UNIVERSITY OF NEBRASKA—LINCOLN

INSTITUTE OF AGRICULTURE AND NATURAL RESORCES

Annual Meeting of Multi-State Project NC-1168

Regulation of Photosynthetic Processes

November 17, 2007

Multi-State Meeting Schedule, Nebraska AES

NC-1168 Regulation of Photosynthetic Processes

Friday, November 16, 2007

6:30 PM	Meet in the lobby of the Chase Suites Hotel (200 South 68th Street). We'll walk to Mazatlan's for dinner (211 North 70th Street).

Saturday, November 17, 2007

7:00 AM	Breakfast at your hotel (Chase Suites) any time after 7:00
8:30 AM	Hotel shuttle departs for the Beadle Center (17th and Vine). Our meeting will be held in room E228.
9:05 AM	Announcements and introductions, Bob Spreitzer
9:15 AM	Welcome from Dr. Gary Cunningham, Dean and Director, Nebraska AES
9:30 AM	Bob Spreitzer, Nebraska AES
9:55 AM	Mary Rumpho, Maine AES
10:20 AM	Archie Portis, Illinois ARS
10:45 AM	Break
11:00 AM	Mike Salvucci, Arizona ARS
11:25 AM	Don Weeks, Nebraska AES
11:50 AM	Lunch in the atrium of the Beadle Center
1:00 PM	Gerry Edwards, Washington AES
1:25 PM	Karen Koch, Florida AES
1:50 PM	Marna Yandeau-Nelson (for Mark Guiltinan), Pennsylvania AES
2:15 PM	Break
2:30 PM	Jack Preiss, Michigan AES
2:55 PM	Wayne Loescher, Michigan AES
3:20 PM	Break
3:30 PM	Glenda Gillaspy, Virginia AES
3:55 PM	Christoph Benning, Michigan AES
4:20 PM	Break
4:35 PM	Irwin Goldman, Administrative Advisor, Wisconsin AES
4:50 PM	Gail McLean, USDA/CSREES Representative
5:05 PM	Business meeting
6:00 PM	Shuttle departs for Chase Suites hotel
6:30 PM	Transportation by Nebraska members and guests to Billy's restaurant (1301 H Street, http://www.billysrestaurant.com/)

Sunday, November 18, 2007

7:00 AM	Breakfast is served at Chase Suites hotel from 7:00 to 10:00 AM. Other restaurants are within walking distance on the north side of O Street. Arrange hotel shuttle or taxi (477-6074) for transport to airport.

Participation List

NC-1168 Regulation of Photosynthetic Processes

Members Attending
Christoph Benning, Michigan State University
Gerald Edwards, Washington State University
Glenda Gillaspy, Virginia Tech
Karen Koch, University of Florida
Wayne Loescher, Michigan State University
Archie Portis, USDA, University of Illinois
Jack Preiss, Michigan State University
Mary Rumpho, University of Maine
Michael E. Salvucci, USDA-Arizona
Robert J. Spreitzer, University of Nebraska
Donald P. Weeks, University of Nebraska
Marna Yandeau-Nelson (for Guiltinan), Pennsylvania State University

Administrative Advisor
Irwin Goldman, University of Wisconsin

CSREES Representative
Gail McLean, USDA

Guests Attending
Raymond Chollet, University of Nebraska
Gary Cunningham, University of Nebraska
John Markwell, University of Nebraska
Julie Stone, University of Nebraska

Members Not Attending
Fred Below, University of Illinois
Hans Bohnert, University of Illinois
John Cushman, University of Nevada
Mark Guiltinan, Pennsylvania State University
Jeff Harper, University of Nevada
Robert L. Houtz, University of Kentucky
Steve Huber, USDA, University of Illinois (*no report provided*)
Jyan-Chyun Jang, Ohio State University
Robert Jones, University of Minnesota (*no report provided*)
Stephen Long, University of Illinois (*no report provided*)
Ron Mittler, University of Nevada (*no report provided*)
Brandon Moore, Clemson University
Thomas Okita, Washington State University
Steven Rodermel, Iowa State University
Martin Spalding, Iowa State University

Arizona ARS, Michael E. Salvucci

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Objective 2: Photosynthetic capture and photorespiratory release of CO_2

Objective 4: Developmental and environmental limitations to photosynthesis

Progress

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme that catalyzes the capture of CO_2 in photosynthesis, becomes inactive during the normal course of catalysis. Restoration of activity requires the action of activase, a specific molecular chaperone that couples ATP hydrolysis to specific conformational changes in Rubisco. Since inactivation occurs every several hundred turnovers, the reactivation process is dynamic requiring the constant intervention of activase to keep Rubisco in an active state. Because of its requirement for ATP, activase functions as a central modulator of Rubisco activity, essential for the control of CO_2 fixation in response to light, temperature and other environmental conditions.

Rubisco-activase interactions (Objective 2):
To better understand the biochemical mechanism of activase, I have continued to collaborate with Dr. Archie Portis (ARS-Urbana) and his group in an effort to understand how Rubisco and activase interact. With an ARS colleague in Arizona, we produced transgenic *Arabidopsis* that express a modified version of the shorter, β-isoform of activase. The modification, a C-terminal S-tag, facilitates rapid isolation of activase from crude leaf extracts using S-Tactin Sepharose. We have crossed this transgenic line with one developed by Portis and colleagues that expresses only the longer, α-isoform of activase. The new line expresses both forms of activase, like the wild type, but with a β-isoform that is affinity-tagged. The new line will be used to examine the effects of temperature on interactions among activase subunits and the effect of these interactions on Rubisco regulation.

Involvement of activase in thermotolerance of photosynthesis (Objective 4):
We have presented evidence that loss of Rubisco activation, a consequence of the inherent thermal lability of activase, represents the initial target of photosynthesis affected by heat stress. In collaboration with Dr. Archie Portis (ARS-Urbana) we have used transgenic *Arabidopsis* plants with reduced amounts of activase to show that inhibition of photosynthesis and loss of Rubisco activation under moderate heat stress is more acute when activase levels are suboptimal for photosynthesis, regardless of the isoform of activase. This finding is consistent with a proposed mechanism for inhibition of photosynthesis by heat stress based on the acute sensitivity of activase to thermal denaturation.

The involvement of activase thermal lability in the loss of photosynthetic activity during heat stress was confirmed in recovery experiments. Compared with wild type plants, photosynthesis, Φ_{PSII} and Rubisco activation were less thermotolerant and recovered more slowly in transgenic *Arabidopsis* plants with reduced levels of activase. Immunoblots showed that nearly 60% of the activase was recovered in the insoluble fraction after heat stress in leaf extracts of transgenic but

not wild type plants, evidence that deactivation of Rubisco was a consequence of thermal denaturation of activase.

The exceptional thermal lability of activase suggests that acclimation of photosynthesis could involve induction of chloroplast proteins like HSPs that might potentially stabilize activase during periods of high temperature. For these studies, we used the transgenic *Arabidopsis* line that expresses the S-tagged version of activase. The activase from these plants can be adsorbed to a Step-Tactin Sepharose column and eluted under gentle conditions with desthiobiotin. Sequence analysis and immunoblotting identified the β-subunit of chaperonin-60 (cpn60β), the chloroplast GroEL homolog, as a protein that affinity adsorbed with activase from leaf extracts prepared from heat-stressed, but not control plants. Analysis of the proteins by non-denaturing gel electrophoresis showed that cpn60β was associated with activase in a high molecular mass complex. Immunoblot analysis established that the apparent association of cpn60β with activase was dynamic, dependent on the duration and intensity of the heat stress and decreased following recovery. Reconstitution experiments using recombinant activase and cpn60β showed that the purified proteins formed a stable binary complex when incubated together at 40°C. Taken together, these data suggest that cpn60β plays a role in acclimating photosynthesis to heat stress, possibly by protecting activase from thermal denaturation.

Usefulness of the findings

Rubisco catalyzes the rate-determining step in photosynthesis, but it does so very inefficiently. Because of this inefficiency, Rubisco presents an obvious target for increasing photosynthetic performance. However, improvements in the properties of Rubisco will only be realized if the interaction with activase is not compromised. Identifying the precise mechanism by which activase interacts with and the changes the conformation of Rubisco, including the key residues involved in protein-protein interaction, is therefore essential for developing strategies to improve the catalytic efficiency of Rubisco.

Heat stress is a major abiotic stress that negatively impacts plant yield. Even when inhibition of photosynthesis is only temporary and relatively minor under moderate heat stress, the overall effects on yield can be substantial when integrated over the entire canopy. Understanding the precise role of activase in the inhibition of photosynthesis under moderate heat stress is essential for developing strategies that improve the thermotolerance of plants. Our findings of a possible interaction of activase with cpn-60 provides a new avenue for research into photosynthetic acclimation to moderate heat stress.

Plans for the coming year:

1) *Activase structure*: In collaboration with Drs. Archie Portis (ARS-Urbana) and Rebekka Wachter (Arizona State University), attempt to crystallize activase to determine the structure of the protein at the atomic level. Successful crystallization will require strategies for overcoming the problem of aggregation. One possible strategy is to use directed mutants with a more homogeneous quaternary structure. Also, conduct experiments to determine the effect of temperature on the association of the α- and β-type subunits of activase.

2) *Interaction of activase with cpn-60 and other proteins*. Determine the involvement of cpn-60 in stabilizing and protecting activase during heat stress and identify the biochemical nature of the complex formed in the interaction. Identify other proteins that bind to activase, albeit less tightly, during heat shock episode, as well as in the dark, under low light, etc.

<u>Publications</u>:

Salvucci, M.E., DeRidder, B.P. and Portis, A.R. Jr. (2006) Effect of activase level and isoform on the thermostability of photosynthesis in Arabidopsis. J. Exp. Bot. 57: 3793-3799

DeRidder, B. P. and Salvucci, M. E. (2007) Modulation of Rubisco activase gene expression during heat stress in cotton (*Gossypium hirsutum L.*) involves post-translational mechanisms. Plant Science 172, 246-254

Portis, A. R., Jr., Li, C., Wang, D. and Salvucci, M. E. (2007) Regulation of Rubisco activase and its interaction with Rubisco. J. Exp. Bot., in press.

Florida AES, Karen Koch

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Objective 3: Mechanisms regulating photosynthate partitioning.

A role for photosynthate flux through sorbitol in developing grains?

Three initial lines of evidence suggested this possibility. The first was early work of Shaw and Dickinson (1984. Plant Physiol. 75:207-211) showing that ^{14}C-photosynthates entering kernels were partitioned rapidly and extensively into sorbitol as well as hexoses. The second was data from the Brazilian MAIZEST genome database and S. de Sousa (in review) identifying a maize cDNA for sorbitol dehydrogenase as one of the most abundant transcripts in developing kernels at 10 and 15 days after pollination. The third was a result of collaborative efforts between our group and IPK-Gatersleben (Rolletschek, Koch, Wobus, and Borisjuk. 2005. Plant J. 42:69-83) indicating that endosperm of developing maize kernels was deeply hypoxic. Oxygen availability was well below that predicted to limit mitochondrial functioning and increase levels of NAD(P)H. Nonetheless, metabolite analyses indicated that adaptive mechanisms and efficient starch biosynthesis continued to function at still lower oxygen levels. Under these conditions, some means of dissipating or shuttling excess reducing power (NAD[P]H) from endosperm cells could be highly advantageous. Earlier work by Rumpho, Edwards, and Loescher (1983. Plant Physiol. 73:869-873) suggested that sugar alcohol metabolism could serve such a function. Collectively, these studies led us to hypothesize a role for sorbitol metabolism and/or shuttling within developing kernels, possibly involving dual contributions to C-transport and modulation of reducing power.

In situ SDH activity and small-kernel phenotype

To address this possibility, we isolated an *sdh1* maize mutant that lacked a functional gene for the single sorbitol dehydrogenase in its genome. We found that mutations in this gene conferred a small kernel phenotype (a mean of 21% less dried-seed weight) when *sdh1* plants were compared to wildtype siblings in a uniform genetic background. The extent of impact for the *sdh1* mutation was initially unexpected because maize does not use sorbitol for its long-distance vascular transport. Instead, kernels synthesize and metabolize sorbitol internally, consistent with a role for these processes in the grain itself. Thus far, evidence is consistent with the suggestion that sorbitol metabolism can contribute prominently to kernel capacity for sucrose import and use. We are further testing this possibility and exploring mechanisms of regulation.

A new method for expression profiling
(Initially developed to examine regulation of C import by young kernels)

Differences in gene expression underlie central questions in plant biology extending from gene function to evolutionary mechanisms and quantitative traits. However, resolving

expression of closely-related genes (e.g. alleles and gene family members) is challenging on a genome-wide scale due to extensive sequence similarity and frequently incomplete genome sequence data. We developed a new expression-profiling strategy that utilizes long-read, high-throughput sequencing to capture the information-rich 3'-untranslated region (UTR) of mRNAs. Resulting sequences resolve gene-specific transcripts independent of a sequenced genome. Analysis of ~229,000 3'-anchored sequences from maize (*Zea mays* L.) ovaries identified 14,822 unique transcripts represented by ≥ 2 sequence reads. Total RNA from ovaries of drought-stressed wild-type and *viviparous-1* mutant plants was used to construct a multiplex cDNA library. Each sample was labeled by incorporating one of 16 unique three-base key codes into the 3'-cDNA fragments, and combined samples were sequenced using a GS 20 454 instrument. Transcript abundance was quantified by frequency of sequences identifying each unique mRNA. At least 182 unique transcripts showed highly significant differences in abundance between wild-type and mutant samples. For a subset of mRNAs, quantitative differences were validated by Q-PCR. The 3'UTR profile resolved 12 unique *Cellulose Synthase* (*CesA*) transcripts in maize ovaries and identified previously-uncharacterized members of a *Histone-1* gene family. In addition, this method resolved nearly-identical paralogs, as illustrated by two *Auxin Repressed Dormancy Associated* transcripts, which showed reciprocal mRNA abundance in wild-type and mutant samples. Our results demonstrate that 3'UTR profiling enables quantitative analysis of gene- and allele-specific transcripts and provides an efficient tool for gene discovery.

Usefulness of findings:

The *sdh1* (*sorbitol dehydrogenase 1*) mutant offers us a means of testing newly-revealed, but little-known roles of sugar alcohols in grain development.

The method for 454-based, 3’UTR profiling resolves expression to the level of individual gene family members and facilitates gene discovery. It is especially useful where a fully-sequenced genome is not available. The cost of using a 454-sequencing approach is reduced by introduction of a bar-coding key for tagging multiple samples (sub-libraries) for a given run.

Plans for the coming year:

We have three overall goals for our studies of roles for sorbitol dehydrogenase (SDH) in maize, and we will initiate these in the coming year. One of these is to determine the physiological responses to altered SDH activity in maize. The second is to identify the metabolic basis for these responses. We will begin our work on both objectives with a characterization of the *sdh1* mutant. Our third goal will be to test effects of other genetic and transgenic perturbations to sorbitol metabolism in maize. The newly-identified *sdh1* mutant will be central to proposed research, but so too will a combination of genetic, molecular, biochemical, and classical approaches.

We are currently comparing expression data from 454-based 3’UTR profiles and microarray analyses of early development by maize kernels (pre and post pollination). We will identify key contributors to changes in growth and C-import by these structures. We will also obtain useful information on the strengths and limitations of 454-3’UTR profiling versus microarrays.

Publications: (*graduate student, **postdoctoral associate)

Eveland A.L.*, D.R. McCarty, and K.E. Koch. 2007. Transcript profiling by 3'UTR sequencing resolves expression of gene families. *Plant Physiol.* (In press for "breakthrough technology" section)

Huang* L-F, PN Bocock*, JM Davis, and KE Koch. 2007. Invertase regulation: A "suite" of transcriptional and post-transcriptional mechanisms. *Functional Plant Biology.* 34:499-507.

Settles, A.M., D.R. Holding*, B-C Tan*, S.P Latshaw, J. Liu*, M. Suzuki*, B. O'Brien***, D. Fajardo*, E. Wroclwaska, J. Lai*, C. Hunter***, W.T. Avigne, S.O. Peacock***, J. Baier, D. Lonon, J. Messing, L.C. Hannah, K.E. Koch, P.W. Becraft, B.A. Larkins, D.R. McCarty. 2007. Sequence-indexed mutations in maize using the UniformMu transposon-tagging population. *BMC-Genomics.* 8:116 (12 pgs on-line)

Illinois AES, Fred Below

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Objective 4: Developmental and environmental limitations to photosynthesis.

We documented a physiological condition known as 'hollow husk,' which occurred in our research plots and in some farmers fields in 2007. Hollow husk is characterized by normal appearing husks that feel hollow due to the cessation in ear development and a lack of silk emergence. It occurred when the foliage of actively growing corn plants was sprayed before the VT growth stage with a chemical treatment that either lowered the level of plant ethylene (i.e. a strobilurin fungicide), or one that decreased the plant's sensitivity to ethylene (i.e. 1-MCP). Our original goal was to alter the senescence trajectory of the leaf by an ethylene-mediated priming process. Hollow husk only occurred on chemically treated plants exhibiting very rapid growth following relief from an extended period of vegetative water stress. The percentage of plants exhibiting hollow husk symptoms depended on the hybrid, the stage of plant growth, and the combination of management conditions that promoted rapid plant growth. Plants sprayed at V15 generally exhibited greater symptoms than those sprayed at V11, and hollow husk successively increased with increases in N supply and decreased with increases in plant population. Almost three times as many plants exhibited hollow husk symptoms when they were grown at the lowest population and with the highest N, compared to those grown with the highest population and with the lowest N. A large difference in susceptibility to hollow husk was observed among a group of ten hybrids with hybrid ranges of 9 to 88% of plants affected by strobilurin fungicide and 2 to 19% of plants affected by 1-MCP. Plants exhibiting hollow husk symptoms retained green leaves below the ear for an extended period. Based on our combination of treatment applications designed to alter the level and/or the sensitivity of the plant to ethylene, we speculate that hollow husk results from a decrease in ethylene due to lower synthesis, and to a lesser extent, decreased ethylene sensing by the plant.

Usefulness of Findings

Knowing how ethylene alters leaf and ear development helps us understand how plants perceive changes in their environment. From a practical standpoint, understanding the cause and conditions that lead to hollow husk is of interest to growers and chemical dealers because it lowers yield. Knowing when to, and when not to, apply chemical treatments to achieve leaf-greening while avoiding hollow husk is clearly important to the agrochemical industry.

Plans for Next Year

Ongoing experiments are designed to elucidate the function of ethylene in causing late-season leaf greening and/or in causing hollow husk. We plan to identify and quantify proteins that are involved in producing ethylene responses/perception in plants. To analyze and quantify the abundance of proteins involved in ethylene response, a 2-dimensional (2D) gel electrophoresis approach will be taken. Such a proteomic approach would also allow us to analyze ethylene perception in maize at a global scale. Another approach would be to design and create antibodies to analyze key enzymes involved in ethylene synthesis in maize.

Publications

Uribelarrea M., Moose SP, and Below FE (2007). Divergent selection for grain protein affects nitrogen use in maize hybrids. Field Crops Res. 100:82-90.

Wessel, JR, Ruffo, ML, and Below FE (2007). Spring rainfall dictates the success of nitrogen applied to corn in fall and winter. *In*: Managing Crop Nitrogen for Weather. International Plant Nutrition Institute, Norcross, GA 30092-2837 USA.

Illinois AES, Hans J. Bohnert

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Objective 1: Photochemistry and the biogenesis of the photosynthetic apparatus.

Nothing to report here.

Objective 2: Photosynthetic capture and photorespiratory release of CO_2

Nothing to report here.

Objective 3: Mechanisms regulating photosynthate partitioning

- The idea of working on this topic presented itself based on the work with Arabidopsis in elevated [CO_2] (and ozone). The plants cannot make efficient use of the increased substrate for increasing biomass and this effect is also observed in other (crop) species. It seems, however, that soybean can escape the effect to a large degree, with indications that the escape is based on an increase in nitrogen fixation.
- Arabidopsis ecotypes grown in elevated [CO_2] seem to sense and respond to imaginary nitrogen deficiency. Our analysis of Col-0 and Cvi-0 in SoyFACE elevated [CO_2] indicated a number of genes that were regulated irrespective of local weather in 2002, 2003 and 2005 in the same direction. Among the ecotypes, Cvi-0 (adapted to a dry climate) seemed to show N-deficiency early in the FACE rings but then acclimated, while Col-0 failed to significantly acclimate over time (Li et al., submitted).
- When analyzing the genes that responded (and responded differently in the ecotypes over time) we identified several transcripts for proteins that have by others been associated with N-sensing and N-signaling. When these genes are modeled into the Arabidopsis whole genome GGM-based gene network we seem to identify the node that may be affected by elevated [CO_2] (Ma and Bohnert, 2007; Ma et al., 2007; see below).

Objective 4: Developmental and environmental limitations to photosynthesis

Abiotic stress in its many manifestations is one focus of the lab. That focus is represented by several projects:

- The analysis of T-DNA tagged Arabidopsis, Thellungiella and Oryza lines for altered stress perception and response (1),
- Metabolite profiling of soybean and Arabidopsis in SoyFACE rings (2), and
- Arabidopsis ecotypes and *Thellungiella halophila* in SoyFACE (3).
- <u>Can we use the accumulated data on Arabidopsis transcript profiling to generate gene networks?</u> (4)

(1) We have identified by tail-PCR some 240 tagged Arabidopsis lines that show altered responses to stress, as reported by a RD29A-luciferase construct. A large number of the genes that have been tagged has, or seems to have, a relationship to intracellular transport

processes, including RNA, protein and metabolite entities. We are characterizing several of these mutants at present, but, owing to a lack of funding, much of this work is now carried out by colleagues in China.

(2) Soybean metabolite profiles are taken in SoyFACE under ambient, high CO2, elevated ozone, and CO2 + O3 conditions (one graduate student). There is a lot that has still to be analyzed. We are collecting (as a part of the entire SoyFACE group) leave disks during day-night cycles in multiple samples from 16 rings during the entire growing season. Obvious are developmentally programmed changes in metabolites and signaling intermediates that are modulated by the different treatment conditions. Similar analyses are carried out with Arabidopsis Col-0 and Cvi-0

(3) Arabidopsis ecotypes and *Thellungiella halophila* in SoyFACE show very different responses to elevated ozone and/or CO2. We used *Arabidopsis thaliana* ecotypes Col-0, Cvi-0, WS and a closely related species *Thellungiella halophila* to apply the advantages of this model to analyze changes in gene expression and metabolite profiles of plants grown in "SoyFACE" (http://www.soyface.uiuc.edu/), a system of open-air rings within which CO_2 is elevated to ~550ppm. Data from multiple rings, comparing plants in ambient air and elevated CO_2, were analyzed by a mixed model ANOVA and data-mining tools. In elevated CO_2, decreases in the expression of genes related to chloroplast functions characterized all lines but individual members of distinct multi-gene families were regulated differently between lines. Also, different strategies distinguished the lines with respect to the regulation of genes related to carbohydrate biosynthesis and partitioning, N-allocation and amino acid metabolism, cell wall biosynthesis, and hormone responses. The evolutionary adaptation of species to their habitat and intrinsic genetic plasticity seem to determine the nature of responses to elevated CO_2. Irrespective of the underlying genetic diversity, common signature processes and pathways appear to determine organismal responses in FACE. Some points:

(i) Among the "signature" genes that changed in all ecotypes, we find several signal transduction intermediates, and also, genes for enzymes that seem to divert carbon from the commonly accumulating carbohydrate into complex carbohydrates, fatty acids and amino acids.

(ii) High CO2 that leads to altered stomatal conductance can generate a heat stress in some lines that are less adapted to high light.

(iii) The different ecotypes showed different growth behavior in the field, compared to growth chamber conditions, but neither CO2 nor ozone changed the ecotype-specific growth patterns.

(iv) Irrespective of different growth behavior of the ecotypes, the transcript profiles report the CO2 response and differences in this response between lines/ ecotypes that are independent of developmental differences.

(v) Thellungiella turns out to be superior to the Arabidopsis ecotypes. As mature plants, they are less affected than Arabidopsis ecotypes by a rise in ABA/JA/SA, high light, or temperature. The species is a "compulsive" accumulator of nearly everything the metabolite profiles detected.

(4) An Arabidopsis Gene Network. This new project is based on the data that have been deposited by many groups on Arabidopsis transcript profiling under many different conditions.

Thousands of *Arabidopsis thaliana* transcript profiling studies report transcript dynamics based on abiotic and biotic stresses, chemical treatments, or development. Organizing these datasets could reveal the structure of responses and cross-talk, and in which cells the plants perceive, signal, respond to and integrate environmental inputs. We have clustered *Arabidopsis* transcript profiles for >22,000 genes and >150 treatments, comprising abiotic, biotic and chemical stresses. A new clustering procedure resulted in ~180 clusters that explain the response dynamics of the Arabidopsis genome. In particular, we identify ubiquitous stress responses in *Arabidopsis* - similar to those of fungi and animals - that employ genes in pathways related to Snf1- or MAP-kinases, vesicle transport, mitochondrial functions, and the transcription machinery. The ABA-dependent transcriptome is clearly delineated in well-defined clusters, while functions dependent on reactive oxygen species are widely distributed, possibly indicating evolutionary pressures conferring distinction to different stresses in time and space.

In further studies, the Graphical Gaussian Model (GGM) was used to assemble a gene network for the *Arabidopsis* transcriptome. Based on partial correlation (pcor), GGM infers co-regulation patterns between gene pairs conditional on the behavior of other genes. We used 'regularized' GGM, coupled with iterative random samplings, to expand the network to cover the entire *Arabidopsis* genome. Several network variants include up to 50% of the *Arabidopsis* transcriptome. When querying for selected genes, locally coherent sub-networks emerge that are often related to metabolic functions and stress responses. GGM recovers interactions with biological significance that typically escape capture by Pearson correlation networks. Finally, the network reconciles individual sub-networks in a topology joined at the whole genome level, and provides a general framework that can instruct future studies on plant metabolism and stress responses. One example network, related to chloroplast functions is appended below.

The project started from my frustration – having carried out some 500 microarray hybridizations in the lab – about the often meaningless representation of results in the form of boring tables or "heatmaps". The mathematics behind the GGM program we owe to Dr. K. Strimmer, U. Leipzig, Germany, whose group worked on the GGM in 2005, and I owe the assembly of the Arabidopsis whole genome GGM to Dr. Shisong Ma, a student in my lab, now a postdoctoral fellow at Yale University.

References for this project:

Gong et al. (2005) Plant Journal 44:826. (Fuzzy k-means analysis)

Ma S, Gong Q, Bohnert HJ (2006) Journal Experimental Botany, 57: 1097. (Fuzzy k-means analysis)

Ma S, Bohnert HJ (2007) Genome Biology 8: R49. (Fuzzy k-means and root cell lineage specificity)

Ma S, Gong Q, Bohnert HJ (2007) Genome Research 17: epub October 2007. (Graphical Gaussian Model for the Arabidopsis genome)

Accomplishments

See publications, lectures at conferences and workshops. Teaching of graduate students in China (CAU, SNU, Guangxi U), August - September 2007.

Usefulness of findings

- The availability of the Arabidopsis genome sequence, of microarray platforms and metabolite profiles has permitted the analysis of ecological adaptive diversity within this species. Different ecotypes have very different lifestyles – the phenotypes that these ecotypes exhibit is increasingly visible in the results (at least: data) that can be sampled by genomics-type tools. Towards Arabidopsis haplotype maps?

- Arabidopsis is an excellent, maybe the best, model for conducting FACE experiments owing to the many tools, including more than 300 ecotypes that are available for this species. These ecotypes represent an immense evolutionary adaptive diversity that can be harnessed. The superior performance of the extremophile Thellungiella is a good sign for stress biology: It will be possible to transfer the entire Thellungiella genome into Arabidopsis, in the form of BAC clones.

Plans for the coming Year

Considering the funding situation – and maybe some "blacklisting" for not being "pliable" – I might decide that I should not waste more time writing grant proposals; after life after science is possible.

Publications

Poroyko V, Spollen W, Hejlik LG, Hernandez AG, LeNoble M, Davis G, Nguyen HT, Springer GK, Sharp RE, Bohnert HJ (2007) Comparing regional transcript profiles from well-watered and water-stressed maize primary roots. **Journal of Experimental Botany 58**: 279-289.

Lee J, Nam J, Park HC, Na G, Miura K, Jin JB, Yoo CY, Baek D, Kim DH, Jeong JC, Kim D, Lee SY, Salt D, Mengiste T, Gong Q, Ma S, Bohnert HJ, Kwak SS, Bressan RA, Hasegawa PM, Yun DJ (2007) Salicylic acid-mediated innate immunity in Arabidopsis is regulated by SIZ1 SUMO E3 ligase. **Plant Journal** 49**:** 79-90; e-pub, Dec 2007.

Talamè V., Oztürk ZN, Bohnert HJ, Tuberosa R (2007) Dynamics of water loss affects the differential expression of drought-related genes in barley. **Journal of Experimental Botany** 58: 229-240 (July), e-pub; November 2006.

Li P, Bohnert HJ, Grene R (2007) All about FACE – Plants in a high [CO_2] World. **Trends in Plant Sciences 12**: 87-89. epub (December 2006).

Sato K, Ohsato H, Izumi S, Miyazaki S, Bohnert HJ, Moriyama H, Fukuhara T (2007) Diurnal expressions of five protein phosphatase type 2C genes in the common ice plant, *Mesembryanthemum crystallinum*. **Functional Plant Biology 34**: 581-588.

Li, X., Wen Z, Bohnert, H.J., Schuler, M., Kushad, M. (2007). Cloning of a myrosinase cDNA from horseradish (*Armoaracia rusticana*) root and its heterologous expression in *Spodoptera frugiperda* insect cells. **Plant Science 172**: 1095-1102.

Ma S, Bohnert HJ (2007) Integration of *Arabidopsis thaliana* stress-related transcript profiles, promoter structures, and cell-specific expression. **Genome Biology 8**: R49; e-pub (April 2007).

Oh D-H, Gong Q, Ulanov A, Zhang Q, Li Y, Bressan RA, Yun D-J, Bohnert HJ (2007) Sodium stress in the halophyte *Thellungiella halophila* and transcriptional changes in a *thsos1*-RNAi line. **Journal of Integrative Plant Biology 49**: in press.

Burey SC, Poroyko, V, Oztürk, ZN, Fathi-Nejad, S, Schüller, C, Ohnishi N, Fukuzawa H, Bohnert, HJ and Löffelhardt, W (2007) Acclimation to low [CO_2] by an inorganic carbon concentrating mechanism in *Cyanophora paradoxa.* **Plant Cell & Environment**, in press.

Ma S, Bohnert HJ (2007) Gene Networks for the Integration and better Understanding of Gene Expression Characteristics. **Weed Science Journal**, in press (contribution to a symposium issue, peer reviewed).

Ma S, Gong Q, Bohnert HJ (2007) An Arabidopsis Gene Network based on the Graphical Gaussian Model. **Genome Research** 17: epub Oct 5, 2007.

Kim J-I, Shakhun A, Li P, Jeong J-C, Baek D, Lee S-Y, Blakeslee JJ, Murphy AS, Bohnert HJ, Hasegawa PM, Yun D-J, Bressan RA (2007) Activation of the Arabidopsis *HYPERTALL (HYT1/YUCCA6)* locus affects several auxin-mediated responses to a staygreen phenotype. **Plant Physiol 145**: in press (e-pub Sept. 2007).

Jonathan I. Watkinson, Lori Hendricks, Allan A. Sioson, Lenwood S. Heath, Hans J. Bohnert, Ruth Grene (2007) Tuber development phenotypes in adapted and acclimated, drought-stressed *Solanum tuberosum* ssp. *andigena* have distinct expression profiles of genes associated with carbon metabolism. **Plant Physiology and Biochemistry**. In press.

Submitted

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Bohnert HJ (2007) Abiotic Stress. In „**Encyclopedia of Life Sciences**“. (ed. R. Roberts), John Wiley & Sons, Chichester, UK; **http://www.els.net/ [DOI: 10.1002/9780470015902.a0020087.**

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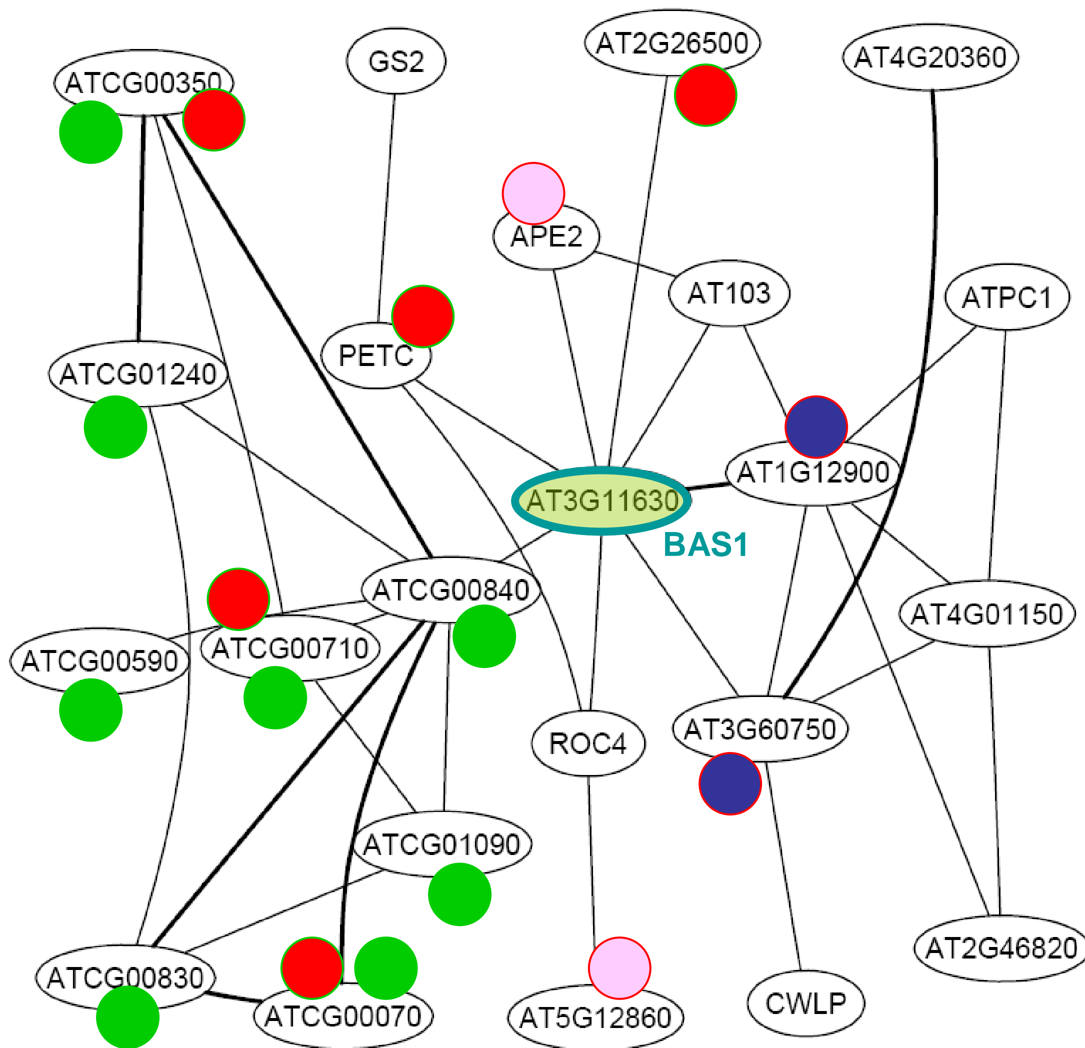
Fathinejad, S**,** Steiner JM, Reipert S, Marchetti M, Allmaier G, Burey SC, Ohnishi N, Fukuzawa H, Löffelhardt W, and Bohnert HJ (2007) A carboxysomal CCM in the cyanelles in the “coelacanth” of the algal world, *Cyanophora paradoxa*? **Physiologia Plantarum**, in press.



The figure shows a gene network (within two edges (connections) of BAS1, a chloroplast peroxiredoxin. Transcripts from chloroplast DNA are highlighted in green, photosystem components in red, transporters in pink, and carbohydrate metabolism in blue. For other transcripts see the table.

It must be realized that the GGM (which is significantly superior to the typically used Pearson correlation algorithm) shows all genes that are, at very high statistical significance, correlate with a seed gene (here: BAS1) considering expression under >150 experimental conditions and conditional to other genes in these experiments. In the example chosen one will find many genes that are known to be regulated by redox state.

Our GGM exists in three versions of different statistical stringency. It is particularly useful (and corroborated by many single-gene studies) for biochemical pathways and stress responses. Its best feature, however, may be that the GGM places isoforms of genes in families into different context. For example, the peroxiredoxins all associate with different other genes. As a second example, nine of the 11 trehalose-6-phosphate phosphatases in the Arabidopsis genome are visible in the network – 7 of 9 associate with different other genes.

Table. List of genes whose transcripts are associated with BAS1 by two connections.

APE2	1	Phosphate/triose-phosphate translocator, putative,
AT103	1	Dicarboxylate diiron protein, putative (Crd1), similar to leucine containing zipper protein At103; Copper response defect 1 (CRD1)
AT1G12900	1	Glyceraldehyde 3-phosphate dehydrogenase, chloroplast, putative / NADP-dependent glyceraldehydephosphate dehydrogenase
AT2G26500	1	Cytochrome b6f complex subunit (petM), putative; alternative spliceforms exist
AT2G46820	1	Expressed protein
AT3G11630	2	2-cys peroxiredoxin, chloroplast (BAS1), identical to SP:Q96291 2-cysperoxiredoxin BAS1
AT3G60750	2	Transketolase, putative, strong similarity to transketolase 1
AT4G01150	1	Expressed protein
AT4G20360	1	Elongation factor Tu / EF-Tu (TUFA)
AT5G12860	1	Oxoglutarate/malate translocator, putative,
ATCG00070	28	PSII K protein
ATCG00350	28	Encodes psaA protein.
ATCG00590	28	Hypothetical protein
ATCG00710	28	Encodes a 8 kD phosphoprotein that is a component of the photosystem II oxygen evolving core. May play a role in electron transfer between the secondary quinine acceptors, QA and QB, associated with the acceptor side of PSII.
ATCG00830	28	Encodes a chloroplast ribosomal protein L2,
ATCG00840	28	One of two chloroplast genes encoding chloroplast ribosomal protein L23
ATCG01090	28	Encodes a subunit of the chloroplast NAD(P)H dehydrogenase complex
ATCG01240	28	30S chloroplast ribosomal protein S7
ATPC1	1	ATP synthase gamma chain 1, chloroplast (ATPC1)
CWLP	2	Protease inhibitor/seed storage/lipid transfer protein (LTP) familyprotein,
GS2	2	Glutamine synthetase (GS2), chloroplast precursor
PETC	1	Cytochrome B6-F complex iron-sulfur subunit, chloroplast / Rieske iron-sulfur protein / plastoquinol-plastocyanin reductase (petC)
ROC4	2	Peptidyl-prolyl cis-trans isomerase, chloroplast / cyclophilin / rotamase

The numbers reflect the cluster number of these genes as determined by fuzzy k-means clustering (Ma and Bohnert, 2007), i.e. some genes are correlated with the seed gene in cluster 1, 2, or 28, etc.

Illinois ARS, Archie R. Portis Jr.

NC-1168 ANNUAL REPORT 2007

Progress on Objective 2: Photosynthetic capture and photorespiratory release of CO_2.

1a) *Genetic engineering of Rubisco in tobacco.* We demonstrated that RNAi directed at the 3' UTR region of the native tobacco small subunit genes was effective in strongly suppressing their expression while allowing the expression of a small subunit gene from the C4 plant *Amaranthus retroflexus*. Transgenic plants with both the RNAi and Amaranthus small subunit gene constructs were able to grow under ambient CO_2 conditions, although photosynthesis was somewhat impaired, whereas the RNAi only plants required elevated CO_2 to achieve sufficient growth to maturity.

1b) Methods to reduce or eliminate native small subunit gene expression from the nucleus may be needed to allow adequate expression of both large and small subunits of a foreign Rubisco, which has properties that can result in more photosynthesis at the higher CO_2 levels associated with global climate change.

2a) *Improving the photosynthesis of plants at moderately higher temperatures*. We demonstrated that photosynthesis and growth by Arabidopsis at moderately higher temperatures could be improved by transforming the *rca* mutant (which does not express activase) with a chimeric and more thermostable activase gene. The chimeric construct consisted of the more thermostable tobacco activase in which the Rubisco recognition domain was replaced with that from Arabidopsis.

2b) Improving the photosynthetic potential of plants may be necessary to maintain crop productivity as global temperatures increase, which is predicted by climate change models.

Research Plans:

(1) Continue attempts to crystallize Rubisco activase using site-directed mutagenesis to modify its properties (in collaboration with Dr. Salvucci, USDA-Arizona)

(2) Develop an activase-Rubisco binding assay using His-tagged tobacco Rubisco to allow studies of the effects mutations and temperature on the interaction.

Publications:

Salvucci, M.E., DeRidder, B.P. and Portis, A.R. Jr. (2006) Effect of activase level and isoform on the thermotolerance of photosynthesis in *Arabidopsis*. J. Exp. Bot. 57: 3793-3799.

Wang, D and Portis, A.R. Jr. (2007) A novel nucleus-encoded chloroplast protein, PIFI, is involved in NAD(P)H dehydrogenase complex-mediated chlororespiratory electron transport in Arabidopsis. Plant Physiol. 144: 1742-1752

Portis, A.R. Jr. and Marry, M.A.J. (2007) Discoveries in Rubisco (Ribulose 1,5-bisphosphate carboxylase/oxygenase): a historical perspective. Photosynth. Res. 94: 121-143

Abstracts:

Kumar, A., Li, C. and Portis, A.R. The rate of photosynthesis remains relatively high at moderately high temperatures in *Arabidopsis thaliana rca* mutant expressing thermostable chimeric Rubisco activase. Plant Biology 2007 Program, ASPB annual meeting, Chicago, IL. url: http://abstracts.aspb.org/pb2007/public/P13/P13007.html

Stessman, D. and Portis, A.R. Expression of a foreign Rubisco small subunit in tobacco with reduced levels of native protein. Plant Biology 2007 Program, ASPB annual meeting, Chicago, IL. url: http://abstracts.aspb.org/pb2007/public/P13/P13012.html

Iowa AES, Steve Rodermel

NC-1168 ANNUAL REPORT 2007

Objective 1. Plastid Function and Intracellular Communication

The *var2* variegation mutant of Arabidopsis arises as a consequence of a lesion in the nuclear gene for a chloroplast AtFtsH metalloprotease. The mutant plants have green- and white-sectored leaves, and they are hypersusceptible to photoinhibition (photosynthesis-induced light stress). Consistent with this phenotype, VAR2 is involved in the D1 repair cycle of Photosystem II, likely by affecting turnover of the photodamaged D1 polypeptide. Because VAR2 plays a central role in protecting plants against light stress, it is critical to understand the function of this protein in photosynthesis and light stress, and how its activity and expression are regulated.

As one approach to address these questions, we have conducted a genetic suppressor screen. These sorts of analyses allow one to define proteins that interact with a protein-of-interest to control its function (directly or indirectly). Second-site suppressor screens of *var2* yielded several lines in which the variegation phenotype of *var2* is significantly altered. Some of these lines have a "central yellow" (CY) phenotype, in which the basal part of younger leaves on the rosette are pale-green or yellow, then turn fully-green as they develop and expand. Two suppressor lines with a CY phenotype, *2484* and *24107*, were chosen for further analysis. Map-based cloning revealed that the suppressor gene in *2484* encodes a plastid Polyribonucleotide Phosphorylase (PNPase), which is involved in RNA processing and in the translation of chloroplast protein. We designated this gene *CY1*. Isolation of the *cy1* single mutant showed that it has a phenotype similar to the *cy1/var2* double mutant, which suggests that *cy1* is epistatic to *var2*. Because *var2* and *cy1* are recessive, the results suggest that *VAR2* and *CY1* act antagonistically in chloroplast biogenesis, and that down-regulation of *CY1* reduces the requirement for *VAR2* in photosynthesis and plastid development.

Map-based cloning has revealed that the mutation in the suppressor line, *24107*, is due to a lesion in the gene *At5g18820*, which encodes a chloroplast GroEL-like chaperone, termed "chaperonin 60 alpha-like protein" (or "Cpn60 alpha-like"). Previous studies have shown that this protein mediates the folding (and/or unfolding) of many chloroplast proteins (including Rubisco), and that it is important in assembling the photosynthetic apparatus during early plant development. The gene for this protein has been designated *CY2*. Independent lines with *cy2* single mutations have been generated, and like *cy1*, *cy2* single mutants have a phenotype similar to the *cy2/var2* double mutant, suggesting that *cy2* is epistatic to *var2*.

Usefulness of findings

Chloroplast proteases, such as the VAR2 FtsH metalloprotease, play an important role in assembling and maintaining the photosynthetic apparatus. Further insight into the function of this protein in photosynthesis, plant development and plant stress responses might lead to the design of strategies to manipulate the photosynthetic capacity and quality of crop plants

Work planned for next year

We will continue to characterize the two VAR2 suppressors described above (*cy1* and *cy2*) and the functions of the *CY1* and *CY2* gene products in chloroplast biogenesis and thylakoid membrane function. We will also undertake genetic complementation, double mutant, and further molecular analyses to elucidate the relationships between *CY1, CY2* and *VAR2*.

Publications (2006-)

Aluru, M.R., F. Yu, A. Fu and S. Rodermel. 2006. *Arabidopsis* variegation mutants: new insights into chloroplast biogenesis. **J. Experimental Botany** 57: 1871-1881. (**Refereed review**).

Rosso, D. A.G. Ivanov, A. Fu, J. Geisler-Lee, L. Hendrickson, M. Geisler, G. Stewart, M. Krol, V. Hurry, S.R. Rodermel, D.P. Maxwell and N.P.A. Hüner. 2006. IMMUTANS does not act as a stress-induced safety valve in the protection of the photosynthetic apparatus of Arabidopsis during steady state photosynthesis. **Plant Physiol**. 142: 574-585.

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Yu, F., A. Fu, M. Aluru, S. Park, Y. Xu, H. Liu, X. Liu, A. Foudree, M. Nambogga and S. Rodermel. 2007. Variegation mutants and mechanisms of chloroplast biogenesis. **Plant Cell & Environment** 30: 350-365. (**Refereed review**) (**Cover Article**).

Varbanova, M., S. Yamaguchi, Y. Yang, K. McKelvey, A. Hanada, R. Borochov, F. Yu, Y. Jikumaru, J. Ross, D. Cortes, C.-J. Ma, J. P. Noel, L. Mander, V. Shulaev, Y. Kamiya, S. Rodermel, D. Weiss and E. Pichersky. 2007. Methylation of gibberellins by *Arabidopsis* GAMT1 and GAMT2. **Plant Cell** 19: 32-45.

Taber, H., S. Li, W. White, S. Rodermel and Y. Xu. 2007. Enhancement of tomato fruit lycopene by potassium is cultivar dependent**.** **HortScience** 42: 1-7.

Iowa AES, Martin Spalding

NC-1168 ANNUAL REPORT 2007

Objective 2. Photosynthetic Capture and Photorespiratory Release of CO_2

Microalgae grow under extremely low CO_2 concentrations via operation of an inducible CO_2-concentrating mechanism (CCM) that uses active inorganic carbon (C_i) transport to increase their internal CO_2 concentration. The microalga *Chlamydomonas reinhardtii*, a robust genetic model, exists in 3 CO_2 regulated states, a high CO_2 ($\geq$0.5% CO_2) state lacking CCM induction, a low CO_2 state (0.4% - 0.03% CO_2) with CCM induction and a very low CO_2 ($\leq$0.01% CO_2) state with a more active CCM. The distinction between the low CO_2 state and the novel very low CO_2 state is highlighted by a mutant, *pmp1*, which grows well in high or very low CO_2 but dies in low CO_2.

Many photosynthetic microorganisms are capable of acclimating to CO_2 limited environments by induction and operation of CCMs. In spite of their central role in CCM function, Ci transport systems have never been identified in eukaryotic photosynthetic organisms. In *C. reinhardtii*, a mutant, *pmp1*, was described in 1983 with deficiencies in Ci transport, and a Pmp1 protein associated Ci uptake system has thus been proposed to be responsible for the Ci uptake in low CO_2 (air level) acclimated cells. However, even though *pmp1* represents the only clear genetic link to Ci transport in microalgae and is one of only a very few mutants directly affecting the CCM itself, the identities of Pmp1 and the Pmp1 associated Ci transport system have remained unknown. Physiological analyses indicate that *C. reinhardtii* possesses multiple Ci transport systems responsible for acclimation to different levels of limiting CO_2, and that the Pmp1 associated transport system is specifically required for low (air level) CO_2 acclimation. We identified and characterized a *pmp1* allelic mutant, *air dier 1* (*ad1*), which, like *pmp1*, cannot grow in low CO_2 (350 ppm) but can grow either in high CO_2 (5% CO_2 in air) or in very low CO_2 (below 200 ppm). Molecular analyses revealed that the Ad1/Pmp1 protein is encoded by *LciB*, a gene previously identified as a CO_2 responsive gene. The *LciB* gene and three related genes in *C. reinhardtii* compose a unique gene family that encode four closely related, apparently soluble plastid proteins with no clearly identifiable conserved motifs.

Usefulness of findings

Understanding the mechanisms that allow *Chlamydomonas* to acclimate to such low CO_2 concentrations and identifying the genes involved is important for evaluating the potential for transfer of all or part of this CCM into higher plants, as well as to increase our understanding of CO_2 assimilation and its regulation in this key group of photosynthetic organisms.

Work planned for next year

We will continue to investigate the LCIB family of proteins and their role in Ci transport and accumulation in *C. reinhardtii*. Our plans include investigation of the localization of the members of this family of proteins, identification and characterization of proteins that interact

with members of this protein family and characterization of *pmp1/ad1* suppressors already identified.

Relevant Publications (2006-)

Wang, Yingjun, MH Spalding 2006 An inorganic carbon transport system responsible for acclimation specific to air levels of CO_2 in *Chlamydomonas reinhardtii*. Proceedings of the National Academy of Sciences USA 103:10110-10115.

Grossman, AR, M Croft, VN Gladyshev, S Merchant, M Posewitz, S Prochnik, MH Spalding 2007 Novel metabolism in *Chlamydomonas* through the lens of genomics. Current Opinion in Plant Biology 10:190-198.

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Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, Terry A, Salamov A, Fritz-Laylin LK, Marechal-Drouard L, Marshall WF, Qu LH, Nelson DR, Sanderfoot AA, Spalding MH, Kapitonov VV, Ren Q, Ferris P, Lindquist E, Shapiro H, Lucas SM, Grimwood J, Schmutz J, Cardol P, Cerutti H, Chanfreau G, Chen CL, Cognat V, Croft MT, Dent R, Dutcher S, Fernandez E, Fukuzawa H, Gonzalez-Ballester D, Gonzalez-Halphen D, Hallmann A, Hanikenne M, Hippler M, Inwood W, Jabbari K, Kalanon M, Kuras R, Lefebvre PA, Lemaire SD, Lobanov AV, Lohr M, Manuell A, Meier I, Mets L, Mittag M, Mittelmeier T, Moroney JV, Moseley J, Napoli C, Nedelcu AM, Niyogi K, Novoselov SV, Paulsen IT, Pazour G, Purton S, Ral JP, Riano-Pachon DM, Riekhof W, Rymarquis L, Schroda M, Stern D, Umen J, Willows R, Wilson N, Zimmer SL, Allmer J, Balk J, Bisova K, Chen CJ, Elias M, Gendler K, Hauser C, Lamb MR, Ledford H, Long JC, Minagawa J, Page MD, Pan J, Pootakham W, Roje S, Rose A, Stahlberg E, Terauchi AM, Yang P, Ball S, Bowler C, Dieckmann CL, Gladyshev VN, Green P, Jorgensen R, Mayfield S, Mueller-Roeber B, Rajamani S, Sayre RT, Brokstein P, Dubchak I, Goodstein D, Hornick L, Huang YW, Jhaveri J, Luo Y, Martinez D, Ngau WC, Otillar B, Poliakov A, Porter A, Szajkowski L, Werner G, Zhou K, Grigoriev IV, Rokhsar DS, Grossman AR 2007 The *Chlamydomonas* genome reveals evolutionary insights into key animal and plant functions. Science 318:245-250.

Spalding, MH (in press) Microalgal carbon-dioxide-concentrating mechanisms: *Chlamydomonas* inorganic carbon transporters. Journal of Experimental Botany. (refereed, invited mini-review)

Kentucky AES, Robert L. Houtz

NC-1168 ANNUAL REPORT 2007

Objective 2: Photosynthetic capture and respiratory release of CO_2

Accomplishments - Processive versus distributive methyl group transfer was assessed for pea Rubisco large subunit methyltransferase, a SET domain protein lysine methyltransferase catalyzing the formation of trimethyllysine-14 in the large subunit of Rubisco. Catalytically competent complexes between an immobilized form of des(methyl) Rubisco and Rubisco large subunit methyltransferase were used to demonstrate enzyme release that was co-incident with and dependent on formation of trimethyllysine. Catalytic rate constants determined for formation of trimethyllysine were considerably lower (~10-fold) than rate constants determined for total radiolabel incorporation from [^{3}H-*methyl*]-*S*-adenosylmethionine. Double-reciprocal velocity plots under catalytic conditions favoring monomethyllysine indicated a random or ordered reaction mechanism, while conditions favoring trimethyllysine suggested a hybrid ping-pong mechanism. These results were compared with double-reciprocal velocity plots and product analyses obtained for *Hs*SET7/9 (a monomethyltransferase) and *Sp*CLR4 (a dimethyltransferase) and suggest a predictive ability of double-reciprocal velocity plots for single versus multiple methyl group transfers by SET domain protein lysine methyltransferases. A model is proposed for SET domain protein lysine methyltransferases in which initial binding of polypeptide substrate and *S*-adenosylmethionine is random, with polypeptide binding followed by deprotonation of the ε-amine of the target lysyl residue and subsequent methylation. Following methyl group transfer, *S*-adenosylhomocysteine and monomethylated polypeptide dissociate from monomethyltransferases, but di- and trimethyltransferases begin a successive and catalytically obligatory deprotonation of enzyme-bound methylated lysyl intermediates, which along with binding and release of *S*-adenosylmethionine and *S*-adenosylhomocysteine is manifested as a hybrid ping-pong-like reaction mechanism.

The polypeptide substrate specificity determinants for pea Rubisco large subunit methyltransferase were determined using a fusion protein construct between the first 23 amino acids from the large subunit of Rubisco and human carbonic anhydrase II. A total of 40 conservative and non-conservative amino acid substitutions flanking the target Lys-14 methylation site (positions P_3 to P_3) were engineered in the fusion protein. The catalytic efficiency (*k*cat/ *Km*) of *Ps*LSMT was determined using each of the substitutions and a polypeptide consensus recognition sequence deduced from the results. The consensus sequence, represented by *X*-(Gly/Ser)-(Phe/Tyr)-Lys-(Ala/Lys/Arg)-(Gly/Ser)-π, where *X* is any residue, Lys is the methylation site, and π is any aromatic or hydrophobic residue, was used to predict potential alternative substrates for *Ps*LSMT. Four chloroplast-localized proteins were identified including γ-tocopherol methyltransferase (γ-TMT). *In vitro* methylation assays using *Ps*LSMT

and a bacterially expressed form of γ-TMT from *Perilla frutescens* confirmed recognition and methylation of γ-TMT by *Ps*LSMT *in vitro*. RNA interference-mediated knockdown of the *Ps*LSMT homologue (*Nt*LSMT) in transgenic tobacco plants resulted in a 2-fold decrease of α-tocopherol, the product of _γ-TMT. The results demonstrate the efficacy of consensus sequence-driven identification of alternative substrates for *Ps*LSMT as well as identification of functional attributes of protein methylation catalyzed by LSMT.

My laboratory continues to characterize the significant decrease in catalytic efficiency of non-methylated fructose-1,6 bisphosphate aldolase from tobacco Rubisco LSMT knock-down plants, and have determined that the loss is due to decreases in both K_m and k_{cat}. Currently we are conducting metabolic profile analyses to determine the ramifications of decreased FBP aldolase activity *in vivo*.

We also study plant-specific peptide deformylase and the structure of Arabidopsis peptide deformylase 2 (*At*DEF2; EC 3.5.1.88), has been determined at a resolution of 2.4 Å. The overall fold and symmetry of DEF2 is similar to eubacterial and mitochondrial forms of DEF (DEF1). DEF2 is essential in plants and has preferred substrate specificity towards the photosystem II D1 polypeptide. A comparative sequence analyses coupled with structural overlays between DEF2, DEF1, and eubacterial DEFs, identified (perhaps we can put here the exact number of residues) several residues which may contribute to the unique catalytic properties of DEF2. Molecular docking models of the N-terminal five amino acids from the D1 polypeptide into the active-site of DEF2 suggest an influence of Tyr-178 in DEF2 as a structural determinant for polypeptide substrate specificity through hydrogen bonding with Thr-2 in the D1 sequence. Kinetic analyses using a polypeptide mimic of the D1 N-termini was performed on DEF2 mutagenized at Tyr-178 to Ala, Phe, or Arg (equivalent residue in DEF1). The results suggest that while Tyr-178 can influence catalytic activity, other residues contribute to the overall preference for the D1 polypeptide.

Usefulness of Findings – Understanding the role and structure/function relationships for Rubisco LSMT is uncovering a potentially significant and unknown mechanism for the regulation of several chloroplast-localized enzymes. These findings may be useful for understanding how carbon reactions in the chloroplast can be altered to enhance photosynthetic efficiency. Understanding peptide deformylase provides the unique opportunity to explore potential alternatives to anti-biotic resistance markers in plant transformation vectors as well as alternative broad-spectrum herbicides.

Plans for the coming year – We will continue structure/function studies of Rubisco LSMT and peptide deformylase. Additional studies will characterize the molecular basis for the changes in enzyme activity observed in the absence of site-specific methylation for specific chloroplast-localized enzymes.

Publications –

Magnani,R., N.R.Nayak, M.Mazarei, L.M.Dirk, and R.L.Houtz. 2007. Polypeptide substrate specificity of PsLSMT. A set domain protein methyltransferase. J. Biol. Chem. 282:27857-

27864.

Dirk,L.M., E.M.Flynn, K.Dietzel, J.F.Couture, R.C.Trievel, and R.L.Houtz. 2007. Kinetic manifestation of processivity during multiple methylations catalyzed by SET domain protein methyltransferases. Biochemistry 46:3905-3915.

Maine AES, Mary E. Rumpho

NC-1168 ANNUAL REPORT 2007

Objective addressed: Obj. 1. Plastid Function and Intracellular Communication

Background: My lab group is studying the endosymbiotic relationship between the marine mollusc *Elysia chlorotica* (sea slug) and chloroplasts of the heterokont alga, *Vaucheria litorea*. The sea slug has acquired the ability to sustain itself photosynthetically as a result of acquiring chloroplasts from the alga and retaining them intracellularly in cells lining the digestive diverticula. The retained chloroplasts, or “kleptoplasts,” remain intact and photosynthetically competent in direct contact with the animal nucleo-cytosol for up to ten months, despite the absence of any algal nuclei (1). The association between *E. chlorotica* and *V. litorea* chloroplasts is not heritable. Biochemical, microscopic, and molecular analysis has yielded no evidence for the presence of chlorophyll, plastids, or cpDNA in the sea slug eggs (1, 2). It is estimated that between 1,000 and 5,000 nuclear-encoded chloroplast-targeted proteins are needed to sustain the full metabolic capability of chloroplasts. The consequences of severing the chloroplast-alga, nucleo-cytosolic communication network in the sea slug cells are unknown. However, one would expect it to lead at a minimum to uncoordinated chloroplast activity and more likely, to the rapid demise of the organelles, unless the sea slug can provide the essential nuclear encoded gene products to the chloroplasts through horizontal gene transfer (HGT). There is growing interest in HGT, the transfer of genetic material between organisms that do not mate. HGT is common among prokaryotes and is becoming more commonly reported for mitochondrial genes of parasitic and epiphytic plants (3-5). However, HGT between eukaryotic, multicellular organisms of two different kingdoms has not been convincingly demonstrated. We have obtained preliminary evidence for horizontal transfer of genes encoding two algal nuclear encoded chloroplast proteins from the alga to the sea slug and are pursuing confirming their integration into the sea slug chromosome. *E. chlorotica* provides a rare opportunity to examine how genetic and biochemical components from two extremely divergent organisms have evolved to form a functional and productive photosynthetic union. Understanding the process of endosymbiotic plastid acquisition and retention offers unique insight into the establishment of regulatory processes that coordinate nuclear and plastid functions.

Accomplishments: We have successfully reared *E. chlorotica* in the laboratory and demonstrated that *V. litorea* can serve as the sole source of kleptoplasts. However, sequencing of the *V. litorea* chloroplast genome revealed that it does not contain all the genes necessary to sustain the observed activity in the sea slug. A more plausible source for these essential proteins is horizontal gene transfer (HGT) from the algal nucleus to the sea slug. We focused on the genes for two essential nuclear-encoded chloroplast-localized photosynthetic proteins, phosphoribulokinase (PRK) and the photosystem II Mn-stabilizing protein (PsbO or oxygen

evolving protein). We first cloned and characterized *prk* from *V. litorea*. Using PCR and primers designed against the algal *prk* sequence, two copies of partial PRK genes were detected in sea slug DNA. Each copy contains the nucleotide region spanning exon 1 and part of exon 2 of *V. litorea prk*, including the bipartite chloroplast transit peptide. However, the larger *prk* fragment possesses intron 1 while the smaller fragment is intronless. Both *prk* copies were detected in adult animals containing kleptoplasts as well as in plastid-free eggs produced by the sea slugs in culture. The exon and intron sequences of *prk* in *E. chlorotica* animals and eggs, and that of *V. litorea*, are almost identical. These data suggest that at least a portion of the *V. litorea* PRK gene was horizontally transferred at least once from the algal nuclear genome to *Elysia*, and it was a very recent event. In a similar manner we cloned the algal PsbO gene and used primers and PCR to demonstrate *psbO* in both sea slugs and sea slug eggs. In addition, using RT-PCR and N-blotting, we have shown that the gene is also expressed in adult animals. Sequencing of the sea slug mitochondrial genome ruled out insertion of the foreign genes into this organellar genome. Rather, we hypothesize that they are integrated into the sea slug chromosomal DNA and under the control of the animal's nucleus. We are in the process of verifying that the algal gene fragment sequences are integrated into the animal chromosomal and flanked by sea slug DNA. Further identification and characterization of DNA elements that have been horizontally transferred to the sea slug, integrated, expressed and targeted to the foreign chloroplasts, may explain how the engulfed plastids are maintained.

Usefulness of Findings: Characterization of this endosymbiotic association may provide a further understanding of chloroplast autonomy and the requirements for nuclear-cytosolic interactions to sustain chloroplast structure and function. Ultimately, this may add to the information necessary for maintaining isolated organelles in culture or foreign hosts for long periods of time, enabling large scale production of chloroplast secondary products in isolation and/or alternative forms of photosynthetic energy production to be developed. On a much broader scale, this is an opportunity to study inter-kingdom horizontal gene transfer, its propensity for widespread occurrence, and the potential for evolution of photosynthesis in a typically non-photosynthetic lineage.

Plans for Next Year: 1) Identify HGT on a more global scale using 454 GS-FLX sequencing of over 25,000 sea slug transcripts enhanced for photosynthesis-related messages. 2) qRT-PCR will be used to analyze the expression of several chloroplast genes, and the two nuclear genes encoding chloroplast proteins already identified in the sea slug (*psbO*, *prk*), in both alga and sea slug RNA. This analysis will help illuminate the role of the nucleo-cytosol in regulating chloroplast gene expression and vice versa. 3) We will examine PRK at the enzyme activity level to characterize redox regulation in the alga and compare with the sea slug (no algal-nuclear regulation) over the course of the animal's life-span. 4) Finally, we hope to find a collaborator to pursue characterizing the chloroplast thylakoid proteome of the sea slug over time (0 to 10 months). The results will be compared with the alga to determine the stability and capacity for *de novo* synthesis of kleptoplast/chloroplast proteins.

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Michigan AES, Christoph Benning

NC-1168 ANNUAL REPORT 2007

Objective 3: Mechanisms Regulating Photosynthate Partitioning

The conversion of photosynthate in the form of sugars into triacylglycerol in developing seeds is a classic example for the developmental and tissue-specific regulation of metabolism. Regulation of this pathway occurs at multiple levels (Hills, 2004). Global analysis of mRNAs in developing seeds of Arabidopsis provided a snapshot of specific expression profiles of genes encoding enzymes predicted or known to be involved in the underlying pathways (Ruuska et al., 2002). Typically, transcription factors affect these expression profiles. For example, WRINKLED 1 (WRI1), proposed to be involved in controlling oil biosynthesis was recently identified in Arabidopsis (Baud et al., 2007a; Cernac and Benning, 2004; Masaki et al., 2005). The corresponding *wri1* mutant is deficient in seed oil biosynthesis and shows a reduction in the activity of glycolytic enzymes, impaired seedling germination and seedling establishment (Focks and Benning, 1998; Cernac et al, 2006; Baud et al., 2007a). Under Objective 3 we are currently investigating the function and molecular biology of WRI1 and the genes controlled by this transcription factor. We and others have identified plastid pyruvate kinase as one of the potential target genes and have characterized its central role in carbon partitioning in oil seeds (Andre et al., 2007; Baud et al. 2007b). Efforts are currently under way to understand the molecular biology of WRI1 and to use the WRI1 transcription factor to alter carbon partitioning in different plant tissues with the goal to increase oil yields.

Plastid pyruvate kinase plays a central role in seed oil carbon partitioning.
Glycolysis is a ubiquitous pathway thought to be essential for the production of oil in developing seeds of Arabidopsis and oil crops. Compartmentation of primary metabolism in developing embryos poses a significant challenge towards testing this hypothesis and for the engineering of seed biomass production. It also raises the question whether there is a preferred route of carbon from imported photosynthate to seed oil in the embryo. Plastidic pyruvate kinase catalyzes a highly regulated, ATP-producing reaction of glycolysis. The Arabidopsis genome encodes 14 putative isoforms of pyruvate kinases. Three genes encode subunits α, β1, and β2 of plastidic pyruvate kinase. The plastid enzyme prevalent in developing seeds likely has a subunit composition of 4α4β1, is most active at pH 8 and is inhibited by glutamate. Disruption of the gene encoding the β1 subunit causes a reduction in plastidic pyruvate kinase activity and 60% reduction in seed oil content. The seed oil phenotype is fully restored by expression of the β1 subunit-encoding cDNA, and partially by the β2 subunit-encoding cDNA. The *pkp1* mutant showed delayed germination, and seedling establishment was dependent on an exogenous sugar supply. However, it appears that these phenotypes are not exclusively caused by a lack of seed

oil and may be related to reduced PKp activity in germinating seeds. Increasing the sucrose concentration in the medium further inhibited germination of *pkp1*, possibly due to the accumulation of soluble sugars in seeds. Germinating seeds of *pkp1* were unable to metabolize storage oil and cannot utilize applied sucrose for hypocotyl elongation in the dark. Moreover, *pkp1* contained less tocopherol and chlorophyll than wild type. Taken together, the identified pyruvate kinase catalyzes a crucial step in the conversion of photosynthate into oil suggesting a preferred plastid route from its substrate phosphoenolpyruvate to fatty acids. The results are consistent with a model in which PKp is required for the efficient conversion of sugar into precursors for different anabolic pathways.

The Role of Glucose 6-Phosphate Dehydrogenase in Carbon Partitioning.
Oil biosynthesis not only requires carbon but also reducing equivalents in the form of NAPH/H^+ or NADH/H^+. Glucose-6-phosphate dehydrogenase (G6PDH) has been implicated in the supply of reduced nicotine amide cofactors for biochemical reactions and in modulating the redox state of cells. In plants, identification of its role is complicated due to the presence of several isoforms in the cytosol and plastids. We focused on G6PDHs in the cytosol of *Arabidopsis thaliana* using single and double mutants disrupted in the two cytosolic G6PDHs. Only a single G6PDH isoform remained in the double mutant and was present in chloroplasts, consistent with a loss of cytosolic G6PDH activity. The activities of the cytosolic isoforms G6PD5 and G6PD6 were reciprocally increased in single mutants with no increase of their respective transcript levels. We hypothesized that G6PDH plays a role in supplying NADPH for oil accumulation in developing seeds in which photosynthesis may be light limited. G6PDH activity in seeds derived from G6PD6 and a plastid G6PDH isoform and showed a similar temporal pattern as oil accumulation. Seeds of the double mutant but not of the single mutants had higher oil content and increased weight compared to those of the wild type, with no alteration in the carbon/nitrogen ratio or fatty acid composition. A decrease in total G6PDH activity was observed only in the double mutant. These results suggest that loss of cytosolic G6PDH activity affects the metabolism of developing seeds by increasing carbon substrates for synthesis of storage compounds rather than by decreasing the NADPH supply specifically for fatty acid synthesis.

Altering Carbon Partitioning using WRI1.
The availability of transcription factors controlling genes involved in seed maturation and storage accumulation provides targets to engineer carbon partitioning into oil in different tissues or to adopt a process to convert sugar into oil by seedling fermentation. As the ectopic expression of WRI1 leads to embryos or embryo-like tissues producing oil when fed with sugars, one could envision a process in which, for example, seeds over-expressing WRI1 are germinated in a reactor that can be supplied with sugar containing liquid substrate. Under those conditions embryo like tissues or seedlings should be able to accumulate oil as we have already shown for Arabidopsis seedlings expressing a WRI1 cDNA (Cernac and Benning, 2004). Sugars could be provided as lignocellulose digests as it appears that plant tissues can tolerate and utilize lignocellulosic products better than microorganisms currently employed for fermentative production of biofuels. Towards this goal we are currently optimizing the conditions under which transgenic Arabidopsis seedlings produce oil following germination. Testing different sugars we found that xylose, a major ingredient in lignocellulosic digests is converted. In comparison, microorganisms do not readily metabolize pentoses. We have also generated transgenic canola (*Brassica napus*) lines expressing the *AtWRI1* cDNA under the control of

different promoters. The T2 seedlings will be soon tested for their ability to convert sugars into oil. Combining agricultural and industrial processes based on plant seedlings might provide an alternative to the production of high yielding oil crops in the field. The overall process should generate approximately 5-fold more oil per land used than canola itself. A second avenue to ectopically produce oil in field grown crops is the expression of the *AtWRI1* cDNA in rutabaga (*Brassica napus*) storage roots. Efforts to generate transgenic lines are currently under way.

Usefulness of the Results

Understanding the conversion of photosynthetic sugars into triacylglycerols will be essential to the engineering of novel biofuel crops. Triacylglycerols found in plant oils are the feed stock for biodiesel, fatty acid methylesters derived from plant oils. Current seed oil crops have not sufficient yields per area to be considered a reasonable alternative for replacing a substantial fraction of fossil liquid transportation fuels. However, by developing schemes and new biofuel crops which lead to improved oil yield per area of five-fold or more, biodiesel from plant oils could make up a significant fraction of renewable fuels. Developing rutabaga as a biofuel crop might provide Michigan's sugar beet farmers with an alternative biofuel crop, that could be handled, harvested, and processed using methods established for sugar beets.

Plans for the Upcoming Year

A major focus will be on the development of a WRI1 antibody needed for the study of the WRI1 protein and its turnover in transgenic plants. Identification and direct demonstration of target genes of WRI1 remains a high priority. We expect that a number of transgenic canola and rutabaga lines will become available and will be tested for the expression of WRI1 and the possible production of oil. Optimization of the seedling fermentation protocol will continue in Arabidopsis. In addition, as transgenic canola seeds become available, the system will be transferred from Arabidopsis to canola and a rigorous feasibility study will be conducted.

Publications

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Michigan AES, Wayne Loescher

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In one project we (Rebecca, lead PI, myself, and Jim Hancock) seek to examine fitness and secondary effects of alternate approaches to genetic engineering of salt stress resistance. We are comparing effects of constitutive expression of the abiotic stress responsive transcription factor gene *CBF3*, the mannitol biosynthetic enzyme gene, *M6PR*, which is associated with osmotic adaptation, and the plasma membrane sodium antiporter gene, *SOS1* in transgenic Arabidopsis. Three expression-verified lines are being studied for each gene. Growth chamber experiments established growth conditions and salt stress levels to examine effects of the genes and compare extent of protection offered against salt stress. Global gene expression analyses will be performed on the different transgenic lines and controls by Affymetrix gene chip assay. Studies of fitness costs or benefits of the different genes will be performed for multiple generations in the field, and in control and salt stress conditions in the greenhouse. More details can be provided.

In another project, focusing on stress tolerance and sugar alcohols, we have also performed further characterization of the effectiveness of the *M6PR* gene both in Arabidopsis and its original source, celery. Arabidopsis studies demonstrating effectiveness of M6PR in conferring salt tolerance and reducing loss in photosynthetic activity and PSII efficiency was published this year (Sickler et al., 2007). The work in celery, using antisense *M6PR*, showed that transgenic antisense *M6PR* plants fail to accumulate mannitol and further demonstrated the critical role of mannitol production for salt tolerance. Antisense *M6PR* plants grew normally in the absence of salt stress but, unlike wild type plants, were severely impaired by irrigation with 200 mM NaCl.

In another project not related to this 1168 group we (Amy Iezzoni, lead PI, and myself here at MSU, and Esther van der Knaap at Ohio St) are looking at the genomics of fruit quality in sour cherry. My focus is on evaluating quality and identification of candidate genes involved in determining fruit size and accumulation of carbohydrates, e.g., transporters.

Recent Publications: (note that the first represents a collaborative effort in our 1168 group).

C. M. Sickler, G. E. Edwards, O. Kiirats, Z. Gao, W. Loescher. 2007. Response of mannitol-producing Arabidopsis thaliana to abiotic stress. Functional Plant Biology 34: 382-391.

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Michigan AES, Jack Preiss

NC-1168 ANNUAL REPORT 2007

Evolutionary fate of ADP-glucose pyrophosphorylase gene family in *Arabidopsis thaliana*

ADP-Glucose Pyrophosphorylase (ADP-Glc PPase) catalyzes the first committed step in starch biosynthesis. Higher plant ADP-Glc PPase is a heterotetramer (a_2b_2) consisting of two small and two large subunits. It is widely accepted that the small subunits play a catalytic role and serve as scaffolding to assemble the large ones, which are considered regulatory. In Arabidopsis thaliana two genes encode small subunits (APS1 and APS2) and four large subunits (APL1 to 4). Here we demonstrate that in *Arabidopsis thaliana* APL1 and APL2, besides their regulatory role, have catalytic activity. Heterotetramers formed by combinations of a non-catalytic APS1 and the four large subunits showed that APL1 and APL2 exhibited ADP-Glc PPase activity with distinctive sensitivities to the allosteric activator (3-PGA). To determine the relevance of these activities in vivo a T-DNA mutant of APS1 (aps1) was characterized. aps1 is starchless and lacked of ADP-Glc PPase activity, APS1 mRNA, APS1 protein and was late flowering in long days. Transgenic lines of the aps1 mutant, expressing an inactivated form of APS1, recovered the wild type phenotype indicating that APL1 and APL2 are not only regulatory but also catalytic in nature. Phylogenetic analysis and structural comparisons let us to propose an evolutionary model for the ADP-Glc PPase gene family from a common catalytic and regulatory ancestor. This evolutionary fate may have implications for other a_2b_2 enzymes.

Regulatory Properties of Potato–Arabidopsis Hybrid ADP-Glucose Pyrophosphorylase

In higher plants, ADP-glucose pyrophosphorylase (ADPGlc-PPase) is a heterotetrameric enzyme comprised of two small and two large subunits. Potato–Arabidopsis hybrid ADPGlc-PPases were generated and their regulatory properties analyzed. We show that ADPGlc-PPase subunits from two different species can interact, producing active enzymes with new regulatory properties. Depending on the subunit combinations, hybrid heterotetramers showed responses to allosteric effectors [3-phosphoglycerate (3-PGA) and Pi] in the micromolar or millimolar range. While hybrid potato small subunit (PSS) and the Arabidopsis large subunit APL1 showed an extremely sensitive response to 3-PGA and Pi, hybrid PSS/Arabidopsis APL2 was very insensitive to them. Intermediate responses were determined for other subunit combinations.

Identification of Regions Critically Affecting Kinetics and Allosteric Regulation of the *Escherichia coli* ADP-glucose Pyrophosphorylase by Modeling and Pentapeptide-Scanning Mutagenesis

ADP-glucose pyrophosphorylase (ADP-Glc PPase) is the enzyme responsible for 2 the regulation of bacterial glycogen synthesis. To perform a structure-function relationship study of the *Escherichia coli* enzyme, we studied the effect of pentapeptide insertions at different positions in the enzyme and analyzed the results with a homology model. We randomly inserted 15 bp in a plasmid with the ADP-Glc PPase gene. We obtained 140 modified plasmids with single insertions of which 21 were in the coding region of the enzyme. Fourteen of them generated insertions of five amino acids, whereas the other seven created a stop codon and produced truncations. Correlation of ADP-Glc PPase activity to these modifications validated the enzyme model. Six of the insertions 10 and one truncation produced enzymes with sufficient activity for the *E. coli* cells to synthesize glycogen and stain in presence of iodine vapors. These were in regions away from the substrate site, whereas the mutants that did not stain had alterations in critical areas of the protein. The enzyme with a pentapeptide insertion after Leu102 was catalytically competent but insensitive to activation. We postulate this region as critical for the allosteric regulation of the enzyme, participating in the communication between the catalytic and regulatory domains.

Crystal structure of the closed conformation of *Escherichia coli* glycogen synthase: critical amino acids involved in substrate binding and catalysis.

At present 2 crystal structures of glycogen synthase have been reported. One is from *Agrobacterium tumefaciens* (Buschiazzo et al., 2004) and one from *Pyrococcus abyssi* (Christina et al., 2006). However, both of these enzymes were crystallized in their catalytically inactive "open" conformations. We have crystallized and have determined the crystal structure of the substrate-bound closed form of the *E. coli* glycogen synthase. In addition we have determined the structure of the ADP-glucose-bound, enzymatically inactive E377A mutant glycogen synthase (Yep et al., 2004a). Glu residue 377 is important in binding of the glucose moiety of the ADP-Glc substrate. Asp 137, His 161, Arg300 and Lys 305 are suggested by the structure to be critical catalytic residues in the reaction. Indeed, mutagenesis of these amino acids to Ala causes a decrease of Vmax, respectively, of 8,140-, 710-, 2590-, and 1240-fold (Yep etal., 2004b). The change in Km for ADP-glucose is slight for the Lys305 and Asp 137 mutants and only about 10-fold for the Arg300 and His161 mutants. The structure and mutagenesis studies indicate that the active site of the bacterial glycogen synthase is similar to the active sites of retaining GT-B glycosyltransferases such as maltodextrin phosphorylase and trehalose-6-P synthase.

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Nebraska AES, Robert J. Spreitzer

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Objective 2: Photosynthetic Capture and Photorespiratory Release of CO_2

Accomplishments

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the rate-limiting step of photosynthesis. It is a remarkably slow enzyme (a carboxylation k_{cat} of only several per sec) that is competitively inhibited by O_2 (as much as 50% of net CO_2 fixation). Oxygenation of ribulose-1,5-bisphosphate generates phosphoglycolate, which is the first intermediate in the nonessential photorespiratory pathway that leads to the loss of CO_2. Because of these properties, there has been much interest in engineering Rubisco as a means for increasing agricultural productivity. However, plans for improvement have generally been limited to finding a "better" enzyme in nature or devising procedures for genetically selecting improvements in the enzyme's CO_2/O_2 specificity or velocity. Without a deeper understanding of the structure-function relationships of Rubisco, it will continue to be difficult to develop a rationale for genetic improvement. Our research has relied on various genetic approaches for elucidating the structural basis of Rubisco catalytic efficiency and CO_2/O_2 specificity. The green alga *Chlamydomonas reinhardtii* is the model organism of choice because photosynthesis-deficient mutants can be maintained with acetate as an alternative carbon source. The chloroplast large-subunit gene (*rbcL*) and family of nuclear small-subunit genes (*rbcS1* and *rbcS2*) can also be eliminated or replaced by directed mutagenesis/transformation or by appropriate genetic crosses. In an ongoing collaboration with Dr. Inger Andersson (Swedish Agricultural University), the X-ray crystal structure of *Chlamydomonas* Rubisco has been solved to the highest resolution (1.4 Å) of all Rubisco enzymes, and the structures of mutant enzymes are being solved .

Because the catalytic properties of Rubisco enzymes differ among various species, we have been taking a phylogenetic approach to identify those regions of the Rubisco large subunit that may account for these differences. *Chlamydomonas* Rubisco has a higher k_{cat} for carboxylation but lower CO_2/O_2 specificity than the land-plant enzymes. When 500 large-subunit sequences from flowering plants were compared with the *Chlamydomonas* sequence, only 34 residues were found to be unique to *Chlamydomonas*. However, to change these "phylogenetic" residues to the residues common to land plants (in all possible combinations) would require the creation and analysis of 2^{34} enzymes. Instead, all the phylogenetic residues can be placed in 15 groups based on van der Waals contact in the high-resolution crystal structure of *Chlamydomonas* Rubisco. These 15 mutant enzymes have now been created by directed mutagenesis and chloroplast transformation. All the mutant strains grow photosynthetically, and all but three of the mutant

enzymes have normal catalytic properties. The G168P-L326I-M349L-M375L-A398S-C399V mutant enzyme has decreased CO_2/O_2 specificity, and the G442N-D443E-V444I-S447E and R305K-D470E-T471A-I472M-K474T enzymes have increased and decreased O_2 inhibition of carboxylation, respectively. None of the mutant enzymes has catalytic properties comparable to those of land-plant Rubisco. Because the engineered phylogenetic residues must be complemented by other residues in land-plant Rubisco (which differ from those in *Chlamydomonas*), work is in progress to combine the groups of phylogenetic substitutions.

Previous studies have shown that engineered changes in the small subunit of *Chlamydomonas* Rubisco can alter CO_2/O_2 specificity despite the fact that the active site resides in the large subunit. It would be interesting to determine whether small subunits might play a role in the differences in catalytic properties observed for Rubisco enzymes from different species. However, it has been difficult to introduce foreign small subunits into *Chlamydomonas* because the *Chlamydomonas rbcS* introns are essential for gene expression. We have recently circumvented this problem by engineering the introns into the region of the *rbcS* gene that encodes the transit peptide. It is now possible to express any foreign *rbcS* cDNA by inserting it behind the *Chlamydomonas* transit-peptide region. A hybrid Rubisco comprised of spinach small subunits and *Chlamydomonas* large subunits is expressed at a near-normal level. Relative to *Chlamydomonas* Rubisco, the hybrid holoenzyme has normal CO_2/O_2 specificity. However, carboxylation V_{max} is decreased to a value similar to that of spinach Rubisco, and K_m CO_2 and O_2 are increased to values greater than those of either native enzyme. Previous attempts by others to create hybrid land-plant enzymes resulted in a dramatic decrease in Rubisco carboxylation catalytic efficiency. Based on our observations, it is likely that those previous attempts were hindered by alterations in subunit processing. Using our system, work is in progress to substitute more-divergent large and small subunits.

Usefulness of Findings

Rubisco catalysis depends on a conserved set of large-subunit active-site residues, but catalytic efficiency and CO_2/O_2 specificity vary among Rubisco enzymes from different species. Our work has shown that engineered changes in either the large or small subunit far from the active site can influence carboxylation catalytic efficiency and CO_2/O_2 specificity. These regions may serve as targets for either the design of an improved Rubisco, or for genetic selection following random mutagenesis or DNA shuffling.

Plans for Next Year

Groups of large-subunit phylogenetic residues will be combined into associated groups (separated by no more than one amino-acid residue) to see whether land-plant catalytic properties can be obtained. Now that the *Chlamydomonas* small subunit has been replaced by that of spinach, attempts will be made to also replace the chloroplast-encoded large subunit.

Publications

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Nebraska AES, Donald P. Weeks

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Carbon Concentrating Mechanism (CCM) in *Chlamydomonas reinhardtii*

Background: The carbon concentrating mechanism (CCM) in prokaryotic and eukaryotic algae is essential for growth at ambient levels of CO_2. The ability of algal cells to increase internal levels of inorganic carbon has allowed them the ability to survive and grow in CO_2 and HCO_3^- deficient environments. Indeed, the Rubisco contained in these cells operates as if it were adapted for growth a CO_2 and HCO_3^- levels well above the concentrations found in most natural aqueous environments. The Weeks laboratory has focused on the identification and characterization of a number of the components of the CCM of *Chlamydomonas reinhardtii* during the last few years. This write-up provides a brief summary of progress to date in this research. Some of our more recent research has been in collaboration with our NC-1168 colleague, Dr.. Martin Spalding of Iowa State University.

In the early 1990s, the Weeks laboratory developed an indexed cosmid library of *C. reinhardtii* genomic DNA (Zhang et al., 1994). The approximately 11,000 clones in this library had an average insert size of about 38,000 bp. Thus, the entire library represented a total DNA content approximately four times the DNA content of the *C. reinhardtii* genome (~1.2×10^8 bp). On average each *C. reinhardtii* gene should have been represented in this library, as an intact gene, about 3 to 4 times (Gardner et al., 1999). Transformation efficiency of *C. reinhardtii* at that time was sufficient that there was a good possibility that transformation of mutant cells with a pool of 94 clones from a single microtiter plate of cosmid clones should have allowed a reasonably high probability of cloning the gene involved in a particular recessive mutation by genetic complementation (i.e., restoration of the wild-type phenotype).

Our first practical test of the indexed cosmid library was an attempt to complement the high CO2-requiring (HCR) phenotype of the *ca-1* mutation. It had been demonstrated earlier that the *ca-1* mutant retained the ability to concentrate inorganic carbon intracellularly, but apparently lacked a carbonic anhydrase (CA) needed to convert HCO_3^- to CO_2 for use by Rubisco. We succeeded in obtaining *ca-1* mutants after transformation with certain pools of cosmid DNA that could grow in ambient concentrations of CO_2. Sequencing of DNA in the cosmid clones that were capable of providing complementation, indeed, demonstrated that the target gene encoded a carbonic anhydrase (Funke et al., 1997). Interestingly, this CA , CAH3, appears to be localized to the lumen of the chloroplast thylakoid. Although it appears that this CA is needed to supply CO_2 to Rubisco, the mechanism by which this is accomplished is not yet clear.

The next target of our research was the identification of the gene involved in the *cia5* mutation. The *cia5* mutant is a high CO_2-requiring mutant that neither can concentrate internal inorganic carbon nor induce the synthesis of several "CO_2-responsive genes" that are highly induced when *C. reinhardtii* cells are shifted from an environment of high CO_2 to ambient levels of CO_2. For this and other reasons, it was hypothesized that the *cia5* mutant was a regulatory mutant involved in several processes associated with the CCM. We successfully complemented the *cia5* mutant with DNA from the indexed cosmid library and discovered that the gene involved produced a molecule whose characteristics suggested that it might be a transcriptional co-activator (Xiang et al., 2002). That is, it had no apparent DNA binding domain, but did contain a number of domains that could allow potential interaction with other molecules (perhaps transcription factors) to control the expression of the several genes induced in cells experiencing a decrease in external CO_2 levels. Additional studies verified that CIA5 is a nuclear-localized molecule and that it or an associated protein is likely undergoes rapid posttranslational modification in response to changing CO_2 levels (Sun et al., 2005). Research continues to determine if CIA5 is the target of posttranslational modification or a protein(s) that associates with CIA5 that is the target of posttranslational modification when external CO_2 concentrations change.

More recently our attention has shifted to the identification and characterization of the molecules and molecular systems that allow for the transport of CO_2 and HCO_3^- into *C. reinhardtii* cells (i.e., inorganic carbon transporters). An initial focus of this work has been the *HLA3* gene that encodes a putative ABC transporter. The *HLA3* gene is CO_2 responsive and its mRNA is present in moderately high amounts in cells maintained in air or in very low CO_2 concentrations (i.e., < 100 ppm). Given that one of the high affinity inorganic transporters in cyanobacteria is an ABC transporter specific for HCO_3^-, we have sought to test the hypothesis that HLA3 is a bicarbonate transporter in *C. reinhardtii*. Fortuitously, our collaborator, Dr. Martin Spalding and his laboratory has recently demonstrated that the *pmp1* mutant of *C. reinhardtii* (and the newly characterized allelic mutant, *ad-1*) possesses an unusual phenotype. That is, as expected from earlier studies, these mutants were able to grow in high concentrations of CO_2, but not at ambient levels of CO_2. Unexpectedly, when placed in very low CO_2 levels, the mutants were observed to grow. One possible explanation was that the cells were able to grow at very low CO_2 concentrations because they gained the ability to transport HCO_3^-. If this was so, and if HLA3 was the bicarbonate transporter involved, then RNA interference (RNAi) knockdown of HLA3 expression should lead to a loss of ability to grow at <100 ppm CO_2. When HLA3 RNAi knockdown lines were produced, this, indeed, was the phenotype produced. Moreover, the uptake of ^{14}C-HCO_3^- observed in the *pmp1* and *ad-1* mutants at very low CO_2 concentrations was greatly diminished in the RNAi knockdown lines. These observations have provided initial indications that HLA3 may be a bicarbonate transporter in *C. reinhardtii*. However, additional data are required for confirmation, including expression of the bicarbonate transporter phenotype by the *HLA3* gene in a heterologous system. Those experiments are presently underway.

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Nevada AES, John C. Cushman

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Objective 2: Photosynthetic Capture and Photorespiratory Release of CO_2

Accomplishments:
Research efforts have focused on several different aspects of grape berry development:
1) Developmental mRNA expression in winegrape berries: Grape berry development is a dynamic process that involves a complex series of molecular genetic and biochemical changes divided into three major phases. During initial berry growth (Phase I), berry size increases along a sigmoidal growth curve due to cell division and subsequent cell expansion, and organic acids (mainly malate and tartrate), tannins, and hydroxycinnamates accumulate to peak levels. The second major phase (Phase II) is defined as a lag phase in which cell expansion ceases and sugars begin to accumulate. Véraison (the onset of ripening) marks the beginning of the third major phase (Phase III) in which berries undergo a second period of sigmoidal growth due to additional mesocarp cell expansion, accumulation of anthocyanin pigments for berry color, accumulation of volatile compounds for aroma, softening, peak accumulation of sugars (mainly glucose and fructose), and a decline in organic acid accumulation. In order to understand the transcriptional network responsible for controlling berry development, mRNA expression profiling was conducted on berries of *V. vinifera* Cabernet Sauvignon using the Affymetrix GeneChip® *Vitis* oligonucleotide microarray ver. 1.0 spanning seven stages of berry development from small pea size berries (E-L stages 31 to 33 as defined by the modified E-L system), through véraison (E-L stages 34 and 35), to mature berries (E-L stages 36 and 38). Selected metabolites were profiled in parallel with mRNA expression profiling to understand the effect of transcriptional regulatory processes on specific metabolite production that ultimately influence the organoleptic properties of wine.

Over the course of berry development whole fruit tissues were found to express an average of 74.5% of probes represented on the *Vitis* microarray, which has 14,470 Unigenes. Approximately 60% of the expressed transcripts were differentially expressed between at least two out of the seven stages of berry development (28% of transcripts, 4,151 Unigenes, had pronounced (≥ 2 fold) differences in mRNA expression) illustrating the dynamic nature of the developmental process. The subset of 4,151 Unigenes was split into twenty well-correlated expression profiles. Expression profile patterns included those with declining or increasing mRNA expression over the course of berry development as well as transient peak or trough patterns across various developmental stages as defined by the modified E-L system. These detailed surveys revealed the expression patterns for genes that play key functional roles in phytohormone biosynthesis and response, calcium sequestration, transport and signaling, cell

wall metabolism mediating expansion, ripening, and softening, flavonoid metabolism and transport, organic and amino acid metabolism, hexose sugar and triose phosphate metabolism and transport, starch metabolism, photosynthesis, circadian cycles and pathogen resistance. In particular, mRNA expression patterns of transcription factors, abscisic acid (ABA) biosynthesis, and calcium signaling genes identified candidate factors likely to participate in the progression of key developmental events such as véraison and potential candidate genes associated with such processes as auxin partitioning within berry cells, aroma compound production, and pathway regulation and sequestration of flavonoid compounds. Finally, analysis of sugar metabolism gene expression patterns indicated the existence of an alternative pathway for glucose and triose phosphate production that is invoked from véraison to mature berries (Deluc et al., 2007).

2) Tissue-specific mRNA expression in winegrape berries: Berries of grape (Vitis vinifera) contain three major tissue types (skin, pulp and seed) all of which contribute to the aroma, color, and flavor characters of wine. The pericarp, which is composed of the exocarp (skin) and mesocarp (pulp), not only functions to protect and feed the developing seed, but also to assist in the dispersal of the mature seed by avian and mammalian vectors. The skin provides volatile and nonvolatile aroma and color compounds, the pulp contributes organic acids and sugars, and the seeds provide condensed tannins, all of which are important to the formation of organoleptic characteristics of wine. In order to understand the transcriptional network responsible for controlling tissue-specific mRNA expression patterns, mRNA expression profiling was conducted on each tissue of mature berries of *V. vinifera* Cabernet Sauvignon using the Affymetrix GeneChip® *Vitis* oligonucleotide microarray ver. 1.0. Overall, berry tissues were found to express approximately 76% of genes represented on the *Vitis* microarray. Approximately 60% of these genes exhibited significant differential expression in one or more of the three major tissue types with more than 28% of genes showing pronounced (2-fold or greater) differences in mRNA expression. The largest difference in tissue-specific expression was observed between the seed and pulp/skin. Exocarp tissue, which is involved in pathogen defense and pigment production, showed higher mRNA abundance relative to other berry tissues for genes involved with flavonoid biosynthesis, pathogen resistance, and cell wall modification. Mesocarp tissue, which is considered a nutritive tissue, exhibited a higher mRNA abundance of genes involved in cell wall function and transport processes. Seeds, which supply essential resources for embryo development, showed higher mRNA abundance of genes encoding phenylpropanoid biosynthetic enzymes, seed storage proteins, and late embryogenesis abundant proteins (Grimplet et al., 2007).

Usefulness of findings:

The grape berry development study revealed the first high-resolution picture of the transcriptome dynamics that occur during seven stages of grape berry development. This work also established an extensive catalog of gene expression patterns for future investigations aimed at the dissection of the transcriptional regulatory hierarchies that govern berry development in a widely grown cultivar of wine grape. More importantly, this analysis identified a set of previously unknown genes potentially involved in critical steps associated with fruit development that can now be subjected to functional testing (Deluc et al., 2007).

The grape berry tissue study revealed novel insights into the tissue-specific expression mRNA expression patterns of an extensive repertoire of genes expressed in berry tissues. This work also established an extensive catalogue of gene expression patterns for future investigations

aimed at the dissection of the transcriptional regulatory hierarchies that govern tissue-specific expression patterns associated with tissue differentiation within berries (Grimplet et al., 2007)

Work Plans for the Coming Year:
In the coming year, the focus of our winegrape research will be the analysis of the mRNA, protein, and metabolite differences during berry development in two different cultivars of winegrape. We will also continue our investigations into integrated functional genomics of Crassulacean acid metabolism (CAM) and the molecular genetic changes that have occurred across the evolutionary continuum from C_3 photosynthesis to CAM in neotropical orchids.

Objective 4: Developmental and environmental limitations to photosynthesis.

Accomplishments:
Research efforts have focused on the effects of both long-term and short-term cold, water-deficit and salinity stress in winegrape vegetative tissues and the long-term effects of water-deficit stress on winegrape berry tissues. We also studied the functional role of epidermal bladder cells in the salinity tolerance of the common ice plant.

1) Effects of long-term abiotic stress on winegrape vegetative tissues: Grapes are grown in semi-arid environments, where drought and salinity are common problems. Microarray transcript profiling, quantitative RT-PCR and metabolite profiling were used to define genes and metabolic pathways in *Vitis vinifera* cv. Cabernet Sauvignon with shared and divergent responses to a gradually applied and long-term (16 days) water-deficit stress and equivalent salinity stress. In this first of a kind study, distinct differences between water-deficit and salinity were revealed. Water deficit caused more rapid and greater inhibition of shoot growth than did salinity at equivalent stem water potentials. There was an increase in the transcript abundance of RuBisCo activase very early in the response to water deficit (day 4), but this increase was much later in salt-stressed plants (day 12). As water deficit progressed, a greater number of affected transcripts were involved in metabolism, transport and the biogenesis of cellular components than did salinity. Salinity affected a higher percentage of transcripts involved in transcription, protein synthesis and protein fate than did water deficit. Metabolite profiling revealed that there were higher concentrations of glucose, malate and proline in water-deficit-treated plants as compared to salinized plants (Cramer et al., 2007).

2) Effects of short-term abiotic stress on winegrape vegetative tissues: Cabernet Sauvignon grapevines were exposed to sudden chilling (5°C), water deficit (PEG) and an iso-osmotic salinity (120 mM NaCl and 12 mM $CaCl_2$) for 1, 4, 8 and 24 hours. Stomatal conductance and stem water potentials were significantly reduced following stress application. Microarray analysis of transcript abundance in shoot tips detected no significant differences in transcript abundance between salinity and PEG prior to 24 hours. Forty-three percent of transcripts affected by stress versus control for 1 through 8 hours were affected only by chilling. The functional categories most affected by stress included metabolism, protein metabolism and signal transduction. Osmotic stress affected more protein synthesis and cell cycle transcripts, whereas chilling affected more calcium signaling transcripts, indicating that chilling has more complex calcium signaling. Stress affected many hormone (ABA, ethylene and jasmonate) and

transcription factor transcripts. The concentrations and transporter transcripts of several anions increased with time, including nitrate, sulfate and phosphate (Tattersall et al., 2007).

3) Effects of water-deficit stress on winegrape berry tissue-specific patterns of gene expression: In order to monitor the influence of water-deficit stress on tissue-specific expression patterns, mRNA expression profiles were also compared from mature berries harvested from vines subjected to well-watered or water-deficit conditions. Water-deficit stress affected the mRNA abundance of 13% of the genes with differential expression patterns occurring mainly in the pulp and skin. In pulp and seed tissues transcript abundance in most functional categories declined in water-deficit stressed vines relative to well-watered vines with transcripts for storage proteins and novel (no-hit) functional assignments being over represented. In the skin of berries from water-deficit stressed vines, however, transcripts from several functional categories including general phenylpropanoid and ethylene metabolism, pathogenesis-related responses, energy, and interaction with the environment were significantly over-represented (Grimplet et al., 2007).

4) Role of epidermal bladder cells in ice plant performance under salinity stress: The aerial surfaces of the common or crystalline ice plant, *Mesembryanthemum crystallinum* L., a halophytic, facultative Crassulacean acid metabolism species, are covered with specialized trichome cells called epidermal bladder cells (EBCs). EBCs are thought to serve as a peripheral salinity and/or water storage organ to improve survival under high salinity or water deficit stress conditions. However, the exact contribution of EBCs to salt tolerance in the ice plant remains poorly understood. A *M. crystallinum* mutant lacking EBCs was isolated from plant collections mutagenized by fast neutron irradiation. The light and electron microscopy revealed that mutant plants lacked EBCs on all surfaces of leaves and stems. Dry weight gain of aerial parts of the mutant was almost half that of wildtype plants after three weeks of growth at 400 mM NaCl. The EBC mutant also showed reduced leaf succulence and leaf and stem water contents compared with wildtype plants. Aerial tissues of wildtype plants had approximately 1.5-fold higher Na^+ and Cl^- content than the mutant grown under 400 mM NaCl for 2 weeks. Na^+ and Cl^- partitioning into EBCs of wildtype plants resulted in lower concentrations of these ions in photosynthetically active leaf tissues than in leaves of the EBC-less mutant, particularly under conditions of high salt stress. Potassium, nitrate, and phosphate ion content decreased with incorporation of NaCl into tissues in both wildtype and mutant, but the ratios of Na^+/K^+ and Cl^-/NO_3^- content were maintained only in the leaf and stem tissues of wildtype plants. The EBC mutant showed significant impairment in plant productivity under salt stress as evaluated by seed pod and seed number and average seed weight. These results clearly show that EBCs contribute to succulence by serving as a water storage reservoir and to salt tolerance by maintaining ion sequestration and homeostasis within photosynthetically active tissues of *M. crystallinum* (Agarie et al., 2007).

Usefulness of findings:

The metabolite differences found under long-term salinity or water deficit stress were linked to differences in transcript abundance of many genes involved in energy metabolism and nitrogen assimilation, particularly photosynthesis, gluconeogenesis and photorespiration. Water-deficit-treated plants also appeared to have a higher demand than salinized plants to adjust osmotically, detoxify free radicals (ROS) and cope with photoinhibition (Cramer et al., 2007).

The transcript abundance changes in the short-term study were largely the same as a gradually-applied long-term salinity and water-deficit study (Tattersall et al., 2007), but the reverse was not true, indicating a larger and more complex response in the acclimation process of a gradual long-term stress (Cramer et al., 2007).

The tissue-specific expression profiling study in winegrape berries confirmed that water-deficit stress has a profound effect on mRNA expression patterns particularly associated with the biosynthesis of aroma and color metabolites within skin and pulp tissues that ultimately impact wine quality (Grimplet et al., 2007).

The ice plant study clearly showed that EBCs contribute to succulence by serving as a water storage reservoir and to salt tolerance by maintaining ion sequestration and homeostasis within photosynthetically active tissues of *M. crystallinum* (Agarie et al., 2007).

Work Plans for the Coming Year:

We plan to continue our analyses of salinity and water deficit responses in wine grape berries by examining proteome changes as has been performed for vegetative tissues (Vincent et al., 2007) and berry tissues as well as transcriptomic and metabolomic responses to long-term water deficit in two different cultivars of winegrape. We also plan to continue our investigation of the effects of salinity stress on stress responses including CAM in wildtype and CAM-deficient mutants of ice plant, *M. crystallinum*. Transcriptomic studies have been completed in wildtype ice plant.

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Sultan Ciftci-Yilmaz, Mustafa R. Morsy, Luhua Song, Alicia Coutu, Beth A. Krizek, Michael W. Lewis, Daniel Warren, John Cushman, Erin L. Connolly and Ron Mittler. (2007) The EAR-Motif of the C2H2 Zinc-Finger Protein Zat7 Plays a Key Role in the Defense Response of Arabidopsis to Salinity Stress. J. Biol. Chem. 282:9260-9268

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Nevada AES, Jeff Harper

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Overview of Harper Lab Research. The long-term interest is to understand how a plant can use as few as 30,000 genes to develop and survive under extreme environmental conditions, such as cold, heat, drought, salt, and high-light stress. A primary focus is on calcium signaling. The lab uses Arabidopsis and yeast as model systems. Specific aims are focused on questions of enzyme structure and function for members of the following three gene families. **1) Calcium Dependent Protein Kinases (CDPKs, or CPKs).** CDPKs have a unique structure defined by the presence of a calmodulin-like regulatory domain fused to the C-terminal end of the kinase. They are only found in plants and some protist, including the human parasite *Plasmodium*, the causative agent of malaria. **2) Cyclic Nucleotide Gated Channels (CNGCs).** These ion channels have been implicated in calcium signaling and are regulated by both cyclic nucleotides and calmodulin. **3) P-type ATPase Ion Pumps.** These ion pumps are used to transport across membranes a wide variety of ions, including H^+, Ca^{2+}, Na^+, and heavy metals. They are involved in ion homeostasis, nutrient acquisition from the soil, toxic ion efflux, and signal transduction.

The Harper lab contribution to the NC-1-142 Regional Project has involved multiple collaborations on multiple topics, including signal transduction, source-sink nutrient translocation, and crop yield. Outlined below are brief descriptions of research projects that relate to NC-1168 Objectives 3 and 4.

Objective 3: Partitioning of fixed carbohydrates

14-3-3 Regulation. 14-3-3s are regulatory proteins that have been implicated in several different metabolic pathways, including nitrogen and carbon metabolism (Huber group). Most 14-3-3 interactions are thought to be dependent on the phosphorylation of a mode 1 or mode 2 binding site on a 14-3-3 client protein. These binding sites have a consensus that overlaps with the substrate consensus site for a calcium dependent protein kinase (CDPK). As part of our search for CPK substrates, the Harper lab expressed an affinity tagged 14-3-3 protein (TAP tagged) in transgenic Arabidopsis plants. The tagged 14-3-3 protein was affinity purified and copurifying clients identified by mass spectrometry. As a control for non-specific binding, a parallel experiment was done with the same affinity tag without the 14-3-3.

Our results provide in planta interaction evidence for more than 120 14-3-3 clients, 94 of which have not previously been reported. Many of the newly identified clients are involved directly in

metabolism, such as glucose-6-phophate 1-dehydrgenase, ion transport (e.g. glutamate receptors), transcription (e.g. multiple WRKY transcription factors), vesicle trafficking (e.g. dynamin), flower timing (e.g. Frigida) and hormone signaling (e.g. proteins implicated in ethylene and branssinolide signaling).

Work Plan for the Coming Year: The aim in the coming year is to begin analyzing the 14-3-3 clients to identify which ones are likely targets for CPK kinase regulation.

Objective 4: Developmental and environmental limitations to photosynthesis.

Stress. Biotic and Abiotic stress result in significant limitations to photosynthetic productivity in plants. Calcium signals have been implicated in how a plant responds to stress. The Harper lab has multiple projects designed to understand how calcium signals are coded and decoded in plant cells. A long-term goal is to understand how calcium-signaling systems can be modified to improve stress tolerance in crop plants. This year we have obtained evidence from a double KO of calcium pumps ACA4 and 11 that the vacuolar localized calcium pumps control calcium signals implicated in triggering a salicylic acid (SA) dependent hypersensitivity response (i.e. lesions).

Genomics of Crop Yield. An important practical goal of the Harper lab is to identify potential mechanisms to improve crop yield. This includes identifying genes that affect a plants response to biotic and abiotic stresses (including light stress), as well as developmental timing and plant structure. For example, a current project funded in collaboration with John Cushman and Ron Mittler (other NC-1168 members), is to screen through Arabidopsis mutants to identify genes that affect a plants ability to grow under extreme environments. These genes provide important leads towards a goal of engineering of higher yield crops. During the past year the Harper lab has developed a robust assay for screening KOs to identify genes that affect reproductive success of plants under hot and cold stress. One of the genes identified is a Cation Chloride Co-transporter (CCC).

Work Plans for the Coming Year:

1. Continue efforts to determine the different signaling pathways that are controlled by the activity of the vacuolar calcium pumps ACA4 and 11. In addition to controlling an HR pathway, preliminary evidence indicates that they are also required for controlling stomatal closure, circadian rhythm, and flower timing. A key hypothesis to be tested is that the ACA4 and 11 pumps control the magnitude and duration multiple calcium signals originating from a localized release of calcium from the vacuole

2. Continue screening mutant plants for stress sensitive phenotypes resulting from gene knockouts in stress-response genes. The focus will be on genes of unknown function using pollen as a single cell model for stress tolerance assays.

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6. Frietsch, S, Y-F Wang, C Sladek, LR Poulsen, JI Schroeder, and **JF Harper** (2007) A Cyclic Nucleotide-Gated Channel essential for polarized tip growth of pollen. ***PNAS*** 104: 14531-36.

Ohio AES, Jyan-Chyun Jang

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Objective addressed: #3. *Mechanisms regulating photosynthate partitioning*.

Accomplishment
We continue to investigate the roles of eight interacting AtbZIP transcription factors in sugar signaling network. Since chromatin immunoprecipitation coupled genechip analysis (ChIP-on-chip) will be used to globally identify target genes, it is imperative to determine the expression levels temporally and spatially. Two types of constructs are being used in this study: one is driven by the ubiquitous CaMV35S promoter and the other is driven by the native bZIP promoter. Both constructs carry either 6xHis and GFP or TAPa and GFP. It was surprising that the GFP signals were primarily detected in non-green cells including lower hypocotyls, roots, and trichomes in plants even with the CaMV35S promoter. Because the same pattern was observed in etiolated seedlings, we hypothesized that the differential expression is due to the tissue-specific post-translational regulation. Work is in progress to verify whether or not it is caused by protein instability.

To further optimize chromatin immuno-precipitation condition, we used maize protoplast system to transiently express AtbZIPs. Surprisingly, the construct contained the 5'UTR yielded very low expression. Full expression is restored by deleting the 5'UTR. Because the mRNA levels were comparable between the two constructs, this suggests that the 5'UTR is involved in translational repression of AtbZIPs. To test if AtbZIP 5'UTR works in *trans*, it was fused to the free GFP and expressed in maize protoplasts. There is a dramatic reduction of free GFP expression when fused with AtbZIP 5'UTR. Work is in progress to determine whether or not this translational repression is regulated by any internal or external cues.

Although there are detectable changes of sugar sensitivity in individual AtbZIP knockout and overexpression plants, the phenotypes are subtle. This is likely due to the functional redundancy of other bZIP factors, which form heterodimers with a specific group of proteins in the bZIP family. To overcome this problem, double and triple mutants of these interacting bZIPs are being made. Work is in progress to determine sugar sensitivity of these double mutants. Generation of double mutant combination within C-group is also in progress.

Usefulness of findings
The findings are expected to advance our understanding on sugar signaling mechanisms in plants.

Plans for the coming year
We will fully concentrate on the global identification of AtbZIP target genes using ChIP-on-chip.

Publications
None.

Pennsylvania AES, Mark Guiltinan

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Objective addressed: Obj 3: Mechanisms regulating photosynthate partitioning

Accomplishments
The main long-term objectives of this project are to understand the molecular and biochemical basis for starch biosynthesis and utilization in plants. Towards these goals, this project focuses on the Starch Branching Enzymes (SBE) of maize, which are critical in determining the molecular fine-structure of starch. Our work over the past decade has characterized the maize SBE gene family (SBEI, SBEIIa and SBEIIb) and begun to unravel the specific functions each isoform plays in plant development. It is now clear that each of the maize SBE genes plays unique, and partially overlapping functions. SBEIIb (encoded by the *Ae* gene) is the predominant SBE in seeds, but both SBEI and SBEIIa also play subtle roles. In leaves, SBEIIa is the key if not only SBE, and in its absence, plants exhibit an extreme premature leaf senescence phenotype. Preliminary analysis of diurnal cycling of starch in *sbe2a* and wildtype leaves demonstrated that in wildtype starch accumulates during the light phase and little remained at the end of the dark phase. Although starch accumulation in *sbe2a* and wildtype leaves was similar at the end of the light phase, at the end of the dark phase, starch levels remained high in *sbe2a*, suggesting that starch fails to degrade fully in plants that lack SBEIIa. Additionally, we have show that *sbe2a* starch grains are approximately three times larger than wildtype granules, are abnormal in shape and are more resistant to enzymatic digestion. In pollen, *ae* and *sbe2a* single mutants are normal, but recovery of double homozygous viable kernels is at a rate lower then expected, suggesting that pollen function may be disrupted by the double mutation. The rate of double mutant recovery from progeny of a selfed *Sbe2a/sbe2a* heterozygote in a background homozygous for *ae* was accurately determined to be 5.7% from a population of ~700 seedlings, significantly lower than the theoretical 25%. We hypothesize that the double mutant is impaired in pollen tube growth and is not capable of competing for fertilization with pollen containing one or more of the functional *Sbe2a* or *Ae* genes. Pollen tubes from *sbe2a; ae* homozygous double mutants, however, can successfully grow and pollinate silks as demonstrated by achieving full seed-set on progeny ears. We have begun to investigate the possibility that the SBEs are regulated by redox. Recent data from our laboratory suggests that in wildtype W64A, SBE activity in leaf extracts can be modulated by treatment with reduced DTT or oxidized DTT, which approximates *in vivo* redox reactions. This observed increase in SBE activity in the presence of reduced DTT is consistent with the fact that starch synthesis by SBEs and other enzymes occurs during the light phase when photosynthesis maintains the chloroplast in reducing conditions. We hypothesize that redox regulation works in concert with other modes of regulation (e.g., phosphorylation and protein complex formation) to both

coordinate transitory starch biosynthesis according to the diurnal cycle and to differentiate transitory and storage starch structures and digestibility. We have examined starch produced by our mutants using several approaches, including measurements of the branch chain lengths and distributions of granule size and shape, and have examined functional characteristics of the starches, including resistance to enzyme degradation and gelatinization physical parameters. Our mutant starches show profound changes in various parameters, which reflect changes in starch micro-structure and possibly changes in granule supra-molecular structure. Surprisingly, the double mutant combinations *sbe1; ae* and *sbe2a; ae* produce starch with increased branching as compared to *ae* starch alone, suggesting the possible existence of a feedback mechanism regulating the SBE activity during starch biosynthesis. The *sbe1* mutation, but not the *sbe2a* mutation, produced kernel starch that also exhibited enhanced resistance to enzymatic degradation, somewhat intermediate between *ae* and wildtype controls. The finding that *sbe1* starch produces an appreciable amount of RS in endosperm could be of practical importance since these mutants have a higher starch yield in kernels as compared to *ae* mutants, which is the current commercial source for RS.

Usefulness of findings

This work will contribute to the base of knowledge of the mechanisms of starch biosynthesis and thus will impact the future improvement of crop productivity and utilization of this important biorenewable resource. In particular, knowledge of the mechanisms of branch point formation and its role in starch granule formation could have impact on development of novel starch types for industrial, medical and food processing applications.

Plans for the coming year

We plan to continue our investigations into the leaf and pollen phenotypes of the *sbe2a* mutant. We are examining protein phosphorylation, redox and protein-protein interactions as putative regulation mechanisms for the SBEs in maize leaves and, as time permits, endosperm. We will also continue to study the subtle changes in starch structure conditioned by the mutations and to try to place this knowledge into an integrated model of starch biosynthesis.

Publications

Li, J., Guiltinan, M., and Thompson, D. (2007). Mutation of the maize sbe1a and ae genes alters morphology and physical behavior of wx-type endosperm starch granules. Carbohydr Res In Press., available online.

South Carolina AES, Brandon Moore

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Objective 3: Mechanisms regulating photosynthate partitioning

Arabidopsis hexokinase1 has dual roles in glucose signaling and in the support of metabolism. A current model of plant HXK signaling is that the sensor protein can translocate to the nucleus and somehow modulate transcriptional activity of target gene promoters. We previously showed that F-actin mediates some aspects of glucose signaling by interacting with HXK1 which is bound only to mitochondria. Our non-detection of HXK1 in nuclei might have resulted if it is present there with very low expression levels. To test this, we over-expressed HXK1-GFP in mesophyll protoplasts. This caused much of the HXK1-GFP to aggregate as a large clump within the protoplast, but co-transfection of a nuclear marker protein showed that the aggregated HXK1-GFP did not occur in the nucleus. The aggregation is due to a grouping of mitochondria, mediated by HXK1. This aggregation was blocked by treatment with LatrunculinB. The affect of LatB was not entirely unexpected since plant mitochondria are known to traffic on F-actin. However, this experiment does show in a novel way that perturbations of HXK or F-actin can each influence the cellular behavior of the other. We hypothesize from these and previous data that HXK1 might promote the bundling of actin filaments, while modulated F-actin dynamics might affect the formation and/or stabilization of associated cellular polysomes.

We also are interested in the function of the 6 Arabidopsis HXKs in organismal space. At least 5 are expressed proteins. In one recent series of experiments, we did a detailed amino acid sequence analysis to examine both by inference and by direct manipulation, a number of possible mechanisms by which catalysis might be compromised in the hexokinase-like (HKL) proteins. The HKL proteins are about 50% identical to HXK1. Homologous regions and residues in AtHXKs were assigned based on reported detailed analyses of HXK2 from *Saccharomyces cerevisiae*. The catalytically inactive HXK1, HXK2, and HXK3 proteins are much more similar to each other in the longer motifs designated phosphate 1, connect 1, phosphate 2, connect 2, and adenosine, than were HKL1, HKL2, and HKL3. Most noticeably, both HKL1 and HKL2 have an insert of 10 and 7 amino acids, respectively, in the adenosine binding site. Other residues and motifs examined include 4 loops that enclose the active site, 4 glucose contact residues, 12 conserved hydrophobic residues previously assigned to a channel in the small domain, and 8 conserved glycine residues thought to be located at the end of α-helices or β-sheets. In summary, the amino acid sequence analysis shows there is a general conservation of key motifs and residues among HXK1, HXK2, and HXK3 proteins. The predicted HKL3 protein lacks a number of recognized residues for sugar and adenylate binding and for enzyme catalysis. HKL1 and HKL2 proteins have residues known to be important for sugar binding, but have a few noted

key residue changes and also have the noted insert in the adenosine binding domain. Additionally, HKL1 and HKL2 proteins have changes in many other single residues throughout the protein relative to HXK1 protein.

The lack of glucose phosphorylation activity in HKL1 and HKL2 proteins is not surprising in view of the large number of key amino acid changes that are present. However, we asked whether the loss of HXK1 activity might require a suite of changes or whether there might be one or just a few key changes that are sufficient to make the protein inactive. A further examination of the amino acid sequences revealed that the HKL1 and HKL2 proteins have equivalent changes at 39 positions relative to the conserved residues in the 3 proteins with catalytic activity. We therefore carried out site directed mutagenesis of both HXK1 and HKL1 in order to test whether key amino acid changes might compromise or restore catalytic activity. The target amino acids included Asn106 (located within phosphate 1, next to loop 1), Gly173 (located within the core sugar binding domain), Leu 251 (located within phosphate 2), insertion into HXK1 of the novel 10 amino acid adenosine insert present in HKL1, and removal of the 10 amino acid adenosine insert from HKL1. Cys159 is a possible control since it is one of only 3 amino acids among all 6 HXKs in which there is variation among either 5 or all 6 of the aligned amino acids.

What we observed in the modified proteins is that the 10 amino acid insertion into HXK1 reduced activity 40-50% and removal of the insert from HKL1 did not restore any detectable activity. Changing N106Y, G173A, and L251F reduced enzyme activity by 40, 90, and 100% respectively. Surprisingly, the C159E mutation stimulated activity 2-fold. Since we have previously shown that S177A and G104D also inactivate HXK1, we surmise that there likely are many modified amino acids in the HKL proteins, many of which each could compromise catalytic activity. This supports our genetic studies which indicate that the HKL proteins have specialized roles as regulatory components for glucose signaling. Perhaps the noted 10 amino acid insert is involved in a regulatory function as its presence did not abolish catalytic activity.

Usefulness of Findings

We now have a much improved cellular framework for understanding glucose dependent regulation of gene expression as mediated by hexokinase1. Further elucidating the roles of glucose, HXK1, and F-actin dynamics is needed to improve our understanding of this process. These findings indicate that future experiments should examine the expression of different polysome populations and identify their bound mRNAs following glucose treatments.

The recognition that HKL proteins are not merely compromised in catalytic activity, but have specialized non-catalytic functions is important. Further mapping of modified motifs and amino acids might identify a novel interaction surface presumably required for their regulatory function. Additionally, the observed large increase in HXK1 activity in the C159E mutant merits further consideration as to whether there might occur physiological regulation of activity by modification of this residue in vivo.

Plans for the Coming Year

We will examine the possible regulation of HXK under a variety of physiological conditions. We will continue to collaborate with Ill-AES (S. Huber), which has made available to us a

related transgenic (C159A). Ongoing experiments will further examine the possible link between HXK signaling and mitochondrial biogenesis noted previously.

Refereed Publications

R. Balasubramanian¸ A. Karve, M. Kandasamy, R. B. Meagher, B. d. Moore (2007) A role for F-actin in hexokinase mediated glucose signaling. Plant Physiol, in press.

R. Balasubramanian¸ A. Karve, and B. d. Moore (2007) A cellular framework for glucose signaling by Arabidopsis hexokinase1. Biosignaling, in press.

A. Karve, B. Rauh, M. Kandasamy, R.B. Meagher, B.d. Moore (2007) Expression and evolutionary features of the hexokinase gene gamily in Arabidopsis. Planta, in review.

Virginia AES, Glenda E. Gillaspy

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OBJECTIVES ADDRESSED: My laboratory's work addresses Objectives 3 (Mechanisms regulating photosynthate partitioning) and 4 (Developmental and environmental limitations to photosynthesis).

ACCOMPLISHMENTS

To gain insight into the mechanisms that regulate photosynthate partitioning into pathways of biosynthesis and use of sugar alcohols, my laboratory has been continuing studies on the synthesis and signaling functions of the sugar alcohol *myo*-inositol. *Myo*-inositol has been linked to osmoprotective functions, signal transduction, and ascorbic acid synthesis, among other functions. Plants contain redundancy in the genes that encode the two enzymes that convert glucose-6-P into *myo*-inositol (*myo*-inositol phosphate synthase; MIPS) and (inositol monophosphatase; IMP). We have been interested in whether this gene redundancy is due to unique functions of the individual isozymes and/or whether metabolic channeling provides a *myo*-inositol supply for specific end products in the pathway. To test this, we have examined loss-of-function mutants for each gene, and are examining the subcellular location of the encoded enzymes. Loss-of-function mutants for MIPS1, MIPS2 and MIPS3 genes have different impacts on *myo*-inositol synthesis. Specifically, a loss of MIPS1 function reduces *myo*-inositol synthesis to a greater degree than losses in MIPS2 or MIPS3. In addition, *mips1* mutant leaves undergo cell death that is associated with a reduction in ascorbic acid and phosphatidylinositol content. Localization of MIPS1 protein in plant cells indicates a possible membrane association of the enzyme. In contrast, the second enzyme in the pathway, IMP, and is encoded by three genes in Arabidopsis, and two of these genes (IMPL1 and IMPL2) are embryonic lethal. The IMP gene is most similar to other eukaryotic IMPs, while IMPL1 and IMPL2 encode proteins most similar to prokaryotic IMPs. Examination of plants containing a loss-of-function in IMP revealed that *myo*-inositol and ascorbic acid levels were decreased, however L-galactose levels were increased. In addition, analysis of IMP;GFP transgenic plants indicates a cytoplasmic location for the IMP enzyme. Together, these data support different subcellular locations for the first and second steps in *myo*-inositol synthesis.

To analyze the limitations and environmental factors that influence photosynthetic productivity at the whole plant level, my laboratory has also focused on genes involved in *myo*-inositol signaling that can impact plant stress responses. Specifically, we have been examining the genes that encode enzymes which degrade Ins(1,4,5)P_3 that are called 5PTases. There are two classes of 5PTase enzymes in plants, with the "A" class containing a 5PTase catalytic domain only, and

the "B" class containing this same catalytic domain and 5-7 WD40 repeats (Berdy, S. et al. 2001). We have shown that a class B mutant (*5ptase13*), contains lower Ins(1,4,5)P_3 levels and ABA and sugar-insensitivity. The WD40 repeats contained in the 5PTase13 protein are likely to provide the 5PTase13 protein with ability to form protein:protein complexes with other signaling proteins. We therefore used the WD40 repeats from 5PTase13 as bait in a yeast two hybrid experiment and identified the SNF1-like kinase as a binding partner. *In vitro* binding studies with recombinant proteins verified the interaction of SNF1-like kinase and 5PTase13, supporting the role of 5PTase13 in sugar signaling pathways. Further, subcellular localization of the 5PTase13 protein indicates a possible endosomal location within the plant cell. These data suggest that the 5PTase13 gene functions in sugar and stress signaling to modulate Ins(1,4,5)P_3 levels.

USEFULNESS OF FINDINGS:

Our studies focused on Objective 3 support an alternate pathway for ascorbic acid synthesis from *myo*-inositol in plants. As ascorbic acid is both an essential plant-derived nutrient for humans, and an important anti-oxidant in plants, understanding its synthesis can lead to new strategies to increase both human and plant health.

Our studies focused on Objective 3 add to our understanding of abiotic stress in plants and suggest that modulating cellular Ins(1,4,5)P_3 levels could allow for novel regulation of plant signaling processes.

PLANS FOR THE COMING YEAR:

Our current data on both *myo*-inositol metabolism and signaling support unique subcellular locations for the MIPS, IMP and 5PTase enzymes. During the next year we will utilize co-localization experiments to verify our preliminary results. In addition, we will continue our metabolic profiling work on phosphatidylinositol signaling molecules.

PUBLICATIONS

Gunesekera B, Torabinejad J, Robinson J, Gillaspy, GE. 2007. Inositol polyphosphate 5-phosphatases 1 and 2 are required for regulating seedling growth. *Plant Physiol.* 143:1408-17.

Ercetin, M, Torabinejad, J, Robinson, J and Gillaspy, GE. A Phospholipid-Specific *Myo*-Inositol Polyphosphate 5-Phosphatase Required for Regulating Seedling Growth. Under revision for *Plant Molecular Biology*

Washington AES, Gerry Edwards and Tom Okita

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Objective 2: Photosynthetic capture and photorespiratory release of CO_2

During the last year, we continued studies on unique species in family Chenopodiaceae which perform C_4 photosynthesis without Kranz anatomy. In these species, the spatial separation of C_4 functions occurs by developing two cytoplasmic compartments within individual chlorenchyma cells, with each compartment having a different type of chloroplast. Studies show that there is a transition from C_3 to C_3-C_4 to full C_4 during leaf development. Initially, there is only one type of chloroplast (C_3-default, Rubisco-containing), with subsequent development of two cytoplasmic compartments, each having dimorphic chloroplasts. One type of chloroplast has Rubisco, ADPG pyrophosphorylase, starch biosynthesis and well-developed grana, while the other type of chloroplast is specialized for supporting CO_2 fixation in the C_4 cycle (through the enzyme pyruvate,Pi dikinase). Analysis of the development of the cytoskeleton, using antibodies to actin and microtubules, and use of cytoskeleton-disrupting drugs, shows that microtubules are critical for maintenance of the two cytoplasmic compartments.

Objective 3: Partitioning of fixed carbohydrates – starch metabolism

During the last year, we continued research on ADPglucose pyrophosphorylase (AGPase) and its role in starch biosynthesis, which is directed at both the biochemical and physiological levels. Specifically, we are studying the roles of the two types of subunits that constitute the heterotetrameric enzyme, and the function of this enzyme activity in source (leaves) and sink (seed) tissues in photosynthesis, plant growth and crop production. We are working with Arabidopsis as a model system through manipulation of leaf starch biosynthesis, and rice as an important crop plant through manipulation of both leaf and seed starch biosynthesis. Progress to date is described below.

Structure-function of AGPase subunits. Past studies suggested that the large and small subunits had different roles in AGPase function. The small subunit (SS) was the catalytic subunit, while the large subunit (LS) was regulatory. This view was found to be overly simplistic as the LS was found to have the potential to be catalytic when two mutations were introduced (Ballicora et al. J. Biol. Chem. 280, 10189-10195). Results obtained on enzyme properties from a site-directed mutagenesis study of the effect of six proline residues found within the first 66 amino acids at its N-terminus were consistent with the catalytic potential of the LS. Although non-catalytic, the LS was found to bind ATP at the same efficiency as the catalytic SS. More recent results have verified the catalytic potential of the LS. Introduction of a single mutation elevated the solubility

of the expressed LS, enabling it to form a homotetrameric structure. The LS homotetramer possesses very little enzyme activity at a level 100-fold less than that seen for the unactivated SS homotetramer. Unlike the SS enzyme, however, the LS homotetramer enzyme is not activated by the effector 3-phosphoglycerate or inhibited by Pi. When combined with the catalytically-silenced SS containing a D143N mutation, however, LS shows significantly enhanced enzyme activity and restored 3-PGA activation. Overall, these markedly distinctive properties of the LS, when assembled with itself or with the SS, provide new insights on its role in modulating enzyme catalysis and regulation. These results together with the kinetic data provide evidence that the LS is not only required to serve as a synergistic partner to the SS in affecting the net allosteric properties of the enzyme, but also for catalysis as well.

Manipulation of leaf starch metabolism in Arabidopsis. During the last year, we continued research on Arabidopsis plants which have been genetically modified to produce more leaf starch. The TL46 Arabidopsis mutant, which lacks the large subunit of AGPase, is starch-deficient and has lower rates of photosynthesis than wildtype. As reported last year, complementation of this mutant with up-regulated forms of the large subunit results in higher AGPase and higher starch biosynthesis than in wildtype. This year, a study with several transgenic lines versus wildtype plants, grown under current atmospheric levels of CO_2, was completed, including measurements of AGPase activity, chlorophyll content, leaf starch content, analysis of photosynthetic capacity and plant biomass. These results, combined with earlier studies, show a progressive increase in photosynthetic capacity and biomass production from starch-deficient mutants, to wildtype, to transgenic plants having increased capacity for starch biosynthesis. This indicates starch synthesis in leaves is an important transient sink for the initial products (triose-P) of photosynthesis.

Manipulation of seed starch metabolism in rice. From previous research, we have shown that expression of up-regulated AGPase in leaves of rice resulted in increased synthesis of leaf starch and biomass (from field experiments); and, that expression of an up-regulated AGPase in the cytoplasm, but not amyloplast, of developing rice seeds resulted in an increase (up to 10%) in seed weight. During the last year, reciprocal crosses have been made of these two lines through the F4 generation to develop lines having upregulated AGPase in both leaves and seed endosperm. Also, some initial analyses have been made on lines of transgenic rice having up-regulated AGPase in leaves, which show increase in plant biomass and panicle weight per plant compared to wildtype.

Usefulness of Findings

Our research is directed towards understanding and modifying source-sink relationships. C_4 plants prevent photorespiration and improve photosynthesis under CO_2-limited conditions. The single-cell C_4 system is very unique in developing two types of chloroplasts within individual photosynthetic cells. Defining the pattern of development and this mechanism(s) is important in designing approaches to genetically modify C_3 crops to improve carbon acquisition in source tissue (see ref. 6 for discussion relative to rice). Likewise, development of genetically modified rice to increase the capacity for starch synthesis in both source and sink tissue provides unique lines for testing the potential to improve yields of this economically important crop. Through our

research we have been affiliated with the International Rice Research Institute (IRRI), The Philippines, which has long been interested in improving source-sink relationships in rice.

Plans for next year

We are very interested in the mechanisms for development of cellular organization for a functional single-cell C_4 system. Thus, our future objectives are to elucidate how the single-cell C_4 system develops from young chlorenchyma cells, which includes development of two cytoplasmic compartments containing dimorphic chloroplasts with specific functions associated with C_4 photosynthesis.

Utilizing the AGP transformants of Arabidopsis having higher capacity for leaf starch biosynthesis which have been developed through backcrossing and several generations, we will evaluate their performance in photosynthesis and growth relative to wildtype plants when grown under CO_2-enriched atmospheric conditions, where there is a greater potential for increased carbon fixation (triose-P production by source tissue) due to reduction of photorespiration.

Future studies will be conducted on rice transformants having up-regulated AGPase in leaves to assess starch biosynthesis, photosynthesis and production of biomass compared to wildtype plants. In addition, the lines which have been developed through reciprocal crosses to have up-regulated AGPase in both leaves and seed endosperm will be analyzed for changes in source and sink tissues to determine the impact on carbon partitioning and yield.

Publications

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