METABOLONS: THE INTRACELLULAR METABOLIC HIGHWAYS IN PLANTS

By

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CERTIFICATE

This is to certify that the seminar report entitled **"Metabolons: The intracellular metabolic highways in plants"** has been solely prepared by **FEMINA K (2018-11-094)**, under my guidance and has not been copied from seminar reports of any seniors, juniors or fellow students.

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I, Femina K (2018-11-094) declare that the seminar entitled **"Metabolons: The intracellular metabolic highways in plants"** has been prepared by me, after going through various references cited at the end and has not been copied from any of my fellow students.

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1. Metabolons - An introduction

To optimize growth and development, the metabolic activities of a plant are highly coordinated at the whole plant, organ, tissue, cellular, organellar and molecular levels. At the cellular level, channelling of substrates to their target enzymes is facilitated by the compartmentation of the cell into different organelles and sub structures thereof. This serves to co-localize and optimize the concentrations of enzymes and their substrates. Metabolic activities in cells are compartmented into various structures and organelles. In each compartment [outer cell membrane, Golgi, endoplasmic reticulum (rough and smooth), outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, matrix, lysosome, starch or glycogen particles, nuclear matrix, structural proteins, cytosol (aqueous cytoplasm, endoplasm)] one can locate metabolic enzymes concerned with individual metabolic pathways (Moller, 2010).

Within some metabolic pathways there are stable multi-enzyme complexes and multifunctional enzymes that catalyse sequential reactions in that path. Recent evidence indicates that in addition to these more easily identifiable metabolic sequences there are specific interactions between many "soluble" sequential enzymes of metabolic pathways. Such complexes have been described in prokaryotes as well as eukaryotes. Additional evidence indicates that there are few, if any, free enzymes within cells. It appears also that the complexes of sequential metabolic enzymes are often bound to structural elements of the cell. The advantages associated with the organization of a portion of or of an entire biosynthetic pathway in a metabolon are thus many-fold.

Metabolon formation and metabolic channelling in plant secondary metabolism enable plants to effectively synthesize specific natural products and to avoid metabolic interference. Channelling can involve different cell types, take advantage of compartmentalization within the same cell or proceed directly within a metabolon. New experimental approaches document the importance of channelling in the synthesis of isoprenoids, alkaloids, phenylpropanoids, flavonoids and cyanogenic glucosides. Metabolon formation and metabolic channelling in natural-product synthesis facilitate attempts to genetically engineer new pathways into plants to improve their content of valuable natural products. They also offer the opportunity to introduce new traits by genetic engineering to produce plant cultivars that adhere to the principle of substantial equivalence.

Metabolon formation typically involves specific interactions between several ‘soluble’ enzymes that might be anchored to a membrane either by membrane-bound structural proteins that serve as ‘nucleation’ sites for metabolon formation or by membrane-bound proteins, such as cytochrome P450s (CYPs), that directly catalyse one or more of the sequential channelled reactions carried out by the metabolon. Metabolons vary greatly in physical stability as determined, for example, by the strength by which the individual components are attached. Accordingly, the difference between an enzyme that is composed of multiple subunits that are present in a defined stoichiometric ratio and a metabolon is neither precise nor absolute. Metabolon formation typically involves specific interactions between several ‘soluble’ enzymes that might be anchored to a membrane either by membrane-bound structural proteins that serve as ‘nucleation’ sites for metabolon formation or by membrane-bound proteins, such as cytochrome P450s (CYPs), that directly catalyse one or more of the sequential channelled reactions carried out by the metabolon. Mounting evidence indicates that even pathways that were once thought to encompass only soluble enzymes are subject to subcellular structuring that involves metabolon formation.

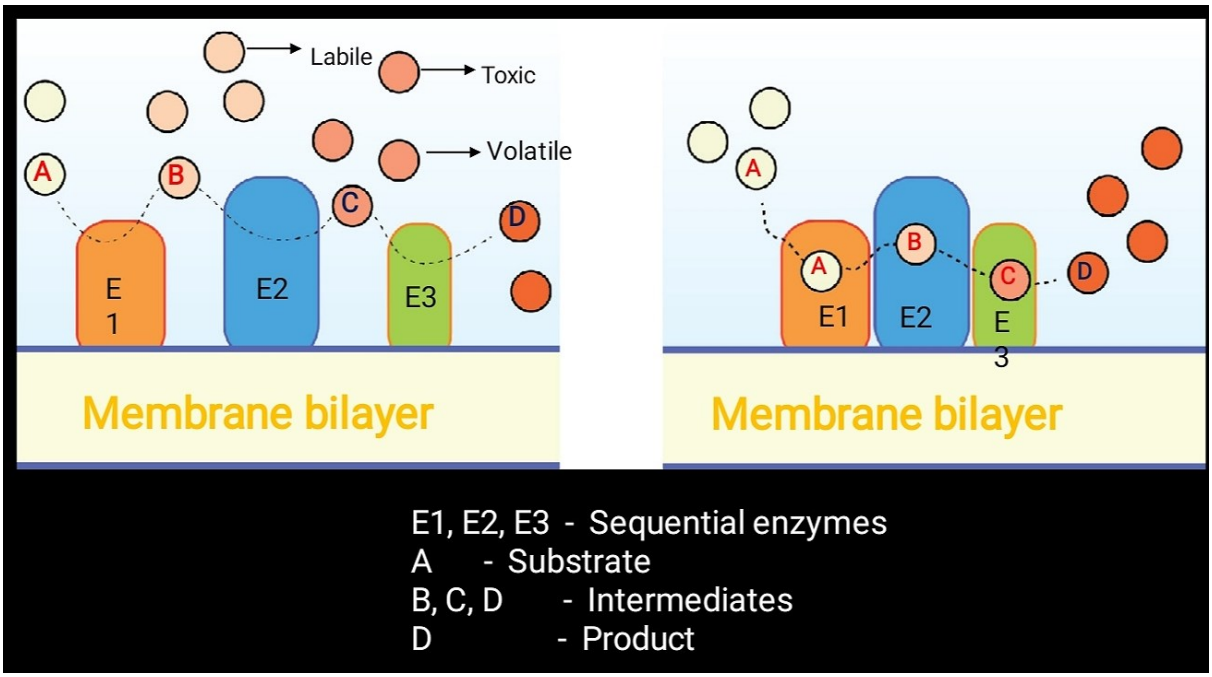

Plate 1. Normal metabolic pathway *vs* metabolon formation

2. Why metabolons?

There may be several reasons for metabolon formation. First, to improve catalytic efficiency by channelling an intermediate that is formed at one active site of an enzyme to the active site of the next enzyme, i.e. to bring co-operating active sites into close proximity and thereby decrease the transit time for intermediates. Second, to relieve kinetic constraints that result from the dilution of intermediates into the bulk phase of the cell. Third, to secure swift conversion of labile and/or toxic intermediates into more stable and less toxic constituents by sequestration and by preventing their diffusion into the surrounding cell matrix where chemical decomposition would take place. Fourth, to prevent compounds that might exert an inhibitory effect on the enzyme from reaching the active site. Fifth, to control and co-ordinate metabolic cross-talk that is mediated either by enzymes that function in different pathways or by intermediates that are shared between different metabolic pathways. And sixth, to provide a possibility for swift re-direction of metabolism by the formation of new metabolons that have altered polypeptide composition and product out-put, as might be demanded, for example, by environmental challenges (Bassard *et al.,* 2017).

3. Isolation

The isolation of dynamic membrane-embedded metabolons is hampered by their destabilization and dissociation upon detergent solubilization of the lipid bilayer. The use of the styrene maleic acid (SMA) copolymer circumvents these issues. The SMA polymer spontaneously integrates into the lipid bilayer and carves out discrete lipid particles (SMALPs) containing the resident membrane proteins and the surrounding lipids.We used the SMALP technology in combination with affinity chromatography to isolate the dynamic dhurrin metabolon (Laursen *et al.*, 2016).

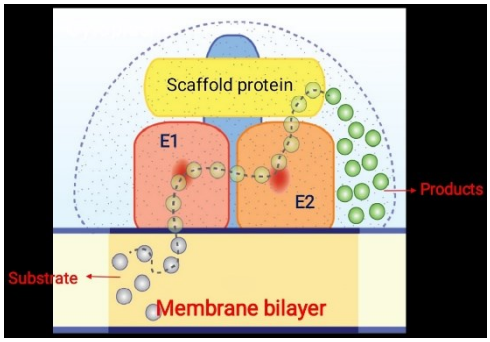

Plate 2. Structure of a metabolon

4. CHARACTERISATION

4.1. Yeast-2-hybrid methods

Probably the most widely used technique to detect protein–protein interaction is the yeast-two-hybrid assay. In the conventional yeast-two hybrid method, protein interactions bring together a DNA-binding domain and a transactivation domain of the GAL4 transcription factor in the nucleus. Spurious self-activators in the nucleus often give rise to false positive interactions. To overcome this problem, the split-ubiquitin system was developed. In brief, the split-ubiquitin principle is based on that the ubiquitin protein can be split into two stable moieties, an N-terminal fragment called Nub and a C-terminal fragment called Cub. The wild-type Nub (referred to as NubI) is capable of spontaneous re-association with Cub to form a full-length ‘‘pseudo-ubiquitin’’ molecule. In the mutated NubG fragment the spontaneous association between Nub and Cub is prevented. The split-ubiquitin approach is based on the detection of the in vivo processing of a reconstituted split ubiquitin. The bait protein—which can be either naturally membrane-bound or artificially membrane-anchored— is fused to a Cub moiety linked to a transcription factor, while a prey protein is fused to the NubG fragment. Upon interaction of bait and prey proteins tagged with either Nub and Cub, ubiquitin reconstitution occurs and leads to the proteolytic cleavage and subsequent release of a transcription factor that triggers the activation of a reporter system enabling easy detection. In this manner, the membrane- based yeast-two-hybrid system enables detection of interactions between membrane proteins in a natural environment

4.2. Co-immunoprecipitation (Co-IP)

The basic principle for any Co-IP is extracting proteins interacting with a given protein in biological samples by immunoprecipitation, followed by identification of the proteins by proteomics. As the interaction between the proteins has to last throughout the extraction procedure, Co-IP experiments typically report robust interactions. To enable more transient interactions to be reported, crosslinking of the proteins prior to the extraction has successfully been used. Due to the challenge in having specific antibodies against a target of interest, using tags to which commercial antibodies are available has become a common practice. The Green Fluorescent Protein (GFP) fluorophore tag is well-documented to form a self-contained and stable structure independent of its fusion partners and often does not interfere despite its large

size. Furthermore, transgenic lines with fluorophore-tagged versions of the protein of interest are often generated for localization purposes and therefore available for Co- IP experiments using commercial GFP antibodies. As an alternative to use of the classical antibodies, there is now available a promising technique, still not adopted in plant research, which use a distinct type of heavy chain- only antibodies that in nature is found in sera of camelids. From these antibodies, the smallest intact functional antigen-binding single domain is the VHH fragment (only 15 KD), also known as a nanobody. In a GFP trap, nanobodies directed towards the fluorophore protein is coupled to a matrix (e.g. agarose beads, magnetic agarose beads, magnetic particles) and used for Co-IP of GFP fusion proteins and their interacting partners.

4.3. Tandem affinity purification (TAP) method

The technique was developed in 1998. The first article that mentions the purification of protein complexes from plant via the TAP method was published in 2004. TAP has become one of the most popular methods for purification of in vivo protein complexes and for identification of their components by mass spectrometry (MS), thanks to regular optimizations of the method to filter hits using database of background proteins from different experiments, the development of several tags, the use of mild detergents (e.g. digitonin, dodecylmaltoside, nonidet P-40), and the advent of high-throughput, ultrasensitive MS and protein sequence databases. The TAP method relies on the application of a two-step affinity purification protocol. The first tag is a fusion protein containing a strong antigenic region (such as Protein G or GFP) fused to a separate smaller tag (such as streptavidin or calmodulin-binding peptide). The two tags are usually linked by a specific cleavage site (such as the Tobacco Etch Virus or Rhinovirus 3C protease cleavage sites). Details on tag variations already used, their limitations, as well as critical conditions for TAP are available elsewhere. For the solubilization and isolation of membrane protein complexes, detergent type and detergent concentration should be chosen and tested regarding the tagged protein to be purified. The two-step purification protocol may wash out partners in weak or transient interactions and thus be problematic for the study of metabolons. The tag may not be exposed to the affinity beads and the protease may in some conditions unspecifically cleave target proteins. Finally, large scale analysis of the interactome using TAP tagging is time-consuming and expensive.

4.4. Fluorescence resonance energy transfer (FRET)-based techniques

To observe FRET, we need two fluorophores with a significant spectral overlap. FRET is based on a dipole–dipole resonance interaction that does not involve any light emission and absorption and in which non-radiative energy is transferred from an excited fluorescent molecule serving as a ‘‘donor’’ to another fluorescent molecule, the ‘‘acceptor’. With appropriate orientation of the fluorophores, FRET is occurring over a range of 1–10 nm. The energy transfer leads to quenching in the fluorescence emission and to reduced lifetime of the donor, concomitantly FRET increases photon emission from the acceptor. FRET-based techniques are unique methods to monitor the functional dynamic changes of (1) biochemical activities, (2) conformation, and (3) particularly transient protein– protein interactions both in vitro and in vivo. Nevertheless, it must be emphasized that FRET techniques do not detect directly the interaction of the two tagged proteins, but the distance between the two fluorescent tags—a distance in the scale at which protein–protein interactions take place.

In recent years, FRET—measured by FLIM methods—has become the method of choice to probe and quantify protein–protein interactions in living cells. Fluorescence lifetime is the average time that a molecule spends in an excited state before returning to the ground state, typically with the emission of photons. FRET efficiency is precisely calculated by measuring the donor lifetime in the presence and the absence of acceptor. The donor lifetime is always shorter in the presence of an acceptor. Fluorescence lifetime measurements are implemented in wide-field, Con focal, and multi-photon excitation microscopes, and determined in either the time domain or the frequency domain methods. The physics underneath these two different methods is identical, only the analysis of the measurements differs. The most accurate, the most employed and also the most time-consuming method—the time domain FLIM—can be measured by time-correlated single photon counting (Bassard *et al.,* 2017)

5. Metabolon formation - Primary and secondary metabolism

5.1. Primary metabolism (Obata, 2019)

Plant primary metabolism needs to be quickly adjusted under rapidly fluctuating environments to meet changing metabolic demands and to maintain metabolic homeostasis. Dynamic metabolons will provide ideal solutions to achieve quick adjustment of metabolic network since metabolons can regulate fluxes and directions of metabolic pathway reactions

without time-consuming processes like protein synthesis/ degradation and post-translational modification of enzymes. Since these processes are energy and resource demanding, dynamic metabolons should also be cost-effective means to respond to repeating environmental events including day/night cycle, shading, temperature and humidity changes. Thus, the dynamic metabolon is a reasonable mechanism to regulate plant primary metabolism and indeed many metabolons have been found in major primary metabolic pathways.

5.1.1. Glycolysis

The finding of the glycolytic metabolon started from an unexpected detection of glycolytic enzymes in a mitochondrial proteome study in Arabidopsis. The mitochondrial localization of glycolytic enzymes is confirmed by fluorescent protein fusion. In a fractionation experiment of subcellular compartments, four of the glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, aldolase, phosphoglycerate mutase, and enolase) are detected in the fraction containing proteins in the inter membrane space or associated with the outer membrane of the mitochondria and a protease protection assay further demonstrated the localization of these enzymes on the outer surface of mitochondria. The enzymes attached to the mitochondria are catalytically active and therefore the isolated mitochondria can utilize 13C-glucose to feed the TCA cycle ending up with 13Clabel accumulation in the intermediates of the cycle and compounds derived from them. In a follow-up study, metabolite channelling between the glycolytic enzymes attached to the mitochondria was confirmed by an isotope dilution experiment. In this experiment, 13C-labled glucose or fructose-1, 6-bisphosphate (F16BP) was fed to the isolated mitochondria and the changes of label accumulation rate in fructose-6-phosphate (F6P) / dihydroxyacetone phosphate (DHAP) or 3-phosphoglycerate were monitored when the non-labeled glycolytic intermediates were added to dilute the 13C-lable. Metabolite channelling is expected to prevent the incorporation of non-labeled intermediates in the reactions and eventually keep the label accumulation rate in the product molecules. The results showed that F16BP, DHAP and glyceraldehyde-3- phosphate were almost fully channelled in the glycolytic enzyme complex whereas only 38 and 63% of glucose-6-phosphate and F6P are channelled, respectively.

5.1.2. TCA cycle

The TCA cycle metabolon is the multi-enzyme complex for which the term 'metabolon' was coined. The initial studies extensively investigated its composition, catalytic properties, tertiary structure, and metabolite channelling both in vitro and in vivo. However, there had been no information on plant TCA cycle metabolon. Additionally, comprehensive assessment of the TCA cycle enzyme complexes had been limited in bacteria and the interactome of the TCA cycle enzymes had not been elucidated in eukaryotic cells until recently. In order to efficiently detect relatively unstable interactions of the TCA cycle enzymes, which is expected for dynamic metabolon, three (semi)quantitative methods, namely affinity purification mass spectrometry (AP-MS), split-luciferase complementation, and yeast-two-hybrid assay, are integrated to avoid both false-positive and -negative detection of interacting protein pair. It should be noted that the interaction of proteins takes place in different living cell systems (Arabidopsis cell culture, Arabidopsis mesophyll protoplasts, and yeast cells in APMS, split-luciferase, and yeast-two-hybrid, respectively) and the detection of interaction is based on independent principles (Zhang *et al.,* 2017)

PATHWAYS	ENZYMES	SPECIES
Glycolysis	GAPDH – aldolase - PGM - enolase	Arabidopsis Potato
TCA cycle	SDH – Fumarase - MDH- CSY- aconitase-IDH	Arabidopsis Potato
Starch synthesis	SBE – SS - DBE - Pho-DPE	Wheat, Maize, Barley Rice, Sweet potato
Polyamine synthesis	SPDS-SPMS	Arabidopsis

Table 1. Metabolon formation in different primary metabolic pathways

5.2. Metabolons and secondary metabolism

Metabolon formation and metabolic channelling in plant secondary metabolism enable plants to effectively synthesize specific natural products and to avoid metabolic interference. Channelling can involve different cell types, take advantage of compartmentalization within the same cell or proceed directly within a metabolon. New experimental approaches document the importance of channelling in the synthesis of isoprenoids, alkaloids, phenylpropanoids, flavonoids and cyanogenic glucosides. Metabolon formation and metabolic channelling in natural-product synthesis facilitate attempts to genetically engineer new pathways into plants to improve their content of valuable natural products. They also offer the opportunity to introduce new traits by genetic engineering to produce plant cultivars that adhere to the principle of substantial equivalence (Xia *et al.,* 2017).

6. Metabolons and phytochemical defense

6.1. Dhurrin metabolon

One class of specialized metabolites is the cyanogenic glucosides, such as dhurrin, which is present in Sorghum bicolor. sorghum plant releases chemicals in response to attacks by plant eaters or pests—one such chemical, a substance called dhurrin, has been found to turn into cyanide when hydrolysed (Laursen *et al.*, 2016).

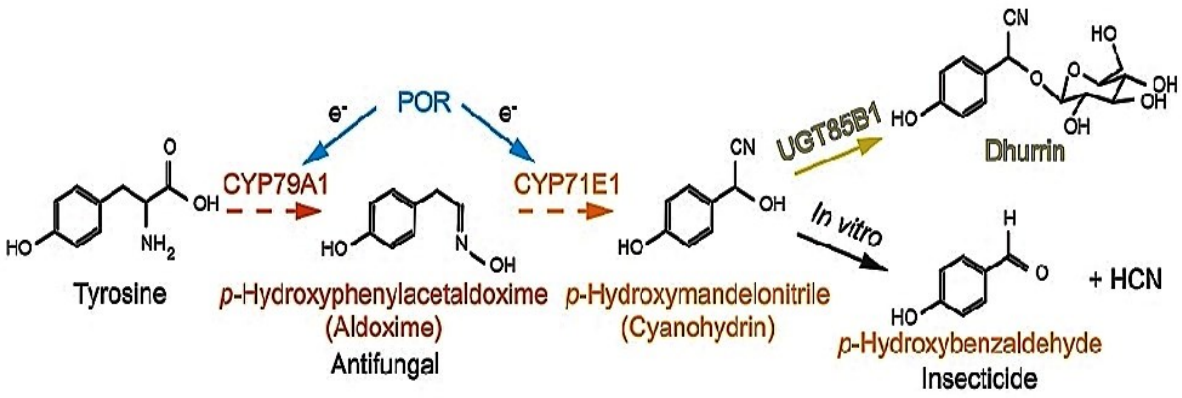

Figure 1. Dhurrin biosynthetic pathway

Protein content in the course of SMALP purification analysed by quantitative mass spectrometry. The protein enrichment was determined on the basis of the content of the three

POR isoforms, the P450 proteins, and other enzymes including UGT85B1, dhurrinase, cytochrome b5 (Cytb5), and Cytb5 reductase. Microsomal and SMA-solubilized fractions had similar protein content. After affinity chromatography, 132 proteins were quantified in the SMALPs, with the P450s CYP79A1 and CYP71E1 among the eightmost abundant proteins. The soluble UGT85B1 could not be quantified in the purified SMALP sample, most likely because of the extensive washing steps during microsome preparation and affinity chromatography. The strong enrichment of the entire complement of membrane-bound dhurrin pathway enzymes in the affinity-purified SMALPs demonstrates that these enzymes are assembled in a metabolon.

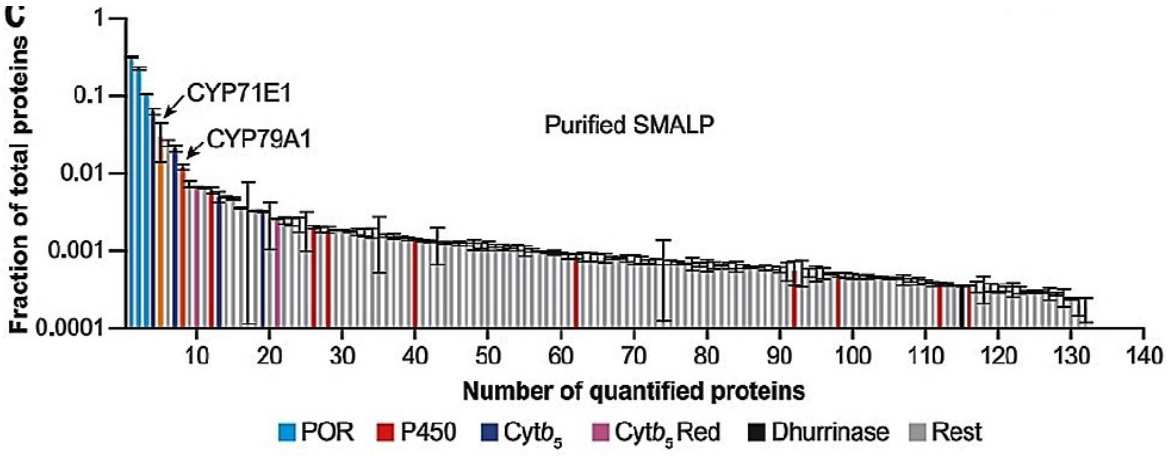

Figure 2. Fraction of total proteins in affinity purified SMALPs

Functional regulation of dhurrin biosynthesis as a response to environmental stresses likely involves dynamic assembly and disassembly of the metabolon. Channelling of L-tyrosine toward dhurrin by in vitro reconstitution of the dhurrin enzymes in liposomes depends on UGT85B1. Catalytic activities of P450s were determined based on the amounts of aldoxime (CYP79A1-mediated) and cyanohydrin (CYP71E1-mediated) produced after administration of radiolabeled L-tyrosine substrate. In the absence of UGT85B1, 50% of the produced aldoxime was further converted into the cyanohydrin.

CYP79A1, CYP71E1, UGT85B1, POR2b, and different combinations of these (including control proteins) were transiently expressed in Nicotiana benthamiana leaf epidermal cells to study organization of the dhurrin pathway enzymes in plants. The expression levels of the heterologous proteins in N. benthamiana were quantitatively comparable to those in S. bicolor

seedlings. Upon coexpression of CYP79A1 and CYP71E1, Several by-products of the dhurrin pathway and some dhurrin accumulated. The formation of dhurrin reflects the inherent capability of N. benthamiana to glycosylate exogenous compounds. Coexpression of UGT85B1 with the two P450s and without S. bicolor POR2b resulted in the accumulation of 263%more dhurrin and a reduction of by-product release to 9% of the level observed in the absence of UGT85B1 (table S3). The efficient channelling of intermediates achieved upon coexpression of UGT85B1 supports the assembly of a metabolon in planta and confirms that endogenous N. benthamiana POR is sufficient to provide reducing equivalents to the P450s.

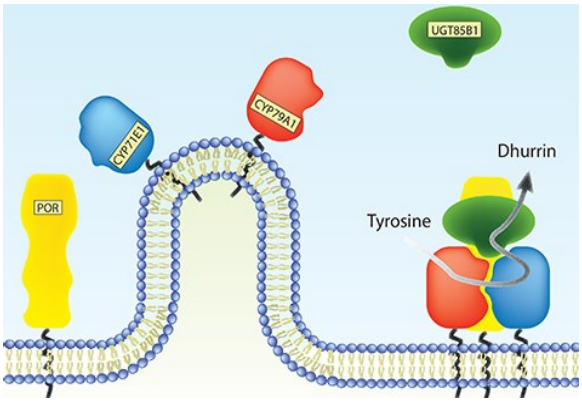

Plate 3. Dhurrin metabolon

CYP79A1, CYP71E1, UGT85B1, and POR2b expressed to evaluate dhurrin metabolon formation as fusion proteins with different fluorescent proteins suitable for plants FLIM and FCS. All possible combinations with enhanced green fluorescent protein (eGFP) fused to two of the three target proteins produced similar amounts of dhurrin and byproducts. The tracking of fluorescent fusion constructs of the dhurrin enzymes with confocal microscopy techniques illustrated their fast movement in the plant cell along the endoplasmic reticulum (ER) network, as has also been observed in studies of other metabolons.

The in planta FRET results demonstrated that CYP79A1, CYP71E1, and UGT85B1 all form homo and hetero-oligomers with FRET values higher than those of the controls. FRET signals for CYP79A1-CYP79A1, CYP71E1-CYP71E1, and CYP79A1-CYP71E1 complexes were unaffected by coexpression of UGT85B1. In contrast, UGT85B1-UGT85B1 oligomerization was enhanced by coexpression of either CYP79A1 or CYP71E1, with the

highest FRET signal observed when the entire dhurrin pathway was expressed, suggesting recruitment of UGT85B1 by the P450s.

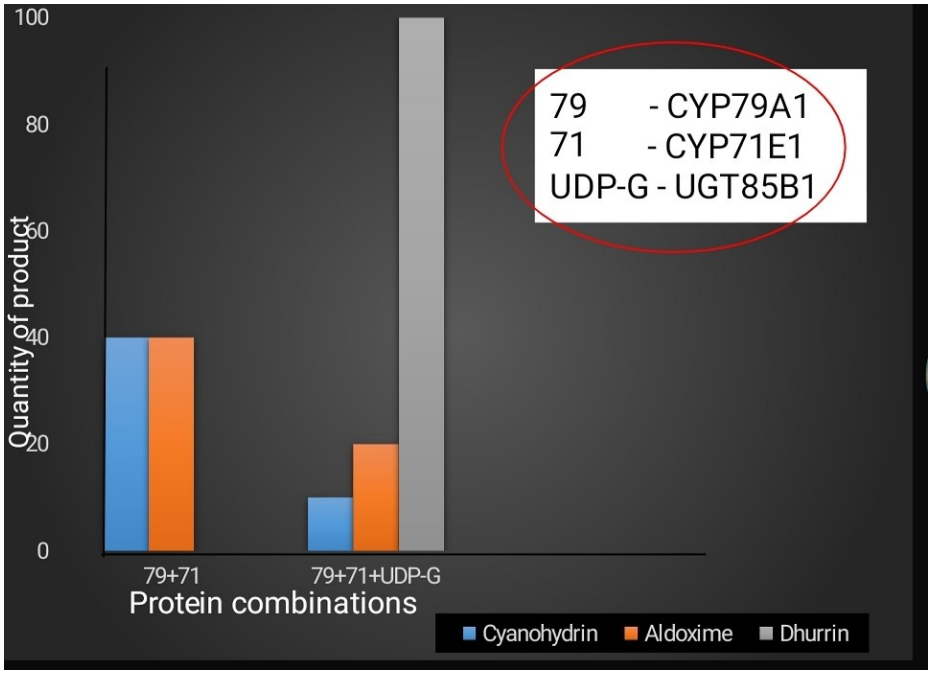

Figure 3. Quantity of products formed from different combinations of dhurrin metabolic pathway enzymes

6.2. Camalexin metabolon

Camalexin is a sulfur-containing tryptophan-derived secondary metabolite, and is considered to be the major phytoalexin involved in biotic responses in *A. thaliana*. The camalexin biosynthesis pathway involves the conversion of tryptophan to indole-3-acetaldoxime (IAOx), through the action of two functionally redundant cytochrome P450 enzymes, CYP79B2 and CYP79B3. This followed by the dehydration of IAOx to indole 3 acetonitrile (IAN), catalysed by CYP71A13 and CYP71A12. IAN is then conjugated to glutathione by the glutathione-S-transferase GSTF6 to synthesize GSH (IAN) then metabolized to Cys (IAN) by g-glutamyl peptidases GGP1 and GGP3. Finally, the PAD3/CYP71B15 enzyme catalyses the last two reactions of the biosynthesis pathway leading to camalexin.

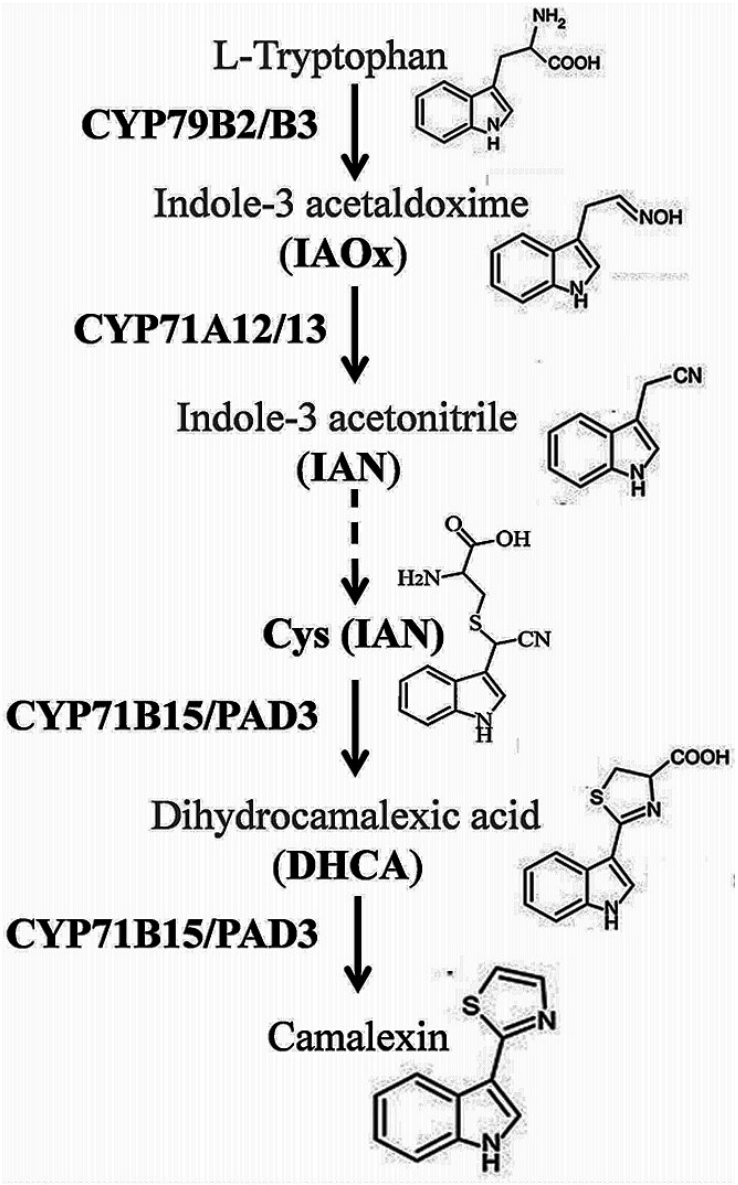

Figure 4. Camalexin biosynthetic pathway

Many genetic approaches confirmed that camalexin plays a positive role in resistance. For instance, camalexin accumulation was correlated with resistance to necrotrophic fungi such as *Alternaria brassicicola*, *Botrytiscinerea* and *Plectosphaerella cucumerina*. Camalexin has also been reported to play a defensive role against hemibiotrophic fungus (Lemarie *et al.,* 2015)

CYP71A12, CYP71A13, CYP71B15 and ATR1, we transiently expressed different combinations of these proteins in *Nicotiana benthamiana* to confirm the physical interaction of the camalexin biosynthetic enzymes as C-terminally YFP- and FLAG-tagged proteins (Mucha *et al*., 2019).

Solubilized microsomes were applied to GFP-beads and IP and co-IP was monitored by protein gel blot analysis with GFP- and FLAG-specific antibodies, respectively. CYP71A13 interacted with CYP71B15 and ATR1, and CYP71B15 interacted with CYP71A12. In addition, we observed interactions CYP71A13 and CYP71B15 with the glutathione transferase.

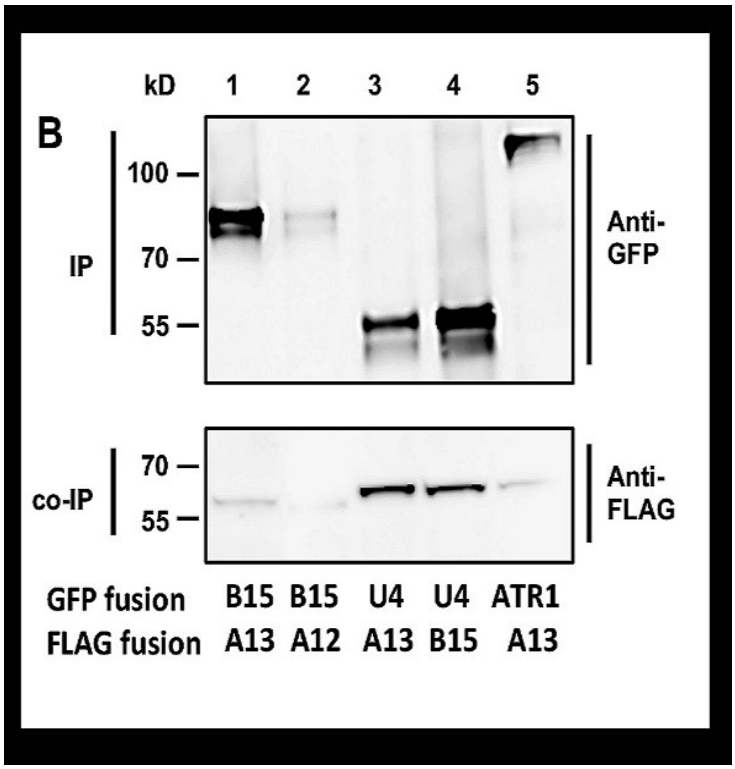

Figure 5. Western blot analysis of immunoprecipitation sample

7. Synthetic metabolons

Synthetic multienzyme complex formation to mimic nature's strategy to increase metabolic and signaling efficiencyIn metabolic engineering where a natural endogenous biosynthetic pathway is manipulated to increase productivity and yield of a valuable molecule, several challenges have to be overcome. One is the expression level of heterologous enzymes. In bacterial systems, expression levels are often much higher compared to endogenous enzymes. this can lead to cellular stress responses, for example, due to the huge amount of proteins produced that are even unfolded or misfolded, this imbalances coupled with unpredictable and non-controllable metabolic changes and high energy and molecule (amino acids, nucleotides)

consumption. An additional challenge is to balance expression of consecutive enzymes. If the reactions are not balanced in a manipulated pathway, toxic intermediates are likely to accumulate which can lead to death of the expression host. With nature's strategies to increase metabolic efficiency in mind, metabolic engineers are trying to engineer artificial multi-enzyme complexes, where the enzymes performing consecutive reactions are spatially organized (directed enzyme organization)

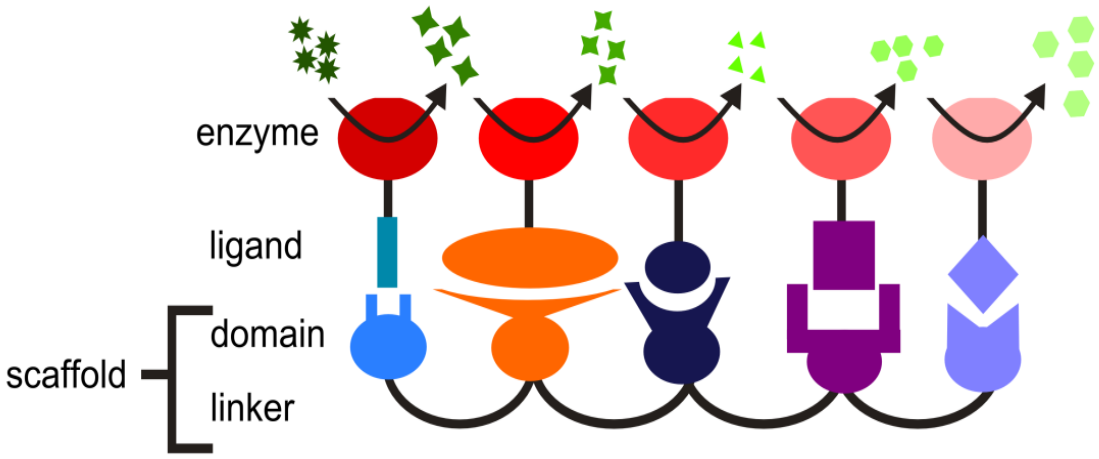

Plate 4. Artificial scaffolding of consecutive enzymes of a metabolic pathway (IWT Phd project)

The idea is to co-localize functional enzymes into complexes. Due to the enforced proximity of the enzyme active sites and the formation of enzyme microdomains built as a consequence of co-clustering of multiple enzymes into higher aggregates, catalytic efficiency and metabolic pathway performance are improved. Overall, pathway balancing involves several layers, including DNA copy number, transcriptional and translational regulation, scaffolding and compartmentation, as well as inclusion of metabolic sensors balancing the flux through synthetic pathways. In addi-tion to balancing protein amount and organization, enzyme engineering allows to improve activity, selectivity, and stability of enzymes

Employing metazoan machinery for modular control over pathway flux. The genes encoding the mevalonate pathway enzymes (HMGS and HMGR) were taken from yeast (*S. cerevisiae*) and inserted into *E. coli* along with the *E. coli* gene encoding AtoB. These enzymes

have different levels of activity, creating a bottleneck that results in accumulation of the intermediate HMG-CoA, which is toxic to *E. coli* at high concentrations. (Proschel *et al.,* 2015)

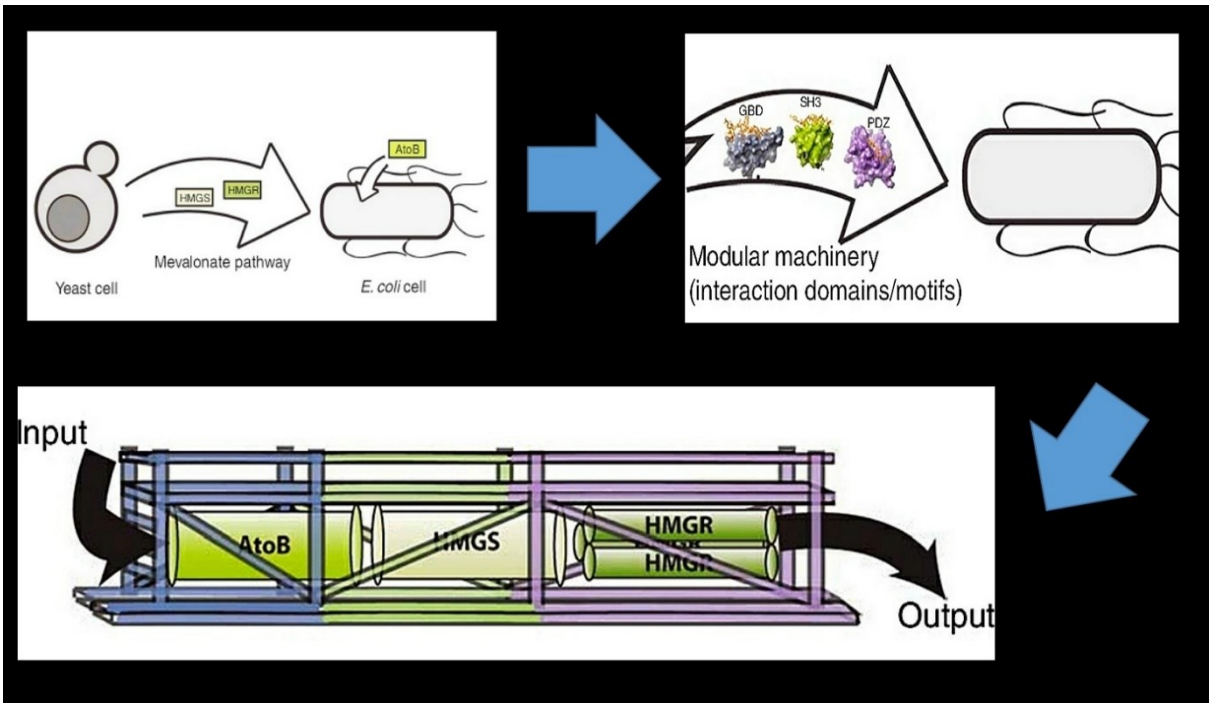

Plate 5. Scaffolding enzymes of mevalonate biosynthetic pathway

Fluxes through each enzymatic step can be compared to pipes of different cross-sectional areas put together to make a pipeline. The thickness of the arrows connecting these enzymes represents the flux through these respective steps, and the resultant relative accumulation of intermediates. Protein-protein interaction domains and ligands from metazoan cells (mouse SH3 and PDZ domains and rat GBD) were used to design regulation machinery and inserted into these same *E. coli* cells. Thus, both the mevalonate pathway enzymes and the regulation machinery are heterologous to *E. coli*. The scaffolded pathway is more efficient as a result of co-localizing the mevalonate enzymes to the same complex as well as optimizing the enzyme stoichiometry to balance the units of activity at the complex (Dueber *et al.*, 2009).

8. Summary

In plants, hundreds of chemical reactions occur simultaneously to process energy sources to provide precursor molecules for metabolism and defense compounds. To facilitate rapid

production of metabolites and to avoid leakage of potentially toxic and labile intermediates, many of the biosynthetic pathways in plants are organized in multienzyme clusters known as metabolons. Metabolon is the supramolecular complex of sequential metabolic enzymes and cellular structural elements. Metabolon formation involves protein-protein interactions, solvent effect, scaffold proteins and organization on structural elements such as endoplasmic reticulum, mitochondria *etc*. As metabolons are stabilized by weak protein–protein interactions, their isolation by detergents will cause dissociation of these enzyme complexes. Instead, a co-polymer of styrene and maleic acid (SMA) is commonly used for isolation, which spontaneously inserts into membranes and forms discrete lipid particles.

Dynamic metabolons provide an ideal solution to plants to adjust quickly to the changing environment. Metabolon formation has been identified in various primary metabolic pathways such as glycolysis, TCA cycle, starch synthesis and polyamine synthesis and also in the production of secondary metabolites like lignin, anthocyanin, sporopollenin, flavonoids, isoflavanoids, tannins *etc*.

Dhurrin is a tyrosine derived cyanogenic glucoside in *Sorghum bicolor,* whose biosynthesis involves two CYP enzymes (CYP79A1 and CYP71E1), cytochrome P450 oxidoreductase and a UDP-glucosyl transferase (UGT85B1). They physically interact to form a metabolon which helps to detoxify by-products formed from intermediates like cyanohydrin and aldoxime. A metabolon formed by the interaction between the enzymes CYP79B2, CYP79B3, CYP71A13, CYP71A12, PAD3 and CYP71B15 helps in the instant mass production of camalexin, a tryptophan-derived phytoalexin present in *Arabidopsis thaliana*.

Currently, metabolic engineers are trying to develop artificial multienzyme complexes by emulating nature's strategies, in which the enzymes involved in consecutive reactions are spatially organized by specific scaffold proteins. To simulate the mevalonate pathway of *Saccharomyces cerevisiae* in *E. coli*, a scaffold consisting of protein interaction domains and corresponding peptide ligands were introduced, wherein, the product titre was increased 77-fold in comparison with the unscaffolded enzyme system. RuBisCO and carbonic anhydrase (CA) can be scaffolded in *E. coli* to increase the carboxylase activity of RuBisCO. Knowledge of metabolons has opened up new avenues in metabolic engineering, where synthetic metabolons form efficient tools for manipulating biosynthetic pathways.

9. Future line of work

Attempts can take to genetically engineer new pathways into plants to improve their content of valuable natural products. Different combinatorial uses of the individual proteins can expand the product output profile. Involvement of post-translational regulation in metabolon formation needs to be investigated in much more detail. Studies should be conducted more on microcompartments - Proteinaceous shells encapsulating functionally related enzymes.

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Discussion

1) What happened to the third enzyme UGT8 B1 of Dhurrin biosynthetic pathway after purification?

Answer – It lost during extensive washing steps of microsome preparation and affinity chromatography

2) What is co-immunoprecipitation?

Answer – Widely used technique to identify protein-protein interactions by using target protein- specific antibodies to indirectly capture proteins that are bound to this specific target protein. Co-IP experiments typically report robust interactions.

3) Scaffolding or microcompartments - Which is better choice?

Answer – Scaffolding is arranging consecutive enzymes of metabolic pathway in a sequencial manner and microcompartments are proteinaceous shells encapsulating functionally related enzymes. I think microcompartments will be a better choice as they encapsulate the whole metabolic pathway from bulk of cell.

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COLLEGE OF HORTICULTURE, VELLANIKKARA

Department of Plant Physiology

PP 591: MASTER'S SEMINAR

Name : Femina K.

Admission No : 2018-11-094

Major Advisor : Dr. T. Girija

Venue : Seminar Hall

Date : 05-12-2019

Time : 10:00 am

Metabolons: The intracellular metabolic highways in plants

Abstract

In plants, hundreds of chemical reactions occur simultaneously to process energy sources to provide precursor molecules for metabolism and defense compounds. To facilitate rapid production of metabolites and to avoid leakage of potentially toxic and labile intermediates, many of the biosynthetic pathways in plants are organized in multienzyme clusters known as metabolons. Metabolon is the supramolecular complex of sequential metabolic enzymes and cellular structural elements (Srere, 1985).

Metabolon formation involves protein-protein interactions, solvent effect, scaffold proteins and organization on structural elements such as endoplasmic reticulum, mitochondria *etc*. (Laursen *et al.,* 2015). As metabolons are stabilized by weak protein–protein interactions, their isolation by detergents will cause dissociation of these enzyme complexes. Instead, a co-polymer of styrene and maleic acid (SMA) is commonly used for isolation, which spontaneously inserts into membranes and forms discrete lipid particles.

Metabolon formation has been identified in various primary metabolic pathways such as glycolysis, TCA cycle, starch synthesis and polyamine synthesis and also in the production of secondary metabolites like lignin, anthocyanin, sporopollenin, flavonoids, isoflavanoids, tannins *etc*. (Obata, 2019).

Dhurrin is a tyrosine derived cyanogenic glucoside in *Sorghum bicolor,* whose biosynthesis involves two CYP enzymes (CYP79A1 and CYP71E1), cytochrome P450 oxidoreductase and a UDP-glucosyl transferase (UGT85B1). They physically interact to form a

metabolon which helps to detoxify by-products formed from intermediates like cyanohydrin and aldoxime (Laursen *et al.,* 2016). A metabolon formed by the interaction between the enzymes CYP79B2, CYP79B3, CYP71A13, CYP71A12, PAD3 and CYP71B15 helps in the instant mass production of camalexin, a tryptophan-derived phytoalexin present in *Arabidopsis thaliana* (Mucha *et al.,* 2019).

Currently, metabolic engineers are trying to develop artificial multienzyme complexes by emulating nature's strategies, in which the enzymes involved in consecutive reactions are spatially organized by specific scaffold proteins. To simulate the mevalonate pathway of *Saccharomyces cerevisiae* in *E. coli*, a scaffold consisting of protein interaction domains and corresponding peptide ligands were introduced, wherein, the product titre was increased 77-fold in comparison with the unscaffolded enzyme system (Dueber *et al.,* 2009). In another study, Smirnoff (2019) scaffolded RuBisCO and carbonic anhydrase (CA) in *E. coli* to increase the carboxylase activity of RuBisCO. Knowledge of metabolons has opened up new avenues in metabolic engineering, where synthetic metabolons form efficient tools for manipulating biosynthetic pathways.

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