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Metabolons in plant primary and secondary metabolism

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Abstract Metabolons are multi-enzyme protein complexes composed of enzymes catalyzing sequential reactions in a metabolic pathway. Metabolons mediate substrate channeling between the enzyme catalytic cores to enhance the pathway reactions, to achieve containment of reactive intermediates, and to prevent access of competing enzymes to the intermediates. These provide unique advantages in metabolic regulation. The discovery of plant metabolons has been accelerated by the recent technical developments and a considerable number of metabolons involved in both primary and secondary metabolism have been indicated in the last decade. These findings related with plant metabolons are comprehensively reviewed in this review, indicating metabolome-wide engagement of metabolons. However, there are still unexplored frontiers remaining for further discovery of metabolons in plant metabolism. Pathways with high potential of novel metabolon and technical issues to be solved for the future discovery will also be discussed.

Keywords Metabolon · Metabolite channeling · Substrate channeling · Multi-enzyme complex · Protein–protein interaction

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Abbreviations

TCA	Tricarboxylic acid
F16BP	Fructose-1,6-bisposphate
F6P	Fructose-6-phosphate
DHAP	Dihydroxyacetone phosphate
VDAC	Voltage dependent anion channel
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PEPC	Phospho*enol*pyruvate carboxylase
AP-MS	Affinity purification mass spectrometry
MDH	Malate dehydrogenase
IDH	Isocitrate dehydrogenase
SDH	Succinate dehydrogenase
CS	Citrate synthase
SBE	Starch branching enzyme
Pho	Starch phosphorylase
SS	Starch synthase
DBE	Starch debranching enzyme
DPE	Disproportionating enzyme
IAA	Indole-3-acetic acid
ER	Endoplasmic reticulum
SMALP	Styrene maleic acid lipid particle
FLIM	Fluorescence-lifetime imaging microscopy
FRET	Fluorescent resonance energy transfer
PAL	Phenylalanine ammonia lyase
C4H	Cinnamate 4-hydroxylase
CHS	Chalcone synthase
CHI	Chalcone isomerase
F3H	Flavanone 3-hydroxylase

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DFR	Dihydroflavonol 4-reductase
ANS	Anthocyanidin synthase
FLS	Flavonol synthase
F3′H	Flavonoid 3′-hydroxylase
C4′GT	Chalcone 4′-glucosyltransferase
FNS	Flavone synthase
LAR	Leucoanthocyanidin reductase
IFS	Isoflavone synthase
ADT	Arogenate dehydratase
CHR	Chalcone reductase
HCT	Hydroxycinnamoyl transferase
C3′H	*p*-Coumaroyl shikimate 3′-hydroxylase
CCoAOMT	Caffeoyl CoA *O*-methyltransferase
COMT	Caffeic acid *O*-methyltransferase
F5H	Ferulate 5-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
CCR	Cinnamoyl CoA reductase
4CL	4-Coumaroyl-CoA ligase
MSBP	Membrane steroid-binding protein
PT	Prenyltransferase
CHIL	Chalcone isomerase like
GGPP	Geranylgeranyl diphosphate
GGPS	Geranylgeranyl diphosphate synthase
PSY	Phytoene synthase
BiFC	Bimolecular fluorescent complementation
ACOS5	Acetyl-CoA synthetase
PKS	Polyketide synthase
TKPR	Tetraketide α-pyrone reductase
OPPP	Oxidative pentose phosphate pathway

Introduction

'Metabolon' is the term proposed by Paul A Srere for a "supramolecular complex of sequential metabolic enzymes and cellular structural elements" (Srere 1985). Enzymes composed of a metabolon often mediate metabolite channeling (termed also as substrate channeling, or metabolic channeling) by which the intermediate metabolites are kept within the enzyme complex and catalyzed by the sequential enzymes without diffusing into bulk phase. Srere supposed supramolecular assemblies formed by quinary (transient and protein-surface structure dependent) interactions; capable of substrate channeling; regulate metabolic flux by association and dissociation of components; involve interactions with structural elements of the cell; restrict the localization of the enzymes; have common features in gene expression for constituting enzymes (Srere 1985). Initially the term 'metabolon' is used to describe the complexes of the enzymes of the Krebs' tricarboxylic acid (TCA) cycle, glycolysis, and purine biosynthetic pathways. A considerable number of so called 'metabolon' have been found in various organisms including bacteria, fungi, plants, and mammals to date. However, strictly to say, none of them have been proved to fulfill all criteria Srere expected. Hence the use of this term slightly differs among researchers depending on the features they put the most values on. Some include protein complexes composed of more than one enzyme mediating a part of pathway reactions (e.g. Li et al. 2015), but others expect higher order organization which include whole pathway enzymes (Sweetlove and Fernie 2018). Some call all protein complexes of sequential enzymes as metabolon (Du et al. 2018), but others require strict proof of metabolite channeling and/or association to structural elements (Fernie et al. 2018). Heteromeric enzyme complexes such as pyruvate dehydrogenase complex and 2-oxoglutarate dehydrogenase complex are sometimes called also as metabolons (Sheu and Blass 1999). Here in this review, a protein complex (or a part of a protein complex) composed of multiple enzymes which independently catalyze sequential reactions of a metabolic pathway is called as a metabolon. A metabolon should also be expected or proved to mediate some type of metabolite channeling.

Plant metabolism needs to be rapidly, flexibly, and coordinately regulated in response to fluctuating environments, which can be drastically changed sometimes within an order of seconds. The metabolon is a mechanism which can achieve the metabolic network regulation to meet such demands. Spivey and Merz (1989) nicely summarized the potential functions of metabolite channeling in a metabolon. The functions are mainly due to the creation of microenvironment for the catalytic reactions and containment of intermediate metabolites inside a metabolon, which are closely related each other. By providing a specific microenvironment inside the protein complex, a metabolon can (1) increases local concentrations of intermediates independent of total metabolite contents in the cell; (2) circumvents unfavorable equilibria (Noor et al. 2014); (3) avoids solvation of metabolites

which often permits faster reaction rates. Additionally, by containing the intermediates and preventing their diffusion into bulk matrix phases, a metabolon can (4) prevents intermediates from competing reactions; (5) protects unstable intermediates from degradation and reduces the cytotoxicity of them; (6) reduces lag-times between coupled reactions (Spivey and Merz 1989). Thus, dynamic formation of a metabolon can regulate the metabolic flux in a pathway and replacement of the components of a metabolon can mediate re-direction of the metabolic flux into another reaction (Fig. 1). Since changes in degree of association and composition of a metabolon necessarily require neither of energy inputs, synthesis and degradation of proteins, nor additional regulatory proteins, they are ideal mechanisms to regulate rapid and frequent changes of metabolic fluxes in response to fluctuating environments. Metabolons are also suitable to coordinate fluxes into multiple metabolic pathways by regulating the ratio of reactions at branching points of metabolic pathways (Figure 1, Spivey and Merz 1989; Sweetlove and Fernie 2013). These features are suitable for the regulation of the metabolic network of central carbon metabolism. Importance of metabolon is also

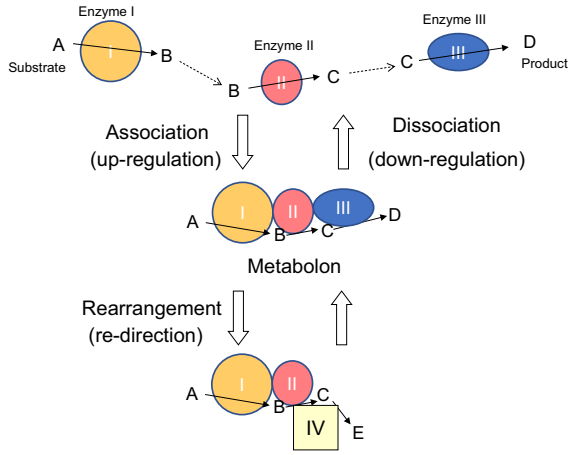

Fig. 1 Metabolic regulation by a dynamic metabolon. A schematic diagram showing the regulation of metabolic pathways by a dynamic metabolon. A pathway in which product D is synthesized from substrate A via the reactions catalyzed by enzyme I, II, and III (arrows) is assumed. B and C are the pathway intermediates. Association of the metabolon will enhance the pathway reactions and is expected to up-regulate the pathway. On the other hand, dissociation of metabolon will down-regulate the pathway. When the enzyme III is replaced with enzyme IV which catalyze conversion of C to E, the metabolic flux is re-directed to the production of E

discussed in plant secondary metabolism (specialized metabolism), especially on the needs to achieve high concentration of toxic and unstable intermediate metabolites, to dynamically regulate branching pathways, and to ensure high metabolic flux enabling high production of functional phytochemicals (Winkel 2004, 2009; Jørgensen et al. 2005; Møller 2010; Laursen et al. 2015; Knudsen et al. 2018). In addition to the biological significance, metabolon draws attention in the application to metabolic engineering and synthetic biology (Siu et al. 2015; Wheeldon et al. 2016; Abernathy et al. 2017; Li et al. 2018; Smirnoff 2019) due to its advantages in regulation of metabolic reactions both in vitro and in vivo. There are attempts to introduce artificial metabolons into engineered pathways to enhance pathway reactions. Even though experimental proof of metabolite channeling in synthetic metabolon is scarce, the metabolic enzymes connected by synthetic scaffold and the expression of chimeric enzymes successfully enhance the reactions of metabolic pathways (Dueber et al. 2009; Ke et al. 2016; Lin et al. 2017a).

Thanks to the recent advances in protein–protein interaction assays and theoretical modeling approaches, a lot of potential metabolons are demonstrated and proposed (Table 1). This review article will provide a comprehensive review of these studies showing involvement of metabolons in plant metabolic pathways. Since there are excellent comprehensive reviews on plant metabolon in mid to end of the 2000s (Winkel 2004, 2009; Jørgensen et al. 2005; Ralston and Yu 2006), the findings in the last decade will mainly be reviewed in this article. Metabolons are apparently involved in most of the major metabolic pathways in plants, indicating ubiquitous existence of metabolons in entire metabolic network of plants. However, there are many unexplored metabolic pathways which have strong potential to involve metabolons for their regulation. These pathways and the technical issues to enhance the discovery of novel metabolons will also be discussed in this review.

Metabolons in plant primary metabolism

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

Table 1 Metabolons in plants

Pathways	Enzymes	Co	In	Cn	Fu	Species	Methods	References
Primary metabolism								
Glycolysis	GAPDH, aldolase, PGM, enolase	+		+	+	*Arabidopsis* Potato	AP	Giegé et al. (2003), Graham et al. (2007)
TCA cycle	SDH, fumarase-MDH-CS-aconitase-IDH		+	+		*Arabidopsis* Potato	AP, Split-LUC, Y2H, BiFC	Zhang et al. (2017, 2018)
Starch synthesis	SBE, SS, DBE, Pho-DPE	+	+		+	Wheat Maize Barley Rice Sweet potato	CoIP XC CoIP Y2H, AP, SEC	Tetlow et al. (2004, 2008), Hennen-Bierwagen et al. (2009), Ahmed et al. (2015), Crofts et al. (2015), Lin et al. (2017b)
Polyamine synthesis	SPDS-SPMS	+	+			*Arabidopsis*	SEC	Panicot et al. (2002)
Secondary metabolism								
Dhurrin	CYP79A1-CYP71E1-UGT85B1	+	+	+		Sorghum	SMALP, FLIM/FRET FCS	Laursen et al. (2016)
Auxin	TAA-YUC		+			*Arabidopsis*	FLIM/FRET	Kriechbaumer et al. (2017)
Phenylpropanoid core pathway	PAL-C4H		+	+		Tobacco	FRET CoIP	Rasmussen and Dixon (1999), Achnine et al. (2004)
Anthocyanins Flavonols	CHS-CHI-F3H-DFR CHS-CHI-F3H-FLS		+			*Arabidopsis* Snapdragon Torrenia	CoIP, AP, Y2H	Burbulis and Winkel-Shirley (1999), Crosby et al. (2011)
Isoflavonoids	C4H, 4CL, CHS-CHR-CHI-IFS	+	+			Soybean	CoIP, BiFC, CoIP, Y2H	Dastmalchi et al. (2016), Mameda et al. (2018)
Lignin	C4H-4CL-CHS, HCT-C3′H					*Arabidopsis*		Bassard et al. (2012), Gou et al. (2018)
Prenylchalcones	CHS_H1, PT		+*			Hop	Y2H	Ban et al. (2018)
Isoprenoids	GGPS-PSY		+			*Arabidopsis*	Y2H	Camagna et al. (2018)
Bitter acids	PT1L-PT2		+		+	Hop	Y2H, CoIP	Li et al. (2015)
Monoterpene indole alkaloids	SGD-THAS		+			*Catharanthus*	BiFC	Stavrinides et al. (2015)

Table 1 continued

Pathways	Enzymes	Co	In	Cn	Fu	Species	Methods	References
Sporopollenin	ACOS5-PKSA-PKSB-TKPR1	+	+			*Arabidopsis* *Brassica napus*	CoIP, Y2H, FLIM/FRET	Lallemand et al. (2013), Qin et al. (2016)

Co, In, Cn and Fu indicate that the complex formation, interactions of specific protein pairs, metabolite channeling, and functions are experimentally proved. Hyphnated enzymes are shown to directly interact. +* indicates indirect interactions. PGM, phosphoglycerate mutase; SPDS, spermidine synthase; SPMS, spermine synthase; TAA, TAA family amino transferases; YUC, YUC family flavin monooxygenases; PT1L, PT1 like; SGD, strictosidine glucosidase; THAS, tetrahydroalstonine synthase; Split-LUC, split luciferase complementation; Y2H, yeast-two-hybrid assay; BiFC, bimolecular fluorescent complementation assay; Co-IP, co-immunoprecipitation; XC, crosslinking; SEC, size exclusion chromatography; SMALP, styrene maleic acid lipid particle; FLIM, fluorescent lifetime measurement; FRET, fluorescent resonance energy transfer

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 (Fig. 1). 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 (Table 1; Fig. 2).

Glycolysis

The finding of the glycolytic metabolon started from an unexpected detection of glycolytic enzymes in a mitochondrial proteome study in *Arabidopsis* (Giegé et al. 2003). The mitochondrial localization of glycolytic enzymes is confirmed by fluorescent protein fusion (Giegé et al. 2003). 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 (Giegé et al. 2003). The enzymes attached to the mitochondria are catalytically active and therefore the isolated mitochondria can utilize ^{13}C-glucose to feed the TCA cycle ending up with ^{13}C-label accumulation in the intermediates of the cycle and compounds derived from them (Giegé et al. 2003). In a follow-up study, metabolite channeling between the glycolytic enzymes attached to the mitochondria was confirmed by an isotope dilution experiment (Graham et al. 2007). In this experiment, ^{13}C-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 ^{13}C-lable. Metabolite channeling 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 channeled in the glycolytic enzyme complex whereas only 38 and 63% of glucose-6-phosphate and F6P are channeled, respectively (Graham et al. 2007). The authors also showed the dynamics of the protein complex by assessing the ratio of total cellular glycolytic enzyme activities and those associated with the isolated mitochondria under situations with different respiratory demands (Graham et al. 2007). More enzyme activities are associated with mitochondria when the mitochondrial respiration is stimulated by an uncoupler, overexpression of invertase, and aging, while less activities are associated with mitochondria when mitochondrial respiration is inhibited by KCN (Graham et al. 2007). A co-immunoprecipitation assay using anti-aldolase antibody demonstrated the possible interaction between

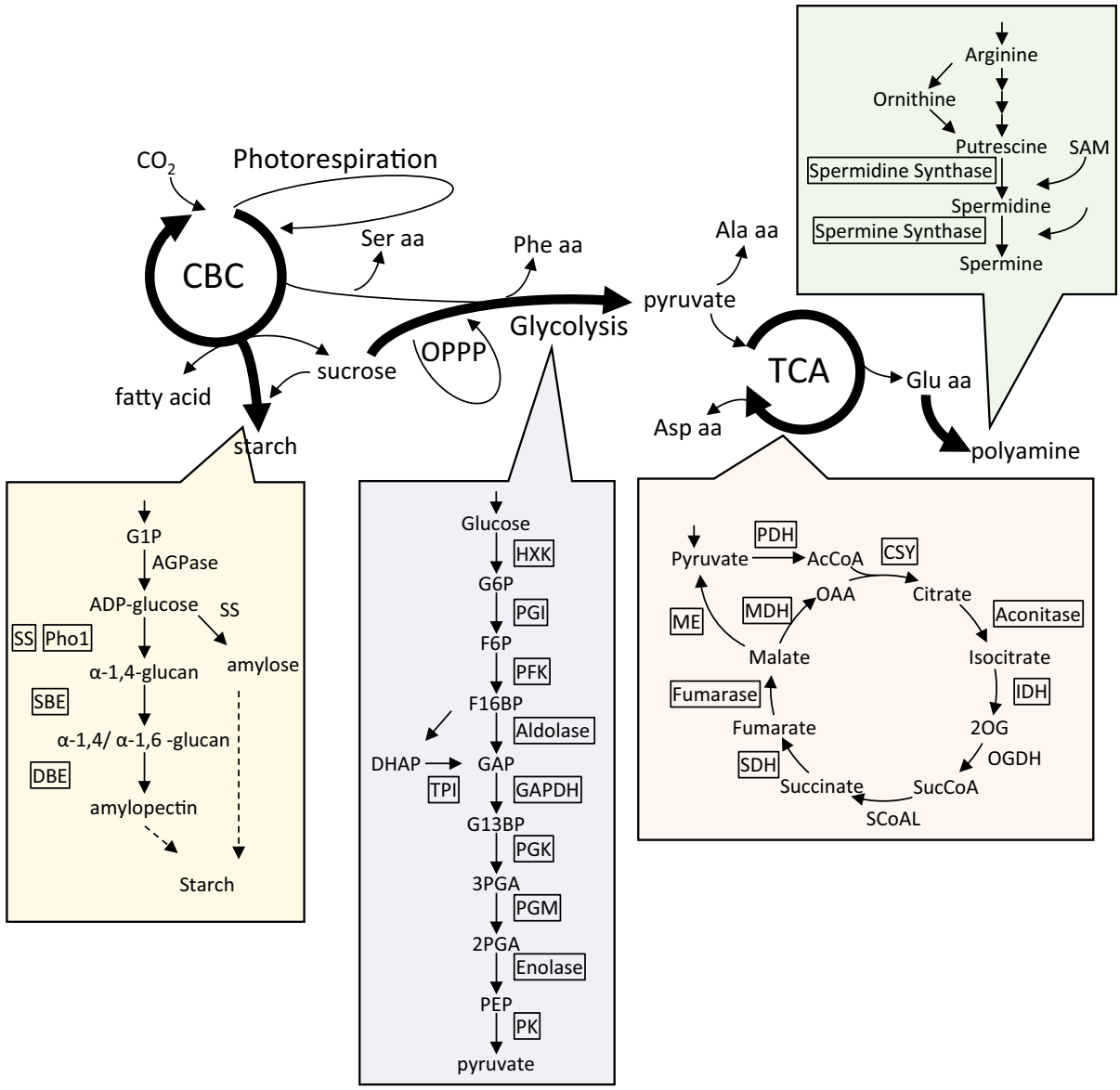

Fig. 2 Metabolons in plant primary metabolism. A schematic diagram of plant primary metabolism and the detailed pathways involving metabolons. The pathways with any evidence of metabolon are represented by bold arrows. Details of the pathways discussed in this review are shown in the squares. Solid and dashed arrows represent single and multiple reactions, respectively. The outlined enzymes are indicated to be involved in a metabolon. *CBC* Calvin–Benson cycle, *OPPP* oxidative pentose phosphate pathway, *aa* amino acid, *TCA* tricarboxylic acid cycle, *SAM S*-adenosyl methionine, *G1P* glucose-1-phosphspate, *AGPase* ADP-glucose pyrophosphorylase, *SS* starch synthase, *SBE* starch branching enzyme, *DBE1* starch debranching enzyme, *HXK* hexokinase, *PGI* phosphoglucose isomerase, *PFK* phosphofructokinase, *TPI* triosephosphate isomerase, *GAPDH* glyceraldehyde-3-phosphate, *PGK* phosphoglycerate kinase, *PGM* phosphoglycerate mutase, *PK* pyruvate kinase, *G6P* glucose-6-phosphate, *F6P* fructose-6-phosphate, *F16BP* fructose-1,6-bisphosphate, *DHAP* dihydroxyacetone phosphate, *GAP* glyceraldehyde-3-phosphate, *G13BP* 1,3-bisphosphoglycerate, *3PGA* 3-phosphoglycerate, *2PGA* 2-phosphoglycerate, *PEP* phospho*enol*pyruvate, *PDH* pyruvate dehydrogenase, *CSY* citrate synthase, *IDH* isocitrate dehydrogenase, *OGDH* oxoglutarate dehydrogenase, *SCoAL* succinyl-CoA lyase, *SDH* succinate dehydrogenase, *ME* malic enzyme, *MDH* malate dehydrogenase, *AcCoA* acetyl-CoA, *2OG* 2-oxoglutarate, *SucCoA* succinyl-CoA, *OAA* oxaloacetate

glycolytic enzymes with voltage dependent anion channel (VDAC) located in the mitochondrial outer membrane, which can serve to anchor the glycolytic enzyme complex on the mitochondrial surface (Graham et al. 2007). Further study demonstrated the interaction between VDAC and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Holtgrawe et al. 2005). The results from these studies indicated the presence of the glycolytic metabolon on the outer surface of mitochondria and it mediates metabolite channeling to efficiently provide substrate (pyruvate) to the mitochondrial TCA cycle in response to respiratory demands.

Interestingly, an enzyme closely related to glycolysis, the cytosolic phospho*enol*pyruvate carboxylase (PEPC) of a developing castor oil seed form hetero-octamer and interacts with mitochondrial outer surface in response to the decreased respiratory activity in mitochondria (Park et al. 2012). PEPC functions in NAD(P)H production required for fatty acid biosynthesis (Park et al. 2012). The functional association to mitochondrial outer surface might be a mechanism to connect cytosolic and mitochondrial metabolism although metabolite channeling is not indicated in the case of PEPC.

A recent study on a glycolytic enzyme, cytosolic GAPDH (GapC), indicated the function of the glycolytic metabolon in stress responses. Biotic and abiotic stresses can cause cellular redox-imbalance and oxidation, leading to the inhibition of enzymatic activity of GapC by reversible oxidation of the catalytic cysteine residue (Hildebrandt et al. 2015). Inhibition of GapC is proposed to induce redirection of metabolic flux from glycolysis to the oxidative pentose phosphate pathway (OPPP) to enhance production of NADPH being used for reactive oxygen scavenging (Hildebrandt et al. 2015). In addition to the catalytic inhibition, cellular oxidation can cause the dissociation of the glycolytic metabolon on mitochondrial outer surface, which is indicated by the smaller number of mitochondria co-localized with GapC in oxidized condition than in reduced condition (Schneider et al. 2018). This dissociation is likely due to the lower affinity of oxidized GapC with VDAC which is shown in vitro (Schneider et al. 2018). The dissociation of the glycolytic metabolon likely enhances nuclear translocation of GapC to exert its moonlighting function as a transcriptional regulator (Schneider et al. 2018). This is an intriguing example of a synergistic function among dynamic metabolon, post-translational enzyme modification, and protein translocation to regulate metabolic fluxes.

TCA cycle

The TCA cycle metabolon is the multi-enzyme complex for which the term 'metabolon' was coined (Srere 1985). The initial studies extensively investigated its composition, catalytic properties, tertiary structure, and metabolite channeling both in vitro and in vivo (D'Souza and Srere 1983a, b; Moore et al. 1984; Robinson et al. 1987; Sumegi et al. 1993; Vélot et al. 1997; Morgunov and Srere 1998; Shatalin et al. 1999). 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 (Meyer et al. 2011) 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 pairs (Zhang et al. 2017). 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 AP-MS, split-luciferase, and yeast-two-hybrid, respectively) and the detection of interaction is based on independent principles. The signals from each method are statistically integrated and used to screen binary protein–protein interactions of all 38 enzyme proteins composing the TCA cycle and related pathways in *Arabidopsis thaliana*, including mitochondrial pyruvate dehydrogenase complex and malic enzymes (Zhang et al. 2017). As a result, 158 binary protein interactions were detected in this method, which include not only the interactions of enzymes catalyzing sequential reactions but also those between non-sequential ones (Zhang et al. 2017). AP-MS data further suggest 125 interaction between TCA cycle enzymes and the proteins not directly related to the cycle (Zhang et al. 2018). These results indicate a dense protein–protein interaction network in plant mitochondria. The identified interactions include

those between catalytic subunit of enzymes including fumarase and malate dehydrogenase (MDH), MDH and citrate synthase (CS), CS and aconitase, and aconitase and isocitrate dehydrogenase (IDH), indicating the possible metabolite channeling from fumarate to 2-oxoglutarate (Fig. 2). Only one of two mitochondrial isozymes of MDH, aconitase, and IDH are involved in the interactions of fumarase/MDH and aconitase/IDH, which might be related to the functional differentiation of isozymes (Zhang et al. 2017). Metabolite channeling is further tested by isotope dilution experiment using isolated potato mitochondria. In this case, ^{13}C-labled pyruvate or glutamate were fed to the isolated mitochondria and the decrease of label accumulation in succinate and citrate were monitored, respectively. To minimize the complexity arose from the cyclic reactions, the TCA cycle was linearized by adding specific inhibitors of an enzyme. Although channeling of the oxaloacetate, isocitrate, and succinyl-CoA was not tested and channeling rate could not be determined due to the limitations in the experimental system, efficient channeling of citrate and fumarate were successfully demonstrated (Zhang et al. 2017). This channeling result extend the potential metabolon including also succinate dehydrogenase (SDH). Although the interactions of fumarase and catalytic subunits of SDH was not detected, the interaction between fumarase and non-catalytic subunits may serve as a scaffold allowing fumarate channeling. As a whole, these results indicate the occurrence of the TCA cycle metabolon which can mediate channeling between succinate and 2-oxoglutarate (Zhang et al. 2017). The composition of the TCA cycle metabolon is quite similar among mammals (Srere 2000), bacteria (Meyer et al. 2011), and plants (Fernie et al. 2018). Especially the interactions of MDH/CS/aconitase have been detected in all organisms tested, suggesting this as a "core" component of the TCA cycle metabolon. The interaction between aconitase and IDH indicate a plant specific part of the TCA cycle metabolon although the channeling of isocitrate is not shown. Thus, our knowledge on plant TCA cycle metabolon has been greatly advanced in the last few years. However, dynamics of these metabolons in response to the metabolic demands and their in vivo metabolic functions are the subjects of future studies.

Starch synthesis

The involvement of a protein complex in plastidial starch metabolism had been expected from close genetic interactions among genes coding enzymes of starch biosynthesis and degradation. The protein complex of starch metabolic enzymes was first indicated in the study to identify the phosphorylated enzymes in the amyloplasts of developing wheat endosperm, in which three starch branching enzymes (SBEs; SBEI, SBEIIa, and SBEIIb) were shown to be phosphorylated (Tetlow et al. 2004) (Fig. 2). Co-immunoprecipitation analysis was performed to test the possibility that the phosphorylation of SBEs regulate the formation of protein complex involving SBEs. The results actually indicate the phosphorylation dependent formation of SBEI/SBEIIb/starch phosphorylase (Pho) protein complex (Tetlow et al. 2004). Further components of the enzyme complex were identified by employing protein cross-linking strategy resulted in the identification of two types of phosphorylation dependent protein complexes composed of starch synthase (SS) I and SSIIa and either of SBEIIa or SBEIIb (Tetlow et al. 2008). These protein complexes are catalytically active and showed higher affinity to the glucan substrate than the monomeric enzymes in vitro (Tetlow et al. 2008). These complexes are formed 10–15 days after pollination whereas only monomeric forms of these enzymes are detected at the earlier stage (Tetlow et al. 2008) while SBEI/SBEIIb/Pho complex is observed specifically at the later stages of grain development (Tetlow et al. 2004). These results indicate dynamic nature of the enzyme complexes of starch metabolism.

Similar multi-enzyme complexes have been identified in various crop species. Association between isoforms of SS and SBE in maize are detected using the yeast two-hybrid and affinity purification approaches using the recombinant proteins (Hennen-Bierwagen et al. 2008). The elution profiles of native enzymes isolated from the amyloplast in developing endosperm on size exclusion chromatography indicate the formation of a 600 kDa protein complex which is composed of SSIII, SSIIa, SBEIIa, and SBEIIb and another 300 kDa complex lacking SSIII. Any one of these subunits are required to form these protein complexes and the mutants lacking one of them cannot form these protein complexes (Hennen-Bierwagen et al. 2009). The analysis of multi-enzyme complexes

in the *amylose extender* (*ae*$^{-}$) mutant, which lacks SBEIIb, indicated that SBEIIa and SSI form the core of the multi-enzyme complex associated with starch granules (Liu et al. 2009). Formation of these multi-enzyme complexes are dependent on the phosphorylation of multiple sites alike their counterparts in wheat (Makhmoudova et al. 2014). Interestingly, the enzyme complexes are co-purified also with pyruvate orthophosphate dikinase, subunits of ADP-glucose pyrophosphorylase and sucrose synthase isoform SUS-SH1, which might be involved in the regulation of carbon partitioning in developing maize seeds (Hennen-Bierwagen et al. 2009). The protein complex composed of SSI, SSIIa, SBEIIa, and SBEIIb is also found to be associated with starch granules in barley endosperm amyloplasts (Ahmed et al. 2015). The comparison of different mutants deficient in each amylopectin synthesis genes and their composition of starch granule types suggested the relationship between the composition of multi-enzyme complexes and the formation of different granule types in barley (Ahmed et al. 2015). The amylopectin biosynthetic enzymes in developing rice seeds also form two types of protein complexes but these are shown to include more variety of enzymes (Crofts et al. 2015). In addition to SSs and SBEs, starch (amylopectin) synthesis also requires debranching enzymes (DBEs) and Pho to remove misplaced branches and to initiate starch biosynthesis, respectively (Nakamura 2015). The large amylopectin biosynthetic multi-enzyme complex of rice is larger than 700 kDa and composed of SSIIa, SSIIIa, SSIVb, SBEI, SBEIIb and pullulanase, a DBE. The small 200–400 kDa complex contains SSI, SSIIIa, SBEI, SBEIIa, SBEIIb, DBEs (isoamylase 1 and pullulanase), and Pho1 (Crofts et al. 2015; Hayashi et al. 2018). Rice Pho1 also interacts with a disproportionating enzyme (DPE1) involved in the synthesis of malto-oligosaccharides (Hwang et al. 2016). Formation of this Pho1-DPE1 protein complex allows Pho1 to use wider variety of malto-oligosaccharides with different chain length, which may lead to facilitate the chain elongation of malto-oligosaccharides at the initial stages of starch biosynthesis (Hwang et al. 2016). This multi-enzyme complexes of Pho1 and DPE1 is also found in the tuberous roots in sweet potato. This Pho1/DPE1 complex can use maltotriose and maltotetraose for the Pho1 activity and shows higher Dpe1 activity than free DPE1 for maltotetraose, which may enhance the amylopectin biosynthesis by recycling glucosyl units (Lin et al. 2017b).

These studies clearly show that the enzymes of starch biosynthesis form multi-enzyme complexes in the tissues storing starch as starch granules depending on the developmental ques. These complexes have influences on quality and quantity of the starch granules. However, the precise molecular mechanisms by which these multi-enzyme complexes regulate starch biosynthesis is obscure and the occurrence of metabolite channeling unproved. Structural information on these complexes associating with oligosaccharides will greatly help to understand the functions of them. Additionally, the formation and the dynamics of multi-enzyme complexes are unexplored in the tissues transiently accumulating starch including leaves. These insights will greatly improve our understanding on the functions of multi-enzyme complexes in starch biosynthesis and how they regulate this complex process.

Polyamine synthesis

Polyamines are molecules containing more than two amino groups. Putrescine, spermidine, and spermine are the most abundant plant polyamines but plants also produce uncommon polyamines including homocaldopentamine and homocaldohexamine (Møller and Conn 1980). Polyamines play an important role in plant development and environmental responses and serve also as precursors of various secondary metabolites including alkaloids and phenylpropanoids (Shoji and Hashimoto 2015). Higher order polyamines like a triamine, spermidine, and a tetraamine, spermine, are synthesized by elongating putrescine using *S*-adenosylmethionine as a donor of amine unit (Fig. 2). The enzymes catalyzing these elongation reactions show very strict substrate specificity and each reaction step is catalyzed by a specific enzyme. For example, spermidine synthase, which catalyze the addition of an amine to putrescine, uses only putrescine as the substrate and other polyamines such as spermidine and spermine cannot be elongated by this enzyme. However, the enzyme purified from Alfalfa synthesize not only spermidine but also a tetraamine, spermine, and other uncommon polyamines (Bagga et al. 1997). Interestingly, the enzymes purified from the Alfalfa cultivar tolerant to water deficit stress produce these unexpected polyamines which might be related to the

functions of polyamine in abiotic stress resistance (Bagga et al. 1997). Panicot et al. (2002) hypothesized that plant spermidine synthase forms a metabolon which facilitate further elongation of polyamines and the formation of uncommon polyamines. The authors identified spermine synthase gene in *Arabidopsis* and showed the interaction between spermidine synthase and spermine synthase (Panicot et al. 2002). These enzymes compose a protein complex of 650–750 kDa, which is much higher than the estimated size of homo- and hetero-dimers, suggesting the metabolon formation. This polyamine metabolon is expected to facilitate the formation of higher order polyamines and to regulate the synthesis of polyamine species including uncommon ones, although other components of this protein complex have not been identified so far.

Metabolons in plant secondary metabolism

In plant metabolism, the metabolon is rather recognized as the system to control production of secondary (specialized) metabolites. Plants produce vast variety of secondary metabolites in a coordinated manner and accumulate them to very high contents. Although biosynthetic pathways of secondary metabolites largely rely on transcriptional regulation (Patra et al. 2013; Schluttenhofer and Yuan 2015; Chezem and Clay 2016), branching nature of secondary metabolism makes the metabolon a suitable system to restrict the metabolic flux into desired routs or to 'switch' the pathways producing different molecules by exchanging its components. Metabolons can also enhance biosynthetic pathway activities by achieving high concentration of intermediates within protein complexes. The containment function of metabolons is particularly important in plant secondary metabolism since many of the pathway intermediates are either fragile or toxic and should be isolated from the cellular matrix. As expected, many metabolons have been identified in various pathways of plant secondary metabolism especially in the last decade (Table 1; Fig. 3).

Biosynthesis of glycosides

Metabolon of biosynthetic enzymes of dhurrin, a cyanogenic glucoside in sorghum and other plants, is the most well-characterized metabolon involved in plant secondary metabolism. Dhurrin is synthesized from tyrosine via aldoxime and cyanohydrin by the sequential reactions catalyzed by two cytochrome P450 enzymes (CYP79A1 and CYP71E1) and a UDP-glucosyltransferase (UGT85B1; Jørgensen et al. 2005) (Fig. 3). Dry sorghum seeds contain almost no dhurrin but they accumulate 40 mg g^{-1} dry weight of dhurrin during the first 3 days of germination (Akazawa et al. 1960). Additionally, the intermediates of dhurrin biosynthesis are labile and highly cytotoxic (Winkel 2004). Therefore, researchers hypothesized that a metabolon is formed in sorghum seedlings to support the very rapid synthesis of dhurrin and to isolate intermediate from other cellular components. In vitro isotope dilution experiments in 80s revealed that the dhurrin biosynthesis pathway is highly channeled (Møller and Conn 1980). Recent studies made great advances in revealing the structure and dynamics of the dhurrin biosynthesis metabolon in vivo. Localization of the YFP-fused dhurrin biosynthesis enzymes heterologously expressed in *Arabidopsis* supports the metabolon formation in living cells (Nielsen et al. 2008). While YFP-fused UGT85B1 diffuses in the cytosol when it is solely expressed in *Arabidopsis*, the localization of YFP-UGT85B1 alters to the endoplasmic reticulum (ER) surface by the co-expression with both of the cytochrome P450 proteins. This result also indicates the function of the cytochrome P450 proteins to anchor dhurrin metabolon on the ER surface (Nielsen et al. 2008). Homology modeling of the tertiary structures of membrane bound cytochrome P450 proteins and a NADPH-dependent cytochrome P450 reductase indicates the interaction and possible docking sites among these three proteins and the ER membrane (Jensen et al. 2011). A hallmark study by Laursen et al. (2016) conclusively showed the occurrence of dhurrin metabolon in living cells. As the dhurrin metabolon requires the ER membrane to anchor its components, the application of detergent disrupt the metabolon. The dhurrin metabolon was successfully isolated from the etiolated sorghum seedlings by overcoming this problem using styrene maleic acid lipid particle (SMALP) technology. SMALPs partially purified from sorghum seedlings by the affinity chromatography using the affinity of cytochrome P450 reductase are significantly enriched with components of dhurrin metabolon. This is a very strong evidence of the naturally occurring dhurrin metabolon in a sorghum seed highly active in dhurrin

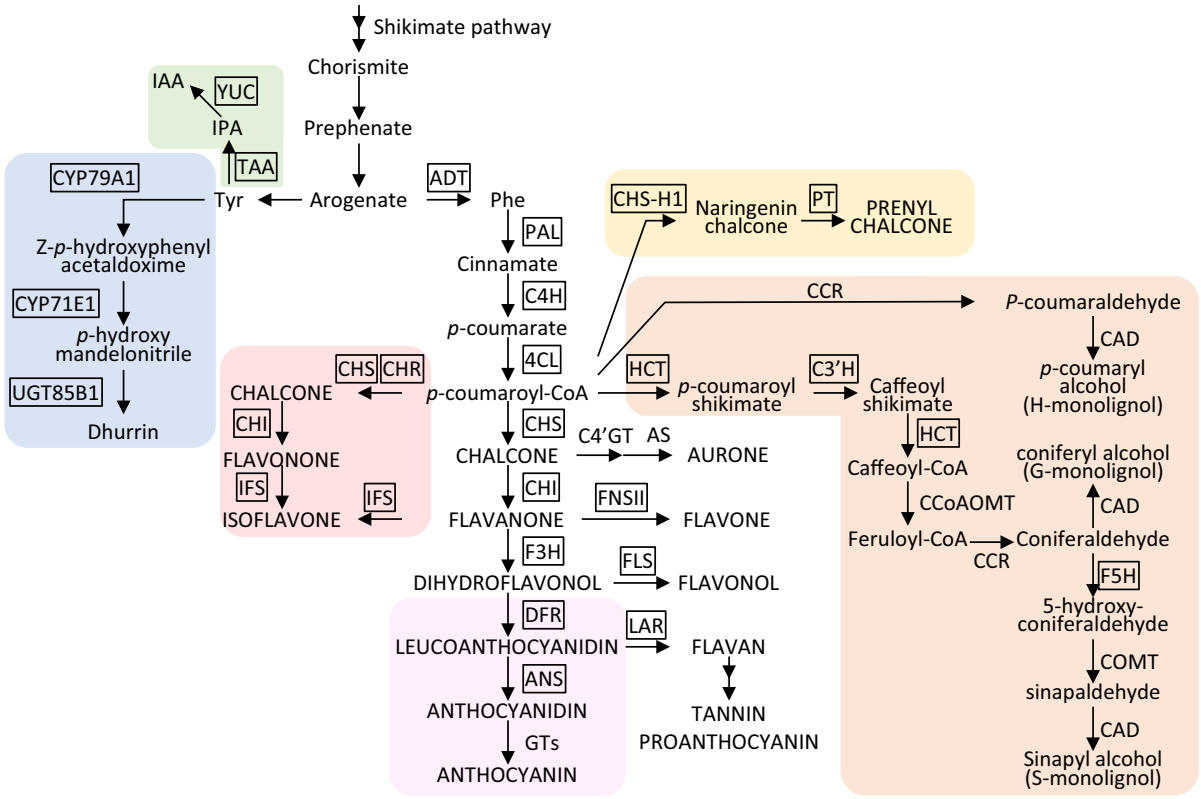

Fig. 3 Metabolons in biosynthetic pathways of phenolic compounds. A schematic diagram of plant secondary pathways for the biosynthesis of phenolic compounds derived from tyrosine and phenylalanine with any evidence of metabolon. Some pathways are species-specific and whole the pathways do not exist in a single plant species. Single and double arrows represent single and multiple reactions, respectively. Compound names in capital are the classes of metabolites. The outlined enzymes are indicated to be involved in a metabolon. *ADT* arogenate dehydratase, *PAL* phenylalanine ammonia lyase, *C4H* cinnamate-4-hydroxylase, *4CL* 4-coumaroyl-CoA ligase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone 3-hydroxylase, *DFR* dihydroxyflavanone 4-reductase, *ANS* anthocyanidin synthase, *GT* glycosyltransferase, *PT* prenyltransferase, *CHR* chalcone reductase, *IFS* isoflavone synthase, *HCT* hydroxycinnamoyltransferase, *C3′H* *p*-coumaroyl shikimate 3′-hydroxylase, *CCR* cinnamoyl CoA reductase, *CCoAOMT* caffeoyl CoA *O*-methyltransferase, *F5H* ferulate 5-hydroxylase, *COMT* caffeic acid *O*-methyltransferase, *CAD* cinnamyl alcohol dehydrogenase, *IPA* indole-3-pyruvate, *IAA* indole-3-acetate

synthesis (Laursen et al. 2016). The interaction between the components of dhurrin metabolon is further confirmed by the fluorescence correlation spectroscopy which demonstrates the reduced diffusion of the fluorescence fused proteins when they are co-expressed with other components of dhurrin metabolon. Metabolon formation is sensitive to the lipid environment and dependent on the concentration of negative charges of the lipid headgroups. Finally, the composition and the possible structure of dhurrin metabolon is presented by comprehensively assessing interactions among individual components using fluorescent lifetime measurement (FLIM)-fluorescent resonance energy transfer (FRET). Additionally, metabolon formation enhances the production of intermediates in vitro even when the last enzyme does not catalyze dhurrin production due to the lack of UDP-glucose, suggesting the structural advantage of metabolon (Laursen et al. 2016). Cumulatively, this study clearly demonstrated the metabolon formation in vivo.

Remaining questions regarding dhurrin metabolon is its dynamics and functions in living cells. The dynamics of dhurrin metabolon is postulated since dhurrin is synthesized in response to environmental stresses (Gleadow and Møller 2014). However, currently no study clearly shows the association and dissociation of dhurrin metabolon. A modeling study

indicates the P450 proteins associate with P450 reductase to form a transient platform determining the dynamics of metabolon (Bassard et al. 2017). This requires experimental validation and the factors regulating the dynamics are totally unknown.

The dhurrin metabolon is also investigated toward the application of metabolon to synthetic biology. A bottleneck to produce large amount of dhurrin is the provision of reducing power to the cytochrome P450s. One way to overcome this bottleneck for metabolic engineering is to relocate dhurrin biosynthetic pathway into chloroplast, where the ferredoxin reduced by photosynthetic light reaction serves as an efficient electron donor to the P450s (Nielsen et al. 2013; Mellor et al. 2017). However, it disrupts metabolon formation leading to reduced yield and the accumulation of intermediate metabolites (Laursen et al. 2015). To facilitate metabolon formation in the chloroplasts, the enzymes of dhurrin biosynthesis are fused with the components of Tat complex, which is a naturally occurring protein complex in the thylakoid membrane, using flexible linkers (Henriques de Jesus et al. 2017). The fusion with Tat proteins successfully improved dhurrin production in three to four times and reduced accumulation of side products (Henriques de Jesus et al. 2017). This research has a strong potential to improve efficiency of synthetic metabolic pathways involving reduction steps and also indicates the crucial function of metabolon in efficient production of plant secondary metabolites.

Aldoximes, intermediates of dhurrin biosynthesis, are also precursors for the biosynthesis of glucosinolates (Jørgensen et al. 2005). Aldoximes are highly reactive molecules, inhibit enzymes, and thus potentially cytotoxic (Sakurada et al. 2009). *Arabidopsis* mutants lacking a cytochrome P450 oxygenase, CYP83A1, catalyzing the conversion of aldoximes into thiohydroximates, accumulate large amount of aldoximes (Hemm et al. 2003; Weis et al. 2014). These lines show both phenylpropanoid and glucosinolate phenotypes most likely due to the inhibition of an enzyme in phenylpropanoid pathways, caffeic acid *O*-methyltransferase by aldoximes (Hemm et al. 2003). Interestingly, CYP83A1 interact with BAX INHIBITOR-1 (BI-1), an ER resident cell-death inhibitor protein (Weis et al. 2013). BI-1 also interacts with cytochrome b5 and modulates plant metabolism (Ishikawa et al. 2011). Therefore, BI-1 might serve as a scaffold for the glucosinolate metabolon formation on the ER surface although there is no direct evidence supporting the formation of glucosinolate metabolon yet (Grubb and Abel 2006; Weis et al. 2014).

Auxin biosynthesis

Indole-3-acetic acid (IAA) is the major form of naturally occurring auxin, a plant hormone involved in the regulation of plant growth and development. IAA is synthesized from tryptophan by two reactions catalyzed by TAA family amino transferases and YUC family flavin monooxygenases (Zhao 2012) (Fig. 3). Metabolite channeling and metabolon formation between these enzymes are postulated based on their weak substrate specificity (Müller and Weiler 2000; Kriechbaumer et al. 2006; Pollmann et al. 2009). These enzymes are shown to associate with ER and ER microsomal fraction has significant portion of auxin biosynthesis activity in *Arabidopsis* seedlings (Kriechbaumer et al. 2017). Binary protein–protein interaction assays by FLIM/FRET indicates the interactions between a TAA family amino transferase, TAR2, and two YUC proteins, YUC5 and 8. These two YUC proteins are localized on the ER-membrane and may work to anchor enzymes of auxin biosynthesis on ER surface (Kriechbaumer et al. 2017).

Phenylpropanoid pathway

Phenylpropanoid is a major group of plant secondary metabolites derived from phenylalanine. It includes flavonoids, sinapate esters, stilbenes, and lignins, which play important roles in acclimation to abiotic and biotic environments, as pigments and signaling molecules, and as structural components. These metabolites often accumulate to very high contents. A mechanism to achieve the large production of phenylpropanoids is needed to quickly accumulate these compounds in response to environmental and developmental demands. The phenylpropanoid pathway is composed of core pathway, which convert phenylalanine into *p*-coumarolyl-CoA, and the biosynthetic pathways for specific classes of phenylpropanoids (Fig. 3). *p*-coumarolyl-CoA is a common precursor for many compounds and the metabolic flux distribution into biosynthetic pathways for each compound must be tightly regulated. Additionally, some intermediates of the phenylpropanoid pathways are

highly reactive and should be contained in a specific compartment. Given these requirements for phenylpropanoid biosynthesis, it is reasonable to assume the involvement of metabolon in these pathways. Indeed, the metabolon formation in the phenylpropanoid pathway was proposed in 1974 (Stafford 1974) and many pioneering studies provide the evidence of multi-enzyme complexes and metabolite channeling (Rasmussen and Dixon 1999; He and Dixon 2000; Winkel 2004, 2009; Achnine et al. 2004; Bomati et al. 2005; Jørgensen et al. 2005). These studies demonstrated the multi-enzyme complex composed of the core phenylpropanoid pathway enzymes, phenylalanine ammonia lyase (PAL) and cinnamate 4-hydroxylase (C4H), and metabolite channeling of *trans*-cinnamic acid between these enzymes (Rasmussen and Dixon 1999; Achnine et al. 2004). Recent studies showed the multi-enzyme complexes of branching pathways to produce specific classes of phenylpropanoid and the interaction between core and branching pathways in *Arabidopsis* (Fig. 4) and other plant species, which likely provide regulatory

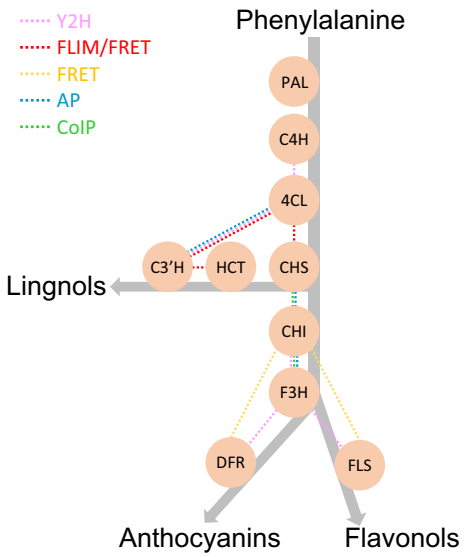

Fig. 4 Current model of the phenylpropanoid metabolon in *Arabidopsis thaliana*. Spheres are enzymes catalyzing pathway reactions. Pathway directions producing each class of phenylpropanoid are shown by gray arrow. Dashed lines indicate experimentally shown binary protein–protein interactions. Colors of dashed lines represent the methods used to show the interactions; pink, yeast-two-hybrid; red, FLIM/FRET; yellow, FRET; blue, affinity purification; green, co-immunopurification. The interaction between PAL and C4H has not been demonstrated in *Arabidopsis* but in tobacco. (Color figure online)

capability to determine the flux distribution among the pathways.

Anthocyanins are pigments to give often purplish colors and comprise a major class of flavonoids widespread in plants (Liu et al. 2018). They are rapidly accumulated at particular developmental events including flower and fruits development, as well as in response to biotic and abiotic stresses (Liu et al. 2018). Anthocyanins are produced by glycosylation of anthocyanidin, which is synthesized from *p*-coumaroyl-CoA by the sequential reactions of chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) (Figs. 3, 4). The interactions among CHS, CHI, F3H, and DFR were already demonstrate in 1999 (Burbulis and Winkel-Shirley 1999). Recent studies addressed precise composition of the multi-enzyme complex, their functions and dynamics, and diversity among species (Burbulis and Winkel-Shirley 1999; Crosby et al. 2011; Diharce et al. 2016; Fujino et al. 2018; Owens et al. 2008). Dihydroflavonols are the intermediates of anthocyanin biosynthesis as well as the precursors of flavonols including kaempferol. DFR and flavonol synthase (FLS) mediate the conversion of dihydroflavonols for the production of anthocyanins and flavonols, respectively, and these enzymes are known to physically interact with CHI catalyzing the production of flavonols, direct precursors of dihydroflavonols (Burbulis and Winkel-Shirley 1999; Owens et al. 2008) (Figs. 3, 4). CHI interacts also with F3H (Burbulis and Winkel-Shirley 1999), the enzyme producing dihydroflavonols, although direct interactions between F3H and neither DFR nor FLS have been reported. These results raise a question if the branches of phenylpropanoid pathway can be regulated by switching the interaction partner of CHI between DFR and FLS. This hypothesis is supported by the observation that the interactions of CHI/DFR and CHI/FLS competes each other and in the presence of DFR or FLS, the interaction between CHI and the other enzyme is competitively inhibited (Crosby et al. 2011). These studies indicated the function of phenylpropanoid metabolon to organize metabolic fluxes into branching pathways. Recently, comprehensive protein–protein interaction analysis was carried out for the enzymes of flavonoid biosynthesis in snapdragon (*Antirrhinum majus* L.) and a related species toenia (*Torenia hybrida*) (Fujino et al. 2018). The flower

petal colors of these species are based on the different compositions of flavonoids including anthocyanins, flavones, and aurones, which is also distinct from the major flavonoids in previously tested species, namely *A. thaliana* and *Glycine max*. Binary interaction assays among CHS, CHI, F3H, DFR, flavonoid 3′-hydroxylase (F3′H), chalcone 4′-glucosyltransferase (C4′GT), and flavone synthase II (FNSII) (Fig. 3) rediscovered the interactions of CHS/CHI and CHI/DFR while F3H did not interact with any other enzymes. Interestingly, FNSII, an ER membrane anchored cytochrome P450 to produce flavones from flavanones, is found to interact with all CHS, CHI and DFR although these enzymes catalyze non-consecutive reactions in the flavonoid pathway. These results, together with additional localization experiments, indicates the existence of the flavone metabolon and the function of FNSII to tether the components of the flavonoid metabolon on the ER membrane (Fujino et al. 2018). It should also be noted that C4′GT, which is involved in the aurone synthesis, showed interaction with no other enzymes of the flavonoid pathway, indicating that aurones may be synthesized independent of a metabolon (Fujino et al. 2018). The composition of flavonoid metabolons is slightly different between snapdragon and torenia. In the latter, CHS interacts with neither CHI nor FNSII and the interaction between FNSII and ANS are detected (Fujino et al. 2018). These compositions of flavonoid metabolon are also distinct from that in *A. thaliana* which lacks FNSII gene (Crosby et al. 2011). Such species-specific organization of flavonoid metabolon may lead to the unique composition of flavonoids in plant species. It should be noted that some intermediates of phenylpropanoid pathways are revile in aqueous solution and metabolite channeling is proposed to be necessary to complete pathway reactions. Leucoanthocyanidins are the precursors of anthocyanin biosynthesis but also converted into proanthocyanidins. Leucoanthocyanidin reductase (LAR) catalyzes the reaction following DFR which produces leucoanthocyanidin. However, it is unstable in water and need to be protected when diffusing to the active site of LAR enzyme (Diharce et al. 2016). A molecular structural modeling study indicated that the association of DFR and LAR complex can open the DFR active site to facilitate the diffusion of leucoanthocyanidin. Additionally, the dissociation of the complex leads to the conformation change to close the DFR active site to prevent the diffusion of the intermediate into the bulk phase (Diharce et al. 2016).

Isoflavonoids are a group of flavonoids synthesized almost exclusively by legumes. They play roles in plant–microbe interactions and have positive effects on human health and nutrition (Du et al. 2010). Isoflavonones are synthesized from flavanones by the reaction catalyzed by isoflavone synthase (IFS) and further used to generate other isoflavonoids in soybean (Dastmalchi et al. 2016) (Fig. 3). IFS is a cytochrome P450 enzyme localized on the ER membrane and interacts with both CHS and CHI which catalyze sequential reaction to produce flavanones (Dastmalchi et al. 2016; Waki et al. 2016). IFS interact with another ER anchored P450 enzyme, C4H, which catalyze an upstream reaction on the flavonoid pathway (Dastmalchi et al. 2016). Combination of binary protein–protein interaction assays revealed that cytosolic enzymes catalyzing the reactions of flavonoid pathway, namely arogenate dehydratase (ADT), CHS, chalcone reductase (CHR), and CHI, interact each other together with C4H and IFS. These results strongly support that two ER anchored enzymes, C4H and IFS, tether the isoflavonoid metabolon on the ER surface (Dastmalchi et al. 2016). IFS interacts with specific isoforms of CHRs in soybean, which might lead to the functional difference among isozymes (Mameda et al. 2018).

Lignin is a complex polymer of phenylpropanoid units composing plant secondary cell walls. Phenylpropanoid units are derived from phenylpropanoids called monolignols (Du et al. 2018). *p*-coumaroyl-CoA is the precursor of monolignols and hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase (HCT), *p*-coumaroyl shikimate 3′-hydroxylase (C3′H), caffeoyl CoA *O*-methyltransferase (CCoAOMT), caffeic acid *O*-methyltransferase (COMT), ferulate 5-hydroxylase (F5H), cinnamyl alcohol dehydrogenase (CAD), and cinnamoyl CoA reductase (CCR) are involved in the lignin pathway (Vanholme et al. 2010) (Fig. 3). C3′H and F5H colocalizes with C4H, which catalyze the production of *p*-coumaric acid, on the ER membrane to form homo- and heteromers (Bassard et al. 2012; Gou et al. 2018). These cytochrome P450 enzymes enhance the localization of soluble HCT and 4-coumaroyl-CoA ligase (4CL) enzymes on the ER surface, indicating their function to anchor the lignin metabolon on the ER (Bassard et al. 2012). However, they do not

directly interact with each other (Gou et al. 2018). It was revealed that membrane steroid-binding proteins (MSBPs) interact with these P450 proteins and serve as scaffolds to organize the lignin metabolon (Gou et al. 2018). This is an interesting example showing the function of naturally occurring proteins which are not the enzymes catalyzing pathway reactions serve as scaffolds to form a metabolon. Thus, the rapid synthesis of lignin to support plant mechanical growth can be mediated by the lignin metabolon (Fig. 4). Additionally, different organization of lignin metabolons may contribute to determine lignin composition since two independent channels for the guaiacyl (G) and syringyl (S) lignin monomers are proposed to be necessary for optimal lignin synthesis in Medicago (Lee et al. 2012). However, the protein complexes involving CCR, CAD, COMT, CCoAOMT have not been identified yet.

Hops (*Humulus lupulus*) highly accumulate a set of secondary metabolites, called terpenophenolics, in glandular trichomes of female cones, which provide unique flavor and aroma of beer (Van Cleemput et al. 2009). Prenylchalcones are a major group of terpenophenolics and xanthohumol and demethylxanthohumol are the major components of the group (Van Cleemput et al. 2009). Naringenin chalcone is produced by a hop specific CHS, CHS_H1, and prenylated by an aromatic prenyltransferase (PT) to be demethylxanthohumol. CHS_H1/PT metabolon has been proposed considering the high production rate of demethylxanthohumol in glandular trichomes (Ban et al. 2018). Nevertheless, CHS_H1 and PT appeared not to directly interact in a yeast two hybrid assay (Ban et al. 2018). Instead, the non-catalytic type IV CHI-fold (CHI-like; CHIL) proteins interact with both CHI_H1 and PT (HlPT1L) to stabilize the ring-open configuration of chalconoids and enhance the production of prenylchalcones (Ban et al. 2018). This is an interesting example of species-specific evolution of metabolons. The involvement of catalytically inactive CHIL protein is reasonable since the direct CHS/CHI interaction is common among plant species but CHI competes naringenin chalcone with PT and the inactive version of CHI.

Isoprenoid biosynthesis

Isoprenoids are the group of metabolites with diverse biological functions including sterols, carotenoids, chlorophylls and phytohormones. Geranylgeranyl diphosphate (GGPP) is an intermediate of isoprenoid biosynthetic pathway, which is a precursor of various isoprenoids including chlorophylls, carotenoids, phylloquinone, gibberellins, and tocopherols (Lange et al. 2015). Due to the multi-functionality of the GGPP, it has been postulated that the isoprenoid pathway enzymes form metabolons and the metabolic flux into the production of each class of compounds are regulated by exchanging their components (Cervantes-Cervantes et al. 2006). Recently, the interaction between *Arabidopsis* GGPP synthase (GGPS) and phytoene synthase (PSY) was demonstrated by yeast two hybrid assay (Camagna et al. 2018). As phytoene is the first intermediate in biosynthesis of carotenoids, this interaction has potential capability to draw metabolic flux into biosynthetic pathways of carotenoid competing with those of chlorophylls, phylloquinones, and tocopherols. The PSY-GGPS fusion protein produces phytoene in an engineered *E. coli* strain, showing in vivo functionality of this protein complex (Camagna et al. 2018). Interestingly, the purified fusion enzyme produces very limited amount of GGPP in vitro, while it is efficiently produced when equimolar of independent enzymes are added to the reaction mixture (Camagna et al. 2018). These results indicate the possible regulation of isoprenoid pathways by a dynamic metabolon although occurrence and functions of this complex have not been tested *in planta*. It would be interesting if at least the initial enzymes of the biosynthetic pathways for other isoprenoids could also interact with GGPS and channel GGPP into specific pathways. These enzyme interactions will provide a mean to regulate isoprenoid production by exchanging the interaction partner of GGPS in response to the demands for each metabolite.

Bitter acids are isoprenoids composing another major group of terpenophenolics in hop. Two and three sequential prenylation steps are required to produce α- and β-bitter acids from acylphloroglucinols derived from branched-chain fatty acids, respectively. PT1L catalyze the first prenylation and PT2 is responsible for the latter two reactions. These two enzymes are shown to form a metabolon by a direct physical interaction (Li et al. 2015).

Alkaloid biosynthesis

Alkaloid is another large group of plant derived secondary metabolites which are related only by the occurrence of a nitrogen atom in a heterocyclic ring. Therefore, it includes variety of metabolites with diverse origins (Ziegler and Facchini 2008). Nevertheless, there is only one study reporting involvement of a metabolon in plant alkaloid biosynthesis. Strictosidine aglycone is a highly reactive intermediate in the biosynthesis of monoterpene indole alkaloids (Guirimand et al. 2010) which should be protected from degradation. Therefore, the interaction between strictosidine glucosidase and tetrahydroalstonine synthase, producing and consuming strictosidine aglycone, respectively, is tested, and detected by bimolecular fluorescence complementation (BiFC) assay (Stavrinides et al. 2015). This protein complex is considered not only to protect strictosidine aglycone from degradation but also to coordinate the metabolic fluxes into downstream pathways since strictosidine aglycone is a branching point of pathways for the synthesis of various monoterpene indole alkaloids (Stavrinides et al. 2015). The need for metabolite channeling to protect metabolic intermediate is also demonstrated in morphine biosynthetic pathways in opium poppy. In this pathway, a fusion enzyme of a cytochrome P450 and an oxidoreductase is essential for efficient morphinan production and is considered to mediate metabolite channeling (Winzer et al. 2015). This study also indicates an important role of metabolite channeling in alkaloid biosynthesis although it does not directly show the involvement of a metabolon. Further research is expected to elucidate alkaloid metabolons.

Metabolons in wax biosynthesis

Sporopollenin polymer is a major component of the wax coating the outer surface of pollens and spores. Hydroxylated medium- and long-chain fatty acids are major components of this extremely durable biopolymer (Quilichini et al. 2015). Co-localization of four enzymes in sporopollenin biosynthesis in ER was observed by immunogold staining to investigate the subcellular localization of these enzymes in tapetum cells of *A. thaliana* (Lallemand et al. 2013). The interactions between acyl-CoA synthetase 5 (ACOS5), polyketide synthase A (PKSA) and B (PKSB), and tetraketide α-pyrone reductase 1 (TKPR1) are demonstrated by multiple techniques i.e. in vitro pull-down, yeast two hybrid, and FLIM/FRET assays. TKPR2 was not found in the complex. These results indicate the formation of sporopollenin metabolons in the tapetum cells, which may partly explain the efficiency of the pathway to produce the outer surface of the pollen during a short period in pollen development (Lallemand et al. 2013). The composition of this multi-enzyme complex is slightly different in *Brassica napus*, in which TKPR1 is not involved in the multi-enzyme complex (Qin et al. 2016). Direct interaction between ACOS5 and PKSA is not observed in contrast to *Arabidopsis* ACOS5 which directly interacts with all other components (Qin et al. 2016). Although the relevance of the difference in the composition of metabolons is unclear, these results suggest that the metabolon is involved in the synthesis of cuticular wax in land plants. Also related to the cuticular wax, "lipotubuloids metabolon" has been proposed (Kwiatkowska et al. 2014, 2015; Stępiński et al. 2017). However, this is not discussed in this review since it is a compartmented particle containing cellular organelle and does not meet with the definition of the metabolon used here.

Perspectives

The studies in the last decade indicate the involvement of metabolons in many metabolic pathways in plants as reviewed above. However, exploration of novel plant metabolons has just started, and many potential metabolons are remained to be investigated. To elucidate how enzyme proteins are organized to orchestrate the metabolic network at the systems level, further exploration of plant metabolons is required.

Potential plant metabolons

Given that metabolons tend to be conserved in domains of life (Zhang et al. 2017), a metabolon found in other organisms are likely formed in plants too. The metabolons indicated in other organisms but not in plants include lipid biosynthesis (Gillevet and Dakshinamurti 1982; Shuib et al. 2018), purine biosynthesis (purinosome; An et al. 2008; French et al. 2016; Pedley and Benkovic 2017), heme

catabolism (Yanatori et al. 2017), bicarbonate and ion transport (McMurtrie et al. 2004; Tresguerres et al. 2006; Sowah and Casey 2011), propionate metabolism (Pronk et al. 1994), arginine synthesis (de Cima et al. 2012), melanogenesis (Sugumaran et al. 2000), amino acid catabolism (Islam et al. 2007; Sanyal et al. 2015), metabolism of neurotransmitters (Jansen et al. 2001; McKenna et al. 2006; McKenna 2011; McKenna and Ferreira 2016), glycogenolysis (Shmelev and Serebrenikova 1997), cholesterol metabolism (Fan and Papadopoulos 2013; Luu et al. 2015), redox regulation (Ghosh et al. 2017), glyoxylate cycle (Kunze and Hartig 2013), and folate metabolism (Wang and McCammon 2015). Purine biosynthesis metabolon, called "purinosome" is one of the most well-characterized metabolons in mammalian cells (Pedley and Benkovic 2017) although there is an argument on the natural occurrence of it (Zhao et al. 2014). There is no study showing the purinosome in plants, which might be due to lower demand for purines in plants than highly proliferating human cell lines. It may be observed specifically in tissues with actively dividing cells including meristems and callus.

On the other hand, the compositions of metabolons in plant secondary metabolism appeared to be diverse in certain extent depending on plant species, tissues, and developmental stages (Waki et al. 2016; Fujino et al. 2018; Mameda et al. 2018). This is reasonable to achieve production of specific secondary metabolites in response to metabolic demands. As described above, the metabolon is a suitable mechanism to regulate highly branching secondary pathways since it can selectively provide intermediates into specific metabolic pathways to produce desired metabolites by preventing the access of enzymes of other pathways to them. Since many intermediates in secondary metabolism are fragile and/or toxic, metabolons are supposed to be essential to protect them. Given the vast variety of the plant secondary metabolites, the biosynthesis pathways of them are an arsenal of novel metabolons. Especially, identification of alkaloid metabolons will have a great impact as only one metabolon is found in alkaloid biosynthetic pathways despite of their chemical diversity and the evidence on the needs of metabolite channeling (Stavrinides et al. 2015; Winzer et al. 2015). Elucidation of alkaloid metabolon is important not only for the understanding of pathway regulation but also for biotechnological perspectives as many alkaloids are economically high value products and artificial metabolons or stabilization of naturally occurring metabolons have strong potential in metabolic engineering to improve yield and to reduce side products (Winzer et al. 2015; Li et al. 2018). The isoprenoid metabolon is also a promising frontier for the same reasons and there have already been several metabolons identified (Li et al. 2015; Camagna et al. 2018).

Additionally, previously proposed metabolons in some metabolic pathways are remained to be analyzed. In the early study, the potential multi-enzyme complex in Calvin–Benson cycle which involve five pathway enzymes, ribose-phosphate isomerase, phosphoribulokinase, ribulose-bisphosphate carboxylase/oxygenase, phosphoglycerate kinase, and glyceraldehyde-phosphate dehydrogenase was proposed (Gontero et al. 1988). In the photorespiratory pathway, channeling of tetrahydrofolate between mitochondrial glycine decarboxylase system T protein and serine hydroxymethyltransferase has been expected due to the sensitivity of tetrahydrofolate to oxidation. Although in vitro enzyme kinetics showed no channeling between these two enzymes (Rebeille et al. 1994), this might happen in vivo, considering condensed microenvironment in mitochondrial matrix (Rebeille et al. 1994; Douce et al. 2001). Additionally, complex formation and metabolite channeling among the components of glycine decarboxylase system is still unclear (Oliver et al. 1990; Douce et al. 2001). Association and dynamics of these multi-enzyme complexes in vivo may reveal the additional regulatory mechanisms for these pathways.

Researchers can also predict the involvement of metabolons in particular metabolic pathways by considering the requirement of potential functions of metabolons (Spivey and Ovádi 1999). Novel metabolons will be discovered by targeting on the pathways with high potential of involvement of metabolons. Metabolons can protect reactive metabolic intermediates by containing them within the protein complexes (Spivey and Ovádi 1999). The interaction between monoterpene indole alkaloid pathway enzymes was tested based on the assumption that reactive strictosidine aglycone should be protected to prevent degradation (Stavrinides et al. 2015). Metabolon is also considered as a mechanism to achieve high metabolic flux and to allow accumulation of specific metabolites. This was a motivation to explore the metabolons in secondary pathways, including dhurrin, lignin, bitter

acid, and sporopollenin metabolons (Møller and Conn 1980; Lallemand et al. 2013; Li et al. 2015). Additionally, several multi-enzyme complexes involving the enzymes in multiple pathways have been reported. This type of interaction is particularly interesting since they are potential 'switches' quantitatively regulating the re-direction of metabolic flux into multiple pathways by changing the composition of the multi-enzyme complexes. This type of regulation is suggested by the competitive interaction of CHI with DFR and FLS, which compete for flavanone for the anthocyanin and flavonol pathways, respectively (Crosby et al. 2011). It can also be speculated that the metabolic fluxes into glycolysis and OPPP is regulated by the exchange of metabolon components since these pathways should be quickly regulated in response to the fluctuating demands for metabolic precursors and NADPH which are exclusively produced by OPPP (Schneider et al. 2018). While the metabolite channeling in both pathways has been demonstrated (Debnam et al. 1997; Graham et al. 2007), the dynamics of the components of metabolon must be shown to support this hypothesis. Even when there is no possible metabolite channeling between enzymes, the physical interaction of enzymes can be associated with the functional relationship between pathways (Obata et al. 2011, 2016). Co-purification-MS is a suitable approach to identify possible multi-enzyme complexes and has strong potential to extend our view in the connection between pathways by metabolons (Dastmalchi et al. 2016; Zhang et al. 2018). Thus, the metabolic enzymes catalyzing the reactions with (1) a fragile intermediate, (2) high metabolic flux, and (3) competing reactions may be promising targets for the discovery of novel metabolons.

Technical issues in metabolon discovery

Recent development of protein–protein interaction assays provides us various options to test protein–protein interactions (Xing et al. 2016). Indeed, the studies discussed here employ various techniques to show protein complex formation and interactions between specific enzymes (Table 1; Fig. 4). However, there is still no perfect method which can identify protein–protein interactions without any false positive and negative detections. This makes the validation and screening of novel metabolons complicate. Yeast two hybrid assays, colocalization of fluorescently tagged proteins, BiFC, and co-purification are commonly used to identify multi-enzyme complexes (Bassard and Halkier 2018; Sweetlove and Fernie 2018). These methods tend to produce a lot of false positive results and must be conducted with special care, especially the use of proper negative control is essential (Kudla and Bock 2016; Xing et al. 2016). Additionally, given the relatively high false positive and negative rates of these assays, multiple proof is required to avoid argument on the presence of the metabolon (Zhao et al. 2014). Sophisticated fluorescence microscopy-based methods including FLIM/FRET, fluorescence correlation spectroscopy, fluorescence cross-correlation spectroscopy, are used to verify the existence of metabolon (Crosby et al. 2011; Laursen et al. 2016; Bassard and Halkier 2018). These contribute greatly to reduce the false positive results and to improve the reliability of the data. However, these techniques require special equipment and expertise and used by limited research groups. When the purpose of an experiment comes to the screening of interacting protein pairs or protein complexes within a large set of enzymes, it becomes increasingly complicate to obtain reliable results. The use of multiple techniques is essential to avoid not only false-positives but also false-negatives. Given the transient nature of metabolons, its association is most likely dependent on the microenvironments of the enzymes. Single method may provide an inappropriate microenvironment leading to false-negative results. Nevertheless, problems then raise to integrate the results from multiple techniques and to set proper threshold to declare the protein pair interact. In the screening of protein–protein interaction within the plant TCA cycle, combination of three rather conventional methods, yeast two hybrid, split luciferase complementation, and AP-MS is used (Zhang et al. 2017). The protein–protein interaction occurs in vivo in all of these three methods and the principle of detection is different from each other. Importantly, they produce semi-quantitative scores which can be statistically combined to generate single reliability score. This approach successfully identified expected and novel components of plant TCA cycle metabolon (Zhang et al. 2017) and can be a milestone in the screening of metabolons.

Functional analysis of metabolons

Metabolons should be supported by both physical protein interactions and their physiological functions. To date, majority of the proof of metabolons is solely based on their physical complex formation except for some examples. First of all, the metabolite channeling should be experimentally validated ideally in vivo. There are multiple ways to evaluate metabolite channeling, including isotope dilution and enrichment, enzyme competition, transient time analysis, and the protection of enzyme from competitive inhibiter, which are described in recent reviews (Wheeldon et al. 2016; Sweetlove and Fernie 2018). However, applicability of these experiments is dependent on the localization of enzymes, substrate stability and availability, stability of the multi-enzyme complex, and many other factors especially in vivo. Therefore, the method should be carefully chosen taking the nature of the metabolon into consideration. Next, stability and dynamics of the multi-enzyme complex needed to be shown to prove its function in the regulation of metabolic pathways (Facchini 2016; Knudsen et al. 2018). This should also be linked with the metabolic flux and demands. The on-demand association of metabolon has been shown in the glycolytic metabolon in *Arabidopsis* and potato (Graham et al. 2007), dhurrin metabolon in sorghum (Laursen et al. 2016) and purinosome in human cell culture (An et al. 2010). A quantitative method to monitor protein–protein interaction in living cells is desired to analyze the dynamics of metabolons and to correlate it to metabolic flux on particular pathways. Further extension of the method to analyze higher order organization of protein communities among enzyme complexes will greatly improve our understanding of the functions of enzyme micro-compartmentation in the regulation of metabolic network (Kastritis et al. 2017). Finally, the metabolic and physiological functions of metabolons should be elucidated. Reverse genetic approaches are conventional yet still powerful ways to reveal gene functions and can also be applied for the functional analysis of metabolons. The study by Gou et al. (2018) is a good example showing the function of a metabolon by a reverse genetic approach. The formation of the lignin metabolon was disrupted and the stability and activity of the P450 proteins involved in the lignin biosynthesis were substantially impaired by the downregulation of MSBP scaffold proteins without compromising the expression levels of P450 genes. In consequence, lignin deposition and accumulation of intermediates of lignin pathways are significantly decreased with little effect on total flavonoid contents (Gou et al. 2018).

Conclusions

The recent studies providing novel insights in the functions of metabolons in plant metabolism are comprehensively reviewed in this article with the special focus on the studies conducted during the last decades. To date, metabolons have been found in most of the major metabolic pathways spanning from primary to secondary metabolism. This indicates that a metabolon is a ubiquitous cellular organization involved in metabolic regulation throughout the metabolic network. The coming decade will witness discoveries of many novel metabolons and elucidation of the functions of them. To this end, it is crucial to set a consensus in the level of proof needed to declare the existence of a metabolon. Unfortunately, current evidence supporting individual metabolons vary among them, some are supported by more than three techniques while others are based on only one unreliable assay. Although all the studies are covered in this review, some of them requires additional support by multiple techniques. Solid proof of in vivo multi-enzyme complex formation and metabolic functions is essential to gain more advocates on the concept of the metabolon and to establish it as a novel layer of mechanism for metabolic regulation.

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