Structural and functional diversity in plant specialized metabolism signals and products: the case of oxylipins and triterpenes

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Abstract

Metabolic enzymes tend to evolve towards catalytic efficacy, precision and speed. This seems particularly true for ancient and conserved enzymes involved in fundamental cellular processes that are present virtually in every cell and organism and converting and producing relatively limited metabolite numbers. Nevertheless, sessile organisms like plants have an astonishing repertoire of specific (specialized) metabolites that, by numbers and chemical complexity, by far exceed primary metabolites. Most theories agree that early gene duplication, subsequent positive selection and diversifying evolution have allowed relaxed selection of duplicated metabolic genes, thus facilitating the accumulation of mutations that could broaden substrate/product specificity and lower activation barriers and kinetics. Here, we use oxylipins, oxygenated fatty acids of plastidial origin to which the phytohormone jasmonate belongs, and triterpenes, a large group of specialized metabolites whose biosynthesis is often elicited by jasmonates, to showcase the structural and functional diversity of chemical signals and products in plant metabolism.

Introduction

Metabolites provide more than just structural roles. They can carry different types of information, not only because they may act as signaling molecules *sensu stricto,* but also because their physiochemical properties may dictate how enzymes have to evolve to interact with handed-down templates of biomolecular structures [1]. As a result, metabolic and genetic complexity (i.e. comprising both the number of coding sequences and regulatory connections) frequently go hand in hand, and linking structures to functions means deciphering another layer of communication and evolution of plants. Although new organisms may emerge or go extinct during evolution, membranes will typically be built the same way, hormones will frequently work identically and, ultimately, the showcase metabolites of this review, i.e. oxylipins and triterpenes, will remain present in every member of the Viridiplantae.

Conventionally, plant metabolites are often divided into primary and specialized metabolites. The first are ubiquitously present in most organisms, being products of enzymes that normally have a high substrate and product specificity and are kinetically efficient. These are indispensable for plant growth and survival, and constitute the metabolic heritage of evolution and the results of macro-evolutionary processes. In contrast, enzymes involved in specialized metabolite biosynthesis, which ancestrally originated from duplication of genes involved in primary metabolism [2], tend to be more promiscuous and kinetically slower, and sometimes produce not only one but a bouquet of metabolites, only some of which will confer evolutionary advantages [2]. In this perspective, we speculate that once a certain metabolite confers a specific advantage to plant fitness, it may become a fixed part of the core metabolic toolkit, allowing evolution to select enzymes that will produce this product more efficiently [2]. Also, the more we move away from primary metabolism, the more frequently enzymes can have multiple substrates or products that will allow to explore metabolic possibilities and sometimes develop new functions. In this review, we will highlight these hypotheses by discussing the evolution of plant oxylipin and triterpene metabolites.

Structural and functional diversity of oxylipins

Oxylipins are molecules that originate from oxygenated fatty acids [3]. In humans, oxylipins are critical bioactive mediators of physiology and inflammation, and more than 500 distinct oxylipin entities have recently been detected in human samples [4]. In plants, oxylipins play essential roles in stress signaling and development. One of the best-characterized examples is the phytohormone jasmonate (JA), regulating responses to (a)biotic stresses, the production of specialized metabolites (including most triterpenes), but also several aspects of plant growth and development [3,5,6].

JA biosynthesis in plants starts in the chloroplast and ends in the peroxisome. In *Arabidopsis thaliana* (Arabidopsis), α-linoleic acid is the predominant unsaturated fatty acid composing the lipid bilayer of the thylakoid membrane and it represents the starting molecule for the biosynthesis of JA. In the chloroplast, α-linoleic acid is oxidized by 13-lipoxygenase, forming 13-hydroperoxy-octadecatrienoic acid, which is cyclized and rearranged by allene oxide synthase and allene oxide cyclase to form 12-oxo-phytodienoic acid (OPDA). Then, OPDA is exported to the peroxisome where it undergoes reduction by the 12-oxophytodienoate reductase 3 (OPR3) enzyme and rounds of β-oxidations forming JA that is released in the cytosol [3]. It is worth mentioning that recent studies on *OPR3* loss-of-function mutants revealed that parallel OPR3-independent pathways are capable of circumventing peroxisomal OPR3-dependent biosynthetic steps so that, in the absence of OPR3, β-oxidized derivatives of OPDA are released and reduced to JA in the cytosol by the action of the OPR3-related

enzyme OPR2 [7]. JA represents the basic structure that is metabolized into multiple bioactive compounds. For example, JASMONATE RESISTANT 1 (JAR1) conjugates JA with isoleucine to form jasmonoyl-isoleucine (JA-Ile), which binds to the coreceptor complex consisting of the F-box protein coronatine-insensitive 1 (COI1) [8] and a repressor jasmonate-ZIM domain (JAZ) protein [9,10]. JA-Ile perception unleashes key transcriptional regulators, such as MYC2, from inhibition by JAZ, ultimately leading to the onset of defense responses, such as biosynthesis of specialized metabolites [6,11,12]. Whereas JA-Ile is considered as the main and most efficient bioactive form of JA [13,14], several other JA conjugates, mostly with hydrophobic amino acids, have been identified [15,16]. Yet, the latter conjugates are less potent than JA-Ile in triggering JA-mediated responses and are the products of enzymes that show higher promiscuity than the canonical JAR1 [15]. However, the bioactivity of JA derivatives may go beyond the binding to the COI1–JAZ coreceptor complex and structural JA variants may have a specific function. A suitable example is the methylated form of JA (MeJA), which is not considered active *per se* because it does not bind the coreceptor complex. Yet, this highly volatile JA ester may be involved in the communication between plants or in the systemic triggering of a prompt JA response [17-20]. Notably, whereas hydroxylation of JA to 12-OH-JA was initially proposed as a mechanism linked to hormone catabolism and thus inactivation [21], recent findings have shown that, in its conjugated form, 12-OH-JA-Ile also has bioactive properties promoting the formation of the COI1–JAZ coreceptor complex and thereby inducing defense responses [22,23]. Nevertheless, upon stimulus perception, the timing of the production of oxidized JA derivatives and their strength in inducing the JA response may differ from the canonical JA-Ile pathway, hence the formation of these derivatives may be ways to tune the intensity of the JA response. Furthermore, conversion of 12-OH-JA into cis-jasmone triggers volatile defenses against aphids in potato [24]. These examples illustrate that novel JA variants have been arising during evolution (Fig. 1), and plausibly the identity and function of many of them remain to be revealed and elucidated.

Some recent studies on oxylipin signaling in non-vascular plant lineages revealed that JA-Ile was not the ancient bioactive oxylipin in plants. Earlier lineages of land plants, such as the bryophytes *Marchantia polymorpha*, *Physcomitrella patens,* and *Calohypnum plumiforme,* use OPDA instead of JA as the actual signaling hormone, given they miss most of the JA biosynthetic machinery [25-28]. Here, the OPDA derivative dinor-12-oxo-phytodienoic acid (dn-OPDA) exerts the function of JA-Ile in binding and activating the COI1–JAZ coreceptor complex, suggesting dn-OPDA to be the ancestral phytohormone. Contrary to JA derivatives, little is known about OPDA derivatives, except for a few reports that found C20-OPDA and iso-12-OPDA to accumulate upon wounding in *M. polymorpha* and *P. patens,* respectively [29,30].

Accordingly, recent studies revealed that in contrast to most vascular plants, bryophytes such as *Marchantia* are rich in long-chain C20 and C22 polyunsaturated fatty acids. In this species, the exogenous application of C20-OPDA is able to activate COI1-dependent and -independent responses, as it is the precursor of Δ^4-dn-OPDA, the direct ligand of the MpCOI1-MpJAZ coreceptor [30].

As life gained complexity, so did the signals necessary to fine-tune developmental programs. For example, differently from liverworts, the genome of the lycophyte *Selaginella moellendorffii* already contains JA biosynthetic enzymes, similar to those of Arabidopsis [31]. Besides the enzymes, also the components of the COI1–JAZ coreceptor complex coevolved to accommodate the new ligands. In fact, a single amino-acid change in the receptor protein MpCOI1 (V377A) of *M. polymorpha* was found to be responsible for switching ligand specificity from dn-OPDA into JA-Ile [25]. Similarly, specific residues of the JAZ proteins in bryophytes and lycophytes enable the perception of dn-OPDA ligands, which does not occur in vascular plants in which JAZ proteins recognize only JA-Ile [32]. MpMYC from *Marchantia* was shown

to be capable of directly interacting with the Arabidopsis JAZ proteins and to upregulate defense responses in *Marchantia* by binding G-boxes of promoters of genes encoding biosynthetic enzymes involved in the production of specialized metabolites such as sesquiterpenes [33]. So, although the components and dynamics of COI–JAZ–MYC regulatory modules are functionally conserved, different ligands determine their (specific) behavior.

The fact that different plant lineages use related but non-identical molecules and molecular complexes to address the same type of developmental and environmental challenges, raises intriguing questions regarding the advantage for angiosperms to evolve and produce JAs. Do OPDA derivatives work in the same way as JA derivatives? Has OPDA lost its function in flowering plants? Chances are that OPDA as well as other, non-OPDA linoleic acid derivatives have partially conserved stress signaling functions in angiosperms as well. For example, oxylipins produced from linoleic acid by 9-lipoxygenase, such as (9S,10E,12Z,15Z)-9-hydroxy-10,12,15-octadecatrienoic acid, regulate defense responses against the nematode *Meloidogyne* spp. in tomato [34], while in the monocot *Epipremnum aureum*, OPDA displays a scavenging activity that reduces reactive oxygen species levels in the chloroplast [35]. Likewise, OPDA has recently been found to tune the activity of thioredoxins and peroxiredoxins in plant stress responses by covalently binding thiols. This type of post-translational modification has been termed OPDAylation [36]. It is therefore likely that OPDA in vascular plants is not merely a JA precursor [37], and that some of its ancient functions, such as a COI1-independent function in thermotolerance [38], may have persisted. Considering that OPDA-Ile was proposed as a new alternative and independent signal from JA [39], it is clear that many structure–activity relationships of plant oxylipins remain to be discovered.

Structural and functional diversity of triterpenes

It has been reported that specialized metabolic enzymes are on average thirty times less active than those involved in central metabolism [1]. Therefore, varying levels of substrate permissiveness, catalytic promiscuity and reduced kinetics would explain how single enzymes could perform multiple reactions and synthesize multiple compounds [1]. For instance, squalene-derived triterpenes are among the largest group of plant specialized metabolites, accounting for thousands of distinct structures [40-42] with disparate functions, ranging from structural or defensive roles [40,43] to the modulation of root microbiota and plant growth [44,45]. Ultimately, these molecules result from three main biosynthetic steps: cyclization of the terpene precursor 2,3-oxidosqualene by oxidosqualene cyclases (OSCs) yielding apolar aglycones such as sterols and triterpenes, oxidation of specific positions of the carbon backbone by cytochrome P450s (P450s) and decoration of the resulting aglycone with, e.g. sugar moieties by UDP-glycosyltransferases (UGTs) (Fig. 2). This last step confers polarity and turns triterpenes into amphipathic saponins. Generally speaking, a genome of an angiosperm plant would harbor around ten OSCs and between hundred and several hundreds of P450s and UGTs, the latter being active on many other substrates beside triterpenes. In turn, this translates into hundreds of mono- to hexacyclic scaffolds deriving from OSC activities [46], thousands of different oxygenated aglycones produced by P450s and tens of thousands of glycosylated saponins resulting from UGT activities [41]. Importantly, here not only enzyme promiscuity, but also the combinatorial interconnection between and within the biosynthetic networks greatly expands the number of possible metabolic outcomes. Although intricate dynamics render it hard to infer metabolic from genetic complexity, genomic analysis of the unicellular algae *Chlamydomonas reinhardtii* and the moss *P. patens* revealed the presence of only one OSC resembling a cycloartenol synthase required for sterol biosynthesis [47]. Simplifying, we could speculate, as experimental evidences are mostly lacking, that from this

ancestral, yet essential monofunctional enzyme, cycles of gene duplication and fixation have recurrently first tested newly arisen multifunctional enzymes (and their products) and then embedded in the basic metabolic core only those enzymes whose products would prove advantageous [2].

These evolutionary dynamics are clearly found among the P450s performing oxidations of pentacyclic triterpenes. P450s that oxidize the C28 position are among the most represented members of this family and show a relatively high degree of specificity and efficiency [48,49] as compared with others that target less common positions of the carbon skeleton. C28 oxidation is indeed the first and most common decoration found in saponins and a prerequisite for further oxidizing or glycosylating steps. Therefore, C28 oxidases of the CYP716A subgroup are considered the earliest members of triterpene-modifying enzymes in land plants [48], whose products became part of the plant's default metabolic repertoire. Accordingly, evolution had enough time to select more efficient C28 oxidases. In contrast, P450s active on other positions are sometimes slower and more promiscuous [50], because their products may still be developing peculiar functions, conquering a functional niche. The promiscuity of some triterpene-modifying enzymes becomes evident, especially when enzymes are tested for functional characterization in heterologous systems or *in vitro*. Here, we frequently see that in addition to a main substrate or product, enzymes are active on several substrates and/or accumulate by-products or intermediates [51] that in endogenous systems would be scarcely produced or promptly catabolized through conversion by competing pathways.

It should be noted though that chemical diversity does not just arise when duplicated enzymes gain new functions towards completely new substrates or residues. Sometimes redundant enzymes can perform the same reaction but with different kinetics/affinity, thus accumulating different products. For example, some P450s catalyze a three-step sequential oxidation going from the hydroxyl to the carboxyl moiety via an aldehyde intermediate [48,51]. Redundant enzymes catalyzing the same reaction may have less proclivity to finalize the three oxidative steps, thus resulting in the preferential accumulation of specific intermediates.

For years, *Medicago truncatula* has been the benchmark for triterpene studies. This model legume accumulates among others triterpenes with β-amyrin derived oleanane (OA) aglycone backbones such as hederagenin (C23-OH OA), gypsogenic acid (C23-COOH OA) or medicagenic acid (C23-COOH, C2-OH OA). Aglycones carrying the aldehyde moiety at C23, such as gypsogenin (C23-COH OA) or quillaic acid (C23-COH, C16-OH QA), are barely detectable as intermediates or absent in *M. truncatula* extracts, meaning that the carbon on this position is fully oxidized to the carboxyl moiety [51]. In contrast, plants belonging to the Caryophyllaceae family tend to predominantly accumulate gypsogenin and quillaic acid characterized by the aldehyde moiety on C23 [52]. Together, this suggests that plants evolved multiple versions of C23 oxidases, some of which will complete the oxidation to the carboxyl group, while others, having reduced affinity for the third oxidative step, accumulate aldehyde-bearing compounds. Although little is known about the functions of these different but related molecules in plants, studies on structure–activity relationships revealed that subtle changes can have a big impact on bioactivity [53]. For instance, hederagenin monoglucoside, bearing a hydroxyl on C23 and a glucose on C3, is more toxic to herbivorous caterpillars as compared with gypsogenic acid or oleanolic acid monoglucoside carrying a carboxyl or hydrogen on position 23 [54]. 3D modeling predicted that the C23 hydroxyl group may cause a 90° rotation of the sugar residue at C3 with respect to the aglycone plane, thus increasing its lytic properties towards insect membranes [40].

The ability of plants to synthesize saponins that can disrupt the integrity of biological membranes while avoiding self-toxicity through compartmentalization, relies on a different affinity of these compounds towards plant (e.g. β-sitosterol, campesterol, stigmasterol) or

animal (e.g. cholesterol) sterols. Studies with different plant saponins revealed that plasma membrane cholesterol is essential for its permeabilization activity [55-57]. Accordingly, it was shown that by replacing cholesterol with other sterols, such as Δ7 and Δ9 sterol, the cell membranes of sea cucumbers, which represent some of the few animal clades capable of synthesizing triterpene saponins, can tolerate their own cytotoxic triterpenes [58]. Cholesterol and phospholipid composition, therefore, seems to dictate the toxicity of these types of saponins. Considering that Golgi and endoplasmic reticulum membranes contain a lower amount of cholesterol than the plasma membrane [59], this would also explain why the lytic activity of saponins is particularly effective towards the plasmalemma [60,61]. Although sugar residues are essential to confer lytic properties to saponins [40,56,62,63], functional moieties on aglycones also play a role [53]. For example, carboxyl and hydroxyl groups on aglycones are likely priming cholesterol-independent electrostatic binding to positively charged choline heads of phospholipids [57,62], while the backbone types seem to drive the depth and orientation of their insertion within the bilayer [57]. Hence, the great structural diversity in aglycones may result in different affinities for membranes (i.e. bacterial, fungal or animal) based on their lipid composition. Yet, the permeabilizing activity of saponins seems to be related mainly to their extent and type of glycosylation. Comparison between α- and δ-hederin, having respectively two or one sugar residue(s) on the C3, indicated that the first is much more effective for pore formation, likely because the two sugars confer a bigger hydrophilic surface and upon insertion cause curvature until disruption of the lipid bilayer [62]. Also, bidesmosidic saponins, in which the carbohydrate chains are linked to the C3 and C28 via glycosidic and ester bonds, respectively, display a lower toxicity than their monodesmosidic counterparts, having sugars only on C3 [53,57,61]. Accordingly, chemical or enzymatic hydrolysis of glycosyl residues on C28, which restores the carboxyl moiety, greatly enhances saponin toxicity, for instance against fungal pathogens [64,65]. Although saponins are mainly known as defensive compounds that are mostly toxic in their monodesmosidic form, plants still synthesize highly glycosylated bidesmosidic saponins [66,67]. One may therefore wonder what the function of such complex compounds could be. Can they have a dual purpose: being synthesized when resources are not limiting as energetic investment by storing sugars on defensive compounds to be promptly hydrolyzed upon cell damage, releasing toxic monodesmosidic saponins together with sugars to sustain energy metabolism? This question is still far from being answered as the structural and biosynthetic complexity of highly glycosylated saponins represents major hurdles for functional studies.

Outlook

Although technical advances considerably increased the throughput of metabolomics compared with proteomics, transcriptomics and genomics, the unambiguous identification of metabolites remains scarce and labor intensive because it largely relies on purification followed by structure elucidation via nuclear magnetic resonance [41].

Platforms like Global Natural Product Social Molecular Networking (GNPS) [68] are innovative open-source repositories for interconnecting mass spectrometry datasets of natural compounds with streamline functional annotation. However, especially for plant metabolites, the efficiency falls a long way short for the hundreds of thousands of estimated compounds [69]. Likewise, with regard to both structural and functional characterization, large-scale screenings for metabolic phenotypes are hampered by the lack of development of efficient read-out methods or by the limited availability of collections of pests, such as microbial pathogens or insects, both in numbers and species [70]. Nonetheless, new techniques keep on emerging that will help us to tackle this huge challenge. Particularly noteworthy are the many emerging protein–metabolite interactomics platforms, such as limited proteolysis-coupled mass spectrometry or protein–metabolite interactions using size separation [71-73],

which will help reveal how the interactome of diverse metabolites evolves, thus shedding new light on how structures and functions are connected to create new variability and increase in the complexity of the plant's developmental programs.

Acknowledgements

We thank Annick Bleys for critically reading the manuscript. This research was supported by the European Union's Horizon 2020 research and innovation program under Grant Agreements No 825730 (Endoscape) and the Research Foundation Flanders (FWO) through the projects G008417N and G004320N, all assigned to A.G.

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This remarkable work revealed how the JA coreceptor complex involving COI1 is conserved in the bryophyte *Marchantia polymorpha* to perceive the endogenous ligands, i.e. the two OPDA derivatives dn-*iso*-OPDA and dn-*cis*-OPDA. This research demonstrates that bryophytes and vascular plants share the signaling machinery but use different signaling molecules. The authors also show how a single amino-acid change in COI1 switches ligand specificity, allowing late diverging plants such as Arabidopsis to use JA as ligand but not dn-OPDA or vice versa.

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This research revealed the bioactive oxylipins in *Marchantia polymorpha*. These are derived from 20- and 22-long chain polyunsaturated fatty acids (LCPUFAs), in contrast to the bioactive jasmonates from

vascular plants that derive from polyunsaturated fatty acids containing 16 or 18 carbons. The authors also demonstrate that one of these bioactive compounds, a dn-OPDA-like molecule derived from LCPUFAs, is a ligand of COI1, one of the proteins of the JA coreceptor complex, thus activating stress responses.

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This report shows that JASMONATE-ZIM-DOMAIN (JAZ) proteins, which form part of the JA coreceptor complex, are essential for ligand recognition. The authors discovered that specific residues of JAZ proteins in bryophytes and lycophytes enable the perception of dn-OPDA ligands, but in vascular plants beyond lycophytes, JAZ evolved to bind JA-Ile, thus impeding dn-OPDA recognition. Additionally, they found that the lycophyte *Huperzia selago* represents an intermediate evolutionary step presenting JAZ features from the ancestral bryophytes but COI1 features from angiosperms.

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Figures and figure legends

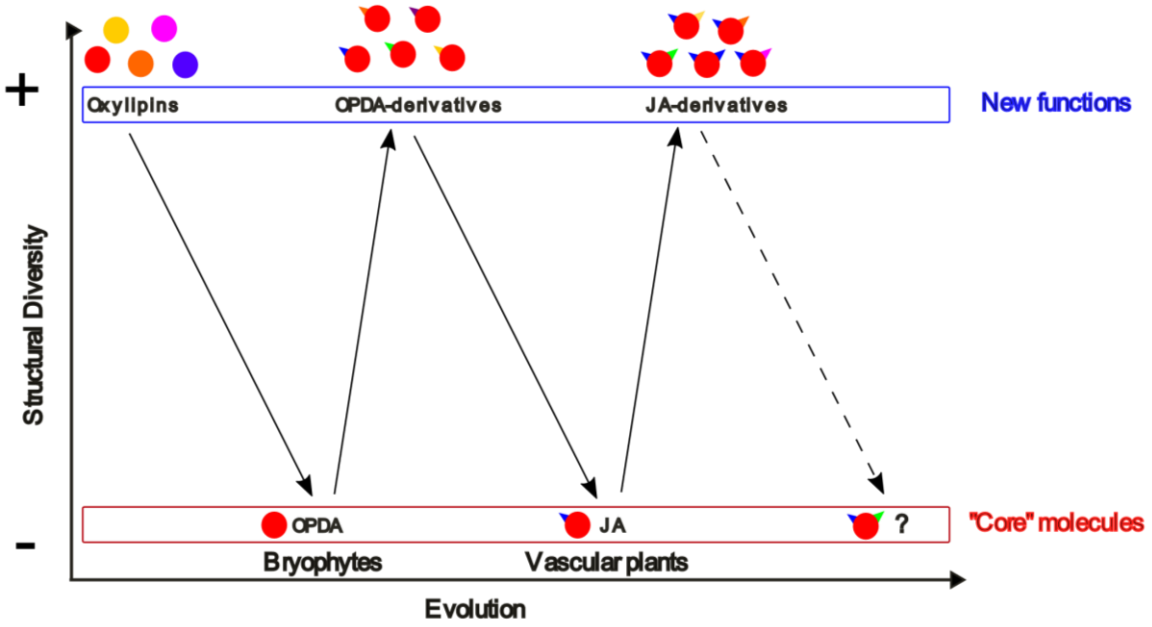

Figure 1. Evolution model of oxylipins. Concept of how plant metabolites may evolve new structures and (signaling) functions. Once a molecule gains new functions, it becomes (i) part of the core elements of the system and (ii) the chemical skeleton that can be further modified to produce a variety of new molecules. In the given example, out of many oxylipins, 12-oxo-phytodienoic acid (OPDA) (i) gained essential functions and became part of the core oxylipin signaling module for non-vascular plants, and (ii) constituted the starting point for a multitude of OPDA derivatives. Afterwards, among all OPDA derivatives, jasmonic acid (JA) became the core signaling molecule in vascular plants and continued to diversify into new derivatives that will “explore” new evolutionary possibilities and functions. During evolution, cycles of expansions and fixations have likely produced well-established signaling molecules as well as numerous derivatives that may have partially redundant or complementary functions, or are still gaining new ones. Colored dots represent metabolites and their derivatives that cyclically arose and got embedded into developmental programs along evolution.

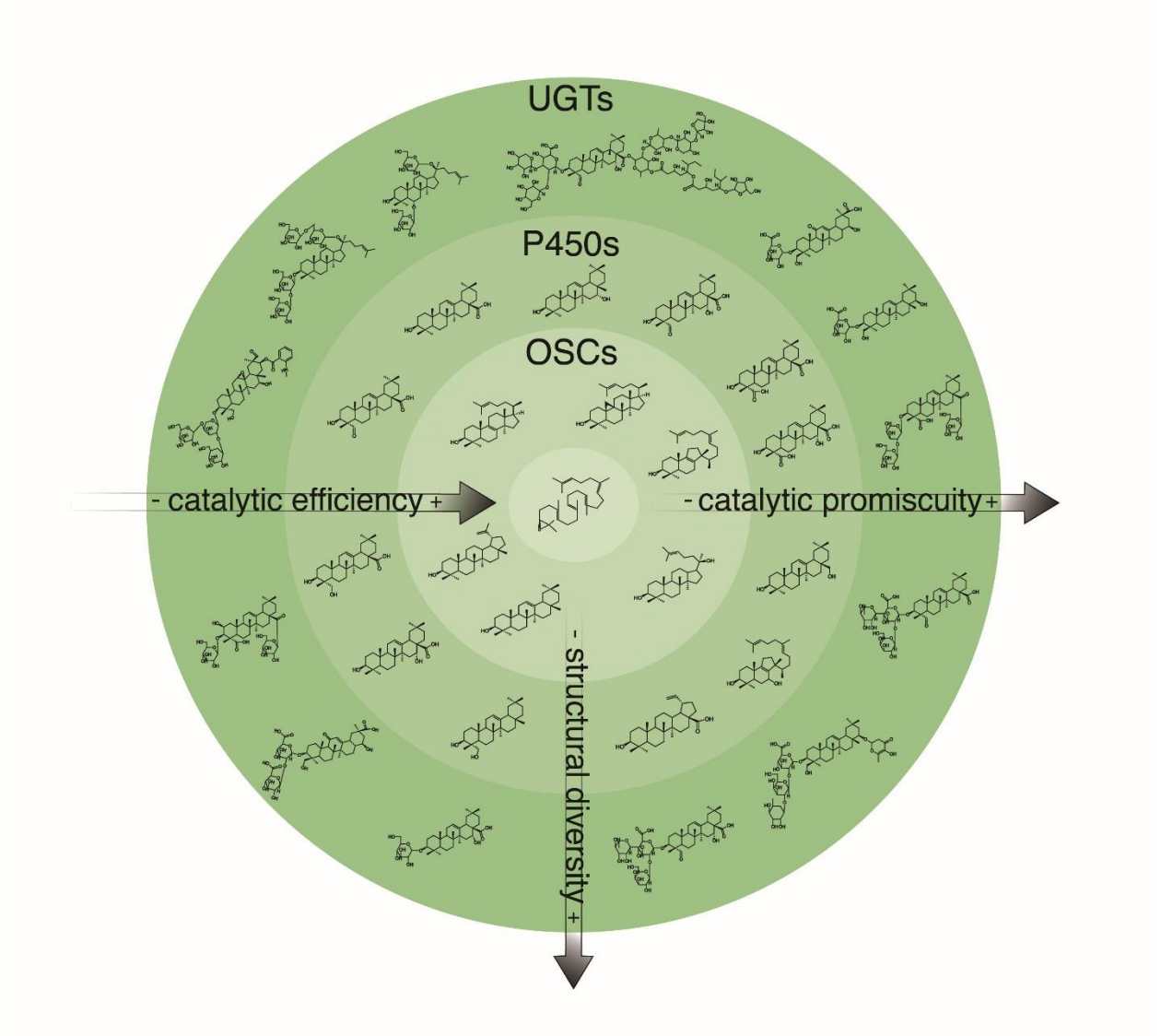

Figure 2 Snapshot of the diversity of 2,3-oxidosqualene-derived metabolites. The figure reports an exemplifying portion of the virtually infinite array of triterpenes that can derive from 2,3-oxidosqualene (central circle) by the consecutive action of cyclases (OSCs), cytochrome P450s (P450s) and UDP-glycosyltransferases (UGTs). Both the diversity of the structures and the catalytic promiscuity of the enzymes involved in their biosynthesis increase while moving from the center to the periphery. This figure should be considered as a general concept, and we would like to point out that there may be cases where P450s have more promiscuity, and conversely, that UGTs are more specific. Yet, with regard to those involved in triterpenes, in most cases P450s are more specific than UGTs, with the notable exception of those involved in 'check-points', such as for instance the UGTs that initiate the glycosylation path, being the first enzymes to grant higher solubility to the hydrophobic oxidized aglycone.