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The Commemorative International Symposium for the 50th Anniversary of **KSABC**

- Applied Biological Chemistry Toward Centennial -

August 25-27, 2010, Hotel Hyundai Gyeongju, Korea



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Dates : August 25 - 27, 2010 | **Venue** : Hotel Hyundai Gyeongju

Program

■ Plenary Lecture (PL)

Dr. Robert Huber (Max-Planck-Institut fuer Biochemie, Germany)

Nobel Laureate in Chemistry 1988

■ International Symposium (IS)

IS1	Plant Molecular Biology
IS2	Natural Products
IS3	Pesticides/Environmental Science
IS4	Antibody Engineering
IS5	Genetically Modified Organisms
IS6	Biological Chemistry
IS7	Soil Science/Fertilizer

■ Symposium for Annual Meeting (S)

S1	Biogreen 21: Bioactive Materials
S2	National Research Group on Residual Pesticide Safety Control in Food
S3	Biogreen 21: GMO Development



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■ Young Scientist Presentation (YS)

YS1	Biochemistry • Molecular Biology
YS2	Bioactive Materials • Natural products
YS3	Environmental Science • Food Science/Microbiology

■ Poster Session

PBC	Biochemistry • Molecular Biology
PBM	Bioactive Materials • Natural products
PES	Pesticides/Environmental Science
PFM	Food Science/Microbiology
PBB	Biogreen 21: Bioactive materials
PBG	Biogreen 21: GMO Development

Schedule

August 25 (Wednesday)			
Time	Convention Hall (B1)		Diamond Hall (B1)
	Hall A	Hall C	
09:00 - 10:00	Registration		
10:00 - 11:20	YS1	YS2	YS3
11:20 - 12:30	lunch (Crystal Hall, 1F)		
12:30 - 13:10	Opening Ceremony (Hall A)		
13:10 - 14:10	Plenary lecture (Hall A)		
14:10 - 18:10	IS1	IS2	IS3
	Poster Session I (Multi-Purpose Hall, B2)		
	IS1	IS2	IS3
18:40 - 20:40	Welcome Reception I (Convention Hall)		
20:40 - 22:00	Poster Session I (Multi-Purpose Hall, B2)		

August 26 (Thursday)				
Time	Convention Hall (B1)			Diamond Hall (B1)
	Hall A	Hall B	Hall C	
9:00 - 12:10	IS1	IS2	S1	
	Poster Session I (Multi-Purpose Hall, B2)			
	IS1	IS2	S1	Forum
12:10 - 13:00	lunch (Crystal Hall, 1F)			
13:00 - 18:20	IS4	IS5	S1	S2
	Poster Session II (Multi-Purpose Hall, B2)			
	IS4	IS5	S1	S2
18:20 - 20:00	Beer Party for Students (Roof Garden, 2F) Welcome Reception II (Topaz Hall, B1)			
20:00 - 22:00	Poster Session II (Multi-Purpose Hall, B2)			

August 27 (Friday)				
Time	Convention Hall (B1)			Hall C
	Hall A	Hall B	Hall C	
9:00 - 12:10	IS6	S3		IS7
	Poster Session II (Multi-Purpose Hall, B2)			
	IS6	S3		IS7
12:10 - 13:00	General Assembly Members Meeting & Closing Ceremony (Hall A)			



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Plenary Lecture

August 25 (Wed), 13:10 - 14:10, Hall A

Intracellular protein degradation, basic research and application

Dr. Robert Huber

Session chair: Sangkee Rhee (Seoul National University, Korea)

Max-Planck-Institut fuer Biochemie, Germany

Universität Duisburg-Essen, Zentrum für Medizinische Biotechnologie, Germany

Cardiff University, School of Biosciences, UK

International Symposium

IS1

Plant Molecular Biology : Plant Cell Communication and Development

8/25 (Wed)

Session chair: Jae-Yean Kim (Gyeongsang National University, Korea)

14:10 - 14:50 William J. Lucas (University of California, Davis, USA)

14:50 - 15:20 Myeong Min Lee (Yonsei University, Korea)

15:20 - 16:00 David P. Jackson (Cold Spring Harbor Laboratory, USA)

16:00 - 16:20 *Coffee break*

Session Chair: David P. Jackson (Cold Spring Harbor Laboratory, USA)

16:20 - 16:50 Hyung-Taeg Cho (Seoul National University, Korea)

16:50 - 17:30 Ykä Helariutta (University of Helsinki, Finland)

17:30 - 18:00 Jae-Yean Kim (Gyeongsang National University, Korea)

8/26 (Thu)

Session chair: Leslie Sieburth (University of Utah, USA)

09:00 - 09:40 Hong Gil Nam (POSTECH, Korea)

09:40 - 10:15 Peter M. Gresshoff (The University of Queensland, Australia)

10:15 - 10:35 *Coffee break*



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Session chair: Peter M. Gresshoff (The University of Queensland, Australia)
10:35 - 11:05 Giltsu Choi (KAIST, Korea)
11:05 - 11:40 Leslie Sieburth (University of Utah, USA)
11:40 - 12:10 Gyung-Tae Kim (Dong-A University, Korea)

IS2 Natural Products : New trends in Natural Products

8/25 (Wed)

Session chair: Soo-Un Kim (Seoul National University, Korea)
14:10 - 14:50 Jonathan D. Walton (Michigan State University, USA)
14:50 - 15:30 Masahiro Miyashita (Kyoto University, Japan)
15:30 - 16:10 Norman G. Lewis (Washington State University, USA)

Session chair: Nam-In Baek (Kyung Hee University, Korea)
16:10 - 16:50 Mattheos A. G. Koffas (State University of New York, USA)
16:50 - 17:30 Ikuro Abe (The University of Tokyo, Japan)
17:30 - 18:10 Thomas Vogt (Leibniz-Institute of Plant Biochemistry, Germany)

8/26 (Thu)

Session chair: Jaehong Han (Chung-Ang University, Korea)
09:00 - 09:40 E. Neil G. Marsh (University of Michigan, USA)
09:40 - 10:20 William Helferich (University of Illinois, USA)
10:20 - 11:00 Nicki J. Engeseth (University of Illinois, USA)

Session chair: Chi-Hwan Lim (Chungnam National University, Korea)
11:00 - 11:30 Young Joon Surh (Seoul National University, Korea)
11:30 - 12:00 Ho Jeong Kwon (Yonsei University, Korea)

IS3 Pesticides/Environ. : Recent advance in pesticide and environmental science

8/25 (Wed)

Session Chair: Jeong-Han Kim (Seoul National University, Korea)
14:10 - 14:50 James N. Seiber (University of California, Davis, USA)
14:50 - 15:20 Hor-Gil Hur (Gwangju Institute of Science and Technology, Korea)
15:20 - 15:50 Young Soo Keum (Konkuk University, Korea)
15:50 - 16:20 *Coffee break*



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Session Chair: Jang-Eok Kim (Kyungpook National University, Korea)
16:20 - 17:00 Takayuki Shibamoto (University of California, Davis, USA)
17:00 - 17:30 Duk-Hee Lee (Kyungpook National University, Korea)
17:30 - 18:00 Su-Il Kang (Gwangju Institute of Science and Technology, Korea)

IS4 Antibody : Recent trends in antibody engineering and therapeutics

8/26 (Thu)

Session chair: Yong-Sung Kim (Ajou University, Korea)

13:00 - 13:30 James D. Marks (University of California, San Francisco, USA)
13:30 - 14:00 K. Dane Wittrup (Massachusetts Institute of Technology, USA)
14:00 - 14:30 Gou-Young Koh (KAIST, Korea)
14:30 - 15:00 Jin-San Yoo (PharmAbcine Co., Korea)
15:00 - 15:20 *Coffee Break*

15:20 - 15:50 Louis M. Weiner (Georgetown University Medical Center, USA)
15:50 - 16:20 Se-ho Kim (Greencross Co., Korea)
16:20 - 16:50 Junho Chung (Seoul National University College of Medicine, Korea)
16:50 - 17:20 Eun-Kyung Lee (NeoPharm Co., Korea)
17:20 - 17:50 Dewey Ryu (University of California, Davis, USA)

IS5 GMO : Current Developments in Genetically Engineered Plants

8/26 (Thr)

Session Chair: Yong-Hwan Kim (National Academy of Agricultural Science, RDA)
13:00 - 13:40 Richard G. F. Visser (Wageningen UR, Netherlands)
13:40 - 14:20 Peter Palukaitis (Scottish Crop Research Institute, UK)
14:20 - 15:00 Tsutomu Kawasaki (Kinki University, Japan)
15:00 - 15:30 Youngsook Lee (POSTECH, Korea)
15:30 - 16:00 *Coffee Break*

Session Chair: Richard G. F. Visser (Wageningen UR, Netherlands)
16:00 - 16:30 Donghern Kim (NAAS, RDA, Korea)
16:30 - 17:00 Ju-Kon Kim (Myongji University, Korea)
17:00 - 17:40 Raymond Layton (Pioneer Hi-Bred International, Inc., USA)
17:40 - 18:20 Xun Wang (Syngenta Biotechnology (China) Co., Ltd., China)



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IS6

Biological Chemistry : Recent advances in biological chemistry

8/27 (Fri)

Session Chair: Won Bae Jeon (Daegu Gyeongbuk Institute of Science & Technology, Korea)

09:10 - 09:50

Michael R. Freeman (Harvard Medical School, USA)

09:50 - 10:20

Kwang Pyo Kim (Konkuk University, Korea)

10:20 - 10:50

Coffee Break

10:50 - 11:30

Catharina Svanborg (Lund University, Sweden)

11:30 - 12:10

K. Hun Mok (Trinity College Dublin, Ireland)

IS7

Soil Science/Fertilizer : It's an urban soil you stand on, isn't it?

8/27 (Fri)

Session Chair:

09:00 - 09:40

Jean-Louis Morel (Nancy University, France)

09:40 - 10:10

Kye-Hoon Kim (The University of Seoul, Korea)

10:10 - 10:40

Dongwook Kim (Phygen Inc., Korea)

11:00 - 11:40

Thomas Nehls (Berlin Institute of Technology, Germany)

11:40 - 12:10

Dohaeng Heo (Seoul Metropolitan Government, Korea)



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Plenary Lecture

(PL)

Intracellular protein degradation, basic research and application

Robert Huber

Max-Planck-Institut für Biochemie, Am Klopferspitz 18, D-82152 Martinsried, Germany

Universität Duisburg-Essen, Zentrum für Medizinische Biotechnologie, D-45117 Essen, Germany

Cardiff University, School of Biosciences, Cardiff CF10 3US, UK

Within cells or subcellular compartments misfolded and/or short-lived regulatory proteins are degraded by protease machines, cage-forming multi-subunit assemblages. Their proteolytic active sites are sequestered within the particles and located on the inner walls. Access of protein substrates is regulated by protein subcomplexes or protein domains which may assist in substrate unfolding dependent of ATP. Five protease machines will be described displaying different subunit structures, oligomeric states, enzymatic mechanisms, and regulatory properties.

Proteasome

Groll, M., Ditzel, L., Löwe, J., Stock, D., Bochtler, M., Bartunik, H. D. and Huber, R. (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* **386**, 463-471.

Groll, M., Heinemeyer, W., Jäger, S., Ullrich, T., Bochtler, M., Wolf, D. H. and Huber, R.(1999) The catalytic sites of 20S proteasomes and their role in subunit maturation: A mutational and crystallographic study. *Proc. Natl. Acad. Sci. USA* **96**, 10976-10983.

Groll, M., Bajorek, M., Köhler, A., Moroder, L., Rubin, D. M., Huber, R., Glickman, M. H. and Finley, D.(2000) A gated channel into the proteasome core particle. *Nature Struct. Biol.* **7**, 1062-1067.

Groll, M., Schellenberg, B., Bachmann, A. S., Archer, C. R., Huber, R., Powell, T. K., Lindow, S., Kaiser, M. and Dudler, R. (2008) A plant pathogen virulence factor inhibits the eukaryotic proteasome by a novel mechanism. *Nature* **452**, 755-758.

HslV/HslU

Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G., Bartunik, H. and Huber, R.(2000) The structure of HslU and the ATP-dependent protease HslU-HslV. *Nature* **403**, 800-805.

Song, H. K., Hartmann, C., Ramachandran, R., Bochtler, M., Behrendt, R., Moroder, L. and Huber, R. (2000) Mutational studies on HslU and its docking mode with HslV. *Proc. Natl. Acad. Sci. USA* **97**, 14103-14108.

Ramachandran, R., Hartmann, C., Song, H. J., Huber, R. and Bochtler, M.(2002) Functional interactions of HslV(ClpQ) with the ATPase HslU(ClpY). *Proc. Natl. Acad. Sci. USA* **99**, 7396-7401.

Tricorn

Brandstetter, H., Kim, J. S., Groll, M. and Huber, R. (2001) Crystal structure of the tricorn protease reveals a protein disassembly line. *Nature* **414**, 466-470.

Kim, J. S., Groll, M., Musiol, H. J., Behrendt, R., Kaiser, M., Moroder, L., Huber, R. and Brandstetter H.(2002) Navigation inside a protease: substrate selection and product exit in the tricorn protease from *Thermoplasma acidophilum*. *J. Mol. Biol.* **324**, 1041-1050.

Goettig, P., Groll, M., Kim, J. S., Huber, R. and Brandstetter, H. (2002) Structures of the tricorn interacting aminopeptidase F1 with different ligands explain its catalytic mechanism. *EMBO J.* **21**, 5343-5352.

Dipeptidyl peptidase IV

Engel, M., Hoffmann, T., Wagner, L., Wermann, M., Heiser, U., Kiefersauer, R., Huber, R., Bode, W., Demuth, H. U. and Brandstetter, H. (2003) The crystal structure of dipeptidyl peptidase IV (CD26) reveals its functional regulation and enzymatic mechanism. *Proc. Natl. Acad. Sci. USA* **100**, 5063-5068.

DegP(HtrA)

Krojer, T., Garrido-Franco, M., Huber, R., Ehrmann, M., and Clausen, T. (2002) Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine. *Nature* **416**, 455-459.

Krojer, T., Pangerl, K., Kurt, J., Sawa, J., Stingl, C., Mechtler, K., Huber, R., Ehrmann, M. and Clausen, T. (2008). Interplay of PDZ and protease domain of DegP ensures efficient elimination of misfolded proteins. *Proc. Natl. Acad. Sci. USA* **105**, 7702-7707.

Krojer, T., Sawa, J., Schäfer, E., Saibil, H. R., Ehrmann, M., and Clausen, T. (2008). [Structural basis for the regulated protease and chaperone function of DegP](#). *Nature* **453**, 885-890.



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Invited Lectures

IS1

Plant Molecular Biology

Plant Cell Communication and Development

(IS1-1)

1. Speaker

William John Lucas (wjucas@ucdavis.edu)

2. Affiliation

Professor, University of California, Davis, USA

3. Appointments on Editorial Boards

The Plant Cell (Co-editor, 2003 – present)

The Plant Cell (Assigning Editor, 2006 – present)

Open Plant Science Journal (Editorial Board, 2007 – present)

Journal of Integrative Plant Biology (Associate Editor, 2007 – present)

Journal of Plant Biology (Overseas Editor, 2007 – 2011)

Plasmodesmata and the Phloem: Partners in Cell-to-Cell & Long-Distance Trafficking of Information Macromolecules

William J. Lucas, Byung-Kook (Brian) Ham, Gang Li, Ping-fang (Gloria) Li, Lijun (June) Liu, Yi Ma, Eriko Miura,
Michitaka Notaguchi, Soo-Cheul (Charles) Yoo

Dept. of Plant Biology, College of Biological Sciences, University of California, Davis CA 95616, USA

The sieve tube system of the phloem is comprised of two main cell types, the sieve elements (SE) and their associated companion cells (CC). Specialized, branched plasmodesmata (PD) interconnect these two cell types, thereby forming the CC-SE complex. At maturity, SEs are devoid of nuclei and most cytoplasmic organelles have been removed, or are greatly reduced in number. This simplified nature of the SE has given rise to the notion that these cells are incapable of synthesizing proteins. Thus, as the phloem translocation stream has been shown to contain a complex population of proteins (approx. 2,000 for the cucurbits), it would appear that the CC-SE PD mediate in the entry of these proteins into the SE. Support for this hypothesis has been provided by detailed studies on a number of phloem proteins. In general, proteins located in the phloem translocation stream have the capacity to interact with PD to mediate their cell-to-cell trafficking and, thus, entry/exit of these phloem proteins is likely regulated by the CC-SE PD. Our studies on the phloem RNA-binding protein, *Cucubita maxima* phloem protein16 (CmPP16), will be used to provide an example of such regulated non-cell-autonomous protein (NCAP) trafficking.

Identification of the proteins that function in the NCAP trafficking pathway is a major goal of our research program. We will describe experiments in which we used an antibody directed against the phloem NCAP, CmPP16-1, to perform co-immunoprecipitation (co-IP) experiments to identify interacting proteins contained within a plasmodesmal enriched cell wall preparation (PECP) isolated from tobacco BY-2 suspension cultured cells. Seven candidate proteins were identified

and four of these candidate proteins, when fused with RFP, appeared to be co-localized with both *Cucumber mosaic virus* (CMV)-MP:GFP and *Tobacco mosaic virus* (TMV)-MP:GFP being expressed in transgenic *Nicotiana benthamiana*. These results are consistent with their being components of the plasmodesmal NCAP pathway.

One candidate protein, named *N. tabacum* PLASMODESMAL GERMIN-LIKE PROTEIN1 (NtPDGLP1), was chosen for further study. NtPDGLP1 appears to be targeted to plasmodesmata through the secretory pathway in a brefeldin A-sensitive manner. The N-terminal signal peptide on NtPDGLP1 was necessary and sufficient for this PD targeting. A GST-pull down assay showed that NtPDGLP1 directly interacts with CmPPI16 and that the C-terminal region of NtPDGLP1 is involved in this CmPPI16 binding. NtPDGLP1 has 29 *Arabidopsis* orthologs and 2 members, named AtPDGLP1 and AtPDGLP2, exhibited similar PD localization patterns; however, the other members of this gene family accumulated along the length of the plasma membrane. PDGLP overexpression plants displayed slightly enhanced growth rate compared with wild-type plants. PDGLP also appeared to interact with a range of PECP proteins indicating that it may function within PD as a larger complex. These findings will be discussed in terms of the role of the PDGLP in NCAP trafficking and the strategy for building a comprehensive model of the NCAP pathway.

The phloem translocation stream contains a complex sub-population of RNA molecules (both mRNA and si/miRNA). Although a role for some of these RNA species has been demonstrated, the majority have yet to be fully characterized. In addition, molecular studies on the ribonucleoprotein (RNP) complexes which operate in the delivery of mRNA to target sink tissues are in their infancy. Insights into the complexity of such RNP complexes will be illustrated by our ongoing studies on the *Cucubita maxima* RNA-binding protein50 (CmRBP50) system.

In order to develop a comprehensive study of phloem proteins and RNA, we have been working towards the development of genomics resources for plants from which analytical quantities of phloem sap can be collected. Ideally, at a minimum, we need the genomes of two graft-compatible species. To this end, we recently completed the sequencing of the cucumber genome; the recent re-sequencing of several additional cucumber cultivars will provide an excellent range of genetic materials for grafting experiments. It is anticipated that the pumpkin genome will be completed in the near future. Ongoing studies are being directed at developing the following cucumber phloem/vascular databases: (a) vascular and phloem transcriptomes, (b) vascular and phloem proteomes, (c) a phloem metabolome. Our cucumber data is currently being used to develop a metabolic network that will map the enzymes detected in the phloem translocation stream into biochemical pathways that produce the identified metabolites. Once the pumpkin genome has been completed, an equivalent set of databases will be generated. Separation of phloem proteins and RNA into locally-acting and long-distance trafficking agents will be accomplished using a pumpkin (stock):cucumber (scion) heterografting system.

Interrogation of our phloem databases has provided interesting new insights into the functioning of the sieve tube system. We anticipate that availability of the cucumber and pumpkin genomes and associated omics databases will establish a powerful platform for the study of PD and the phloem as an information superhighway.

Acknowledgments: These studies are funded by NSF grants IOS-0918433 and IOS-0752997 and by National Research Initiative Grant 2006-35304-17346 from the USDA Cooperative State Research, Education and Extension Service.

(IS1-2)

1. Speaker

Myeong Min Lee (mmlee@yonsei.ac.kr)

2. Affiliation

Associate Professor, Yonsei University, Korea

3. Appointments on Editorial Boards

Developmental Dynamics (Editorial Board Member, 2004. 02 – present)

WEREWOLF, an R2R3 MYB-type transcription factor enhances the non-hair cell fate specification in a dosage sensitive manner in the *Arabidopsis* root epidermis

WEREWOLF (WER), a MYB-type transcription factor is known to play a pivotal role in specifying the fates of root epidermal cells of *Arabidopsis* based on the previous molecular genetic studies. However, it is difficult to fully understand how WER functions in this genetic pathway because the over-expression of WER under the control of cauliflower mosaic virus 35S (CaMV 35S) promoter failed to induce any discernible phenotype in the wild-type background. In this study, we have identified several enhancer trap promoters inducing distinctive hairless phenotypes by using the GAL4-*pUAS:WER* trans-activation system. We show that the ectopic expression of Q2610>>WER, exhibiting a strong hairless phenotype, positively regulates the expression of *GLABLA2 (GL2)* and *AtMYB23*, genes specifying non-hair cell fate, together with *CAPRICE (CPC)*, a gene specifying hair cell fate. WER directly enhances the expression of *GL2* by the interaction with specific cis-elements localized in the *GL2* promoter. Q2610>>WER negatively regulates the expression of *ENHANCER OF GLABLA3 (EGL3)*, *AtMYC1*, and *TRANSPARENT TESTA GLABLA1 (TTG1)*. We also find that WER requires EGL3 and EGL3, bHLH proteins for its proper function while it does not require TTG1, a WD40 protein. Furthermore, we show that the Q2610>>CPC, exhibiting strong hairy phenotype, suppresses the phenotype induced by Q2610>>WER whereas *p35S:CPC* does not. Both WER and CPC interact with the bHLH proteins in vivo. These results suggest that WER and CPC determine the cell fate in a dosage sensitive manner by competing with each other in the regulation of *GL2* expression in *Arabidopsis* roots.

(IS1-3)

1. Speaker

David P. Jackson (Jacksond@cshl.org)

2. Affiliation

Professor, Cold Spring Harbor Laboratory, USA

3. Editorial Board and Scientific Review Appointments

Editorial Board: Plant Signaling and Behavior (2005-), Plant Cell (2008-).

USDA Developmental Mechanisms (2000, 2002, 2006).

DOE Energy Biosciences (2003).

NSF Developmental Mechanisms (2004, 2008).

Contributing member, Faculty of 1000 (2004-).

How do non-autonomous signals traffic through plasmodesmata?

Xianfeng (Morgan) Xu, Yoselin Benitez, Jing Wang and Dave Jackson.

Cold Spring Harbor Lab.,

1 Bungtown Rd., Cold Spring Harbor, New York, NY 11724.

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Cell-to-cell communication plays critical roles in specifying cell fate and coordinating development in all multi-cellular organisms. A new paradigm for such communication in plants is the selective trafficking of transcription factors and other signals through plasmodesmata (PDs), regulated channels that traverse the cell wall to connect cells with their neighbors. We have taken genetic approaches to dissect the mechanism of PD trafficking. In one screen, we used the free diffusion of GFP as an assay to find mutants that restrict PD size exclusion limit, leading to the discovery of a meristem-expressed thioredoxin gene that maintains redox status and callose levels in meristems (Benitez Alfonso et al., 2009). These findings support an emerging model that dynamic regulation of PD permeability throughout plant development is achieved through regulated callose deposition.

The maize KNOTTED1 (KN1) homeodomain protein was the first plant protein found to selectively traffic through PD, and its' trafficking appears to be important for its function in stem cell maintenance. A gain-of-function trafficking assay in *Arabidopsis* was developed to demonstrate that the C-terminal region of KN1 is necessary and sufficient for trafficking in vivo (Kim et al., 2005). This system provides a simple and tractable model to understand how proteins traffic, and to isolate mutants defective in trafficking.



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As a proof of concept for our strategy, a mutant with attenuated KN1 trafficking has been identified, and was found to be defective in a chaperonin gene. This chaperonin appears essential for PD trafficking of some but all non-cell-autonomous proteins, and biochemical evidence suggests a physical association between chaperonin and KN1. Proteins are thought to undergo partial unfolding during PD translocation, which makes the discovery of this chaperonin particularly exciting. A functional characterization of the role of chaperonins in will further our understanding of developmental regulation and mechanisms of selective cell-to-cell trafficking. In addition, it may give mechanistic insights into this elaborate protein folding machinery, which is not well understood in any system.

References:

- Kim, J.Y., Rim, Y., Wang, J. and Jackson, D. (2005). A novel cell-to-cell trafficking assay indicates that the KNOX homeodomain is necessary and sufficient for intercellular protein and mRNA trafficking. **Genes Dev.** 19: 788-793.
- Benitez-Alfonso, Y., Cantrill, L. and Jackson, D. (2006). Plasmodesmata: Cell-Cell Channels in Plants. In: **Cell-Cell Channels**. (Landes Bioscience eds.)
- Bolduc, N., Hake, S. and Jackson, D. (2008). Dual Functions of the KNOTTED1 Homeodomain: Sequence-Specific DNA Binding and Regulation of Cell-to-Cell Transport. **Science Signaling** 1, pe28.
- Benitez-Alfonso, Y., Cilia, M., SanRoman, A., Thomas, C., Maule, A., Hearn, S. and Jackson, D. (2009). Control of *Arabidopsis* Meristem Development by Thioredoxin-dependent Regulation of Intercellular Transport. **Proc. Natl. Acad. Sci** 106(9):3615-20.

(IS1-4)

1. Speaker

Hyung-Taeg Cho (htcho@snu.ac.kr)

2. Affiliation

Associate Professor, School of Biological Sciences, Seoul National University, Korea

3. Appointments on Editorial Boards

Physiologia Plantarum (Editor, 2004 – 2011)

Molecules and Cells (Editor, 2005 – 2008)

Journal of Plant Biology (Editor, 2007 – 2009)

Phospholipase A₂ Affects the Intracellular Trafficking of PIN Proteins in the *Arabidopsis* Root

Ok Ran Lee^{1,2}, Soo Jin Kim³, Hae Jin Kim⁴, Jeum Kyu Hong⁴, Stephen Beungtae Ryu⁴, Sang Ho Lee^{1,5}, Anindya Ganguly¹,
and Hyung-Taeg Cho¹

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Phospholipase A₂ (PLA₂), which hydrolyzes a fatty acyl chain of membrane phospholipids, has been implicated in several biological processes in plants. However, its role in intracellular trafficking in plants has yet to be studied. Here, using pharmacological and genetic approaches, the root hair bioassay system, and PIN-FORMED (PIN) auxin efflux transporters as molecular markers, we demonstrate that plant PLA₂s are required for the trafficking of PIN proteins to the plasma membrane (PM) in the *Arabidopsis thaliana* root. The PLA₂α protein co-localized with the Golgi marker. Impairments of PLA₂ function by PLA₂α mutation, PLA₂-RNAi, or PLA₂ inhibitor treatments significantly disrupted the PM localization of PINs, causing internal PIN compartments to form. Conversely, supplementation with lysophosphatidylethanolamine (the PLA₂ hydrolytic product) restored the PM localization of PINs in the *pla2α* mutant and the ONO-treated seedling. Suppression of PLA₂ activity by the inhibitor induced a greater accumulation of *trans*-Golgi network vesicles. Root hair-specific PIN overexpression (PINox) lines grew very short root hairs most likely due to lowered auxin levels in root hair cells, but PLA₂ inhibitor treatments, PLA₂α mutation, or PLA₂-RNAi restored the root hair growth of PINox lines by disrupting the PM localization of PINs and thus reducing auxin efflux. These results suggest that PLA₂, likely acting in Golgi-related compartments, modulates the trafficking of PIN proteins.

(IS1-5)

1. Speaker

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3. Appointments on Editorial Boards

Plant Cell and Physiology (JPN, since 2008-); *Physiologia Plantarum* (DEN, since 2009-);

Plant Molecular Biology (NED, since 2009-)

Analysis of cell signalling during vascular morphogenesis in *Arabidopsis*

Ykä Helariutta, Anthony Bishopp, Jan Dettmer, Satu Lehesranta, Annakaisa Elo, Kamil Ruzicka, Jing Zhang, Shri Ram Yadav, Shunsuke Miyashima, Anne Honkanen, Juha Immanen, Sedeer El-Showk, Hanna Help, Raffael Lichtenberger, Robertas Ursache

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The long-term goal of my group is to understand how the plant stem cell population, the vascular cambium, is genetically controlled to pattern and promote the formation of the conductive tissues in a trunk of a tree.

During recent years our laboratory has been able to make significant progress in understanding cambium by investigating primary development of the vascular tissue (procambium) in the *Arabidopsis* root, which is a powerful anatomical and genetic model. Much of the activity of our laboratory has been focusing on the role of cytokinin phytohormones during vascular development. This has been based on the earlier discovery and identification of CRE1/WOL/AHK4 as a cytokinin receptor required for stem cell identity (Mähönen et al. 2000, Inoue et al. 2001, Suzuki et al. 2001). My presentation will focus on two aspects following this observation.

1. The role of cytokinins in regulating procambial patterning during root development in *Arabidopsis*

Through the identification of the *AHP6* gene as a negative regulator of cytokinin signalling, we have published a spatial model on the role and regulation of cytokinin signalling during procambial development (Mähönen et al. 2006). In this model we postulate that high cytokinin signalling promotes procambial cell identity and low cytokinin signalling (due to

inhibition by AHP6) promotes protoxylem identity. Furthermore, we showed that cytokinin signalling regulates the spatial domain of AHP6 and protoxylem formation. The AHP6 domain is expanded in *wol* and the cytokinin receptor double mutant, *cre1ahk3*. Treatment with cytokinin results in loss of AHP6 expression and protoxylem identity. (Mähönen et al., 2006). Together these data indicate the involvement of a spatial interaction loop.

More recently we have been able to establish the involvement of this interaction loop as part of a more complex network involving other regulators of plant development. Firstly, we have identified new components within this interaction loop and show that it also includes another phytohormone auxin (Bishopp, Help, Lehesranta, El-Showk, Vaten et al., in preparation). Secondly, in a collaborative project with Philip Benfey's, Ji-Young Lee's and Annelie Carlsbecker's laboratories (Duke University, Boyce Thompson Institute, Uppsala University, respectively) we have recently identified another regulatory cascade that controls the patterning of marginal protoxylem and central metaxylem within the xylem axis (Carlsbecker et al., Nature, *in press*). In this cascade the gene expression level of the five class III HD-ZIP genes coding for transcription factors *ATHB-8*, *CORONA*, *PHABULOSA*, *PHAVOLUTA* and *REVOLUTA* (Prigge et al. 2005) is modulated by two *miR165/166* species. This causes the expression of the transcription factor genes to be lower at the protoxylem position and higher at the epicentre of the root. Low and high HD-ZIP III expression result in protoxylem and metaxylem identity respectively. The evidence for this is the phenotypic consequences of the miR resistant mutations that result in ectopic *PHABULOSA* expression and the subsequent specification of metaxylem at the position normally occupied by protoxylem. One of the gain-of-function alleles, a new microRNA resistant allele of *PHABULOSA*, *phb-dva1*, was identified in a genetic screen for misexpression of a phloem marker *AtSUC2::GFP*. Additional evidence comes from the opposite combinatorial loss-of-function mutations of the HD-ZIP genes which result in ectopic protoxylem specification in the central metaxylem position. In the most extreme case, a knock-outs of all the five genes, xylem does not developing. The unequal effect of the miR165/166 species on the class III HD-ZIP genes at the periphery and central region of the root is based on their mobility. The miR165/166 genes are transcribed in the surrounding endodermal tissue and they are direct targets of the transcription factors SCR and SHR (the latter of which moves to endodermis from vascular tissue). The miR species then move inside the vascular tissue. As protoxylem (which is located at the periphery of vascular bundle) is closer to the source of the miR, HD-ZIP III expression is lower at this position. The class III HDZIP gene dosage appears to also regulate AHP6 expression (and thereby cytokinin signalling). AHP6 is ectopically expressed at the metaxylem position when levels of HD-ZIP III expression are low and is abolished at the protoxylem position when there is high ectopic HD-ZIP expression. We have also determined that expression of other xylem specific genes, such as *ACL5* which encodes an enzyme in spermine biosynthesis (Brady et al. 2007), are promoted by high HD-ZIP III expression at the central metaxylem positions only.

2. The role of intercellular communication during root procambial patterning

The regulatory interactions described above indicate an interplay between several spatially specific key regulators (proteins, hormones, miRNA species) which control each other's distribution through various processes of cell-to-cell communication.



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How do these regulators move between the cells to obtain their positions in the procambium?

Through identification of dominant mutations affecting callose biosynthesis, we have engineered a temporally and spatially controlled system to control plasmodesmatal trafficking during root procambial development. The mobility of the various signals is discussed based on the analysis with this system.

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Prigge et al. (2005) *Plant Cell* **17**:61-76;
Suzuki et al. (2001) *Plant Cell Physiol* **42**, 107-13;

(IS1-6)

1. Speaker

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3. Appointments on Editorial Boards

Plant Biotechnology Report (Associate Editor, 2009. 3 – present)

Journal of Applied Biological Chemistry (Managing Editor, 2007. 1-2008. 12)

Plasmodesmal callose regulation as a key modulator of intercellular movement of signaling molecules

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Plant cells evolved a unique intercellular information channel termed plasmodesmata (PD). PD allow not only the movement of small molecules such as ions, water, nutrients, but also macromolecules (proteins, RNA). Callose is a beta-1,3-glucan cell wall component that is found in diverse vegetative and reproductive cell types. PD permeability can be mediated by callose, which is deposited at the neck region of PD. However, how this callose deposition is controlled at the molecular level and its biological function remain to be elucidated. Bioinformatic and phylogenetic studies suggested *Arabidopsis* has 12 members of glucan synthase genes. To identify callose synthase gene that is involved in PD callose accumulation, we isolated T-DNA insertional lines. Among 12 members of callose synthase, 11 *gsl* homozygous mutants were isolated except for *gsl10*. All *gsl* plants showed pretty much normal developmental phenotype compared to WT, except *gsl8*. *gsl8* showed highly stunted and retard growth. And it was seedling lethal suggesting that *GSL8* is critical for plant growth and development.

We screened PD callose defective mutants using aniline blue staining of callose from wounded cotyledones. *Gsl8* homo mutants only showed little callose deposition as shown the cell wall fluorescent spots, while other all mutants showed



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similar level of PD-like callose deposition compared to WT. Electronic microscopy showed high amorphous callose layer in differentiating phloem cells of the root tip was disappeared in *gsl8* mutant. Confocal and electronic microscopies indicated that *gsl8* mutant lines has imperfect cell wall with cell wall stubs, two nuclei in a cell, suggesting that *gsl8* has cytokinetic defects. To dissect PD function of GSL8, we developed dexamethasone inducible GSL8 RNAi line using a 300 bp specific dsRNA. When grown in dex media, RNAi line showed similar phenotype to *gsl8* homozygote mutant; GSL8 RNAi lines lacked callose deposition at PD. Our data also revealed that GSL8 RNAi lines have dramatically increased PD permeability as judged by enhanced movement of GFP. However, SHR-GFP movement pattern from stele to endodermal layer was maintained, although its level was reduced in GSL8 RNAi line. Coordination between PD regulation and cell elongation was well reported in cotton fiber elongation. Active cell elongation requires high turgor pressure for cell wall expansion that needs the closure of PD channels. Thus we hypothesized that defect in maintenance of PD callose inhibits cell elongation. Since GSL8 is involved in cytokinesis, we observed hypocotyl, an embryonic organ of which growth depends on fully cell elongation, but not cell division.

Interestingly, *GSL8* RNAi lines also displayed a defect in cell elongation that could be mimicked by treatment with the callose synthase inhibitor, 2-deoxy-glucose. Rapid cell elongation usually requires high turgor to drive the expansion of the cell wall, suggesting that turgor maintenance for cell elongation might be compromised in our RNAi lines. These finding will be discussed in terms of likely mechanisms by which a change in PD permeability could result in aberrant growth responses in the *gsl8* background.

Acknowledgments: Supported by EB-NCRC, BK21, NRL and WCU programs

(IS1-7)

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Dec, 2004 - Current: Editorial member, *Physiologia Plantarum*

Nov, 1998 - Dec, 2004: Editorial member, *Journal of Plant Biology*

Jan, 1998 - Dec, 2004: Editorial member, *Plant Cell and Physiology* (Japanese Society of Plant Physiology)

Understanding and controlling plant growth: Systems and chemical genomic approaches

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Dae Hee Hwang^{2,3,†} and Hong Gil Nam^{1, 2, 4, ‡}

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Final output of plant growth is an orchestration of various physiological responses. Controlling plant growth has a great potential for sustaining human beings on Earth, including food, energy, and environment. Controlling plant growth can be approached in three ways. First, we will need to understand and reveal the materials and principle involved in processing of endogenous and exogenous information regarding plant growth ion of various physiological responses. The next step would include synthetic biology approach where one synthesizes new genetic pathways and regulation modes. The other approaches may utilize small molecule chemicals to modulate plants' genetic pathways or regulatory modes

We are studying the materials and principle involved in processing of endogenous and exogenous information regarding plant growth. One of our area is when and how plants or leaf organ of plants die. We have isolated many key molecules involved in this process and are trying to understand the process at the systems level as well as at molecular and cellular level. In our recent research, we identified the trifurcate feed forward pathway for age-dependent cell death involving EIN2, ORE1, and miR164. We found that in young stage of Arabidopsis leaves, miR164 suppresses ORE1 which functions



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positively in aging-induced cell death. But in old stage, EIN2 suppresses miR164 and induces ORE1 expression, which leads to age-dependent cell death in leaves. To understand a function of the trifurcate pathway of age-dependent cell death, we developed a mathematical model.

We previously reported that the plant hormone, cytokinin, defies age-dependent cell death through two component signaling system. The two component signaling in Arabidopsis include 11 type B ARR and 10 type A ARRs. We are trying to understand how they function in cytokinin signaling at the systems level. We also previously reported that ORE7, when overexpressed, can greatly enhance leaf longevity and productivity in Arabidopsis. We are generating transgenic crop plants to see their effect in crop plants.

Chemical genomics is an excellent complementation to the genetic mutation in revealing the information processing principles and materials. We developed and established a chemical screening system using Arabidopsis to identify new compounds controlling growth and development, in collaboration with Markus Geisler (University of Zurich, Switzerland), Young Tae Chang (National University of Singapore) and KRICT (Korea Research Institute of Chemical Technology). We will describe our progress on screening, identification of chemicals controlling plant development.

We found BUM as a novel auxin transport inhibitor. BUM efficiently blocks auxin regulated plant physiology and development. Physiological analysis, auxin transport as well as binding assays identified ABCB1, as key target of BUM.

Plump3, identified from tagged chemical library, mainly inhibit root elongation in dose-dependent and reversible manner. Importin β was identified as a direct binding target of plump3 and cellular movement from cytosol to nucleus of importin β was inhibited by plump3.

(IS1-8)

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3. Professional Activities

Chairman: Third International Conference of Legume Genomics and Genetics (ICLGG-3); Brisbane April 2006

Chairman: RIRDC Plant and Animal Genome Conference workshop: Functional Genomics Technologies. San Diego (2001 to present).

Genetic and biochemical components of local and systemic long-distance signalling during nodule regulation in legumes

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All plants regulate the number of organs through sensing environmental and internal signals. For example, legumes regulate the number, size and activity of nitrogen fixing nodules through local and systemic processes. Prior nodulation events (as in Autoregulation of Nodulation, AON), phytohormones, acid soil (pH) and nitrate are major controls. Repeated cell divisions initiating the nodule primordium are autoregulated leading the characteristic nodulation on the upper root system. AON in all legumes functions through an ubiquitously phloem-expressed LRR receptor kinase gene (*GmNARK*, *MtSUNN*, *LjHARI*), that requires the sensing within the root of *Rhizobium*-(Nod-factor)-based infection. Yet inoculation-related AON functions exclusively in the leaf phloem parenchyma where NARK and interacting proteins result in blockage of further cell divisions in the root cortex/pericycle. We have discovered candidate molecules for the long distance signals moving upwards and downwards. The presentation will synthesize a combined genetic, functional genomics and biochemical view of our present understanding of the sensing stages, the systemic and local signals, leaf and root roles of receptors, and related response pathways.



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(IS1-9)

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Inhibition of phytochrome-interacting factors by phytochromes

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Phytochromes are red and far-red light receptors that activate plant responses to light. They achieve this in part by interacting with and degrading negatively acting bHLH transcription factors called phytochrome-interacting factors (PIFs) within the nucleus. Many aspects of phytochrome signaling, however, remain unclear. In the present study, we investigated two unresolved questions regarding phytochrome signaling. We first investigated whether cytosolic PIF3 can be phosphorylated and degraded by cytosolic phytochromes. We found that the nuclear localization of PIF3 is determined by a cluster of basic amino acids localized outside the bHLH domain. Cytosolic PIF3 generated by a mutation in this nuclear localization signal sequence (NLS) is neither phosphorylated nor degraded by light, even in the presence of cytosolic phyB. We then investigated whether PIF3 can be phosphorylated and degraded by the N-terminal domain of phyB. We found that PIF3 is partially phosphorylated, but not degraded by the N-terminal domain of phyB. Our preliminary data indicate that the N-terminal domain of phyB induces light responses through its gain-of-function mode.

(IS1-10)

1. Speaker

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3. Professional Activities

Panel Member: NSF Plant and Microbial Developmental Mechanisms, Served on panel between 2004 and now. Site

Visit Team Member, NSF Plant Genomics Panel, 2002

Root-to-Shoot Signaling: the *bypass1* mobile signal confers root-to-shoot signaling

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The development of plants incorporates organ-intrinsic programs that lead to stereotyped patterns of specialized cell types, and extrinsic signals that coordinate the developmental programs of distant organs. Despite dramatic advanced in understanding of key regulators of many organ systems, how plants coordinate development, and the identity of longdistance signals, remains largely unknown. The focus of our research is long distance root-to-shoot signaling under control of the BYPASS1 pathway.

The *bypass1* (*bps1*) mutant of *Arabidopsis* was originally isolated because of its leaf defects; *bps1* mutants initiate leaf primordia but these either arrest development or undergo only minimal development to produce a small leaf with disorganized vascular tissues. The mutant also shows root defects. The root meristem arrests shortly after germination, lateral roots initiate but then undergo similar loss of the root apical meristem, yet the root continues to expand radially.

A role for BPS1 in long-distance signaling was recognized following grafting and root excision experiments. Cutting off the *bps1* root allowed the mutant's arrested leaf primordia to resume apparently normal development. In addition, grafting the mutant root to a wild-type shoot caused that shoot to arrest leaf development. Thus, the root is both necessary and sufficient for shoot arrest. These data indicate that the root is the source of a mobile substance that induces developmental arrest. The data also suggest that the normal role of BPS1 is to prevent over-production of this active substance. Because

transportation is from the root and to the shoot, its most likely route is through the xylem.

To understand how BPS1 functions, we cloned the gene. BPS1 encodes a 349 amino acid protein with no functionally characterized domains, and no robust localization signals.

BPS1 homologs are found in genomes of other plants, including some non-vascular plants such as *Physcomitrella*, however it is restricted to the plant kingdom. We propose that BPS1 functions to modulate the amount of the mobile root-to-shoot signal that is produced in *bps1* mutants, and that it is normally produced when reduced leaf number or reduced leaf size would be advantageous for the growing conditions encountered by the roots. In *bps1* mutants, an excess amount of a normal mobile compound is produced, and the response is a rapid, yet reversible, arrest of shoot development.

A critical question is the identity of the *bps1* signal. It appears to behave like a plant hormone (modifying development at a distance), however until we know its chemical identity, we will not be sure that normal plants use this compound to modulate development. Assays using genetics, hormone supplementation, and inhibitors suggest that the mobile compound is not any of the recognized plant hormones. However, treatment of mutants with herbicides that normally kill plants because they disrupt carotenoid biosynthesis actually suppress both the leaf and root phenotypes of *bps1* mutants.

The partial suppression from carotenoid biosynthesis inhibitors indicates that the biosynthetic pathway leading to the *bps1* signal requires carotenoids. One way that carotenoid biosynthesis could be required that it could serve as the mobile compound's precursor. Two known mobile signals arise from carotenoid biosynthesis - abscisic acid and strigolactones. However, we know that the *bps1* signal is neither of these compounds because the *bps1* phenotype is not suppressed in double mutants where ABA or strigolactone biosynthesis is disrupted. Thus, the *bps1* mobile signal could be a novel carotenoid cleavage product. Alternatively, the carotenoid biosynthesis requirement could be indirect. Blocking of carotenoid biosynthesis leads to bleached plants because the plastids undergo photooxidation. Thus, all pathways with steps that are localized to plastids are blocked by these inhibitors. Current efforts to identify the mobile signal include analysis of metabolite and development of bioassays, which will allow us to follow active metabolite fractions.

Another crucial question is how the *bps1* mobile signal affects shoot development. Because developmental arrest is reversible, the signal might be a competitive inhibitor of a signaling process. Our current data suggests that the mobile signal inhibits auxin responses. These data come from experiments investigating the auxin-inducible DR5:GUS transgene in *bps1* mutants. In normal plants, DR5 confers GUS expression at the apex of a leaf primordium, and then progressive expression through the vascular system. In *bps1* mutants, the early distal signal is present, however it fails to expand its expression, and vascular tissues typically fail to differentiate. Moreover, when exogenous auxin is supplied, the wild type and *bps1* mutants show even greater differences. The wild type responds to exogenous auxin by activating DR5:GUS throughout its developing leaves. By contrast, exogenous auxin fails to activate DR5:GUS in *bps1* mutants.



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We have linked the loss of auxin responsiveness to the *bps1* root by carrying out root excision experiments. We know that cutting off the root allows the *bps1* shoot to rapidly resume normal development. Similarly, root excision also allows the *bps1* shoot to regain DR5:GUS responses to exogenous auxin. Restored response is observed within 3 hours of root excision. These data suggest that the *bps1* signal actively arrests shoot development by repressing auxin responses. We are now investigating the molecular basis of auxin repression by the *bps1* signal.

A long-distance signal that modulates shoot development in response to environmental perturbations sensed by the root would be consistent with physiological experiments carried out using split root design. These experiments have shown that roots subjected to drought produce an unknown signal that moves to the shoot and decreases the number of leaves, and the size of leaves, produced. Moreover, that signal is reversed rapidly by removal of the drought-treated root. The *bps1* mutant's signal shows many similarities to this signaling pathway. Future work identifying the mobile substance will allow us to test this model.

(IS1-11)

1. Speaker

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3. Awards

2001 The Young Scientist Award for Plant Morphology from the Japanese Society of Plant Morphology

Regulatory mechanisms of cell division during shoot apical meristem and organs development

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Plant has unique control system for indeterminate growth and repetitive organogenesis which derived from plant stem cells. To do this, cell cycle plays an important role to achieve proper plant architecture for developmental progression and environmental adaptation. In this study, we focused and discussed the cell division patterning of shoot apical meristem (SAM) maintenance and cellular differentiation during organ development.

Cell cycle progression is regulated by reversible phosphorylation of cell cycle regulatory factors. In particular, interaction of cyclin (CYC) and cyclin-dependent kinase (CDK) is core positive regulatory mechanism to progress cell cycle. Furthermore, CDK inhibitors called Kip-related proteins (KRPs) and SIAMESE are the inhibitory proteins blocking CYC/CDK interaction. Genome of *Arabidopsis thaliana* contains seven KRPs (KRP1-KRP7) which have low sequence similarity and distinct spatial and temporal expression patterns. We focused on the negative regulator of cell cycle, KRPs of *Arabidopsis* to understand complex cell division controls for SAM maintenance and for organ development.

Firstly in this study, we have identified the expression patterns of KRPs in *Arabidopsis* with KRP-GUS fusion proteins. As a result, we found that KRP1 and KRP3 were strongly expressed in SAM and leaf primordia, whereas KRP6 and KRP7 were expressed weakly in leaf primordia. Secondly, we have generated transgenic plants overexpressing KRPs family (KRP1, KRP3, KRP6, and KRP7) under the promoter of 35S CaMV and SAM-specific genes, *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*), and analyzed various morphological alterations in transgenic plants. As results, transgenic plants overexpressing KRPs showed common phenotypes, such as reduction of whole plant and organ size, serration in leaves,



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reduction of fertility, and inhibition of root growth. In cell level, overexpression of KRP triggered remarkable reduction of cell number in SAM and leaves. On the other hand, cell size in transgenic plants was enlarged, indicating a compensatory mechanism at work when cell division is inhibited. Interestingly, overexpression of KRP1 and KRP3 resulted in the structural alteration of SAM, such as the destruction of layer arrangement and the modification of dome shape, suggesting that KRP3 might regulate SAM regulators, including *WUS* and *CLV3*. On the other hand, overexpression of KRP6 resulted in severe morphological alternations of leaves, including upward curling and rough surface. Statistical analyses and microscopic observance of epidermal cells in leaves indicated that KRP6 might regulate the balance of cell division between adaxial side and abaxial side of leaf epidermis.

In conclusion, our study indicated that the regulation of cell division by KRP family affects SAM maintenance as well as organ size and shape. Taken together, we discussed the cell division controls in SAM activity and cellular differentiation during organ development.



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IS2

Natural Products

New Trends in Natural Products

(IS2-1)

1. Speaker

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3. Appointments on Editorial Boards

Editor-in-Chief, Intl. Society for Molecular Plant-Microbe Interactions (IS-MPMI) *Reporter*, 2000-2003.

Editorial Board, *Eukaryotic Cell*, 2001-2006

Editor-in-Chief, *Molecular Plant-Microbe Interactions*, 2007-2010

Member of Publications Board, APS, 2007-2010.

Biosynthesis of the Cyclic Peptide Toxins of Amanita Mushrooms

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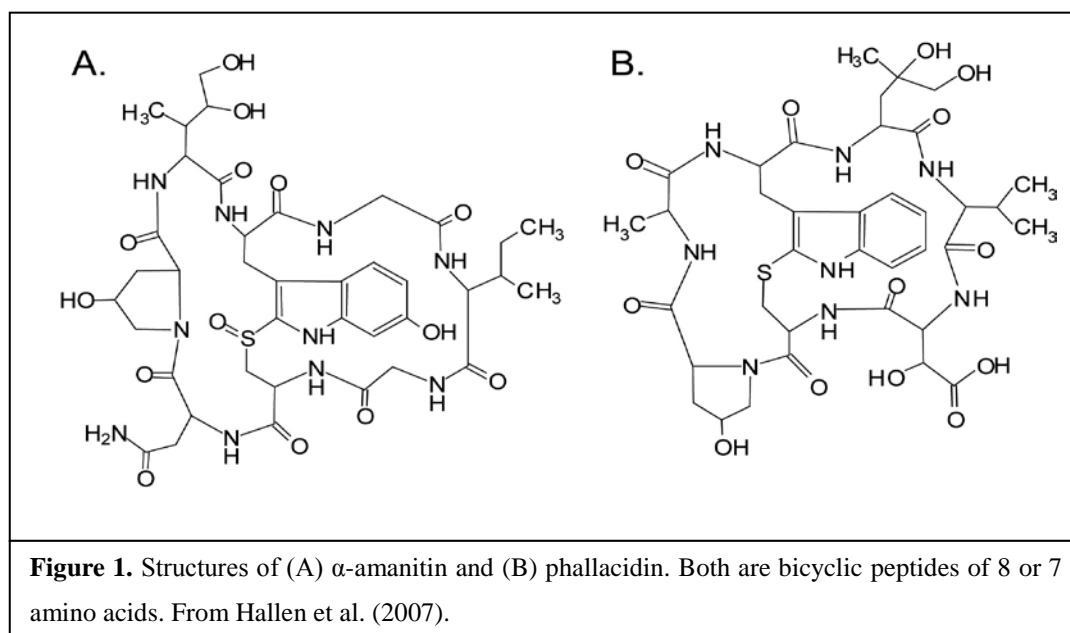
Natural products have historically been the source of most of our medicines. There is currently a critical need for new pharmaceutical agents against a wide variety of old and emerging human diseases, such as AIDS, antibiotic-resistant pathogens such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, fungal diseases, and many types of cancer (Walsh and Fischbach, 2010). However, most of our medicines are based on only a few fundamental molecular scaffolds, which have been heavily exploited by generations of tailoring. For example, no new major classes of antibiotics were introduced between 1962 and 2000 (Fischbach and Walsh, 2009; Newman and Cragg, 2007).

The natural world provides multiple examples of novel chemical classes with medically relevant biological activities. One is exemplified by the cyclic peptide toxins of *Amanita* mushrooms (Fig. 1). In several respects, these compounds have unique and desirable properties among known natural products, including other natural cyclic peptides. First, they contain a Cys-Trp crossbridge (called tryptathionine), that has not been found in any other natural product (May and Perrin, 2007). Second, they are the smallest known peptides synthesized on ribosomes. Third, because their amino acid sequences are genetically encoded, they can be readily engineered to produce thousands to millions of novel compounds. Fourth, the known *Amanita* toxins have specific and important sites of action, which has led to their widespread use as reagents for biochemical research. Based on these properties and precedents, it is likely that other molecules based on the same general scaffold will have biomedically useful properties.

One of the ecologically and medically most important classes of microbial secondary metabolites are the small peptides. They can be linear or cyclic and often contain unusual or modified amino acids (Felngale et al., 2008; McIntosh et al., 2009; Walton et al., 2004). Consistent with their potent biological potential, small peptides have evolved multiple times for multiple purposes in all branches of life, including bacteria, fungi, plants, mollusks, amphibians, snakes, spiders, and even primates (Selsted, 2004). Small cyclic peptides have a variety of known or potential medical applications (Cole et al., 2004; Craik et al., 2010). Their intrinsic advantages include thermal and pH stability, resistance to proteolysis, and superior amphipathic solubility (Clark et al., 2005).

Mushrooms in section *Phalloideae* of the genus *Amanita* account for >90% of all fatal mushroom poisonings. The human LD₅₀ for α -amanitin is ~0.1 mg/kg, and one mushroom can contain a fatal dose of 10-12 mg (Wieland, 1986). Amatoxins survive the digestive tract and are actively imported into liver cells. They are potent inhibitors of RNA polymerase II. Without prompt intervention, amatoxins cause liver failure and death in 3-7 days. Liver transplants are often the only treatment option.

In addition to amatoxins, most *Amanita* mushrooms make phallotoxins (e.g., phalloidin and phalloidin), which are chemically related but have only 7 amino acids (Fig. 1B).



Previously, all known fungal cyclic peptides were thought to be made by nonribosomal peptide synthetases (NRPSs) (Walton et al., 2004). However, we discovered from genome survey sequencing that the *Amanita* toxins are synthesized on ribosomes, initially as 34 or 35 amino acid propeptides (Hallen et al., 2007). Although α -amanitin and phalloidin have only three amino acids in common, the flanking regions of the encoding genes, *AMA1* and *PHA1*, are highly conserved (Fig. 2). The genome survey sequence of *A. bisporigera* contains at least 30 sequences related to the

conserved upstream and downstream regions of *AMA1* and *PHA1*. An alignment of these sequences indicates that the central "toxin" region is hypervariable compared to the flanking conserved regions. The toxin regions vary from 6 to 10 amino acids.

MSDINATRLP <u>IWGIGCNP</u> CI GDDVTLLTRGEALC	[α -amanitin]
MSDINATRLP W C PC GDDV LLTRGE LC	[consensus]
MSDINATRLP <u>AWLVDC-PC</u> VGDDVNRLLRGESLC	[phalloidin]

Figure 2. Alignment of the predicted proproteins (deduced from cDNAs) of α -amanitin (*AMA1*) and phalloidin (*PHA1*) from *Amanita bisporigera*. The amino acids in the mature toxins are underlined. A gap has been introduced into the *PHA1* sequence to emphasize the downstream alignment (phalloidin has 7 amino acids compared to 8 in amanitin). From Hallen et al. (2007).

We have also identified genes encoding β -amanitin (IWGIGCDP) and phalloidin (AWLATCP) in *A. ocreata* and *A. phalloides* (Hallen et al., 2007). The sequence FFQPPEFRPP, found in *A. phalloides* (Hallen et al., 2007), is 70% identical to the cyclic decapeptide antamanide, cyclo(FFVPPAFFPP), which was isolated from *A. phalloides* by its ability to protect mice against phalloidin (Wieland, 1986).

Based on the conservation of a Pro residue immediately upstream of the toxin region, and of a Pro as the last amino acid in the toxin region (Fig. 2), it seemed likely that cleavage of the proprotein was catalyzed by a Pro-specific protease. Of the known Pro-specific proteases, prolyl oligopeptidase (POP) (EC 3.4.21.26) has the most likely characteristics (Cunningham and O'Connor, 1997). In order to identify the cleavage enzyme directly, we purified the cleavage enzyme from *Conocybe albipes*, which produces phalloidin. The assay was HPLC-monitored cleavage of a synthetic phalloidin precursor. Based on proteomics and cDNA cloning of the encoding gene, the cleavage enzyme is a POP. The same enzyme cleaves at both Pro residues, cutting the second Pro preferentially. The enzyme also cleaves a synthetic phalloidin precursor, but cuts an amanitin precursor only partially (Luo et al., 2009).

Following translation and excision, the steps in *Amanita* toxin biosynthesis are predicted to include cyclization, hydroxylation, formation of the Trp-Cys cross-bridge (i.e., tryptathionine biosynthesis), and epimerization in the case of the phallotoxins, not necessarily in that order. The genes may be clustered, as are secondary metabolite genes in other fungi (e.g., Ahn et al., 2002), and currently we are testing this hypothesis through screening lambda genomic libraries and deep genome sequencing. Another interesting question that we hope to address is the origin of amatoxin production in mushrooms unrelated to *Amanita*, such as *Galerina*, *Lepiota*, and *Conocybe* (Kaneko et al., 2001).

Small, modified, biologically active peptides that are synthesized on ribosomes have been identified from many sources but not previously from fungi (Craik et al., 2007; Escoubas, 2006; McIntosh et al., 2009; Olivera, 2006; Simmaco et al., 1998; Willey and van der Donk, 2007). Like the *Amanita* toxins, these peptides are synthesized as precursor proteins

and some undergo post-translational modifications similar to those of the *Amanita* toxins, such as hydroxylation and epimerization. Examples include cone snail toxins (conotoxins); cyclotides such as kalata B1 and SFTI-1, which are made by plants; and lantibiotics, thiocillins, patellamides, and cyanobactins from prokaryotes.

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Walton JD, DG Panaccione, H Hallen (2004) Peptide synthesis without ribosomes. In: *Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine*. J Tkacz and L Lange, eds, Kluwer Academic, New York, pp 127-162.

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(IS2-2)

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Screening for peptides active against insect and plant from natural and synthetic libraries

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Peptides are composed of a linear combination of 20 proteinogenic amino acids, which exhibit tremendous structural diversity. Peptides are key molecules in various biological events, and many organisms use peptides as hormones, neuropeptides or antimicrobials. In addition, certain types of animals such as scorpions, spiders, bees and snakes have venom in which a wide variety of peptide toxins are included to subdue prey and defend against predators. Since venoms contain peptides with various structures and biological activities, they are recognized as “natural peptide libraries” for pesticide and drug discovery. On the other hand, “synthetic peptide libraries” can be readily prepared by utilizing combinatorial chemistry approaches. Millions of peptide sequences generated by this method are tested for biological activity of interest using a high-throughput screening system. In this presentation, we will report the isolation and characterization of novel insecticidal peptides from scorpion venom, and plant immunity-activating peptides from synthetic random libraries.

Insecticidal toxins from scorpions in Japan

Scorpion venoms are composed of a number of peptides, many of which show neurotoxicity against insects and/or mammals. The scorpion *Liocheles australasiae* belonging to the Hemiscorpidae family inhabits the western Pacific region including Japan. We characterized the chemical components of the venom of *L. australasiae* using mass spectrometry, and found that it contains more than 200 components with molecular masses ranging 500-10,000 Da. From this venom, which showed significant insect toxicity, we isolated the insecticidal peptide, LaIT1, by bioassay-guided HPLC separations. Edman sequencing and mass spectrometric analysis revealed that the toxin is composed of 36 amino acid residues and cross-linked by two disulfide bridges. LaIT1 shows no sequence homology to any other known toxins, suggesting that this toxin adopts a novel structural motif. To determine the amino acid residues important for the toxicity, we synthesized LaIT1 analogs and measured their insect toxicity. We further explored insect toxins included in the venom



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of *L. australasiae*, and isolated the novel toxin, LaIT2. The amino acid sequence of LaIT2 was determined by Edman sequencing and cDNA cloning. LaIT2 is composed of 59 amino acids with three disulfide bridges, and shares sequence homology with the scorpion α -KTx peptides. These toxins will be useful for a better understanding of the evolution of scorpion toxins as well as the development of novel insecticides.

Plant immunity-activating peptides from combinatorial libraries

Chemicals that induce plant defense responses against pathogens attract attention as "plant defense activators". Several peptides derived from microbes are known to induce a series of defense responses in plant. However, since these peptides are relatively large (~20 residues), they are difficult to use as lead compounds for the development of plant defense activators. In this study, to obtain small peptides that can activate the plant defense system, we screened a combinatorial random peptide library using a cell-based "lawn" format assay that we developed. A hexapeptide random library containing a photocleavable linker was synthesized by the split-mix method. High-throughput screening was performed against tobacco suspension cells by detecting H₂O₂ generation as a defense response. After screening of the library containing approximately 1,000,000 sequences of peptides, several "hits" were obtained. Among them, one positive peptide was identified as YGIH₂TH-NH₂, which showed no sequence homology to any previously identified elicitor peptides and was named plant-immunity activating peptide-1 (PIP-1). This peptide, showed an EC₅₀ value of 2.2 μ M for the H₂O₂ generating activity. To further investigate the mechanism of defense responses induced by PIP-1, we analyzed its effect on secondary metabolite biosynthesis and expression of defense-related genes in tobacco cells. Biosynthesis of phytoalexin (capsidiol) was significantly induced by PIP-1. Since this biosynthesis was inhibited by salicylic acid, which is known to act antagonistically against jasmonic acid (JA), the contribution of JA to this defense reaction as a signaling molecule was suggested. The expression of defense-related genes involved in the JA pathway was also induced by PIP-1, indicating that PIP-1 activates the plant defense reactions via the JA pathway. This peptide will be useful for the development of novel plant defense activators as well as the investigation of plant defense mechanisms.



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The wonderful world of plant phenolics: Arogenate dehydratases (ADTs), dirigent proteins (DPs) and their downstream anticancer substances; medicinal plant transcriptome and metabolome profiling; and new solutions to petrochemicals from biomass plant phenolics

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Introduction: The last 50 years has witnessed enormous advances in understanding and exploiting how many of our plant medicinals, flavors/fragrances, as well as plant fibers/commodities (such as cellulose), are biochemically generated. In this regard, emphasis in our laboratory is now being placed upon furthering knowledge in several of these key biochemical areas, as well as extending investigations towards finding new solutions to replace petrochemicals in a sustainable manner from biomass.

In celebration of the 50th anniversary of the *Korean Society for Applied Biological Chemistry*, this contribution summarizes some of the recent exciting work from our laboratory in moving these various fields substantially forward. The areas of emphasis to be discussed include: our pioneering work with arogenate dehydratases (ADTs), the final step in phenylalanine biosynthesis, and its importance in differentially controlling carbon allocation to both lignins and proteins; our new discoveries with dirigent proteins (DPs) which extend the stereoselectivities in lignan biosynthesis, including that affording the widely utilized anticancer compound, podophyllotoxin, as well as possibly extending the DP metabolic range to diverse biochemical pathways other than lignans; transcriptome and metabolome profiling of selected medicinal plants,

in order to help identify and exploit currently unknown biochemical steps to important medicinals; our recent progress extending our discoveries of how flavor/fragrance plant chemicals, such as eugenol/isoeugenol are formed, and their potential role as new commodity chemicals; our discoveries and progress to date in generating new woody plant lines accumulating selected sustainable petrochemical replacements.

Results and Discussion: Each of the above various topic areas are described below.

Aroenate dehydratases: An upstream biochemical step differentially modulating carbon allocation into the downstream lignin pathway. Pioneering work by Roy Jensen and colleagues,¹ as well as by Eric Conn and coworkers,² provisionally established that the final step in phenylalanine biosynthesis in plants was catalyzed by aroenate dehydratase (ADT), although attempts to purify the enzyme(s) involved were unsuccessful. Following the genome sequencing of *Arabidopsis* in 2000, we then both discovered and validated that a 6-membered multigene family in this plant species encoded genes for ADTs.³ One reason for our interest in this enzymatic step was as to whether individual isoforms might differentially control carbon allocation into phenylpropanoid (lignin) metabolism vs. that of, for example, protein synthesis. In our more recent work,⁴ we have now generated a variety of ADT mutants, in order to probe and resolve this question. In this regard, ADT T-DNA mutants were obtained for 5 of the 6 ADT genes, with the sixth not being fully suppressed. Each of the 5 ADT mutant lines, following screening to obtain them in homozygous form, was initially examined to assess effects on lignin deposition. In addition, double mutants were generated in all possible permutations, as well as triple and quadruple mutants. The analysis of these different plant lines, over the life-span of growth and development of *Arabidopsis*, has now provided exciting new insights into how carbon allocation can be differentially controlled into the lignin-forming apparatus and the corresponding vasculature.

Specifically, the various mutants so obtained gave phenotypes that were differentially reduced in lignin content, from up to nearly 75 to 80% reduction of lignin content in some lines to others having essentially little change in overall lignin amounts when compared to the wild type (WT) line (Figure 1). As a result, this is the first example of not only a step-function reduction in lignin content through manipulation of different members of a multi-gene family, but perhaps even more interestingly that carbon can be differentially allocated by manipulation of an upstream enzymatic step (namely aroenate dehydratase, ADT). In addition, histochemical staining for the presence/absence of guaiacyl (G) and syringyl (S) lignins was also most informative. In this regard, histochemical staining using phloroglucinol-HCl for guaiacyl (G) lignin indicated that the interfascicular fiber (*if*) regions may have been most greatly affected in these manipulations. Furthermore, pyrolysis GC/MS analyses of micro-dissected (*if*) and vascular bundle (*vb*) tissues were also carried out, with this providing novel insight into the nature of the lignins in these different cell wall types.

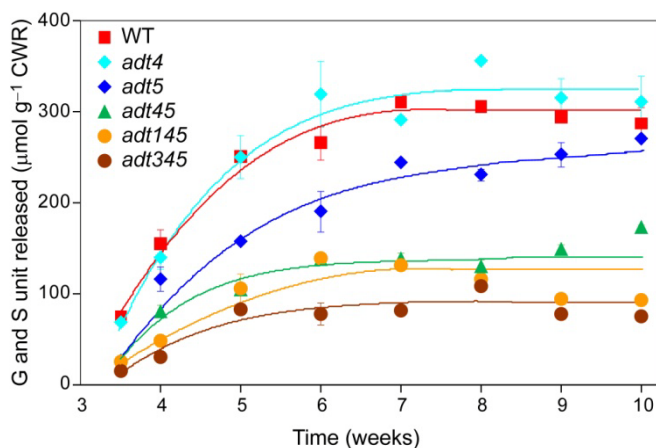


Figure 1. Step-function reduction of lignin contents in ADT mutants. G + S derived lignin monomeric cleavage products were released by thioacidolysis over a 3 to 10 weeks for *adt4*, *adt5*, *adt4/5*, *adt1/4/5*, *adt3/4/5* and WT lines. Reductions in lignin contents are as much as 70 – 80%.

Dirigent Proteins: Differing Stereoselectivities. Following our discovery of (+)-pinoresinol forming dirigent proteins, DPs, the first protein known able to control the outcome of phenoxy radical radical coupling,⁵ this work has now been extended to the study of both its substrate binding and mechanism of action, as well as in comparing this DP with the corresponding (–)-pinoresinol forming, DPs (Figure 2). For the latter, both *Arabidopsis* and flax have been used to study formation of (–)-pinoresinol.⁶ In this context, the recent discovery of pinoresinol reductases⁷ and their enantioselectivities in root tissue of *Arabidopsis* initially provided some preliminary clues as to this species potentially harboring a (–)-pinoresinol forming DP.⁶ The latter DP was obtained by first establishing which DP genes were expressed in *Arabidopsis* root tissue, and then cloning the corresponding homologs in insect cell cultures, and assaying same for stereoselective coupling.^{6,8} In this way, we established that *Arabidopsis* has a (–)-pinoresinol forming DP (Figures 2D and 2E), this also being confirmed by others in a separate investigation.⁹ Additionally, we have over-expressed the (–)-pinoresinol forming DP in *Arabidopsis* and have shown that this manipulation results in an increase in levels of (–)-pinoresinol and its downstream products *in vivo*.⁸ Accordingly, we now have in hand both DP's engendering opposite stereoselectivities. This, in turn, raises fascinating questions as to how these different proteins bind and orientate their substrates in order to engender the formation of these distinct/opposite enantiomeric forms. This is also discussed in terms of our current understanding of how these processes might occur.

It is also important to note that DP involvement has been extended to considering the stereoselective formation of the terpenoid, (+)-gossypol, from its achiral precursor, hemi-gossypol.¹⁰ We are thus poised to begin to understand the biochemical mechanism of DP's in general, as well as in obtaining a much better understanding of their distribution (and different stereoselectivities) that have evolved during land plant adaptation over the eons.

Transcriptome and metabolome profiling of selected medicinal plant species: Over the past 50 years or so, the investigation of the biosynthesis of plant-derived medicinal compounds has been largely based on attempting to identify possible biochemical steps in complex biochemical pathways. Yet most of these highly valued medicinals are: structurally very complex; cannot be obtained readily/economically by total synthesis; are often found in very small amounts, and many of their biochemical pathways remain poorly understood. This, in turn, has limited the biotechnological exploitation of

their pathways to increase the production levels in transgenic cell cultures and/or plant lines of these substances. However, more recently, genome-wide studies of model plant species have resulted in an explosive increase in our knowledge of, and capacity to understand, basic biological processes. Thus, amongst a group of selected medicinal plants, we discuss herein our next generation Illumina-based sequencing, coupled with targeted metabolomic analyses, of the important medicinal plants, *Podophyllum*, *Linum*, and *Larrea* species, which produce podophyllotoxin (derivatives) and *nor*-dihydroguaiaretic acid derivatives, respectively. As will be discussed, this approach now gives the most efficacious means to build the long awaited and urgently needed foundational infrastructure to efficiently probe and exploit these poorly understood plant medicinal compound biosynthetic pathways. Our data obtained is expected to expedite biochemical and molecular genetic research on other diverse species, thereby advancing the entire field of plant-derived pharmaceuticals.

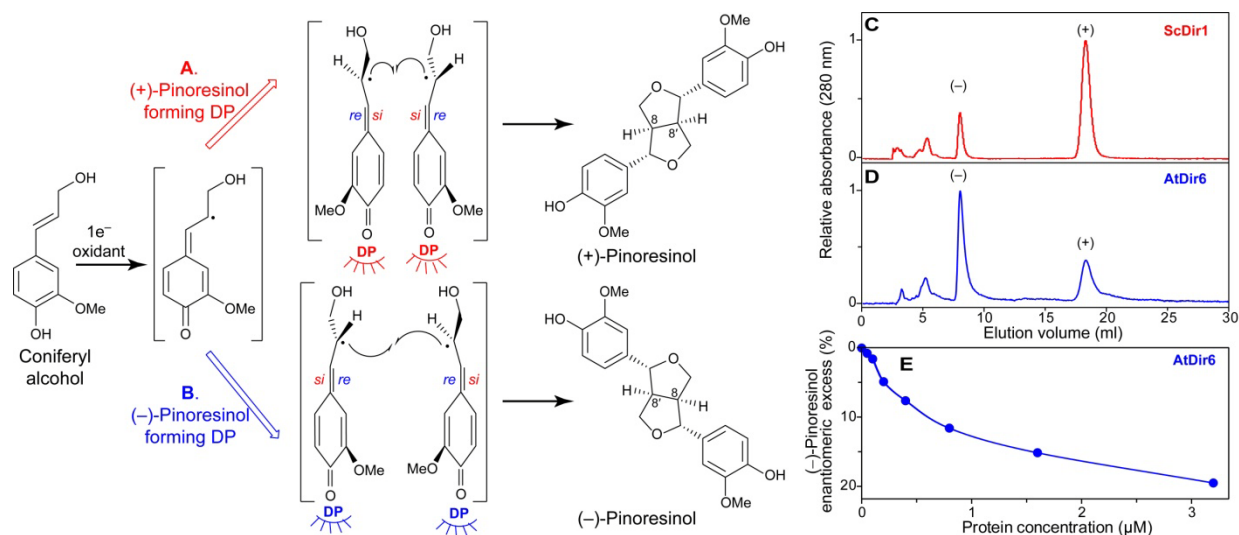


Figure 2. Stereoselective coupling of coniferyl alcohol in presence of (+)-pinoresinol (A) and (-)-pinoresinol (B) forming dirigent proteins (DPs). Chiral chromatographic separations of (+)- and (-)-pinoresinols formed upon incubation of *Schizandra chinensis* recombinant DP (ScDir1, C) and *Arabidopsis* recombinant AtDir6 (D) with coniferyl alcohol and laccase. (E) Effect of varying AtDir6 protein concentration on (-)-pinoresinol formation (enantiomeric excess).

Plant phenolic flavor/fragrance and related phenyl chemicals as sources of commodity chemicals/petrochemical substitutes in cell-culture and transgenic woody plants: Monomeric allylphenols/propenylphenols and related phenyl derivatives can be present as important constituents of essential oils/flavors of several herbs, spices, flowers, and many woody species. As products of the phenylpropanoid (C₆C₃) pathway, we have been investigating whether the genes and enzymes involved can be used as a source of commodity chemicals/petrochemical substitutes. In this regard, isolation of a creosote bush (*Larrea tridentata*) cinnamyl alcohol acyltransferase (CAAT) catalyzing conversion of monolignols into their corresponding monolignol esters, as well as an allylphenol synthase (APS) and a propenylphenol synthase (PPS) converting monolignol esters into their corresponding allyl- and propenyl-phenols, respectively, has been achieved.^{11,12} In this

context, the co-expression of CAAT/APS and CAAT/PPS in *Escherichia coli* was carried out, where it was established that various monolignol substrates examined could be efficiently converted into their allyl/propenyl phenol counterparts without addition of cofactors (e.g., acetyl-CoA or NADPH). This, therefore, provides proof of concept for their efficacious conversion in host systems, as well as providing an alternate means to source to these important plant phytochemicals, whether for flavor/fragrance and fine chemicals, or as commodities, e.g. for renewable energy purposes. This work is also discussed, as well as their potential as sources of petrochemical substitutes. Additionally, related studies on alteration of the lignified vasculature are summarized.13-15

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(IS2-4)

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Open Biotechnology Journal (Editorial Board, 2007-onwards)

Chromadex Inc. (Member, Scientific Board, 2007- onwards)

Firstwave Technologies Inc. (Member, Scientific Board, 2007-onwards)

Chair of the Session on Metabolic Engineering for Tissues and Organs at the Annual Meeting of the American Institute of Chemical Engineers (Salt Lake City UT, November 2007).

Title: Engineering Plant Secondary Metabolites in Microorganisms

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Abstract: The overall goal of our work is the development of production platforms for the biosynthesis of plant secondary metabolites, such as flavonoids, using well-characterized microbial species, such as *Escherichia coli* and *Saccharomyces cerevisiae* (yeast). This is achieved first through episomal introduction of the flavonoid metabolic circuits from various plant species in these organisms and subsequent optimization by rational strain design using computational metabolic models that allow us to identify genetic targets for overexpression or deletion, optimization of expression of the recombinant pathways and finally by the characterization and improvement of the biochemical properties of some critical enzymes in the recombinant pathways. Using the constructed recombinant strains, we have also achieved the mutasynthesis of novel, unnatural flavonoid analogous with potent antibacterial and antifungal properties.

Introduction. Tailored for the production of specific compounds, novel *E. coli* and *S. cerevisiae* strains, co-optimized with entire imported biosynthetic pathways, are able to convert low-cost feed stocks into high-value specialty and commodity chemicals. The promise of “industrial biotechnology” has been slowly but steadily emerging into the marketplace. Its

advantages over conventional synthetic chemistry include: 1) inexpensive, unpurified feedstocks; 2) reduced reliance on hazardous solvents; 3) little or no reliance on metal catalysts; 4) operation at low temperature (lower power requirements, lower CO₂ emissions); 5) non-hazardous wastes; 6) chiral synthesis; and 7) simpler purification.

Plant secondary metabolites, such as Flavonoids- the largest group of polyphenols in nature- have historically been harvested by extraction from seeds, flowers or leaves; they literally “grow on trees”. But extracts from plants have several disadvantages including: 1) seasonal and regional variation; 2) low specific yields (g product/kg wet weight of plant); 3) difficult purification; 4) undesirable or uncharacterized contaminants.

It is the explicit premise of our work that *E. coli* and *S. cerevisiae*-based production of flavonoids will soon be more consistent, less expensive, more versatile and better for the environment than current extractive methods or synthetic chemical approaches. For doing so, we have developed an array of Metabolic Engineering tools that allow us to optimize the recombinant production platforms in order to achieve commercially viable processes. In this talk we will present some of our recent efforts towards production optimization using a stoichiometric based, genome-wide metabolic model that allows us to predict genetic modifications in the host strain for the purpose of improving production of flavanones, the global precursors of flavonoids. In addition, we will also present production of resveratrol and its optimization through protein engineering and transcription optimization approaches.

Flavanone Production optimization in Escherichia coli.

Development of efficient recombinant production platforms for natural product biosynthesis is often limited by the availability of precursors and cofactors derived from the host's native metabolism. This limitation is generally addressed through modifications based on ad hoc predictions or random genetic perturbations, often overlooking the myriad of interactions within the global metabolic network. Advances in computational systems biology have yielded more systems-based approaches, providing a means to analyze genome-wide reaction networks using limited parameters and assumptions. Using constraints developed from the network architecture to define a metabolic flux space and optimizing these by means of a prescribed metabolic objective, such as biomass, known as flux balance analysis (FBA), one can explore unique aspects within the solution space. Gene deletion mutants are typically investigated using a quadratic objective, termed minimization of metabolic adjustment, although numerous other optimization routines have also been developed. All routines face the same challenges in application, particularly those related to computational costs associated with the combinatorial explosion of phenotypic possibilities in large networks. FBA has largely remained at the theoretical level with few examples presenting its successful experimental verification, especially toward heterologous product biosynthesis.

We will present an alternative, independently derived model that allows us to investigate the impact of multiple gene deletions in microorganisms by coupling an evolutionary search to constraint-based modeling, termed the cipher of evolutionary design (CiED) model. Furthermore, we will present the experimental validation of genotypes predicted by CiED for the optimized biosynthesis of flavanones in *E. coli*. Such genotypes were identified by both flavanone production potential and a minimal growth requirement by evolving random populations of in silico strains under an artificial selection

process. Finally, since the CiED algorithm predicted carbon flux increases through the CoA biosynthetic pathway in concert with gene deletions, sequential overexpression of native *E. coli* enzymes responsible for CoA biosynthesis were performed, resulting in an optimal strain with improved flavanone production levels.

Results. The CiED stoichiometric model was prepared by expanding an existing *E. coli* model (EciJR904) to include additional biochemical processes identified since that model was published. In addition, reactions of the heterologous flavanone pathway (4CL, CHS, and CHI), assumed diffusion fluxes for metabolites within this pathway, and exchange fluxes for those metabolites were also introduced. The additional genes were identified by manual comparison of the EciJR904 model to the available online databases. Our expanded model (EciZF922) was compiled and solved using CiED for the steady-state FBA solution and, in the case of gene deletions, the minimization of metabolic adjustment solution. The built-in genetic algorithm function within MatLab 7.1 (Mathworks, Inc.) was employed with certain modifications. Formulated linear programming and quadratic programming problems were solved in CPLEX 9.1 (ILOG, Inc.) via a MEX file. All simulations were performed on an HP Compaq dc5000 system running a 2.80-GHz Pentium 4 processor with 512 Mb of RAM.

Predictions made by CiED appeared to result to improvement of intracellular malonyl-CoA, based on the topology of the mutant predictions. To verify that the CiED predictions did in fact improve malonyl-CoA and CoA levels, intracellular concentrations were measured from both the *E. coli* mutant and wild-type strains while overexpressing ACC and BPL. These overexpressions were included because a previous study suggested flavanone production increased as a result of increased malonyl-CoA levels due to ACC and BPL overexpression. Here we indeed found a significant change for the intracellular concentration of malonyl-CoA during growth post-IPTG induction when comparing the parent strain BL21Star to the same strain harboring genes for overexpression of ACC and BPL (strain E2M).

To exploit the increased malonyl-CoA levels within the gene deletion strains, we extended the application of these mutant genotypes to the production of flavanones by incorporating the recombinant plant biosynthetic pathway. Indeed, the increased number of genetic modifications resulted in increased flavanone production levels. Of the single gene deletions performed, the *sdhA* mutant (Z11N), as predicted by CiED, performed the best with an increased production of 102 mg/liter of naringenin. Continued improvement was seen in higher-order deletion strains, with the best producer, a quadruple deletion strain, achieving production levels of 215 mg/liter of naringenin and 114 mg/liter of eriodictyol. Perhaps more importantly than total production, the specific flavanone production from these recombinant strains was increased by over 530% for naringenin (15 to 80 mg/liter/OD) and by over 320% for eriodictyol (13 to 42 mg/liter/OD).

Conclusions. The results of the CiED suggested that the deletion of citrate cycle genes *sdhCDAB* and *citE*, the amino acid transporter *brnQ*, and the pyruvate consumer *adhE*, generate an efficient genotype for the production of flavanones. CiED predictions included altering distant metabolic routes rather than neighboring pathways that directly impact the formation of

malonyl-CoA, the major indigenous precursor in *E. coli* used for flavanone biosynthesis. Batch fermentations confirmed that simulation predictions led to improved phenotypes with the most promising strain increasing specific flavanone yields by over 500%. Importantly, it was demonstrated that the combination of the two best single deletions, *sdhA* and *glyA*, resulted in lower levels of flavanone biosynthesis and minimal growth in glucose-supplemented medium. This highlights two important points. First, since the genotype was also predicted as one of the best double deletions when using only production as a fitness function, coupling growth to production when scoring the genotype's fitness may be critical to identifying beneficial deletions for pathways tied to growth rate. Second, the mere stacking of predicted primary deletions leads to only limited increases in recombinant production, as global rearrangements in the carbon flows are not considered for the added genetic modifications. Therefore, algorithms such as CiED that look at global changes offer important advantages in designing optimal genotypes.

Resveratrol Production optimization in Escherichia coli.

Resveratrol is a stilbene-class, polyphenolic compound of high concentrations in red wine (up to 6.8 mg/L) and speculated to be responsible for a decreased risk of heart disease and diabetes. Yet resveratrol may have additional health benefits as numerous studies have described its biological activities to include anti-oxidative, anti-inflammatory, anti-cancer and chemopreventative abilities. However the most interesting activity attributed to resveratrol may be the ability to prevent aging and possibly prolong life spans.

Previous efforts to engineer microorganisms for stilbene biosynthesis have generated only relatively low titers and little effort has been made to vary expression constructs or explore different STS proteins. We examined multiple constructs for resveratrol production by varying the 'construct environment' through different *E. coli* strains (BL21Star and BW27784), promoter systems, specifically the strong T7 promoter (pT7) and the constitutive promoter of glyceraldehyde-phosphate dehydrogenase (GAP), and gene combinations between both 4CL and STS. Additionally, we did a more detailed investigation of stilbene biosynthesis in microorganisms by conducting both sequence and protein-structure analysis as well as biochemical comparisons using a selection of stilbene synthases to identify highly active enzymes. With these optimization efforts, high yields of resveratrol molecules up to gram per liter scale from their precursor *p*-coumaric acids were achieved.

An initial resveratrol construct. Based on previous efforts for flavonoid biosynthesis, a similar strain design was used as the initial resveratrol production platform. This construct consisted of *E. coli* strain BL21Star harboring plasmid pCDFDuet-1 that encoded a 4CL enzyme from *Petroselinum crispum* (Parsley, *Pc4CL*) and a STS from *Arachis hypogaea* (Peanut, *AhSTS*). Previous studies revealed that *Pc4CL* was significantly more robust in its substrate specificity than other 4CL enzymes, while the STS from peanut is well-studied and known to be active under recombinant *E. coli* expression with broad substrate specificity. Batch fermentations resulted in resveratrol production of up to 33 mg/L in shake flask cultures.

Screening for new STS via bioinformatics and homology modeling. To further expand the production potential of this construct, the characterization of an array of STS proteins was performed as it was postulated a more active STS should increase production. An initial phylogenetic tree built from a BLAST search led to the selection of an additional seven different STS sequences from different tree groups for further analysis. Those selected include *Vitis vinifera* (grape, VvSTS), 2 sequences from *Polygonum cuspidatum* (Japanese Knotweed, Pc1STS and Pc3STS), *Psilotum nudum* (Whisk Fern, PnSTS), *Pinus masoniana* (Chinese Red Pine, PmSTS), *Pinus strobus* (Eastern White Pine, PsSTS), and *Pinus densiflora* (Japanese Red Pine, PdSTS). After being realigned using their protein sequences, all STS enzymes used in this study were used to construct a new phylogenetic tree. Inspection of the protein alignment revealed a highly conserved active site pocket with greater than 90% similarity. On the other hand, regions downstream and adjacent to the residues involved in the hydrogen-binding network of the STS protein show a large variance in conservation.

To investigate this further, homology models were built using SwissPdb Viewer for each of the STS proteins identified by mapping the translated amino acid sequence to the previously published peanut STS crystal structure (PDB identification number 1Z1F), the known highly active STS. After building the homology models, a considerable variance in the hydrogen-binding network compared to that of peanut STS can be seen in five of the plant species (VvSTS, PsSTS, PmSTS, PdSTS and PnSTS) when looking from the back side of the catalytic pocket. Specifically, Met-98 (*Ah*STS numbering) has been changed to a variety of residues of both increasing and decreasing size as well as increased electron withdrawing ability. In contrast, a significant difference is seen in the supporting residues for both *Polygonum* STS versus the *Ah*STS when looking at the protein structure through the CoA-binding tunnel. Phe-265 is lost in Pc1STS completely while in Pc3STS the Gly-256 is changed to a Leu that extends into the catalytic site.

Diversification of STS and 4CL gene library. VvSTS was isolated from its native plant while the six remaining STS gene sequences were sent for synthesis following codon optimization. Similar to *Ah*STS, other STS genes were simultaneously cloned into the second multiple cloning site of the pCDFDuet-1 vector with the first being occupied by Pc4CL. Functional expression of each STS was verified by fermentation. Out of all STSs, only two STSs in addition to the *Ah*STS produced resveratrol above detectable limits: VvSTS (0.74 mg/L) and PsSTS (2.01 mg/L). To further validate the expression of each STS, crude *E. coli* extract after expressing each STS under pT7 constructs in pACYC vector were separately mixed with another crude extract harboring an active Pc4CL. This led to the identification of a new active STS from *P. masoniana* in addition to previously verified functional proteins: *Ah*STS, VvSTS and PsSTS. Other STS enzymes failed to form resveratrol above detectable limits and were not investigated further.

The four active proteins were further characterized *in vitro* using pure proteins. They were purified using a N-terminal 6x histidine tag on a Ni⁺ affinity column, a method previously used for STS purification. Following purification and buffer exchange to remove excess imidazole, *in vitro* assays with purified STS established differing kinetic parameters for each synthase. Corresponding to the levels of resveratrol in plant material, the k_{cat}/K_M ratios for *Ah*STS and VvSTS were significantly higher than those of PsSTS and PmSTS. Additional *in vitro* assays using the purified STSs were performed while varying the acetyl-CoA concentration to investigate any substrate inhibitory activity. Significant reductions in

production were seen for all STS enzymes tested at concentrations above 1 mM indicating a degree of inhibitory activity, although well above normal cellular levels of acetyl-CoA. Due to the complexity of the kinetic system (substrate binding, three successive substrate condensations and product cyclization and release) only the apparent inhibitory constants for competitive and uncompetitive inhibition are reported.

Based on the kinetics study with purified proteins, both *Vv*STS and *Ah*STS seem to have higher enzyme efficiency compared to the other STSs, especially in the case of *Vv*STS with catalytic turnover of almost twice as fast as that of *Ah*STS. Surprisingly, this observation was not reflected on the overall production, in which *Ah*STS (33mg/L) performed much better than *Vv*STS (0.7mg/L). In order to eradicate the differences in terms of gene expression for 4CL and STS, another cloning approach was attempted, in which both 4CL and STS were cloned into the same operon promoted by one pT7 promoter. In this case, partial activity of *Vv*STS can be recovered and the production, 15.5 mg/L was greater than *Ah*STS, 0.1mg/L.

Apart from STSs, 4CL diversification was also addressed. 4CL from *Arabidopsis thaliana* (*At*4CL1) was employed in addition to *Pc*4CL because *At*4CL1 has also been shown to be functionally assembled in recombinant *E. coli* for the flavonoids biosynthesis. In the pT7 system, both 4CL gave similar production, indicating 4CL is able to convert the substrate, *p*-coumaric acid to its coumaroyl-coA ester while STS, the possible rate-limiting enzyme is not efficient enough to turn over the ester to resveratrol.

High production resveratrol strains by changing the expression construct. Since the pCDF vector requires the expression of the T7 RNA polymerase of BL21Star for induction of resveratrol biosynthesis, a new expression construct was needed for the use in strain BW27784. To contradict the use of inducible promoter (pT7) in the initial expression system, a constitutive GAP promoter (pGAP) was selected. This constitutive promoter allows continual transcription of both 4CL and STS because its induction occurs in the presence of a hexose sugar, which is an absolute carbon source requirement for the host strain. Two approaches: one by expressing both genes under separate GAP promoter and the other by expressing both in an operon were again introduced to the new strain BW27784, under a high copy plasmid pUC19. Fermentation titers revealed the inefficiency of the two promoters construct in this new expression compared to the one with a bicistronic operon. The production of resveratrol in this new system of STSs coupled with either *Pc*4CL or *At*4CL was much higher compared to the pCDF system. *Pm*STS, *Ps*STS, *Ah*STS and *Vv*STS were all active and their shake flask fermentations coupling with *At*4CL expressed under an operon resulted in final volumetric production levels of 23.7 mg/L, 61.5 mg/L, 404 mg/L and 1706 mg/L respectively. The highest production number represents an astounding increase of more than 30-fold for the production of resveratrol when compared to the starting strain pCDF-*Pc*4CL-*Ah*STS in BL21Star of merely 33 mg/L. These result also confirmed the accuracy of the *in vitro* assay with purified proteins, in which the catalytic turnover of *Vv*STS is more efficient than *Ah*STS and both their k_{cat}/K_m were significantly higher than those of *Ps*STS and *Pm*STS. In the case of 4CL, *Pc*4CL limited resveratrol production compared to *At*4CL.

High-production resveratrol strains by increasing the precursor supply. Additional modifications to the fermentative strategy and construct design were also attempted in preliminary trials. Since malonyl-CoA is the major metabolite



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harnessed from the native metabolism of *E. coli*, increasing its availability should lead to improved production. As a simple means to increase the intracellular malonyl-CoA level, cerulenin, a specific inhibitor of the FabB/FabF gene product, was introduced to the fermentation medium post induction. In the pCDF system, cerulenin was able to improve the production up to 2-fold to 65 mg/L. For the pOM system, additional coumaric acid was needed (increase from 6mM to 15mM) to maintain a substrate driving force for the formation of resveratrol. Production, as a result of the cerulenin treatment and increased substrate level, was improved to 2337 mg/L.

(IS2-5)

1. Speaker

Ikuro Abe

2. Affiliation

Professor, University of Tokyo

3. Professional experience

Investigator, PRESTO, Japan Science and Technology Agency (2005)

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Engineering Plant Polyketide Synthases

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In the last 10 years, there have been significant advances in understanding the structures and functions of the chalcone synthase (CHS) superfamily of type III polyketide synthases (PKSs). Discoveries of increasing number of enzymes with novel and unexpected catalytic functions have significantly expanded the biosynthetic repertoire of type III PKSs. It is now established that many classes of plant secondary metabolites are produced by type III PKSs. Further, the crystallographic and structure-based mutagenesis studies on functionally diverse type III PKSs have begun to reveal intimate structural details of the enzyme-catalyzed processes. The enzymes share a common three-dimensional overall fold and catalytic machinery. Only a small modification of the active-site architecture leads to the remarkable functional diversity of the enzymes. Further, steric factors that shape the active-site cavity control the number of condensation reactions, and the conformation and thus the cyclization fate of polyketide intermediates. It is remarkable that the functional diversity of the type III PKSs evolved from such a simple steric modulation of the active-site architecture.

In addition, type III PKSs exhibit unusually broad, promiscuous substrate specificities, accepting a variety of non-physiological substrates to produce an array of chemically and structurally divergent unnatural novel polyketides. These findings have revolutionized our understanding of the catalytic potential of the structurally simple type III PKSs. Manipulation of the enzyme reactions by combining the structure-based and precursor-directed approaches should lead to further production of chemically and structurally divergent unnatural novel polyketides. Because of the remarkable catalytic potential and the substrate promiscuity, the structurally simple type III PKS can be an excellent platform for engineering to design and develop supra-natural enzyme with novel catalytic functions. Since it is now possible to



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manipulate the substrate specificity and the number of the malonyl-CoA condensations, the next goal is how to control the mechanism of the cyclization reactions to generate polyketides with desired ring systems. The poly- α -keto intermediates are highly reactive and readily react with amines to yield Schiff bases, which makes it possible to introduce additional C-C or C-N bond forming chemistry to generate more complex enzyme reaction products. Combining structure-based protein engineering and precursor-directed biosynthesis with rationally designed nitrogen-containing substrate analogues is thus expected to generate unnatural novel "alkaloid" scaffolds with further promising biological activities. In this lecture, recent progress of our studies on engineering of plant type III PKS enzymes will be discussed.

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(IS2-6)

1. Speaker

Thomas Vogt

2. Affiliation

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3. Professional Activities

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Structure and function of plant cation-dependent *O*-methyltransferases

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The enzymes required for methylation of hydroxyl groups are usually *S*-adenosyl-L-methionine (AdoMet)-dependent *O*-methyltransferases (OMTs). In this group a subset of bivalent cation-dependent enzymes can be distinguished that exhibit activity towards compounds containing aromatic *cis*-diols. Representatives of this family are common in pro- and eukaryotes. Prominent members include the mammalian catechol OMTs, with important roles in methylation and inactivation of neurotransmitters in the brain, as well as detoxification of reactive, hydroxylated aromatic ring systems in the liver (1). Among the most prominent representatives in plants are the caffeoyl CoA *O*-methyltransferases (CCoAOMT) generally accepted to be involved in lignin monomer biosynthesis. In principle, two subtypes of CCoAOMTs can be distinguished with respect to acceptor specificity: a specific and promiscuous subtype. The latter are able to methylate a variety of natural compounds with vicinal dihydroxy groups, like phenylpropanoids, flavonoids, and anthocyanidins (2,3). Crystal structures of both types of enzymes are known (4,5). Although differences in the acceptor specificity occur, both are usually specific for the *meta*-position. The three-dimensional structures suggest that N-terminal and a C-terminal loops are relevant for the substrate specificity of this type of enzymes (6). The corresponding domains display the lowest degree of sequence similarity among the members of AdoMet-dependent enzyme subclusters. Site-directed mutagenesis, N-terminal truncations and the use of chimeric enzymes between a specific CCoAOMT from alfalfa and a promiscuous subtype cloned and expressed from *Mesembryanthemum crystallinum* allowed a detailed investigation of the role of individual amino acid residues. The results indicate that the observed changes in substrate specificity *in vitro* are based on relatively small differences in otherwise conserved region which makes prediction of the acceptor specificity difficult (5).

Arabidopsis thaliana contains a set of seven CCoAOMT-like enzymes which all display the characteristic *O*-methyltransferase signature motifs. Likewise, several members of this CCoAOMT-like enzyme family are found in the genomes several plant genomes. The specific CCoAOMT1 in *Arabidopsis*, besides lignin biosynthesis, was recently shown to be involved also in the biosynthesis of soluble phenylpropanoids like scopoletin and sinapoylmalate, methylating the common precursor caffeoyl coenzyme A (7,8). Annotation of the *in vivo* function of the remaining six genes and enzymes was recently initiated. One of the genes, encoded by the *Atlg67990* locus, was exclusively expressed in flowers of *Arabidopsis*. The corresponding enzyme, AtTSM1, was functionally expressed and showed the predicted broad acceptor specificity. By the use of specific antibodies and knockout mutants, localization of this enzyme exclusively in the tapetum was demonstrated (9). During late stages of stamen and tapetum development, AtTSM1 appears only to be required for a single methylation step, methylating (tris)5-hydroxyferuloyl spermidine to a mono-sinapoylated conjugate. Methylation of the single 5-hydroxy feruloyl moiety is considered the final step in a recently established phenylpropanoid spermidine biosynthetic pathway, specifically expressed only in the *Arabidopsis* tapetum (9-12). In this pathway, CCoAOMT1 again is assumed to catalyze the required methylating step from caffeoyl to feruloyl CoA earlier in the phenylpropanoid part of the pathway. The biological relevance of spermidine phenylpropanoid conjugates which accumulate on the surface of *Arabidopsis* pollen grains remains to be established. Possible functions include a role as UV-shields of the developing male gametophyte and/or protection against biotic and abiotic stressors. AtTSM1 is the only promiscuous CCoAOMT from *Arabidopsis*, with a clearly established function. The remaining five genes and enzymes, although partially characterized by knockout mutations and transcript profiling remain to be functionally annotated. Elucidation of their function may help to establish a niche among the large set of cation-independent OMTs which display a much broader acceptor and position specificity, due to less restrictions in the catalytic center.

Due to a broad acceptor specificity and high expression yields, some CCoAOMT-like enzymes can be used as bioorganic catalysts in the modification or biofermentation of pharmacologically interesting hydroxylated aromatic compounds. A current update of our research with respect to putative biotechnological applications of CCoAOMT-like enzymes will conclude this presentation.

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(IS2-7)

1. Speaker

E. Neil G. Marsh

2. Affiliation

Professor, Chemistry and Biological Chemistry, University of Michigan

3. Professional Activities

Fellow of the Royal Society of Chemistry (2005)

Co-Chair Bio-organic Chemistry Gordon Research Conference (2005)

Living on Toluene – Studies on the Mechanism of Benzylsuccinate Synthase, a Key Enzyme in the Anaerobic Metabolism of Toluene by Denitrifying Bacteria

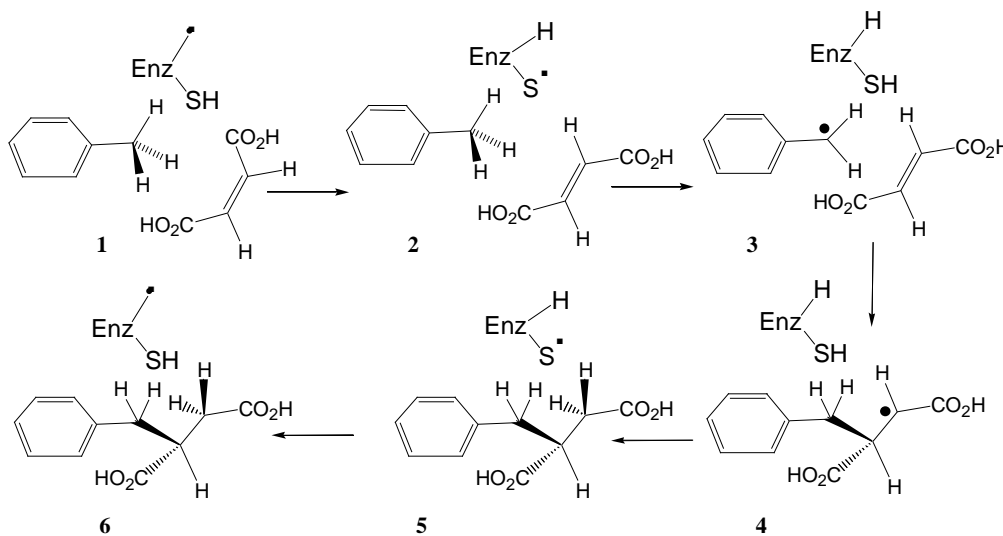
E. Neil G. Marsh, Lei Li and Dustin P. Patterson

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Aromatic compounds such as toluene are an important class of long-lived pollutants that have relatively high water solubility. They are a particular problem in environments where oxygen is scarce, such as ground water supplies, because most pathways to degrade such compounds require molecular oxygen to functionalize otherwise inert aromatic hydrocarbons. Recently, however, various denitrifying and sulfate-reducing bacteria such as *Thauera aromatica* and *Desulfobacula toluolica* have been discovered that can live on toluene and related aromatic compounds as their sole source of carbon and energy under anaerobic conditions. This discovery has aroused much interest, both for the potential such bacteria offer for bio-remediation in situations where oxygen is scarce, and because of the novel chemistry involved.

The first step in the anaerobic metabolism of toluene is its reaction with fumarate to produce (*R*)-benzylsuccinate. This remarkable reaction is catalyzed by benzylsuccinate synthase (BSS), which is a member of the glycyl radical family of enzymes that include pyruvate formate lyase and anaerobic ribonucleotide reductase. There is good evidence that the BSS-catalyzed reaction involves radical intermediates, but its extreme oxygen sensitivity and lability have hampered efforts to investigate the mechanism of this unusual enzyme. In the proposed mechanism, shown below, the glycyl radical is first transferred to an active site cysteine that then abstracts a hydrogen atom from the methyl group of toluene to generate a

benzylic radical. The benzyl radical undergoes addition to the double bond of fumarate to form a benzylsuccinyl radical and in the last step the hydrogen is replaced from cysteine and the glycyl radical regenerated.



Proposed mechanism for the reaction catalyzed by benzylsuccinate synthase

My talk will describe recent experiments in our laboratory that aim to elucidate the structure and mechanism of this unusual enzyme. We have taken a variety of approaches to understanding the mechanism that include determining the stereochemistry of hydrogen transfer; kinetic isotope effect measurements and using alternate substrates to probe the free energy profile of the reaction. These studies have provided evidence for several of the radical intermediates postulated in the mechanism. We have also examined the subunit structure of the enzyme and made the unexpected discovery that the enzyme contains iron-sulfur clusters that are important for its structural integrity.

(IS2-8)

1. Speaker

William Helferich

2. Affiliation

Diet, Women's Health and Aging Professor, Professor of Nutrition, U. of Illinois, Urbana, IL.

3. Professional Activities

Babcock-Hart Award from the IFT (2002).

Oser Food Ingredient Safety Award from the IFT (2009)

DIETARY GENISTEIN & BREAST CANCER: A COMPLEX STORY

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Soy is an important healthy food and it is important that soy protein be incorporated into the American diet. In October 1999, the FDA approved a health claim for soy protein and reduction of cardiovascular disease (CVD). Research has extended past CVD and is focused on other chronic diseases such as breast cancer. The data on CVD is clear, with obvious health benefits to humans. However, the safety of isoflavone-containing soy ingredients and breast cancer is less clear. Soy contains high concentrations of the plant estrogenic isoflavone, genistein. There are numerous reports in the literature that genistein can act as a chemoprevention agent to reduce carcinogen-induced mammary tumors in the rat. Studies like this have sparked widespread acceptance of soy components. In fact, several companies produce ingredients that are highly enriched with isoflavones and have GRAS status by the FDA. Many of these products are currently used as food ingredients and are being marketed to post-menopausal women as a potential substitute for hormone replacement therapy (HRT). It is well known that estrogens and estrogen-like compounds stimulate the growth of estrogen-dependent tumors. *This presents a paradox, since genistein may be beneficial as a chemopreventative agent when consumed early in life and may be detrimental to individuals with existing estrogen-dependent breast cancers.* Dr. Helferich's research has demonstrated that diets with isoflavone-containing ingredients can stimulate growth of estrogen-dependent tumors. For example, with estrogen-dependent tumors may take genistein believing they are slowing tumor growth, when they may actually be doing themselves harm. Dr. Helferich's current hypothesis is that there are at least two mechanisms by which the estrogenic isoflavone genistein alters carcinogenesis: a mechanism based on cellular differentiation, which acts as a chemoprotective

agent against mammary cancer, and an estrogenic, growth-stimulatory effect which is active after tumors exist.

The following outlines the key investigations conducted in Dr. Helferich's laboratory on the soy ingredients containing isoflavones over the past ten years. One of the first investigations that Dr. Helferich conducted with the synthesized genistein was an evaluation of its effects in mammary gland development. In critical studies in laboratory rodents, he demonstrated that genistein was estrogenic in the mammary gland and was able to enhance mammary gland development, increase serum prolactin levels, and induce estrogen-responsive gene expression. Because these estrogenic effects on the mammary gland suggested that genistein might have an effect on breast cancer, the Helferich laboratory then conducted numerous studies to determine the effect of dietary phytoestrogens on growth of both estrogen-dependent and independent tumors. There has been a significant increase in the use of soy protein isolates (SPI) as food ingredients in part due to the FDA-approved health claim and the popularity of low carbohydrate foods containing SPI. Dr. Helferich has evaluated SPI that were processed by different methods to contain various concentrations of isoflavones and has demonstrated that tumor growth is related to the dietary dosage of genistein, but it does not matter if the source is from soy protein or in pure form. This is an important observation because SPI are a soy ingredient that is currently being added to many foods, especially the low carbohydrate foods where soy protein is used to reduce the carbohydrate content. This work demonstrated that pure genistein, as well as various food ingredients and dietary supplements containing genistein, could stimulate estrogen-dependent tumor growth. Working in well-established pre-clinical animal models, Dr. Helferich then made the remarkable discovery that dietary phytoestrogens stimulate growth of estrogen-dependent human breast tumors transplanted into mice sufficiently to negate the inhibitory effects of current anti-estrogen therapies such as tamoxifen and aromatase inhibitors. *Results from these landmark studies are of major importance, and they are providing critical information on the safety and efficacy of these widely used bioactive food ingredients that will allow nutritionists and scientists working in the food industry and regulatory agencies to make appropriate decisions on the use of these ingredients in human foods.*

In follow-up investigations, Dr. Helferich evaluated the effect of other bioactive components present in soy on the activity of genistein. Mice were fed equal concentrations of the soy isoflavone genistein, allowing his team to determine the influences that various bioactive soy compounds had on genistein's ability to stimulate estrogen-dependent breast tumor growth. Very interesting results were observed—as bioactive compounds were removed, they observed an increase in estrogen-dependent tumor growth. If genistein had been the only biologically active compound, all diets would have resulted in similar tumor growth, but that was not the case. *These results suggest that other components in the soy flour were able to blunt the stimulatory effect of dietary genistein and that the removal of these components might cause soy to lose its beneficial effects.*

He has also conducted studies to investigate why complex soy diets, which are high in genistein, inhibit growth of mammary tumors. His approach will be to determine whether there are other, as yet unidentified, components in soy that possess potent anti-tumor activity. It is his philosophy that there are differences between consumption of a whole food as opposed to the purified individual components, and that in order to achieve health benefits, one should strive to consume whole foods as part of a healthy diet and not try to remedy a poor diet by using supplements with unproven safety. For



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example, his team has demonstrated that phytosterols can reduce estrogen-stimulated tumor growth. It is also likely that other components may have anti-tumor growth potential. However investigations have been limited because of the lack of a model with low levels of estrogen – similar to that observed in postmenopausal women. In order to overcome this critical limitation, the Helferich laboratory developed a pre-clinical model with low levels of circulating estradiol and that the weak dietary estrogen agonist, genistein can act in an additive manner with estradiol to stimulate estrogen-responsive breast tumor growth.

(IS2-9)

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Honey as a natural source of dietary antioxidants

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Lipid oxidation is a major deteriorative reaction in foods. Lipid hydroperoxides, and their breakdown products, have been implicated in a number of deleterious effects, including off-flavor and color development in foods, known as oxidative rancidity. Also, nutritional quality of foods may be compromised due to loss of lipophilic vitamins, loss of essential fatty acids, damage to protein and DNA, and potential toxicity of certain lipid oxidation products. This has ultimately led to concern over potential health-related problems such as heart disease and cancer, in addition to healthy aging. Typically in lipid containing foods, synthetic antioxidants such as BHA and BHT are utilized to minimize oxidative rancidity and minimize nutritional degradation. However, consumers are demanding the use of “natural” as opposed to synthetic substances. Thus, there is a need to develop natural sources of antioxidants.

Honey has been used since ancient times and has gained appreciation as the only concentrated form of sugar available worldwide. Traditionally its use in food has been as a sweetening agent. However, it has been valued throughout history for its role in traditional medicine. Unfortunately, honey has so far been neglected as a therapeutic agent in modern medicine due to the lack of systematic scientific studies. However, interest in the therapeutic potential of honey is growing and scientific evidence for the effectiveness of honey in several experimental and clinical situations is



beginning to emerge. Honey has been reported effective in wound and burn healing, in gastrointestinal disorders, and as an antimicrobial agent. There may be an association of these ailments with oxidative stress; however, there are only limited numbers of studies to examine the potential benefit of honey in oxidative disorders.

Honey is available from a variety of floral sources, each unique in color and flavor. Initially honeys from different floral sources were examined, resulting in the finding that the color of honey is positively correlated with antioxidant capacity; i.e., darker colored honeys exhibit the strongest antioxidant capacity. Also tested was the ability of different honeys to protect against oxidative deterioration reactions in foods. We demonstrated that honey was effective at inhibiting oxidative enzymatic browning in fruits and that honey inhibits lipid oxidation in cooked, ground turkey patties (at 0.5% w/w).

Differences in honey's ability to protect against oxidation led to chemical characterization of potential antioxidant components of honey. There was marked variation in antioxidant capacity of different honeys, strongly correlated with the phenolic and protein content. Phenolic profiles of a number of honeys were characterized. Although marked differences exist between honeys from different floral sources, it was not possible to select specific phenolic components to explain the antioxidant effectiveness of individual honeys. The total antioxidant capacity is likely the result of the combined activity and interactions of a wide range of components, including phenolics, peptides, organic acids, enzymes, Maillard reaction products, and possibly other minor products.

To determine the relevance of honey as a dietary source of antioxidants we compared the antioxidant capacity (measured by the ORAC – oxygen radical absorbance capacity – assay) with the ability to protect against *in vitro* LDL oxidation. Linear correlation was observed with ORAC and LDL oxidation assays, demonstrating the potential for honey to be used as a healthy alternative to sugar and provide a source of dietary antioxidants. This was later confirmed by a dietary intervention study demonstrating the ability of honey to enhance the antioxidant capacity of human blood.

The importance of honey as a natural source of antioxidants is thus being well illustrated. An apparent obstacle to incorporation of honey into food products is resistance to usage of honey in large quantities by the food industry. One major reason for industry's lack of use of honey in food products is the notion of honey strictly as a sweetening agent and also the cost of honey in comparison to sugar. Thus, research was needed to establish honey's effectiveness in food systems as a natural antioxidant source and to demonstrate the functional properties of honey in certain food products.

We demonstrated the ability to substitute honey for sweetening agents (i.e., high fructose corn syrup, HFCS) in commercial salad dressing formulations. Salad dressing oils are chosen for moderate flavor, light color and low cost. Soybean oil is used in the largest volumes for this purpose in this country. Soybean oil has high levels of polyunsaturated fatty acids, which are highly susceptible to oxidation. Strategies to minimize oxidation are thus desirable (e.g., use of antioxidants). Although honey had been demonstrated to have antioxidant capacity against oxidation of various lipid-based systems; oil-based emulsions are different than straight oil systems. The mechanics of oxidation and catalysis of oxidation appear to be quite different between oils and oil-based emulsions. Thus, properties of antioxidants to function in such systems may be slightly different as well. We demonstrated the capability of honey to effectively replace both HFCS as a sweetening agent and also EDTA (ethylenediaminetetraacetic acid), a common ingredient for metal chelating



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antioxidant action in salad dressings. By substituting honey the label becomes much more acceptable to consumers, removing HFCS, often associated with obesity issues, and EDTA, a synthetic chelator.

The impact of honey processing and storage on nutritional impact was not clearly understood. Our laboratory demonstrated the impact of processing and storage on honey antioxidant capacity. Although changes were complex, processed and stored honey was effective in serving in the role of a food/dietary antioxidant. This study led us in the direction of studying the impact of processing and storage on other natural sources of antioxidants, such as grapes, raisins and papaya. As a result of studying these different natural products we were intrigued by their overall nutritional impact and tremendous variation in chemical composition. We were also motivated by research demonstrating that combinations of chemical constituents commonly found in highly nutritious foods may reduce potential negative or toxic impacts that may be observed when administered singly. Thus, we selected a series of chemicals commonly found in honey, papaya and grapes/raisins to conduct a three stage analysis to explore combinations of five components of these antioxidant rich foods, both before and after acid/base digestive processes, and four compounds that have been quantified in human serum. We used the ORAC antioxidant capacity assay in combination with Electron Paramagnetic Resonance to study the ramifications of consuming a complex diet.

There is much “folklore” surrounding the concept of honey improving human health. These studies complement a growing body of research to demonstrate the science behind much of this “folklore”. We have demonstrated honey to be a significant source of natural antioxidants, going beyond its potential sweetening power to protect against oxidative deterioration in foods and to serve as a dietary source of antioxidants.

(IS2-10)

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Chemoprevention of Inflammation-Associated Carcinogenesis by Dietary and Medicinal Phytochemicals: NF- κ B and Nrf2 as Prime Targets

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Chemoprevention with Edible Phytochemicals

Chemoprevention is an attempt to use either naturally occurring or synthetic substances or their mixtures to intervene in the progress of carcinogenesis. A vast variety of phytochemicals present in our daily diet, including fruit, vegetables, grains, spices, and seeds, have been shown to possess substantial anti-mutagenic and anti-carcinogenic activities (**Figure 1**). The chemopreventive effects that most edible phytochemicals exerts are likely to be the sum of several distinct mechanisms. These include blockage of metabolic activation and DNA binding of carcinogens, stimulation of detoxification, repair of DNA damage, suppression of cell proliferation and metastasis or angiogenesis, induction of differentiation or apoptosis of precancerous or malignant cells, etc. as illustrated in **Figure 2**. Recently, it has been known that common dietary chemicals act on the human genome, either directly or indirectly, to alter gene expression, thereby regulating carcinogenic



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processes.

The implication of inflammation in carcinogenesis

Recent progress in our understanding of molecular biology of cancer highlights the intracellular signal transduction pathways, including those involved in mediating inflammatory response, which often become awry during carcinogenesis. One of the key players in the inflammatory signaling is cyclooxygenase-2 (COX-2). Aberrant upregulation of COX-2 has been frequently observed in various precancerous and malignant tissues. A growing body of evidence indicates that the ubiquitous eukaryotic transcription factor NF- κ B plays a central role in general inflammatory as well as immune responses. The promoter region of human COX-2 gene harbours two putative NF- κ B binding sites. Thus, NF- κ B has been shown to be a positive regulator of COX-2 expression in diverse types of cells challenged with a wide array of proinflammatory stimuli. Under normal physiologic conditions, NF- κ B is retained in the cytoplasm by binding to one of the inhibitory I κ B proteins (I κ B α , I κ B β , I κ B ϵ , p105, and p100), which blocks the nuclear localization sequences of NF- κ B.

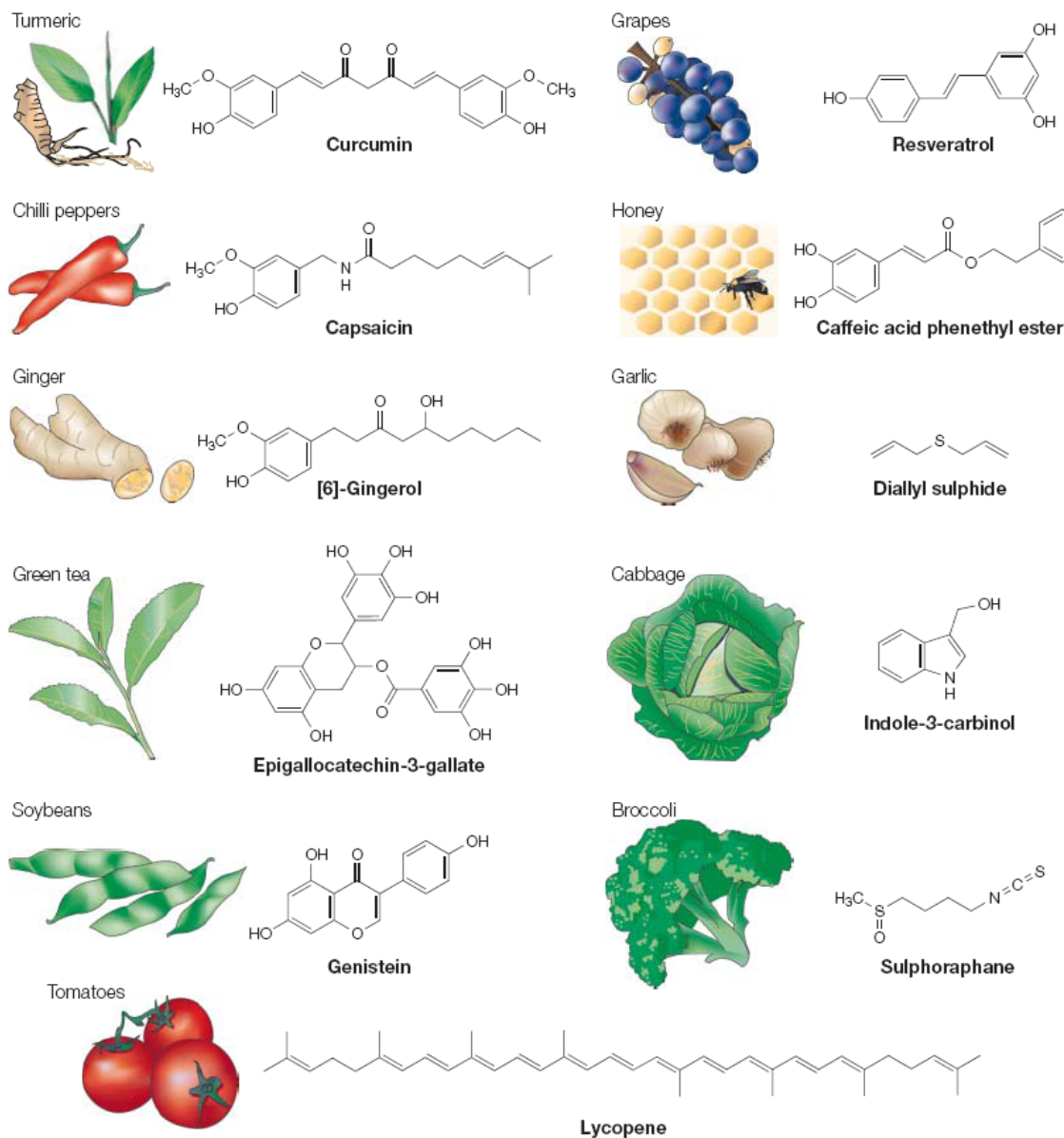


Figure 1. Representative chemopreventive phytochemicals and their dietary sources. Adopted from: Y.-J. Surh (2003) *Nature Reviews Cancer*

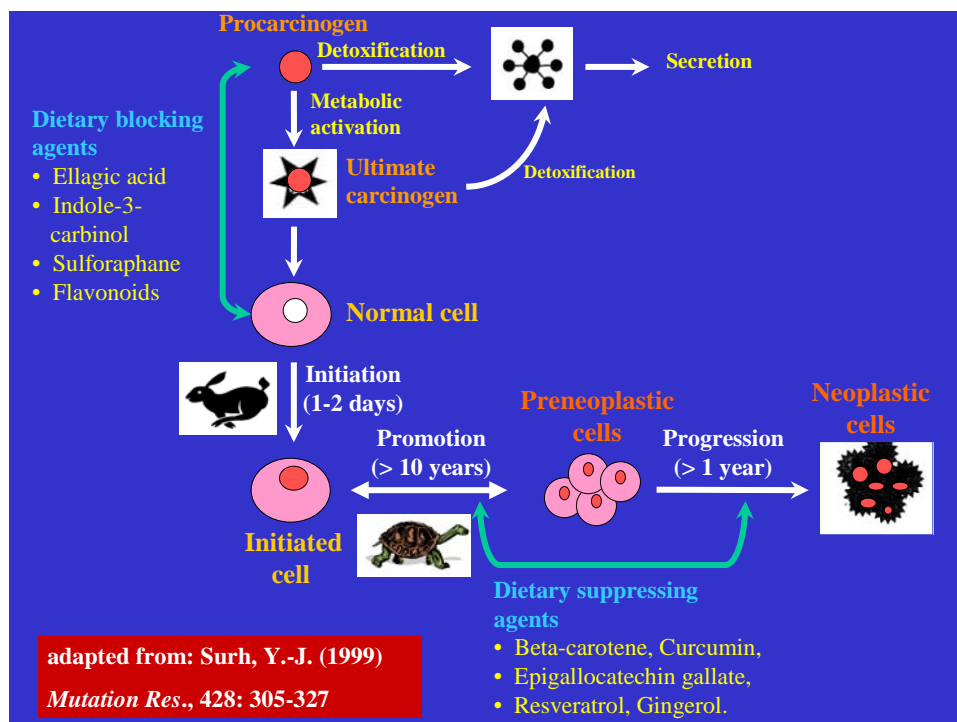


Figure 2. Effects of dietary chemopreventives on multistage carcinogenesis.

NF- κ B is activated in response to a vast variety of stimuli that promote the dissociation of I κ B phosphorylation followed by ubiquitination, and degradation. Thus unmasking of the nuclear localization sequence of NF- κ B allows NF- κ B to enter the nucleus and bind to κ B-regulatory elements. The phosphorylation of I κ B, as a critical event in NF- κ B activation, is catalyzed by an IKK complex. The core IKK complex consists of a heterodimer of IKK- α and IKK- β and two IKK- γ subunits. IKK- α and IKK- β mediate the phosphorylation of I κ B, whereas IKK- γ links the core to the upstream signaling molecules. While there is none or very little expression of COX-2 and activation of NF- κ B and IKK in non-neoplastic cells and tissues, all these proteins were found to be often coexpressed in the malignant epithelium of certain tissues, such as colon. Therefore, the normalization of inappropriately overamplified signaling cascades implicated in chronic inflammation-associated carcinogenesis by use of specific inhibitors of COX-2 or its upstream regulators, especially IKK and NF- κ B, may represents a rational and pragmatic strategy for molecular target-based chemoprevention of inflammation-associated malignancies.

The intracellular signal network as a prime target of chemopreventive phytochemicals

Over the past few decades, there has been a growing body of interest in identifying naturally occurring chemopreventive agents, particularly those present in our diet. Dietary chemopreventives are present predominantly in fruits, vegetables,

grains, spices and herbs and have diverse chemical structures. Chemical substances derived from such plant-based diet are collectively called ‘phytochemicals’. Research directed toward elucidating molecular mechanisms underlying chemopreventive or chemoprotective actions of dietary phytochemicals has recognized components of signal transduction pathways as potential targets. Since the cellular signaling network often goes awry in carcinogenesis, it is fairly rational to target intracellular signaling cascades for achieving chemoprevention. Numerous molecules and events are involved in relaying intracellular signals. Both external and endogenous stimuli turn on or switch off critical events of this relay, thereby transmitting the appropriate signals to diverse downstream target molecules in a highly sophisticated fashion for the fine-tuning of cellular homeostasis. Components of upstream or cytoplasmic signaling networks include protein kinases, such as the family of proline-directed serine/threonine kinases named mitogen-activated protein (MAP) kinases, protein kinase C, phosphatidylinositol-3-kinase, protein kinase B/Akt, glycogen synthase kinase, etc. Chemopreventive phytochemicals can work as modifiers of signal transduction pathways to elicit their beneficial effects. Oxidative stress and inflammatory injuries are closely linked to each other in the process of multi-stage carcinogenesis. Thus, compounds with anti-inflammatory activities are anticipated to inhibit oxidative stress, and *vice versa*. We have investigated chemopreventive effects of some edible phytochemicals, with the focus on those with antioxidative and anti-inflammatory properties, and their underlying molecular mechanisms. Our research program has attempted to unravel common events mediated by two major redox-sensitive transcription factors, NF- κ B and Nrf2, which mediate inflammatory and antioxidant signaling.

Nuclear factor kappa B (NF- κ B)

A growing body of data indicates that NF- κ B, a ubiquitously expressed eukaryotic transcription factor, plays a central role in general inflammatory as well as immune responses. NF- κ B is regarded as a potential link between inflammation and cancer. NF- κ B, predominantly a heterodimer of p65 and p50 proteins, is normally sequestered in the cytoplasm as an inactive complex with the inhibitory protein, I κ B. Upon stimulation with mitogens, pro-inflammatory cytokines, UV radiation, viral infection, bacterial toxins etc. it gets phosphorylated by activated I κ B kinases (IKKs) or MAP kinases. Phosphorylated I κ B, upon ubiquitination, is directed to proteasomes for degradation. The degradation of I κ B allows NF- κ B to translocate to the nucleus, and to bind to a κ B element located in the promoter regions of *cox-2* and other proinflammatory genes, thereby controlling their expression. The transcriptional activation of NF- κ B depends on the phosphorylation of its active subunit p65/RelA. The upstream IKK signalsome has been shown to phosphorylate both I κ B and p65.

Nuclear factor erythroid 2 p45 (NF-E2)-related factor (Nrf2)

The induction of phase II detoxifying or antioxidant enzymes represents one of the most important components of cellular defense mechanisms whereby a diverse array of electrophilic and oxidative toxicants can be eliminated from the cell before



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they damage genomic DNA. Some representative detoxifying/antioxidant enzymes include glutathione peroxidase, glutamate cysteine ligase, glutathione *S*-transferase, NAD(P)H:quinone oxidoreductase 1, and heme oxygenase-1. The induction of these enzymes is primarily regulated by Nrf2 as evidenced by negation of their expression in the genetically engineered Nrf2-deficient mice. Under normal physiological conditions, a cytoskeleton binding protein called Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (Keap1) binds to Nrf2 thereby repressing its activation. Exposure to inducers of antioxidant/phase II detoxifying enzyme expression diminishes the affinity of Keap1 for Nrf2, which allows for the translocation of Nrf2 into the nucleus. Following nuclear translocation, Nrf2 binds to the specific consensus *cis*-element called the antioxidant-response elements (ARE) or the electrophile-responsive elements (EpRE) that are present in the promoter region of genes encoding many antioxidant enzymes. Therefore, Nrf2-Keap1 signaling has been recognized as an important target for chemoprevention and chemoprotection. While the molecular mechanisms involved in the Nrf2-derived transcriptional activation of antioxidant enzymes are not fully understood, it has been hypothesized that covalent modification or oxidation of critical cysteine residues contained in Keap1 may facilitate the dissociation of the Keap1-Nrf2 complex or increase the stability of Nrf2. It has been suggested that cysteine residues present in Keap1 could serve as a molecular sensor for the recognition of the altered intracellular redox status triggered by electrophiles or reactive oxygen species (ROS).

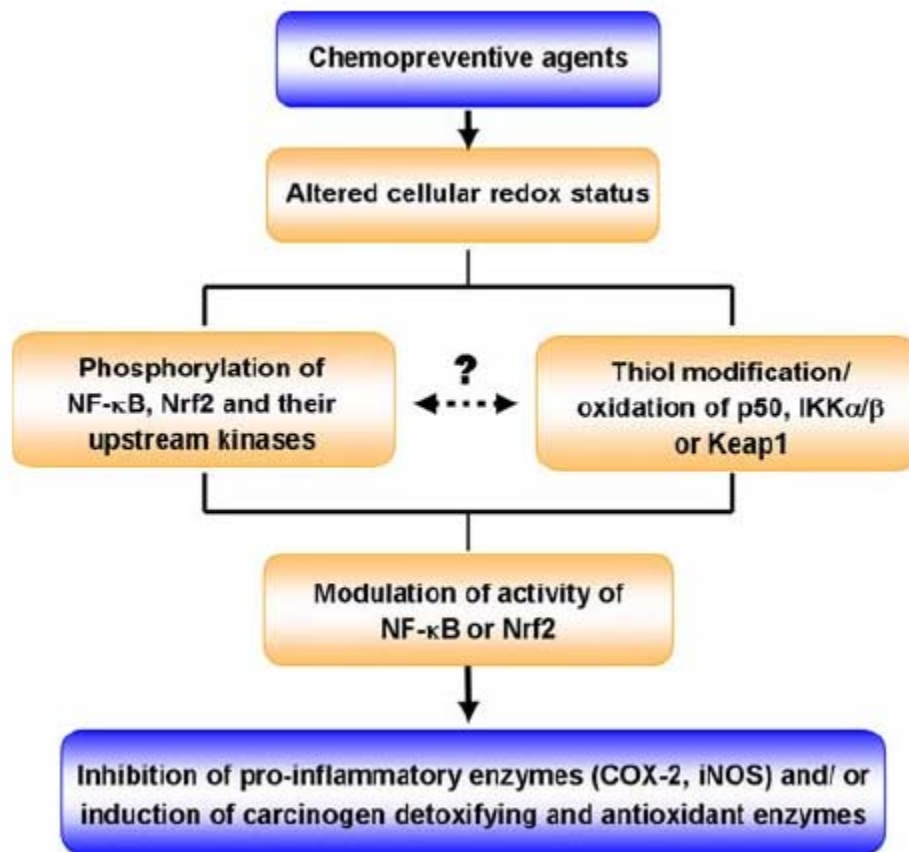


Figure 3. Regulation of NF- κ B and Nrf2-ARE signaling by chemopreventive agents via phosphorylation and thiol modification/oxidation of cellular signaling molecules or their regulators. Adopted from: H.-K. Na and Y.-J. Surh (2008).

Chemopreventive phytochemicals targeting NF- κ B and Nrf2

Some naturally occurring cancer chemopreventive agents can suppress inappropriate overactivation of NF- κ B signalling and/or activate Nrf2. Cysteine thiols, as presented in various transcription factors and their regulators, are recognized to function as redox sensors involved in the fine-tuning of transcriptional regulations of many genes essential for maintaining cellular homeostasis. Thus, oxidation or covalent modification of the thiol groups present in redox-sensitive transcription factors and their regulating molecules can provide a unique strategy for molecular target-based chemoprevention and cytoprotection that utilizes anti-inflammatory and antioxidant phytochemicals (**Figure 3**). Some examples of representative chemopreventive phytochemicals that modulate NF- κ B and/or Nrf2 signaling will be presented.

Acknowledgments

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(IS2-11)

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Terpestacin, A New Chemical Code of Angiogenesis

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Small molecules have been successfully utilized as molecular probes to decipher molecular and cellular functions of their binding proteins in a given biological phenotype of interest. In other words, small molecules are chemical codes to explore the mystery of biology (1, 2). The small molecule and its receptor information further facilitate structure based better drug design and development. Our mission is to apply this powerful potential of small molecules to address the complicated and multi-components involved biological system such as angiogenesis (2). We performed a large scale screen of our synthetic and natural products library to identify small molecules that could inhibit the angiogenic response to pro-angiogenic stimuli, such as hypoxia, in endothelial cells. We identified terpestacin as a small molecule with unique bicyclic sesterterpene structure capable of inhibiting the angiogenic response at concentrations below the toxic threshold (3). Terpestacin strongly inhibits the functional response to hypoxia of human umbilical vein endothelial cells *in vitro*, and angiogenesis within the embryonic chick chorioallantoic membrane (CAM) *in vivo*. In addition to this anti-angiogenic activity, terpestacin has previously been reported to inhibit syncytium formation during HIV infection and has been chemically synthesized (4, 5). However, neither the molecular target of this compound nor the cellular mechanism of its anti-angiogenic activity has been identified.

Herein, we identified the binding protein of terpestacin, and clarified the cellular mechanism underlying its effects on

angiogenesis. We find that terpestacin specifically binds to the 13.4 kDa subunit (UQCRB in human, Accession NM_006294; QCR7 in yeast; Sub 6 in bovine or chicken) of Complex III in the mitochondrial respiratory chain (6). The biological activity of terpestacin correlates significantly with the response to UQCRB knock-down in mammalian cells. The discovery that a small molecule targeting UQCRB in mitochondrial Complex III can prevent the angiogenic response *in vivo* and *in vitro* without inducing cell death implies that UQCRB plays a key role in the cellular oxygen sensing and transduction system. This study provides new insight into the oxygen sensing role of UQCRB in mitochondrial Complex III and a small molecule targeting that system provides a powerful tool for regulating tumor angiogenesis (7).

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IS3

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(IS3-1)

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Pesticide Residue Chemistry and its Contributions to Understanding the Environmental Fate of Pesticides

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The principles of modern pesticide residue chemistry were well articulated by Francis Gunther and Roger Blinn and others in the 1950s. These early authors pointed out the advantages of systematizing and standardizing analytical methods for pesticides so that they could be widely practiced and the results could be reproduced from one laboratory to the next. The availability of improved methods has led to a much more complete understanding of pesticide behaviour and fate in foods and the environment. In the U.S., and increasingly worldwide, expertise in pesticide residue chemistry has emerged in federal (EPA, USDA, FDA, others), state, university and industry laboratories. Using methods based upon gas chromatography (GC) and high performance liquid (HPLC) coupled increasingly with mass spectrometry (MS) as the detection tool of choice, residues can be measured at ppb levels and below, in a variety of food and environmental matrices. Development of efficient extraction and cleanup methods, screening techniques based upon techniques such as ELISA, and automated lab and field instrumentation has also contributed to the tools available for use in modern pesticide residue analysis



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As a result, great strides have been made in improving food and worker safety, particularly in the areas of removing and replacing some of the more toxic and persistent chemicals with lower rate, less residual chemicals and biorationals, accompanied by better education and enforcement of regulations. While pesticide residues in foods are still of concern, food monitoring programs routinely show less than 1% violation of tolerances, and many of these are technical violations not posing a significant hazard to consumers. This does not mean that there are not still very real food safety concerns, from naturally occurring toxicants such as aflatoxins, adulterants such as melamine, reaction products such as acrylamide, and pathogenic microorganisms, but the frequency and severity linked to pesticide residues has decreased significantly in most parts of the world. Worker safety records have, at least in the industrial world, improved, again with better residue monitoring programs coupled with safer chemicals and better application technology.

In the area of understanding environmental behaviour and fate that there is still much to be accomplished, although the situation has improved in these areas too, in large part as a direct result of monitoring programs that surfaced unacceptable persistence and exposure behaviours, leading to phaseout of some of the legacy pesticides of the 1940s, 50s, 60s, and 70s.

Much of the work of the author and his colleagues at the University of California, Davis and the University of Nevada, Reno, was on residues in air and water, particularly in non-target areas, i.e. removed from agricultural fields and other use areas. Fumigants like methyl bromide, chloropicrin, 1,3-D and MITC are quite volatile, and following their residue movement in the air proved to be challenging. By a combination of method improvements for trapping and releasing airborne residues for analysis, and the use of better computer-based modeling techniques, knowledge of fumigant behaviour has improved to the point where control measures can be implemented to minimize exposures to people downwind.

But even semivolatile pesticides, which include the majority of the chemicals in use today, can volatilize or drift and be carried to sensitive areas downwind, where they can contact fish, other wildlife, and species important to ecosystem health and well being. In one study, the volatilization of organophosphorus insecticides like parathion and chlorpyrifos resulted in unacceptable exposures to raptor species, like red-tailed hawks. In another study, those airborne residues were shown to move many kilometers from the areas where the pesticides are used, contaminating the air, deposition rain and snow, forest canopies and streams and lakes. Even though the levels are quite low, it is hard to prove that they are toxicologically insignificant given the lack of critical dose-response data for organisms like tadpoles, frogs, fish, and other wildlife and for organisms that are critical to the food chain for these indicator species. Coupling good analytical chemistry with biologically based assays will help considerably in pinpointing significant exposure situations that require mitigation.

There are many challenges remaining in the field of pesticide residue chemistry that will continue to require the attention of analytical chemists. New chemistries are emerging, such as the avermectins and other biobased, biorational chemicals patterned after complex natural products. Analyzing for the parent chemicals, and potentially multiple breakdown products will require analytical ingenuity and careful attention to premarket screening of these new products. The



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development of more sensitive bioassays, and knowledge of unintended side effects will challenge residue chemistry as well, as in the case of following the fate of environmental endocrine disruptors associated with some pesticides as well as non-pesticide ingredients of packaging materials and other familiar articles.

Better training, international cooperation, and accelerated research and development activities are in constant need. The *Journal of Agricultural and Food Chemistry* has been an important medium for reporting new analytical methods and new findings regarding residues in food and the environment. Many of these studies are conducted by scientists (faculty, postdoctoral fellows, and students) in universities with strong programs in agricultural and environmental sciences, and those in regulatory agencies. These programs are in jeopardy in days of economic downturn such as we have experienced the past 2 or 3 years. It is important that Societies such as KSABC call attention to the need for strong R & D efforts in pesticide residue chemistry, to avoid unintended consequences of pesticide use and maintain public confidence in a system of pest control that has served us well in safeguarding the food supply from pest and disease problems.

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(IS3-2)

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Inorganic Nanobiofactory of *Shewanella* Strains

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Dissimilatory metal-reducing bacteria are able to couple oxidation of hydrogen or organic compounds to the reduction of Fe^{3+} and gain energy for growth. These bacteria include species from several genera, including *Geobacter*, *Desulfuromonas*, *Pelobacter*, *Shewanella*, *Ferrimonas*, *Geovibrio*, *Geothrix*, and *Bacillus*. The use of Fe^{3+} and other metals by certain microbial groups as terminal electron acceptors for anaerobic respiration is of particular relevance to bioremediation of heavy metals and radionuclides. Dissimilatory metal-reducers and other microorganisms can reduce mineral-associated iron to produce reactive sites within the minerals or directly reduce contaminants, such as arsenic and selenium.

Arsenic is a trace element in terms of its abundance in nature. Nevertheless, arsenic has a common presence within the earth's crust. Among arsenic compounds, arsenate [As(V)] and arsenite [As(III)] are the most predominant species found in the environment. Arsenate was recognized as an anaerobic terminal electron acceptor capable of supporting the respiratory growth of diverse bacteria. Currently, two bacterial systems that specifically reduce As(V) are known, namely, a respiratory system (encoded by the *arr* genes) and a detoxification system (encoded by the *ars* genes). Apart from respiratory arsenate reduction, arsenate can also be reduced by various bacteria using a soluble arsenate reductase which is part of an arsenic resistance system (*ars*). Selenium is found in fossil fuels, shales, alkaline soils and as a constituent in over 40 minerals. Selenate [Se(VI), SeO_4^{2-}] and selenite [Se(IV), SeO_3^{2-}] are the predominant species in aqueous environments, and occur

as soluble oxyanions. The transformation of selenium in nature occurs primarily by biotic processes. It has been shown that phylogenetically diverse bacteria from genera *Thauera*, *Sulfurospirillum*, *Bacillus*, *Ralstonia*, *Desulfotomaculum*, *Desulfovibrio*, and *Shewanella* are able to respire selenium as an electron acceptor, thus to reduce selenate or selenite to elemental selenium. The discovery of microbial arsenate and selenium reduction for anaerobic respiration has implications across several disciplines of environmental importance, including microbiology, biochemistry, toxicology, and geochemistry. Furthermore, diverse inorganic minerals such as metallic chalcogenide, etc. have been known to be synthesized by microorganisms.

Shewanella sp. HN-41 was used to precipitate As(V) by making sulfide mineral. In addition to examination of the formation of arsenic sulfide through reduction of As(V) and $S_2O_3^{2-}$ by *Shewanella* sp. HN-41, the fibrous type of arsenic sulfide was characterized in terms of structural features during the formation by X-ray absorption spectroscopy (XAS), and electrical or optoelectronic properties for perspective purpose of the biogenic arsenic sulfide nanowires that have unique physical and chemical properties as well as morphologies that are not produced by abiotic processes. We report the production of an extensive extracellular network of filamentous, arsenic-sulfide (As-S) nanotubes (20–100 nm in diameter by $\sim 30\mu\text{m}$ in length) by the dissimilatory metal-reducing bacterium *Shewanella* sp. HN-41. The As-S nanotubes, formed via the reduction of As(V) and $S_2O_3^{2-}$, were initially amorphous As_2S_3 but evolved with increasing incubation time toward polycrystalline phases of the chalcogenide minerals realgar (AsS) and duranusite (As_4S). Upon maturation, the As-S nanotubes behaved as metals and semiconductors in terms of their electrical and photoconductive properties, respectively. The As-S nanotubes produced by *Shewanella* may provide useful materials for novel nano- and opto-electronic devices.

To determine if the ability that producing novel, photoactive, biogenic As-S nanotubes was unique to *Shewanella* sp. HN-41, ten different *Shewanella* strains, including *Shewanella* sp. HN-41, *Shewanella* sp. PV-4, *S. alga* BrY, *S. amazonensis* SB2B, *S. denitrificans* OS217, *S. oneidensis* MR-1, *S. putrefaciens* CN-32, *S. putrefaciens* IR-1, *S. putrefaciens* SP200 and *S. putrefaciens* W3-6-1, were examined for production of As-S nanotubes under standardized conditions. Only 3 of the 10 strains examined formed as As-S nanotubes like strain HN-41. While *Shewanella* sp. HN-41 and *S. putrefaciens* CN-32 rapidly reduced As(V) to As(III) coupled to consumption of lactate in incubations with thiosulfate and formed As-S precipitates in 7 days, strains *S. alga* BrY and *S. oneidensis* MR-1 reduced As(V) much slower and formed yellow-colored As-S after 30 days. Electron microscopy, EDX, and EXAFS analyses showed that the morphological and chemical properties of As-S formed by strains *S. putrefaciens* CN-32, *S. alga* BrY, and *S. oneidensis* MR-1 were similar to those previously determined for *Shewanella* sp. HN-41 As-S nanotubes. Results of these studies indicate that formation of As-S nanotubes was not unique and was closely related to bacterial growth and rate of As(V) and thiosulfate reduction. However, since the detailed mechanisms of formation of As-S is still unclear, in addition to the required arsenate reducing reactions, other abiotic and biotic factors influencing the formation of photoactive As-S nanotubes by *Shewanella* sp. strains should be investigated.

Semiconducting nanostructures have become intensively investigated by both experimentalists and theoreticians because of their unique size dependent electronic and optical properties. One group of the most investigated semiconductors is chalcogenide compounds (MX , $M=As, Cd, Zn$; $X = S, Se, Te$) because their band gap can be easily fine-tuned from zero

(like the semi-metal HgTe) to large band gap (e.g. ZnS ($E_g = 3.8$ eV)). In addition to composition, the properties of chalcogenide can be further “tuned” by controlling the dimension of materials in nanoscale. It has been reported that diverse semiconducting inorganic hybrid nanotubes were synthesized via ion exchange reaction to enhance the functionality and applicability. It is also known that electrical conduction is closely associated with the structures such as the grain size, defects and impurities. Especially, the conduction of semiconductors is mainly governed by the grain boundary scattering where amorphous/nanocrystalline materials have much lower carrier concentration and mobility than single or polycrystalline materials with larger grains. As the grain size increased, the contribution of grain resistance would be reduced, resulting in smaller thermal activation energy, E_A . This suggested that the biological photoactive As-S nanotubes can be transformed into tunable structure with varying composition and ideal electrical property via kinetically controlled solution-phase ion exchange reaction and crystallization. Compare to physical-chemical methods, bio-inspired or biomimic routes allow synthesizing nanoengineered materials with “greener” precursors under mild ambient conditions. Thus, various biological activities of dissimilatory metal-reducing bacteria, including formation of the selenium nanoparticles from Se(IV) reduction and the photoactive As-S nanotubes, were applied for synthesis of the versatile ternary and quaternary chalcogenide (i.e. As-S-Se, As-Cd-S and As-Cd-S-Se) nanotubes with aid of biological and/or abiological activities. Se and/or Cd were incorporated either by biogenic deposition or ion exchange onto As-S nanotubes to control their electrical properties, which may open-up the possibility to integrate these nanotubes in nanoelectronics, optoelectronics, and solar cells. The mineralogical, crystal structure, morphology and electrical properties of nanotubes were characterized, thereby understanding the influence of the ratio and different elemental composition.

Shewanella sp. HN-41 also showed reduction of selenite [Se(IV)] to elemental selenium with sphere-like structure. Elemental selenium (Se) is a member of the semiconductor family of elements and possesses band gaps of 1.85 eV (indirect) and 1.95 eV (direct). Se has several important chemical and physical properties, including: a relatively low melting point (~490 K), photovoltaic properties, high piezoelectricity, high photoconductivity ($\sim 8 \times 10^4$ S cm^{-1}), superconductivity, thermoelectric properties, linear and nonlinear optical properties, and high reactivities toward a variety of chemicals that are useful for the conversion of selenium into other functional materials. These diverse properties allow for Se to be utilized in the production of solar cells, rectifiers, photographic exposure meters, xerography, pigments, glasses, and steel. It has been widely accepted that one-dimensional (1D) Se nanostructures (nanorods, nanowires, nanoribbons, and nanotubes) may be used as important building blocks for fabricating nanoscale electronic, optical, optoelectronic, electrochemical, and electromechanical devices. This is in part due to their high aspect ratios and unique size-dependent properties. There are several biologically-mediated methods for the synthesis of 1D Se nanostructures. The resulting reduced selenium, which is in a suspended state in the solution, likely precipitates from the solution, forming an amorphous Se phase that spontaneously forms spherical nanostructures with a uniform diameter and smooth surface. The formation of these α -Se nanoparticles or nanospheres have been shown to be dependent on the concentration of Se, temperature, and reaction time. Among these experimental parameters, the concentration of precursor and reductant are likely key factors controlling the dimension of the amorphous Se structures. This microbial reduction of soluble toxic Se(IV) by strain HN-41 may



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prove to be a useful strategy to recover elemental Se from the environment, reducing contamination and producing valuable Se nanomaterials. We describe the use of the iron-respiring facultative anaerobic bacterium *Shewanella* sp. HN-41, which forms amorphous Se nanospheres, to produce 1D Se nanostructures, nanowires, and nanoribbons at ambient conditions in 80% dimethyl sulfoxide solution. Amorphous selenium nanospheres, originally produced by *Shewanella* sp. strain HN-41 under the anaerobic conditions, can be rapidly transformed into extensive, long and thin, polycrystalline Se nanowires and nanoribbons ($>100\ \mu\text{m} \times 57\ \text{nm}$) in 80% DMSO with bacterial pellets at physiological temperature. Scanning and transmission electron microscopic analyses indicated that the Se nanowires and nanospheres were crystalline structures indexed into the hexagonal plane of Se. The structures possessed an unusually high crystalline peak (100), suggesting a preferential [001] growth direction. Electron micrographic analyses and incubation studies suggested that the cell membrane of *Shewanella* sp. strain HN-41 likely plays an important role in the formation of amorphous Se nanospheres from soluble Se (IV) and the formation of long and thin *h*-Se nanowires and nanoribbons. The formation of zero- and one-dimensional *h*-Se nanostructures by this bacterium may provide a facile strategy to recover soluble Se (IV) from the environment and generate new materials that will be useful for advanced nanotechnologies. More importantly, because the crystallinity and shape of the Se nanostructures produced can be controlled by using different ratios of DMSO with water in the presence of *Shewanella* sp. HN-41, our results suggest that this process will likely be useful for the production of novel semiconductors and other shape-dependent functional materials.

(IS3-3)

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Metabolomic Responses of Xenobiotic-Degrading Bacterium

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System-wide evaluations of numerous organisms are now becoming more and more common with rapid accumulation of genomic information. As a part of “omics” tools, metabolomics is frequently used to speculate biological response against external stresses. Xenobiotic toxicants are one of the most commonly encountered chemical stressors for animals, plants, and microorganisms. Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are ubiquitous contaminants which originated from various industrial processes. Because of their toxicological importance, these persistent organic pollutants (POPs) are regulated in many countries. Several sophisticated remediation approaches are applied in contaminated area or under development. Bacteria or its gene products are considered to be possible candidates for bioremediation of these pollutants. However, it is generally known that the practical applications are difficult and frequently failed to achieve the expected results. Many obstacles, including scarce information of system-wide evaluation of target organism are observed. Although intensive researches have been attributed to “omics” of microorganism, systematic evaluation of microorganisms under chemical stress are highly limited.

This study is a part of integrated omics (proteomics and metabolomics) researches of PAH-degrading bacterium from tropical area. *Sinorhizobium* sp. C4 was isolated from PAH-contaminated area in Hawaii, USA. From the screening, it was confirmed that strain C4 is highly efficient PAH degrader. Phenanthrene (PHE) was one of the most preferred substrates for this strain. Metabolomic responses of strain C4 are given in this presentation with details of PHE metabolism. Strain C4 was cultivated with several different carbon sources (nutrient broth, glucose, pyruvate, and PHE). Both culture media and cells were harvested to determine the metabolism of phenanthrene and metabolomes of bacterial cells.

Comprehensive profiling of PHE metabolites were performed with several synthetic standards. Metabolites from primary metabolism ranged from water-soluble metabolites to lipids (fatty acids and polyhydroxyalkanoates). Quantitative evaluations of metabolites were performed with GC-MS with or without derivatisation.

According to the instrumental analysis, strain C4 could metabolize PHE through complex pathways (Figure 1). For example, several PHE diol or dihydrodiols were observed, where 3,4-dihydroxylation were the most common starting point of PHE metabolism. Transformation of PHE-3,4-diol was occurred in meta-cleavage pathway. Consecutive metabolism follows a common metabolic pathway of PHE by other bacteria, in which phthalate is produced as a final metabolite. Relative concentrations of PHE metabolites indicate that compounds with carboxylic acid were the most abundant metabolites. Kinetic analysis of 17 metabolites suggests that phthalate was further degraded into protocatechuate, and eventually incorporated into primary metabolism. From the literatures and current study, several metabolites, including pyruvate, glyoxalate, carbon dioxide, and muconates were expected to be primary building blocks of other metabolites during PHE-metabolism. Several metabolites (e.g., *o*-hydroxynaphthotes, naphthalene-1,2-dicarboxylates, phthalates) have shown weak to strong growth inhibitory effects in replacement cultures.

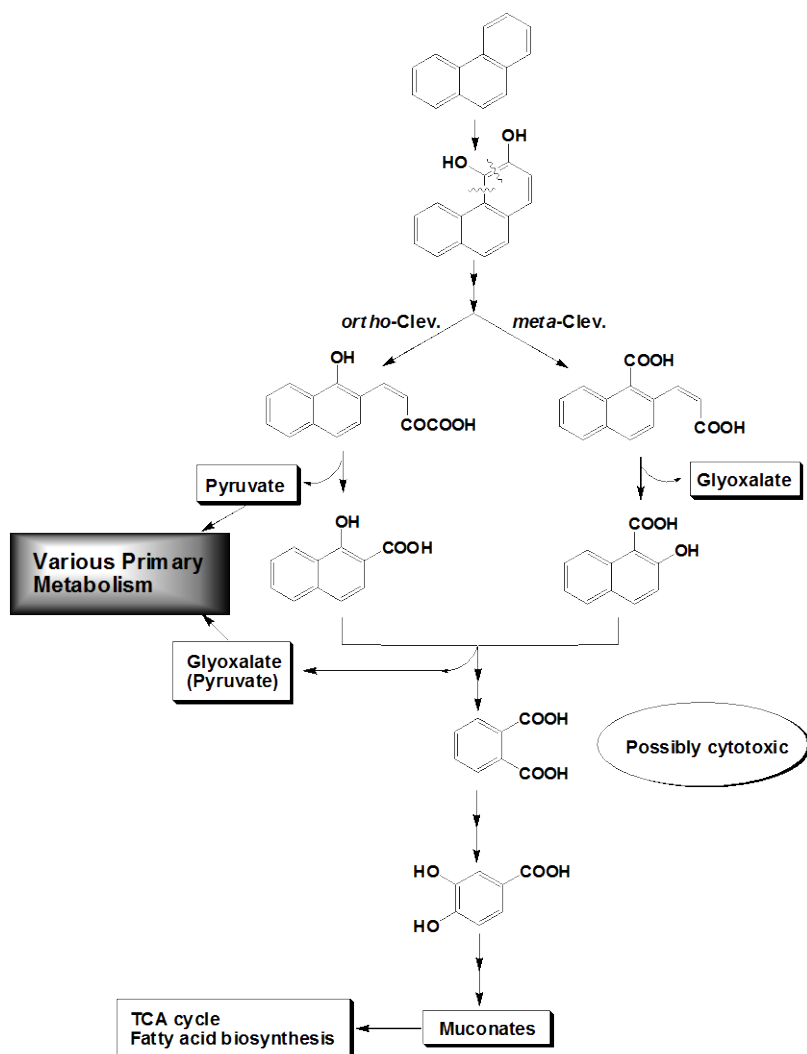


Figure 1. Metabolism of phenanthrene by *Sinorhizobium* sp. C4 and connection to primary metabolism

Biomass of PHE-supplementation increases very slowly than other carbon sources. Metabolomics with both hydrophilic- and hydrophobic primary metabolites have shown interesting difference of patterns, depending on carbon sources and time. After deconvolution and peak alignment, approximately 1200 components were observed in metabolome data, which was reduced to 312 metabolites after careful re-examination. Diversities of metabolites were one of the most noticeable differences between carbon sources. For example, approximately 290 metabolites were observed in nutrient broth while 140-230 metabolites were detected in other carbon sources. Nutrient broth (NB) is complex mixtures of natural metabolites and minerals. Higher complexities of metabolite profile of NB-supplemented cells indicates that the cellular processes are adjusted to manipulate more diverse chemical sets while the cells with single carbon source (PHE, glucose, and pyruvate) are optimized to more simplified metabolic pathways. Principal component analysis (PCA) of metabolomes gives clear discrimination between carbon sources (Figure 2).

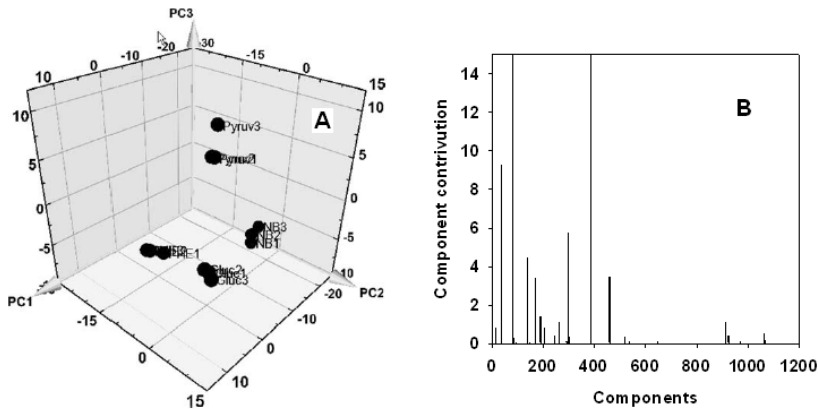


Figure 2. Principal component analysis of water soluble metabolome of *Sinorhizobium* sp. C4, grown with different carbon sources. Loading plot (A) and case contribution of each metabolites in principal component 1 (PC1, B). Abbreviations: Glu, glucose; PHE, phenanthrene; NB, nutrient broth; Pyruv, pyruvate.

Careful investigation of each principal components, many metabolites were selected as possible markers to discriminate the carbon sources. Those metabolites include branched amino acids, intermediates in TCA cycles, sugars, sugar alcohols, nicotines, and lipids. For example, high amount of polyhydroxyalkanoates (PHA) were observed in glucose-supplemented culture, while the levels were 5-10 fold less in other carbon sources. The biomass production with glucose and NB was almost same. The difference indicates that PHA accumulation may have higher correlation with glucose than other primary metabolites.

In comparison with other carbon sources, several characteristic differences were found in strain C4, supplemented with PHE. For example, high amount of methionine was observed while the concentration of cysteine was reduced in PHE.



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It was not clear whether the reduction of cysteine pool is the consequence of high catabolic rate. It has to be mentioned that serine, a precursor of cysteine was accumulated in PHE. Serine is versatile metabolite in primary metabolism, where pyruvate can be produced from serine. Rapid and continuous accumulation of lysine is another characteristic pattern of PHE-treated cultures. Because lysine is an important precursor of peptidoglycans in bacterial cell wall, changes of cell-wall morphology can be expected during PHE metabolism. Modifications of cell membrane (metabolite profiles, abundance) are one of the most characteristic responses of bacterial cells, treated with xenobiotics. Analysis of lipid profile has shown a shift to longer chain fatty acid during PHE metabolism. Nicotinic acid and quinolinate levels were generally increased with PHE. Because many catabolic steps of PHE or related xenobiotics require NAD(P)H as cofactors of oxidative enzymes, accumulation of these cofactors indicate the increased demand and supply of selected metabolites. Accumulation of some organic acids, especially pyruvate was expected during PHE catabolism. However, no significant changes were observed between carbon sources, even in pyruvate-supplemented cultures. This observation may indicate a rapid turnover of pyruvate to other metabolite. Oxalate, a key intermediate of TCA cycle was accumulated in PHE-culture. The facts may suggest that large proportion of pyruvate from PHE metabolism may be introduced to primary metabolism through TCA cycle. Among several sugars and phosphates, rapid accumulation of ribose and 5-phosphate was observed during PHE metabolism. Both metabolites are important precursors of nucleotides. It is commonly known that bacterial cells in chemical stress or starvation increase nucleic acid contents while the amount of proteins decrease.

In overall, the metabolomic studies of PHE-degrading bacterium give interesting adjustment of cellular metabolism to toxic xenobiotics. The characteristic points include a) a simplified metabolic profile, b) TCA cycle as an entry to primary metabolism, c) Increased demand of some cofactors, especially those for redox-enzyme and d) extensive changes of storage lipid and fatty acids. Numerous additional metabolites also give interesting difference between carbon sources. Integrated approaches, however, are required to clarify the relationship between metabolites, genes, or proteins.

(IS3-4)

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Formation of Dioxin from Chloride Containing Substances Combusted in an Incinerator under Various Conditions

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Dioxins—polychlorinated dibenzo dioxins (PCDDs), polychlorinated dibenzo furans (PCDFs), and polychlorinated biphenyls (PCBs) — are a class of chemical contaminants that are formed during combustion processes such as waste incineration, forest fires, and backyard trash burning, as well as during some industrial processes including paper pulp bleaching and herbicide manufacturing. There has been great concern about dioxin contamination in the environment because the adverse effects of these chemicals toward human health have been known many years. Humans are primarily exposed to dioxins by eating food contaminated by these chemicals. Dioxins are known to form during the combustion of industrial and domestic wastes and escape into environment *via* exhaust gases from incinerators. They accumulate in plant foodstuffs, which are subsequently consumed by humans and animals. Possible dioxin contaminations have received much attention recently not only by environmental scientists but also by the public.

People who have been exposed to high levels of dioxin have developed chloracne, a skin disease marked by severe acne-like pimples. Animal studies indicate that dioxins are potent carcinogens. Consequently, there is a pressing need to investigate the formation mechanisms or reaction pathways of these chlorinated chemicals to reduce their environmental contamination.

A well-controlled small-scale incinerator has been used for the experiments in the core references of this review. These articles report on the results of investigations of dioxin formation from the combustion of various waste-simulated samples,

including different kinds of paper, various kinds of wood, fallen leaves, food samples, polyethylene (PE), polystyrene (PS), polyvinyl chloride (PVC), polyvinylidene chloride, polyethylene terephthalate (PET), and various kinds of plastic products. These samples were also combusted with inorganic chlorides (NaCl, KCl, CuCl₂, MgCl₂, MnCl₂, FeCl₂, CoCl₂, fly ash, and seawater) or organic chlorides (PVC, chlordane, and pentachlorophenol) in order to investigate the role of chlorine content and different metals in the dioxin formation. Some samples, such as newspapers, were combusted after they were impregnated with NaCl or PVC, as well as when they were co-combusted with chlorides. The role of combustion conditions, including chamber temperatures, O₂ concentrations, and CO concentrations, in dioxin formation were also investigated. Dioxins (PCDDs, PCDFs, and coplanar-PCBs) formed in the exhaust gases from a well-controlled small-scale incinerator, where experimental waste samples were combusted, were analyzed by gas chromatography/mass spectrometry.

Formation of total PCDFs was much higher than that of PCDDs in all samples. The total PCDFs composed 70–90% of the total dioxin formed. Amount of total PCDFs formed ranged from 0.78 ng/g (newspaper) to 8,490 ng/g (PVC combusted in high CO concentration). Amount of total PCDDs formed ranged from 0.02 ng/g (newspaper) to 430 ng/g (PVC). Coplanar PCBs were found at the lowest levels among the dioxins formed. Their formation levels ranged from 0 (newspaper) to 77.6 ng/g (PVC). It is obvious that the samples with either inorganic or organic chlorides produced much more dioxin than the sample without chlorides upon combustion under similar conditions. It is not clear that how inorganic and organic chloride contribute differently to the dioxin formation. Among the metals examined, copper seems to have higher activity toward dioxin formation than other metals. It acted not only as a catalyst but also as a transmitter of heterogeneous chlorine.

The TEQ values, generally, correlated with the amount of chlorine content in combustion samples and the amount of dioxin formed in exhaust gases from an incinerator. When the same sample was combusted at different temperatures, however, the sample combusted at low temperature yielded a higher TEQ value than the sample combusted at high temperature did. The samples that did not contain chlorine or were not combusted with chlorides exhibited low levels of TEQ values. In contrast, samples with high chlorine content, such as PVC (51.3%), gave a high level of TEQ values.

Combustion temperatures may play an important role in dioxin formation in exhaust gases from incineration of waste materials. However, no significant relationship between dioxin formation and chamber temperatures was reported in the core articles. On the other hand, it is obvious that dioxin formation occurred at temperatures above 450 °C and reduced significantly at temperatures above 850 °C. The reaction occurring in an incinerator is extremely complex and there are many factors in addition to combustion temperature influencing dioxin formation. Even though it is possible to hypothesize reasonable formation mechanisms of dioxins formed in exhaust gases according to the results obtained from experiments in classical chemistry, the reactions involved in an incinerator are extremely complex and heterogeneous. More detailed investigation of the many individual factors influencing dioxin formation is needed in order to find ways to reduce their formation in individual and municipal incinerators.

(IS3-5)

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Associations of low dose persistent organic pollutants with type 2 diabetes and related metabolic dysfunction

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Persistent Organic Pollutants (POPs) are a group of chemicals with common properties such as persistence, lipophilicity, and biomagnification in the food chain. POPs include a variety of man-made chemicals, including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and several organochlorines used as pesticides (OC pesticides). POPs are linked to cancer, neurobehavioral disorders, impaired immunity, endocrine problems, and reproductive disorders in various species. However, most epidemiological findings to date have focused on people with high exposure to POPs in occupational or accidental settings, while people without such high exposure have been much less studied; with few exceptions, this approach has uncovered only modest associations with various health outcomes.

There is emerging evidence that the background exposure to POPs may be critically involved in the pathogenesis of type 2 diabetes. A current paradigm for type 2 diabetes rests on a sequence of events. Obesity occurs due to energy imbalance between energy input and output, insulin resistance is due to obesity, and exhaustion of pancreatic beta cells is due to overproduction of insulin to compensate for insulin resistance, ultimately progressing to type 2 diabetes. However, it has been recently reported that serum concentrations of POPs were strongly associated with the prevalence of type 2 diabetes in the U.S. general population. After adjusting for known risk factors for diabetes, compared to people with very low concentrations of POPs, the prevalence of type 2 diabetes among those with detectable concentrations of POPs



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increased by 15 to 40 fold. Also, the associations were more strongly observed among obese than nonobese persons. Although POPs with dioxin property have been widely studied as the most toxic chemical among POPs, OC pesticides and PCB congeners, not dioxins, were strongly associated with type 2 diabetes. In particular, it was striking that when POPs concentrations were very low, prevalent type 2 diabetes was rare even among the obese (persons with body mass index (BMI) ≥ 30 kg/m²). Serum concentrations of these POPs were also associated with insulin resistance and adverse lipid profiles, metabolic dysfunctions commonly observed before developing overt type 2 diabetes. Similar associations of some POPs with type 2 diabetes have been reported in other cross-sectional studies.

We recently confirmed these cross-sectional associations through a prospective study. Some OC pesticides and PCBs predicted insulin resistance, dyslipidemia, and type 2 diabetes. Importantly, we observed that some POPs predicted even future obesity. In fact, some of the numerous environmental pollutants that cause perturbations in endogenous hormonal regulation involved in weight homeostasis have been recently proposed as possible risk factors for obesity under the heading of “environmental obesogens” although most studies have performed among animals, focusing on effects of fetal exposure to chemicals.

Previous prospective studies of selected POPs performed in occupational or accidental high exposure settings reported inconsistent results, particularly for 2,3,7,8-TCDD. Furthermore, the decreasing trend of organochlorine POPs during recent decades is inconsistent with the current trend towards an increased prevalence type 2 diabetes. However, adverse metabolic effects due to POPs could tend to occur more at concentrations due to low, but persistent exposures rather than high exposures, for effects in higher dose (producing an inverted U-shaped graphic appearance), are proposed as possible biological responses to endocrine disruptors. In this scenario, current background POPs serum concentrations could be even more biologically active than were the higher serum concentrations typically experienced before many POPs were banned.

The past two decades have observed an explosive worldwide increase in the number of people diagnosed with type 2 diabetes. Obesity is regarded as a primary cause of the current epidemic of type 2 diabetes. However, some POPs at low doses similar to current exposure levels may increase the risk of diabetes and obesity-related metabolic dysfunction, possibly through endocrine disruption, suggesting that POPs may play a role in the current epidemic of diabetes. example, if the adverse effects were caused by endocrine disruption. In fact, strong biological effects in low dose, but weak or no

(IS3-6)

1. Speaker

Su-Il Kang

2. Affiliation

Associate Research Professor, International Environmental Research Center (IERC), Gwangju Institute of Science and Technology (GIST)

3. Professional Activities

Visiting research associate, Michigan State University (2000 – 2004)

Determination of Pharmaceuticals and Personal Care Products (PPCPs) from Surface Water and Drinking Water in Ho Chi Minh City, Vietnam

Pornpimon Buachaoko¹⁾, Seo-Young Kang²⁾, Sungyun Lee³⁾, Kannika Sajjaphan¹⁾, Le Thi Hong Tran⁴⁾, Jaewon Cho³⁾, and
Su-Il Kang²⁾

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Korea*

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Vietnam*

The International Environmental Research Center has been operating full-pledged United Nations University (UNU) & Gwangju Institute of Science and Technology (GIST) Joint Programme on Science and Technology for Sustainability toward the establishment of the UNU research and training programme or center to be known as the “UNU Institute on Science and Technology for Sustainability (UNU-ISTS)” since 2004.

Science and Technology for Sustainability (STS) is a metadiscipline which integrates economical, social and environmental processes in a global context (Mihelcic et al., 2003). The key concepts for research in STS are problem-driven and place-based research. The project entitled as “determination of pharmaceutical and personal care products from surface and drinking water in Ho Chi Minh City (HCMC), Vietnam” is a part of efforts to implement the concepts of STS.

The presence of pharmaceuticals and personal care products (PPCPs) in the aquatic environment has become a subject

of emerging concern in the past decades. They have been consistently detected in wastewater and surface waters at ng/L to µg/L levels throughout the world (Kolpin *et al.*, 2002; Kim *et al.*, 2007; References in Kang *et al.*, 2009) and also have been found in drinking water (Benotti *et al.*, 2009). Their occurrence and levels of contamination are of recent environmental concerns due to the possibility of bioaccumulation and toxicological effects of PPCPs in living tissues and organisms (References in Kang *et al.*, 2009). The application of advanced measurement technologies to environmental analysis has provided the opportunity to quantify many PPCPs down to ng/L levels.

Ho Chi Minh City (HCMC) is located in the south of Vietnam and is facing many environmental issues emerged from rapid urbanization and economical growth. Its population was more than 8 million in October 2008. Three main water resources such as the Dong Nai river, the Sai Gon river and groundwater have been used for water supply in HCMC (Figure 1).

The aim of this study is to develop analysis method for 20 target PPCPs using triple quadrupole tandem mass spectrometry (LC/MS/MS) and determine the concentration of PPCPs in the water samples collected from canals, hospital wastewater, Drinking Water Treatment Plants (DWTPs) and Wastewater Treatment Plants (WWTPs) in HCMC. It would provide useful information on the water quality of HCMC based on PPCPs levels and make it possible to transfer technology to researchers in HCMC.

Various compounds of pharmaceuticals and personal care products (PPCPs) selected for this study are shown in Table 1. All compounds were kindly gifted from Professor Jaewon Cho, School of Science and Engineering, GIST. Samples were collected in 1L Amber Boston Round glass bottles (VWR International LLC., West Chester, PA) and sample bottles were preserved with 1 g/L sodium azide to prevent microbial degradation and 50 mg/L ascorbic acid to quench any residual oxidant (e.g., chlorine, ozone, chloramine). The samples were packed in special sample boxes with ice packs, shipped to the laboratory, and stored in the dark at 4°C in the refrigerator until filtration. Solid phase extraction was done by the method in Southern Nevada Water Authority laboratory (Vanderford *et al.*, 2006). Analytes were extracted with hydrophiliclipophilic balance (HLB) glass cartridges from Waters Corporation (Milford, MA, USA). All extractions were performed on an Auto Trace automated SPE system (Caliper Life Science Inc., Hopkington, MA). Before extraction, samples were spiked with isotopically labeled surrogates solution and a normal standard solution. For LC/MS/MS, analyses were conducted using a Waters 2695 Separations Module (Waters, Milford, MA) coupled with a Micromass Quattro Micro triple-quadrupole tandem mass spectrometer (Waters) in electrospray ionization mode (ESI). Multiple reaction monitoring (MRM) was employed to identify and quantify the analytes.

To determine the concentration of target PPCPs in water samples, optimal separation and detection conditions with LC/MS/MS were determined (Table 1). Determining the concentration of PPCPs for the water samples from HCMC is under investigation. At the same time, we are trying to expand the number of target compounds including antibiotics and endocrine disrupting chemicals.

Table 1. Optimized parameters of selected PPCPs

Compound (Uses)	Cone voltage (v)	Collision energy (eV)	Parent ion (<i>m/z</i>)	Daughter ion (<i>m/z</i>)	Retention time (min)
<i>ESI positive</i>					
Acetaminophen (Analgesic)	28	17	152	109.9	3.98
Amlodipine (Ca ²⁺ channel blocker)	12	10	409	238	6.98
Atenolol (beta blocker)	35	23	267	145	2.25
Atenolol-d ₇	35	25	274	145	2.24
Caffeine (Stimulant)	35	20	195.	138	6.11
Carbamazepine(Anticonvulsant)	35	18	237	194	8.70
Carbamazepine-d ₁₀	35	18	247	204	8.74
Cefaclor (Antibiotic)	15	12	368	174	2.35
Clopidogrel (Antiplatelet agent)	25	20	322	184	12.05
Dilantin (Anticonvulsant)	35	13	253	182	8.68
Dilantin-d ₁₀	30	12	263	192	8.63
Glimepiride (Anticholesterol)	28	13	491	352	11.34
Glimpiride-d ₅	32	23	494	364	11.29
Sulfamethoxazole (Antibiotic)	30	18	254	156	7.83
Sulfamethoxazole-d ₄	25	15	258	160	7.80
TCEP (Fire retardant)	30	16	285	161	9.12
<i>ESI negative</i>					
<i>N</i> -Acetyl-sulfamethoxazole	30	13	294	198	7.71
<i>N</i> -Acetylsulfamethoxazole-d ₄	35	14	298	202	7.65
<i>O</i> -Desmethyl-naproxen	15	12	215	171	8.10

Diclofenac (Analgesic)	15	10	294	249	11.27
Diclofenac-d ₄	15	12	298	254	11.23
Florfenicol (Antibiotic)	11	25	356	336	7.84
Flofenicol-d ₃	25	11	359	339	7.69
2-Hydroxyl-ibuprofen	17	7	221	177	8.17
Ibuprofen (Analgesic)	15	8	205	161	11.63
Ibuprofen-d ₃	15	8	208	164	11.63
Ibuprofen carboxylic acid	12	5	235	191	8.04
Iopromide (X-ray contrast)	20	14	790	127	2.13
Naproxen (Analgesic)	10	8	229	185	9.84
Naproxen-d ₃	10	6	233	189	9.83



Figure 1. Location of Thu Duc, Tan Hiep Water Treatment Plants and Hoa An and Hoa Phu Pumping Stations in Ho Chi Minh City, Vietnam

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IS4

Antibody

Recent trends in Antibody Engineering and Therapeutics

(IS4-1)

1. Speaker

James D. Marks

2. Affiliation

Professor, Anesthesia and Pharmaceutical Chemistry, UCSF

3. Professional Activities

Director, Medical-Surgical Intensive Care Unit, San Francisco General Hospital (1997-present)

Development of recombinant human antitoxin for the treatment of types A, B, and E botulism

Consuelo Garcia-Rodriguez¹, Jianlong Lou, Isin N. Geren¹, Fraser Conrad¹, Weihua Wen, W.¹, Terry J. Smith², Jennifer Brown², William H. Tepp³, Eric A. Johnson³, Leonard A. Smith², and James D. Marks, J.D.^{1,4}

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Botulism is caused by botulinum neurotoxin (BoNT) the most poisonous substance known. The crystal structure of BoNT shows three functional domains comprising a heavy and a light chain. The C-terminal portion of the heavy chain (H_C) is the binding domain which docks the toxin to sialoganglioside receptors and a protein receptor on presynaptic neurons, resulting in toxin endocytosis. The translocation domain (H_N), at the N-terminal portion of the heavy chain, mediates escape of the toxin light chain (L_C) from the endosome. Depending on serotype, the L_C cleaves one or more members of the SNARE complex of proteins, blocking acetylcholine release.

Human botulism is caused by BoNT serotypes A, B, E, and F and is characterized by flaccid paralysis which, if not fatal, requires prolonged hospitalization in an intensive care unit and mechanical ventilation. Besides causing naturally occurring botulism, BoNTs are also classified by the Centers for Disease Control and Prevention as one of the 6 highest-risk threat agents for bioterrorism. Both Iraq and the former Soviet Union produced BoNT for use as weapons and the Japanese cult Aum Shinrikyo attempted to use BoNT for bioterrorism.

As a result, there is an urgent need for rapid and very sensitive diagnostic assays that can detect BoNTs, as well as therapies that are safe, effective, and can be produced in large quantities. There are a number of assays under development, and many of these rely on high affinity polyclonal or monoclonal antibodies (mAbs). The current mainstay of treatment

for botulism is antibody based antitoxin. Thus mAbs are an important resource for the detection, diagnosis, and treatment of botulism. Such mAbs should ideally be of high affinity, for more sensitive BoNT detection and more potent BoNT treatment. For therapeutic use, mAbs should also be human in sequence. Finally, mAbs need to be able to bind to the different BoNT subtypes, variability of the BoNT protein sequence within each of the serotypes. A recent analysis of 174 toxin producing Clostridial strains indicates the presence of four BoNT/A, five BoNT/B subtypes, and five BoNT/E subtypes which differ from each other by 5% to 16% at the amino acid level. Such variability impacts antibody binding and neutralization..

Antibody products, such as equine antitoxin and human botulism immune globulin, are used to treat adult and infant botulism respectively. While human botulism immune globulin has been shown to be both safe and effective for treating infant botulism, scaling of this product for treatment of adult botulism is not feasible. Equine antitoxin is a polyclonal antibody produced from horses immunized with BoNT. When administered, there is a 9% incidence of acute or delayed hypersensitivity reactions, including serum sickness (3.7%) and anaphylactic shock (1.9%). An alternative to equine antitoxin is serotype specific monoclonal antibody (mAb) based antitoxin. Given the extraordinary toxicity of BoNTs, such antitoxin must be of high potency.

For the work described here, we sought to develop human mAb based antitoxin for the treatment of types A, B, and E botulism using antibody gene diversity libraries and display technologies. First, peripheral blood lymphocytes from botulinum-toxoid-immunized humans were used to create immune single chain Fv (scFv) antibody libraries displayed on the surface of yeast. Yeast displayed libraries were sorted for binding to BoNT/A, BoNT/B, and BoNT/E using fluorescent activated cell sorting (FACS). Panels of lead antibodies were generated to each of the three BoNT serotypes (BoNT/A: 14 scFv, average $K_D = 9.1$ nM; BoNT/B, 22 scFv, average $K_D = 8.5$ nM; and BoNT/E: 11 scFv, average $K_D = 8.2$ nM). Lead antibodies were characterized with respect to their ability to bind the different BoNT subtypes. Those binding multiple subtypes underwent affinity maturation by chain shuffling and yeast display. The affinity of chain shuffled scFv increased on average 16 fold, from an initial average $K_D = 4.6$ nM to a final $K_D = 0.29$ nM. Affinity increase a further 4.8 fold when the scFv were converted to IgG. The resulting IgG had an average $K_D = 60$ pM (range 460 pM to 1 pM).

The resulting IgG were studied with respect to epitope bound and their ability to neutralize BoNT in vivo. The toxin domain recognized by antibodies was determined using yeast displayed toxin domains. In vivo, no single mAb neutralized BoNT with a potency greater than 8000 mouse LD50s/mg. Combining mAbs led to synergistic increases in mAb potency, which was maximal when three mAbs binding non-overlapping epitopes were combined. For BoNT/A, BoNT/B, and BoNT/E, a combination of three mAbs could completely neutralize 40,000 mouse LD50s of BoNT with as little as 1 ug of combined mAbs. This potency translates to a human therapeutic dose of 1 mg of antibody. Further studies indicated that the mechanism for the synergistic toxin neutralization observed upon combining mAbs was rapid hepatic clearance of the immune complexes that was dependent on an intact Fc. We believe this mechanism of neutralization would be general for antibody combinations to any antigen in solution.

(IS4-2)

1. Speaker

Dane Wittrup

2. Affiliation

J.R. Mares Professor of Chemical Engineering and Bioengineering
Massachusetts Institute of Technology

3. Professional Activities

Professor, University of Illinois at Urbana-Champaign, Urbana, IL (1989.08 – 1999.12)
Amgen, Inc., Yeast Molecular Biology Group, Thousand Oaks, CA (1988.09-1989.08)

IgG-based multispecific anti-EGFR antibodies efficiently downregulate surface receptor levels

K. Dane Wittrup

*Dept. of Chemical Engineering & Biological Engineering,
Massachusetts Institute of Technology, USA.*

Due to its common dysregulation in epithelial-based cancers and extensive characterization of its role in tumor growth, epidermal growth factor receptor (EGFR) is a highly validated target for anti-cancer therapies. There has been particular interest in the development of monoclonal antibodies (mAbs) targeting EGFR, resulting in two approved mAb-based drugs and several others in clinical trials. It has been reported that treatment with combinations of non-competitive mAbs can induce receptor clustering, leading to synergistic receptor downregulation. We find the mechanism underlying downregulation to be consistent with recycling inhibition. No single engineered protein has been shown previously to robustly downregulate epidermal growth factor receptor (EGFR). A panel of fibronectin-based domains was engineered to bind with picomolar to nanomolar affinity to multiple epitopes of EGFR. Monovalent and homo- and hetero-bivalent dimers of these domains were tested for EGFR downregulation. Selected orientations of non-competitive heterodimers decrease EGFR levels by up to 80% in multiple cell types, without activating receptor signaling. These heterodimers inhibit autophosphorylation, proliferation, and migration, and are synergistic with the monoclonal antibody cetuximab in these activities. These small (25 kDa) heterodimers represent a novel modality for modulating surface receptor levels.



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(IS4-3)

1. Speaker

Gou Young Koh

2. Affiliation

Professor, Graduate School of Medical Science and Engineering,
Korea Advanced Institute Science and Technology

3. Professional Activities

Director, National Creative Research Initiatives for Endothelial Cells, Korean Minister of Science and Technology (1997-2003)

Associate Professor, Department of Life Sciences, Pohang University of Science and Technology (2001-2003)

Development and Application of Double Anti-angiogenic Trap Protein

Gou-Young Koh

Graduate School of Medical Science and Engineering, Korea Advanced Institute Science and Technology (KAIST), Korea

Two vascular growth factor families, VEGF and the angiopoietins, play critical and coordinate roles in tumor progression and metastasis. A single inhibitor targeting both VEGF and angiopoietins is not available. Here, we developed a novel chimeric decoy receptor, namely double anti-angiogenic protein (DAAP), which can simultaneously bind VEGF-A and angiopoietins, blocking their actions. Compared to VEGF-Trap or Tie2-Fc, which block either VEGF-A or angiopoietins alone, DAAP is a highly effective molecule for regressing tumor angiogenesis and metastasis in implanted and spontaneous solid tumors; it can also effectively reduce ascites formation and vascular leakage in an ovarian carcinoma model. Thus, simultaneous blockade of VEGF-A and angiopoietins with DAAP is an effective therapeutic strategy for blocking tumor angiogenesis, metastasis, and vascular leakage.

(IS4-4)

1. Speaker

Jin-San Yoo

2. Affiliation

PharmAbcine, Inc., Korea

3. Professional Activities

Research Associate, the Howard Hughes Medical Institute at Stanford University School of Medicine

Director, LG Life Sciences R&D Park, Therapeutic Antibody Division

Development of Fully Human Antibody Neutralizing VEGFR-2

Jin-San Yoo

PharmAbcine, Inc., Korea

Vascular endothelial growth factor (VEGF) and its receptors (VEGFR) have been implicated in promoting solid tumor growth and metastasis via stimulating tumor-associated angiogenesis. Models of murine tumor angiogenesis and receptor-specific antibodies are required to evaluate roles of VEGF receptors in mouse xenograft models of human cancer. Human VEGFR-2 (also known as kinase insert domain-containing receptor, KDR) and murine VEGFR-2 (or Fetal liver kinase-1, Flk-1) share 85% amino acid sequence identity in their extracellular domain. However, sequence homology of VEGF binding domain of KDR and Flk1 is less than 75%. Until now, none of available KDR neutralizing antibodies has species crossreactivity. We first describe here the development of fully human antibodies that cross-react with mouse, rat and human VEGFR-2. High-affinity, species cross-reactive, ScFv antibodies specific for KDR/Flk-1 were selected from fully human naïve antibody phage display library we constructed. The selected and converted fully human IgG antibodies were found to bind to purified KDR with sub-nanomolar affinity. Their binding epitope locate in the IgG like domain 3 of extracellular domain of VEGFR-2, which is responsible for neutralizing effect of KDR function. I will discuss about recent in vivo data and part of preclinical data of anti-KDR antibody.



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(IS4-5)

1. Speaker

Louis M. Weiner

2. Affiliation

Georgetown-Lombardi Comprehensive Cancer Center, Washington, DC, USA

3. Professional Activities

Antibody-initiated cancer immunotherapy

Louis M. Weiner, Yong Tang, Shangzi Wang, Rishi Surana, Wei Xu, Yu Zhou, James D. Marks¹, Erica Golemis²

Georgetown-Lombardi Comprehensive Cancer Center, Washington, DC;

¹*University of California, San Francisco;* ²*Fox Chase Cancer Center, Philadelphia, PA.*

We have previously described the critical structural determinants of anti-tumor antibodies that promote in vivo tumor targeting and antibody-dependent cellular cytotoxicity (ADCC). We now describe treatment strategies in murine models that 1) improve in vitro and in vivo ADCC, 2) dissect the relative contributions of tumor signaling perturbation and ADCC for in vivo therapeutic efficacy, and 3) identify therapy conditions that promote the induction of host-protective adaptive immunity. We also have identified tumor-intrinsic factors that sensitize tumor cells to EGFR-directed antibody therapy, using a siRNA library focused on genes functionally linked to the EGFR signaling pathway. We predict that these findings will be important for the improved efficacy of EGFR family-directed antibody therapies.

Supported by CA50633, CA121033.

(IS4-6)

1. Speaker

Se-Ho Kim

2. Affiliation

Research Director, Green Cross Corp., Yongin, Korea

3. Professional Activities

Postdoctoral research fellow. Antibody Engineering lab., Massachusetts General Hospital/Harvard Medical School, Boston, MA, USA (1997-1999)

HBV neutralizing human monoclonal antibody for liver transplantation & chronic hepatitis B treatment

Se-ho Kim

Greencross Co., Korea

Hepatitis B virus (HBV) is one of the main pathogens responsible for hepatitis and hepatocellular carcinoma. Human plasma-derived hepatitis B immune globulin (HBIG) is being used for prophylactic and liver transplantation currently. However, it may be necessary to replace a HBIG with a recombinant one because of limited availability of human plasma with high anti-HBs antibody titer.

In order to meet these requirements, we selected human antibodies to the HBsAg from a phage-display library and a Chinese hamster ovary (CHO) cell line was established which produces a fully human IgG1 that binds the HBV. The rHBIG exhibits ~3500 units/mg of antibody and ~7 fold higher affinity than that of Hepabig (an HBIG from Green Cross Corp., Korea). It recognizes a conformational “a” determinant of HBsAg and binds to HBV-infected human liver tissue but does not to normal human tissues. Neutralization of the HBV by rHBIG was proved in a chimpanzee model. In addition neutralization of the HBV mutants, especially a typical G145R mutant, by the rHBIG was manifested in a hydrodynamic mouse model.

Mass production and purification processes for the rHBIG were established and the safety of rHBIG was investigated in monkeys. In phase I clinical trial for healthy volunteers, the rHBIG was well tolerated without any serious adverse events up to 8 folds of clinical dose, and current stage is in clinical phase II/III for liver transplantation in Korea. This rHBIG has several advantages compared to plasma-derived Hepabig such as activity, safety and availability.

(IS4-7)

1. Speaker

Junho Chung

2. Affiliation

Chairman & Professor, Department of Biochemistry and Molecular Biology, Seoul National University School of Medicine.

Cancer Research Institute, Seoul National University

3. Professional Activities

President, Korean Society for Antibody Engineering. (2007 – 2009)

Vice president, Korean Society for Antibody Engineering. (2002 – 2007)

Executive member, Korean Society for Molecular and Cellular Biology

Development of anti-hepatocyte growth factor antibody as an anti-glioblastoma agent

Junho Chung

Department of Biochemistry and Molecular Biology, Seoul National University College of Medicine, Korea

In human glioblastoma nude rat orthotopic xenograft model, genetic or pharmacological inhibition of VEGF or its receptor promote a more invasive and aggressive tumor behavior (Neoplasia 2000 Vol. 2 p 306 & Cancer Res 2001 Vol. 61 p 6624) and distant metastasis (Cancer Cell 2009 Vol. 15 p 220). In clinical setting, increased non-enhancing tumor with decreased or stable enhancing disease is observed after bevacizumab treatment especially in responders (Neurology 2008 Vol. 70 p 779). Furthermore glioblastoma progression on bevacizumab was observed in 35% of patients, which were associated with shorter survival. (Neurology 2009 73 1200). In this aggressive transformation of glioblastoma, HGF/cMet signaling plays a critical role as they are directly under control of hypoxia inducing transcription factors (HIFs), of which overexpression would be induced by inhibition of angiogenesis (Nature Medicine 2003 Vol. 9 p 822).

By immunizing rabbits with HGF/cMet complex, we successfully generated a monoclonal antibody that inhibits HGF/cMet interaction, and blocks the biological function mediated by HGF *in vitro* and *in vivo*. To define epitope, we screened out an epitope-mimicking peptide, KSLSRHDHIIHHH, from a phage display of combinatorial peptide library. In molecular mimicry this peptide bound to cMet and inhibited HGF/cMet interaction. No humoral response was induced to this epitope-mimicking peptide when immunization was done with HGF alone. The humanized version of this antibody showed potent inhibition of human glioblastoma growth in nude mouse subcutaneous tumor xenograft model.



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(IS4-8)

1. Speaker

Eunkyung (Kyung) Lee

2. Affiliation

Neopharm Inc (Ari Sciences was acquired by NeoPharm), CSO

3. Professional Activities

2002-2008: Amgen, Senior Research scientist in Protein Science department

1999-2002: Postdoctoral fellow at Yale University School of Medicine and Howard Hughes Medical Institute, New Haven, CT

What antibodies can do better than small molecules

Eun-Kyung Lee

NeoPharm Co., Korea

The program NP-B1 at NeoPharm is to discover antagonistic antibodies against proprietary receptor involved in glucose homeostasis. It is very well validated target in diabetes field, however multiple attempts to develop small molecule therapeutics have not been successful so far and there is no drug available in the market.

Using phage display of human antibody libraries, multiple human monoclonal antibodies have been identified. The lead candidate NPB112 showed impressive glycemic control in various animal models, such as high fat diet induced obesity mouse model (DIO) and db/db mice. Its efficacy was also confirmed in glucose tolerance test (ipGTT). To further validate mechanism of action of NPB112, hyperinsulinemic-euglycemic clamp was performed. In this model, insulin level is increased and glucose level is maintained at constant to mimic the status after the meal. NPB112 reduced hepatic glucose output by 42%, and increased glucose infusion rate by 37%, most likely due to reducing insulin resistance. Since insulin resistance is the most common causes of Type II diabetes, the result strongly suggests that NPB112 may be an excellent candidate to treat Type II diabetes. We also confirmed that hypoglycemia is not observed with NPB112.

Another program NP-B3, targets also well known protein without marketed drug to treat allergy related diseases, such as asthma, hay fever, and atopic dermatitis. We have a recombinant protein drug candidate, as well as human monoclonal antibodies with quite strong in vitro potency with IC50 of 10 to 100pM. They are in the process of being tested for in vivo efficacy in mouse asthma models and atopic dermatitis models.



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(IS4-9)

1. Speaker

Dewey Ryu

2. Affiliation

Professor and Director of Biochemical Engineering Program

Professor, Department of Chemical Engineering and Material Science, College of Engineering

Professor, College of Agriculture and Environmental Science, University of California, Davis, CA 95616

3. Appointment on Editorial Boards

Enzyme and Microbial Technology (1978- Present)

Applied Biochemistry and Biotechnology (1981- Present)

Journal of Bioscience and Bioengineering (1986- Present)

Journal of Biochemical Engineering and Biotechnology (1996- Present)



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IS5

GMO

Current Developments in Genetically Engineered Plants

(IS5-1)

1. Speaker

Richard Gerardus Franciscus Visser

2. Affiliation

Head & Professor, Wageningen UR Plant Breeding, Wageningen University and Research Centre

3. Appointments on Editorial Boards

Editor in chief of Euphytica

Editor of Annals of Applied Biology, Molecular Breeding, Theoretical & Applied Genetics and Potato Research

Guest editor, Plant Molecular Biology

GM potatoes: latest developments in the field of resistance to late blight (*Phytophthora infestans*)

Richard GF Visser

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Potato is the world's third largest food crop yet it continues to endure late blight (LB), a devastating disease caused by the Irish famine pathogen *Phytophthora infestans*. Exploiting genetic resistance is the best disease management strategy but current approaches have been unsuccessful. The first introgressed resistance genes from *S. demissum* were quickly broken and other alternatives were investigated including partial resistance by using race-nonspecific sources of resistance. However, under long day conditions, breeders using this strategy have achieved little progress, the major draw-back being the strong linkage between foliage resistance and late foliage maturity. We therefore anticipate that breeding for LB resistance in potato, aiming at substantially contributing to disease management, requires, by one way or another, the deployment of strong and new *Rpi* genes. In an attempt to identify novel *Rpi* genes and to gain insight into *Rpi* gene diversity we have carried out an extensive germplasm screen and are currently mapping *Rpi* loci with the ultimate goal to clone complementary *Rpi* genes. To date, more than 10 different *Rpi* genes have been identified, mapped and isolated from a number of different species. All these genes belong to the NB-LRR class of *R* genes and are members of homologous gene clusters of varying sizes. As more and more *Rpi* genes are identified and cloned, the chance increases that new *Rpi* gene alleles reside at known and well-characterized loci, enabling the use of comparative genomics, and thus the development of efficient allele mining strategies. Moreover ongoing potato and tomato genome sequencing projects by

international consortia are providing a (complete) survey of the distribution of *R* gene clusters in the *Solanaceae*, enabling even faster cloning of *Rpi* genes. Challenges that remain are how to predict *Rpi* gene durability and how to introduce durable combinations of *Rpi* genes into existing and future varieties in the most efficient and sustainable manner.

Knowledge on the evolution and distribution of late blight resistance genes is important for a better understanding of the dynamics of these genes in nature. We analyzed the presence and allelic diversity of several late blight resistance genes, including *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3*, originating from *Solanum bulbocastanum* in a set of tuber-bearing *Solanum* species, comprising 196 different taxa. The 3 genes were only present in some Mexican diploid as well as polyploid species closely related to *S. bulbocastanum*. Sequence analysis of the fragments obtained from the *Rpi-blb1* and *Rpi-blb3* gene suggests an evolution through recombination and point mutations. For *Rpi-blb2* only sequences identical to the cloned gene were present in *S. bulbocastanum* accessions, suggesting that it has emerged recently. The three resistance genes occurred in different combinations and frequencies in *S. bulbocastanum* accessions and their spread is confined to Central America. A selected set of genotypes was tested for their response to the avirulence effectors IPIO-2, Avr-blb2 and Pi-Avr2, which interact with *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3*, respectively, as well as by disease assays with a diverse set of isolates. Using this approach some accessions could be identified that contain novel, yet unknown, late blight resistance factors in addition to the *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes.

We believe that knowledge of effector diversity will inform likely durability of *Rpi* genes. We have pioneered the exploitation of putative effector genes predicted computationally from the *P. infestans* genome to accelerate the identification, functional characterization, and cloning of *Rpi* genes. An initial set of over 100 effectors containing a signal peptide and an RXLR motif were profiled for activation of innate immunity (avirulence or Avr activity) on wild *Solanum* species using the PVX expression system. This approach proved successful and resulted in amongst others the discovery of *Avr-blb*. We tested over 130 *Solanum* genotypes. Our study revealed notable trends in the recognition of effectors which could be related to the phylogenetic and geographic origin of *Solanum*, suggesting that many of the identified responses emerged as a result of coevolution between host and pathogen. One example is the high rate of recognition of a PexRD effector by genotypes of the phylogenetic clade demissum and semidemissum, which consists of natural hosts of *P. infestans* in Mexico. Close inspection of the results matrix suggested that this PexRD is also recognized by cultivars containing *R2* family genes, thus leading to the hypothesis that PexRD might interact with the *R2* family. Analysis of the genome sequence of *P. infestans* strain T30-4 revealed 18 members of the *Avr2* family, including PexRD, with a low degree of similarity. Functional tests between non-redundant *Avr2* family members and *R2* family genes by co-expression in *N. benthamiana* revealed a complex pattern of specificity. We hypothesize that the high degree of variation observed for the *R2* genes in *Solanum* with the *Avr2* family in *P. infestans* may have been driven by a co-evolutionary arms-race between host and pathogen.

The traditional way of introducing resistance is introgression breeding by inter-specific crosses and repeated backcrosses



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with cultivated potato, which is a very slow and inefficient process. The result is introgression of a piece of DNA with the gene of interest, surrounded by other alien genes of the donor species. This is called linkage drag and is almost always connected with linked genes coding for traits with a negative impact, such as increased glycoalkaloid content. Furthermore, since potato is a heterozygous tetraploid, few out-crossed progeny retain all of the desirable parental traits, and much pre-breeding is required to remove the most important linked genes coding for negative traits. It can be said that introgression breeding is a multiple step approach resulting in the introduction of a target gene, e.g. an *Rpi* gene, with linkage drag. For durable LB resistance strategies, efficient stacking of *Rpi* genes from one or several species is essential, but in practice this will enlarge the linkage drag problems considerably. Their introduction by genetic modification (GM) is a much more efficient way to improve resistance in one step and in a short period. It can even be applied to existing varieties with a long history of safe use. Currently, *Rpi* genes of natural origin, so called cisgenes, can be introduced using marker-free transformationsystems leading to cisgenic plants with only the gene(s) of interest and without linkage drag.

(IS5-2)

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Associate Editor for *Molecular Plant-Microbe Interactions* (1987-1996, 2003-2005)

Editorial Board Member, *Journal of General Virology* (1997-2001; 2008-2012)

Advisory Board Member, *The Plant Pathology Journal* (2001-2006)

Associate Editor for *Virus Research* (2005-2007)

Virology Ed. Board Member for *Encyclopedia of Life Sciences* (2006-2010)

Multiple Virus Resistance in Transgenic Plants Achieved by Pyramiding Multiple Viral Sequences in a Single Transferred Gene

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Potato is an important horticultural crop world-wide and is subject to a number of viruses, the most important of which, in terms of effects on crop yields, are Potato leaf roll virus (PLRV), Potato virus Y (PVY), Potato virus A (PVA), Tobacco rattle virus (TRV) and Potato mop-top virus (PMTV). PVY and PVA are potyviruses transmitted by aphids; PLRV is a poliovirus, also transmitted by aphids, as well as in seed tubers; TRV is transmitted by nematodes as well as in seed tubers; and PMTV is transmitted by a fungus-like vector. Thus, different strategies are implemented to reduce infection of potato crops by these viral pathogens. However, as pesticides become less available, or less effective, other strategies are needed for crop protection. One such strategy is to use resistance conferred by genetic modification.

Genetically modified plants have been engineered in the past to express resistance to one or two viruses (reviewed by Prins

et al., 2008). In addition, resistance to three viruses [watermelon mosaic virus 2, zucchini yellow mosaic virus and cucumber mosaic virus (CMV)] together in the same plant was generated in transgenic cantaloupe (Fuchs et al., 1997) as well as in transgenic lines of squash (Fuchs et al., 1998) (both named CZW-3). These plant lines were deregulated by the U.S. Department of Agriculture and were sold commercially. Using the strategy of expressing inverted repeat sequences separated by an intron from a transformation vector, which provides a much higher percentage of resistant regenerated transformants, resistance to three viruses [tobacco mosaic virus, PVY and CMV] also was obtained in transgenic tobacco (Zhu et al., 2008) and against four tospoviruses (Groundnut ringspot virus, Tomato chlorotic spot virus, Tomato spotted wilt virus, and Watermelon silver mottle virus) in transgenic *Nicotiana benthamiana* (Bucher et al., 2006). Resistance to the four tospoviruses was achieved by fusing fragments of sequences of the four viruses arranged in linear order so that the homologous sense-antisense or antisense-sense sequences would base pair to form inverted double-stranded RNA molecules on transcription (Bucher et al., 2006). This illustrates that multiple virus resistance in a single plant can be obtained and can be extended to crop plants as well as model systems.

Based on these previous successes, we transformed and regenerated tobacco plants (*Nicotiana tabacum* cv. Samsun NN) with a transformation vector expressing inverted repeat constructs of fused, 200-bp segments of viral sequences encoding the PVY coat protein, the PVA cylindrical inclusion and the PLRV coat protein. The PVY strain O sequences were selected for greatest similarity to PVY strain N; previously, good resistance had not been achieved to one PVY strain using sequences derived from the other PVY strain. The viral-derived sequences in the constructs and transgenic plants were either in a sense-intron-antisense (IN) or an antisense-intron-sense (OUT) orientation. Regenerated T₂ seedlings of transgenic tobacco lines expressing the above viral gene sequences were evaluated for resistance to PVY-N, PVY-O, PVA and PLRV using both mechanical inoculation (PVY-O, PVY-N and PVA) and transmission by aphids (*Myzus persicae*) against all four viruses; PLRV is not transmissible mechanically. We obtained resistance to all of these viruses, by both forms of inoculation, although plants of some IN transgenic lines showed a lower degree of resistance than the OUT transgenic lines, for which resistance occurred in 100 % of the plants.

Subsequently, we transformed and regenerated both tobacco plants and potato (*Solanum tuberosum* cv. Vales Sovereign) plants with transformation vectors expressing inverted repeat double-stranded RNA segments of five viruses (PVY, PVA, PLRV, PMTV and TRV). We obtained transgenic lines of both IN and OUT orientation for tobacco, but only OUT orientation lines from potato. The plants of these lines are still being evaluated for resistance to some virus introduced by the various natural vectors, although resistance to infection has been assessed in the transgenic tobacco plants to mechanical inoculation of PVY-O, PVY-N, PVA and TRV. The IN transgenic tobacco lines showed no resistance to PVA, PVY-O, or PVY-N, and only 70 % of IN transgenic tobacco plants showed resistance to infection by TRV. However, the OUT transgenic tobacco lines showed 100 % resistance to PVA, PVY-O, PVY-N and TRV. PMTV could not be evaluated thusly, as it is not transmissible mechanically to tobacco. The same lines were evaluated also for resistance to the aphid transmissible viruses and while the OUT transgenic tobacco lines showed 100 % resistance to infection by PVA, PVY-O,

PVY-N, and PLRV transmitted by aphids, the IN transgenic tobacco line lines showed variable resistance to infection by these viruses: PVA (73 % resistance), PVY-N (0 % resistance), PVY-O (34 % resistance), and PLRV (43 % resistance). Non-transgenic as well as transgenic potato plants were not susceptible to infection by mechanical inoculation of any of the viruses tested, but were assessed for resistance to three viruses (PVY-O, PVY-N and PLRV) transmitted by aphids. In this case, plants of these transgenic lines were 100 % resistant to infection by PVY-O, PVY-N and PLRV. The results for resistance to natural transmission of the other viruses will be presented.

Northern blot hybridization analysis showed that the expression of fused, viral double-stranded RNA, in two independent OUT lines and two independent IN lines of transgenic tobacco, and three independent OUT lines of potato, led to the generation of silencing-inducing RNAs, characteristic of RNA silencing. Thus, the mechanism of resistance to these various viruses is due to sequence-specific, RNA silencing, mediated by various plant enzymes involved in this natural form of virus resistance and RNA turnover in both plants and animals.

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(IS5-3)

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Control of plant immunity by *Xanthomonas* effectors and GM crop research in Japan

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Plants developed cell surface-localized immune receptors to recognize various pathogen / microbe-associated molecular patterns (PAMPs / MAMPs). Recognition of PAMPs by the receptors triggers an array of immune responses to restrict pathogen growth. PAMP-triggered immunity (PTI) is critical for the survival of plants under constant threat from various pathogenic microbes. To overcome such immunity, pathogens secrete virulence effector molecules into plant cell. However, how the effectors inhibit PTI is not well understood.

Bacterial blight, a disease affecting rice and caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is a significant agronomic problem in many rice-growing regions and is an ideal model system for the study of the interaction between plants and bacterial pathogens. So far, more than 20 effectors of *Xoo* have been found to be incorporated into plant cell through the Type III secretion system (TTSS). They contain transcription-activator-like (TAL) effectors, which function as DNA binding proteins to control transcription of host gene. However, the functions of most of effectors in plant remain to be elucidated.

To identify the effectors that are able to inhibit host immune responses, transgenic rice plants over-expressing 10 *Xoo* effectors were generated by *Agrobacterium*-based transformation. These transgenic plants were inoculated with TTSS-deficient mutant *Xoo hrpX*. Since *Xoo hrpX* is not able to suppress PTI by the TTSS effectors, disease lesions and growth of *Xoo hrpX* are strongly inhibited in wild type plant. Transgenic plants expressing the effectors exhibited different levels of susceptibility to *Xoo hrpX*. Especially, transgenic plants expressing 4 of them (*Xoo5*, *Xoo7*, *Xoo8*, *Xoo10*) developed



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severe disease lesions of *Xoo hrpX*, suggesting that these effectors strongly suppress PTI in rice. The *Xoo8-ox* plant showed dwarf phenotype which is similar to that of brassinosteroid(BR)-insensitive mutants lacking functional BR receptor (BRI1) or BAK1 which was originally identified as BRI1-associated receptor kinase mediating BR signaling. BAK1 is also required for the flagellin receptor FLS2, indicating that BAK1 is involved in PAMPs recognition. We analyzed interaction of *Xoo8* with BRI1, BAK1, and PAMPs receptors and found that *Xoo8* specifically interacts with BAK1. Thus, it is likely that *Xoo8* suppresses PAMPs recognition by inhibition of BAK1.

Although most of *Xoo* effectors are conserved in *Xanthomonas* genus, *Xoo5* is specific for *Xoo*. To identify host factors interacted with *Xoo5*, we did two-hybrid screening and found several candidates including receptor-like cytoplasmic kinase (RLCK) and uncharacterized transcription factor. Functional analysis of these proteins in immune response is in progress.

Chitin is known to induce a series of immune responses in rice, which is triggered by recognition of chitin by the chitin receptor, CERK1. The chitin-induced immune response was inhibited in transgenic rice cells expressing *Xoo10*, suggesting that *Xoo10* targets host factors which are essential for chitin signaling.

In the talk, I also introduce recent progress of GM crops research. To aim to produce disease-resistance crops, we use two approaches; one is to use new identified host immune factors that are targeted by the TTSS effectors. During evolution, the TTSS effectors greatly contribute to overcome host immunity, suggesting that the immune factors targeted by the TTSS effectors play important roles in plant immunity, and could be useful as activator of immune responses. Another one is to use small GTPase Rac signaling. Rac plays important roles in immune responses in rice, barley, and other species. In rice, OsRac1 is known to regulate a series of immune response including cell death, reactive oxygen species production, activation of pathogenesis-related genes, lignification and phytoalexin production. It is therefore expected that the factors involved in OsRac1-mediated signaling are useful tools to engineer plant immunity.



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(IS5-4)

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Transgenic Poplar Plants for Phytoremediation

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Poplar plant is an ideal system for plant biotechnology. It is amenable for transformation to express foreign genes; once individuals with desirable characteristics are generated, they can be propagated by cutting; poplar plants grow fast into large biomass; many varieties of poplar are naturally resistant to stress and pollutants. Thus, this species of plants are good candidates for further improvement and use for specific purpose or as a bioenergy source. Finally, we have a natural mutant poplar that does not flower (no pollen or seeds), and therefore, do not spread pollen or seeds to the ecosystem.

Our environment is contaminated by diverse pollutants including industrial by-products, waste matters, fertilizers and pesticides. The conventional method to remedy polluted environment, such as the physical or chemical cleanup, is costly and can often further damage the environment. Phytoremediation is a low-cost, environmentally-friendly way to remove pollutants from contaminated soils. The use of plants usually costs less than the use of any other forms of organisms, since plants obtain energy from sunlight to synthesize complex molecules which can be used for tolerance to the pollutants. Moreover, plants are aesthetically pleasing, and have good influence on the physical, chemical and biological aspects of the environment.

In the USA and Europe, plants that naturally hyper-accumulate toxic materials are used for the purpose of remediation of the polluted environment. However, phytoremediation using these natural plants has limitations. The plants may suffer

toxicity symptoms, or even die of toxicity of the pollutants, and it takes long time for the plants to grow to certain size to effectively extract the pollutants. In this respect, the potential for genetic engineering of plants to improve their cleaning capacity is enormous. Many transgenic plants with new characteristics due to expression of foreign genes have been reported in scientific journals. Once an individual plant with suitable phenotypes is produced, it is easy to multiply the numbers of plants by asexual reproduction methods, such as cuttings and somatic embryogenesis. Moreover, many trees grow indefinitely as long as the conditions are suitable.

We already found many genes that enhance tolerance to cadmium, lead, or zinc, when overexpressed in Arabidopsis (Kim et al., 2006, Kim et al., 2007, Kim et al., 2008, Kim et al., 2009, Lee et al., 2003, Lee et al., 2005, Shim et al., 2009, Song et al., 2003, Song et al., 2004). We expressed these heavy metal tolerance genes in a line of poplar that does not flower (a natural mutant of *Populus alba* X *P. tremula* var. *glandulosa*), and are in the process of testing them in greenhouse and in the field. For test tube trials, the young plantlets of the wild-type and transgenic poplar at similar sizes were chosen for comparison. As a result, we observed that many genes improved the plant's tolerance to Cd, Pb, or As. For example, YCF1, an ABC transporter of baker's yeast (*Saccharomyces cerevisiae*), enhanced Cd-tolerance in poplar, similarly as in Arabidopsis plants (Song et al., 2003). We selected transgenic poplar lines with enhanced heavy metal tolerance and accumulation, based on such experiments in controlled environment, and planted them in our field test site for multiple-year field trial. Our test site is located in a closed mine area, which is contaminated by heavy metals such as arsenic, cadmium, zinc and other toxic elements. To protect the transgenic poplars from wild life and also to prevent escape of the fallen leaves or branches from the test site, we erected a barbed-wire fence with a dense net around the test site. Many plants survived the harsh conditions of the test site, and are growing. We plan to evaluate their growth and heavy metal contents soon.

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(IS5-5)

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Working on R&D for the genetic improvement of crops with enhanced photosynthesis

Member of Novel Food Expert Group under Food Safety Policy Committee

Shepherd of RDEAB subgroup for ATCWG of APEC (2006 – 2008)

Current Status of GMO and Perspective in Korea

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In 2009, world-wide hectareage of genetically modified crops such as corn, soybean, canola and cotton reached 134 million hectares in 25 countries, which is 48 fold increase as compared to the total area of GM planting in 1986. As evidenced by the recent policy decision made by Chinese Government to approve GM rice, the commercialization of GM crops is expected to expand continuously. Korean Government recognized in 1990s that agricultural biotechnology would be a key factor for the success of Korean agriculture and bio-industry, and has made extensive investment in agricultural biotechnology through national R&D Programs such as Bio-Green 21 and Crop Functional Genomics Center. Currently, more than 100 GM crop plants are under development, although exact statistic numbers are not available.

In RDA, agricultural biotechnology and GM crops are developed mainly at the Department of Agricultural Biotechnology, NAAS. Improvements of crops traits, such as resistance against abiotic and biotic stress, nutritional improvement, increased yield productivity as well as the production of novel functional materials are major targets for the Agro-biotech R&D. Structural and functional genomics research for rice, Chinese cabbage and a couple of microbes are performed together with molecular analyses of gene functions to excavate valuable genes. Novel genes developed by genomic and molecular biological researches are evaluated through transgenesis. Target traits as well as other agricultural traits of transgenic model crops are examined under greenhouse and field condition. Genes proved to be valuable and transgenic lines with improved target traits are then used for the development of GM crops. Currently, 88 GM events in 18 crop

species are under development. Among them, six events are at the stage of risk assessment and expected to go forward to the regulatory stage.

Korea has never allowed for the commercial growing of GMO in her territory while she has been one of major GMO importing countries in the world. In 2008, the total amounts of GMO imported for feed and food were 7.7 and 0.8 million metric tons for GM maize and soybean, respectively.

There is a variety of visions on GMO in Korea in that GMO is considered as one of driving forces for the national economic growth in 21st century while negative public perception for GMO and anti-GMO movement of NGO activist groups have been major obstacles for the commercial expansion of GMO industry. In order to public demands conflicting each other, Korean Government has promulgated a series of laws to promote biotech R&D and industrialization at the same time to secure food and environmental safety of GM products, such as 'the Biotechnology Promotion Act', 'the Food Sanitation Act' and the Act on Trans-boundary Movement of LMO (LMO Act)'. As Korea ratified the Cartagena Biosafety Protocol in 2007, the LMO Act became in effect from January 1st of 2008 and the legal basis for the national framework for GMO regulation.

Although six Ministries participate in the national regulatory framework, the Korean Food and Drug Administration (KFDA) under the Ministry of Health, Welfare and Family Affairs (MHWFA) and the Ministry of Food, Agriculture, Forestry and Fisheries (MFAFF) play major roles in GMO regulation. KFDA and the Rural Development Administration (RDA) under MFAFF are responsible for the food safety assessment and the environmental risk assessment and have authorities to approve GMO for its commercial use (such as commercial growing and food/feed use). GMO safety evaluation committee has been in operation to review the safety assessment and evaluation data of GM foods for KFDA. By the end of 2008, 54 GM events including soybean(1), maize (28), cotton (13), canola (6), sugar beet (1), potato (4) and alfalfa (1) have been approved for the use as food and nine events were under review process. For the environmental risk assessment, RDA established expert review committee in 2004. By April of 2009, 65 GM events including one for domestic field release for commercial purpose were submitted for review and the review for 48 events was finished. Currently, environmental risk assessments for 7 GM events including herbicide tolerant GM rice, insect resistant GM rice and Chinese cabbage are under progress to collect data for the review for environmental release for commercialization. GMOs approved for commercial use by national authorities need to get another approval for import. KFDA has an authority to approve GMO for food and the National Agricultural Products Quality Management Services (NAQS) under MFAFF is responsible for the approval for GM feed import. KFDA and the National Plant Quarantine Service (NPQS) under MFAFF are supposed to inspect imported food and feed for the surveillance of the entry of unapproved GMO. Once GM products enter Korea, they are subject to be managed by mandatory labeling system. GMO labeling system has been established to offer consumer's right to choose.

Korea's national framework for the risk assessment and management of GMO is based on four basic principles such as



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'science based', 'case by case', 'comparative' and 'substantial equivalence and familiarity'. We believe that sound and transparent regulation would be one of the key factors for the success of modern biotechnology.

In conclusion, Extensive investment and R&D activities have been made to develop agricultural biotechnology and GM crops in Korea, even though social environment is not favorable for GMO. Strict regulation would be necessary to change public's negative conception on GMO and biotechnology. We believe that it is inevitable chasing two rabbits such as developing GMO that is acceptable to Korean consumers and strictly regulating GM commercialization for the success of agro-biotech in Korea.

(IS5-6)

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Journal of the Korean Society for Applied Biological Chemistry (2009 – present)

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Making the Drought Tolerant GM Rice Plants

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Drought poses a serious threat to the sustainability of rice yields in rainfed agriculture. Here we report the results of a functional genomics approach that identified a rice NAC-domain gene, *OsNAC10*, which improved performance of transgenic rice plants under field drought conditions. A group of *OsNAC* genes were pre-screened for enhanced stress tolerance when overexpressed in rice. The *OsNAC10*, one of the effective members selected from pre-screening, is expressed predominantly in roots and panicles, and induced by drought, high salinity and abscisic acid. Overexpression of *OsNAC10* in rice under the control of the constitutive promoter *GOS2* and the root-specific promoter *RCc3* increased the plant tolerance to drought, high salinity and low temperature at the vegetative stage. More importantly, the *RCc3:OsNAC10* plants showed significantly enhanced drought tolerance at the reproductive stage, increasing grain yield by 25-42% and 5-14% over controls in the field under drought and normal conditions, respectively. Grain yield of *GOS2:OsNAC10* plants in the field, in contrast, remained similar to that of controls under both normal and drought conditions. These differences in performance under field drought conditions reflect the difference in expression of *OsNAC10*-dependent target genes in roots as well as in leaves of the two transgenic plants, as revealed by microarray analyses. Root diameter of the *RCc3:OsNAC10* plants was thicker by 1.25-fold than that of the *GOS2:OsNAC10* and NT plants due to the enlarged stele, cortex and epidermis. Overall, our results demonstrated that root specific overexpression of *OsNAC10* enlarges roots, enhancing



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drought tolerance of transgenic plants, which increases grain yield significantly under field drought conditions.

Over the past several years, consumer and environmental groups have expressed concerns about the use of antibiotic-and herbicide-resistance genes against ecological and food safety perspective. Although no scientific basis has been established for these concerns, generation of marker-free plants would certainly contribute to the public acceptance of transgenic crops. Here we present a technology that allows us to make transgenic plants without using selectable markers. This technology relies on an efficient *Agrobacterium*-mediated transformation method and PCR-based selection of transformants. After co-cultivated, transformed cells were allowed to regenerate on MS medium without any antibiotics or herbicides. In about 2-3 weeks after regeneration, a leaf of regenerated plants from one callus was cut and pooled. We isolated genomic DNAs from the pools in 96-well microplate and performed PCR for the presence of transgene in the pools. We then select pools that are positive and grew individual rice plants from the positive pools. Second PCR on genomic DNAs from individual plants of the PCR-positive pools led us to identify individual transgenic plants. This series of process was referred to as the Clean T-DNA technology. Such transgenic rice plants are marker-free and further analyzed after grown in a paddy field. These include genotyping by gDNA PCR, insertion site and Southern blot analysis. Thus, our Clean T-DNA technology may provide marker-free transgenic rice plants that can be used directly for agricultural practices.

(IS5-7)

1. Speaker

Raymond J. Layton

2. Affiliation

Research Fellow, Pioneer Hi-Bred International, Inc.

3. Professional Activities

DuPont/Pioneer Hi-Bred International, Coordinator Product Safety Group (2002-2010)

DuPont Crop Protection, Environmental Risk Assessment Competency Leader (2000-2002)

Served as panelist for FAO expert consultation on GM crops and USDA Biotech Risk Assessment Grant programs

Risk Assessment of Biotech Crops

Raymond Layton, Ph.D.

Research Fellow, Regulatory Science and Registration, Pioneer Hi-Bred International, Inc., Ankeny, IA 50021, USA

Crops have been genetically modified for over 10,000 years. Although early farmers did not understand genetics, they actively selected genotypes that provided desirable phenotypes. For example, the ancient maize teosinte was improved to become primitive maize and then improved again to become modern maize. Significant genetic changes occurred to move from a plant with a few exposed kernels prone to shattering, to the husk enclosed maize varieties we take for granted today. Over the past 200 years, an ever increasing knowledge of genetics has allowed us to push the phenology of crops in new ways. For example, the use of hybrid maize and other directed breeding techniques have increased yield to three or four times higher than open pollinated varieties used 100 years ago. Use of chemicals or radiation to cause new mutations and laboratory methods such as embryo rescue techniques (to allow genetic crosses of normally incompatible species), continue to provide new varieties of crops new traits and increased yield. Each of these methods results in significant changes in crop genetics – all of these crops were modified genetically from their original natural state.

In the 1990s biotechnology emerged as a useful method to produce crops with new traits. Rather than using cross breeding that might shift hundreds of genes between varieties, or mutagenic chemicals or radiation to develop potentially useful mutations, biotechnology methods allow for the insertion of one or a few specific genes into a plant. Since their introduction, biotech crops have been grown on an increasing number of hectares around the world. Current crops include:



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soybean, maize, cotton, canola, squash, sugar beet, papaya, and tomato. These crops have been modified using modern genetic techniques so that they are tolerant to herbicides, resistant to insects or diseases, or have some new trait such as a modified oil profile. The number of different crops and the cultivation area will continue to increase. In future years we can expect to see transgenic varieties of rice, brinjal (egg plant), sorghum, and other crops.

The world population is expected to grow to nine billion by the year 2050. Biotechnology is one tool that can be used along with conventional crop breeding to help meet the demand for food, feed, and fuel in a sustainable manner. For example, in the United States, USDA figures indicate that average maize yields which increased about 10 kg per hectare during each of the 25 years prior to 1996, have increased about 170 kg per hectare since the introduction of insect resistance traits in 1996. This means that biotechnology in tandem with improved germplasm has contributed to an average yield that is 1700 kg per hectare greater than just a decade ago. Herbicide tolerant crops allow use of erosion reducing tillage practices. Drought or heat resistant crops, or crops developed for use with poor quality soils will help maximize productivity where land is scarce and to maintain yields with less nitrogen. Advances in biotechnology allow farmers to benefit from greater productivity and output with less cost, effort and economic burden to the environment. Biotechnology products also have the potential to help children around the world who suffer from nutritional deficiencies as well as play an increasing role in providing healthier foods for all consumers.

The first step in producing a biotech crop is developing a genetic construct that will be inserted into the plant cells. The construct contains the genetic materials (promoter, gene, terminator, etc.) needed to produce the desired phenotypic change. The insertion can be made using either a biolistic approach or using a plant bacteria. The transformed cells are then grown into full sized plants. The step of growing the transformed cells into full sized plants is the first of many steps that selects out transformed plants with any unexpected effects (e.g. an unacceptable phenotype). Once the full sized plant is grown and seeds are produced, the plants can be grown in the greenhouse and later in the field. The composition and agronomy are compared to the untransformed plant with a similar genetic background (the “isoline”) and also to the range of results obtained for commercial varieties.

Unlike crops developed using traditional breeding techniques, biotech crops undergo a rigorous deregulation process prior to commercialization. This process includes extensive characterization and product safety evaluations. Laboratory and field studies are conducted to examine environmental fate and potential toxicity to wildlife, humans, livestock, and allergenicity. The biotech crops can only be cultivated commercially after the studies have been reviewed and found to be acceptable by regulators.

Characterization of the crop includes composition and agronomic trials to evaluate whether the biotech crop is substantially equivalent to the non-biotech crop - meaning that the biotech crop is essentially identical to the non-biotech crop except for the presence of the inserted trait. These trials provide data on approximately 100 different composition and agronomic



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endpoints. These results can then be compared to data from non-biotech lines of the same crop grown at the same location or to ranges of results found in the literature. These trials are replicated at several different locations (often 6-10) and for multiple growing seasons. Composition variables include amino acids, carbohydrates, fatty acids, fiber, proximate, and vitamins. Agronomic variables include many different plant growth parameters. The compositional and agronomic comparison conducted for biotech crops is much more rigorous than the composition conducted for crops using traditional breeding.

The environmental safety assessment utilizes both effects and fate data. For an insect-resistant crop, the effects data include the mode of action and the spectrum of activity on various arthropod species. For example, the mode of action of the insecticidal Cry proteins in Bt maize is a binding to receptors in the gut lining of certain insects and subsequent pore formation. The spectrum of activity tends to be restricted to only a few species of within a certain insect order – for example Lepidoptera. Laboratory, and when necessary, field tests are conducted to evaluate the possibility of effects on other types of arthropods. The environmental fate of the protein is also studied; most proteins degrade within only a few hours or days in soil. The effect and fate data are then used to assess any potential environmental risks. When compared to conventional insecticides, insect resistant crops have a very narrow spectrum of activity and can be considered environmentally friendly (Bt based insecticides have been used in organic agriculture for decades).

The potential for unintended effects of the transgenic protein or in whole foods obtained from individual crops are evaluated using toxicology and livestock feeding studies. One study that is typically conducted early in development is an acute toxicology study using purified protein administered at high concentrations to mice. In this test mice are usually exposed to a dose of the protein that is hundreds or even thousands of times greater than a person could possibly consume in any given day from the biotech crop. Other studies include feeding of the grain from the biotech crop to animals to assess the potential for unintended adverse effects that may not have been detected during compositional analysis. For example, soybeans or corn from biotech crops have been processed into edible fractions and fed to broiler chickens – an animal known to be very sensitive to nutritional changes – for 42 days to assess whether nutritional changes could have occurred that were not detected during compositional analysis. Similarly, these fractions have also been fed to rats for 90-days to assess the potential for adverse health effects. As part of the development of a biotech crop, studies on various kinds of livestock (cattle, dairy cows, swine, laying hens, etc.) are conducted. The consumption of biotech crops and processed commodities is then determined to evaluate potential short-term and long-term exposure. To date, no biologically significant effects have been found in the studies that we have conducted using grain from biotech crops.

In addition to the toxicology and nutrition studies, the biotech crop is also evaluated to see if there is any change in allergenic potential – either through production of a new allergen, a cross-reactive allergen, or an increase in the levels of an already existing allergen. Results from several areas of analyses support a weight of evidence approach: the origin of gene is from a non-allergenic source, the produced protein degrades readily under conditions of heating or simulated digestive or



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intestinal fluids, a lack of glycosylation, and a bioinformatics comparison with known allergens that does not indicate significant amino acid sequence homology. Significant bioinformatic matches are supplemented with studies conducted using human sera obtained from patients allergic to the suspect allergen. Tests conducted on commercialized biotech crops have indicated that they are no more allergenic than non-biotech crops.

In summary, the risk assessment process for biotech crops follows a well defined, step by step approach starting with a definition of the potential issues and problems. This is then followed by rigorous screening to eliminate unexpected phenotypes and studies to evaluate the composition and agronomy profiles of the biotech crop and to ensure that it is substantially equivalent to the non-biotech crop. Laboratory and field studies are used to look for potential effects on humans, livestock, and the environment. Data from all of these studies are used by regulators to evaluate biotech crop safety before allowing cultivation or import. As the world population continues to increase, we need to provide more resources. Biotechnology alone cannot solve this problem, but biotechnology does promise some potential solutions to global food security and environmental protection, and the current risk assessment processes indicate that it can do so in a safe manner.



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(IS5-8)

1. Speaker

Xun Wang

2. Affiliation

President, Syngenta Biotechnology (China) Co., Ltd

3. Professional Activities

Head of R&D for Syngenta China (2006-2008)

Section Head of Trait Research & Technologies for Syngenta Biotechnology Inc. North Carolina, USA (2003-2006)

Director of Genome Technology for Torrey Mesa Research Institute, California, USA (1998-2003).

Accelerating Agricultural Biotechnology Innovation through Win-Win Partnerships

Xun Wang, PhD,

President, Syngenta Biotechnology (China) Co., Ltd.

Head, Biotechnology R&D, Syngenta APAC

To address the many challenges facing today's agriculture in terms of growing populations despite decreasing arable lands, limited water resource and climate change, it's commonly agreed that we have to pursue a sustainable increase in crop yields. This requires the use of agricultural technologies within farming systems that incorporate the latest techniques and practices.

Biotechnology has been proven to a key technology to help deliver the required step-change in crop productivity whilst using natural resources more efficiently. It is reported to be one of the fastest adopted technologies in modern agricultural history without a single reliably documented safety issue. Over the past 14 years, farmers in 25 countries have seen how biotechnology can improve yield and quality of their crops, 90% of whom are small, resource-poor farmers from developing countries.

In 2006 it was estimated that without the yield benefits of biotechnology, an additional 3.9 million hectares of soybeans and 1 million hectares of corn would have to have been planted to deliver the same harvested output. But it is not just biotech that can benefit farmers. The widespread adoption by farmers of hybrid rice varieties has seen farmers benefit from substantial yield gains, allowing farmers to grow more from less and in many cases move from subsistence to commercial



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farming. And the use of genetic markers in breeding has increased the accuracy of plant breeding, bringing certainty to trait identification and in some cases reducing the time it takes for a new variety to be developed from more than 15 years to less than 10 years. This efficiency reduces investment timelines and allows farmers to access new technology quicker.

Over the past a few years, Asian countries including South Korea have emerged as a pivotal force in advocating the use of novel agricultural technologies. The Chinese government's recent issuance of biosafety certificates for the Bt rice and phytase corn is a landmark decision that will stimulate biotechnology development.

The process of developing an effective biotech crop is long and resource-intensive, often taking 8-10 years and up to USD 100 million from initial discovery to commercialization. To make a product available on the market requires close linkage and collaboration between the public and private sectors. In China, Korea and many other Asian countries, biotechnology research programs are overwhelmingly implemented by the public sector. Global companies like Syngenta can provide the critical mass necessary for adequate investment in technology and product development.

Syngenta is a world-leading agribusiness with a strong investment in R&D, and a proven track record in delivering innovative products to market. We are uniquely positioned to develop and deliver solutions by incorporating technology applications across genetics, biotechnology, seed treatment and crop protection.

Biotechnology R&D at Syngenta unites world-class scientists, state-of-the-art facilities, including the newly established biotech research center in Beijing, China, as well as cutting-edge platform technologies to bring plant potential to life. At Syngenta we invest approximately 10 percent of our sales in research and development every year, and our over 4,500 dedicated scientific staff work closely with farmers across the world to understand their needs and concerns. Syngenta is also a company with a long history of collaborating with academics worldwide based on mutual understandings and win-win principles. In Korea, we have built a strong partnership with the Crop Functional Genetics Center, which is designed to leverage Korean universities' expertise in plant functional genomics research and Syngenta's capability and platform for further development and commercialization.

We must also recognize that nobody has all of the answers and so we must work in partnership with governments, researchers, academic institutions, farmers and consumers because everyone benefits from the responsible adoption of advanced technology. We believe with a fundamentally common vision to help farmers enhance crop productivity and quality through biotechnology innovation, partnerships based on win-win principles are crucial in our joint journey towards sustainable agriculture.



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IS6

Biological Chemistry

Recent Advances in Biological Chemistry

(IS6-1)

1. Speaker

Michael R. Freeman

2. Affiliation

Professor, Urological Diseases Research Center, Departments of Surgery and Biological Chemistry and Molecular Pharmacology, Children's Hospital Boston, Harvard Medical School

3. Professional Activities

- 2008 NCI Site Visit Panel for the Mayo Clinic Comprehensive Cancer Center Core Grant
- 2009 2009/10 ZRG1 DKUS-A (58) R, Challenge Grant Editorial Panel 18
- 2009 ARRA AREA grant ZRG1 EMNR-H 52R study section
- 2010 UKGD (Urology, Kidney, Genitourinary Development) study section

Lipid Metabolism and Castrate-Resistant Prostate Cancer

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Children's Hospital Boston

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Aggressive tumor cells exhibit metabolic derangements in which anabolic lipid metabolism is constitutively upregulated. This is one manifestation of the "Warburg effect," where aerobic glycolysis is stimulated, mitochondrial respiration is suppressed, and biosynthetic pathways that produce macromolecules necessary for rapid cell division operate at increased rates in comparison to normal cells. Overexpression of fatty acid synthase (FASN), a "metabolic oncogene," which is the primary source of long-chain fatty acids (primarily palmitate) in tumor cells, is one emblematic feature of this metabolic profile. FASN overexpression occurs in prostate cancer and levels of the protein positively correlate with increasing Gleason grade.

The aerobic glycolytic/biosynthetic metabolic phenotype has recently been recognized as an important, yet often overlooked, aspect of tumor progression.



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Our laboratory has published the results of a series of studies designed to understand the consequences of altered lipid metabolism in prostate cancer cells and tissues. Our findings suggest that prostate tumor cells use lipid-dependent pathways for cell cycle transit, resistance to apoptotic triggers, and metastatic dissemination. We have shown that “lipid rafts,” plasma membrane microdomains enriched in cholesterol and glycosphingolipids, are critical regulatory sites for signaling events relevant to progression of prostate cancer to the hormone-refractory state. Our studies have demonstrated that the AKT pathway, which is upregulated in greater than 50% of castrate-resistant prostate tumors, is highly sensitive to perturbations of cholesterol metabolism, and that raising cholesterol in vivo stimulates prostate tumor growth. Conversely, suppressing cholesterol levels inhibits prostate tumor growth. We have shown that hypercholesterolemia results in AKT pathway activation and stimulation of tumor angiogenesis. Neoplastic lipogenesis also appears relevant to metastasis, and we are now using the concept of abnormal lipid metabolism to identify new proteins and pathways that are relevant to metastatic dissemination. We have recently demonstrated that the “amoeboid” tumor phenotype, a series of tumor cell behaviors characterized by highly dynamic membrane deformations, as well as rapid cell movement in three-dimensional matrices, appears to be a feature of tumor cells that display lipogenic metabolism. Our studies indicate that the amoeboid phenotype may be directly relevant to castrate-resistant prostate cancer. Consistent with this hypothesis, we recently identified and reported on a novel signaling protein that inhibits amoeboid properties, which is encoded by a gene that shows a high frequency of chromosomal loss (~50%) in metastatic prostate cancer.

Our conclusions from animal models, cell culture and biochemical experiments, and analyses of human tissues directly, suggest that prostate cancer, and possibly other solid tumors, might be inhibited by targeting cholesterol and other lipid-dependent signaling mechanisms. This hypothesis is consistent with a series of recent prospective epidemiologic studies from independent groups indicating that HMG-CoA reductase inhibitors (generically known as “statins”), widely used cholesterol-lowering drugs, can inhibit aggressive prostate cancer in humans. Recent published data suggest that reduced risk of castrate-resistant human prostate cancer arising from long-term statin use may originate principally from sustained lowering of circulating cholesterol levels, consistent with our findings in model systems. Other agents that target neoplastic lipogenesis, such as FASN inhibitors, also represent attempts to exploit the lipid dependence of aggressive tumors as novel means of cancer therapy.

Recent relevant publications from our group:

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Adam, R.M., Mukhopadhyay, N.K., Kim, J. Di Vizio, D., Cinar, B., Boucher, K., Solomon, K.R., and Freeman, M.R. (2007) Cholesterol sensitivity of endogenous and myristoylated Akt. Cancer Research 67:6238-6246.

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- Cinar, B., Fang, P.-K., Lutchman, M., Di Vizio, D., Adam, R.M., Pavlova, N., Rubin, M.A., Yelick, P., and Freeman, M.R. (2007) The proapoptotic kinase Mst1 and its caspase cleavage products are direct inhibitors of Akt1. EMBO Journal 26:4523-4534. PMID: 17932490.
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- Freeman, M.R., Di Vizio, D., and Solomon, K.R. (2010) The rafts of the medusa: cholesterol targeting in cancer therapy. Oncogene [May 3 – Epub ahead of print].

(IS6-2)

1. Speaker

Kwang Pyo Kim

2. Affiliation

Professor, Konkuk University Department of Biotechnology

3. Professional Activities

Post-doctoral fellow, Harvard Medical School Dept of Cell Biology (2002 – 2004)

Systematic Discovery of Nitrated motifs in Nitroproteomics data by Computational Methods

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Protein tyrosine nitration (PTN) is a covalent protein modification that occurs in several diseases such as various cancers, diabetes, Alzheimer's disease, and Parkinson's disease. This modification has been considered as a non-random chemical process. Until now, however, little is known about conserved PTN motifs. Hence, we tried to identify special features of PTN sequences. To achieve such goal, we performed sequence alignment of enriched nitropeptides of our own nitrated peptide data set which is composed of 360 peptides identified from the samples of Non Small Cell Lung Cancer(NSCLC), mitochondria with type II diabetes, liver cancer cell line, Huh7, and ischemic rat brain by a novel proteomics method. After sequence alignment and redundancy elimination, we analyzed PTN sequence property of the nitrated peptide data set. Motif finding programs, GLAM2 and MEME, were utilized to find the gap-allowed sequence motifs for the data set. The revealed motifs were classified by clustering and three dimensional structures of the motifs. Through this process, we finally detected PTN sites with specific feature containing specific amino acids set: {Y, G, P}, {Y, A, I, F}, {Y, L, S|A}, {Y, A|L|Q, D}.

(IS6-3)

1. Speaker

Catharina Svanborg

2. Affiliation

Professor of Clinical Immunology and Adjunct Professor in Oncology, Lund University.

3. Appointments on Editorial Boards

Infection and Immunity / European Journal of Clinical Microbiology / Journal of Infectious Diseases /
Microbial Pathogenesis / International Journal Molecular Medicine / Microbial Drug Resistance /
Current Opinion in Microbiology

HAMLET (Human α -lactalbumin made lethal to tumor cells) A partially unfolded protein-lipid complex with tumoricidal activity

Catharina Svanborg and the HAMLET group, MIG, Institute of Laboratory Medicine, Lund University, Sweden

HAMLET is a complex consisting of partially unfolded α -lactalbumin and oleic acid that exhibits potent tumoricidal activities. Structural studies have shown that partial unfolding is essential for the potent tumoricidal activity of HAMLET, illustrating that partial unfolding of a previously native protein is a mechanism to generate functional diversity. HAMLET exemplifies how unfolding in response to specific environments may define a novel protein structure and how the binding of cofactors may adjust the structure to local conditions in different tissues.

HAMLET kills tumor cells and immature cells, while healthy, differentiated cells survive in the presence of HAMLET. HAMLET shows broad anti-tumor activity (>40 different lymphoma and carcinoma cell lines *in vitro*), suggesting that very basic cell death pathways are identified and activated in tumor cells. Furthermore, HAMLET has multiple, intracellular targets, and the destruction of their function contributes to cell death, but so far, no single target has been found to account for death. We have used the “hydra” metaphor to illustrate the nature of HAMLET. The hydra in Greek mythology is a serpent like beast with many heads, which kills its prey by combining several lethal properties.

The resistance of healthy cells to HAMLET may appear paradoxical, and the mechanism not known. We have compared the transcriptional response to HAMLET in normal human kidney cells and kidney carcinoma cells and shown that most of the strongly regulated genes were unique for either cell type. While healthy cells showed a strong innate immune response, the tumor cells activated genes implicated in cell death, chromatin re-modulation and ER stress. Results for candidate genes

from the most regulated classes were confirmed at the transcriptional or the protein level. Our results show that the transcriptional response to HAMLET differs between normal and cancer cells. Rather than being inert to HAMLET, healthy cells appear to protect themselves and escape the death response, like Heracles. The identified genes constitute a basis to understand basic differences between tumor cells and healthy cells and to elucidate the mechanism of tumor cell death in response to HAMLET.

Older studies have shown that HAMLET binds to the surface of tumor cells, and that large amounts are taken into the cytoplasm. There is a rapid mitochondrial response and HAMLET binds directly to mitochondria *in vitro*. Furthermore, HAMLET interacts directly with the proteasomes, causing initial proteasome activation and later destruction of the proteasomes. HAMLET impairs the transcriptional and translational machinery of the cell, by interacting with ribosomes and with chromatin, after translocation to the nuclei. In parallel, the cells activate apoptotic and autophagic death responses. Cell death is not due to either of those responses, however, as blocking of apoptosis, autophagy or proteasome activation does not rescue the cells from death. We therefore propose that HAMLET activates a new combination of highly conserved death mechanisms in tumor cells, making this molecule a fascinating therapeutic agent.

In vivo studies in animal models of glioblastoma and bladder cancer have confirmed that HAMLET is tumor selective and has therapeutic efficacy. Furthermore, our clinical trials have shown that HAMLET is an efficient topical agent against skin papillomas and kills bladder cancer cells, resulting in a reduction in tumor size after about one week.

The talk will address information on structural, functional and therapeutic properties of the complex.

Selected references

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- 5) Gustafsson, *et al*, *N Engl J Med*, 2004.
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- 7) Petterson-Kastberg *et al*, *J Mol Biol*, 2009.



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(IS6-4)

1. Speaker

K. Hun Mok

2. Affiliation

Professor, Trinity College Dublin

3. Professional Activities

ULTRA PROGRAMME ADVISOR, 2008-present

Republic of Korea, Ministry of Education, Science and Technology

Protein Folding/Misfolding in Medicine:

Probing the attainment of natively- or alternatively- folded three-dimensional molecular structures

Mok, K. Hun

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School of Biochemistry and Immunology
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Abstract

Amyloidoses are diseases involving abnormal protein aggregation and deposition in tissues by normally soluble proteins, and are well documented in more than twenty clinically defined cases¹. Protein folding research – the study of how the information contained within a given amino acid sequence leads to a functional, three dimensional structure - has evolved to seek how and why such certain amino acid sequences misfold or aggregate to the detriment of the host. Despite very significant advances (e.g. the method of phi-value analysis for studying folding transition states², the concept of energy

landscapes³, the generic nature of amyloid fibrils⁴, and proof confirming the self-replication of infectious scrapie prions⁵), conclusive and concrete answers that can provide predictive and therapeutic powers in ameliorating these diseases have only just begun to show promise. One serendipitous finding is that partially – or alternatively - folded molecules such as HAMLET (Human Alpha-lactalbumin Made LEthal to Tumour cells)⁶ may serve to act as agents in selectively killing tumorigenic cells. Hence along with our understanding that misfolding may incur harm, it is becoming evident that misfolding may also yield benefit to its host, further underscoring the need for detailed, biophysical investigations.

In obtaining atomic-level resolution of the protein folding-misfolding process, NMR spectroscopy has continuously played a significant role⁷. For the past several years, we have been involved in developing novel methodologies in laser-polarized photo-CIDNP⁸ (Chemically- Induced Dynamic Nuclear Polarization) and NMR spectroscopy, with the aim of characterizing ill-defined partially-folded intermediates and denatured states previously intractable with other methods⁹. Such partially folded or denatured molecules have been shown to form protein aggregates, some with morphologies that are strikingly similar to amyloid fibrils found in the tissues of neurodegenerative disease patients¹. To this end, we have designed and constructed an *in situ* injection device that permits rapid homogenous mixing of solutions in the NMR magnet within 50 ms, permitting the characterization of transient, kinetic intermediate species present in real-time protein folding experiments¹⁰. In addition, by CIDNP pulse-labelling the denatured state of the 20-residue Trp-cage (TC5b)¹¹ and transferring its magnetization to the well-defined native state, we have been able to obtain direct NOE-based distances of side chains involved in the residual structure of the ill-characterized denatured state¹².

HAMLET^{6,13}, a potentially new biomolecular assembly that selectively induces apoptosis in tumor cells, is being explored from a biophysical, structure-and-dynamics-based perspective¹⁴. HAMLET, BAMLET (the bovine analogue), and other analogues represent a new type of tumoricidal molecular complex, exhibiting broad activities against tumors from > 40 different lymphomas and carcinomas, yet leaving healthy, differentiated cells unharmed¹⁵. Using a wide range of biophysical techniques (e.g. NMR, EM, AFM, SAXS, etc), the ultrastructural and physico-chemical properties of this complex are being determined and the common structural assembly rules deduced. In addition, the molecular basis of cytotoxic activity – in comparison with those of amyloid pre-fibrils – will be presented.

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IS7

Soil Science/Fertilizer

It's an urban soil you stand on, isn't it?

(IS7-1)

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Urban soils: a new frontier for soil science

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“Increasing urbanization is a worldwide phenomenon. According to the United Nations Population Division, one-half of the world’s population now lives in cities and towns, and by 2030 this will reach 60 percent. Currently there are 20 megacities with more than 10 million inhabitants, many in developing countries. An understanding of the properties, processes, and the ecosystem services of the disturbed soils that are an inevitable consequence of this urbanization has important quality-of-life implications for a large segment of our population. Methods of characterization, classification, and mapping of disturbed soils are required to keep soil science relevant in a changing world. The study of urban and human-altered soils has emerged as one of the important frontiers in the field.” (Introduction to the New York City – 2009 – SUITMA congress – <http://www.suitma2009.com>).

Indeed, for several decades, soil survey and research were largely focused on agricultural and forest land, and intensively managed and disturbed soils in urbanized areas were not much investigated as revealed by the white areas representing most cities on soil maps (De Kimpe and Morel, 2002). Urban soils can be defined as soils resulting from deep transformations of natural soils which have been deepened or augmented through addition of man-made materials (e.g. waste). Their profile composition has been strongly altered by human action, and the original horizon sequence has been generally destroyed. Urban soils are undoubtedly heterogeneous and their characteristics vary considerably over relatively

small geographical centers of urbanization, which make them difficult to study and map.

In this communication, main characteristics of urban soils will be presented, with a special attention to their usage, diversity, classification, functions, evolution, contamination, and remediation. The presentation will show examples to illustrate this various features of urban soils, and will stress on the need for research in this area.

Use of soils in urban areas

Obviously, urban soils are characterized by a wide array of usages (*i.e.* housing, transport, industry, parks, urban forestry, urban agriculture, recreation). A large portion of urban soils are sealed soils. They host cables (*e.g.* electricity, telephone, television), and pipes of various size, age and composition (*e.g.* drinking water, waste water, gas). Open urban soils are the substrate for plants (*e.g.* isolated trees, public parks, ornamental, gardening, urban and sub-urban agriculture). Forestry and agriculture are rather different than in rural zones. Also, urban soils have been used since the beginning for waste disposal which generally occurred at the periphery of residential areas or industrial activities. A main feature of urban soils is the high frequency of usage change with time as a result of economic and politic constraints. An example is the conversion of former industrial sites (brownfield land) to new activities, including residential, public and recreational activities. Changes are often brutal and may cause secondary problems for residents as a result of the possible soil contamination, requiring costly remediation operations. Landfills located at the periphery of cities are, knowingly or not, often converted to residential development as a result of the pressure of increasing urban population. This, of course, may result in increasing risks for residents from contamination through direct contact, inhalation and/or consumption of gardening products.

Diversity, heterogeneity of urban soils

Urban soils are characterized by a strong spatial heterogeneity resulting from the various inputs of exogenous materials, excavation and mixing operations with original soil material. They are generally composed of coarse elements, derived from natural and anthropogenic material (*e.g.* mortar, concrete, asphalt). There are numerous soil types in urban areas as a result of the history of the cities (*e.g.* input of technogenic material, mixing, compaction). In urban areas, a gradient of anthropization can be observed: urban forests are characterized by light human impact, and, on the opposite, in built areas soils are deeply transformed, and ultimately sealed. In general, artificiality increases from the periphery to the center of the city. In older cities, waste material has accumulated over the years, and houses are mostly built on several meters of anthropogenic materials which hold prints of former human activities (*e.g.* Paris, Moscow). Hence, soils are developed on several layers of materials of increasing age with depth. Each layer contains products which are characteristics of the technological evolution of human societies. Urban soils are polycyclic, resulting from the superposition of several young soils. As a result, urban soils hold anthropologic material of great historical interest. Heterogeneity prompts a number of questions regarding the methods of investigation (description of the soil profile, sampling, material, analyses). Despite, mapping has been developed in some cities to providing a tool for land planning.

Classification of urban soils

“Urban soils” can be accepted as a generic expression to define soils deeply affected by human action. Soil classifications refer to anthropic soils, anthroposols or anthrosols, meaning soils extensively influenced by human activities, found mostly in urban areas but not only. They include i) soils composed of a mixture of materials differing from those in adjacent

agricultural or forest areas, and that may present a surface layer greater than 50 cm, deeply transformed by human activity through mixing, importing and exporting material, and by contamination, ii) soils in parks and gardens that are closer to agricultural soils but offer different composition, use and management, and iii) soils that result in the various construction activities in urban areas and which are often sealed.

The WRB has introduced in its revised 2006 edition the group of Technosols (Lehmann, 2007; Rossiter, 2007) which represents soils containing 20 percent or more (by volume, by weighted average) [artefacts](#) in the upper 100 cm from the soil surface or to [continuous rock](#) or a cemented or indurated layer, or a continuous, very slowly permeable to impermeable, constructed geomembrane of any thickness starting within 100 cm of the soil surface, or [technic hard rock](#) starting within 5 cm of the soil surface and covering 95 percent or more of the horizontal extent of the soil. Hence, Technosols are a specific group of urban soils which present characteristics generally not found in natural or slightly modified soils in urban areas. This group deserves special attention regarding its properties and evolution which might be rather different than “normal” soils.

Another definition has been given referring to the use of urban soils, SUITMAs which stands for *Soils in Urban, Industrial, Traffic, Mining and Military Areas*. This definition is also the name of the working group of IUSS dedicated to the study of SUITMAs. SUITMA organizes a series of conferences (*i.e.* Essen 1998, Nancy 2003, Cairo 2005, Nanjing 2007, New York City 2009, Marrakech 2011), and is active at the IUSS congress (*e.g.* Brisbane "*Pedogenesis and functioning of soils in urban and industrial areas*") and EGU Conference. SUITMA is associated to the *Journal of Soils and Sediments* since 2008.

http://maquettewicri.loria.fr/en/wicri-t-sols_urbains/index.php5?title=Soils_of_Urban_Industrial_Traffic_Mining_and_Military_Areas

Functions of Urban soils

Except housing and transport, SUITMAs fulfill several functions and services in urban ecosystems: resource for food through urban agriculture (*e.g.* horticulture, private gardening), fundamental component of urban ecosystems (*e.g.* parks), sink for resources (*e.g.* P), mitigation of global change (*e.g.* C storage, vegetation, green roofs), resource for social stability (*e.g.* gardens, art). However, the high degree of surface sealing limits the possibility to perform the water partitioning that normally exists under natural conditions, causing a high surface run-off and floods. Increasing rainwater infiltration, groundwater recharge, by reducing the rise of discharge are challenges that faces management of urban soils. Urban soils are characterized by a great ecological heterogeneity. They are habitats for plants and soil organisms, and for their filtering, buffering, and transforming capacity of organic and inorganic pollutants. Root depth is often limited due to abrupt horizon transitions, especially in the presence of a large percentage of coarse material (> 2mm). As a medium for plant growth, soil supports a large population of amenity vegetation in diversified habitats, including parks, gardens, roadsides and turf areas. Much of the urban vegetation is cultivated, but there are also relics from the natural vegetation or spontaneous infestation by opportunistic species.

Evolution of soils in urban areas

The fundamental processes of formation and evolution of soils apply to urban soils. The evolution of urban soils is controlled by the same factors as natural soils, but the « human factor » imposes extremely rapid transformation cycles by

comparison with those dominant under natural conditions. The “human factor” often creates a new “parent rock”, and modifies the conditions of its evolution (transformation, mixing, compaction, aeration, excavation, addition of exogenous materials). The kinetics of evolution is very rapid in comparison to natural conditions. Urban soils often hold pollutants which represent a threat for human health as they may be transformed under available forms. Oxidizing conditions generally dominate in urban soils as a result of a rather lack of water in the soil profile. However, soil compaction and leakage of drinking or waste water from pipes may induce locally strong changes in redox and water flux conditions.

Urban soils may exhibit specific pedogenetic processes controlled by the specific parent material. An example is the aggregation process. In Technosols non natural water-stable organo-mineral associations can be found, as observed in samples collected from a former coking plant (Monserié *et al.*, 2009). Thoses aggregates result from both former industrial activities (*e.g.* slags, glass) and neo-aggregation phenomena involving technogenic organic matter, such as hydrocarbons with high C/N values. Also, uncommon particles can be identified, *i.e.* Fe oxide beads, complex mineral materials (slags) as a result of industrial processes at high temperature. Aggregates contribute to the temporary sequestration of contaminants which can be released in the long term as a result of pedogenetic processes causing an environmental risk.

Soil contamination in urban areas

Because of their diversified origin, urban soils may contain pollutants which prompt a strong interest because of the growing public concern about environment and human health (*e.g.* urban horticulture). Also, children are often in direct contact with soil material which may bear pollutants and pathogens (*e.g.* playgrounds). Some soils, in particular those previously used as the support for the industry, may contain amounts of organic (*e.g.* hydrocarbons, polycyclic aromatic hydrocarbons, polychlorobiphenyls, herbicides) as well as inorganic pollutants (heavy metals, As). Heavy metals are present at higher concentration in urban soils than in the corresponding agricultural soils (Cd, Cu, Pb, Zn). In general, alkaline reaction of urban soils limits the mobility of heavy metals.

Gardens are specific sites with strong human influence. They are characterized by a very intensive agriculture, resulting in a high fertility. Garden soils exhibit a large diversity as a result of gardener inputs and actions. Soil quality (nutrients, pollutants) is controlled by parent material, the various anthropogenic inputs (*e.g.* amendments, fertilizers, pesticides, household waste, industrial wastes), and by the modifications of the soil profile (Schwartz and Morel, 1995). In general, rates of application are far higher than in traditional agriculture production, and garden soils have a thick upper horizon with a high content in organic matter and nutrients. Pollutants may accumulate in garden soils, and be transferred to the food chain, by direct consumption of vegetables. Heavy metals accumulate in garden soils at concentration twice that found in agricultural soils, probably because of the input of various amendments to the garden soils which poses risks for those who rely on a sole garden for their vegetable supply.

Soil remediation in urban areas

To reduce risks for populations, control of pollutants in urban soils is of utmost importance. When soils are declared “contaminated” in relation to regulations or to a specific usage, they must be treated. In general, there are three main approaches to deal with soil contaminants: extraction, degradation or immobilization. All have the same goal: to decrease the transfer risk. Treatment can be *in situ*, on site or off site. In the case of high pressure from urban development (*e.g.*

housing, industries), contaminated soils are generally removed and treated on site with mobile units (*e.g.* thermal treatment, biopile) the soil being reused on the site, or off site with transport to decontamination plants. However, for large contaminated areas, there is no economically and technically feasible technique except natural attenuation and phytoremediation. In such case, safety measures must be taken to protect populations and adjacent ecosystems (*e.g.* water bodies). These sites may provide services such as C storage, biomass production, biodiversity.

In urban areas, it is more and more necessary to substitute natural processes by anthropogenic actions to construct soils designated to reclaim derelict land (*e.g.* brownfield sites), to complete civil engineering operations (*e.g.* ditches along roads, lawns), and to fulfill specific functions (*e.g.* recreation areas). A sustainable soil construction relies on the use of material derived from urban and industrial activities (*e.g.* by-products, organic wastes, green compost, treated soil material) instead of using agricultural top soil (Séré *et al.*, 2008). In the process, materials are combined in order to create favorable conditions for plant growth in the long run allowing ecological reclamation. An example is a process which combines thermally treated soil, paper mill sludge, and green waste compost. The soil profile undergoes a rapid evolution as a result of a composition in fine material with high specific surface, and a behavior similar as natural soils (*i.e.* water cycle, filtration, biomass production). Restoration of soil functions according to this process is the first step for ecological reclamation of derelict lands.

Another pedo-engineering operation under strong development is green roof technology. Green roofs have been used for several centuries, but, according to the recent social demand in “green” management in cities for landscape, water cycling, and mitigation of heat waves, they are extending dramatically. Beside plant growth, green roofs are expected to fulfill other functions, such as water filtration, particle retention, biodiversity, and food production through roof gardening. Their increasing importance is connected to the lack of available space on the ground for plant growth.

Conclusion

Urban soils are diverse and heterogeneous, and they fulfill primary functions of utmost importance in urban ecosystems. However, knowledge related to urban soils is only fragmentary, which hinders their fundamental roles and impairs the management in urban areas. Urban soils are a part of the answer imposed to humankind to meet major challenges (food, health, prosperity). It is only through a multi-disciplinary approach that urban soils will be better understood, and their use optimized to protect human health and quality: disciplines directly connected to soil science (soil, geology, agronomy, chemistry, physics, environment, forestry, horticulture, ecology), or to other communities (*e.g.* economists, lawyers, policy-makers, engineers, health, land planners, companies, citizens). Advance in knowledge will increase the impact of soil science in the decision-making process for urban land management. Among the most urgent issues related to urban soils are the competition for land which leads to the loss of arable land, soil degradation, urban agriculture, health-soil related problems, soil management, soil remediation, land planning, health, sociology.

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(IS7-2)

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Are roadsides suitable for trees?

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Trees in urban area have significant meaning to people. They provide greenness to urban people, remove carbon dioxide, provide oxygen, etc. Therefore people, especially urban people plant trees along the streets. The qualification for roadside trees are stateliness, symmetry of growth, large and abundant leaves, healthiness, ease for transplanting, rapid growth, patience under difficulties, beautiful and fragrant flowers, leaves that have rich autumn tints, etc. The objectives of this study were to investigate the present contamination level of the soils along the major roadsides of Seoul, the capital of Korea and to prepare base-line data for the future. Five districts (“gu” in Korea; Jungnang-, Gangdong-, Gwangjin-, Nowon-, Sungdong-gu) among 25 in Seoul were selected for this study. For each gu, 50 sampling sites were selected along the major roadsides based mostly on traffic. At each site both topsoil (1-5 cm) and subsoil (20-50 cm) samples were taken. The number of total samples was 250 for topsoil and 250 for subsoil. The collected samples were air-dried, passed through 2 mm sieves, and analyzed to determine the physicochemical properties of the soils including pH, the contents of heavy metals (Cd, Cu, Pb, Zn) and anions (Cl, NO₃, SO₄). Soil textures of topsoils and subsoils were mainly loamy sand and sandy loam, respectively. The range of the pH was 4.5-10.0 with an average of 7.5 for both topsoils and subsoils, which is much higher than that of the forest soils in Seoul. The ranges of 0.1 N HCl extractable Cd, Cu, Pb and Zn contents for both topsoils and subsoils were N.D. (not detected)-1.19, N.D.-228.99, N.D.-352.54 and 2.97-511.56 mg kg⁻¹, respectively (Table 1). Most of the average heavy metal contents were lower than the warning standard of the Soil Environment Conservation Act of Korea whereas they were much higher than those of the forest soils in Seoul. There were some sites

whose heavy metal contents were higher than those in the warning standard. In particular, the average Cu content in Sungdong-gu was much higher than the warning standard, 50 mg kg⁻¹. Anion concentrations were also much higher than those of the forest soils in Seoul (Table 2). A careful management of the soil to prevent the aggravation of the present contamination level, to help trees to live in better soil environment and to prevent the dissemination of contamination is highly recommended.

Table 1. Distribution of Cd, Cu, Pb and Zn concentration in soils from the study area.

Area	Depth	Cd Cu Pb Zn				
		----- mg kg ⁻¹ -----				
Jungnang-gu	Topsoil	Mean	0.17	23.83	20.63	162.74
		Range	0.02-0.56	0.51-52.46	0.57-352.54	15.90-332.96
	Subsoil	Mean	0.08	12.80	13.69	39.36
		Range	N.D * .-0.48	2.90-48.95	1.33-143.53	3.52-206.98
Gangdong-gu	Topsoil	Mean	0.28	15.04	17.89	68.66
		Range	0.18-0.42	1.65-44.51	2.39-98.55	9.32-197.80
	Subsoil	Mean	0.22	6.17	8.69	19.21
		Range	0.11-0.31	0.69-29.83	N.D.-55.69	4.23-55.69
Gwangjin-gu	Topsoil	Mean	0.21	17.17	23.91	129.36
		Range	0.01-0.71	0.32-50.32	0.49-221.45	9.01-198.37
	Subsoil	Mean	0.12	11.21	15.71	89.31
		Range	0.03-0.61	2.28-37.95	1.54-161.91	12.03-161.91
Nowon-gu	Topsoil	Mean	0.16	15.08	14.20	93.94
		Range	0.01-0.54	N.D.-36.86	1.59-41.42	2.97-301.18
	Subsoil	Mean	0.08	8.80	11.09	36.80
		Range	N.D.-0.18	1.27-33.39	2.15-123.20	3.66-123.20
Sungdong-gu	Topsoil	Mean	0.58	50.05	14.66	184.99
		Range	0.45-1.19	3.03-228.99	N.D.-70.01	25.37-511.56
	Subsoil	Mean	0.53	45.54	25.61	106.57
		Range	0.41-0.79	N.D.-171.51	N.D.-239.47	27.36-239.47

Table 2. Concentration of anions in soils from the study area.

Depth		Cl	NO ₃	SO ₄
		----- mg kg ⁻¹ -----		
Topsoil	Mean	79.85	286.80	134.31
	Range	13.78-224.20	32.62-624.46	24.88-403.84
Subsoil	Mean	32.12	23.95	55.75
	Range	14.16-66.44	N.D* -.56.36	21.98-106.02

* Not detected.

(IS7-3)

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Soil material for Green Roofs

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The urbanization of the land brings significant changes in the physical properties of land surface, since it replaces vegetation and soils with hard and impervious surfaces such as buildings, streets, driveways and so on. These impermeable surfaces block or distort the natural circulation of water, material and energy. Green roofs have been installed in urban areas to mitigate the adverse effects of the urbanization. A green roof is a roof of a building that is partially or completely covered with vegetation and a growing medium, planted over a waterproof membrane. So they are called vegetated roofs or living roofs. Green roofs provide many benefits as compared to traditional roofs including reduction of rainfall runoff, mitigation of the urban heat island effect, energy conservation, increased longevity of roofing membranes, reduction in noise and air pollution, increased urban biodiversity, as well as aesthetically pleasing living environment.

Green roofs usually are categorized as intensive, semi-intensive or extensive, based on the depth of planting medium (or soil/substrate layer) and the amount of maintenance they need. Intensive green roofs are similar to traditional roof gardens which require a reasonable depth of soil (more than 150mm) to grow large plants or conventional lawns. They are labour-intensive, require irrigation, fertilization and other maintenance. So they require high cost. Extensive green roofs, by contrast, are designed to be virtually self-sustaining and should require only a minimum of maintenance. Extensive roofs are usually only accessed for maintenance. They can be established on a very thin layer of soil (less than 150mm). Semi-intensive green roofs in terms of requirements fall in between extensive and intensive green roofs. More maintenance, higher costs and more weight are the characteristics for the intermediate green roof type compared to that of extensive green roofs.

Table 1. General features of the green roof types

	Extensive	Semi-intensive	Intensive
Use	Ecological landscape	Garden/ecological landscape	Garden/park
Vegetation	Moss, sedum, herbs, grass	Grass, herbs, shrubs	Lawn, shrubs, trees
Soil depth	< 150mm	120~250mm	> 150mm
Weight	60~150kg/m ²	120~200kg/m ²	180~500kg/m ²
Maintenance	Low	Periodical	High
Cost	Low	Middle	High

Soils (or substrates) are major component of green roofs. Green roof soils (or substrates) are required to be light weight, chemically and physically stable, hold adequate amounts of water and nutrients for plant survival, but also to be free draining so plant roots don't become saturated. Recycled crushed brick and roofing tiles, steel mill slag and light expanded clay aggregate are utilized extensively in Europe. In North America, pumice and heat expanded shale and slate are common materials. In Korea, perlite and expanded vermiculite are used nationwide. Recently bottom ash, mainly produced from the burning of coal for electricity, is considered.

Since green roof soils must be designed in concert with all other major design elements of a green roof project, it is difficult to make any standard for the proper green roof soils. Soil specifications for a green roof are determined by a number of factors, including allowable depth and weight, climate and whether the roof will be irrigated. To keep costs down and ensure success it should specify the use of locally available materials and be suited to the specific plants that are to be grown. The criteria for growing media specifications for green roofs are prepared in Germany; grain size distribution, density, water and air capacity, pH, lime and salt content, organic matter content, nutrient condition and cation exchange capacity. German criteria are the standards worldwide. However many countries are researching to make their own criteria on the basis of German ones.

Green roofs are becoming more and more prevalent as their sustainability, energy saving properties and ecological benefits are becoming better understood. Due to the specialization required for green roofs, researchers and developers of engineered soil need to fully understand the importance of all factors that will spell success or failure of a green roof media. It is sure that soils on green roofs are not only subject but also opportunities to soil scientists.



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(IS7-4)

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Urban Soils - paved but still functioning

Urban soils are anthropic soils, which are situated inside (recent or former) urban areas (Morel, 2005). They may contain technogenic materials or they may be sealed. Both heavily influence their functions for the urban ecosystem.

Soil sealing is a very widespread soil alteration during urbanisation (Wessolek et al., 2010). The paved surface not only changes the water but also the gas exchange of the underlying soils. However, partly sealed soils, or so called permeable pavements still allow some exchange of water and gases and therefore are to favour before other types of sealing like tar or impervious concrete.

In this contribution, the following questions are focussed: (i) how is the water affected by pavings? What are the typical contaminants, how is the water re-distributed after reaching the surface? What is the impact on flow behaviour and how does this affect the infiltration and quality of groundwater recharge? (ii) What are the options to treat rainwater in urban areas? What is the status quo, where are the potentials to increase the functionality of the ecosystem?

(i) Paved surfaces are often situated near streets or other sources of contaminants such as heavy metals (Nehls et al, 2008) or pesticides, which are applied for sidewalk maintenance (Klingelmann, 2009). Due to the geometric forms of permeable pavings, the impervious pavers and the open pavement seams, water is likely to infiltrate in preferential pathways, as found by dye tracer experiments (Nehls, 2007). This may lead to preferential leaching of contaminants as it was shown for glyphosate (Klingelmann, 2009). More relevant, the contaminants reach surface water bodies by runoff and combined sewage systems. Especially the combined sewage systems cause severe ecological problems in urban water bodies (Heinzmann, 1998).

(ii) Therefore, different initiatives are started to reduce the amount of water, which runs off of paved surfaces. Among the options of rainwater treatment in urban areas there is infiltration but also evaporation. Evaporation may be reached by façade greening or just by substituting traditional pavers by especially ecologically designed pavers. A measure like this can enhance the evaporation dramatically as this will be shown in the talk.

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(IS7-5)

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Past, Present and Future of the Worldcup Park, a Case Study

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Parks play very important roles to human being, especially people who live in urban area. Nanjido which had been a beautiful island filled with orchids and other seasonal flowers was converted into a landfill in the middle of the rapid urbanization of Seoul in 1978. From that time to 1993, 92 million tons of garbage including household wastes and construction and industrial wastes were dumped on the island, resulting in two massive mountains of garbage measuring over 90 meters in height. In this presentation I want to share the invaluable experience of changing a former landfill site into an environmentally- as well as eco-friendly park, the Worldcup Park, as a case study.

1. Story of Nanjido

Scent of an Orchid

Nanjido was once a beautiful island filled with orchids, gromwells and other flowers of the season. It was also known for its crystal-clear brooks and varied aquatic plants and animals. In the winter, it was a haven for scores of migratory birds including swans and ducks.

Island of Spotted Cantaloupe and Peanuts

Prior to 1978, the year the island was changed into a landfill, Nanjido was a peaceful island where various flowers, cabbage, radish and peanuts were widely cultivated. In particular, the size of the spotted cantaloupes grown on Nanjido was so big that an adult could not wrap their arms fully around their diameter. As well, Nanjido's peanut crops were considerable enough to occupy 30 percent of the nation's total peanut production.

Mountains of Garbage

Nanjido was converted into a landfill in the middle of the rapid urbanization of Seoul in 1978. From that time to 1993, 92 million tons of garbage including household wastes and construction and industrial wastes were dumped on the island, resulting in two massive mountains of garbage measuring over 90 meters in height.

Rethinking Nanjido

As time went on, the Seoul government began to rethink Nanjido, recognizing that the island could not and should not be passed down to the next generation in the state it was in. Thus, efforts to transform Nanjido into an environment-friendly park began. World Cup Park is the stunning result of those efforts.

2. Story of The Landfill Recovery Project

The Landfill Recovery Project is extremely meaningful as our first effort to create a new history of Seoul. The project consisted of treatment of landfill gas and polluted water discharged from waste and the stabilization process of reclaimed land. Starting with a master plan in 1994, we completed facilities for extraction and disposal of gas and contaminated water and covered up the unstable slant through August 2001.

Extraction and Recycling of Landfill Gas

Since November 2001, methane gas generated from the Nanjido landfill has been recycled as fuel for Seoul World Cup Stadium and an apartment complex nearby Nanjido. Thus, the recycling of methane gas has achieved two things: environmental improvement of the Nanjido area and the realization of a new energy source. The recycled gas will be supplied for the Sangam area and the Digital Media City as well.

3. Worldcup park

Pyounghwa Park (446,000 m²)

The entire Pyounghwa Park (= peace park) stands as a symbol of the peace that is achieved when humans make the effort to coexist harmoniously with nature. In particular, the park consists of UNICEF(United Nations Children's Fund) Plaza, a futuristic open space, and Nanji Lake, which is a tributary of the Han River measuring 24,000 square meters. Various aquatic plants live in the lake. Himang Forest (= hope forest), created from the Planting of 10 Million Trees of Life, provides a wonderful resting place as well as opportunities for the public to expand their knowledge of the park and the environment in general.

Haneul Park (192,00 m²)

Haneul Park (= sky park) is located in the highest place within World Cup Park. A View Point is available from which visitors can look over Seoul's great scenery such as Mt. Bukhan, Mt. Namsan, and the Han River. Vast grasslands were created by placing eulalia and cogongrass in the northern part of the park and sunflowers and buckwheat in its southern part. A natural ecosystem was finally established through releasing 30 thousand butterflies across the park.

Wind Power Generator

The five vanes installed in Haneul Park are wind power generators which produce electricity by wind. The electricity from the generators is used as a nonpolluting energy resource for the street lamps at World Cup Park and the guard post.

Noeul Park (340,000 m²)

Noeul Park (= red sky park) was originally constructed as a golf course when it first opened in 2002. Then citizens of Seoul and civil groups started an activity to change this golf course into a park. Seoul Metropolitan Government changed its original plan so that citizens of Seoul can enjoy this area for ecological and family park. Camp sites, nature experiencing area were open. Facilities such as public toilets and shades were expanded.

Nanjicheon Park (294,000 m²)

Nanjicheon Park is located where several branches of the Han River, like Bulgwangcheon, Hongjecheon, Nanjicheon and Hyangdongcheon, converge. Formerly an abandoned brook polluted from waste, Nanjicheon Park has been revived as a natural creek where diverse plants grow in groups and birds and fish visit and stay.

The park is filled with reeds and willows and a forest of 50,000 trees thickly covers the site. In addition, about 5,000 tons of water is streamed into the brook per day from Nanji Lake. Along the promenade, there are bicycle paths and an in-line skating rink, as well as separate resting spaces for handicapped people, senior citizens, youths, and the general public.

4. The Revival of Nanjido

Waste was not the only thing thrown away. After the landfill, Nanjido was abandoned, too. However, the people's abandonment of Nanjido was the key to its survival. Nobody expected life to spring up here because leachate and methane gas continued to seep out after the landfill was closed. However, the power of nature proved to be beyond our imagination. After closing the Nanjido landfill in 1993, the Seoul Metropolitan Government covered the waste with a 1m layer of soil mainly in order to block the odors. But a miracle took place. Slowly at first, but steadily, small lives began to spring up. The speed of rejuvenation would later become amazing.

The first creatures which came to the Nanjido were naturalized plants. Naturalized plants that were moved from foreign countries to our country by the people and increased and lived by themselves for several generations played the role of pioneer in abandoned lands or developed areas. The reason why the variety of naturalized plants were grown up in Nanjido was seeds in waste had the characteristics of being grown well in dry soils like the ones in there.

Plants of Nanjido

Nanjido has turned green since the Landfill Recovery Project began. On the plateau which crowns the mountains of waste is natural grassland and young willow trees, three to four meters tall, standing with their branches surrendered to winds.



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Eighty-five varieties of naturalized plants live there, including annuals like *Ambrosia artemisiifolia* var. *elator*, *Ambrosia trifida*, *Lactuca scarida* L., *Erigeron annuus*, and sundrops and perennials like *Helianthus tuberosus* and *Phragmites japonica*. In addition, indigenous plants such as eulalias and mugworts are abundant.

Animals of Nanjido

Not only plants, but also animals began to visit Nanjido after the closure of the landfill. Because plants grew and human traces could not be found, Nanjido soon became a haven for animals. In the winter season, migratory birds visit Nanjido. Small meadow buntings like black-faced buntings, yellow faced buntings, flycatchers, red-tailed robins and parrotbills, are found in the winter bush warblers, cuckoos, Korean nightingales, and white herons are observed in summer. Residents such as redstart, parrotbill, yellow-throated bunting, shrike and skylark are seen in Nanjido through four seasons, and water birds such as teals and spot-billed ducks flock in Nanjicheon. As the number of birds increased, even birds of prey could be found at Nanjido. Surprisingly, endangered species such as eagle owls have been spotted here. Rare and endangered species including kestrels, Korean buzzards and upland buzzards inhabit Nanjido.

Amphibia and reptile that are very sensitive to surrounding environmental conditions are found to live diversely in Nanjido. Including Korean narrow-mouthed frog protected by the Ministry of Environment, black-spotted frog, tree frog, viper, garter snake and grass snake are stationed in Nanjido. Insects are no exception to this phenomenon. According to the "Survey on the Distribution Change of Species in Seoul" prepared by the Seoul Development Institute in 1999, more than 70 species of insects were found, including *Fabriciana nerippe*, which was designated as an insect to be protected by the Ministry of Environment. The Revival of Nanjido. The Seoul Metropolitan Government started the project to develop Nanjido here. The project intends to turn Nanjido into a beautiful park so that the revived ecosystem can be preserved for citizens.