of reduced cellulose synthesis may help to explain why the monocot grasses tend to be more resistant to herbicides, such as DCB and Isoxaben, which inhibit cellulose synthesis.

It is also very interesting that adapted cells of both the dicots and monocots cannot be plasmolyzed (either with 1.5 M mannitol or 4 M NaCl), yet turgor pressures are normal. This indicates the presence of very strong wall-plasma membrane connections which could be analogous to similar connections mediated by integrins in animal cells. The fact that these connections in both the dicots and monocots can be broken by CDTA suggests that they are stabilized by divalent cations.

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MOLECULAR MECHANISMS THAT REGULATE THE EXPRESSION OF GENES IN PLANTS

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The steady-state level of an mRNA depends both on its rate of synthesis (transcription) and its rate of degradation. Rapidly accumulating data indicates that rates of degradation of mRNAs in eukaryotes vary over a wide range and can be regulated by a variety of stimuli. However, in contrast to the mechanisms that control transcription, the mechanisms that control mRNA stability are largely unknown. A major objective of our work is to identify and characterize the molecular components (e.g., RNases and the mRNA sequences that they recognize) that control the rates of mRNA degradation in plants and determine how those components interact. Another goal is to understand how certain of those components respond to plant growth regulators and to environmental stimuli so as to alter selectively the rates of mRNA degradation. We plan to make maximal use of decay measurements in transgenic plants and the isolation and analysis of mutants with defects in mRNA degradation. Similar experiments are not likely to be feasible in animal model systems in which much of the pioneering work on mRNA stability has been done. Therefore, this work has the potential to identify new components that are unique to plants or of broad significance to higher eukaryotes. Beyond its contribution to basic knowledge, this research should suggest solutions to practical problems resulting from the instability of foreign mRNAs in genetically engineered crop plants.

Measurement of rates of mRNA degradation in stably transformed tobacco

A method has been developed to allow the direct measurement of rates of mRNA decay in suspension cultures of stably transformed *Nicotiana tabacum* cells (NT cells; Nagata *et al., 1981*). To allow mRNA degradation to be monitored in the absence of synthesis, NT cells are treated with Actinomycin D (ActD), a transcription inhibitor commonly used for this purpose in animal cells (e.g. Lindsten *et al.*, 1989; Schuler and Cole, 1988). The effective concentration of ActD for inhibition of mRNA synthesis in NT cells was determined by pulse-labeling. For mRNA degradation measurements, samples are withdrawn from a transformed cell line at various times after ActD treatment, and RNA is isolated. The fate of specific mRNAs is then monitored by hybridizing Northern blots with appropriate probes (see Fig. 1 for an example). We have used this system to measure the rates of decay of β-glucuronidase (GUS) and chloramphenicol acetyltransferase (CAT) transcripts containing 3' end sequences from pea *rbcS-3C* (3C) and *rbcS-E9* (E9) genes, respectively (Fang et al., 1989). Our data showed that GUS and CAT mRNAs had similar half-lives in NT cells (about 70-80 min) and that the kinetics of mRNA degradation were first order over the duration of our experiments. In the same cells, endogenous nuclear encoded transcripts for the mitochondrial β-ATPase and HSP-70 did not decay appreciably during the 2.5-h time course, and therefore are representative of more stable mRNAs. The ratio of GUS and CAT mRNA decay rates was highly reproducible in multiple experiments. This result suggests that GUS and CAT will serve as an effective

test-gene/reference-gene system for the identification of sequences that cause instability of mRNA in NT cells (i.e., the insertion of a destabilizing sequence into the GUS transcript would be expected to cause the transcript to decay more rapidly than an unmodified CAT transcript in the same cell line). Moreover, our experiments performed with NT cells were consistent with those performed with

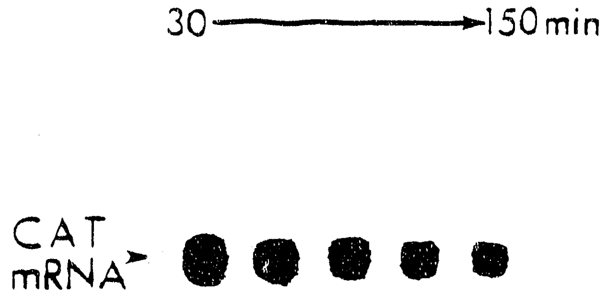

Figure 1. Degradation of the CAT transcript in stably transformed tobacco cells. A cell line that was stably transformed with a CAT-E9 gene, under the control of a Cauliflower mosaic virus 35S promoter, was used for this experiment. RNA was isolated from samples harvested at 30-min intervals following treatment with ActD, and used to prepare a northern blot (20μg/lane). The blot was hybridized with a CAT probe to monitor the degradation of the corresponding mRNA.

transgenic plants. For the latter, we placed GUS-3C and CAT-E9 genes under the control of copies of the wheat Cab-1 promoter, which is induced transiently each morning by an endogenous circadian clock (Nagy et al., 1988). By harvesting transgenic tobacco seedlings during the afternoon and evening, we observed that both transcripts disappeared at approximately the same rate, again indicating that the half-lives of GUS and CAT transcripts are similar in tobacco. (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 (McClure et al., 1989). 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 (McClure and Guilfoyle, 1989). 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. Between amino acids 27 and 76, this clone is 80% homologous at the amino acid level and 74% at the nucleotide level, with the prototype SAUR 15A gene from soybean. 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. It has been suggested that the DST sequence may be involved in the posttranscriptional control of SAUR genes, and the finding of a DST sequence in SAUR-AC1 supports this contention. (Supported, in part, by USDA grant #9001167.)

Properties of the major ribonucleases (RNases) of *Arabidopsis*

We have begun to characterize the RNases of *Arabidopsis* as a first step towards identifying the RNases that play a role in mRNA stability. The profile of RNases in *Arabidopsis* can be resolved into as many as 16 bands of RNase activity using a substrate-based gel assay (Yen and Green, 1991). Several properties of these RNases were investigated, including their apparent molecular weight, Zn^{2+} dependence, EDTA sensitivity, pH optimum, and ability to digest DNA. These characteristics of the *Arabidopsis* enzymes provide a basis for comparison to enzymes from other plants. Classically, the RNA-degrading enzymes of higher plants have been grouped into four main enzyme "families" as described in Table 1 (Farkas, 1982; Wilson, 1982). The RNase I class is RNA-specific, has a pH optimum of 5 to 5.5, a molecular mass of 20 to 25 kD, and a low sensitivity to EDTA; the RNase II enzymes are also RNA specific, but have pH optima between 6 and 7, molecular masses between 17 and 21 kD, and low sensitivity to EDTA. The third class is comprised of endonucleases that digest RNA and DNA, with a pH optimum of 5 to 6.5, a molecular mass of 31 to 35 kD, and a high sensitivity to EDTA. Finally, enzymes in the exonuclease class digest DNA and RNA, have a pH optimum between 7 and 9, molecular masses of ca. 100 kD, and a high sensitivity to EDTA. As indicated in Table I, the first three categories appear to be represented among the major RNA-degrading enzymes that we have identified from *Arabidopsis*. It should now be possible to use the gel assay to screen M2 *Arabidopsis* plants for mutants that lack any of the major RNases that we have characterized. (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 alata* (McClure et al., 1989). 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 approach we have identified a set of three putative RNase genes in *Arabidopsis*, which is a self-compatible plant (Taylor and Green, 1991). These *Arabidopsis*

Table 1. Major types of RNA-degrading enzymes of higher plants.

	RNase I	RNase II	Endonuclease	Exonuclease
Mwt.	20-25 kD	17-21 kD	31-35 kD	100 kD
Specificity	RNA	RNA	RNA & DNA ss preferred	RNA & DNA ss preferred
EDTA sensitivity (ion requirement)	Low	Low*	High	High
Localization	Soluble*	Microsomal*	Particle Bound*	Soluble*
pH optimum	5.0-5.5	6.0-7.0	5.0-6.5	7.0-9.0
***Arabidopsis* enzyme**	**22.6 kD**	**17.7 kD**	**33.6 kD**	

Arabidopsis enzymes that have properties representative of the major classes of RNA-degrading enzymes classically described for higher plants are highlighted in bold. Asterisks indicate properties that were not examined for the *Arabidopsis* enzymes.

genes, designated RNS1, RNS2, and RNS3, do not cross hybridize to each other and their products are homologous to both the S-gene RNases (McClure et al., 1989) and to a set of fungal enzymes (Horiuchi et al., 1988; Kawata et al. 1988) (see Figure 1). 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. We have isolated a cDNA clone containing the entire coding region of RNS2 and compared the deduced amino acid sequence to that of a number of S-RNases (see Figure 2). Our results show that homology between RNS2 and the S-RNases extends throughout the coding region. In particular, RNS2 contains the five regions that are the most highly conserved among the S-RNases. However, subtle differences also exist, such as the absence of an asparagine at position 39 of RNS2 within the second conserved box. The most conspicuous difference among the sequences is the C-terminal extension contained in RNS2 but absent from the S-RNases. This C-terminal extension resembles a C-terminal propeptide sequence recently shown by Bednarek and Raikhel (1991) to function as a vacuolar targeting signal. Therefore, it is possible that the cellular location of RNS2 differs from that of the S-RNases which are secreted. In any event, 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. (Supported, in part, by NSF grant #DCB9105968 and a grant from the McKnight Foundation.)

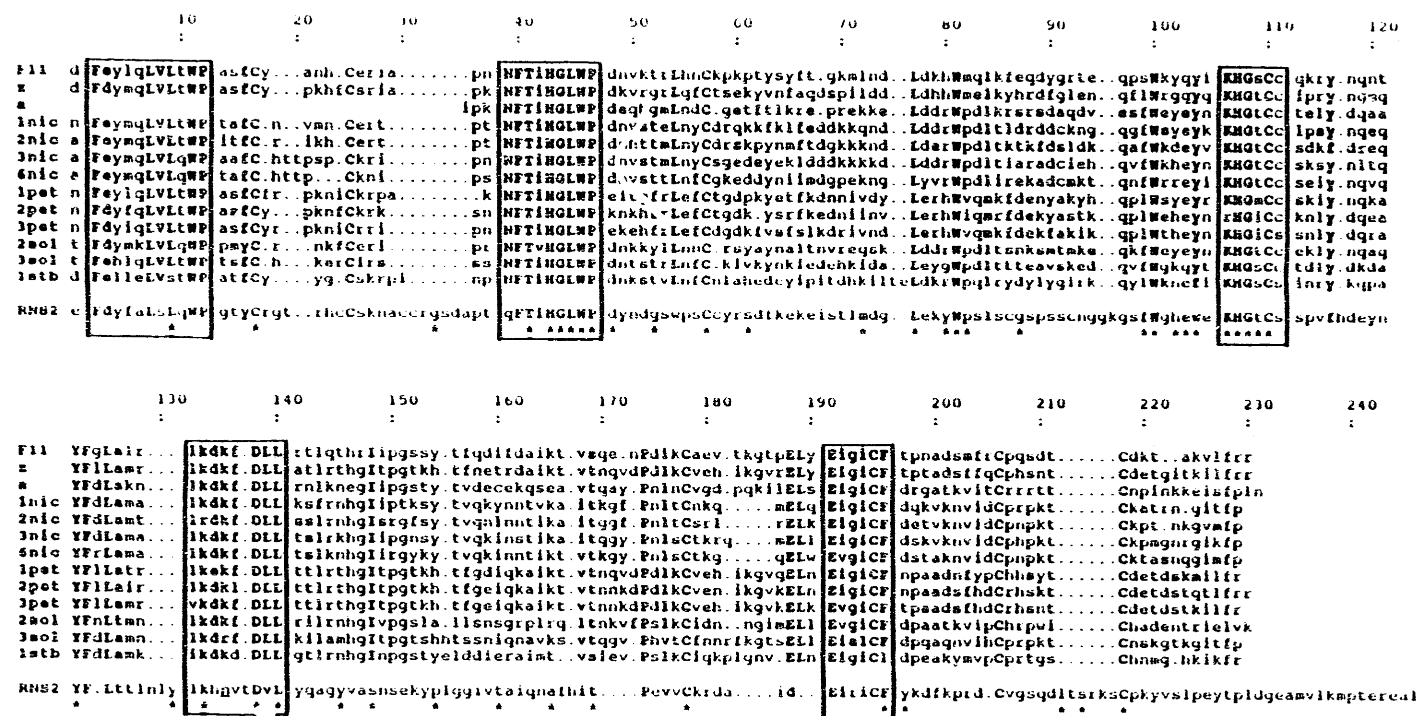

Figure 2. RNS2 contains all five regions that are most highly conserved among S-gene RNases. Boxes indicate the homology of RNS2 with conserved regions C1-C5 (Ioerger et al., 1991) of the S-RNases. Homology with the fungal enzymes T_2 (Kawata et al., 1988) and Rh (Horiuchi et al., 1988) (indicated by asterisks) is concentrated in the active site region between positions 40 and 110. S-RNases F11, z, a, 1nic, 2nic, 3nic, and 6nic, are from *N. alata* (Haring et al., 1990; Ioerger et al., 1991; McClure et al., 1990); RNases 1pet, 2pet, and 3pet are from *Petunia inflata* (Ai et al., 1990); 2sol and 3sol are from *Solanum chacoense* (Xu et al., 1990); and 1stb is from *Solanum tuberosum* (Haring et al., 1990).

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RESISTANCE OF PLANTS TO ENVIRONMENTAL STRESS

Andrew D. Hanson. *Pierre Lafontaine, Kent F. McCue, Jean Rivoal and Bala Rathinasabapathi*

There is much scope for improving crop adaptation to unfavorable environments (Boyer, 1982). However, the mechanisms responsible for adaptation to stresses are poorly understood at the genetic and biochemical levels (McCue and Hanson, 1990), and this is a barrier to conventional plant-breeding and to recombinant DNA (rtDNA) approaches to improving stress resistance. By making possible the transfer of DNA across species boundaries, rtDNA technology has greatly expanded the pool of stress-resistance genes that might be used for crop improvement. The problem is to identify these genes. We are addressing this problem, using a biochemical strategy. First, metabolic pathways which contribute to stress adaptation are sought. Second, the key enzymes of these pathways are found and the corresponding genes isolated. Third, these genes are manipulated in order to evaluate their impact on plant stress resistance.

Two types of metabolic adaptations to stress are being investigated: the accumulation of quaternary ammonium compounds (QACs) induced by salt- or water-stress; and lactate glycolysis induced by hypoxia. QACs such as glycine betaine can act as non-toxic cytoplasmic osmolytes, and hence permit metabolic function at low water potential (Wyn Jones, 1984). Lactate glycolysis complements ethanol glycolysis during O_2 deficit in roots and seeds of many plants (Roberts et al., 1984), and may be critical to survival in flooded soils.

Regulation of expression of betaine aldehyde dehydrogenase in sugar beet

Betaine aldehyde dehydrogenase (BADH) catalyzes the last step in glycine betaine synthesis, and is induced several-fold by salinization (Weretilnyk and Hanson, 1990). Because salt stress is applied to roots and BADH induction occurs in leaves, inter-organ signalling is implied. To investigate this signalling, BADH enzyme activities and mRNA levels were analyzed in leaves of sugar beet plants subjected to various stress regimes. In plants which had adjusted osmotically to growth at various NaCl concentrations, the steady state level of enzyme rose almost linearly between 0 and 500 mM NaCl, whereas that of BADH mRNA reached a plateau at 100 mM. Following a salt shock (transfer from 0 to 400 mM NaCl), the BADH mRNA level first decreased for several h, then increased; BADH enzyme activity rose slowly for several days. When salt was leached from the rooting medium of salinized plants, the level of BADH mRNA declined sharply with an apparent half-life of 2 h; enzymatic activity also declined, but with a half-life of more than 4 days. These data indicate that (a) transcription of the BADH gene or the stability of BADH mRNA in leaves can respond sensitively and dynamically to changes of salinity around the root; and (b) the

mRNA responses are consistent with a non-hydraulic signal or signals coming from the root. The signal is unlikely to be NaCl, as leaf disks exposed to NaCl concentrations typical of the apoplast of salinized leaves did not accumulate BADH mRNA. A biochemical messenger is thus implied and, consistent with this, abscisic acid application to leaf disks elicited modest increases in the level of BADH mRNA. (Supported, in part, by a grant from USDA-CRGO.)

Expression of betaine aldehyde dehydrogenase in transgenic tobacco

Certain members of the Solanaceae accumulate glycine betaine in response to osmotic stresses but tobacco does not, apparently because it lacks both enzymes of glycine betaine biosynthesis--choline monooxygenase (CMO) and BADH (Weretilnyk et al., 1989). Tobacco is thus a good candidate species for manipulation of glycine betaine biosynthesis. As a step towards this aim, cDNAs containing the full coding sequences of spinach and sugar beet BADH were placed under the control of the CaMV 35S promoter and introduced into tobacco, using *Agrobacterium tumefaciens*-mediated transformation. Transgenic plants with leaf BADH enzyme activities comparable to those in spinach and sugar beet were obtained. Leaf disks of these transformants oxidized supplied betaine aldehyde to glycine betaine as rapidly as did spinach disks. When supplied with betaine aldehyde, the transgenic tobacco disks had smaller endogenous pools of betaine aldehyde than spinach disks, indicating that the introduced BADH enzyme has ready access to supplied betaine aldehyde. The difference may reflect a difference in targeting and compartmentation between the transgenic product in tobacco and BADH in the native plant, in which it is a predominantly chloroplastic enzyme (Weretilnyk and Hanson, 1990). (Supported, in part, by grants from USDA-CRGO, and the Rockefeller Foundation.)

Constitutive expression of lactate dehydrogenase in transgenic tomato roots

Most evidence suggests that although there is a brief phase of lactate glycolysis during the onset of hypoxia, the long-term glycolytic flux to lactate is very small compared to that to ethanol (Roberts et al., 1984). However, the roots of many plants have hypoxically inducible lactate dehydrogenase (LDH) enzymes which do not begin to increase in activity until after the initial phase of lactate production. This inconsistency highlights a gap in our understanding of lactate metabolism and its control. To clarify the role of LDH, cDNAs containing the protein-coding sequence of barley LDH were placed under the control of the CaMV 35S promoter and inserted into tomato roots via *Agrobacterium rhizogenes*-mediated transformation. Transgenic root cultures were obtained that expressed high levels of LDH in aerobic conditions. When aerobically grown transgenic roots were incubated with [^{14}C]glucose for 6 h under anoxic conditions, ethanol was the major labeled metabolite, and the lactate/ethanol ratio differed little from that in untransformed controls. This establishes that a high catalytic potential of LDH is not sufficient for lactate glycolysis. Interestingly,

we have found that roots of species of *Limonium* (Plumbaginaceae) synthesize more lactate than ethanol, and that most of the lactate is secreted into the medium. Lactate production may therefore depend on the activity of a lactate transport system.

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STUDIES ON HORMONE BIOSYNTHESIS AND ACTION

Hans Kende. *Susanne Hoffmann, David Olson, Scott Peck, Margret Sauter, Joseph White, Ronda White and Renate de Zacks*

Knowledge of the biosynthesis of ethylene has expanded at a rapid pace during the past year. The molecular cloning of 1-aminocyclopropane-1-carboxylate (ACC) synthase led to the realization that this enzyme is encoded by a small multigene family (Olson et al., 1991; Rottmann et al., 1991; Van Der Straeten et al., 1990). The problem as to whether different factors that induce ethylene biosynthesis activate different ACC synthase genes can now be investigated using gene-specific probes. We have taken this approach and found that the level of mRNA encoding ACC synthase 1 and 2 increases as tomato fruits ripen, but that expression of only ACC synthase 1 is enhanced by wounding of the fruit tissue (Olson et al., 1991). We have now extended this work by investigating the expression of ACC synthase 1 and 2 under other stress conditions. Last year, the ethylene-forming enzyme, ACC oxidase, was cloned (Hamilton et al., 1991; Spanu et al., 1991), and its enzymatic properties were determined in tissue homogenates (Ververidis and John, 1991). We have cloned the gene encoding ACC oxidase from peas and tomatoes and have studied the expression and localization of tomato ACC oxidase in transformed yeast. The aim of our experiments is to gain an understanding of the manner in which different endogenous and environmental factors regulate ethylene biosynthesis.

We have been investigating the regulation of growth in deepwater rice for the past ten years. We have learned that rapid internodal elongation induced by submergence is based on enhanced cell division activity in the intercalary meristem and on increased cell elongation (Bleecker et al., 1986; Métraux and Kende, 1984). We have also learned that the growth response is, ultimately, elicited by gibberellin (GA) (Raskin and Kende, 1984). We are trying to determine what the primary action of GA is, stimulation of cell division or promotion of cell elongation. This problem has been approached by determining the time course of GA action on the cell division cycle and on cell growth. At the same time, we have also investigated the effect of GA on cell wall architecture and have examined secretory processes related to formation of the cell wall or cuticle in the outer epidermis. We hope that our investigations will lead to an understanding of component processes of growth and their interrelationships.

Work on the action of cytokinins focuses on the mechanism by which cytokinins induce nitrate reductase activity in embryos of *Agrostemma githago*. In most plants, nitrate reductase is the rate-limiting enzyme in nitrate assimilation. For this reason, it is important to gain an understanding of its hormonal regulation. We are mainly interested in finding out whether the cytokinin-regulated enzyme is encoded by a gene that is different from the one that encodes the enzyme induced by nitrate.

Expression of ACC synthase mRNA

We examined the timing of expression and the stability of ACC synthase mRNAs from tomato fruits using gene-specific cDNA probes (Olson et al. 1991) for the detection of ACC synthase 1 and 2 mRNA on RNA (Northern) blots. In wounded pericarp tissue, the level of ACC synthase 1 mRNA started to increase after 40 min and continued to rise over the next 2 h. ACC synthase 2 mRNA showed no significant change in signal intensity during the same time period. It has been a standard procedure to boost ACC synthase levels in tomato fruit tissue by incubation in LiCl (Boller, 1984; Ramalingam et al., 1985). ACC synthase was induced by wounding over a 6-h period in the presence and absence of LiCl. No increases in the level of ACC synthase 1 or 2 mRNA could be detected as a result of LiCl treatment. The discrepancy between the effects of LiCl on ACC synthase activity and on ACC synthase mRNA levels has several possible explanations. LiCl may increase the level of an ACC synthase mRNA that is not recognized by our gene-specific probes. It is also possible that LiCl is acting at the post-transcriptional level, either by stimulating the synthesis of ACC synthase or by reducing its rate of degradation.

Infection of tomato leaves with *Phytophthora infestans* induces ethylene biosynthesis and enhances the activity of both ACC synthase and ACC oxidase (Spanu and Boller, 1989). Tomato seedlings were infected with a zoospore suspension of *Phytophthora infestans*, and induction of ACC synthase activity and ethylene formation were monitored along with the level of ACC synthase mRNAs (Fig. 1). Poly(A)$^+$ RNA was prepared from leaves prior to and at different times after infection, and mRNA levels were assessed by Northern blotting using the gene-specific probes for ACC synthase 1 and 2. No ACC synthase activity or expression of ACC synthase mRNA was detectable in freshly harvested leaves. ACC synthase 2 mRNA was not expressed in tomato leaves at any time following infection. In contrast, the abundance of ACC synthase 1 mRNA increased greatly as a result of infection (Fig. 1). Thus, two different stress conditions, wounding of fruits and infection of leaves, enhance expression of the same ACC synthase mRNA. This result poses the question whether all biotic and abiotic stresses known to induce ACC synthase activate the same gene, and whether the transduction chains leading to different stress responses share common elements. (This work was performed in collaboration with P. Spanu and T. Boller during the sabbatical leave of H.K. at the Friedrich Miescher-Institut in Basel, Switzerland.)

ACC synthase has an apparent half-life of 30 to 60 min in wounded tomato pericarp tissue (Acaster and Kende, 1983; Kende and Boller, 1981). We examined whether this short half-life of the enzyme is reflected in an equally short half-life of ACC synthase mRNA. This was investigated by incubating wound-induced tomato pericarp tissue in actinomycin D, an inhibitor of RNA transcription. Both

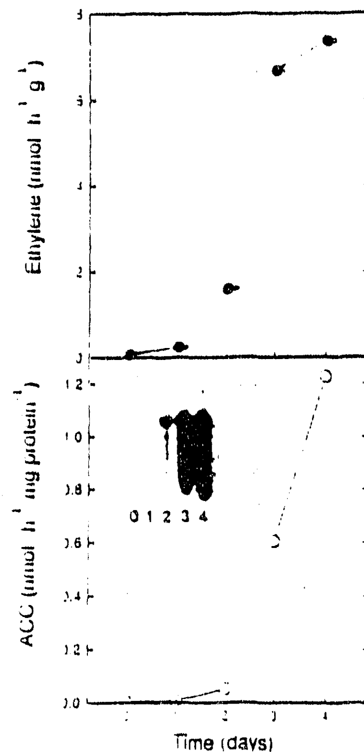

Figure 1. Ethylene biosynthesis, ACC synthase activity, and levels of ACC synthase 1 mRNA (inset) in tomato leaves infected with *Phytophthora infestans* on day 0. Poly(A)$^+$ RNA (3 μg per lane) was probed with the gene-specific probe Len17 of Olson et al. (1991). The numbers in inset refer to days after infection.

ACC synthase 1 and 2 mRNAs declined in abundance after a 2-h lag period with half-lives of approximately 4 h. There was no significant difference in the half-lives of these two mRNAs.

Localization of tomato ACC oxidase in transgenic yeast

The ethylene-forming enzyme, ACC oxidase, converts ACC to ethylene. Earlier work indicated that at least a fraction of ACC oxidase activity was associated with the central vacuole of plant cells (Guy and Kende, 1984) and that the activity of this enzyme required membrane integrity (Mayne and Kende, 1986). Sequence analysis of the recently cloned cDNAs encoding ACC oxidase did not show any vacuolar targeting signals or membrane association sites (Hamilton et al., 1991; Spanu et al., 1991). Furthermore, ACC oxidase appears to be a soluble enzyme in homogenates of melon fruits (Ververidis and John, 1991). We decided to study the localization of ACC oxidase further by expressing it in yeast and determining its subcellular localization. We chose yeast for two reasons. First, other groups have failed to express ACC oxidase activity in *E. coli*. This indicates that a eukaryotic system may be necessary for the expression of functional ACC oxidase. Second, it has been shown that plant proteins are correctly targeted to the yeast vacuole (Tague and Chrispeels, 1987). A PCR-generated fragment of tomato ACC-oxidase was used to isolate a full-length clone from a tomato cDNA library. This clone, pTE2, was subcloned into the yeast expression vector

pYES2.0, yielding pYTE2. Yeast strain F808 was then transformed with pYTE2 by electroporation to create transgenic yeast that expressed functional ACC-oxidase when grown on galactose. In transgenic yeast containing pYTE2, ACC-oxidase activity began to increase 2 h after induction with galactose and continued to increase for at least 5 h. Immunoblot (Western) analysis using rabbit antibodies raised against ACC oxidase showed that the appearance of ACC oxidase protein paralleled that of the enzyme activity.

We isolated yeast protoplasts and vacuoles according to Boller et al. (1989) and examined the distribution of ACC oxidase between the vacuole and the rest of the cell. Protoplasts retained 84% of the ethylene-forming activity found in intact cells, indicating that removal of the cell wall did not affect enzyme activity. Isolated vacuoles did not produce ethylene from ACC, nor did they contain any ACC-oxidase protein as judged by Western blotting. Carboxypeptidase and α-mannosidase assays were performed to confirm that the vacuolar fraction did contain both the contents of the vacuolar lumen and the tonoplast, and visual inspection showed that the vacuoles were intact. Since yeast vacuoles contain lytic enzymes, it is possible that ACC oxidase is rapidly degraded in the vacuole. Thus, we cannot conclude that ACC-oxidase from tomatoes is not taken up by the vacuole. We can conclude, however, that ACC-oxidase from tomatoes does not need to be localized in the vacuole in order to be active. Further cell fractionation showed that most of the ACC oxidase protein is associated with the particulate fraction. At this point, it is not known whether this reflects localization of ACC oxidase, e.g., in a membrane, or whether the enzyme binds non-specifically to particulate fractions during homogenization. (This work was performed in collaboration with Dieter Reinhardt and Thomas Boller, Botanical Institute, University of Basel, Basel, Switzerland. At MSU it was supported, in part, by USDA Grant No. 89-37261-4461.)

Regulation of Growth in Deepwater Rice

We are studying the role of ethylene and other plant hormones in the regulation of growth in deepwater rice, which has the capacity to elongate rapidly when submerged. This adaptive feature permits deepwater rice to survive prolonged flooding during the monsoon season. The environmental signal for growth is the lowered level of oxygen within the submerged tissue. Low oxygen tensions enhance the activity of ACC synthase, thereby promoting ethylene synthesis. Plants kept either under low oxygen tensions or in air containing ethylene elongate as much as do submerged plants. Ethylene does not stimulate growth directly but it increases the plant's sensitivity to gibberellin (GA) (for a review see Kende, 1987). The basis for this change in sensitivity may be an ethylene-mediated reduction in the level of abscisic acid, which is a potent inhibitor of GA action in rice (Hoffmann-Benning and Kende, in press). We are now investigating processes in the cell wall that are related to cell elongation and the effect of GA on the cell division cycle. (Supported, in part, by NSF Grant No. DCB-9103747.)

Characterization of osmiophilic particles in the internodes of rapidly growing deepwater rice

Electron-microscopic examination of the growing zone of internodes of deepwater rice showed that osmiophilic particles are secreted into the outer epidermal walls of submerged but not of air-grown plants (Kutschera and Kende, 1989). These particles are similar to those observed by Kutschera et al. (1987) in corn coleoptiles and may be related to growth of the outer epidermal wall. They may contain cell wall constituents, enzymes or components of the cuticle. Their occurrence in rice has been quantitated in the basal 5-mm region of the internode, which includes the intercalary meristem and part of the elongation zone. The ratio of particles in submerged or GA-treated versus control plants is on the order of 4:1. The size of osmiophilic particles in rice is about 80 nm. In corn coleoptiles treated with auxin, they can reach a diameter of up to 300 nm. They are also two to three times more abundant in corn than in rice. Therefore, we continued the characterization of these particles in corn coleoptiles.

Coleoptile tissue before secondary fixation or sections after embedding were incubated in proteinase K, cutinase or buffer. In tissue treated with proteinase K, the number of osmiophilic particles was reduced by a factor of 2-3 when compared to the control. Their number did not change after treatment with cutinase. We also attempted to characterize the osmiophilic particles by using an enzyme-gold labeling technique similar to the one described by Bendayan (1984a,b). Incubation with cutinase gold showed gold accumulation mostly in the subcuticular layer. The osmiophilic particles became densely labeled after incubation with proteinase K-gold (Fig. 2). Sections that were incubated with proteinase K-gold plus unlabeled proteinase K or BSA did not show any labeled particles. Antibody against the extensin-like protein (threonine-hydroxyproline-rich glycoprotein, TRGP) from corn (Kieliszewski and Lamport, 1987) coupled to gold bound to the cell wall but not to the osmiophilic particles. From the above experiments we can conclude that the contents of the osmiophilic particles are, at least in part, proteinaceous; they do not seem to contain TRGP, however. Since the osmiophilic particles are likely to be related to cell wall or cuticle formation, they may contain cell wall enzymes such as peroxidase or lipid transfer protein.

Analysis of the rice cuticle

In conjunction with our investigations on the nature of the osmiophilic particles, we examined the synthesis and composition of the cuticle in rice internodes. Rice stem sections were treated with GA or water (as control) for varying periods of time and labeled during the last 2 h with [^{14}C]palmitate. The incorporation of palmitate into the cuticle was up to 25-fold higher in stem sections that had been treated with GA. This increase in incorporation was already evident after 2 h and was most pronounced after 48 h. When the cuticle was depolymerized and its monomeric components separated by thin layer chromatography, several

Figure 2. Osmiophilic particle at the plasma membrane-cell wall interface of a corn coleoptile labeled with proteinase K-gold. The coleoptile had been treated with indole-3-acetic acid.

radioactive bands were detected. The relative distribution of radioactivity indicated qualitative changes in cuticle composition between rapidly and slowly growing internodes. The labeled cuticle components are being analyzed further by mass spectrometry.

Gibberellin-induced growth and regulation of the cell division cycle in deepwater rice

Excised stem sections of deepwater rice containing the highest internode were used to study induction of rapid internodal elongation by GA. It has been shown previously that this growth response is based on enhanced cell division in the intercalary meristem and on increased cell elongation (Bleecker et al., 1986; Métraux and Kende, 1984). Our investigations were aimed at establishing the temporal sequence of these GA-regulated processes and to determine the primary target of GA action: does GA promote cell elongation or cell division first or are these two processes induced concurrently? Cell sizes were determined by scanning electron microscopy, DNA synthesis by incorporation of [^{3}H]thymidine, and the phases of the cell cycle by flow cytometry. The lag time for the onset of GA-induced growth was 40 min. Treatment with GA promoted cell elongation in the intercalary meristem within 2 h (Fig. 3, top curve). Meristematic cells in the G2 phase were the primary target of GA action with respect to acceleration of the cell division cycle. These cells were induced to pass through mitosis, resulting in a decline in the population of G2 cells within 4 h of GA treatment (Fig. 3., bottom curve). Subsequent activation of DNA replication led to an overall acceleration of the cell division cycle. This acceleration was evident from an increase in the number of S phase cells and from enhanced incorporation of [^{3}H]thymidine into DNA between 4 and 7 h of GA treatment. An increase in the final cell length and an expansion of the internodal elongation zone contributed to the growth response after 16 h of GA application.

Thus, GA-induced elongation in deepwater rice internodes can be separated into three components. The initial growth response is based on cell elongation in the intercalary meristem. This increase in cell size is followed by an acceleration of the cell division cycle. Cells that emerge from the intercalary meristem during GA treatment reach a final length that is three-fold greater than that of cells in control internodes.

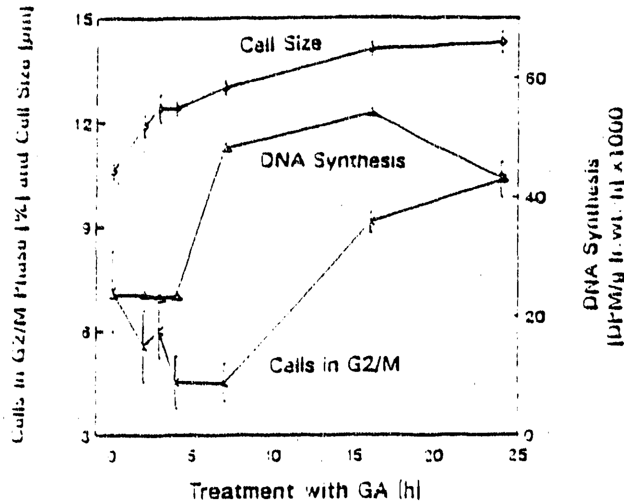

Figure 3. Size of cells, DNA synthesis and percent of cells in the G2/M phase in the intercalary meristem of deepwater rice internodes treated with GA. The first response to GA is an increase in cell size, followed by mitosis (reduction in G2/M phase cells) and DNA synthesis.

Cell elongation and orientation of the cellulose microfibrils in rapidly growing internodes of deepwater rice

The orientation of the innermost layer of cellulose microfibrils is a determining factor in cell growth. For a cell to elongate, its cellulose microfibrils have to be oriented predominantly in transverse direction (90°) to the axis of elongation (Green, 1980). The epidermis limits growth of many tissues including that of rice internodes (Kutschera and Kende, 1988). To determine whether orientation of cellulose microfibrils may limit growth of deepwater rice internodes, we examined the direction of cellulose microfibril deposition in rapidly growing and control internodes using the fluorescence-brightener calcofluor. The orientation of cellulose microfibrils remains transverse in parenchymal cells of GA-treated and control internodes, indicating that the walls of these cells do not limit growth. The cellulose microfibrils of epidermal cells in the intercalary meristem of control internodes are also transversely oriented. However, they reorient to an oblique direction (45°) just above the meristem (Fig. 4). This oblique orientation is maintained throughout the remainder of the internode. In epidermal cells of GA-treated internodes, a predominantly transverse orientation of the cellulose microfibrils is

maintained throughout the growing zone which is located above the intercalary meristem and extends over a distance of 35 mm above the node (Fig. 4). The epidermal cells in the oldest, non-growing part of GA-treated internodes have obliquely oriented cellulose microfibrils. We propose that cell elongation in control internodes is limited because of the mechanical restraint imposed by

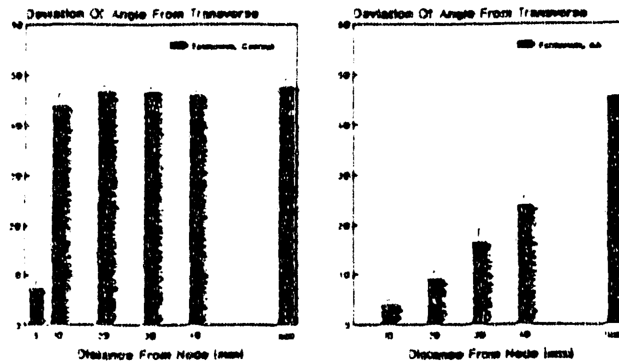

Figure 4. Orientation of cellulose microfibrils in the outer epidermal wall of control and GA-treated internodes of deepwater rice. The intercalary meristem is in the first 5-mm region above the node.

obliquely oriented cellulose microfibrils in the epidermal cell walls. GA promotes elongation of only those cells that are in the intercalary meristem when GA treatment starts and that maintain a transverse orientation of the cellulose microfibrils. This conclusion is supported by measurements of final cell lengths and the fact that internodes whose intercalary meristem has been excised do not elongate in response to GA. (These experiments were performed in collaboration with Robert Seagull, USDA-ARS Southern Regional Research Center, New Orleans, LA.)

Regulation of Nitrate Reductase by Cytokinins

Little is known about the mode of action of cytokinins in higher plants. We are studying the regulation of nitrate reductase (NR) by cytokinins and nitrate in embryos of *Agrostemma githago* at the physiological, biochemical and molecular levels with the aim of determining how cytokinins control the activity of this enzyme. While cytokinins enhance NR activity in several plants in the presence of nitrate, only in *Agrostemma* have cytokinins been shown to do so in the absence of nitrate. This and the fact that the effect of nitrate and cytokinins is additive both with respect to induction of NR reductase activity and the level of NR mRNA indicate that the two effectors act independently of each other. To localize the effect of nitrate and cytokinins, isolated embryos were incubated either in cytokinin, nitrate, or water as control. After the incubation period, the roots and the cotyledons, were assayed separately for NR activity. Cytokinin

enhanced NR activity primarily in the cotyledons, while nitrate induced the enzyme about equally in the roots and the cotyledons. Poly(A)$^+$ RNA was isolated from embryos induced with cytokinin and nitrate. The RNA was fractionated according to size by centifugation through a 15-30% sucrose gradient. Fractions containing NR mRNA were identified by RNA blot analysis. Poly(A)$^+$ RNA in these fractions was pooled and used to prepare a cDNA library. We have screened about 200,000 recombinant plaques using a partial tobacco NR cDNA as a heterologous probe (Calza et al., 1987). We have isolated several positive clones and are currently sequencing and analyzing them to verify whether they encode NR. Sequence comparison of *Agrostemma* NR cDNA and genomic DNA clones with the mRNAs transcribed in response to nitrate and cytokinin will indicate whether or not the substrate and the hormone increase expression of the same or different NR genes. NR cDNA probes will also be used to localize the expression of the NR gene(s) by tissue printing and *in situ* hybridization.

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PLANT CELL WALL PROTEINS

Derek T.A. Lamport, *Renate de Zacks, Cynthia Fong, Abdolreza Kamyab, Marcia Kieliszewski, Linda Schnabelrauch and Brad Upham*

The primary cell wall plays a profound role in the development and maintenance of plant tissues. The wall responds to cellular instructions to grow or differentiate, and informs the cell about the external status, such as the presence of pathogens, or pollen incompatibility. Because the wall self-assembles, questions concerning the function of its components include questions about biological recognition and self-organization (Lindsey, 1991): how do complex molecules spontaneously assemble into organized, 3-dimensional, living structures?

We maintain that the highly repetitive structural motifs which characterize the extensin family define functional domains (Barker et al., 1978; Baron, et al., 1991; Kieliszewski and Lamport, 1992), many of which are intimately involved in wall organization and self-assembly (Lindsey, 1991). Some general examples include: crosslinking domains, ligand- and carbohydrate-binding domains, recognition domains, and domains that nucleate self-assembly and direct growth (i.e., molecular auxiliaries) (Weissbach et al., 1991). In an effort to identify the most important of those domains (which must include examination of the extensin family's extensive posttranslational modifications, as well as polypeptide sequences), we have taken two routes: 1) comparative biochemistry to identify the most highly conserved polypeptide domains and posttranslational modifications (Fong et al., 1992; Kieliszewski et al., 1992a,b); and 2) *in vitro* assays of wall self-assembly, in particular, enzymic crosslinking of wall proteins.

Extensin peroxidase from tomato cell suspension cultures and chemical identification of extension crosslinks

Extensin networks have been defined biochemically using several major approaches; two of these have been the focus of research in this past year: 1) the characterization of an *in vitro* crosslinking system in terms of substrate specificity, pH optimum, cofactor requirements and other parameters leading towards the purification and identification of the enzyme involved; and 2) identification of crosslink domains and specific intermolecular crosslinks of extensin networks generated *in vitro* by the enzymic system, which specifically crosslinks monomeric extensin precursors. The isolation and characterization of an acidic peroxidase isozyme capable of crosslinking extensin monomeric precursors has obvious implications for cell wall biosynthesis and the creation of an accurate cell wall model.

This lab previously described crosslinking activity of a crude preparation obtained from salt-eluates of intact cells (Everdeen et al., 1988). Currently, we can isolate

a preparation of similar crosslinking activity directly from the culture medium. Isoelectric focusing showed that both sources contained multiple peroxidases. However, while basic isozymes predominated in the cell eluates, the growth medium contained a small but significant complement of acidic peroxidases (~3% in 8-day-old cultures). Initial assays of crosslinking activity after isoelectric focusing showed that for a given peroxidase activity [determined by oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)], the specific crosslinking activity of the acidic isozyme (pI 4.6) was about three orders of magnitude greater than of the basic (pI 9.0) isozyme (Everdeen et al., 1988).

We have characterized an acidic peroxidase from tomato suspension cultures by determining the optimal growth stage of cells for enzyme production; demonstrated crosslinking activity associated with the acidic rather than the major basic isozyme present in the medium and cell eluates; outlined a purification protocol; characterized the molecular size, spectrum, pH optimum, substrate specificity and kinetics of the acidic isozyme; and detailed inhibition by ascorbate and absence of extensin crosslinking by other peroxidases. When an extensin monomer, P1, isolated by elution from intact cells is used as substrate, the acidic extensin peroxidase becomes saturated between 9 and 12 μg/μl P1, with maximal reaction rates of about 4 μg/μl of P1 monomer polymerized/min using 0.25 μg/ml acidic peroxidase.

The salt-elutable extensin monomers are true precursors of the wall-bound network based on pulse-chase kinetics and peptide maps, i.e., the sequences of major peptides of monomers were identical to those previously isolated from the extensin network. Recent work has shown that the crosslinks in extensin oligomers eluted from intact cells (natural wall crosslinks) are stable after deglycosylation by anhydrous hydrogen fluoride, indicating a protein-protein crosslink. Extensin oligomerized *in vitro* by the acidic extensin peroxidase (synthetic wall crosslinks) is being tested for similar stability to HF.

The general strategy developed for identifying intermolecular linkages of extensin includes: 1) controlled enzymic degradation of crosslinked substrate,
2) chromatographic separation of the resulting fragments,
3) isolation and characterization of putative crosslinked peptides, and
4) identification of the precise crosslink.

One putative intermolecular crosslink domain is the hexapeptide Val-Lys-Pro-Tyr-His-Pro from the tomato extensin monomer P1. This identification was based on two considerations: first, the hexapeptide has a structure consistent with its suggested role, i.e., it contains reactive side chains in an open and repeated nonglycosylated domain; and second, the parent peptide (H20) is released from the parent monomer but not from the cell wall itself. The presence of these putative crosslink domains in a highly periodic structure supports the warp-weft hypothesis of an extensin network with defined porosity (Everdeen et al., 1988).

Identification of crosslink domains and of the precise chemical linkage is a major thrust of our research program.

We know that the intermolecular crosslink created by the acidic peroxidase is not an isodityrosine bridge, because a chromatograph of the hydrolysate of crosslinked material showed no absorbance in the area of chromatographed, authentic IDT. We currently suspect that there may be crosslinks of tyrosine-lysine, lysine-lysine, tyrosine-histidine, or variations thereof.

Current work involves the identification of tryptic peptides from deglycosylated monomer to simplify the search for crosslinked peptides after tryptic digestion of deglycosylated, crosslinked monomers. A comparison of monomer and oligomer peptides will help indicate "new" (putative crosslinked) peptides in the oligomers created *in vitro*. Amino acid analysis, peptide sequence analysis, GC-MS and FAB MS/MS will help to identify the crosslink involved. Sequencing of the crosslinked peptides will definitively identify the amino acids directly involved in extensin crosslinking.

A repetitive proline-rich protein from the gymnosperm Douglas fir

We purified two extensin monomers, the first hydroxyproline-rich glycoproteins (HRGPs) to be isolated from a gymnosperm (Kieliszewski et al., 1992a), from Douglas fir, *Pseudotsuga menziesii* (Mirbel) Franco. The smaller monomer (discussed below) was similar in composition to angiosperm extensins such as tomato P1. The larger monomer had a simple composition reminiscent of repetitive proline-rich proteins (RPRPs) from soybean cell walls (Marcus et al., 1991) and contained proline, hydroxyproline, and sugar; hence, it was designated a proline-hydroxyproline-rich glycoprotein (PHRGP). The simple composition of the PHRGP implied a periodic structure which was confirmed by the simple chymotryptic map and 45-residue partial sequence of the major chymotryptide PC5: Lys-Pro-Hyp-Val-Hyp-Val-Ile-Pro-Pro-Hyp-Val-Val-Lys-Pro-Hyp-Hyp-Val-Tyr-Lys-Pro-Hyp-Val-Hyp-Val-Ile-Pro-Pro-Hyp-Val-Val-Lys-Pro-Hyp-Hyp-Val-Tyr-Lys-Ile-Pro-Pro(or Hyp)-Hyp-Val-Ile-Lys-Pro. Chymotryptide 5 contained an 18-residue tandem repeat devoid of tetra(hydroxy)-proline or serine; it also contained two instances of the five-residue motif Hyp-Hyp-Val-Tyr-Lys and five of the general Pro-Pro-X-X-Lys motif, thereby establishing its homology with typical angiosperm RPRPs (Marcus et al., 1991) and extensins from tomato, petunia, carrot, tobacco, sugar beet, and *Phaseolus* (Kieliszewski et al., 1992a). Unlike the nonglycosylated soybean RPRP, the Douglas fir PHRGP was lightly glycosylated, as confirmed by a quantitative hydroxyproline glycoside profile, indicating that extensins can range from having hydroxyproline highly glycosylated to having little or no glycosylated hydroxyproline. Comparison of extensin sequence data strongly suggests that a major determinant of hydroxyproline glycosylation specificity is hydroxyproline contiguity: extensins with tetrahydroxyproline blocks

are very highly arabinosylated (>90% hydroxyproline glycosylated), whereas extensins with predominantly tri- and dihydroxyproline blocks are less arabinosylated, and extensins most of whose hydroxyproline residues are non-contiguous are very little or not at all arabinosylated. Despite high yields of extensins eluted from intact cells, the Douglas fir cell wall itself was both hydroxyproline poor and remarkably rich in protein (>20%), emphasizing the possible existence of other structural cell wall proteins that are neither HRGPs nor glycine-rich proteins.

A Gymnosperm extensin contains the serine-tetrahydroxyproline motif

The extensin family is a diverse group of hydroxyproline-rich glycoproteins (HRGPs) characterized by repetitive peptide motifs glycosylated to various degrees. The origin of this diversity and its relationship to function led us earlier to compare extensins of the two major groups of angiosperms; we concluded that the highly glycosylated Ser-Hyp_4 motif was characteristic of advanced herbaceous dicots, occurring rarely or not at all in a representative graminaceous monocot (*Zea mays*) or in a chenopod (*Beta vulgaris*) that is representative of primitive dicots (Kieliszewski et al., 1992b). As these results could arise either from loss or from acquisition of a characteristic feature, we chose to investigate a typical gymnosperm representing seed-bearing plants more primitive than the angiosperms. Salt eluates of cell suspension cultures of Douglas fir yielded two monomeric extensins differing in size and composition. The larger extensin reported earlier lacked the Ser-Hyp_4 motif, was rich in proline and hydroxyproline (hence designated PHRGP), and contained peptide motifs similar to the dicot repetitive proline-rich proteins (RPRPs) (Kieliszewski et al., 1992a). The smaller extensin monomer (Fong et al., 1992) was compositionally similar to typical dicot extensins like tomato P1, mainly consisting of Hyp, Thr, Ser, Pro, Val, Tyr, Lys, His, abundant arabinose and a small but significant content of galactose. A chymotryptic peptide map (on Hamilton PRP-1) of HF-deglycosylated fraction SP2 yielded eight peptides that were sequenced after further purification on a high-resolution, fast-sizing column (Polyhydroxyethyl Aspartamide; Poly LC). Significantly, two of the eight peptides contained the Ser-Hyp_4 motif, consistent both with the SP2 amino acid composition and with the presence of hydroxyproline tetraarabinoside (HA_4) as a small (4% of total Hyp) component of the hydroxyproline arabinoside profile. The presence of HA_4 and Ser-Hyp_4 corroborate our earlier observation (see above) that Hyp-contiguity and Hyp-glycosylation are positively correlated (Kieliszewski et al., 1992a). Interestingly, other peptide sequences indicate that SP2 contains motifs such as Ser-Hyp_4-Thr-Hyp-Tyr-Lys, Ser-Hyp_4-Lys and $(Ala\text{-}Hyp)_n$ repeats that are related to or typify dicot extensins P1, P3 and arabinogalactan-proteins, respectively. Overall, these peptide sequences confirm our previous prediction that Ser-Hyp_4 is an ancient motif, and strongly support our suggestion (Kieliszewski et al., 1992a,b) that the extensins comprise an extraordinarily diverse, but nevertheless phylogenetically related, family of cell wall HRGPs.

A histidine-rich extensin from *Zea mays* is an arabinogalactan protein

Unlike the walls of many dicots, graminaceous monocots such as *Zea mays* are relatively poor in extensins. Earlier, we isolated a threonine-rich extensin (THRGP) from *Zea mays* (Kieliszewski et al., 1990). Here we report that cell suspension cultures of *Zea mays* yield a new extensin rich in histidine (hence HHRGP) that also has characteristics of arabinogalactan proteins (AGPs). Chymotryptic peptide maps of HF-deglycosylated HHRGP showed repetitive motifs related to both extensins and AGPs as follows: HHRGP contains Ala-Hyp_3 and Ala-Hyp_4 repeats which may be related to the classical dicot Ser-Hyp_4 extensin motif by the single U --> G (Ser --> Ala) base change. Furthermore, HHRGP also contains the repetitive motif, Ala-Hyp-Hyp-Hyp-His-Phe-Pro-Ser-Hyp-Hyp, related to the Ser-Hyp_4-Ser-Hyp-Ser-Hyp_4 motif of P3-type dicot extensin. However, HHRGP also has AGP characteristics, notably: an elevated alanine content, near sequence identity with the known *Lolium* AGP peptide Ser-Hyp-Hyp-Ala-Pro-Ala-Pro, the presence of glucuronoarabinogalactan, and precipitation by Yariv antigen. Although HHRGP might be a chimera of two "different" proteins, i.e., of an extensin and an AGP, this is unlikely: one can account for the apparent chimera by the codon relationships of the five common HRGP amino acid residues, Ser, Pro, Thr, Ala (UCx, CCx, ACx, GCx) and His (CAU or CAC). That is, these relationships facilitate interconversion of major motifs by single point mutations. We therefore propose that the extensin family of wall proteins consists of a highly diversified phylogenetic series ranging from basic minimally glycosylated repetitive proline-rich proteins (RPRPs) to the highly glycosylated acidic AGPs (Kieliszewski et al., 1992b).

To relate this diversity of form and function at the molecular level, we identified structurally conserved regions and functional domains hypothetically involved in processes such as reptation, recognition, adhesion, intermolecular crosslinkage, and self-assembly (Barker et al., 1978; Baron et al., 1991; Kieliszewski and Lamport, 1992; Lindsey, 1991; Whitesides et al., 1991). Peptide palindromes which are not (as far as we know) previously noted in any other proteins, feature prominently, e.g., in HHRGP: Hyp-Hyp-Ala-Ala-Asn-Ala-Ala-Hyp-Hyp and Hyp-Hyp-Hyp-His-His-His-Hyp-Hyp-Hyp; in P3: Hyp_4-Ser-Hyp-Ser-Hyp_4; and others in other extensins. Such palindromes would enhance glycoprotein stereoregularity, thereby possibly promoting quasi-crystalline interactions between wall components (Kieliszewski et al., 1992a,b; Kieliszewski and Lamport, 1992; Weissbach et al., 1991).

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INTERACTION OF NUCLEAR AND ORGANELLE GENOMES

Lee McIntosh. *Shawn Anderson, Neil Bowlby, Carrie Hiser, Mary Kakefuda, Roxy Nickels, David Rhoads, Idah Sithole and Lawrence Smart*

The currency of growth and development is energy, and in plants this currency is maintained by a balance between chloroplast energy capture and mitochondrial oxidative reactions. The flow of energy through these organelles is maintained by expression of genes encoding membrane complexes embedded in organelle membrane systems responsible for charge separation and energy conversion. It is possible to unravel the molecular events surrounding energy balance - and thus growth and yield- in plants through isolation and manipulation of genes encoding critical protein components of photosynthesis and oxidative phosphorylation.

Photosynthetic organisms contain unique membrane complexes, performing energy transduction, that are ultimately responsible for the production of the oxygen in our atmosphere. Until recently, our understanding of photosynthesis at a molecular level had been limited both conceptually and technically. However, studies that combine molecular genetics and biochemistry in model cyanobacterial organisms now allow elucidation of the molecular bases for photosynthesis.

Plant mitochondria differ from those of most other eukaryotes in that their genomes contain relatively massive amounts of DNA (Levings and Brown, 1989) that is organized in complex and little-understood patterns (Palmer and Shields, 1984). However the most important differences relative to higher plants, specifically crop plants, are the functional differences (Amthor, 1984; Lambers, 1985). Possibly the most striking functional difference between plant and animal mitochondria is the presence of **two** terminal oxidases in higher plants. Plants contain a "normal" cytochrome c oxidase (Hawkesford et al., 1989) along with an "alternative" oxidase which was first identified by its resistance to cyanide (Genevois, 1929). The electrons flowing through this alternative pathway are not linked to the production of a transmembrane potential and thus are lost, or wasted, for the production of ATP. We wish to understand how respiration is controlled in higher plants and, in particular, how the distribution of electron flow between the two terminal oxidases is regulated.

Molecular Genetics of Photosynthetic Function

How is oxygen made?: Photosystem II

Approximately two billion years ago, oxygen-producing photosynthesis evolved, our oxygen-based atmosphere arose, and higher eukaryotes, plants and animals,

began their ascension. The molecular basis of oxygen evolution through photosystem II (PSII) is, however, still not well characterized at the molecular level. We have chosen to investigate this critical reaction through "reverse genetics"; the use of mutations to analyze the polypeptides thought to be involved.

Previously, as a first step, we demonstrated that both $D^{\cdot}$ and $Z^{\cdot}$, the first electron acceptors from the oxidation of water, are tyrosine radicals located on the D2 and D1 polypeptides of the PSII reaction center, respectively (Debus et al., 1988a,b). It is well known that 4 Mn ions are required for water oxidation (Babcock et al., 1989); however, the mechanism of water oxidation involving these Mn atoms is unknown. Therefore, we initiated two lines of investigations: first, a series of experiments which expands our investigation of $Y_D^{\cdot}$ and $Y_Z^{\cdot}$; second, experiments aimed at the identification of the amino acid residues in the PSII complex which act as Mn ligands.

A series of amino acid mutations in both D1 and D2 (the reaction center "core" of PSII) have been made in the areas surrounding and including the redox-active tyrosines, $Y_D^{\cdot}$ and $Y_Z^{\cdot}$. 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 amino acid ligands to Mn. 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 33% and 66% of wild-type rates. Further analysis of these mutants has begun and includes experiments measuring the effect of light intensity on oxygen evolution and kinetics of fluorescence induction. Both of these approaches have been undertaken to study the quantum efficiency of cells with altered photosystems.

More detailed analyses of PSII mutants require biologically active and stable preparations of PSII from *Synechocystis 6803*. We have been able to obtain preparations similar to those reported in the literature (Burnap et al., 1989; Metz et al., 1989); however, these preparations still lack some properties required for all of our projected analyses. At this time, our efforts to gain a purified PSII preparation are advancing but remain at a developmental stage.

Engineering a non-photosynthetic cyanobacterium: a new host for PSII mutagenesis

One of the main problems in the study of PSII in the model system *Synechocystis 6803* is that cyanobacteria contain many times more Photosystem I (PSI) than PSII in their membranes. Another problem is that the photosynthetic reaction centers are evenly distributed along the thylakoid membranes. There is no

enrichment for PSII in grana stacks as there is in higher plant chloroplasts, due to the lack of such structures in cyanobacteria. As described above, there are new and evolving procedures being developed for PSII preparations from cyanobacteria; however, these preparations are extremely time consuming and, at this time, are not adequate to fulfill all of our analytical needs. In order to overcome this problem we have taken a combined genetic and biochemical approach.

Recently, we have shown it was possible to inactivate PSI in cyanobacteria and to recover cells which lack the PSI core complex and all of its biophysical signals (Smart et al., 1991). It is interesting to note that this cell line still contains wild-type levels of PSII. Therefore, we have constructed a genetic host which lacks the PSI core and two of the three *psbA* gene copies, copies 1 and 3 (Smart and McIntosh, unpublished; Debus et al., 1988a,b). Copy two of *psbA* is only partially present and thus serves as a recipient for DNA that has been site-specifically modified to give specific amino acid changes in PSBA-1. To date, all of our site-directed mutants, mutations directed at understanding water oxidation, have been moved into the PSI$^-$ background. Experiments are under way to purify modified PSII complexes from these strains for biophysical and biochemical analyses.

Photosystem I: Reaction center formation and function

The reaction center of PSI (Fig. 1) contains the electron transfer components P700 (a specialized chlorophyll); a primary chlorophyll electron acceptor, AO; a quinone intermediate electron acceptor, A1; and three iron-sulfur clusters, FX, FB, and FA (for review see Golbeck, 1989; Golbeck and Bryant, 1991). The core of the reaction center is usually thought to be composed of two 64-kDa polypeptides and a 9-kDa polypeptide, encoded by the *psaA*, *psaB* and *psaC* genes, respectively. FA and FB are [4Fe-4S] clusters within the 9-kDa polypeptide (Dunn and Grey, 1988) and FX is a [4Fe-4S] cluster possibly residing *between* the two 64-kDa polypeptides (Golbeck, 1989). A major question arises concerning the mechanism of electron transfer between these three [4Fe-4S] centers.

In order to analyze the structure and function of PSI, some reaction center genes encoding PSI will be employed to probe function. These experiments are dependent on the existence of a biological system where mutants in PSI may be isolated. In brief, a photosynthetic organism which can be grown without a functional PSI is required so that mutants altered in PSI capacity may be segregated from wild type cells. Such a biological system must have the ability to be grown in the absence of PSI activity. Until recently, such a unicellular system, one whose genes are *easily* manipulable, has not been available. However, we have recently found that the strain of cyanobacteria most frequently employed for PSII mutational analyses, *Synechocystis 6803*, may be grown heterotrophically (Anderson and McIntosh, 1991b). It was shown that a single, five-minute pulse of light every 24 hours allows *Synechocystis 6803* to grow in

complete darkness (with 5 mM glucose). Further experiments have shown that this is a blue-light requirement; however, it is not known how this pulse of light facilitates heterotrophic growth.

Figure 1. The photosystem I core reaction center. Plastocyanin (PC) transfers electrons from the cytochrome b_6f to complex P700. PSA-A and PSA-B make up a heterodimer where all of the intermediate electron transfer events and charge separation take place. The L's represent the leucine residues proposed to form a leucine zipper. The boxes represent the three [4Fe-4S] centers F_X, F_B and F_A. PSA-C is a 9-kDa protein which contains the two iron-sulfur centers F_B and F_A. PSA-D is a peripheral polypeptide which has been proposed to be necessary for PSA-C presence in the photosystem I complex.

The ability to grow *Synechocystis 6803* heterotrophically has now allowed us to begin a molecular analysis of the reaction center of PSI in this organism. The reaction center genes *psaC*, *psaA* and *psaB* have been cloned from *Synechocystis* 6803. *psaC, psaA and psaB* have been sequenced and their expression studied in *Synechocystis 6803* (Anderson and McIntosh, 1991a; Smart and McIntosh, 1991).

In *Synechocystis 6803, psaA* and *psaB* are arranged in an operon as they are in higher plants (Kirsch et al., 1986) and in another cyanobacterium, *Synechococcus 7002* (Cantrell and Bryant, 1987). The *psaA* and *psaB* gene products are 88%

and 93% identical when compared with the products of the same genes from *Synechococcus 7002*, and are 81% and 80% identical to the corresponding gene products from spinach (Kirsch et al., 1986). The genes are transcribed as a 5.0-kb transcript which is rapidly turned over in *Synechocystis 6803* (Smart and McIntosh, 1991).

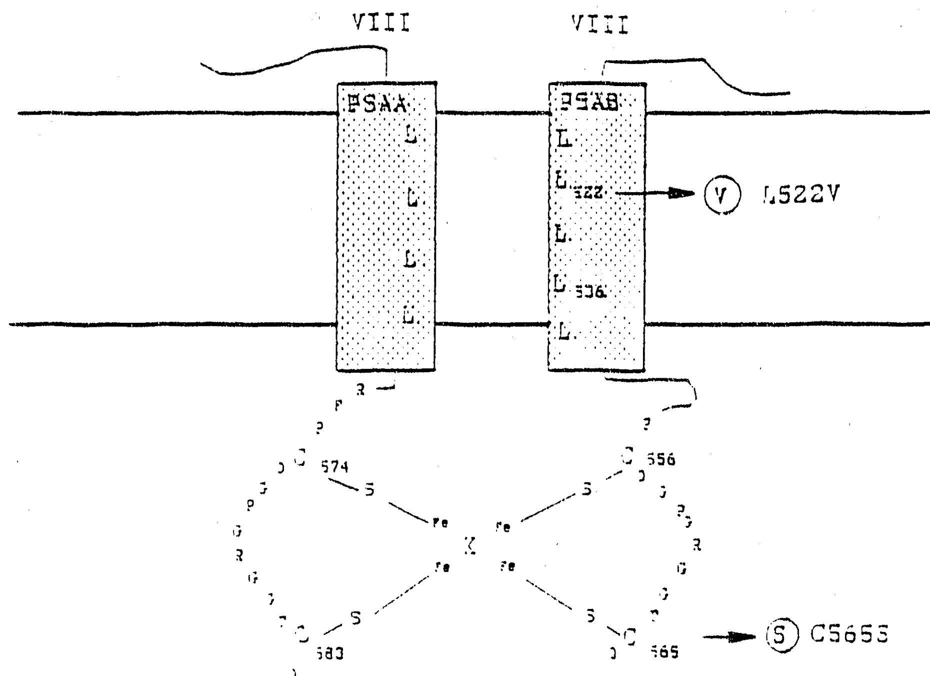

Figure 2. A schematic representation of part of photosystem I: F_X and the proposed leucine zipper reside between the eighth membrane helices of the core polypeptides PSA-A and PSA-B. The first two site-directed mutations in photosystem I, PSA-B/L522V and PSA-B/C565S, are encircled.

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 (Golbeck and Bryant, 1991). 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* (Golbeck and Bryant, 1991). 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 is present as a possible "leucine zipper" shared between these two membrane proteins (Webber and Malkin, 1990). The functional significance of these leucine residues is not known, although it is possible that they may play a role in of the biogenesis of the PSI reaction center.

As a first step in a molecular genetic analysis of PSI in *Synechocystis 6803*, we have genetically deleted the PSI core and recovered viable mutants grown under our blue-light-activated heterotrophic growth conditions. The *psaA* gene was interrupted with a bacterial gene encoding neomycin phosphotrameferase. A mutant, selected under heterotrophic growth conditions, revealed complete segregation of a mutated *psaA* gene (Smart et al., 1991). This strain apparently lacks the chlorophyll molecules associated with PSI and thereby appears blue in color. PSII activity is present in approximately wild-type levels and the growth rate of this mutant strain is indistinguishable from wild type grown under heterotrophic conditions.

In order to investigate the formation and structure of the [4Fe-4S] center F_X and the proposed leucine zipper present in the PSI core, we have begun site-directed mutagenesis of the *psaA* and *psaB* genes (Fig. 2). Recently, we have obtained preliminary results for two mutations: PSA-B cysteine #565 changed to serine and PSA-B leucine #522 changed to valine. The L522V mutant appears to grow normally, photosynthetically, and, as yet, we have found no major differences between it and wild-type cells. This is not unexpected since it has been shown that such single mutations within leucine zippers usually are not enough to disrupt these structures (Turner and Tjian, 1989). However, double mutations of leucines spaced 14 residues apart do have dramatic effects on protein structure and function where such leucine zippers were present (Turner and Tjian, 1989). We are in the process of making such mutants.

The C565S mutation was made to ascertain if this cysteine residue was indeed a ligand of F_X and also whether it would completely disrupt F_X or whether this iron-sulphur center would still form albeit in some altered form. Our initial results indicate that, while this cell line will not grow photoautotrophically, there is still some forward electron transfer to the F_B/F_A centers resident in PSA-C. (Results were obtained as part of a collaboration with Dr. John Golbeck and P. Warren, Biochemistry Department, University of Nebraska.)

Our initial mutagenesis experiments on the PSI core have confirmed—for the first time—at least one ligand (C565) of F_X. However, much further study is needed concerning this iron-sulfur center and also this particular mutant. How can such a center still function with only three ligands? Moreover, our first leucine mutant has shown that it is possible to make a mutation at residue 552 in the membrane helix of PSA-B and not disrupt photosynthetic function. It will be left to further experiments to show whether a membrane-buried leucine zipper is a structural requirement for formation and stability of a PSI reaction center.

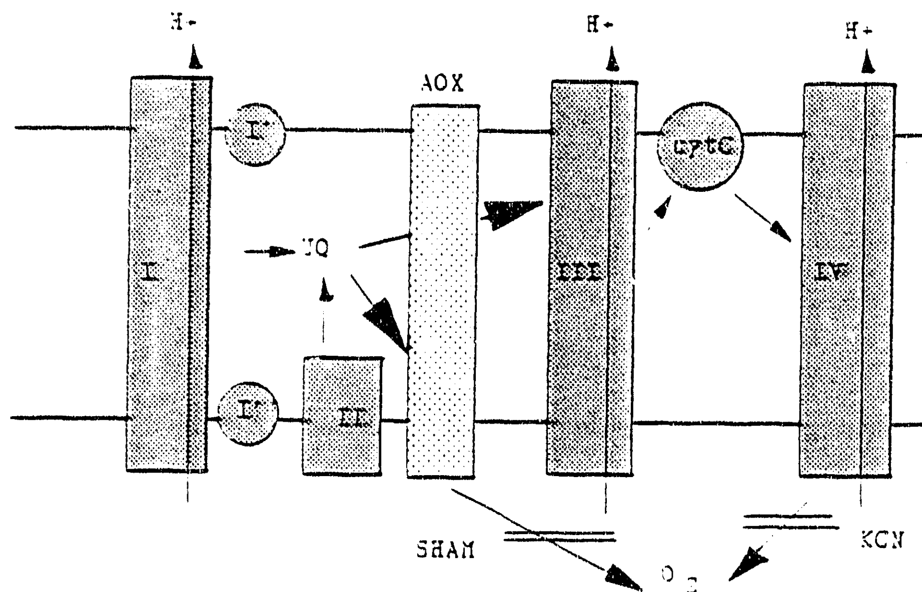

Figure 3. A schematic representation of the membrane complexes controlling plant mitochondrial electron flow. Complex I (NADH-Ubiquinone Oxidoreductase), Complex II (Succinate dehydrogenase), Complex III (UQ-Cytochrome c Oxidoreductase) and Complex IV (Cytochrome c Oxidase) and Cytochrome c are designated. I' and I" are "additional" NADH-Ubiquinone Oxidoreductase complexes proposed for plant mitochondria. AOX shows the position of the alternative pathway in the mitochondrial electron transport pathway. It accepts electrons from ubiquinone (UQ) and transfers them directly to O_2. The alternative oxidase is inhibited by salicylhydroxamic acid (SHAM) and the cytochrome pathway is specifically inhibited by cyanide.

Biochemical and Molecular Analyses of Plant Mitochondrial Respiration

Control of plant respiratory capacity: a molecular approach

Higher plants contain two mitochondrial respiratory pathways: a "normal" cytochrome pathway which is linked to the production of a transmembrane potential, and an "alternative" pathway which is *not* linked to a membrane potential and thus "wastes" energy (Fig. 3; for review, see Laties, 1982). These pathways branch at ubiquinone and this common pool of ubiquinone is kept reduced via substrate oxidation by a number of dehydrogenase complexes (Fig. 3). A functional role of the alternative pathway is agreed upon only for plants which display thermogenic, CN-resistant respiration in their inflorescences. Heat produced by electron flow through the alternative pathway in these inflorescences

is used to volatilize insect attractants, thus facilitating pollination (Meeuse, 1975).

A more general but hypothetical physiological role of the alternative pathway is that this pathway acts as an "overflow" and is active only when the cytochrome pathway is limited or saturated (Lambers, 1982). However, recent evidence (Dry et al., 1989) demonstrates that the overflow hypothesis cannot be correct in its entirety and thus the mechanism(s) regulating the alternative pathway remains obscure. At many points during development, ATP may be in excess, whereas carbon skeletons for metabolic processes may be limiting (Hunt and Fletcher, 1976; Moore and Rich, 1985). From this viewpoint it could be assumed that the alternative pathway would allow the TCA cycle to continue operating, thus ensuring the production of carbon skeletons for biosynthetic purposes. Although the alternative pathway has been investigated extensively at the physiological level, relatively little biochemical characterization has been reported. In order to evaluate the role of the alternative pathway for overall carbon balance in plants, the complete biochemical characterization and, ultimately, genetic manipulation of the alternative oxidase is required.

Genes for the higher plant alternative oxidase

We have recently employed antibodies to the *S. guttatum* alternative oxidase to clone the corresponding gene from a *S. guttatum* cDNA library (Rhoads and McIntosh, 1991). This gene, *aox1*, has been sequenced and the protein sequence deduced from the DNA sequence. Gene *aox1* is encoded by nuclear DNA and codes for a 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 α-helical regions (Figure 4). 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. It is interesting to note that previous work (Elthon et al., 1989a,b) demonstrated that salicylic acid induces the appearance of the alternative oxidase proteins.

The *S. guttatum* gene has been employed as a probe to obtain cDNA from potato and rice. A partial clone from rice has been obtained (Nickels and clonesMcIntosh, unpublished) and a full-length clone from potato has been isolated and sequenced (Hiser and McIntosh, unpublished). Comparison of the deduced amino acid sequence from potato 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.

Expression of *aox1* in *Sauromatum guttatum*

The capacity of the alternative pathway of respiration rises in *S. guttatum* during anthesis along with the production of heat (Meeuse, 1975; Elthon et al., 1989a,b). It is possible to excise tissue sections from pre-anthesis of inflorescences *S. guttatum* (the appendix) and these immature sections may be induced to "flower"

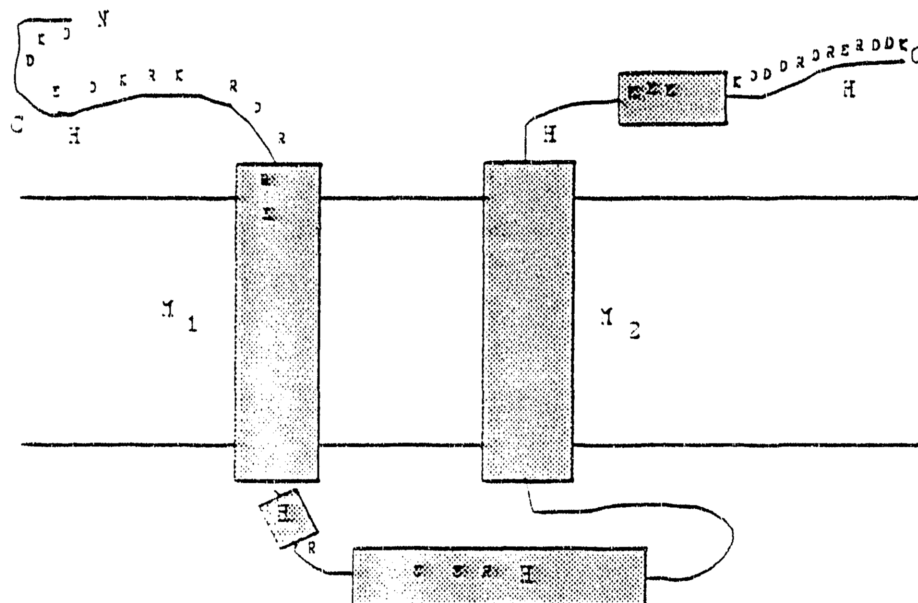

Figure 4. A representation of the structure of the alternative oxidase within the inner mitochondrial membrane. There are two predicted membrane helices, M_1 and M_2. The other shaded portions are predicted form α-helices but not within the membrane. Some conserved residues (Rhoads and McIntosh, 1991; Sakajo et al.,1991) are shown along the polypeptide chain.

(display the thermogenic response and increased alternative pathway) through the application of salicylic acid, the proposed *in vivo* trigger for flowering (Raskin et al., 1987). We have recently shown that induction by salicylic acid is dependent upon synthesis of both mRNA and protein (Rhoads and McIntosh, unpublished). This was demonstrated through the use of the RNA synthesis inhibitor Actinomycin D and the protein synthesis inhibitor cycloheximide. However, it is interesting to note that a constitutive alternative oxidase protein is present in immature inflorescences (Elthon et al., 1979) well before the increase in salicylic acid in the tissues (Raskin et al., 1987). Even though salicylic acid appears to trigger an increase in alternative oxidase capacity and to increase the pool of *aox1* messenger RNA, there appears to be an overlying developmental control for the expression of the alternative oxidase.

Regulation of respiration in tobacco: lowered temperatures and salicylic acid

We are investigating alternative-pathway respiration in suspension cell cultures of tobacco of *Nicotiana tabacum NT1*. Preliminary results indicate that the alternative capacity of these cells is approximately 10% of the total respiratory capacity and it may fluctuate during the growth period. Monoclonal antibodies to the *S. guttatum* alternative oxidase recognize the oxidase from this cell line, it usually appears as a single 36-kDa polypeptide, or possibly a doublet. This cell line is transformable (Mitra and An, 1989) and grows non-photosynthetically. In cell cultures of this type, the amount of "carbon in" and "carbon out" (carbon balance) may be measured (Schnapp et al., 1990).

It is difficult to employ the thermogenic inflorescences of *S. guttatum* to clearly define how salicylic acid is employed to regulate respiration in these tissues. We have now shown that the *NT1* cell line responds to salicylic acid similarly to tissue slices of the immature inflorescence of *S. guttatum*. When 1mM salicylic acid is added to the an actively growing suspension cell culture of *N. tabacum NT1*, the capacity of the alternative pathway increases, and this rise in capacity is coincident with *de novo* synthesis of the alternative oxidase protein (Rhoads and McIntosh, unpublished). This cell line may prove valuable in our understanding of how salicylic acid influences the regulation of plant respiration.

The environment affexts mitochondrial function and, though all plant growth and development; therefore, a specific stress such as cold may allow us insight into both the function of the alternative pathway and its regulation. In plants such as maize (Stewart et al., 1990), potato (Hemrika-Wagner et al., 1983), soybean (Leopold and Musgrave, 1979) and other species, lowered growth temperatures appear to increase the capacity for alternative oxidase. Taking a "model system" approach, we have asked whether cold would have a similar effect on the *N. tabacum NT1* line that we have used for our salicylic acid work. In this cell line, when the growth temperature is lowered from 30° C to 18° C, a five-fold increase in alternative oxidase capacity is obtained (Vanlerberghe and McIntosh, 1992). This increase in alternative oxidase capacity is dependent upon *de novo* synthesis of alternative oxidase and it disappears upon transfer of the cells back to a growth temperature of 30°.

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SENSORY TRANSDUCTION IN PLANTS

Kenneth Poff. *Thérèse Best, Bertha Bullen, Radomir Konjevic, Abdul-K. Janoudi, Vladimir Orbovic and Elizabeth Rosen*

Plants acquire information concerning their environment by processing a number of environmental stimuli such as light, gravity, water, touch, CO_2 and temperature. Until recently, the responses of plants to these stimuli have been known almost totally at the phenomenological level. Ultimately, the sensory physiologist wishes to understand the reception and processing of sensory information at both the behavioral/physiological and molecular levels. In this laboratory, we have been studying gravitropism and phototropism, tropistic responses to two different stimuli. We are moving the level of understanding of these responses more to the mechanistic level largely through the physiological/biophysical characterization of mutants with alterations in specific components of the transduction sequences.

For each sensory pathway, three questions need to be answered: What is the identity of the receptor molecule(s)? What is the mechanism for obtaining directional information? What are the steps in the transduction sequence? Answers to these three questions are not yet available for gravitropism. However, the descriptive physiology is now well known, and we have recently described a set of mutants with altered capacity to respond to gravity (Bullen et al., 1990).

Compared with gravitropism, phototropism is reasonably well understood. Although the photoreceptor pigment has not been isolated and identified, it is thought to be a flavoprotein (Poff and Butler, 1974; Pohl and Russo, 1984; Vierstra and Poff, 1981). It is now known that the plant obtains directional information by measuring light on its lighted and shaded sides, with the shading being provided by scatter and absorption primarily by carotenoids (Piening and Poff, 1988). One major impediment to the understanding of phototropism has been the complexity of the stimulus-response relationship. We have shown that this complexity is derived from a process of adaptation which proceeds along with the phototropic response itself and which alters the capacity of the organism to respond to the light stimulus (Janoudi and Poff, 1990,1991). Unfortunately, not one step in the sensory transduction pathway has been identified. We are approaching this problem by selecting and characterizing mutants with alterations in specific steps of the transduction sequence. We are characterizing the mutants physiologically, biophysically, and genetically to identify those which are altered in some critical step in phototropism. It is our hope that we will be able to use these mutants to clone the genes of interest and thereby determine the proteins associated with particular steps in the transduction sequence. One step toward this goal is reported below. In collaboration with Reymond and Briggs of the Carnegie Institution; Department of Plant Biology at Stanford University, we have now associated an apparent decrease in a photophosphorylatable plasma membrane protein with the mutation altering phototropic sensitivity in one

phototropism mutant. (Supported, in part, by grant NAGW-882 from NASA, and a NASA pre-doctoral fellowship to E. Rosen.)

Adaptation in phototropism of *Arabidopsis thaliana*: desensitization and recovery of phototropic responsiveness

We have previously shown that a blue light irradiation initiates the process of adaptation in phototropism of *Arabidopsis thaliana* (Janoudi and Poff, 1991). Immediately following an exposure to a high fluence of blue light, the etiolated seedling loses its capacity to respond phototropically. We refer to the process by which this capacity is lost as desensitization. Responsiveness is recovered with time in darkness. We have further characterized the processes of desensitization and recovery following several fluences of blue light. The results indicate that the degree of desensitization and the recovery time increase with increasing fluence of blue light. In addition, the fluence of blue light required for complete desensitization is considerably higher for red light pre-irradiated seedlings than for etiolated seedlings. These data are all consistent with the hypothesis that the descending arm of first positive phototropism is a consequence of desensitization competing with phototropic induction.

Adaptation in phototropism: red light pre-irradiation decreases the time threshold for second positive phototropism of *Arabidopsis* and *Nicotiana*

The time threshold for second positive phototropism of dark-grown seedlings of *Arabidopsis thaliana* is about 15 min. This time threshold is decreased to about 4 min by a 669-nm pre-irradiation. Similarly, the time threshold for second positive phototropism of dark-grown seedlings of *Nicotiana tabacum* is about 60 min and is reduced to about 15 min by a 669-nm pre-irradiation. Since the decrease in time threshold is induced by blue light also, it is our working hypothesis that this shift is regulated by phytochrome. The time threshold for second positive phototropism and the dependence of this threshold on the irradiation history of the seedling contribute to the complexity of the fluence response relationship for phototropism.

Distribution of growth during first positive phototropism of *Arabidopsis thaliana* seedlings

In plant shoots where it has been studied, curvature is a consequence of different rates of elongation of the two sides of that shoot. We have measured the elongation of two sides of etiolated *A. thaliana* seedlings during first positive phototropism. Elongation was monitored using an IR-imaging system for 3 hr following a unilateral irradiation with blue light. The results (Fig. 1) demonstrate that the growth rate of the shaded side of the seedling is greater than that of the lighted side for 80 min following the actinic blue light irradiation. During this 80 min period, phototropic curvature increases steadily (Fig. 2). After curving for

approximately 80 min, the seedling then straightens for about 70 min, during which the growth rate of the lighted side is greater than that of the shaded side. Finally, about 150 min following the blue light-irradiation, the growth rates of the lighted and shaded sides are equal and curvature ceases. Thus, it is clear that etiolated seedlings of *A. thaliana* curve phototropically as a result of differences in growth rates of the sides of the irradiated seedling.

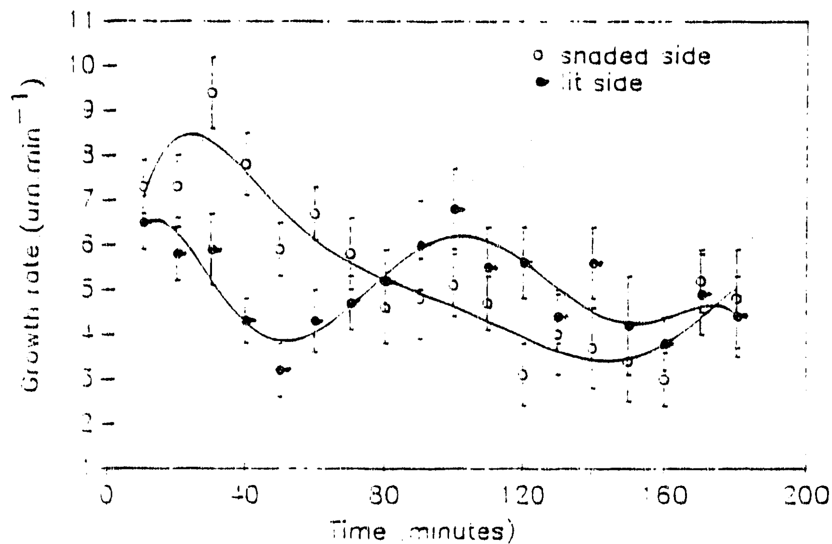

Figure 1. Growth rate of *Arabidopsis* seedlings curving in response to unilateral blue light.

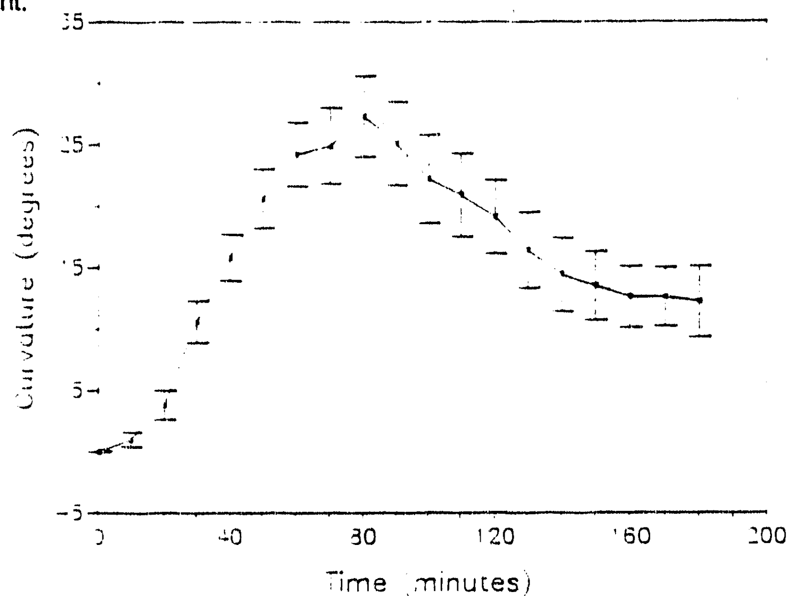

Figure 2. Phototropic curvature of *Arabidopsis* seedlings in response to unilateral blue light.

Phototropism and gravitropism mutants of *Arabidopsis* obtained using gamma-irradiation mutagenesis

Genomic subtraction (Straus and Ausubel, 1990) can be used as a cloning procedure for mutants with deletion mutations. To take advantage of this strategy, we have screened a collection of m2 seedlings from seed exposed to gamma irradiation which induces a high percentage of deletion mutations. A collection of 10,000 seed was exposed to gamma irradiation from a cobalt source. This produced an m1 population of about 6,000 viable seedlings, which were allowed to self-fertilize, producing an m2 generation. Approximately 46,000 m2 seedlings have been screened for their capacity to respond phototropically to blue light. From this screen, 410 individual seedlings were identified with aberrant phototropic curvature. The m3 families from these 410 m2 individuals have been re-screened, and five lines identified with clearly altered phototropism (for one example, see Fig. 3).

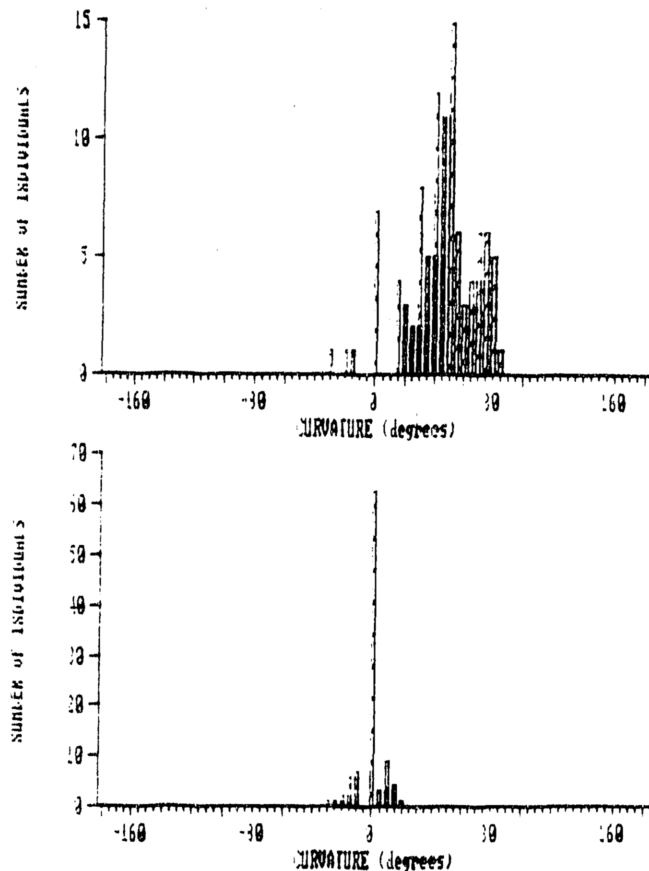

Figure 3. Frequency distribution for phototropic curvature by hypocotyls of a wild-type population (upper histogram) and of a population of seedlings with an altered phototropism phenotype (lower histogram).

Approximately 50,000 light-grown, m2 seedlings from the gamma-irradiation collection have been screened for gravitropism mutants. About 200 putative mutants have been identified, and are being subjected to a secondary screen. The mutants altered in phototropism and gravitropism as a result of gamma irradiation will be analyzed using the genomic substraction technique.

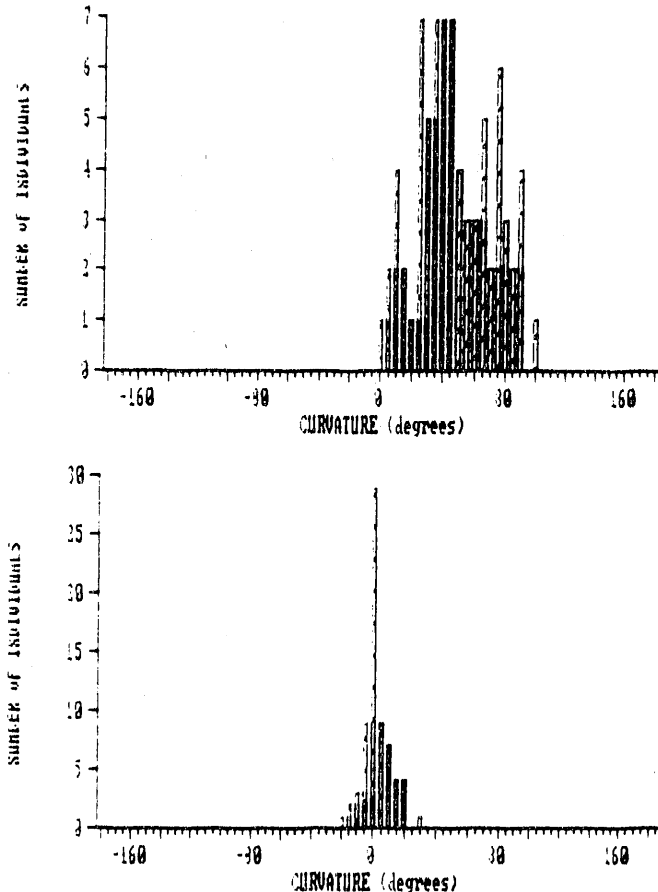

Figure 4. Frequence distribution for gravitropic curvature by roots of a wild-type population (upper histogram) and of a population of seedlings with an altered gravitropism phenotype (lower histogram).

Genetic analysis of gravitropism mutants in *Arabidopsis thaliana*

Three starch-deficient mutants originally identified for their random gravitropism phenotype have been further characterized genetically. Based on biochemical analysis and on complementation studies between these mutants and strain TC18, which is altered in phosphoglucomutase (pgm) (Caspar and Pickard, 1989), all three of our starch-deficient mutants appear to carry mutations in phosphoglucomutase in addition to a separate locus imparting the gravitropism

phenotype. Based on F2 segregation ratios, the altered locus imparting the gravitropism phenotype and pgm are within 20 map units of each other.

Light-induced phosphorylation of a membrane protein is a step in signal transduction for phototropism in *Arabidopsis thaliana*

Blue light is known to cause the rapid phosphorylation of a membrane protein in etiolated seedlings of several plant species. The blue light-induced phosphorylation has been proposed on the basis of correlative evidence to be an early step in the signal transduction chain for phototropism. In the *Arabidopsis* mutant, JK224, the sensitivity to blue light for the induction of first positive phototropism is about 20- to 30-fold lower than in the wild type. Second positive curvature in JK224 appears to be normal. Although light-induced phosphorylation can be demonstrated in crude membrane preparations from hypocotyls of the mutant, the level of phosphorylation is dramatically lower than in the wild-type parent. We conclude from these data that blue light phosphorylation is indeed a step in the signal transduction chain for phototropism. (Collaboration with Phillippe Reymond and Winslow Briggs at the Department of Plant Biology, Carnegie Institution of Washington, Stanford, CA.)

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MOLECULAR MECHANISMS OF TRAFFICKING IN THE PLANT CELL

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Maintenance of separate subcellular compartments in eukaryotic cells depends on the correct sorting and targeting of newly synthesized proteins. Thus, mechanisms must exist in the cell to assure that these proteins are targeted to, and subsequently translocated across, the correct intracellular membranes (Fig.1). We are working with proteins destined for different compartments: vacuoles, cell wall and nucleus. There is increasing evidence that proteins destined for subcellular organelles contain a "sorting signal" which dictates the compartment to which the protein is targeted. We are interested in understanding the molecular determinants of differential protein compartmentalization and in identifying the components of the molecular machinery which carry out the sorting process. Our goals include the analysis and characterization of protein sequences responsible for sorting to the vacuole and to the nucleus, and the identification and isolation of putative receptors which recognize these sorting sequences and subsequently mediate protein transport to the vacuoles and nucleus. The nature of the sorting signal and receptor structure will provide essential background for our understanding of targeting mechanisms in cells and allow us to redirect proteins of interest either to the vacuoles or to the nucleus.

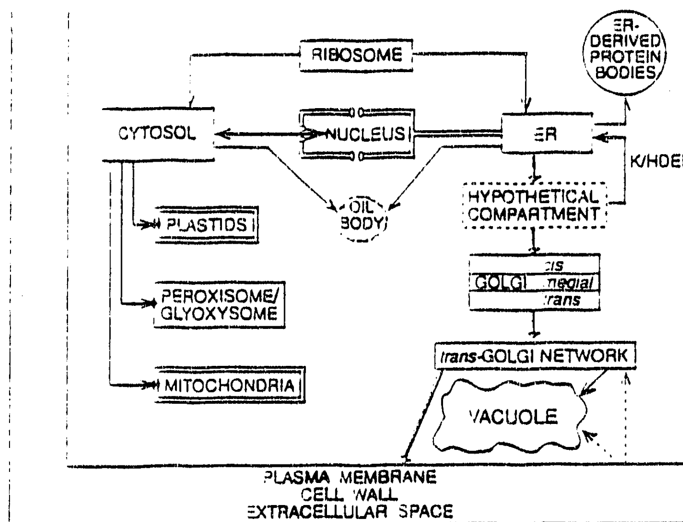

Fig.1. Schematic representation of intracellular sorting in plant cells.

Plants possess a large arsenal of both constitutive and inducible biochemical and mechanical defense mechanisms against various potential fungal pathogens and insect pests. Several arguments support the hypothesis that chitin-binding proteins are involved in host-defense: 1) these lectins exhibit high binding affinity for poly-N-acetylglucosamine (chitin), which is a major component of the fungal cell wall and the peritrophic membrane of the insect midgut; 2) in *in vitro* experiments, chitin-binding proteins show insecticidal and antifungal activities; 3) chitin-binding proteins are predominantly located in the protective peripheral tissues of plant organs and almost all of them are wound-inducible (Chrispeels and Raikhel, 1991). We hope that by studying the molecular mechanisms of regulation and expression of genes for chitin-binding proteins we shall come to understand the endogenous function of the proteins that they encode.

The barley lectin carboxyl-terminal propeptide is a vacuolar protein-sorting determinant in plants

We are interested in the molecular mechanisms regulating vacuolar sorting of the Gramineae lectins. Within the ER, the Gramineae lectin polypeptide is modified by the addition of a high mannose glycan to the carboxyl-terminal propeptide (CTPP) and is assembled to form an active N-acetylglucosamine-binding proprotein (see Raikhel and Lerner, 1991 for review). During transport to or after deposition in the vacuole, the glycosylated CTPP is removed from the proprotein to yield the mature lectin. Previously, we have demonstrated the correct processing and accumulation of barley lectin (BL) in leaves and roots of transgenic tobacco plants (Wilkins et al., 1990). It was also shown that the glycan of the barley lectin proprotein (proBL) is not essential for correct processing and targeting of this protein to the vacuoles (Wilkins et al., 1990). Recently work from our laboratory has established that the 15 amino acid carboxyl-terminal propeptide of probarley lectin is necessary for the proper sorting of this protein to the plant vacuole. A mutant form of the protein lacking the carboxyl-terminal propeptide is secreted (Bednarek et al., 1990). To test whether the carboxyl-terminal propeptide is the vacuole-sorting determinant of probarley lectin, we examined the processing and sorting of a series of fusion proteins containing the secreted protein, cucumber chitinase and regions of probarley lectin, in transgenic tobacco. Pulse-labeling experiments demonstrated that the fusion proteins were properly translocated through the tobacco secretory system and that cucumber chitinase and cucumber chitinase fusion proteins lacking the carboxyl-terminal propeptide were secreted. The cucumber chitinase fusion protein containing the carboxyl-terminal propeptide was properly processed and sorted to the vacuole in transgenic tobacco, as confirmed by organelle fractionation and electron microscopy immunocytochemistry. Therefore, the barley lectin carboxyl-terminal propeptide is both necessary and sufficient for protein sorting to the plant vacuole. (Supported, in part, by NSF grant DCB-9002652.)

Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*) and co- and post-translational processing of the hevein preproprotein

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 (Broekaert et al., 1990). 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. To investigate the mechanisms involved in processing of the protein encoded by HEV1, three domain-specific antisera were raised against fusion proteins harboring the amino-terminal domain, carboxyl-terminal domain, and both domains. Translocation experiments using an *in vitro* translation system showed that the first 17-amino-acid sequence functions as a signal peptide. In immunoblotting analysis of *in vivo* proteins from the lutoid body-enriched fraction, a 5-kD protein comigrates with purified mature hevein and crossreacts with antibodies to the amino-terminus and to the two domains (Fig.2). A 14-kD protein is recognized by antibodies to the carboxy-terminus and to the two domains. A 20-kD protein is cross-reactive with all three antibodies. It appears that the 5-kD (amino-terminal domain) and 14-kD (carboxyl-terminal domain) proteins are posttranslational cleavage products of the 20-kD protein (both domains) which corresponds to the proprotein encoded by HEV1. In addition, it is found that the amino-terminal domain can provide chitin-binding properties to the protein bearing it either amino-terminally or carboxyl-terminally. 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.

Monocot regulatory protein *Opaque 2* is localized in the nucleus of maize endosperm and of transformed tobacco plants

Protein targeting to the nucleus has been studied extensively in animal and yeast systems; however, nothing is known about nuclear targeting in plants. The *opaque 2* (O2) gene produces a regulatory protein that is responsible for inducing transcription of the α-zein class of storage proteins in maize kernels. The cloned O2 gene encodes a protein that contains a leucine zipper DNA binding domain that can interact with zein gene promoters. We have used immunolocalization to show that the O2 protein is present in nuclei in the maize endosperm tissues known to produce α-zeins. In addition, neither embryonic tissue from wild-type

Fig.2. Immunoblot analysis of proteins in a lutoid body-enriched fraction using domain-specific antibodies. Approximately 10 μg of total proteins from a lutoid body-enriched fraction were loaded on a 17.5% SDS gel. After electrophoresis, proteins were either stained with Coomassie Blue (lane 1) or immunoblotted with antibodies specific for the N (lane 3), C (lane 4) or N-C (lane 5) domain. As a control, HPLC-purified hevein was immunoblotted with N domain-specific antibody (lane 2).

kernels nor endosperm from kernels harboring a null *o2* allele contain the O2 protein. Analysis of a transposable element-induced *o2* allele, *o2-m20*, revealed that sectors of endosperm cells contained the nuclear-localized O2 protein, indicating excision of the transposable element. To further study the transport of the O2 protein into the nucleus, we have transformed this gene, under the control of a constitutive promoter, into tobacco. Plants were shown to have detectable steady state levels of *O2* mRNA (data not presented) and O2 protein (Fig.3). Immunolocalization of O2 protein in transformed tobacco plants indicated that the O2 protein was transported into tobacco nuclei. Therefore, we have developed a system to study nuclear targeting in plants and have established that the nuclear transport machinery is similar in monocots and dicots (Varagona et al., 1991).

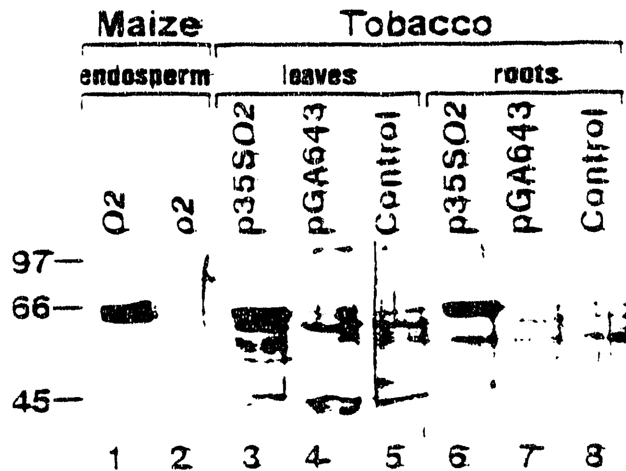

Fig.3. Analysis of steady-state levels of O2 protein in maize and tobacco plants.

The protein gel blot contains 5 μg of partially purified proteins from maize endosperm tissue harboring the O2 allele (lane 1), maize endosperm tissue harboring the null o2 allele (lane 2), crude extracts from 15 μg of tobacco leaves and roots from plants transformed with the p35SO2 construct (obtained by insertion of the O2 cDNA into the HindIII site of the binary plant transformation vector of pGA643; see An et al., 1988) (lanes 3 and 6), plants transformed with the Agrobacterium vector pGA643 (An et al., 1988) (lanes 4 and 7), and untransformed W38 control plants (lanes 5 and 8). Immunodetection was performed using polyclonal anti-O2 sera and IgG-conjugated alkaline phosphatase. Molecular mass standards are indicated on the left in kilodaltons. O2 protein migrates at 65 kD and is present in O2 maize endosperm and in p35SO2-transformed tobacco tissues.

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PHYSIOLOGICAL GENETICS OF *ARABIDOPSIS*

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The major research projects in the laboratory concern molecular genetic investigations of the regulation of membrane and storage lipid biosynthesis, the structure and function of the cell wall, and the production of novel aliphatic polymers by genetic engineering of higher plants. These and various other projects are interrelated by the application of related genetic and biochemical methodologies, similar underlying concepts, and the use of *Arabidopsis thaliana* (L.) as the primary experimental organism. *Arabidopsis* is ideally suited as a model plant species for genetics and molecular biology because of its short life cycle, prolific seed production, extensive genetics, and the fact that it has the smallest genome known for any higher plant. This last feature greatly facilitates the analysis of nuclear genes and can be exploited to permit chromosome walking using yeast artificial chromosomes (Grill and Somerville, 1991), something which is currently not generally feasible in other plant species because of the abundance of repetitive DNA and the large genome size. In a preliminary analysis of the utility of the available YAC libraries, YACs covering approximately one third of the total genome were identified and placed on the genetic map (see Figure 1; Hwang et al. 1991. Thus, it seems likely that it will be possible to develop a complete physical map of the genome – an accomplishment that will greatly facilitate the isolation of genes by map-based cloning methods. The utility of this approach will be proportional to the degree to which mutations affecting all aspects of plant biology have been identified.

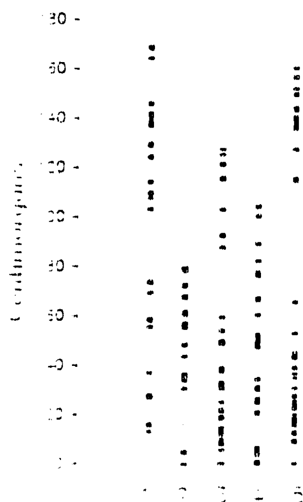

Figure 1. Schematic representation of the approximate regions of the genetic map of *Arabidopsis* which have been mapped onto yeast artificial chromosomes. The map was constructed from data in Hwang et al. (1991).

Regulation of lipid desaturation

The membrane lipid composition of plants is believed to be an important determinant of tolerance to chilling and, in oilseeds, lipid composition determines the quality of the oil. In both cases, one of the most important parameters associated with lipid composition is the degree of fatty acid unsaturation, which is generally different for each membrane lipid (Figure 2). It is not known, in any organism, how the degree of unsaturation is regulated or what is the functional significance of the varying levels of unsaturation.

Lipid Composition of Arabidopsis Leaves

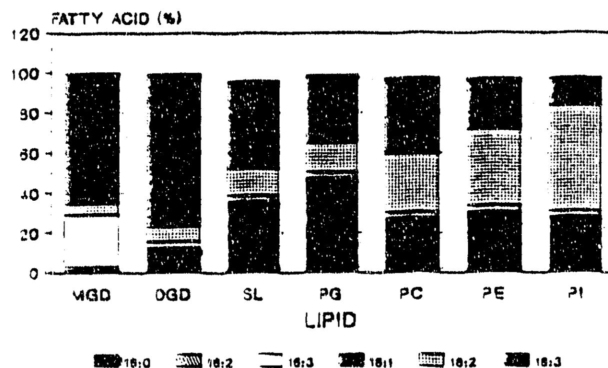

Figure 2. The fatty acid composition of the major membrane lipids of *Arabidopsis*. Minor deviations from 100%, in some cases, are due to experimental error in measurements.

In order to determine the functional significance of lipid desaturation, and to develop methods for genetic manipulation of desaturase genes in oilseeds, we are characterizing a series of mutants of *Arabidopsis* which are deficient in activity of various enzymes involved in lipid metabolism, particularly fatty acyl desaturases. Mutants are available in *Arabidopsis* for all of the known desaturases except for the 18:0-ACP desaturase (Somerville and Browse, 1991). The mutants were isolated by screening more than six thousand individuals from an unselected population of mutagenized plants for individuals with alterations in leaf fatty acid composition by gas chromatography (Somerville and Browse, 1991). These mutants have proven very useful for determining the substrate specificities of the various desaturases and have provided new insights into the role of lipid composition in regulating chloroplast biogenesis and function (Browse and Somerville, 1991). For instance, analysis of the effects of these mutations in heterozygotes provided evidence that one copy of an active gene is generally adequate to obtain wild type fatty acid composition. Since the level of unsaturation is typically less than 100%, it is inferwed that, for many of the desaturases, activity is not present in excess. Thus, the observation that one active copy of the gene results in the same level of unsaturation as two copies implies that at least some of the mechanisms that regulate fatty acid composition are expressed at a posttranscriptional level. The mutants had very minor effects on the 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 (Hugly et al., 1989; Kunst et al., 1989b). In the case of the *fadB* and *fadC* mutations, which control the activity of a chloroplast $\omega 3$ and an $\omega 6$ desaturase, respectively, enhanced thermal stability of membranes *in vitro* was correlated with a significantly higher growth rate of the mutants than of 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. Analysis of the effects of *fadB* and *fadC* mutations on the growth and development of plants at low temperature revealed that tissue of the mutants which develops at low temperature is chlorotic (Hugly and Somerville, 1992). The chlorosis is associated with impaired chloroplast development.

Because of the small genome size the very reduced amount of repetitive DNA in *Arabidopsis*, it is possible to isolate genes from *Arabidopsis* by chromosome walking. Because the current RFLP maps have a relatively low density of mapped RFLPs, it is necessary to walk hundreds of kilobases from a mapped RFLP in order to clone most genes. In order to facilitate walking, we constructed a library of the *Arabidopsis* genome as minichromosomes (YACs) in yeast (Grill and Somerville, 1991). Several loci of interest (i.e., *fad2, fad3, fadA, fadB, fadC, fadD, fae1, act1*) were genetically mapped relative to visible markers (Hugly et al., 1991) or to flanking RFLPs (S. Gibson, K. Iba and V. Arondel, unpublished). In the case of the *fad3* gene, the RFLPs were then used to isolate a family of overlapping YACs covering the region of the chromosome where the gene is located. One of these YACs was then used to probe a cDNA library from developing seeds and a cDNA clone corresponding to the *fad3* locus was identified (V. Arondel, unpublished). This work represents the first instance of the isolation of a plant gene by map-based cloning. The *fad3* gene was used as a hybridization probe to isolate a family of related desaturase genes. In order to determine the specific functions of these various cloned genes, we have used the genes to identify RFLPs. These RFLPs are currently being placed on the genetic map in order to correlate the genetic map location of the cloned genes with that of the mutations which alter fatty acid composition. The availability of the cloned genes will permit a detailed analysis of the mechanisms which regulate membrane lipid fatty acid composition in response to developmental and environmental stimuli. (Supported, in part, by a grant from the National Science Foundation; Sue Gibson was supported by an NIH fellowship; Vincent Arondel was supported by an EMBO fellowship.)

The role of lipid transfer proteins

One class of mutants (*act1*) lacks activity for the chloroplast enzyme glycerol-3-phosphate acyltransferase (Kunst et al. 1988). Therefore, these mutants are largely unable to synthesize lipids by the plastid pathway which normally provides approximately 50% of total leaf lipids. As with the other mutants with alterations in leaf lipid composition, the *act1* mutation had relatively minor effects on the growth or appearance of the plants under any conditions (Kunst et al., 1989a). Surprisingly, this mutation also 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 raises the question as to how the microsomal pathway allocates lipids to a particular membrane.

In order to understand the mechanisms which regulate lipid synthesis and membrane biogenesis, it is necessary to know how lipids are transported between the two membranes where most lipid synthesis takes place. One 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). In order to explore the role of these proteins in lipid transfer we cloned the genes for a LTP from spinach leaf (Bernhard et al., 1991) and *Arabidopsis*. The sequence of cDNA clones revealed the unexpected presence of a 27-residue signal peptide. 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 (Bernhard et al., 1991). Since there are no known examples of soluble cytoplasmic proteins whose 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 in the cell wall by immunoelectron microscopy (S. Thoma, unpublished). This location effectively excludes a role for the protein in membrane lipid transport and raises new questions about the role of the protein. In addition, the promoter region of the LTP gene was fused to the β-glucuronidase (GUS) gene and the tissue specificity of gene expression was examined histochemically. These studies revealed that the LTP gene is primarily expressed in epidermal tissues and is not expressed in mesophyll or most root cells. This pattern of expression raises the possibility that the LTP is involved in cuticle formation.

In order to examine the role of the protein, we have produced a number of transgenic plants which express antisense copies of the gene. The amount of the protein is strongly reduced in some of these plants. However, the effects on growth and development of the plants is very slight and we have not detected any change in the chemical composition of wax, cutin or lipids. Thus, although we have effectively excluded a possible role for LTP in intracellular membrane lipid transfer, we have not, as yet, been able to establish a role for the 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.)

Characterization of enzymes and genes involved in lipid biosynthesis

As a complementary approach to the genetic work, we have undertaken the characterization of several proteins which are implicated in the regulation of fatty acid modification. In particular, we have purified the stearoyl-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 (Shanklin and Somerville, 1991; Shanklin et al., 1991). 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* greatly facilitates the production of large quantities of enzyme for biochemical studies. We have used this system to obtain preliminary evidence that the enzyme is an iron-sulfur pro-

tein and have initiated a collaboration with Carl Branden and colleagues (Uppsala) to determine the tertiary structure of the protein by X-ray crystallography (Schneider et al., 1992).

The stearoyl-CoA desaturases in vertebrates utilize cytochrome b_5 as the electron donor. 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 Δ12 desaturation of fatty acids in safflower microsomes (Kearns et al., 1991), 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. In order to facilitate the reconstitution of a desaturation reaction in yeast or *E. coli*, we recently completed the cloning of the first cytochrome b_5 gene from a plant (Kearns et al., 1992).

Ricinoleic acid (12-hydroxyoleic acid) is the major fatty acid found in the oil from castor (*Ricinus communis* L.). The presence of the hydroxyl group confers useful properties on the oil which has several hundred non-food industrial uses. Antibodies against cytochrome b_5 completely inhibit the activity of the hydroxylase in microsomal membranes (F. van de Loo, unpublished). In conjunction with evidence that the hydroxylase activity is inhibited by iron chelators, requires molecular oxygen and acts on fatty acids esterified to phosphatidylcholine, this observation raises the possibility that the hydroxylase may be functionally related to a desaturase. Because the enzyme activity is labile during purification, we are currently exploring genetic methods for identifying a cDNA for the protein which do not depend on prior purification of the protein. (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 Hi-Bred 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*. In these bacteria, PHB accumulates as granules which may comprise more than 80% of the cell mass. PHB has attracted interest as a potential biodegradable thermoplastic. Therefore, 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 sequential conversion of acetyl-CoA to acetoacetyl-CoA, hydroxybutyryl-CoA and PHB, respectively. The genes encoding the three enzymes have been cloned from *A. eutrophus* (Slater et al. 1988). The genes have been placed distal to the constitutive 35S promoter from cauliflower mosaic virus and introduced into transgenic *Arabidopsis* plants. Expression of the *A. eutrophus* genes in *Arabidopsis* resulted in the accumulation of granules of PHB in the transgenic plants (Poirier et al., 1992). The granules were similar in size to those observed in PHB-producing bacteria but, unexpectedly, were found in the nucleus, vacuole,

and cytoplasm of the transgenic plant cells. Since the enzymes of the PHB biosynthetic pathway were targeted to the cytoplasm, it is possible that PHB granules are able to pass through certain membranes.

Figure 3. Transmission electron micrographs of thin sections of a leaf mesophyll cell from a PHB-producing transgenic *Arabidopsis* plant. A. Arrows point to agglomerations of PHB granules in the nucleus; B. PHB granules in nucleus (Nu) and vacuole (Va); C. PHB granules in cytoplasm. Bar = 1 μM in A and B. Bar = 0.25 μM in C.

These preliminary experiments indicate that PHB can be produced in plant cells, and that the production of granules is an intrinsic property of the PHB synthase. We are now exploring the possibility that the pathway can be targeted to the plastid. The long-term goal will be to divert the carbon which is normally stored as starch or sucrose in species such as potato and sugar beet into PHB. (Yves Poirier was supported, in part, by a fellowship from NSERC; Christianne Nawrath was supported by a fellowship from Bayer AG; the work was supported, in part, by grant #DMB-9014037 from the NSF SGER program.)

The pathway of sulfolipid biosynthesis

One of the characteristic constituents of chloroplast membranes is sulfolipid. Because of its abundance in the chloroplast lamellae, sulfolipid is one of the several most abundant sulfur-containing compounds in the biosphere. Although the structure of this lipid was determined more than 20 years ago, its 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 retain the capability to grow photoautotrophically but do not contain sulfolipid were identified by screening a mutagenized population by thin layer chromatography of lipid extracts from several thousand independent colonies (Benning and Somerville, 1992). The genes which complement three of the mutants were isolated by complementing the mutants with a cosmid library. Two of these genes were found to flank an open reading frame which is in the same transcriptional unit and, therefore, is probably a fourth gene in the sulfolipid pathway (C. Benning, unpublished). Comparisons of the sequences of these four open reading frames with databanks indicate that one of the genes may be related to a hexose epimerase and another gene may be related to a glucosyl-transferase.

By gene disruption techniques, one of the cloned genes has been used to create a null mutation which is completely deficient in sulfolipid. Preliminary analysis of this mutant in collaboration with T. Beatty (University of British Columbia) and R. Prince (Princeton) suggests that sulfolipid is dispensable for normal growth under laboratory conditions. The availability of the cloned genes and of the null mutant should facilitate future studies of the biosynthesis and function of this ubiquitous constituent of plant cells. (Supported, in part, by a grant from the USDA/NSF/DOE Plant Science Center Program).

Chilling-sensitive mutants of *Arabidopsis*

The effective geographical distribution of many species of plants is limited by the sensitivity of these plants to "chilling temperatures" which do not adversely affect other species. The physiological characteristics that are responsible for chilling-sensitivity or resistance are not known. Attempts to identify relevant biochemical differences between chilling-sensitive and chilling-resistant species are confounded by the many irrelevant differences (Graham and Patterson, 1982). 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. Several of these mutants carry additional mutant alleles of the previously described *chs1* locus (Hugly et al., 1990) indicating that chilling-sensitive mutations at this locus are most likely due to loss of function of a cellular component which is only 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.)

Phosphate-deficient mutants

In order to establish a system to examine the factors which regulate mineral nutrient acquisition, we recently 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., 1991). 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. The mutation is currently being mapped, relative to the RFLP markers, as a prelude to the isolation of the gene by chromosome walking.

Genetic dissection of the cell wall

Because of the structural complexity and insolubility of the cell wall, relatively little is known about the structure and function of this organelle. We have initiated a genetic dissection of the cell wall with the general goal of examining the degree to which the kind or amount of the various polysaccharide constituents can be altered by mutation. A large number of mutants have been isolated by screening 5000 plants from a mutagenized population for individuals which exhibit an altered ratio of the various monosaccharides in a complete digest of the cell wall. The collection of mutants is currently being backcrossed to the wild type as a prelude to detailed biochemical and physiological studies of the effects of the mutations on the structure and function of the cell wall.

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MOLECULAR BASES OF PLANT DISEASE RESISTANCE MECHANISMS

Shauna Somerville, *Ke-Bin Liu, Kent Kerby, Sam Simons, Jun Tsuji and Jennifer Walsh Weller*

The powdery mildew disease of barley is caused by the fungus *Erysiphe graminis* f.sp. *hordei* (Mathre, 1982). For this disease, like many others, the development of an incompatible relationship between pathogen and host can be summarized as consisting of three steps: 1) the generation of a signal indicative of attack by a specific race of *E. g. hordei*; 2) recognition of the signal by the host cells; and 3) transduction of the signal to the cell interior, redirecting cellular metabolism towards defensive responses. In susceptible barley, some aspect of this sequence fails and a compatible relationship is established (see Ellingboe, 1972, for a more complete description of the infection process). In addition, the interactions between barley and *E. g. hordei* are characterized by a high degree of specificity (Moseman, 1966). That is, a specific host resistance allele is capable of mediating resistance to a specific subset of *E. g. hordei* races. This pattern of host resistance is characteristic of plant diseases described by the "gene-for-gene" hypothesis. Flor (1955) proposed this hypothesis as a means of summarizing data on the genetic analysis of race-specific rust resistance in flax and of cultivar-specific virulence in the rust pathogen, *Melampsora lini*. The "gene-for-gene" hypothesis states that incompatible interactions and the expression of resistance develop when a host plant carrying a resistance allele interacts with a pathogen race having a specific, complementary avirulence allele. The barley *Ml-a* locus exhibits the typical features of a race-specific resistance gene. Thus, the characterization of the *Ml-a* gene product is an important step toward determining a key biochemical component of disease resistance. In addition, understanding the genetic mechanism that is responsible for the highly polymorphic nature of the *Ml-a* locus is of both practical and theoretical interest. The genetic structure of this locus will dictate the range of novel resistance alleles that can be created *in vitro* for the purposes of genetically engineering pathogen resistance.

Several articles describing the merits of using *Arabidopsis* for molecular genetic experiments have been written (*e.g.*, Meyerowitz, 1987). The most attractive features of this model plant species are its small size, short generation time, ready crossability and prolific seed yield. As a result, *Arabidopsis* is an ideal species for mutational and genetic studies (Estelle and Somerville, 1986). Furthermore, advanced molecular genetic tools, including dense RFLP maps (Chang et al. 1988; Nam et al., 1989) and physical maps (Hwang et al., 1991) of the *Arabidopsis* genome, molecular tags like T-DNA (Feldman, 1991) and *Ac* (Dean et al., 1992) and efficient transformation methods, can be used for recovering and characterizing genes controlling disease development and the expression of resistance.

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

For most of our experiments with barley, we make use of the congenic barley lines AlgR and AlgS, which differ by the respective presence or absence of the dominant *Ml-a1* resistance allele (Moseman, 1972). These two lines are related by 18 generations of breeding and are estimated to be >99% homologous.

By subtractive hybridization methods, we hope to identify one or a limited number of cDNAs that are unique to AlgR and altered or missing in AlgS. Given that the two isolines are >99% similar (Moseman, 1972) and barley expresses about 30,000 different mRNA species (Goldberg et al., 1978; Willing and Mascarenhas 1984), the estimated number of mRNA species to be recovered in the subtractive cDNA library is about 300. In fact, this number is expected to be substantially less than 300 because few of the genes in the introgressed region of the genome are expected to exhibit substantial differences in sequence or mRNA accumulation between AlgR and AlgS. cDNA populations have been constructed and amplified via PCR to levels sufficient to drive renaturation reactions for the subtractive part of the experiment (Akowitz and Manuelidis, 1989). Currently, we are attempting to improve the efficacy of the renaturations and subtractions (Straus and Ausubel, 1990). Once the subtracted cDNA library has been established, the cDNA's will be tested for co-segregation with the *Ml-a1* resistance allele in F_3 [AlgR x AlgS] families.

In a separate experiment, we are developing a high-density map of the region adjacent to the *Ml-a* locus. For this purpose, we have screened the near-isogenic lines AlgR and AlgS for RAPD (randomly amplified polymorphic DNA) markers (Young et al., 1988). With the polymerase chain reaction, random 10-mer primers can be used to amplify fragments of genomic DNA (Williams et al., 1990). Depending on the level of sequence polymorphism between samples, the fragments that are amplified will vary. These polymorphic PCR products can be utilized as molecular markers in mapping experiments. A protocol was developed in which the amount of genomic DNA template, the concentration of primer, and the PCR cycling temperatures were optimized for use with barley. We have screened 500 Operon Tech primers and 300 Carlson primers for those that give RAPD markers between AlgR and AlgS. Currently, we are testing the reproducibility of the RAPD markers that have been identified. Once confirmed, these RAPD markers will be used to generate a high-density genetic map. The *Hor2* locus is about 7.3 cM distal to *Ml-a* on chromosome 5. In order to integrate the RAPD map with the existing RFLP maps of barley, we developed a pair of 22-mer PCR primers for the *Hor2* locus, based on the sequences for this locus in GenBank. These *Hor2* primers amplify two fragments in AlgS and only one fragment in AlgR. We have also developed a protocol for CHEF gel analysis of barley genomic DNA (Chu et al., 1986). Once RAPD markers have been mapped, we will attempt to link two closely linked markers by physical mapping.

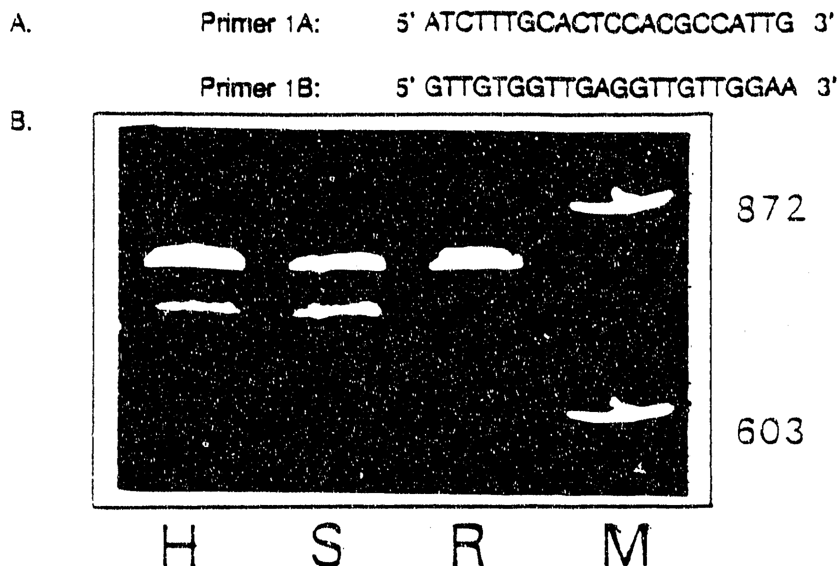

Figure 1. A. Sequence of the pair of *Hor2* primers. B. PCR products amplified from a heterozygote (H), AlgS(S) with the *Hor2* primer set. The PCR products were separated on a polyacrylamide gel and stained with ethidium bromide. M_r standards (M) are also pictured. M_r values are given in bp.

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 (Vance et al., 1980). Notably, wall peroxidases are responsible for initiating the polymerization of hydroxy-cinnamyl alcohol into lignin-like structures (Vance et al., 1980). 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 (Kerby and Somerville, 1989). 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 pI 8.5/8.6 isozyme has been purified to homogeneity (Kerby and Somerville, in press). This isozyme, like other peroxidases, is a heme-containing glycoprotein. It has a M_r of 35,000. Purified P8.5/8.6 was chemically deglycosylated with HF, and then used to produce a highly specific antiserum. In future studies, the localization and

timing of induction of the infection-related peroxidases will be compared to such cytological responses as papilla-formation and the deposition of phenolic compounds in the cell wall adjacent to penetration sites.

RXC1, a locus affecting the development of black rot disease in *Arabidopsis*

Previously, we demonstrated that *Arabidopsis* race Pr0 was distinctly more susceptible than race Columbia to the bacterial pathogen *Xanthomonas campestris* pv. *campestris*. Pr0 develops large, spreading chlorotic lesions following inoculation (Tsuji et al., 1991). Furthermore, the differential response to *X. c. campestris* between these two *Arabidopsis* races is controlled by a single gene, *RXC1*. To map *RXC1* relative to the classical genetic map, crosses were made between the susceptible Pr0 and the resistant Landsberg. In the F_2, a total of 617 individuals were scored for 16 morphological markers and for resistance to *X. c. campestris*. *RXC1* mapped to chromosome 2 near the locus *cp2*. The mapping of *RXC1* relative to RFLP markers is in progress. Once this locus has been placed in an interval between two flanking RFLP markers, chromosome walking to *RXC1* will be initiated.

Synthesis of a phytoalexin in *Arabidopsis*

Based on a purification protocol that consisted of a combination of reverse-phase flash chromatography, thin-layer chromatography, and reverse-phase high-performance liquid chromatography, we purified a phytoalexin from $AgNO_3$-elicited leaves of *Arabidopsis*. The structure of the phytoalexin was elucidated as 3-thiazol-2'-yl-indole on the basis of UV, IR, MS, ^{1}H-NMR, and ^{13}C-NMR spectral data (Tsuji et al., in press).

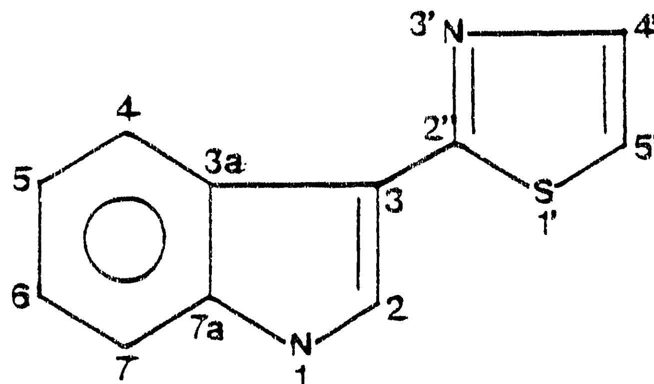

Figure 2. Structure of the *Arabidopsis* phytoalexin, 3-thiazol-2'-yl-indole.

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BIOCHEMICAL AND MOLECULAR ASPECTS OF PLANT PATHOGENESIS

Jonathan D. Walton. *P. Apel, F. Holden, R. Meeley, A. Nikolskaya, D. Panaccione, H. Schaeffer and J. Scott-Craig*

The interactions between a plant and a pathogen are controlled by manifold structural, chemical, and biochemical features of the two organisms. The plant has both pre-formed and induced mechanisms to limit invasion. The pathogen must penetrate, overcome the host defenses, and obtain nutrients. Cell wall-degrading enzymes are examples of general pathogenicity factors. All fungi make them, and in some cases they are required for penetration and nutrient acquisition. Such factors, however, do not account for the exquisite specificity frequently present in host-pathogen interactions, in which compatibility and incompatibility are determined by single genes in the pathogen and single genes in the host. Host-selective toxins are low molecular weight compounds produced by some pathogenic fungi. A fungus that makes a host-selective toxin is pathogenic on just those plants that are sensitive to the toxin, and, conversely, isolates of the fungus that do not make the toxin are not pathogenic on those plants. Host-selective toxins are among the few molecular agents of plant disease specificity known.

This research group is studying the biochemistry and molecular biology of representative examples of general and specific pathogenicity factors. Both types of studies are being done with an important fungal pathogen of cereals, *Cochliobolus* (*Helminthosporium*).

Molecular analysis of the *Tox2* locus from *Cochliobolus 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 (Walton, 1991). 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 *C. 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-AEO), where AEO stands for 2-amino-9,10-epoxi-8-oxodecanoic acid. The toxin and the producing fungus affect only maize that is homozygous recessive at the nuclear *Hm* 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, 1) how single genetic loci can control production of complex

secondary metabolites such as toxins, and 2) the apparently rapid appearance of new toxicogenic races and species of *Cochliobolus* (Leonard, 1973).

We reported the identification, purification, and characterization of two enzymes involved in the biosynthesis of HC-toxin (Walton, 1987; Walton and Holden, 1988). 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 (Walton, 1987). Using antibodies and oligonucleotide probes based on the amino acid sequences of the enzyme, we identified a partial cDNA encoding part of the gene for HTS-1. We found that 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 *Tox2* and hence HC-toxin production did not arise by a simple mutation or by a genetic rearrangement, but rather by a large insertion or deletion event.

In order to explain certain observations, it has been proposed that genes may occasionally move "horizontally" between species. We believe that this probably occurred in the evolution of *C. carbonum*, for several reasons. First, four other fungi unrelated to *C. carbonum* produce compounds closely related to HC-toxin (Walton, 1990). These other compounds are all cyclic tetrapeptides containing AEO. Since secondary metabolites are often complex (as are HC-toxin and its analogs), and their biosynthetic enzymes specialized (as are HTS-1 and HTS-2), the existence of particular secondary metabolites in unrelated organisms has led Luckner (1984) and others (e.g., Kleinkauf and von Döhren, 1990) to propose horizontal gene transfer as an explanation. Second, if such transfer can occur, movement of single "genes" such as *Tox2* is much more likely than the movement of multiple, unlinked genes. Third, a plausible mechanism exists. Filamentous fungi, even of vegetatively incompatible strains, can fuse (form anastomoses) for at least long enough for the transfer of DNA to occur (Collins and Saville, 1990). We are beginning our examination of the hypothesis that *C. carbonum* acquired the capability to make HC-toxin by horizontal gene transfer by using our cloned *Tox2* DNA, and the sequence information derived from it, to search for related DNA in the fungi that make compounds related to HC-toxin.

Our analysis of the 22 kb of *Tox2*-unique DNA has revealed that it is more complicated than originally expected. For example, the entire 22 kb is present in tox^{+} isolates in two identical copies. At the 5' end the two copies are distinguishable only with a single restriction enzyme that cuts approximately 15 kb outside of the 22-kb unique region. In 1991 we disrupted both copies of the 22-kb region by sequential transformation with two selectable markers. (In *C. carbonum*, integration of transforming DNA is homologous.) When only one copy or the other is disrupted, the fungus still makes HC-toxin and is fully pathogenic, although assayable levels of HTS-1 and HTS-2 decline by about half. When both

copies are disrupted, HC-toxin production, HTS-1 and HTS-2 activities, and pathogenicity are lost.

In 1991 we finished sequencing the entire 22-kb tox⁺-unique region. The sequence has revealed some interesting information. First, and surprisingly, it contains a 15.7-kb open reading frame (ORF). There is no evidence for any introns in this ORF. Our earlier conclusion that this 15.7-kb region contains two or more genes was based on the isolation of several cDNAs encoded by it; however, when these cDNAs were sequenced they were found to be fungal artifacts because, although they are polyadenylated and colinear with the genomic DNA, they do not contain stop codons. Only the cDNA at the 3'-most end of the 15.7 kb ORF contains a stop codon. Second, we found in the 15.7-kb ORF the DNA encoding several peptides (which had been obtained from the enzymes by proteolytic digestion) for both HTS-1 and HTS-2. This explains why, when the "gene" for HTS-1 is disrupted, HTS-2 activity also declines. It also suggests that HTS-1 and HTS-2 are normally part of a single, larger enzyme (predicted size 570 kD) and that separation of them into two enzymes might be an artifact of purification. Recent results using modified purification strategies support this hypothesis. Third, the 15.7-kb ORF, which we call simply *HTS1*, contains four copies of an "AMP-binding motif" characteristic of cyclic peptide synthetases. The gene for ACV synthetase, a 400 kD protein that catalyzes the synthesis of the linear tripeptide precursor to penicillins and cephalosporins, contains three such motifs (Smith et al., 1990). Our results support the concept that each such motif corresponds to the activation and/or thioesterification of one constituent amino acid.

A major question still outstanding concerns the biosynthesis of AEO, the amino acid that is required for the toxicity and specificity of HC-toxin (see below). Several strategies are being pursued to identify the genes and enzymes involved in its biosynthesis. First, since HC-toxin biosynthesis is encoded by a single "gene", the AOE biosynthetic genes should be linked, and are probably unique to tox⁺ isolates. We have identified new regions of tox⁺-unique DNA that are genetically linked to the known 22-kb region, and we will analyze their structure and expression. Second, isolates in which one or both copies of the HTS gene have been disrupted (and, to a lesser extent, wild-type tox⁺ isolates, but never wild-type tox⁻ isolates) accumulate a novel epoxide-containing compound. We are purifying and characterizing this putative AEO precursor with the hope that it will provide clues to the AOE biosynthetic pathway. (Supported, in part, by NSF grant #90-16949.)

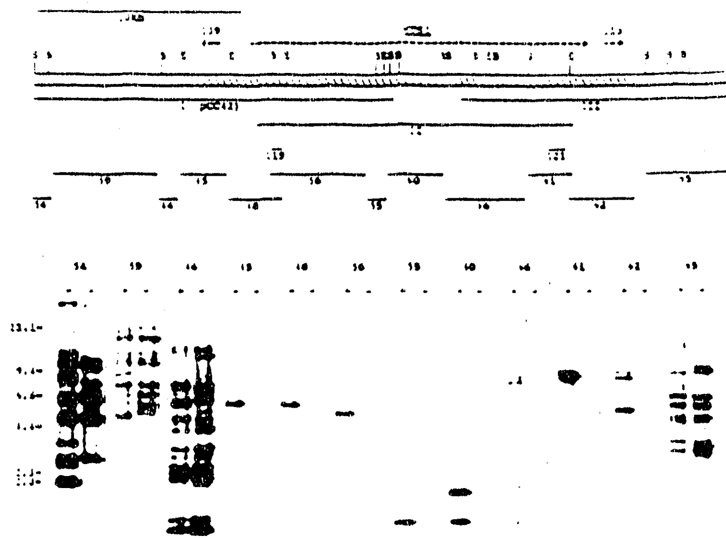

Figure 1. Analysis of the 22 kb of DNA that is associated with HC-toxin production in *C. carbonum* race 1. On top is shown the restriction map of the *C. carbonum* chromosome, based on the three overlapping lambda clones I, II, and III. The hatched area indicates DNA unique to race 1 (tox$^+$) isolates. The large dashed arrow shows the location of the 15.7 kb ORF (*HTS1*) encoding HTS-1 and HTS-2. The arrows labelled 139 and 115 show the location and direction of transcription of two additional genes of unknown function. Underneath the map are shown numbered subcloned fragments of the 22-kb region. These fragments were used as radiolabelled probes against DNA blots of BamHI-digested total genomic DNA from a tox$^+$ and a tox$^-$ isolate, which are shown at the bottom of the figure. Lines at the left of the DNA blots indicate lambda/HindIII size markers.

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 *Hm*. We have been testing the possibility that the *Hm* locus encodes or controls expression of an enzyme that metabolizes HC-toxin to a non-toxic derivative. Tritiated HC-toxin was fed to maize leaves and the resulting products were analyzed by reverse phase HPLC and an in-line radioactivity detector. Maize leaves are able to metabolize HC-toxin very effectively to a single product. Contrary to our expectations, the metabolite is not the diol formed by hydrolysis of the 9,10-epoxide, but rather the 8-alcohol formed by reduction of the 8-ketone (Meeley and Walton, 1991). This compound is non-toxic (Kim et al., 1987).

When tritiated HC-toxin is fed to leaves through the transpiration stream, there are no significant differences in rate or extent of HC-toxin metabolism by resistant and sensitive plants (Meeley and Walton, 1991). 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, we developed an *in vitro* assay for HC-toxin reduction. "HC-toxin reductase" (HCTR) can be partially purified from etiolated maize tissue by ammonium sulfate precipitation and ion exchange chromatography. It prefers NADPH to NADH and is inhibited by p-hydroxymercuribenzoate. Suprisingly, however, we are able to find enzyme activity only in extracts of resistant maize (genotype *Hm/Hm* or *Hm/hm*) and not in susceptible maize (genotype *hm/hm*). Host-selective reaction to *C. carbonum* race 1 (tox⁺) can be assayed by inoculating single etiolated mesocotyls (Heim et al., 1983) and we can assay HCTR in single etiolated shoots. Therefore, individual plants can be tested for reaction to *C. carbonum* race 1 and the presence of HCTR. In 1991, we showed that extractable HCTR activity in etiolated seedlings and resistance to *C. carbonum* race 1 genetically co-segregate. In collaboration with Steve Briggs at Pioneer Hi-Bred International, we assayed HCTR in a variety of experimental maize lines that have transposon-induced mutations to sensitivity at the *Hm* locus, as well as lines that have reverted to HC-toxin resistance due to excision of the transposable element. In all lines, HCTR activity is present in resistant lines and lacking in susceptible lines (Meeley et al., 1992). Therefore, we conclude that the biochemical basis of resistance to *C. carbonum* race 1 controlled by *Hm* is an enzyme, HCTR, that detoxifies HC-toxin.

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

In common with most, and probably all, other filamentous fungi, pathogenic fungi make a variety of extracellular enzymes that can degrade the polysaccharides found in plant cell walls. The available evidence suggests that some of these enzymes have a role in disease, either in penetration and invasion or as triggers of plant defense responses (Cervone et al., 1989). Our goal is to test the involvement of various such enzymes in pathogenesis by constructing strains of fungi specifically mutated in the structural genes for these enzymes by transformation-mediated gene disruption. Using this technique we showed that endo-polygalacturonase (endoPG) is not required for pathogenicity of *C. carbonum* on maize (Scott-Craig et al., 1990). We are now extending our work on endoPG to other cell wall-degrading enzymes. Like endoPG, the enzymes are being purified and partially sequenced, and then corresponding synthetic oligonucleotides are used either as direct probes to screen libraries or as PCR primers to amplify the intervening DNA.

In 1991 we purified and partially sequenced an endo-β 1,4-xylanase (Holden and Walton, in press) and an exo-β 1,3-glucanase (Van Hoof et al., 1991). All land plants contain relatively large amounts of β 1,4-xylan, and fungal xylanases can act as elicitors of plant defense responses (Bailey et al., 1990). Exo-β 1,3-glucanase can degrade callose, a glucose polymer formed in papillae in response to fungal invasion (Bayles et al., 1990), and therefore might have a role in penetration of the epidermis.

Although *C. carbonum* apparently makes two to four "isozymes" of xylanase, our preliminary molecular genetic work indicates that there is a single gene (P. Apel and J. Walton, unpublished). Likewise, *C. carbonum* apparently has a single exo-β 1,3-glucanase gene (H. Shaeffer and J. Walton, unpublished). In 1991, we also began work on the purification of exopolygalacturonase, β 1,3-β 1,4-glucanase, and β-xylosidase. (Supported, in part, by NATO Grant for International Collaboration 0449/88, the MSU Research Excellence Fund, and USDA NRICGP grant 91-01122.)

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DEVELOPMENTAL BIOLOGY OF NITROGEN-FIXING CYANOBACTERIA

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Certain cyanobacteria (blue-green algae) are the only free-living prokaryotes capable of both oxygenic (i.e., higher-plant type) photosynthesis and reduction of nitrogen gas (N_2) to ammonia with light as the sole source of energy and water as the source of reductant. Despite the oxygen (O_2)-lability of the N_2-fixing enzyme system, these microbes are able to fix N_2 while producing O_2 by photosynthesis. Nitrogen fixation in many of these prokaryotes is closely associated with cellular differentiation and intercellular interactions. Vegetative cells--which photosynthesize, grow and divide--can differentiate into two other types of cells: heterocysts and spores. Heterocysts are the sites of N_2 fixation. However, they depend for this activity upon products of photosynthesis that they receive from the vegetative cells, and in return they supply products of N_2 fixation to the vegetative cells. Spores are resistant cells that permit the organisms to survive periods of environmental stress.

We have previously shown that intercellular chemical interactions take place in filamentous cyanobacteria, and that heterocysts control both the differentiation of vegetative cells into additional heterocysts and the juxtaposition of spores and heterocysts. Our objectives based on this work are to define the intercellular interactions and biochemical processes that control cellular differentiation and its localization and, once differentiation has taken place, that underlie nitrogen fixation. Having developed techniques for genetic analysis of these organisms, we are making extensive use of these techniques to approach our objectives.

We have focused on three aspects of heterocyst differentiation:
(1) what activities determine the location of differentiation, and thereby establish patternization;
(2) what regulatory relationships control the sequence(s) of genetic activities during differentiation; and
(3) what metabolic processes specifically support N_2 fixation?
These three aspects will be discussed in reverse order.

Our approach to aspect (3) has been to screen for transposon mutants of *Anabaena* PCC 7120 (Wolk et al., 1991) that are incapable of N_2 fixation under aerobic and anaerobic conditions (i.e., Fix^- phenotype) or only under aerobic conditions (what we call Fox^- phenotype: incapable of N_2 fixation in the presence of oxygen), and then to analyze the mutated genes. We have isolated many such mutants. All strains that showed no obvious differentiation of heterocysts (Het^- phenotype) proved Fix^-, whereas nearly all Het^- mutants proved Fix^+. These

results are consistent with the idea that expression of nitrogenase is developmentally controlled (Elhai and Wolk, 1990). Fix^- mutants having no apparent defect in heterocyst formation may be affected in nitrogenase itself or in the pathway that supplies electrons to the enzyme. We have also identified a mutant that is O_2-sensitive, apparently not because a defect in the heterocyst envelope permits extensive leakage of O_2, but because a deficiency in respiration by the heterocysts prevents reduction of O_2 that enters those cells; yet another mutant reduces acetylene to ethylene and to ethane in a fixed ratio that is reminiscent of an alternative nitrogenase. Developmental arrest at an intermediate stage of heterocyst maturation was also observed, as shown in part by the absence of envelope glycolipid (Hgl^- phenotype) and the lack of rapid oxidation of diaminobenzidine (Dab^- phenotype).

To identify and analyze dependency relationships [aspect (2)], we began by identifying a gene (*hetA*) involved in synthesis or stabilization of the heterocyst envelope and first expressed 5-7 h after nitrogen-stepdown (Wolk et al., 1988; Holland and Wolk, 1990). (In *Anabaena* sp. strain PCC 7120, proheterocysts are first clearly identifiable about 11 h after nitrogen-stepdown.) Expression of *hetA* is localized to spaced cells in a filament; once proheterocysts are discernible, it is in those cells that *hetA* is expressed. We also identified a gene (*tln6*) that is activated between 3 and 4 h after nitrogen-stepdown and that is not required for heterocyst formation. Genes *hetA* and *tln6* have been replaced in the chromosome by fusions to *luxAB* as a reporter, and the resulting strains subjected to transposon mutagenesis. Mutations that alter the regulation of *hetA*, or that both alter the regulation of *tln6* and affect differentiation, are then targeted for in-depth study. First, that part of the chromosome bearing the transposon is cloned from the mutant and transferred (i) to a strain bearing the reporter fusion to test whether the regulatory phenotype in the mutant is due to the insertion of the transposon; and (ii) to a wild-type strain to see whether, and what, developmental differences result. Second, we determine whether expression of the newly mutated gene is spatially and temporally constitutive and if not, in what way not. Third, the mutation is transferred to mutants affected in other developmentally significant genes to determine whether dependency relationships can be identified; and the gene is sequenced in the hope of learning possible function by homology, and is mapped in the genome by pulsed field gel electrophoresis (PFGE). A modification of our earlier technique for preparation of PFGE-quality DNA (Bancroft et al., 1989) permitted the isolation of such DNA more easily and rapidly from a wide variety of mutants.

Mutations (e.g., in mutants HNL2 and HNL4) that lead to fragmentation of filaments generally block differentiation and prevent expression of *hetA*. Other mutations (e.g., in mutants HNL-D and HNL-L) block differentiation completely or nearly completely, without concurrent fragmentation. The phenotype of mutant HNL-D has been regenerated by a clone of the resident transposon fused to contiguous DNA, showing that the phenotype is due to the insertion of the

transposon. Certain other mutations lead to constitutive expression of *hetA*. The phenotype of one of these, HAC-b, has also been regenerated from a clone of the mutated gene. In mutant TTL620 (mutated in gene *hetC*), expression of *tln6* and differentiation of heterocysts are both blocked, but it has not yet been established whether both effects are due to the insertion of the transposon. A mutation in *hetR* (a gene described by Buikema and Haselkorn, 1991) blocks expression of *hetA* and of *tln6*, and also leads to temporally constitutive expression of *hetR* itself, suggesting that *hetR* is autoregulatory. The result with respect to expression of *hetR* is consistent with results of *hetR* transposon mutants T-47 and α-63, both generated with Tn5-1063 oriented to report transcription from the *hetR* promoter. Work with a single-recombinant *hetR*::*luxAB* mutation has shown that *hetR* is weakly expressed throughout the filament in the presence of NO_3^-, is induced within 2 h of nitrogen-stepdown, and is strongly induced in spatially separated cells within 3.5 h after nitrogen-stepdown. This year we also showed that when *rpoT7* is fused to certain genes, including *tln6* (see above), and *luxAB* is put under the control of a promoter recognized by that polymerase, the result is luminescence from vegetative cells, rather than from proheterocysts. This system for transcriptional amplification had previously been used only for genes expressed specifically in proheterocysts. Our present results show that the system is more generally usable.

Our approach to identifying events that establish patternization [aspect (1)] is, in part, intimately related to our approach to aspect (2). That is, we seek to identify, in a dependent sequence of genetic activities that are involved in localizing differentiation, the last such activities that are expressed in a spatially uniform manner. Those activities may affect the differentiation-determining intercellular interactions. We wish also to identify the first genetic activities that are induced upon the onset of differentiation, activities that might be regulated by those interactions.

A final approach that we have taken to the understanding of pattern formation is to screen transposon-mutated colonies for irregularities in pattern. One such mutant (N10) forms proheterocyst-like cells, sometimes in groups of 2 or 3, that appear not to complete differentiation. A spontaneous derivative of this strain proved to form clusters of heterocysts. Whether these phenotypes can be regenerated by transfer of the cloned, Tn-based mutation to a wild-type strain remains to be seen.

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ENVIRONMENTAL CONTROL OF PLANT DEVELOPMENT AND ITS RELATION TO PLANT HORMONES

Jan Zeevaart, *Robert Bensen, Françoise Fantauzzo, Li Li, Christopher Rock, Manuel Talon and Theresa Wilson*

Environmental factors such as photoperiod, light quality, temperature, and water deficits have pronounced effects on plant growth and development. Plant growth substances often play a role as intermediaries between the perception of an intermediary environmental factor and the ultimate morphological expression. The aim of this task is to elucidate how environmental factors regulate the metabolism and action of these plant growth substances. In this context, two topics are under investigations. The first is photoperiodic control by gibberellins of stem growth in long-day rosette plants. The objective is to determine how light regulates gibberellin biosynthesis in these plants, how changes in gibberellin status are brought about at the biochemical and molecular level, and how these changes result in stem growth. These studies on gibberellins in rosette plants, the so-called "physiological dwarfs," are complemented by characterization of a series of single-gene dwarf mutants ("genetic dwarfs") in tomato and *Arabidopsis* which are deficient in gibberellins. The immediate objective is to determine which steps in the gibberellin biosynthetic pathway are blocked in these mutants, and further to use these mutants for molecular genetic studies. As a whole, this research will lead to a better understanding of the environmental regulation of plant growth and productivity. The second subject of investigation is the biosynthesis of abscisic acid and its catabolites induced in response to water stress. The aim is to work out the biosynthetic pathway of abscisic acid, to determine the rate-limiting step that is stimulated by water stress, and to elucidate how a large increase in abscisic acid alleviates the effects of stress. The results of these studies will lead to a better understanding of how plants are able to cope with stress.

Studies on Gibberellins

Effect of BX-112 on metabolism of ^{14}C-gibberellin A_{20} in spinach

We reported previously (Plant Research 1990, pp. 93-94) that the growth retardants AMO-1618 and BX-112 both suppress stem growth in spinach under long-day (LD) conditions. The inhibition by AMO-1618 could be reversed by both GA_{20} and GA_1, but the effects of BX-112 could be overcome only by GA_1, not by GA_{20}. This led to the conclusion that 3β-hydroxylation of GAs is a requirement for bioactivity in spinach. In the present work, the effects of the two growth retardants on metabolism of ^{14}C-GA_{20} applied to spinach were investigated. The radioactive metabolites were initially quantified by HPLC and subsequently identified by GC-MS. As expected, ^{14}C-GA_{20} was much less metabolized in spinach plants treated with BX-112 than in control plants or in plants treated with AMO-1618. The following radioactive metabolites were identified by GC-MS:

GA_1, 3-*epi*-GA_1, GA_{29}, GA_8, and two unidentified isomers of GA_{29}. All of these GAs are native to spinach (Talon et al., 1991). (Supported, in part, by USDA grant #88-37261-3434.)

Only 3ß-hydroxylated gibberellins are active in stem growth of *Arabidopsis*

BX-112 is a new plant growth retardant that inhibits 2-oxoglutarate-dependent dioxygenases. In the GA biosynthetic pathway, BX-112 inhibits the hydroxylation steps after GA_{53}, in particular 3ß-hydroxylation (Nakayama et al., 1990). In the present study, BX-112 was used to determine which GA is active *per se* in stem growth in *Arabidopsis*. Application of BX-112 to both the Landsberg *erecta* and RLD lines of *A. thaliana* resulted in inhibition of stem growth. This growth inhibition could be reversed by 3-hydroxy-GAs (GA_4, GA_7, GA_{36}), and to a lesser extent by the 3,13-hydroxy-GA, GA_1, but non-3-hydroxy-GAs (GA_9, GA_{19}, GA_{20}) did not overcome the inhibition of growth. Analyses of endogenous GAs in plants of the Landsberg *erecta* line treated with BX-112 showed that the levels of the 3-hydroxy-GAs, GA_1 and GA_4, were much reduced, whereas the non-3-hydroxy-GAs, GA_9, GA_{20}, and GA_{19} accumulated. Treatment of *Arabidopsis* with BX-112 is, therefore, a chemical means by which the *ga4* mutation (Talon et al., 1990) can be simulated. These results establish that 3ß-hydroxylation is required for GA activity in *Arabidopsis*. (Supported, in part, by USDA grants #88-37261-3434 and #91-37304-6469.)

Gibberellin conversions in *Arabidopsis thaliana*

Shoots of the Landsberg *erecta* line of *A. thaliana* contain at least twenty GAs that can be arranged in three pathways (Talon et al., 1990). Feeding studies with isotopically labeled GAs with the GA-deficient mutant *ga1-2* (isolation 6.59) were conducted to establish the functioning of these pathways in *Arabidopsis*. Metabolites of labeled GAs were identified by full-scan GC-MS. [^{2}H]GA_{20} was converted to labeled GA_1, GA_{29}, and GA_8, which establishes that the final steps of the early 13-hydroxylation pathway operate in *Arabidopsis*. Applied [^{2}H]GA_4 was converted to labeled GA_1, GA_8, and GA_{34}, indicating that 13-hydroxylation (GA_4-GA_1) can take place at a late stage of GA biosynthesis. [^{2}H]GA_9 was converted to labeled GA_{20}, GA_1, GA_{29}, GA_8, and GA_{51}. In addition, a relatively large amount of labeled GA_{34} (as compared with other metabolites) was also present, indicating that GA_9 was converted to GA_4 which in turn was rapidly 2ß-hydroxylated to GA_{34}. These results show that the three pathways are connected at the C_{19} level and that GA_9 is a precursor of both GA_4 and GA_{20}. The relative importance of 13-hydroxylation of GA_9 to GA_{20} versus 3ß-hydroxylation of GA_9 to GA_4 cannot be assessed on the basis of these results. After feeding [^{14}C] GA_{12}, the following members of the non-3,13-hydroxylation pathway were identified: GA_{24}, GA_{25}, and GA_{51}. Furthermore, identification of labeled GA_{36} and GA_{34} indicates that GA_4 was derived from GA_{12} either via GA_9 (see above) or via the early 3ß-hydroxylation pathway by elimination of C-20 from GA_{36}. Of

the members of the early 13-hydroxylation pathway, only labeled GA_{19} and GA_{20} could be identified by GC-MS. These GAs may have been synthesized via the precursors GA_{53} and GA_{44}, or they may have been derived from GA_{24} and GA_{9}, respectively. Time course studies with labeled precursors are needed to determine which pathway(s) is (are) predominant in GA biosynthesis in *Arabidopsis*.

Since GA_4 is more active than GA_1 (Talon et al., 1990; previous report), it appears that GA_4 is active *per se* in *Arabidopsis*. However, as shown here, GA_4 is converted to GA_1, so that the biological activity of GA_4 may be due to its conversion to GA_1. This leaves unexplained why GA_1, the product, is less active than the precursor, GA_4. As far as tested, 13-hydroxylated GAs are less active in causing stem growth in *Arabidopsis* than the corresponding non-13-hydroxylated GAs. These observations suggest that 13-hydroxylation decreases bioactivity of GAs when tested on *Arabidopsis* and that 3ß-hydroxylation alone is sufficient for maximal bioactivity. (Supported, in part, by USDA grants #88-37261-3434 and #91-37304-6469.)

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

The *ga1*, *ga2*, and *ga3* mutants of *Arabidopsis* are blocked early in the GA biosynthetic pathway (Plant Research 1990, pp. 96-97). In the present investigation, the growth retardant tetcyclacis was used to inhibit *ent*-kaurene oxidation, so that *ent*-kaurene accumulation could be measured in wild type and in the various *ga* mutants of *Arabidopsis*. *Ent*-kaurene accumulation was measured by isotope dilution using combined gas chromatography-selected ion monitoring (Groszelindemann et al., 1991). In the absence of inhibitor, *ent*-kaurene was detectable in shoots of Landsberg *erecta* (Ler) and of the mutants *ga3* and *ga4*. Following treatment with tetcyclacis *ent*-kaurene accumulated to high levels in Ler, *ga3*, and *ga4* plants. However, the specific activity of added ^{14}C-*ent*-kaurene was unchanged in extracts from untreated as well as from inhibitor-treated *ga1* and *ga2* plants, indicating that these plants are incapable of synthesizing *ent*-kaurene. Thus, the blocks in the *ga1* and *ga2* mutants of *Arabidopsis* are at or prior to *ent*-kaurene synthetase. These results are in agreement with the observation that *ga1* and *ga2* plants show a growth response after treatment with *ent*-kaurene and all subsequent intermediates in the GA pathway. In contrast, the *ga3* mutant responds to *ent*-kaurenoic acid and later intermediates, but not to *ent*-kaurene and *ent*-kaurenol. Interestingly, untreated *ga3* plants had a higher *ent*-kaurene content than Ler and *ga4* plants (2000, 75, and 70 pmol/g dry wt., respectively). This accumulation of *ent*-kaurene (and presumably of *ent*-kaurenol as well) is expected if further metabolism of *ent*-kaurenol is blocked by the *ga3* mutation. (Work done in cooperation with D.A. Gage, MSU/NIH Mass Spectrometry Facility. Supported, in part, by USDA grants #88-37261-3434 and 91-37304-6469.)

Stem elongation and changes in the levels of gibberellins in shoot tips of *Silene armeria* induced by differential photoperiodic treatments

In long-day plants, perception of the relative length of day and night occurs in the leaves, whereas stem elongation and formation of the flower primordia take place in the shoot tip (Lang, 1965; Zeevaart, 1983). In the present investigation, the effects of differential photoperiodic treatments applied to shoot tips and mature leaves of the long-day rosette plant, *Silene armeria*, strain S 1.2 (Talon and Zeevaart, 1990), on growth and flowering responses, and on the levels of endogenous gibberellins (GAs), were investigated. Gibberellins were analyzed by GC-MS and the use of internal standards. Exposure of mature leaves to long-day (LD), regardless of the photoperiodic conditions of the shoot tips--short-day (SD), LD, or darkness--promoted elongation of the stem and of the immature leaves attached to it. LD treatment of mature leaves also modified the levels of GA_{53}, GA_{44}, GA_{19}, GA_{20}, and GA_1 in shoot tips. This treatment resulted in an increase in GA_1 in shoot tips growing under SD (2x increase), LD (5x), or dark (8x) conditions. The dark treatment applied to the shoot tips of plants of which the mature leaves were grown in SD promoted elongation of the immature etiolated leaves and caused a three-fold increase in GA_1 in the shoot tips. However, this treatment did not cause stem elongation. The different photoperiodic treatments applied to the shoot tips did not change the levels of GAs in mature leaves. These results indicate that both LD and dark treatments result in an increase in GA_1 in shoot tips. In addition, LD treatment causes the formation of a signal that is transmitted from mature leaves to shoot tips where it enhances the effect of GAs on stem elongation. (Supported, in part, by USDA grant #88-37261-3434.)

Studies on Abscisic Acid

Biochemical and molecular characterization of the *aba* mutant of *Arabidopsis thaliana*

We previously showed that the *aba* mutant of *A. thaliana* is impaired in epoxy-carotenoid biosynthesis and accumulates the epoxy-carotenoid precursor, zeaxanthin (Rock and Zeevaart, 1991). In addition to providing conclusive evidence for the direct pathway of ABA biosynthesis from carotenoids, the *aba* mutation offers a powerful means to study the physiology, biochemistry, and molecular biology of the functions of carotenoids in photosynthesis. Zeaxanthin has been proposed to protect photosystem II (PSII) against photoinhibition through the action of the xanthophyll cycle, a light-induced, reversible de-epoxidation of the diepoxy-carotenoid, violaxanthin (Demmig-Adams, 1990). The epoxy-carotenoids violaxanthin and neoxanthin are necessary for assembly of functional light-harvesting chlorophyll a/b binding complexes (LHPCII) *in vitro* (Plumley and Schmidt, 1987). In order to test the function of these xanthophylls, we compared the rates of photosynthesis of thylakoid membranes from wild type (WT) and *aba-4* plants, as well as *in vivo* chlorophyll (Chl) fluorescence, and

chloroplast ultrastructure of WT plants and plants homozygous for three *aba* alleles.

Oxygen evolution rates (per μg Chl) at various light intensities were lower in *aba-4* than in WT thylakoid membranes. This difference was not due to a reduced quantum efficiency of PSII photochemistry, based on the calculated apparent K_m for the light reactions of *aba-4* and WT thylakoids. Therefore, the number of active PSII centers may be reduced in the *aba* mutant. Measurements of Chl fluorescence in leaves of WT, *aba-1*, *aba-3*, and *aba-4* showed only one significant difference between WT and mutant alleles: the Chl fluorescence was reduced in mutant leaves. This was not due to pleiotropic effects of ABA deficiency in the mutants, since these were reversed by foliar application of ABA. The finding that the fluorescence induction kinetics of the mutants ($F_{var}/F_{max}, t_{½}$) are not significantly different from WT confirms the finding that quantum efficiency and photosynthetic electron transport are not altered by the *aba* mutation. Furthermore, the decreased fluorescence (per unit leaf area) in the mutant plants supports the hypothesis that zeaxanthin is directly responsible for non-photochemical fluorescence quenching.

The role of xanthophylls in the supramolecular structure of PSII was assessed by electron microscopy of WT and mutant leaves and quantification of the thylakoid grana stacks. There were significantly fewer thylakoids per grana stack in *aba* mutant chloroplasts compared to WT, but significantly more grana per chloroplast in the mutants than in WT. The *aba* mutant apparently compensates for reduced thylakoid stacking by increasing the number of grana. Thus, zeaxanthin, violaxanthin and/or neoxanthin function in the organization of the photosynthetic apparatus. Western blot analysis of mutant and WT thylakoid proteins showed that LHCPII is present in normal amounts in the *aba-4* mutant. Taken together, these data suggest that the xanthophyll imbalance in the *aba* mutant plants does not affect the number of PSII complexes or assembly of LHCPII, but rather the number of *active* PSII centers.

With the long-term goal of understanding the molecular mechanisms which regulate epoxy-carotenoid and ABA biosynthesis, the *aba* mutation has been mapped relative to available RFLPs (Chang et al., 1988) in order to clone the gene by chromosome walking. Analysis of over 200 recombinant F_2 and F_3 lines homozygous for *aba* and the linked genes *ttg* and *yi* has given a high resolution map of the DNA surrounding these genes. Progress in the ordering of a contiguous physical map of the *Arabidopsis* genome will facilitate cloning the genes by transformation and complementation of the mutant phenotypes. (Work done in collaboration with N. Bowlby and S. Hoffmann. Supported, in part, by NSF grant #DMB-8703847.)

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