Perspective

Phytochemical Engineering: Combining Chemical Reaction Engineering with Plant Science

Jacqueline V. Shanks
Dept. of Chemical Engineering, Iowa State University, Ames, IA 50011

DOI 10.1002/aic.10418
Published online in Wiley InterScience (www.interscience.wiley.com).

Introduction

Imagine a chemical reactor that only uses CO_2, water from any source, and light as reactants and produces chemicals and materials for the 6 F's sectors in our economy: food for humans, feed for animals, fiber, fuel, pharmaceuticals, and feedstocks for the chemical industry. Now, at the same time, visualize that this same chemical reactor can provide O_2 for fresher air and clean-up xenobiotics (man-made organic chemicals), and metals from soil and groundwater while being aesthetically pleasing. Sounds farfetched? Not really. Plants have evolved to have exquisite and more complicated chemistries than any other living organism. At a minimum, 40,000 chemical compounds have been identified in plants, and estimates of 100K are plausible, given that only a small fraction of the plant kingdom has been analyzed. For example, India, with 8% of the world's biodiversity, has over 45,000 plant species, of which 15,000 are medicinal plants.[1] A given medicinal is typically derived from unique chemistries only observed in one or a few species. Vincristine, perhaps better known as the chemotherapy agent Oncovin®, is only synthesized in the periwinkle plant *Catharanthus roseus*, and the sole source of the compound is extraction from this plant species (Figure 1).

Plants already provide products in the first 5 "F" sectors on the world market. Research in the 6th F, plant biomass as a source of biorenewable feedstocks, has seen a recent renaissance as a result of technological advances, as well as the awareness of the several societal and environmental costs associated with reliance on oil as a feedstock.[2,3] A 7th "F" also has emerged in the last decade—phytoremediation. Plants are being used to remediate contaminated lands and groundwater, and field proof of principle has been demonstrated for Se, TCE, MTBE, PCE, CCL_4 and other organic contaminants.[4] The annual market for phytoremediation is on the order of 10^7 US dollars (in contrast to the pharmaceutical industry at 10^9). Given the exquisite chemistries in plants, and the numerous commercial applications, it is natural for chemical engineers to be involved in exploiting these chemistries.

In 1985, if one asked an attendee of the annual AIChE meeting where they would find out about chemical engineering research in (living) plants, you would be directed to Michael Shuler or Henrik Pederson. Thus began a small but steady nucleus of chemical engineers working in plant tissue culture. The 1993 text by Payne et al.[5] defined the role of chemical engineers in this field at that time. The book mainly highlighted bioreactor operation of plant cells or tissues and strategies that could enhance production of pharmaceuticals. In keeping with the trends in biochemical engineering at that time, fundamentals of plant metabolism was relegated to one chapter. However, mirroring the transition within the chemical engineering profession from biochemical engineering practice to biochemical engineering science,[6] current research in this field focuses on the biochemistry—especially the reaction networks—of the plants themselves. Although multitudes of chemical engineers study microbial reaction networks and their applications, unfortunately the number of chemical engineers specializing in plant reaction networks is very few.

Several issues make research in plant reaction networks more difficult than their microbial counterparts. Plants have evolved to adapt to several environmental conditions. This unique plant property is termed plasticity. Thus, the same reaction network can exist in two or three different intracellular compartments—as shown in Figure 2. Parallel reaction networks can communicate via transporters, thus making intracellular transport processes important. Consequently, efforts to quantify fluxes in a single intracellular compartment model lose validity. Furthermore, plants have different tissues (roots, stems, leaves) and different cell types within a tissue. Competing or co-dependent reaction pathways will exist between cell or tissue types. Plant biochemistry is less understood than for other living systems. Plant reaction pathways and their control architectures are more complex, and also less understood than for their microbial counterparts. The topology of a reaction pathway is often incomplete. Many plant enzymes have not been identified or characterized, and the corresponding genes encoding them have not been cloned, particularly those in pathways that produce plant medicinal compounds.

J. V. Shanks e-mail address is jshanks@iastate.edu.

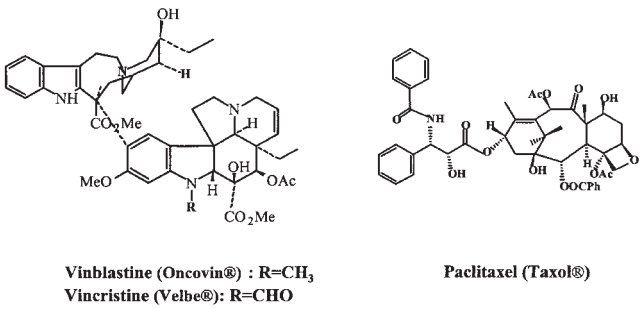

Figure 1. Chemical structures of plant pharmaceuticals vinblastine, vincristine from the periwinkle *Catharanthus roseus*, and paclitaxel from the Pacific Yew *Taxus brevifolia*.

In an attempt to summarize the endeavor of the area of work devoted to plant applications, the terms phytocatalysis and phytochemical engineering are introduced. "Phytocatalysis", coined in this article, is analogous to the plant world, whereas biocatalysis more often has the connotation of microbial systems. Biocatalysis spans the range from fermentations and transformations by pure enzymes. The term "phytocatalysis" is used to denote any reaction(s) in which a substrate is converted to product by a plant enzyme and the engineering concerned with these reactions and reaction networks. Phytocatalysis can take place within a plant cell, or in a different host (*E. coli*, yeast) that expresses a plant enzyme as in the case of carotenoid fermentation in *E. coli*. "Phytochemical engineering", coined by chemical engineer R. A. Mashelkar,[7] Director of the Council of Scientific and Industrial Research (CSIR) in India, refers specifically to the production of pharmacologically active natural products synthesized via phytocatalysis. Phytochemical engineering has a central role in CSIR's vision of the future. In this country, chemical engineers began intensive interactions with microbiologists over 20 years ago, which helped to spawn the field of metabolic engineering. This article will focus on the opportunities for chemical engineers in studying reaction networks within plants or their cells and tissues, and the role that chemical engineers can play in making significant advances in the plant sciences through phytocatalysis and phytochemical engineering.

System-wide analyses of complex reaction pathways

Modern plant breeding programs incorporate "systems biology" efforts in order to understand the interplay of complex genetic traits and environmental conditions on desired plant properties such as seed composition. Systems biology has many definitions, even to chemical engineers.[8] The common elements in all definitions are the blend of theoretical, experimental and computational tools to study the biological system on a large scale. Previous perspective articles in this journal have highlighted the role of chemical engineers in different facets in understanding and manipulating the metabolism of a living organism. Articles in functional genomics,[9] metabolic engineering,[10] proteomics,[11] and the biochemistry-engineering interface[6] have highlighted unique challenges that can be addressed through chemical engineering skills. In plants, several plant genomes have been sequenced (http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html is an entry into this information), and transcriptome analysis has been facilitated by microarray chips manufactured exclusively for plant species, especially for *Arabidopsis*, the *E. coli* of the plant world, and crop plants. Several plant science programs worldwide are advancing proteomic and metabolomics techniques. Clearly, a whole life science arena beyond microbes and mammalian systems exists in which chemical engineers can make significant contributions.

Metabolomics

Metabolomics is another "omics" field in which many opportunities exist for chemical engineers. Metabolomics, a hybrid term from metabolism and genomics, is the science of understanding the entire metabolism of an organism.[12] Metabolite profiling, the goal of which is measuring the levels of all metabolites in the biological material, is the predominant method for obtaining analytical data at the level of metabolism. Being able to measure the levels of multitudes of metabolites at once has been a dream of chemical reaction engineers in the biological arena. The goal of metabolite profiling will likely be reached within a decade. Currently, labs worldwide in plant metabolite profiling are coordinating their efforts in sharing technology and databases of chemical property data.

In metabolite profiling, metabolites are extracted from tissue and then analyzed by one or more detection systems in a high-throughput manner. Technology has now advanced to semi-automatically quantify ≈500 compounds from potato tubers and ≈1,000 compounds from a single leaf extract.[13] However, a high number of peaks in the MS spectra are unknown. Combined GC-MS and LC-MS detection can measure several classes of metabolites: sugars, sugar alcohols, amino acids, organic acids, fatty acids, and some secondary metabolites. Attention to extraction methods and analytical detection methods now allow the quantitative comparison of a given metabolite between biological samples, but quantitative comparison between metabolite A and metabolite B in a given sample is not

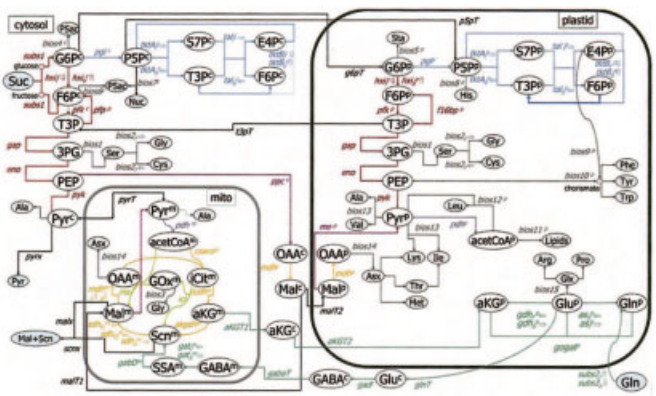

Figure 2. Reaction network for central carbon metabolism in embryos of soybean *Glycine max*.

Parallel pathways for glycolysis and the pentose phosphate pathway exist in the cytoplasm and the plastid and communication between them occur through three transporters: glucose 6-phosphate (g6pT), pentose 5-phosphate (p5pT), and triose 3-phosphate (t3pT).

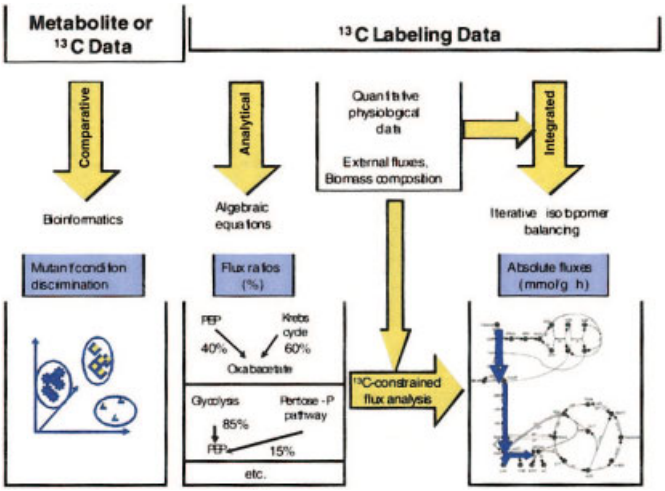

Figure 3. Overview of methods to describe the metabolome.

Adapted from Sauer.[35]

yet possible. Metabolism is even further removed from the genome than the proteome, and this relationship is expected to be more complex.

Metabolite profiling has clearly and comprehensively shown that a "steady-state" level of a given metabolite in a given tissue of the same genotype does not have one value, and the variance cannot be explained by experimental error.[14] These metabolites do not vary independently, but show strong correlations with the concentrations of other metabolites, as metabolite-metabolite scatter plots clearly showed for several metabolite pairs - all at the same time point. Clearly, the underlying biochemical networks provide the dependence. The probable basis for the biological variation observed is that cell metabolism is a complex dynamic system in which small fluctuations can be propagated throughout the network on the way to the steady-state condition.

The major method of analysis of metabolite data exists in the construction of metabolic correlation networks. Metabolite pairs with strong correlations (over some threshold value) are connected graphically, and the strength of the correlation shown in the distance between the pair. However, stochastic theory shows that the correlation matrix cannot be used to deduce the reaction network and gives only partial information about the network.[14] This result highlights the fact that kinetic relationships and fluxes tie together the metabolic reaction network with metabolite levels and that interpretation of metabolite data is problematic in the absence of fluxes. Hypothetically, metabolite profiling data should be able to be connected to enzymes in plant metabolic networks and by extension to gene regulatory networks. The development of proper frameworks presents interesting challenges to the nonlinear dynamics field and to the community of chemical engineers.

Figure 3 indicates the various means to describe the metabolome. Correlative analysis of metabolite data is comparative and not as clear of a link to underlying metabolic pathways as flux analysis, which is a cornerstone in metabolic engineering. Comparative analysis fluxome profiling using raw ^{13}C GC/MS data seeks to overcome that disadvantage and complements the more traditional flux analysis.[15] Increasing levels of information can be obtained from labeling data when coupled with a stoichiometric model of the biochemical pathways and computational methods (both standard chemical engineering tools) to solve for flux data in a larger network. More rigorous analysis is indicated as one moves from analytical to ^{13}C constrained flux analysis (stoichiometric model with a few flux ratios as constraints) to fully integrated determination of fluxes from all the experimental data and the stoichiometric and isotopomer balances. Iterative methods have been used to solve the full relationship of isotopomer balances, and the NMR or GC measurements to provide consistency, and routines to minimize error from the overdetermined data sets are required.

Fluxes, however, are even more difficult to measure than metabolite levels and consequently the quantification of fluxes in plants has been given less attention. Due to the compartmentation in plants, more experimental measurements are needed than in microbial systems, and the number of isotopomer balances increases, further increasing the computational burden. Although flux data is recognized in the plant science community as having value, mathematical difficulty has been a significant barrier to plant scientists. This is easily witnessed in the plant literature, as the few "flux" labeling studies in plants have focused on the identification of metabolic network topology[16,17] or flux quantification using analytical or a highly-simplified ^{13}C NMR constrained analysis.[18-20] The quantitative chemistry and mathematical skills of chemical engineers can be used to overcome these barriers, as shown in the example of central carbon metabolism in soybeans.

Carbon Allocation in Seeds

Many crop plants exhibit storage product metabolism in that large reserves of carbohydrates, oil, or protein accumulate at maturity in seeds. These carbon reserves are highly important to the U.S. economy, both in terms of domestic utilization in the food, feed, industrial and pharmaceutical health sectors, but also in exports. In 2003, U.S. soybean and soybean markets alone were $9.7 billion. Seed composition, including both the specific yield (amount of product/seed dry weight) and quality of the oil, protein, or carbohydrate fraction, is a highly important determinant in the seed value, as separations are a significant portion of the end-use processing costs. Both genetic and environmental factors influence seed composition. Given the plasticity of the seed composition to environmental conditions, the flow of carbon and carbon allocation in the seed are important factors. Not surprisingly, then, the study of central carbon metabolism and physiology has been an important research area in plant breeding for a better part of a century. Plant breeding programs require a large capital investment and substantial organization of plant lines. The chemical engineers' role here is not in the genetic manipulation of pathways or in the housing of biological material - the pathway analysis skills from chemical engineering are integrated in a collaborative team effort.

The developing soybean cotyledon, shown in Figure 4, uses sucrose and glutamine as carbon sources during growth in the seed pod, and can be modeled easily for pseudo-steady-state metabolism. The quantification of fluxes of parallel pathways in two compartments (e.g., cytosolic and plastidic oxPPP, mitochondrial and plastidic malic enzymes) in plants (Figure 2) was achieved through several improvements in the experimen-

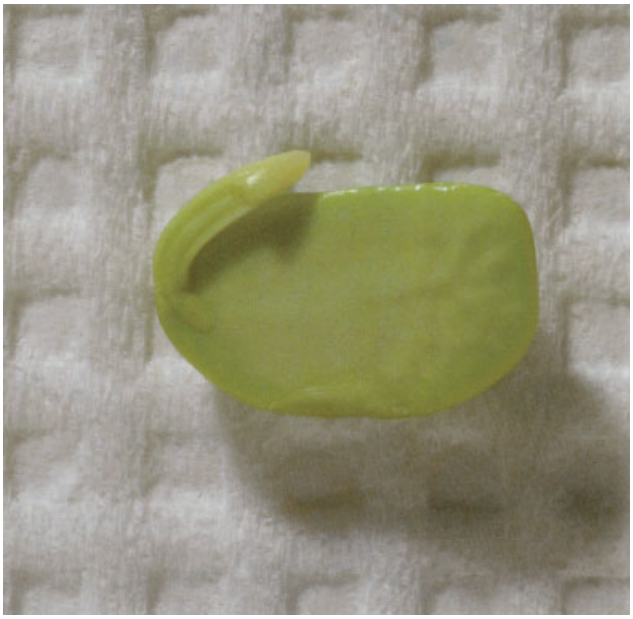

Figure 4. Soybean cotyledon harvested from the pod during the linear fill growth period, seventeen days after flowering.

tal and computational aspects.[21] Two-dimensional (2-D) NMR of the protein hydrolysate provided an overdetermined data set not only from several amino acids, but also from the sugar residues from glycosylated protein. This latter information was the critical piece of new data required to resolve compartmentation of the plastid vs. the cytoplasm. To convert the isotopomer abundances and ^{13}C enrichments in that data set to fluxes, a computer tool was developed, which incorporated recent mathematical and statistical advances, such as Boolean function mapping[22] and explicit solution methods of isotopomer balances.[23] The saving in computation time by using Boolean function mapping with the exact isotopomer solution methods allows additional statistical analysis of fluxes—a key feature for facilitated communication with a plant science community that is wary of models.

A powerful tool should be expected to provide new information to the plant sciences and flux analysis delivers. The metabolic flux maps of soybean provided concrete experimental evidence for the oxidative pentose phosphate pathway model as hypothesized from genetic sequences of enzymes and enzymatic studies.[24] The analysis is also a discovery tool, as an inconsistency was observed in leucine biosynthesis as defined in the biochemical texts, online databases, and the literature. Network topology issues are more common in plants than in microbes, and the use of this tool for other plant species would begin to address some of the gaps in our knowledge of plant reaction pathways. This gap in knowledge also extends to synthesis of natural products in plants through secondary metabolite pathways, as discussed next.

Phytochemical Engineering

Natural products play a large role in the pharmaceutical industry, either as novel lead compounds or as the actual drugs. The international market of medicinal plants-related trade is estimated at $60 billion per year and growing at about 7%.[7] The options to produce natural products in order of pathway complexity are chemical synthesis, microbial fermentation, and extraction from plants. Chemical synthesis is unfeasible for many microbial and plant metabolites. Fermentation of microbes genetically engineered to produce plant metabolites would be an advantageous production system. Chemical engineers are in the forefront in the metabolic engineering of microbial systems to product plant natural products such as carotenoids[25] and terpenoids.[26]

Poor expression of plant enzymes due to rare codon usage, and the toxicity to microbes of plant natural products, which have insecticidal, antifungal or antibacterial activity, have hindered a wider range of plant metabolites produced by microbial fermentation. Medicinal plants then are the only source, but with their exotic chemistries they are often endangered species, and plant production is subject to changes in agricultural and political climate. Plant tissue culture (PTC), the growing of plant cells or tissues in highly controlled bioreactors analogous to microbial fermentation, is consequently attractive but significant engineering advances were needed to make it viable commercially.

The production of paclitaxol (Taxol®), one of the leading drugs for ovarian and breast cancer with a market of approximately $1.5 billion a year, benefited directly from traditional biochemical engineering principles. Commercial sources range from extraction from the Pacific Yew *Taxus brevifolia*, an endangered species, to semisynthesis from a lead compound from a nonendangered plant source, and, recently, plant tissue culture. Michael Shuler's laboratory at Cornell, already conversant with plant tissue culture and bioreactor technology, pioneered the early quantitative and rigorous studies of PTC with paclitaxel.[27,28] A duo of his former students formed Phyton, Inc. and for the first time enabled commercial-scale production of paclitaxel from PTC (DFB Pharmaceuticals, Inc. acquired Phyton, Inc. in November, 2003).

Despite the success of PTC of paclitaxel, commercial production of other medicinals by PTC is very limited. The challenges lie in the complexity of the reaction pathways. The indole alkaloids Vincristine (Oncovin®) and vinblastine (Velbe®), used to treat non-Hodkins lymphoma, Hodgkins disease, leukemias and other cancers since the 1970s, provide a case in point. Numerous attempts to produce these compounds by chemical synthesis or plant tissue culture have been unsuccessful. Commercial production is still by extraction of *C. roseus*. Although the *C. roseus* indole alkaloids and paclitaxel are derived from common terpenoid pathways in plants, the *C. roseus* pathways involve a greater number of reaction pathway steps, exist in three plant organelles plus the cell cytoplasm and in at least two cell types, and are under developmental regulation. Thus, the reaction pathway includes intra- and inter- membrane transport steps, as well as activation of enzymes by an environmental trigger(s). Thus, while paclitaxel can be synthesized in a suspension of single cells,[29] vinblastine and vincristine cannot. However, despite the complexities of the indole alkaloids pathways, substantial flux can be directed to a key metabolite six reaction steps away from the key missing metabolite for vincristine and vinblastine.[30]

The challenges to metabolically engineer such a complex pathway leading toward a particular product are daunting. However, a bright future is taking shape.[31] Key techniques used

to metabolically engineer microbes—cloning, cell culture, metabolite, and flux analysis and modeling—are now being performed by chemical engineers in phytochemical engineering. Genetic transformation by *Agrobacterium* is standard and inducible promoters, a tool in which to change a particular enzyme activity, while having available a genetic control, can be used to more systematically study engineered plant pathways.[32] Tools for flux analyses in primary metabolism,[21] as well as secondary metabolism[33] have been established. Single cell suspensions of otherwise aggregate cultures can now be made for more fundamental studies.[29]

Further progress in phytochemical engineering will rely on additional developments of synthesis and analysis tools. Targeting of an enzyme to a particular compartment or organelle will need to become routine for eliminating intracellular transport limitations. Difficult organelles such as chloroplasts are beginning to be genetically engineered by plant scientists.[34] Concrete identification and quantification of secondary metabolites by metabolite profiling of secondary metabolites will be crucial as genetic manipulation can lead to new sinks or additional flux through unknown pathways—which then have to be identified. The rational and systematic analysis of secondary metabolite reaction pathways by engineers points to branches in which genes and enzymes need to be identified - the domain of plant scientists. Thus, effective collaborations with plant scientists are absolutely necessary for the future of phytochemical engineering.

Concluding remarks

This article has highlighted the opportunities for chemical engineers to make significant advances of the plant sciences by using their chemical reaction engineering and biochemical engineering skills. Rational and systematic methods developed in metabolic engineering are likely to be more important in plant systems than in microbial systems, due to the complexity of plants and their inherently slower growth rate. Thus, chemical engineers with highly developed systems training are perhaps even more critical in plant systems than in microbial systems. Clearly, plant scientists and chemical engineers have complementary expertises, but since there are so few chemical engineers in such collaborations, the dialog has been limited and needs to be fostered.[31] This article provides momentum to continue and accelerate the dialog.

Acknowledgment

Support from the National Science Foundation, the Plant Sciences Institute at Iowa State University, the Iowa Soybean Board, and the Strategic Environmental Research and Development Program (SERDP) is gratefully acknowledged. Thanks to L. K. Doraiswamy for his encouragement and to Vassily Hatzimanikatis and Ramon Gonzalez for their helpful suggestions in the preparation of this article.

Literature Cited

1. Mashelkar RA. Evolution of new knowledge systems by bridging traditional wisdom and modern science, John Bridgewater Symposium, Cambridge, U.K., September 29, 2004.
2. National Research Council Report. *Biobased Industrial Products, Priorities for Research and Commercialization*. Washington, DC: National Research Council, National Academy Press, 2000.
3. Department of Energy. *The Technology Roadmap for Plant/Crop-based Renewable Resources 2020*. 1999: DOE/GO-10099-706.
4. McCutcheon SC and Schnoor JL, eds. *Phytoremediation: Transformation and Control of Contaminants*. Hoboken, NJ: Wiley Interscience, Inc., 2003.
5. Payne G, Bringi V, Prince C, and Shuler ML. *Plant Cell and Tissue Culture in Liquid Systems.* New York: Wiley Interscience, Inc., 1993**.**
6. Khosla C. Biochemistry-engineering interface in biochemical engineering. *AIChE J.,* 2002;48:1366-1368.
7. Mashelkar RA. The new millennium challenges for Indian science & technology. New Delhi: CMS Communication Colloquium National Lecture Centre for Media Studies, July 23, 1999.
8. Henry CM. Systems biology. *C&EN*, 2003;81:45-55.
9. Hatzimanikatis V. Bioinformatics and functional genomics: Challenges and opportunities. *AIChE J.*, 2000;46: 2340-2343.
10. Stephanopoulos G. Metabolic engineering: perspective of a chemical engineer. *AIChE J.,* 2002;48:920-926.
11. Lee K and Reardon R. Proteomics: an exciting new science, but where are the chemical engineers? *AIChE J.,* 2003; 49:2682-2686.
12. Fiehn O. Metabolomics - the link between genotype and phenotype. *Plant Mol. Biol*., 2002;48:155-171.
13. Weckworth W, Loureiro ME, Wenzel K, and Fiehn O. Differential metabolic networks unravel the effects of silent plant phenotypes. *PNAS,* 2004;10:7809-7814.
14. Steuer R, Kurths J, Fiehn O, and Weckwerth W. Observing and interpreting correlations in metabolomics networks. *Bioinformatics,* 2003;19:1019-1026.
15. Zamboni N and Sauer U. Model-independent fluxome profiling from ^{2}H and ^{13}C experiments for metabolic variant discrimination. *BMC Genome Biology*, 2004;5:R99.1–R99.12.
16. Krook J, Vreugdenhil D, Dijkema C, and van der Plas LHW. Sucrose and starch metabolism in carrot (*Daucus carota*) cell suspensions analyzed by ^{13}C labeling: indications for a cytosol and a plastid-localized oxidative pentose phosphate pathway. *J. Exp. Bot.,* 1998;49:1917-1924.
17. Glawischnig E, Gierl A, Tomas A, Bacher A, and Eisenreich W. Starch biosynthesis and intermediary metabolism in maize kernels. Quantitative analysis of metabolite flux by nuclear magnetic resonance. *Plant Physiol.,* 2002;130: 1717-1727.
18. Dieuaide-Noubhani M, Raffard G, Canioni P, Pradet A, and Raymond P. Quantification of compartmented metabolic fluxes in maize root tips using isotope distribution from ^{13}C- or ^{14}C-labeled glucose. *J. Biol. Chem.,* 1995;22: 13147-13159.
19. Rontein D, Dieuaide-Noubhani M, Dufourc EJ, Raymond P, and Rolin D. The metabolic architecture of plant cells: stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. *J. Biol. Chem.,* 2002; 277:43948-43960.
20. Schwender J, Ohlrogge JB, and Shachar-Hill Y. A flux

model of glycolysis and the oxidative pentosephosphate pathway in developing *Brassica napus* embryos. *J. Biol. Chem.* 2003;278:29442-29453.
21. Sriram, G, Fulton DB, Iyer V, Peterson JM, Zhou R, Westgate ME, Spalding MH, and Shanks JV. Quantification of compartmented metabolic fluxes in developing soybean (*Glycine max*) embryos by employing biosynthetically directed fractional ^{13}C labeling, 2-D [^{13}C, ^{1}H] NMR and comprehensive isotopomer balancing. *Plant Physiol.,* 2004;136:3043–3057.
22. Sriram G and Shanks JV. Improvements in metabolic flux analysis using carbon labeling experiments: Bondomer balancing and boolean function mapping. *Metab. Eng.* 2004;6:116-132.
23. Wiechert W and Murzel M. Metabolic isotopomer labeling systems. Part I: global dynamic behavior. *Math. Biosci.,* 2001;169:173-205.
24. Kruger NJ and von Schaewen A. The oxidative pentose phosphate pathway: Structure and organization. *Curr. Opin. Plant Biol.,* 2003;6:236-246.
25. Farmer WR and Liao JC. Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. *Biotechnol. Prog*., 2001;17:57-61.
26. Martin VJJ, Pitera DJ, Withers ST, Newman JD, and Keasling JD. Engineering a mevalonate pathway in *Escherichia coli* for the production of terpenoids. *Nature Biotechnol*., 2003;21:796-802.
27. Srinivasan V, Pestchanker L, Moser S, Hirasuna TJ, Taticek RA, and Shuler ML. Taxol production in bioreactors: Kinetics of biomass accumulation, nutrient uptake and taxol production by cell suspensions of *Taxus baccata. Biotechnol. Bioeng*., 1995;47:666-676.
28. Zhong JJ. Plant cell culture for the production of paclixel and other taxanes. *J. Biosci. Bioeng*., 2002;94:591-599.
29. Naill MC and Roberts SC. Preparation of single cells from aggregated taxus suspension cultures for population analysis. *Biotechnol. Bioeng*., 2004; 86:817-826.
30. Morgan J and Shanks JV. Inhibitor studies of tabersonine metabolism in *Catharanthus roseus* hairy roots. *Phytochemistry,* 1999;51**:**61-68.
31. Hanson AD and Shanks JV. Plant metabolic engineering - Entering the S curve. *Metab. Eng.,* 2002;4:1-2.
32. Hughes E, Hong S-B, Shanks JV, San K-Y, and Gibson SI. Expression of a feedback-resistant anthranilate synthase in *Catharanthus roseus* hairy roots provides evidence for tight regulation of terpenoid indole alkaloid levels. *Biotechnol. Bioeng.,* 2004;86:718-727.
33. Morgan J and Shanks JV. Quantification of metabolic flux in plant secondary metabolism by a biogenetic organizational approach. *Metabolic Engineering*, 2002;4:257-262.
34. Daniell H, Khan MS, and Allison L. Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends Plant Sci.,* 2002;7:84-91.
35. Sauer U. High-throughput phenomics: Experimental methods for mapping fluxomes. *Curr. Opin. Biotechnol*., 2004; 15:58-63.