

# Translational Genomics on a Global Scale: Working at the Plant-Human Interface

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We are in the midst of a golden period for research into plant metabolism in which the ongoing development and integration of various omics-technologies with established biochemical, molecular and genetic approaches have created a near “perfect storm” for plant metabolism research. Plant biochemists can now dissect pathways computationally, genetically and biologically between organisms, genomes and across evolutionary time. My laboratory has taken a leading role in targeting study of the synthesis of nutritionally important components in model plants with the intent of bridging this research into crops, an area we refer to as Nutritional Genomics. This is an exciting area of plant biology that allows one to work at the interface of plant biochemistry, genetics, genomics, human nutrition and agriculture to potentially address long standing problems on a global scale. Current research areas that will be touched upon in this talk include the synthesis and function of the plastid-localized isoprenoids tocopherols (vitamin E) and carotenoids (provitamin A) and the use of natural variation to understand the genetic and biochemical basis of mineral bioavailability from plant foods.

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## Gossypol: Biosynthesis and Plant-Insect Interactions

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Cotton plants accumulate gossypol and related cadinene-type sesquiterpene aldehydes, which act as chemical defenses against pathogens and herbivores. We have characterized cotton farnesyl diphosphate synthase (FDP) (FPS), (+)-delta-cadinene synthase (CAD1, a sesquiterpene synthase) and (+)-delta-cadinene-8-hydroxylase (CYP706B1, a P450 monooxygenase), these enzymes catalyze three consecutive steps from the five carbon isoprenoid unit to 8-hydroxy-(+)-delta-cadinene, a key precursor to gossypol. The genome of *Gossypium arboreum*, a diploid cotton species, contains a complex gene family of *CAD1* but a single copy for *CYP706B1*; this P450 holds a great potential in manipulating gossypol biosynthesis through genetic engineering.

Although cotton sesquiterpene aldehydes are constitutively stored in pigmented glands of aerial organs and in roots, gossypol biosynthesis and expression of gossypol pathway genes are inducible by microbial elicitors and by methyl jasmonate. A WRKY transcription factor, GaWRKY1, has been shown to be able to activate *CAD1-A* gene expression.

Cotton bollworm (*Helicoverpa armigera*) is a devastating insect pest of cotton, despite that gossypol is generally toxic. In insects, cytochrome P450 monooxygenases play a central role in defense reactions against plant secondary metabolites and xenobiotics. We have isolated a gossypol-inducible P450 gene, *CYP6AE14*, from cotton bollworm. *CYP6AE14* is highly expressed in midgut, and its induced expression is correlated with larval growth if gossypol was present in diet. To knock-down *CYP6AE14* expression, we engineered *Arabidopsis* and tobacco plants to express the dsRNA that targets *CYP6AE14*. When cotton bollworm larvae were fed with *AtdsCYP6AE14-3* leaves, the *CYP6AE14* transcript level in midgut was decreased. As a result of *CYP6AE14* suppression, the worms showed stunted growth in comparison with control, and the inhibitive effect of dsRNA-expressing plants became more dramatic when gossypol was administrated. These results suggest that *CYP6AE14* has played a role in cotton bollworm adaptation to cotton plants that contain gossypol.

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# The use of regulatory genes in metabolic engineering to produce ‘healthy foods’

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Plants produce a very broad array of metabolites, which are not essential for growth, but are used to provide protection against stress and pathogens, to attract pollinators and dispersal agents and as signals for development. These are often referred to as ‘secondary metabolites’ but are known more generally as plant ‘natural products’. Natural products have recently been recognised as important components of the diet, offering protection against cardiovascular diseases, certain cancers and age-related degenerative diseases. They are also important components of beauty products and natural remedies for diseases.

Plants often accumulate their natural products to relatively low levels, so there is interest in breeding or engineering plants that produce higher levels. The most effective way to increase the accumulation of secondary metabolites is to increase the activity of genes that regulate the activity of the biosynthetic pathways. The effectiveness of metabolic engineering using genes encoding transcription factors has been demonstrated by the production of high-flavonol and high-anthocyanin tomatoes which have 3-4 fold higher antioxidant capacities. These have the potential to offer protection against a range of diseases when included as part of the diet and are currently being assessed on animal models of cardiovascular disease and cancer.

## Metabolomics-a tool in diagnostics pathway detection and systems biology

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A new quantitative strategy for high resolution metabolomic profiling, based on the combination of Fourier Transform Ion Cyclotron Resonance Mass Spectrometry and <sup>13</sup>C-isotope labelling of entire metabolomes was developed. The strategy is based on the pair-wise comparison of chemical formulas identified from metabolite extracts of *Arabidopsis thaliana* plants grown under identical conditions but with different, namely <sup>12</sup>CO<sub>2</sub> or <sup>13</sup>CO<sub>2</sub>, carbon sources. These differentially isotope labelled metabolite extracts were infused into the mass spectrometer and the derived mass lists were searched against <sup>12</sup>C or <sup>13</sup>C metabolite databases. The Chemical sum formulas matching for both, the <sup>12</sup>C and <sup>13</sup>C samples reveal the biological compounds present in the plants.

Applying this strategy to metabolite extracts of *Arabidopsis thaliana* resulted, not only in the reproducible identification of 1,024 unambiguous chemical sum formulas, but also permitted an accurate (relative) quantification of all detectable <sup>12</sup>C/<sup>13</sup>C peak pairs, without any chromatographic separation.

Furthermore the differential analysis of the dataset allowed for the prediction of thus far un-described pathways in *Arabidopsis thaliana*, based on the accumulation of unambiguously identified compounds, in specific pathways described by the KEGG database. Amongst the most prominent examples is a new lysine degradation pathway, a pathway for alkaloid biosynthesis, arachidonic acid metabolism or tryptophane metabolism.

The application of these tools for diagnostic purposes in both agricultural and medical problems as well as systems biology will be described.

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# Integration of Metabolomics and Transcriptomics for Phytochemical Genomics in Arabidopsis and Non-model Plants

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The completion of the whole genome sequence of *Arabidopsis thaliana* has made it possible to discover the genes involved in metabolism in a high throughput manner by determining gene-to-metabolite correlation through the comprehensive analysis of metabolite accumulation and gene expression [1]. *In silico* co-expression analysis of genes involved in flavonoid metabolism in Arabidopsis was performed using a publicly available transcriptome database of DNA microarrays. We inferred a co-expression framework model of the genes involved in the pathways of flavonol, anthocyanin, and proanthocyanidin synthesis, suggesting specific functions and co-regulation of the genes of pathway enzymes and transcription factors. Changes in flavonoid profiles of wild-type plants and T-DNA insertion mutants of the delimited genes led to the confirmation of gene function [2]. We also applied this strategy to glucosinolate biosynthetic pathway for identification of MYB transcription factors crucial for aliphatic glucosinolate production [3]. Unbiased metabolome analysis of Arabidopsis mutants led to the finding of novel metabolic networks emerged by the knockout mutation of *TT4* (chalcone synthase gene) [4]. These results suggest that the functional genomics approach by integration of metabolome with transcriptome co-expression analysis provides an efficient way of identifying novel gene functions and metabolic networks involved in plant secondary metabolism. This strategy is principally applicable to decipher the function of genes not only for a model plant *A. thaliana* but also for unexplored plants rich with a variety of phytochemicals, such as *Ophiorrhiza pumila* root cultures producing an anti-cancer alkaloid camptothecin [5, 6].

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## **Integration of genome-scale proteome and gene expression information to explore metabolic pathway regulation**

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The analysis of metabolic and gene regulatory networks was greatly advanced by the availability of large data sets from high-throughput technologies such as DNA microarrays, shot-gun proteomics and metabolomic profiling. The genome-wide, parallel monitoring of gene expression at different levels (genome, proteome, metabolome) will increase our understanding of the molecular basis of pathway functions and their cellular network context. In simple eukaryotes or prokaryotes, gene expression data has been combined with two-hybrid data and phenotypic data to successfully predict protein networks and their transcriptional regulation on a large scale. Similar datasets are starting to come online for plants as well. As a first step to integrate gene expression and proteins at a genome scale level, we have developed a comprehensive database of more than 15,000 proteins identified from high throughput proteome analysis of Arabidopsis (<http://www.atproteome.ethz.ch>). This information, when combined with large-scale gene expression data and modeling approaches (<http://www.geneinvestigator.ethz.ch>), allows us to make predictions on pathway abundance and regulatory mechanisms. Hypotheses can then be validated independently using Geneinvestigator<sup>®</sup> V.3, a novel powerful software suite for visualization of microarray and other data in their biological context.

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# Purification and Proteomic Analysis of Plant Trans-Golgi Network (TGN)

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We have recently demonstrated that the secretory trans-Golgi network (TGN) also served as an early endosome in the endocytic pathway, where the TGN was defined by the rice Secretory Carrier Membrane Protein 1 (SCAMP1). To further study the roles of TGN in mediating protein traffic in the secretory pathway, endocytosis and exocytosis, we have developed protocols to purify SCAMP1-enriched TGN fractions from *Arabidopsis* cultured cells for proteomic analysis. LC-MS-MS analysis of the purified TGN has identified more than 300 *Arabidopsis* proteins. Some of these proteins are predicted to play roles in cell wall biosynthesis, indicating their vesicular transport pathway from TGN to reach their destination for the biosynthesis and modifications of cell wall materials. Current studies focus on characterization of selective TGN proteins for their roles in protein traffic and cell wall biosynthesis in *Arabidopsis*. Supported grants from Research Grants Council of Hong Kong (CUHK4307/03M, CUHK4580/05M and CUHK4887/07M), UGC-AoE, NSFC (30529001) and 863 Program (2007AA02Z102) to L. Jiang.

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## **Plant endomembrane system and chemical genomics**

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# Novel Approaches for Inhibitor Screening and Target Identification using *Arabidopsis* Full-Length cDNA Overexpression (FOX) Lines

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Organisms have two possible routes for the biosynthesis of isoprenoids; the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways. The MEP pathway has been recently recognized as important target of drug and agrochemical developments and chemical genetics studies, *e.g.* fosmidomycin shows antimalarial activity by inhibition of 1-deoxyxylulose-5-phosphate reductoisomerase (DXR), and clomazone has been used for herbicide, which metabolite ketoclomazone inhibits 1-deoxyxylulose-5-phosphate synthase. Plants have all of MEP pathway enzymes in plastids, so that the MEP pathway inhibitors generally reduce the levels of carotenoids and chlorophylls and result in a bleached phenotype. However, the transgenic plants overexpressing a target enzyme in the MEP pathway show resistance to bleaching effect of these inhibitors. Taking this advantage, we have developed a phenotypic screening system for the MEP pathway inhibitors using *Arabidopsis* overexpression lines, and indeed we obtained candidates from synthetic chemical libraries.

Target identification is an integral part of chemical genetics studies and its most rate-limiting part. Commonly used approaches in target identification are the pull-down system and the resistant mutant screening system. The pull-down system involves stable immobilization of ligand molecule to affinity matrix, and the resistant mutant screening system often takes long time to identify the target gene from multiple mutant alleles. On the biochemical basis that transgenic plants overexpressing a target protein become resistant to its inhibitor, we have developed a novel system for phenotypic screening of resistant mutants using *Arabidopsis* full-length cDNA overexpression (FOX) lines. By use of fosmidomycin as a model inhibitor, we could efficiently screen-out its target gene (DXR) overexpression mutants as well as other FSM-resistant mutants from FOX lines. Our novel phenotypic screening system using FOX lines can be a conventional method of target identification in chemical genetics studies as well as drug and agrochemical developments.

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# Molecular Biology and Genetics of Nicotine Biosynthesis and Transport

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Nicotine and related pyridine alkaloids are synthesized in the root and then transported to the aerial parts of tobacco plants [1]. Expression of biosynthetic genes for these alkaloids is enhanced 3–4-fold upon insect herbivory to the leaf. Current model suggests that jasmonate acts as a transmissible signal from the damaged leaf to the underground part, where it activates structural genes of nicotine biosynthesis via the conserved COI1-JAZ pathway. In tobacco, regulatory NIC loci specifically control expression of enzyme and transporter genes involved in nicotine biosynthesis and transport [2]. These nicotine-related genes contain G-boxes in their promoter regions, which bind several MYC-related transcriptional factors. It is interesting to know the molecular identity of NIC regulatory genes and their relationship to the conserved COI1-JAZ pathway.

In the root cells, nicotine biosynthesis occurs in multiple subcellular compartments. The pyridine moiety of nicotine is derived from nicotinic acid or its metabolite, which is synthesized from aspartate via a salvage pathway of NAD synthesis [3]. The enzymes involved in the pyridine sub-pathway of nicotine biosynthesis are localized in the plastid. We also found that an oxidoreductase which functions late in nicotine synthesis is localized in the vacuole. Compartmentation of nicotine biosynthetic steps in several subcellular organelles requires intra-cellular transport of pathway intermediates between different compartments. The final product nicotine needs to be efficiently transported from the vacuole to the cytoplasm then to the apoplast in order to enter the xylem for the root-to-shoot transport.

Recently we are trying to tap on natural variations in alkaloid accumulation patterns in wild *Nicotiana* species. Several closely-related wild *Nicotiana* species accumulate different pyridine alkaloids in the leaf. Some species appear to be deficient in transporting alkaloids from the root to the aerial part. Genetic analysis of these natural variations will broaden our understanding on alkaloid synthesis and accumulation.

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## Studies on the regulation of fatty acid metabolism in *B. napus* seeds

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Multiple high-throughput genomic approaches were carried out to study the gene expression profiles during *Brassica napus* seed development and fatty acid (FA) metabolism, as well as the relevant regulatory networks. Serial Analysis of Gene Expression (SAGE) revealed the transcriptome of approximately 35,000 transcripts of *B. napus* developing seeds. Additionally, based on the obtained 8462 uni-ESTs, the FA biosynthesis-related genes of *B. napus* were systemically identified and gene expression profiles were studied through cDNA chip hybridization. Results showed that 17-21 days after flowering (DAF) was a crucial stage for transition of seed to sink tissue, and high expressions of FA biosynthesis-related genes are mainly from 21 DAF. Compared to *Arabidopsis*, more critical roles of starch metabolism are detected for *B. napus* seed FA metabolism and storage components accumulation. Effects of carbon flux, oxidative pentose phosphate pathway (OPPP), photosynthesis, and other regulators in *B. napus* FA metabolism will be discussed.

# Study of Secondary Metabolites from Medicinal Plants in Yunnan

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Yunnan province of China is called “Plant Kingdom” due to its plant biodiversity in the world. Plants elaborated a vast array of natural products (secondary metabolites), which contributed a lot of lead structures for developing new drugs. On the other hand, plants have evolved multiple mechanisms to selectively suppress pathogens by production of secondary metabolites with antimicrobial and antiviral activities. Therefore, direct selections for antiviral compounds from plants can be used to identify new agents with potent antiviral activity but not toxic to hosts. Aim to discovery of new structure/framework or new action mechanism of natural products, our research group devoted ourselves to study of secondary metabolites from medicinal plants distributed in Yunnan Province, China. Here we report some results of our approach of chemical and biochemical studies of secondary metabolites from medicinal plants.

# Strategies in the search for new bioactive plant secondary metabolites

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Despite tremendous progress in the development of new drugs using biotechnology, genetics and genomics, the plant kingdom remains an almost unexploited reservoir of new molecules to be discovered. In the field of cancer therapy, *Taxus* constituents play a major role to treat breast, ovarian and lung cancers and alkaloids issued from the Chinese plant *Camptotheca acuminata* (Nyssaceae) are used worldwide to fight colon, ovarian and bronchial cancers. For slowing down the progression of Alzheimer's disease, Nature afforded galanthamine, from *Galanthus* species (Amaryllidaceae) and huperzine A from the Chinese clubmoss *Huperzia serrata* (Lycopodiaceae). Extracts of *Ginkgo biloba* (Ginkgoaceae) are increasingly consumed to treat senile dementia associated with Alzheimer's disease. *Hypericum perforatum* extracts, despite the fact that some drug interactions have been reported, are still used for the treatment of depression. Its photosensitizing red pigment hypericin, a naphthodianthrone, could become an essential weapon in killing cancer with light (photodynamic cancer therapy). Many drugs and lead compounds which could become drugs will still come from plants.

The approach used to find bioactive plant constituents will be presented. It includes the selection of the plants to be investigated, the preparation of extracts and their biological and chemical screening. Hyphenated techniques such as the coupling of HPLC with UV spectroscopy, mass spectrometry and NMR are essential for the rapid identification of compounds of interest. The whole approach will be illustrated by the search for new antifungal agents against *Candida albicans* and new inhibitors of acetylcholinesterase which could find application in the treatment of Alzheimer's disease. Other examples of plant constituents with a potential to treat problems related to aging will also be presented.

# Plant secondary metabolites: from metabolic networks to volatile emission

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Volatile compounds released from leaves, flowers, fruits, and roots play important roles in plant life as attractants, repellents, and signal molecules. While volatile signaling, repelling or attracting during pathogen or herbivore attack are important for plant survival, floral volatiles play a vital role in the plant reproductive cycle by attracting pollinators to flowers. Floral scent is a diverse blend of low molecular weight (under 300 Da) mostly lipophilic compounds emitted from flowers into the surrounding atmosphere. To date, the chemistry of plant volatiles is well understood, however, little is known about the biosynthesis of this diverse group of compounds. How plants produce volatile compounds and what molecular mechanisms control their accumulation and release pose significant questions in plant biology, with both basic and practical aspects, that remain largely unanswered. In particular, little is known about the entire biochemical pathways leading to the synthesis of the majority of secondary metabolites and the enzymes and genes that control these pathways. Using functional genomic and biochemical approaches we have identified and characterized several genes responsible for the formation of scent volatiles. Diurnally emitting snapdragon and nocturnally emitting petunia were used as model systems to study the flux through the metabolic pathway(s) *in situ*. Petunia flowers produce almost exclusively benzenoid/phenylpropanoid compounds whereas snapdragon floral scent is rich in both terpenoids and phenylpropanoids. Presented results will show how the integration of metabolic profiling, functional genomic approach, targeted metabolic engineering with metabolic flux analysis and modeling can provide a comprehensive understanding of the regulation of flux through the metabolic networks. This presentation will also discuss critical factors that limit floral scent modification and present approaches for rational metabolic engineering of volatile emission based on computer-assisted metabolic flux analysis.

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# Structure-Activity Relationships in the Bioactivities of Gallic Acid Metabolites and Their Interactions with Biopolymers

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Gallic acid metabolites represent a unique family of plant secondary metabolites present widely in the plant kingdom in the forms of polygalloyl esters of polyols, galloyl conjugates of catechins[1,2]. They have many bioactivities such as anti-inflammatory[3], antimutagenesis [4,5], antiviral activities[6]. These metabolites readily bind with collagen and cellulose with hydrophobic interactions[8], and possess outstanding antioxidant and neuroprotective activities[9]. Here, we will report the structure-activity relationships in the neuroprotective activities and the interactions with biopolymers for the GA metabolites. We will further discuss some most recent results from our metabonomic investigation on the effects of gallic acid on the endogenous metabolism of rodent models.

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# Navigating a Path from Metabolomics Methods to Biology

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We have used a non-targeted, mass-spectrometry based approach to metabolomics. The screening platform employed a non-targeted, mass-based metabolomics approach beginning with capillary reverse phase chromatography, electrospray with accurate mass determination. The data was analyzed using the program XCMS, which performs a non-linear alignment of the data, followed by intensity integration. This approach to data analysis can locate very small differences in very large data sets with thousands of features. In some metabolomics experiments multiple methods are combined to increase the coverage, including positive and negative mode electrospray, and positive mode APCI. Molecules were identified using a combination of accurate mass, database searching, and LC/MS/MS using a QTOF.

Other techniques used include transcriptomics using gene chips and quantitative PCR, which were then correlated with the metabolomics. Enzymatic assays have been used to confirm increased activity; thus the entire range of phenomena are examined from transcripts, to protein, to activity, and finally metabolites.

Human diseases, even those which arise from single point mutations, typically manifest in complex downstream effects, involving multiple organ systems and thus potentially affecting multiple biochemical pathways. Animal models provide an experimental system which reflect the level of complexity found in human biochemistry and disease. A global metabolomics approach was used to study the neurochemical effect of SIV infection in rhesus macaques, a model system for HIV and neuroAIDS, and illustrates the potential of metabolomics to address problems in central nervous system biochemistry and neurovirology, as well as neurodegenerative diseases. Cerebrospinal fluid (CSF) was compared before and after viral infection, and more than 3,500 features were measured. There were significant changes in the metabolome, with a general increase in metabolite concentrations during infection. Specific metabolites which changed were identified using database searching and comparison of the MS/MS pattern using a QTOF. Fatty acids, including palmitic, myristic, oleic, and stearic acids increased during infection, as did the corresponding lysophosphocholines. Other molecules that increased significantly during infection include carnitine, and the acylcarnitines, octanoylcarnitine and butyrylcarnitine. All of these molecules are related to fatty acid and lipid metabolism. Gene chip experiments were then performed on the hippocampus of uninfected compared to infected animals. These results indicated that different phospholipase genes, including PLA1 and PLA2 were up-regulated during infection. This increase in transcript levels was confirmed using quantitative PCR. Finally, specific biochemical assays were performed on brain tissue, indicating that phospholipase 2 activity was indeed increased in the brain as a result viral encephalitis. Thus, the metabolomics results were able to generate a testable hypothesis, which was confirmed by using gene chip experiments and biochemistry. These three datasets reinforce each other, and demonstrate the process of going from descriptive metabolomic phenomenology to a testable biochemical hypothesis, even in a disease as complex as neuroAIDS. The combination of metabolomics, transcriptomics and biochemistry, greatly facilitates this process. These methods are general, and can potentially be applied to any living system, including bacteria, plants and animals.

# SCA (lily Stigma/style Cysteine-rich Adhesin) and a SCA-like LTP in *Arabidopsis* function in plant reproduction

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Lily pollen tubes grow adhering to an extracellular matrix (ECM) produced by the transmitting tract epidermis in a hollow style. SCA, a small (~ 9.4 kDa), basic protein, plus low-esterified pectin from this ECM are involved in the pollen tube adhesion event. The mode of action for this adhesion event is unknown. Two SCA isoforms (SCA 1 and SCA 3) were separated from lily stigma preparations. Peptide sequencing analysis allowed us to determine two amino acid variations in SCA3, compared to SCA1. Our structural homology and molecular dynamics (MD) modeling results show that SCA isoforms have the plant non-specific lipid transfer protein (nsLTP)-like structure: a globular shape of the orthogonal 4-helix bundle architecture, four disulfide bonds, an internal hydrophobic and solvent inaccessible cavity, and a long C-terminal tail. The Ala71 in SCA3, replacing the Gly71 in SCA1, has no predictable effect on structure. The Arg26 in SCA3, replacing the Gly26 in SCA1, is predicted to cause structural changes that result in a significantly reduced volume for the internal hydrophobic cavity in SCA3. The volume of the internal cavity fluctuates slightly during the MD simulation, but overall, SCA1 displays a larger cavity than SCA3. SCA1 displays higher activity than SCA3 in the *in vitro* pollen tube adhesion assay. In an attempt to understand the role of SCA-like AtLTPs in *Arabidopsis thaliana*, we utilized SALK T-DNA insertion lines. One mutant showed significantly delayed plant growth with a dwarfed stature at maturity and defects in pollination/fertilization. In reciprocal cross-pollinations to wild-type, *in vivo* growth of the mutant pollen tubes was significantly decreased, resulting in decreased seed set. In addition, the mutant pistil showed defects in fertilization, even when wild-type pollen was used. These phenotypes suggest that this *AtLTP* gene may play a role in both pollen and pistil function in seed production.

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# The Subunit Composition of Hinokiresinol Synthase Controls Both Geometric and Enantiomeric Selectivities in Hinokiresinol Formation

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Norlignans are a class of phenylpropanoids with diphenylpentane carbon skeletons (C6-C5-C6) that are found mainly in conifers and monocotyledons. A norlignan, hinokiresinol, has an *E* or *Z* double bond in its molecule. Interestingly, (*E*)-hinokiresinol is distributed specifically in conifer heartwood, and the coloration of *Chamaecyparis obtuse* (Japanese cypress) heartwood is ascribed to this compound. On the other hand, (*Z*)-Hinokiresinol is detected in herbaceous monocotyledons including *Asparagus officinalis* (asparagus). Both hinokiresinols have antifungal activity and can be produced in response to stress, such as fungal infection and sapwood drying, suggesting that they are synthesized *in vivo* for plant protection [1].

Previously, the mechanism of formation of *E* (*trans*) and *Z* (*cis*) double bonds in hinokiresinols was unknown [1]. However, our findings have indicated that (*Z*)-hinokiresinol is derived from two nonidentical phenylpropane units [2]. Subsequently, (*Z*)-hinokiresinol was found to be synthesized from a dimeric phenylpropanoid ester, 4-coumaryl 4-coumarate, by an enzyme preparation from elicitor-treated *A. officinalis* cells [3]. In addition, (*E*)-hinokiresinol was formed from 4-coumaryl 4-coumarate by an enzyme prepared from *Cryptomeria japonica* (Japanese cedar) cells [4]. Then, we purified (*Z*)-hinokiresinol synthase (HRS) from *A. officinalis*, and isolated cDNAs encoding the enzyme. The enzyme was found to be composed of two subunits (HRSa and HRSb). When each recombinant subunit was incubated individually with 4-coumaryl 4-coumarate, only (*E*)-hinokiresinol was formed. On the other hand, incubating the same substrate with a mixture of the two subunits gave rise to (*Z*)-hinokiresinol that is the geometric isomer accumulated in *A. officinalis*. Thus, the subunit composition can control the geometric isomerism of the product, which is quite interesting from organic chemical aspects [5].

Interestingly, in addition to the *cis-trans* regulation, the enantiomeric composition of hinokiresinol was found to be controlled by the subunit composition of HRS. Thus, (*Z*)-hinokiresinol formed by the mixture of HRSa and HRSb was optically pure (+)-enantiomer. It is noteworthy that the naturally occurring (*Z*)-hinokiresinol in *A. officinalis* is also optically pure (+)-enantiomer. In contrast, (*E*)-hinokiresinol formed following incubation with HRSa or HRSb was not optically pure [(-)>(+) , 20.6 or 9.0% enantiomer excess, respectively]. Therefore, it was demonstrated that the subunit composition of HRS controls not only geometric selectivity but also enantiomeric selectivity in hinokiresinol formation.

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## **Genome-wide characterization of phenylpropanoid biosynthetic enzymes: acyltransferases responsible for modification of plant polyphenolics and cell wall components**

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Plant cell wall polysaccharides and lignin and a variety of plant secondary metabolites are often esterified with different acyl moieties. For example, (iso)flavonoids, a subfamily of phenylpropanoid metabolites, are commonly accumulated as malonylated- or acetylated- glycoconjugates in legumes. BAHD superfamily of acyl-CoA dependent acyltransferases are potentially involved in the acylesterification of phenolics/polyphenolics and non-cellulosic cell wall components in plants. Sequence analysis on the EST databases of the model legume *Medicago truncatula* enabled us to identify about 76 cDNA sequences encoding BAHD superfamily enzymes; nine of them are distinct from the most of the characterized anthocyanin/flavonol acyltransferase genes in other species. Functional characterization revealed that at least three recombinant enzymes specifically recognize malonyl-CoA as an acyl donor and catalyze the malonylation of a range of isoflavone 7-*O*-glucosides; and other two enzymes utilize acetyl-CoA for acetylation of flavonol glycosides *in vitro*. Similarly, analysis of poplar genomic sequences led to identifying about 94 BAHD genes candidates. Bioinformatics analysis, gene expression profiling and biochemical characterization resulted in the recognition of several novel aromatic and aliphatic acyltransferases involved in the modification of a range of phenolic and alcoholic derivatives in wood tissues and a novel acetyltransferase responsible for the modification of cell wall lignin. The identified acyltransferase genes from either legume or poplar plants displayed distinct tissue-specific expression patterns and/or differentially responded to biotic- and abiotic-stresses. Over-expression of these genes in *Arabidopsis* or tobacco caused severe phenotypic alteration or metabolite modification. The GFP fusions exhibited distinct cellular and subcellular localization in living cells.

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# Lignification and Lignin Structure in the Cell Wall and Biofuels: New Strategies for Old Problems

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Lignins, hitherto biopolymers of limited scientific interest, are now of considerable and growing attention. This is largely currently due to the potential of biotechnological manipulation of vascular plant tissue with the purpose of generating biofuels economically from a renewable source.

This contribution describes the progress made in the study of lignin structure, and the effects of manipulation of same. Once viewed as a randomly linked biopolymer, evidence on many fronts now argues against such a simplistic notion. Progress made in establishing lignin primary structure to date is described, as is the predictability of manipulating various biochemical steps in lignin monolignol pathway and downstream metabolism.

In general, however, the biotechnological lignin-reductions effectuated thus far have resulted in compromised vascular apparatus effects and perhaps higher susceptibilities to pathogen attacks. Such plant lines are probably unsuitable for large scale cultivation and on biofuel feedstocks.

Accordingly, new and novel approaches are being both conceptualized and developed if the lignin challenge is to be overcome for purposes of generating biofuels/bioproducts and bioenergy from lignocellulosics. This presentation also addresses some new areas of exploration involving redirection of carbon flux away from the formation of lignin to that of high-value, liquid, plant derived aromatics which can be used for bioenergy/biofuels and other specialty purposes. Discussed herein is the progress made as regards the pertinent enzymes, genes, and specific biochemical precursors involved that have been isolated and which lend themselves to exploitation through bioengineering. Potentially, success in this endeavor could lead to development of a multibillion dollar per year industry within the United States alone.

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## Metabolic engineering of glucosinolates

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Glucosinolates are amino acid-derived natural products present in the economically important Brassica vegetables, such as e.g. oilseed rape and broccoli. In plants, they play a role in plant defence as feeding deterrents for herbivores and pathogens, but also as attractants for specialist herbivorous insects. For humans, they are known for their cancer-preventive properties, their characteristic mustard flavour, and their use as biopesticides. Particularly glucoraphanin has received a lot of attention as a very potent cancer-preventive phytochemical present in high amounts in broccoli. The synthesis of the core structure of glucosinolates involves at least 5 different gene products, probably assembled in a metabolon complex. With the recent identification of the *C-S* lyase, the *S*-glucosyltransferase, and the sulfotransferase, and the previously known cytochromes CYP79 and CYP83 (for review see [1]), transfer of the glucosinolate pathway to heterologous host organisms has become a realistic goal. We have initiated efforts to engineer the whole pathway into tobacco and potato, in order to confer increased resistance. Since the traditional transgene stacking is not convenient (considering the number of genes involved), an alternative method has been adopted. This method utilizes virus-derived sequences as part of one-ORF multicistronic constructs, which allow the expression of several genes from a single promoter sequence. Progress in engineering glucosinolates will be discussed.

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# Engineering and metabolic regulation of vitamin C biosynthesis in plants

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Vitamin C (L-ascorbic acid; AsA) is the major soluble antioxidant found in plants and is also an essential component of human nutrition. Although numerous biotechnological methods have been exploited to increase its yield, the pressures such as commercial competition and environmental concerns make it urgent to find a new way for industrial production of plant-derived AsA.

Engineering plant AsA has now become feasible because its biosynthetic pathway is increasingly understood. Alteration in AsA and its substrates accumulation can be achieved by several possible strategies, such as overcoming the rate limiting steps in the biosynthetic pathway, promoting recycling, and reducing catabolism. However, the effects of the genes involved in AsA pathway on the AsA accumulation are not fully understood.

In this study, we used *Arabidopsis* as the model to study the effects of genes involved in AsA pathway on the AsA accumulation by transgenic technology. We found that by over-expressing AsA biosynthetic pathway genes *gdpmpase* or *galppase*, AsA accumulation in transgenic *Arabidopsis* lines were increased, reaching 1.71-fold and 1.35-fold respectively of the control line, suggesting the promotion of AsA accumulation is not high enough by over-expressing single AsA biosynthetic pathway gene. Through depression of the D-arabinono-1,4-lactone oxidase by RNA interference, total AsA accumulation in the leaf of transgenic lines was not changed much, but the AsA content in apoplasts in transgenic lines was much higher than that in the control line, so as the ratio of AsA/DHA, indicating that depression of AO could reduce the oxidation of AsA in apoplasts, making more AsA maintain in reduction stage.

Over-expressing the gene encoding dehydroascorbate reductase (DHAR) in transgenic plants could effectively activate the AsA recycling. AsA accumulation in transgenic *Arabidopsis* lines over-expressing *dhar* was three times higher than that in control lines, and the ratio of AsA/DHA in transgenic lines was also increased.

We also found that by over-expressing the bacteria hemoglobin from *Vitreoscilla stercoraria* (VHb), which is found to enhance respiration and energy metabolism of bacteria, in transgenic *Arabidopsis* plants, the utilization efficiency for oxygen of plants was enhanced and the AsA content in transgenic lines was increased by 3-fold compared to the control. The tolerance for abiotic stresses of transgenic lines was also improved. This study suggests that several strategies may be required together to achieve the maximum increase of AsA accumulation in plants, which include the manipulation of the AsA pathway by overcoming the rate limiting steps in the biosynthetic pathway, promoting recycling, and reducing catabolism, as well as by over-expressing the genes which could promote the metabolism such as *vhb*.

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## Genetic modifications of *Nicotiana attenuata* reveal functions of plant secondary metabolites in resistance to herbivory

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Plants produce a large variety of secondary metabolites, many of which are thought to have anti-herbivore or anti-pathogen functions. *Nicotiana attenuata* is an annual wild tobacco plant ranges North America, whose interaction with herbivores has been intensively studied. Among the secondary metabolites produced by *N. attenuata*, nicotine, trypsin proteinase inhibitor (TPI), and diterpenoid glycosides (DTGs) have been believed to be compounds that are parts of direct defense. Using genetic tools, we silenced genes involved in these compounds' biosyntheses, and thus downregulated the levels of these compounds in *N. attenuata*. On plants with reduced levels of nicotine, TPI, or DTGS, herbivore performance was measured to examine the defensive roles of these secondary metabolites: both in-glasshouse and in-field studies showed that downregulating these compounds dramatically decreases plants' direct defense levels, demonstrating their important ecological roles in a plant-herbivore interaction [1, 2, 3].

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# Comparative genomics of secretory trichomes – biofactories for production of plant secondary metabolites

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Collectively, plants are a rich source of natural products, chemicals that often function to protect the plant from infection or insect pests. Natural products also form the basis of many currently used drugs, such as aspirin, morphine, taxol or the antimalarial compound artemisinin. Plant natural products are often synthesized and accumulated in secretory trichomes, which are appendages found on the aerial organs of plants. Trichomes have a unique capacity for chemical synthesis and secretion, and have been described as biofactories for the production of natural products. However, with few exceptions, little is known about the molecular aspects of trichome metabolism and secretion. The production of many natural products in specialized trichome cells facilitates genomics-based approaches to characterize biosynthetic and secretory processes.

In this project, we sequenced a large number of expressed sequence tags (ESTs) from trichomes libraries of five species representing different plant families (10,000 ESTs for each species) included *Medicago truncatula* and *M. sativa* (alfalfa), Leguminosae; *Nicotiana benthamiana* and *Lycopersicon esculentum* (cultivated tomato), Solanaceae; and hop (*Humulus lupulus*), Cannabaceae. At the same time, the full spectrum of natural products produced in the trichomes of these five species will be determined. Several subjects are undergoing right now:

1. In hop trichomes, which contain high amounts of terpene rich essential oil and resin (mainly prenylated polyketides, included humulone, lupulone and xanthohumol), candidate enzymes involved in terpenoid and prenylated polyketide biosynthesis have been selected and functionally characterized.

2. Trichomes from potato leafhopper resistant and susceptible alfalfa lines were compared by microarray analysis and metabolite profiling to determine the mechanism of leafhopper resistance.

3. The EST collection is being mined for genes involved in the regulation and transport of natural products, and the functions of a selection of those genes that are common to multiple species will be determined by the virus-induced gene silencing (VIGS) method in *N. benthamiana*.

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# Molecular control of carotenoid and anthocyanin accumulation

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Plant secondary metabolites such as carotenoids and anthocyanins are highly beneficial for humans because they provide important nutrients and antioxidants in our diets. Despite the wealth knowledge regarding carotenoid and anthocyanin metabolism in plants, the regulatory mechanisms underlying their biosynthesis and accumulation remain to be fully elucidated. To gain a better understanding of the mechanisms that control carotenoid and anthocyanin accumulation, we have been using unique orange and purple cauliflower mutants as model systems for isolation of the *Orange* and *Purple* genes responsible for high beta-carotene and cyanidin accumulation in the plant. We found that the *Orange* gene encodes a plastid-associated protein containing a DnaJ cysteine-rich zinc binding domain [1]. Rather than functioning directly in carotenoid biosynthesis, the *Orange* gene exerts a novel mechanism in controlling carotenoid accumulation by inducing the differentiation of non-colored plastids into chromoplasts. Through studying of the *Orange* transgenic potato lines, we not only demonstrate that the *Orange* gene acts as a bona fide molecular switch to trigger chromoplast differentiation, but also provide evidence that manipulation of chromoplast formation offers a very useful strategy to enhance the carotenoid content in food plants for improving their nutritional quality [2,3]. The *Purple* gene in the cauliflower mutant most likely represents a novel mutation of a regulatory gene that controls the expression of other anthocyanin regulators in controlling anthocyanin accumulation. Unlike other anthocyanin accumulating mutants that are caused by constitutive transcriptional activation of many structural genes, the *Purple* gene mutation controls expression of a subset of structural genes in a tissue-specific manner. The *Purple* gene may furnish a potent new genetic tool for nutritional and agronomic improvement of plants.

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## Epigenetic Regulation of the Carotenoid Isomerase

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Carotenoid pigments are critical for the survival of plants and as a consequence carotenoid composition is finely tuned in response to the stage of development, tissue and external environmental stimuli; yet few regulatory components and to date no epigenetic regulatory mechanisms have been identified. We report the cloning of the carotenoid chloroplast regulatory mutant, *ccr1*, which alters carotenoid composition during development and alters plant morphology, including increasing shoot branching. The alterations in the carotenoid and chlorophyll pigment profile, such as reduced lutein in leaves and accumulation of *cis*-carotenes in dark-grown seedlings were caused by a reduction in transcript abundance of the carotenoid isomerase. The *CCR1* gene was identified as a methyl transferase, which is responsible for the epigenetic regulation of transcription. *ccr1* plants show altered methylation surrounding the CRTISO translation start site, which correlates with chromatin compaction and a lower transcript abundance of the carotenoid isomerase. Our results demonstrate that CCR1 epigenetically regulates carotenoid biosynthesis during plant development.

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# Do endophytes regulate phytochemical biosynthesis?

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Growing evidences indicate that the endophyte is ubiquitous in the plant kingdom. Biologically, an endophyte is either bacterium (including representatives in actinomycetes) or fungus spending the whole or part(s) of their life span by colonizing inter- and/or intra-cellularly inside normal tissues of host plants. It has been well documented that the endophyte plays a multiple physiological and ecological roles in the process of endophyte-plant and endophyte-plant-herbivore interactions. The colony of some endophytes has been disclosed to be able to improve or initiate the plant growth through improving the tolerance of the host to environmental stresses such as drought, salinity, heavy metals as well as attacks of or consumptions by microbial pathogens, nematodes, insects and mammal herbivores. Biochemically, an expanding pile of data has demonstrated that the 'host-helping' effects of endophytes are ascribable to the production of phytochemicals. In the viewpoint of mutualism, endophytes could be accepted as a slightly opened reservoir of 'special microorganisms' that must be a rich source of new phytoalexin-like chemicals and a new regulator on the biosynthesis of those important secondary metabolites. This talk will mainly present the latest new findings about the topic as exemplified by authors' characterization of a plant hormone indole-3-acetic acid (IAA) as well as other hormone-like compounds such as fusaricide and oligosaccharide elicitor. The future trend will be mentioned as well.

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# Multiple ecological roles of linalool synthase revealed using transgenic plants

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Linalool is a monoterpene alcohol which is frequently found in the bouquet of flowers and the aroma of fruits. Furthermore, it is a constituent of many herbal oils and induced after herbivore attack in many plant species. The availability of transgenic plants allows us to investigate the multiple roles of this compound in the attraction of pollinators, parasites and predators and in the repellence and deterrence of herbivores of leaves and flowers.

We have transformed the strawberry linalool synthase into many different plant species including Arabidopsis, petunia, potato, lettuce and cultivated chrysanthemum using constitutive promoters. Linalool plants with very high expression levels were lighter green in color and growing less vigorous, but with lower expression levels these phenotypes were hardly visible.

GC-MS analysis of the headspace of leaves showed that in general large amounts of linalool were released from transgenic plants with minor side products such as DMNT. In chrysanthemum we found 106 masses, representing 12.5 % of all the detected masses, significantly different ( $P < 0.05$ , fold change  $> 5$ ) between the transgenic and control group. Among those 106 masses, 87 masses (82%) related to linalool, and 13 masses (12%) related to DMNT ((E)-4,8-dimethylnona-1,3,7-triene). There were no compounds significantly reduced as a result of linalool expression.

LC-MS analysis of leaf extracts of chrysanthemum indicated that under stringent statistical criteria ( $p < 0.01$ ;  $> 5$ -fold change), 2482 negatively ionizable masses among a total of 8968 masses were 93.2% upregulated and 6.8% down-regulated. Detailed analysis shows that linalool and derivatives were conjugated to different sugar residues in transgenic plants.

In choice assays with herbivores such as western flower thrips, *Frankliniella occidentalis*, the transgenic plants were in their first, odor-informed choice significantly attractive, but at later time points ( $> 20$  hours) became significantly deterrent. The peach aphid, *Myzus persicae*, was also deterred by the expression of linalool and the diamondback moth *Plutella xylostella* preferred to oviposit on linalool negative plants. Insects which are beneficial to the plant such as the parasitic wasp *Cotesia glomerata* were attracted more to Arabidopsis plants which also express linalool in response to insect attack.

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# Vitamin biofortification in China: Current and Future

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Vitamins, such as folic acid, vitamin A and vitamin E, are essential for human health. Plant foods are the most important source of the vitamins. Upon recognizing the importance of the vitamins for human health and the prevalence of vitamin deficiency throughout China, especially in the remote underdeveloped rural areas, tremendous efforts have been made to overcome the serious problem through biofortification strategies including conventional breeding and transgenic approaches. A various crops including rice, maize, oilseed rape and potato are set as the target crops.

Folate deficiency results in serious health problems, including neural tube defects (NTD), megaloblastic anemia, and several neurodegenerative disorders. NTD incidence in the poorest regions in China can be up to 10 times higher than that in Western surveillance systems [1]. Biofortification of folates in rice and tomato is being carried out based on the understanding of the existing folate biosynthesis pathway. In addition, to uncover the interactions between folate synthesis and C1 metabolism or other potential pathways, microarray and proteomic approaches are also employed.

Vitamin A deficiency (VAD) is a public health problem in many developing countries, especially affecting the health of infants, young children, pregnant and lactating women, having high vitamin A requirements but diets chronically deficient in vitamin A. In China, the prevalence of VAD (based on serum retinol concentrations  $<0.7\mu\text{mol/L}$ ) among children aged 3~12 years was 9.3%, and that of marginal VAD (based on  $0.7\mu\text{mol/L} = \text{serum retinol concentrations} < 1.05\mu\text{mol/L}$ ) was 45.1% [2]; highest VAD prevalence was found among children in the poor western area [3]. Genetic resource screening and breeding has been successful in obtaining high pro-vitamin A maize and sweet potato, and some QTLs responsible for pro-vitamin A synthesis have been mapped. Human trial studies indicate that consumption of beta-carotene rich sweet potato can efficiently increase vitamin A level in the people suffering from vitamin A deficiency.

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## Folate fortification of rice by metabolic engineering

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Rice is a major staple crop providing 80% of the daily caloric intake to 3 billion people. It is a poor source of micronutrients, including folates (vitamin B9). Therefore, folate shortage is wide-spread, especially in developing countries. Folate deficiency results in serious disorders, including neural tube defects as spina bifida in infants and megaloblastic anemia. Adequate dietary folate intake can prevent onset of these conditions. Folate biofortification through metabolic engineering can complement the current methods to fight folate deficiency, all of which have proven limited success.

We report on metabolic engineering of folate biosynthesis in rice, by overexpression of two genes, GTP cyclohydrolase I and aminodeoxychorismate synthase, involved in the pteridine and para-aminobenzoate branches of the folate biosynthesis pathway, respectively, on a single locus. This resulted in the enhancement of folate content up to 100 times above wild type levels, with 100g of polished raw grains containing up to 4 times the adult daily requirement. The major folate fraction in biofortified rice was represented by 5-methyltetrahydrofolate, which on average accounted for almost 90% of total folate making it superior to folic acid fortified rice as obtained by industrial fortification, mandatory in some countries. The analysis of p-ABA, pterin, and folate content in the transgenic lines also provided new insights into the fundamental aspects of folate biosynthesis.

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## Divergent evolution of triterpenoid biosynthetic pathways in monocots

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Plants produce a large array of steroid and triterpenoid compounds via isoprenoid pathways. Cycloartenol and lanosterol are the precursors to membrane sterols and steroid hormones. Other triterpenes have less well defined function in plant [1], but at least in one case it has been identified that triterpenoid pathway plays an important role in defence against a wide range of microbes in monocot crop, oats [2,3]. 2,3-oxidosqualene is the branch-point in triterpene and sterol biosynthetic pathways. Cyclisation of 2,3-oxidosqualene to diversified sterol and triterpenoid skeletons are conducted by members of the oxidosqualene cyclase (OSC) family. We have identified 11 *OSC* gene homologues in the rice (*Oryza sativa*) genome and 4 of them are like to be the pseudogenes. Analysis of the average ratio of nonsynonymous ( $K_a$ ) to synonymous ( $K_s$ ) nucleotide substitutions per site ( $K_a/K_s$ ) between *indica* and *japonica* subspecies indicates that *OsOSC8* is a functionally conserved gene in monocots but *OsOSC9* has experienced a rapid sequence divergence. The rapid sequence evolution of *OsOSC9* may imply that this gene is potentially involved in pathogen-defence. Comparison of the gene structures, expression patterns and the amino acid sequences between *OSC* genes from rice and other plant species reveals that *OsOSC3* is a candidate gene for cycloartenol synthase involving in the synthesis of sterols, while *OsOSC7*, *OsOSC10* and *OsOSC11* are likely involved in the synthesis of  $\beta$ -amyrin and other *OSC* genes may involved in biosynthesis of other triterpenes. For further characterisation and confirmation of the functions of the rice OSCs, recombinant genes for the seven *OSC*s were transformed into yeast strain. Also we are using RNAi technology to identify the functions of triterpenoid pathways in rice and other cereals.

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## Platforms for Biosynthesis of Aroma-Chemical in Plants

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Plant extracts and essential oils form indispensable sources of natural ingredients for creating flavours and fragrances, and as valuable additives in cosmetics and nutraceuticals. In recent years, there has been increasing demand for such natural ingredients. The demand has raised formidable challenges to the industry. Firstly, very low concentrations of the active compounds in the plant biomass, often as end-point metabolites of secondary pathways, translates to high costs in production. Secondly, quality variations in the extracts are often observed from plants which are grown in different regions or seasons. Thirdly, at the global scale, diminishing agricultural lands and scarcity of water will not tolerate competitions of farming aromatic plants in large scale.

To address these challenges, refining extraction and separation technologies for producing plant extracts alone is not enough. Fundamental understanding of plant secondary pathways and their regulation at gene level will offer us opportunities to develop transgenic cell lines or plants for enhanced production of aromachemicals in the targeted fashion. At Firmenich, we have collaborated with elite academic groups and made the following research progresses: 1) both plant and yeast platforms were tested for commercial production of terpenes for flavour and fragrance industry; 2) more than 30 genes necessary for biosynthesis of target sesquiterpenes were cloned; 3) using patchoulol and the related sesquiterpenes as examples, we have demonstrated it is possible to manipulate plant host for a significant level of elevation of target volatile compounds. Detailed technical progresses and further challenges will be discussed.