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HIGHLIGHT

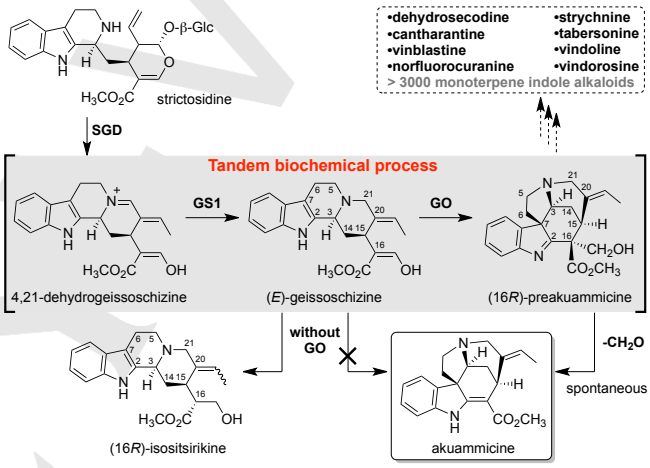
Tandem biocatalysis unlocks the challenging de novo production of plant natural products**

Christophe Duplais* and Yannick Estevez

Dedicated to Michel Rohmer on the occasion of his 69th birthday

Bioengineering the genes of plants and microbes is becoming a very competitive strategy in synthesis to produce pharmacologically relevant plant natural products and their unnatural analogues. During the last 20 years several plant genes have been identified, cloned and expressed successfully unlocking de novo production of plant metabolites such as morphine, strictosidine, artemisinin, taxol® and resveratrol.[1] Future development for accelerating the discovery of gene candidates relies on innovative computational methods combined with transcriptomics and metabolomics analysis to make plant genome mining more feasible.[2] Nevertheless in many cases proteins of interest are produced in low amounts preventing in vivo extraction, or are either inactive or give a different product after heterologous expression in a model system. Our understanding of the biocatalytic cascade or multistep reactions made by enzymes in different cellular compartment remains limited,[3] and yet deciphering new biocatalytic system in nature for future bioinspired synthetic strategies represent a stimulating interdisciplinary field for chemists and biologists. In this context two recent studies on plant alkaloids[4] and plant terpenoids[5] report rare examples of tandem biocatalysis which is an exciting and emerging field in chemical biology.[6]

Strictosidine is an emblematic natural product as it is the common precursor of the vast majority of monoterpene indole alkaloids (MIA) found in the plant families of Apocynaceae, Loganiaceae and Rubiaceae. Although its biosynthesis pathway has been elucidated completely and reconstructed successfully in tobacco and in yeast,[1] the downstream pathways that generate the tremendous diversity of MIA (>3000 structures) from strictosidine remain unknown with almost none of the biosynthetic genes identified. Thus, it is not surprising that the production of MIA anticancer drugs vinblastine and vincristine, as well as cantharantine, is still inaccessible today by protein engineering. Progress has been made recently with the completion of the seven-step pathway from tabersonine to the anticancer drug precursor vindoline with successful assembly in yeast,[7] however these new biosynthetic genes represent only the tip of the iceberg in MIA biosynthesis. The work accomplished by the groups of Courdavault and O'Connor[4] represent a feat of intellect as it reveals for the first time the two proteins responsible for the biotransformation of strictosidine aglycone (4,21-dehydrogeissoschizine) into akuammicine (Scheme 1). The later of which originated from a preakuammicine-type intermediate which is considered the "missing link" in MIA biosynthesis since it is the putative single precursor of all type II and type III MIA and has never been isolated directly.[8]



Scheme 1. Early enzymatic transformations in plant biosynthesis of monoterpene indole alkaloids (MIA) involving strictosidine glucosidase (SGD), alcohol dehydrogenase (GS1), geissoschizine oxidase (GO). GS1 and GO must be added simultaneously in enzyme assays to form akuammicine.

To lift the veil on preakuammicine formation, the authors focused their efforts on the identification of two genes, a reductase and an oxidase. One cytochrome P450 gene candidate (CYP71D1V1, named geissoschizine oxidase GO), displaying correlation with strictosidine glucosidase (SGD), leads to significant decrease in the production of several MIA when silencing in *Catharanthus roseus*. In parallel the protein encoded by an alcohol dehydrogenase gene (named geissoschizine synthase GS1) was assayed with strictosidine in the presence of SGD and the reaction products were identified as (16*R*)-isositsirikine stereoisomers *Z* and *E*. When the same enzymatic reaction is repeated by adding GO protein then the enantiopure akuammicine was successfully isolated, demonstrating that in absence of GO protein, over-reduction of geissoschizine is obtained. Unfortunately the non-enzymatic deformylation of preakuammicine appears to be spontaneous preventing the challenging isolation of preakuammicine. The technique of simultaneously adding both GS1 and GO proteins illustrate a novel example of tandem biocatalysis where the GS1 reaction product, (*E*)-geissoschizine, reacts with GO enzyme with a higher kinetic rate than with GS1 enzyme. Fluorescent labelling of

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proteins shows a nucleocytosolic localization of GS1, whereas GO is found in the endoplasmic reticulum (Figure 1) indicating that both proteins are available in *C. roseus* cells to interact freely in vivo.[4] Mechanistically the formation of preakuammicine relies on a complex rearrangement investigated recently using an elegant biomimetic synthesis approach that provides new evidence of preakuammicine stereochemistry.[9] It is exciting to anticipate additional future insights into the mechanism of this classic biotransformation.

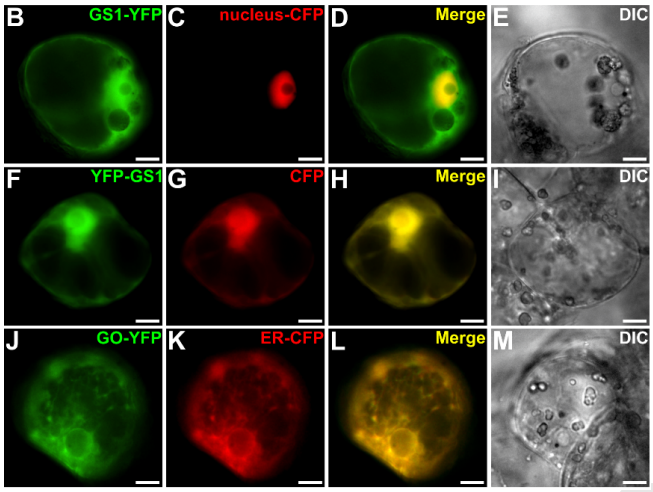

Figure 1. Subcelllular localization of GS1 and GO YFP fusion proteins in *Catharanthus roseus* cells (B-M). Cells were transiently co-transformed with plasmids expressing GS1-YFP (B), YFP-GS1 (F) or GO (CYP71D1V1)-YFP (J) and plasmids encoding a nuclear CFP marker (nucleus-CFP, C), a nucleocytosolic CFP marker (CFP, G) or an endoplasmic reticulum CFP marker (ER-CFP, K). Colocalization of the fluorescence signals appears in yellow when merging the two individual (green/red) false color images (D, H, L). Cell morphology is observed with differential interference contrast (DIC) (E, I, M). Bars, 10 µm (Image from ref [4]).

The second example of tandem biocatalysis concerned the biosynthesis of rare terpenoid sesterterpenes,[5] which represent less than 2% of the diversity within this chemical family. Sesterterpenoids have been found mostly in marine sponges and terrestrial fungi with a few structures having been isolated from plants within the Lamiaceae family. Recent studies[10] show that in fungi sesterterpene synthases (STSs) are bifunctional containing two protein domains: a C-terminal *trans*-prenyltransferase (PT), responsible for the synthesis of the C25 precursor geranylfarnesyl diphosphate (GFPP), and an N-terminal TPS that generates cyclization products. Intrigued by the 2017 identification of terpene synthases (TPSs) synthetizing sesterterpenes in *Arabidopsis thaliana* that were colocalized with PTs,[11] the group of Osbourn and collaborators decided to run a customized genome mining algorithm across 55 plant genomes to identify colocalized pairs of PT and TPS genes. The phylogenetic analysis reveals a clade of 18 colocalized PT-TPS gene pairs using high stringency parameters. These authors investigated five gene pairs from *A. thaliana*, one from *Capsella rubella*, and one from *Brassica oleracea* (Figure 2).

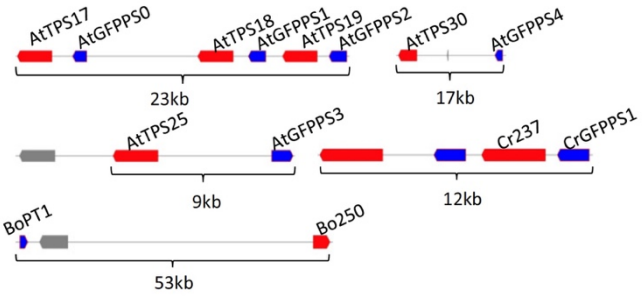

Figure 2. A graphic representation of prenyltransferase (PT, in blue) – terpene synthase (TPS, in red) gene pairs identified in *Arabidopsis thaliana*, *Capsella rubella*, and *Brassica oleracea* plant genomes (Image from ref [5]).

Remarkably only when TPSs were co-expressed individually with PT genes in *Nicotiana benthamiana*, eight sesterterpenes having diverse scaffolds were formed, including 11/6/5 tricyclic (+)-arathanatriene, (−)-retigeranin B, (−)-ent-quiannulatene, (−)-variculartriene A, (+)-astellatene, (−)-caprutriene, (+)-boleracene, and (−)-aleurodiscalene A (Scheme 2). Interestingly interchanging the PT gene did not affect the functionality of PT-TPS gene pairs, although two TPS genes that were part of the same monophyletic clade in the TPS phylogeny, but not clustered with PT genes, did not produce sesterterpenes when coexpressed with PTs in *N. benthamiana*. It is intriguing that PT-TPS gene pairs do not seem to be obviously co-expressed which adds a layer of complexity in this biochemical puzzle. This structural diversity presumably arises from the common bicyclic C12 carbocation precursor which evolves into two tricyclic 11/6/5 and 5/12/5 cations. The authors also carried out site-directed mutagenesis of tyrosine within a conserved amino acid sequence between TPSs with the hope of controlling the shift between the two tricyclic 11/6/5 and 5/12/5 cations and expending the structural diversity. Although results clearly suggest a putative role of tyrosine for stabilizing the carbocation intermediates in pentacyclic (−)-retigeranin B formation, further studies need to be conducted to confirm the carbocation-protein binding within active sites, and to investigate the presence of three-metal ion cluster in STSs.[12] It will be also very informative to get more insights into the relationship between PT-TPS gene pairs for future protein engineering research in biocatalysis.[13] This impressive work opens new avenues for fundamental studies on the trajectory of the cyclization cascade, reaction selectivity and scaffold diversification through paired terpenoid synthase genes. Lastly it is worthy to note that whereas fungal sesterterpene synthases are bifunctional, plants utilize colocalized PT and STS genes that might have evolved independently but produce overall together the same scaffold. For instance the plant metabolite (−)-ent-quiannulatene is an enantiomer of the fungal metabolite (+)-ent-quiannulatene, each are produced by phylogenetically distant STS proteins which represents an extraordinary example of convergent evolution in natural products.[14]

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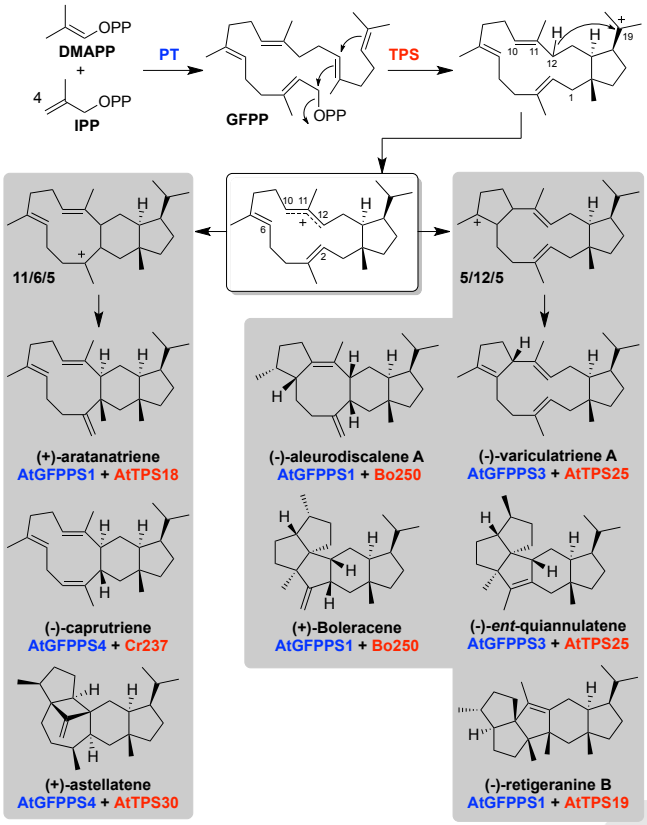

Scheme 2. Biosynthesis of sesterterpenoids from dimethylallyl pyrophosphate (DMAPP) and isopentenyl diphosphate (IPP) precursors. Combination of prenyltransferase (PT, in blue) and terpene synthase (TPS, in red) generate and then transform the acyclic geranylfarnesyl diphosphate (GFPP) intermediate into a structural diversity of sesterterpenes.

Although tandem biocatalysis is not a new concept in biochemistry,[6] fundamental aspect of protein-protein interaction in biocatalysis remains largely unknown. However considering the recent merge of enzymatic and chemical catalysis in tandem reaction,[15] it is exciting to see both in vivo and in vitro examples that could inspire future strategies to unlock the synthesis of complex natural products and unnatural analogues.

Acknowledgements

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Keywords: biosynthesis • alkaloids • terpenes • enzyme catalysis • protein engineering

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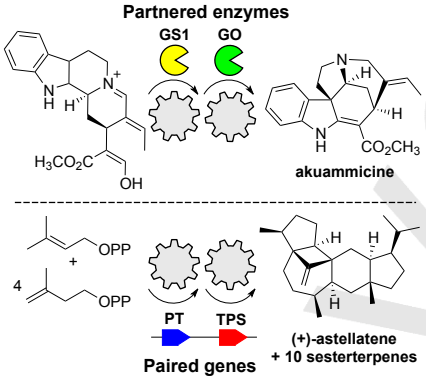
Layout 1:

HIGHLIGHT

Intimate partnership: Two studies report new insights into the biosynthesis of alkaloids and sesterterpenoids in plants. This highlight presents these novel biotransformations to illustrate how tandem biocatalysis could impact the future of natural products production.



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