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Review

Post-translational events and modifications regulating plant enzymes involved in isoprenoid precursor biosynthesis

Andréa Hemmerlin*

Institut de Biologie Moléculaire des Plantes du Centre National de la Recherche Scientifique, IBMP-CNRS-UPR2357, Université de Strasbourg, 28 rue Goethe, F-67083 Strasbourg Cedex, France

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ABSTRACT

Identification of regulatory enzymes is fundamental for engineering metabolic pathways such as the isoprenoid one. All too often, investigation of gene expression remains the major trend in unraveling regulation mechanisms of the isoprenoid cytosolic mevalonate and the plastid-localized methylerythritol phosphate metabolic pathways. But such metabolic regulatory enzymes are frequently multilevel-regulated, especially at a post-translational level. A prominent example is the endoplasmic reticulum-bound 3-hydroxy-3-methylglutaryl coenzyme A reductase catalyzing the synthesis of mevalonic acid. Despite the discovery and the intense efforts made to understand regulation of the methylerythritol phosphate pathway, this enzyme remains a leading player in the regulation of the whole isoprenoid pathway. Strict correlation between this enzyme's gene expression, protein level and enzyme activity is not observed, thus confirming multilevel-regulation. In this context, besides post-translational modifications of proteins, we have to consider feedback of metabolic flow and allosteric regulation, alternative protein structures, targeted proteolysis and/or redox regulation. Such multilevel-regulation processes deliver a range of benefits including rapid response to environmental and physiological challenges or metabolic fluctuations. This review specially emphasizes essential functions of these post-translational events that permit the close regulation of key enzymes involved in plant isoprenoid precursor biosynthesis.

Contents

Abbreviations: DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LC, liquid chromatography; ROS, reactive oxygen species.

* Tel.: +33 0368851858; fax: +33 0368851921.
E-mail address: Andrea.Hemmerlin@ibmp-cnrs.unistra.fr

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1. Introduction

Isoprenoids (or terpenoids) consist of a large variety of metabolites with great diversities in structure and function [1–3]. The biochemistry of this so-called terpenome is based on a C_5 isoprene unit that once elongated into larger molecules are considered as isoprenoid precursors (C_{10} up to C_{20}) being further assembled and metabolized into specific end-of-chain products (Fig. 1). These precursors, including geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), are the major starting metabolites needed to build up specific isoprenoid skeleton families (meroterpenes, monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes). Such a level of complexity requires a subtle organization of the metabolic networks, which has to be coordinated by strict regulatory processes. In fact, pathways that branch into many metabolite families need a tight control of the common core reactions. In addition to isoprenoid precursor biosynthesis, this common core consists also of the isopentenyl diphosphate (IPP) biosynthetic pathways (the mevalonic acid (MVA) and the 2*C*-methyl-D-erythritol 4-phosphate (MEP) pathways) (Fig. 1). Much attention has been given on study the regulatory mechanisms of these metabolic pathways in plants [4], with primary goals of such investigations being to elucidate any positive or negative control of the biosynthesis of specific isoprenoid products. The end-products of many isoprenoids have considerable economic values of many isoprenoid end-of-chain products for the cosmetics, agribusiness and pharmaceutical industries [5–8]. The isoprenoid biosynthesis pathway is an ideal molecular target for numerous herbicides already in use [9,10], or which still await discovery. Some end-of-chain products may also be useful as alternate biofuel sources [11,12]. The regulatory network has to be fully deciphered if there is to be plant overexpression of such metabolites.

It may be too much of a challenge to unravel these pathways, as plants have evolved special ways to adjust and control metabolic alterations of these early steps. First, plants' metabolism might be unique as it is compartmentalized in a particular way [13]. Thus, plants have an unprecedented capacity to regulate isoprenoid biosynthesis since they utilize two differentially compartmentalized pathways to build up the basic isoprene unit: the cytosolic mevalonate pathway and the plastid methylerythritol phosphate pathway. This parallel synthesis leads to an unusual dynamics due to the possibility of exchanging isoprenoid precursors between the cytosolic and plastid compartments, apparently under particular conditions and for the synthesis of defined metabolites (reviewed by Hemmerlin et al., 2012, [14]). The occurrence of numerous enzyme families basically sharing an identical catalytical function are, among additional complexities, associated with plant isoprenoid biosynthesis. These isoforms are expressed under restricted conditions, in defined tissues or organelles, or are associated with specific biosynthesis branches (*cf.* [14]). A substantial body of literature predicts a correlation between transcriptional

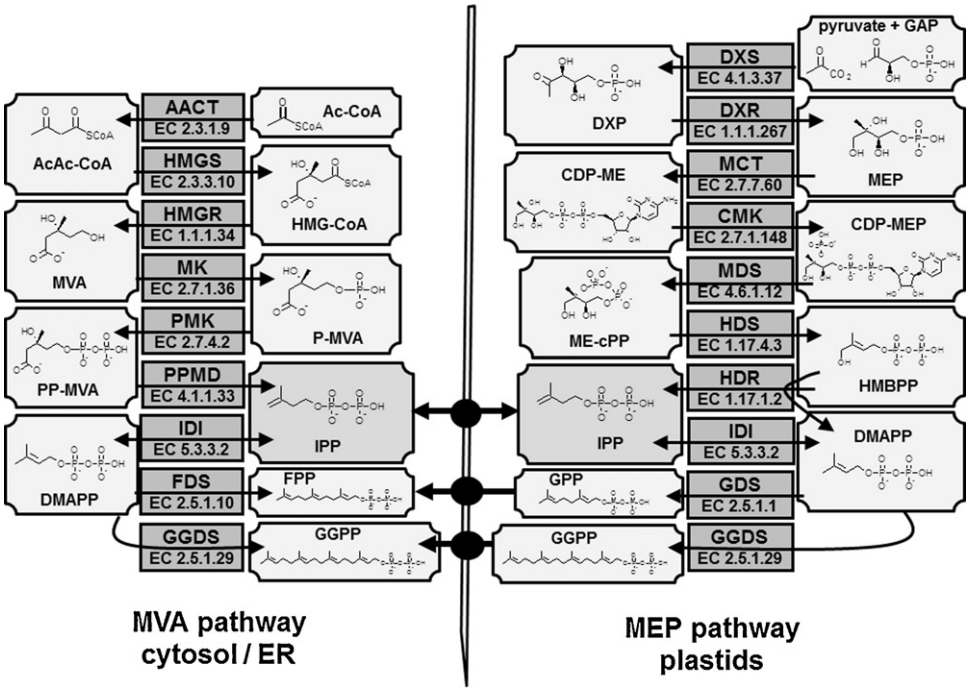

Fig. 1. Biosynthesis of isoprenoid precursors in plants. In plants, isopentenyl diphosphate (IPP) and its chemically active allylic isomer dimethylallyl diphosphate (DMAPP) are synthetized either by the cytosolic MVA pathway or by the plastidial MEP pathway. Substrates and enzyme-products of the MVA pathway are as follow: Ac-CoA, acetyl-coenzyme A; AcAc-CoA, acetoacetyl-coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MVA, mevalonate; P-MVA, mevalonate 5-phosphate; PP-MVA, mevalonate 5-diphosphate. Enzymes of the MVA pathway are as follows: AACT, AcAc-CoA thiolase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PPMD, diphospho-mevalonate decarboxylase. Substrates and enzyme-products of the MEP pathway are as follows: pyruvate; GAP, D-glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2*C*-methyl-D-erythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-2*C*-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate; ME-cPP, 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate. Enzymes of the MEP pathway are as follows: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT, 2*C*-methyl-D-erythritol 4-phosphate cytidyltransferase; CMK, 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase; MDS, 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; HDR, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase. The interconversion of IPP into DMAPP is catalyzed by IDI, isopentenyl diphosphate isomerase. DMAPP is then further elongated into geranyl diphosphate (GPP), farnesyl diphosphate (FPP and geranylgeranyl diphosphate (GGPP) by addition of 1, 2 or 3 IPP units. GPP biosynthesis is believed to occur exclusively in plastids, while *all-trans*-FPP solely in the cytosolic compartment. Exchanges between both cell compartments were described for IPP, GPP and GGPP (reviewed in [14]). The export of GPP is followed by a further elongation into FPP. Transporters are depicted as black dots. These molecules will serve as precursors for the biosynthesis of isoprenoid families: monoterpenes (GPP), sesquiterpenes (FPP), diterpenes (GGPP), triterpenes (FPP), tetraterpenes (GGPP).

expression of the corresponding genes and specific functions. There is an increasing interest in transcriptional regulation, involving RNA binding proteins or miRNAs [15,16]. While this mode of regulation is poorly characterized especially for the isoprenoid biosynthetic pathway, it becomes nevertheless increasingly clear that isoprenoid metabolites (*e.g.* sterols, prenylated proteins and/or polyprenols) are somehow involved [17,18]. In any case, some regulatory enzymes involved in both isoprenoid pathways regulated at multiple levels [19].

This review especially emphasizes the essential function of post-translational regulation of enzymes that are involved in isoprenoid precursor biosynthesis. It focusses as on description of molecular events that might have modulated catalytical capacities of regulatory enzymes. One of the potential consequences of post-translational regulation is the capacity of enzyme activity to rapidly fluctuate in response to signals induced under different environmental or physiological conditions, such as stress or light. Four main categories of post-translational regulation will be illustrated and discussed: allosteric regulation, structural regulation, secondary post-translational modifications and redox regulations.

2. Regulatory enzymes of the isoprenoid biosynthetic pathways

In biosynthesis pathways, the velocity (or rate) of each reaction producing metabolite intermediates, may differ. The slowest step is catalyzed by a so-called pathway regulatory enzyme and is defined as the rate-limiting step controlling the metabolic flux. The identity of such regulatory enzymes needs to be assigned before considering any regulatory process, because they are regarded as bottlenecks controlling metabolic fluxes. Identification of such elements provides essential tools for engineering organisms, which will promote production of specific metabolites [20].

2.1. Identification of regulatory enzymes, a challenging task

The overproduction of specific enzymes *in planta* followed by evaluation of the capacities of transgenic lines to accumulate end-of-chain product, is one of the strategy used to identify and characterize regulatory enzymes. However, overproduction often, results in limited stimulation of metabolite production. With the objective of more precisely characterizing such regulatory enzymes, different state-of-the-art strategies have been adopted by many people. Genomic, metabolomic or proteomic approaches have received considerable attention during the last several years as promising techniques, because they generate large data sets [21–23]. While these techniques may seem appealing at first sight, they have also serious drawbacks: It often is a real stumbling block for distilling essential information from this oversized quantity of data. Genome-scale mathematical modeling has been applied for some primary metabolite pathways, but appears limited in terms of knowledge, but also in terms of relevance to more complex pathways such as the isoprenoid one [24]. Alternative or complementary approaches such as investigation of metabolic networks with flux analysis may be applied. These approaches rely on incorporation studies with ^{13}C-labeled substrates and the analysis of the redistribution of the label into metabolites [25]. Flux rates are subsequently deduced by fitting a network model to the experimental data [25]. They offer the advantages to consider all steps of the metabolic pathway but are not trivial to implement.

Levels of regulation need to be determined to decipher the role of regulatory enzymes. A figure summarizing various level of regulation of enzymes involved in the biosynthesis of IPP in plants is presented (Fig. 2), based on current knowledge recently reviewed

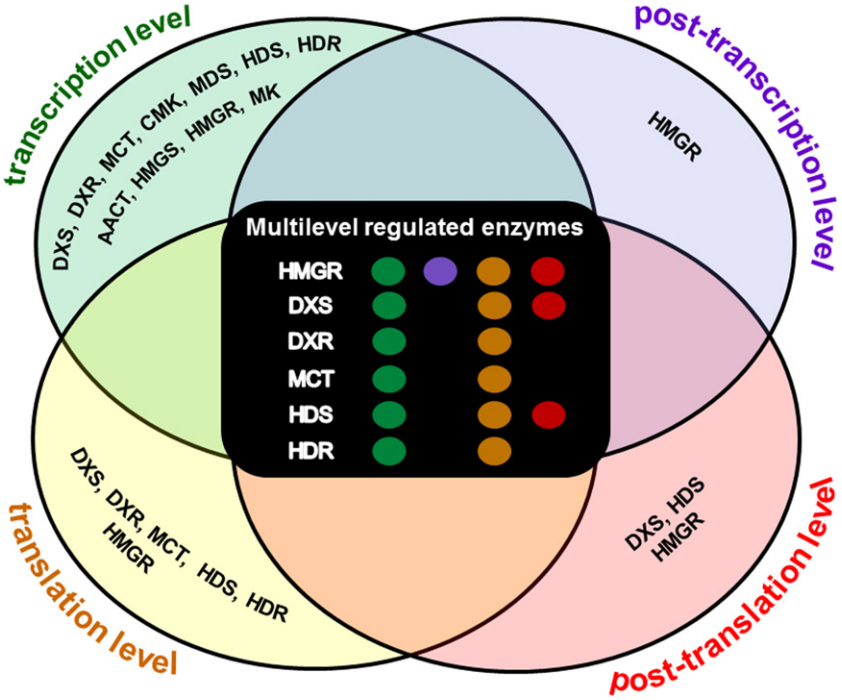

Fig. 2. Level of regulation of enzymes involved in isoprenoid precursor biosynthesis in plants. Enzymes of the MVA pathway are as follows: AACT, AcAc-CoA thiolase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PPMD, diphospho-mevalonate decarboxylase. Enzymes of the MEP pathway are as follows: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT, 2C-methyl-D-erythritol 4-phosphate cytidyltransferase; CMK, 4-diphosphocytidyl-2C-methyl-D-erythritol kinase; MDS, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; HDR, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase. Green color reflects transcriptional level of regulation, blue post-translational level, brown translational level and red post-translational level. The central black square wraps up levels of regulation of enzymes found at least regulated at two levels.

[14] and data collated in 1. It is now clearly established that the regulatory enzymes regulating isoprenoid fluxes are controlled at the level of transcription, post-transcription, translation and also last, but not least, at the post-translation levels. The prime importance of post-translational regulation for key isoprenoid precursor enzymes is also supported by the existence of specific and efficient natural inhibitors of some of the enzymes. Most studies have mainly dealt with gene expression, sometimes protein expression, but only rarely have enzyme activities been measured. Transcriptional data must be carefully related to what happens in the cell, *i.e.* enzyme activity levels. When considering transcriptional regulation, most genes encoding enzymes involved in early biosynthesis steps are found as positive hits in all studies and all plants considered, at least under the tested condition (Fig. 2). This means either that each plant has evolved its own ways to regulate the pathways, or that a set of enzymes control them. The choice of the plant model might constitute a critical point in designing general schemes of metabolic regulation. A third hypothesis would favor a non-transcriptional regulation, *i.e.* a subsequent level of regulation. Many published studies frequently consider gene expression as the sole level of regulation to conclude whether there is a key function of enzymes or of metabolic routes. This strategy may not reflect the real situation *in vivo*, and result sometimes in misleading interpretations. This may be due to experimental easiness, a lack of competence in non-conventional techniques or may be just a result of the available techniques. Little attention has been paid to basic biochemical concepts, such as enzyme characterization or *in vivo* modulation of enzyme activity in response to different endogenous factors. It is anything else but trivial to design systematic studies drawn that focus on all levels of regulation. Two studies with an identical goal but totally different approaches are compared to further illustrate this need to study not only gene expression, but also further steps of enzyme production. Both studies [26,27] aimed at demonstrating "cross-talk" between the methylerythritol phosphate and the mevalonate pathways, *via* the possible exchange of metabolic intermediates such as isopentenyl diphosphate, geranyl diphosphate or geranylgeranyl diphosphate (Fig. 1). Strategies to prove this were based on blocking one pathway and evaluating the possibility to internally complement the deficit by the other pathway. This has been demonstrated with plants treated with specific inhibitors such as mevinolin (blocking HMG-CoA reductase activity) and fosmidomycin (inhibiting 1-deoxy-D-xylulose 5-phosphate reducto-isomerase activity). The first study predominantly used a high-through-put GeneChip (AffyMatrix) to fully characterize the effect of these inhibitors on expression [27]. The second team utilized biochemical methods [26], using incorporation of stable isotope-labeled precursors and evaluation of enzyme activity. They demonstrated that endogenous HMG-CoA reductase activity was highly stimulated when plant cells were inhibited with mevinolin. This stimulation of activity seems surprising at first view, but it appeared when mevinolin was carefully washed out from microsomal fractions. Under such conditions, HMG-CoA reductase activity reflects its apparent capacity to produce mevalonate in the absence of inhibitor and reveals a high-level of catalytical activation. This stimulation does not appear to be transcriptionally regulated. Indeed, accumulation of the corresponding gene transcripts is not visible when *Arabidopsis* seedlings were treated with this same inhibitor [27]. The increase in activity can be related to a translational level of regulation, as mevinolin treatment induces protein accumulation as well. This accumulation is prevented in the presence of mevalonate [26]. One point should be kept in mind to explain why such experimental discrepancies can appear. The plant models differed, what has a direct impact on experimental conditions such as time of treatment or inhibitor concentrations. Nothing guaranties that results obtained with one plant species might be of use to design a general model of regulation.

2.2. Regulatory enzymes of the mevalonate pathway

For some time now, significant efforts have been made to identify regulatory enzymes of isoprenoid pathways. Based on current knowledge (*cf.* [14]), HMG-CoA reductase catalyzing the formation of mevalonate (Fig. 1) appears to be "the" multilevel-regulated enzyme (Fig. 2). Fig. 2 was designed on the basis of all available data described in literature and as compared with the situation of other enzymes, HMG-CoA reductase received some special attention for decades and is the best characterized enzyme of this pathway. Future investigations may reassess the number and identity of multilevel-regulated isozymes. Nevertheless, it is now well accepted that HMG-CoA reductase is the most influential and main regulatory enzyme controlling the biosynthesis of mevalonate-derived isoprenoids (*cf.* [14]). As a consequence, results obtained for this key enzyme appear in all sections of this review. In fact, much interesting data has been obtained for HMG-CoA reductase, because all needed tools are available for determining level of enzyme activity regulation. This is not necessarily true for all other enzymes of the pathway. Gene expression studies have been made using quantitative immunostaining, tissue localization, and more importantly with a reliable enzyme assay using crude protein extracts. In contrast, proteomic quantification approaches are less easy to perform, because such bottleneck enzymes are at lower levels than other proteins, and are usually present in trace amounts. A prominent example is HMG-CoA reductase. A number of studies have not found a strict correlation between protein quantities and enzyme activity [28–30], and even less between mRNA levels and enzyme activities [28,31,32]. This rate-limiting enzyme is regulated at all levels of production demonstrating its regulatory power over the accumulation of several end-of-chain isoprenoid products (*cf.* [14]). This includes modulation of catalytical activities through non-covalent interactions with metabolic intermediates, metabolic flow feedback but also covalent modifications (phosphorylation, ubiquitination, glycosylation). Gene expression must be tightly controlled to assure this permanent multilevel control., but the initiation of HMG-CoA reductase mRNA translation needs as well to be controlled [33]. This mRNA was differentially associated with polysomes in *Phytophthora infestans*-infected potato tubers. The translation into protein is also controlled and the enzyme by itself undergoes a battery of activity modulating modifications, which are discussed later.

2.3. Regulatory enzymes of the methylerythritol phosphate pathway

The identity of regulatory enzymes of the plastid methylerythritol phosphate pathway is still under extensive discussion. It was proposed that the methylerythritol phosphate pathway might be regulated at several control points that compensate for fluctuations in precursor/product equilibrium and redistribute the balance of control within the pathway based on unclear conclusions obtained by comparing results with several plant species [19]. At least three enzymes appear as being regulated at different levels: 1-deoxy-D-xylulose 5-phosphate synthase, 1-deoxy-D-xylulose 5-phosphate reductoisomerase and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase [34], to which 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase should be added as it was described as a possible candidate [35]. One problem in identifying regulatory enzymes of the methylerythritol phosphate pathway can be explained by the use of loss-of-function *Arabidospis* mutants. Indeed, these mutants are characterized by chloroplast developmental disorders, defects that alter nuclear expression of genes encoding chloroplast proteins [36]. Nevertheless, transcription does not appear as the main mode of regulation [27,37–39]. A major obstacle in identifying

methylerythritol phosphate pathway regulatory enzymes is the ability to evaluate enzyme activities in plant extracts. This is even more difficult than with mevalonate pathway enzymes. Intense efforts were made to measure enzyme activities applying radiometric or spectrometric assays, but the enriched protein fractions used from specific tissues [40] or from plastid extracts [41–43] are prone to artifacts. A more invasive but elegant technique was recently utilized to measure deoxy-D-xylulose phosphate synthase activity in poplar leaf extracts [44]. This technique detects the release of $^{13}CO_2$ from labeled pyruvate during the enzyme reaction by liquid chromatography coupled to isotope ratio mass spectrometry. Unfortunately, this method has not yet been validated for use with other plant species expressing 1-deoxy-D-xylulose 5-phosphate synthase at lower rates than a plant emitting high levels of isoprene. Evaluation of the methylerythritol phosphate pathway enzymes is further complicated because they utilize unstable substrates, not always commercialized. Additionally, the substrates are quickly metabolized by enzymes involved in other pathways, for the most part in sugar metabolism (*e.g.* pyruvate or glyceraldehyde 3-phosphate for 1-deoxy-D-xylulose 5-phosphate synthase). Furthermore, methylerythritol phosphate pathway enzymes are often in low abundance and activity (as are most enzymes of the mevalonate pathway) and some of the enzymes are sensitive to oxygen and need to be handled under neutral atmosphere [45].

3. Metabolite-enzyme interactions

Modulation of enzyme activities by molecules is the first level of post-translational enzyme regulation. Many of these molecules (*e.g.* stress inducers, phytohormone treatments) act as activators or inhibitors of gene expression (*cf.* [14]). Some compounds function as activity modulators, by interacting directly with the protein, through non-covalent binding, which modifies the enzyme's catalytical properties. These molecules operate either as inhibitors or as ligands in allosteric regulation [46,47], and assist in understanding feedback inhibition and cooperative binding of ligands by proteins. The direct interaction with an enzyme even modulates its structure and substrate accessibility. Such a strategy of regulation may drastically decrease the time required to adapt the metabolic flux to metabolite biosynthesis. Unfortunately again, generally speaking, the basis of this mode of regulation is less considered than gene expression regulation, and is for this reason clearly poorly understood. Currently, efforts are made to progress in this wide field using allosterome analysis [48] as well as chemical genetics [49]. One example relating to such an approach is a yeast three-hybrid based screening platform which allows identification of interaction between proteins and small signaling molecules [50].

3.1. Feedback modulations of enzyme activities by substrate/product accumulation

Enzyme kinetics might be used as a first step for functional prediction of enzymes constituting a metabolic pathway [51]. It should be noted, however, that usually enzymes act in the linear part of the Michaelis–Menten equation, which means that substrate availability regulates its activity. For that reason, modulation of activity by substrate/product accumulation is more efficient than synthesis and degradation of enzymes. Thus, characterization of enzymes and determination of kinetic constants do not only clarify the enzymatic mechanism, but also provide information on substrate or product inhibition or activation. As a whole, such information might have functional significance *in vivo*.

Relevant information about the regulation of plant methylerythritol phosphate pathway enzymes is rare. Mostly mammalians or bacterial mevalonate pathway enzymes have been characterized [52], but there are some data related to plant enzymes as well. The reaction catalyzed by the alfalfa acetoacetyl-CoA thiolase (the first enzyme of the mevalonate pathway) is inhibited by free CoA [53]. The reaction equilibrium of this thiolase is not conducive to the production of acetoacetyl-CoA [54]. The direct consequence is that under cellular conditions, HMG-CoA synthase must be fully active and decrease the acetyl-CoA pool used to construct isoprenoid molecules. Thus, HMG-CoA synthase appears to control the entry of the mevalonate pathway. The *in vitro* activity of a purified recombinant *Brassica juncea* HMG-CoA synthase isoform 1 (HMGS1) was decreased by its substrate acetoacetyl-CoA, as well as by both products HMG-CoA and free CoA [55]. That means that when HMG-CoA is overproduced, for instance when HMG-CoA reductase is inhibited, the upstream enzyme might stop the production of its substrate. Wild-type and mutant versions of this enzyme defective in acetoacetyl-CoA retro-inhibition and/or an increase of 10-fold in activity, were overexpressed in *Arabidopsis* [56]. Outcomes were diverse, but interestingly, HMG-CoA reductase transcription was induced when HMG-CoA synthase was overexpressed. It can be concluded that an increase of HMG-CoA substrate would be sufficient to activate HMG-CoA reductase production and accordingly activity. Conversely, one can assume that when HMG-CoA reductase is inhibited, its substrate HMG-CoA is accumulated. Regrettably, the activity of the enzyme was not quantified in this study, but one could imagine that it would be drastically stimulated. These results support a hypothesis of a possible regulatory function of HMG-CoA synthase in the metabolic pathway. The next step, leading to the synthesis of mevalonic acid by HMG-CoA reductase, is considered to be the rate-limiting step [14]. Again, determining kinetic parameters provided useful information for concluding on the regulatory function of this enzyme. HMG-CoA reductase activity measured in microsomal fractions of *Pisum sativum* was inhibited by HMG and by free CoA, whereas mevalonate had no effect [57]. Purified radish HMG-CoA reductase was inhibited by its substrate NADPH, it product $NADP^+$, but not by mevalonate [58]. HMG-CoA reductase activity responses to fluctuations of the methylerythritol phosphate pathway [26,59]. HMG-CoA reductase activity is stimulated in plants treated with clomazone [59] that was later identified as an inhibitor of deoxyxylulose phosphate synthase [60]. In addition, the methylerythritol phosphate pathway can provide necessary prenyl diphosphate precursors to complement a mevalonate deficiency [26]. Under those conditions, HMG-CoA reductase activity, which is up-regulated in response to the mevalonate deficiency induced by mevinolin inhibition, is retro-controlled when 1-deoxy-D-xylulose, an intermediate of the methylerythritol phosphate pathway, is provided in sufficient amount to overcome the growth deficiency [26]. However, it remains unknown if HMG-CoA reductase is regulated at a transcriptional, post-transciptional, translational or post-translational level. Still, we may conclude that metabolic exchanges between subcellular compartments (plastids *vs* cytosol), is likely to be correlated directly with regulation of the activity at least of some of the enzymes involved in the early steps of plant isoprenoid biosynthesis (*cf.* [14]).

3.2. Modulation of enzyme activities by isoprenoid intermediates

The enzyme activities necessary to synthesis isopentenyl diphosphate can be modulated by some upstream isoprenoid metabolites. Some prenyl diphosphate precursors interact with several of the downstream enzymes and thereby act as regulators of the metabolic pathway. Principally, two enzymes were described as behaving like this: mevalonate kinase, an enzyme participating in mevalonate -derived IPP biosynthesis and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS), an enzyme of the methylerythritol phosphate pathway. In several

organisms including plants, mevalonate kinase is found feedback-regulated by downstream metabolites [61–63]. Principally FPP, but also IPP, DMAPP or GPP and even phytyl diphosphate inhibit mevalonate kinase. It is surprising that phytyl diphosphate inhibited mevalonate kinase because this metabolite is a methylerythritol phosphate -derived precursor used among others as a precursor for chlorophyll side-chain biosynthesis. The physiological significance of this inhibition remains unknown, but may suggest a key regulatory function of mevalonate kinase under some conditions. FPP acts *in vitro* as a competitive inhibitor of ATP for the *Catharanthus roseus* mevalonate kinase [63]. This regulatory function cannot be identified using only a transcriptomic analysis. Techniques, such as standard protein production from purified crude plant extracts had to be used to characterize the enzyme and its behavior *in vitro*. Recent studies on mevalonate kinase characterization in relation to identification of physiological issues are inexistent. The second enzyme that interacts with a prenyl diphosphate is 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase. Allylic diphosphates (GPP, FPP) interact with this enzyme in a non-covalent manner. This has been highlighted in the crystal structure of the *Escherichia coli* enzyme [64]. Through this interaction, for instance by FPP or GPP entering the enzyme cavity, a feedback regulation that of the non-mevalonate pathway has been suggested [63]. But it has to be noticed that those studies were performed using bacterial enzymes and that plants may have evolved different regulatory mechanisms. Resolution of the *Arabidopsis* 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase structure suggests that such an interaction with GPP or FPP, is not likely to occur in plant enzymes [65]. The homotrimeric Arabidopsis enzyme lacks the possibility to bind a diphosphate-derived molecule in its cavity, but is suited for the binding of molecules with a monophosphate moiety [65]. There is increased evidence that such compounds do not interfere with this enzyme activity in bacteria [66]. None of the prenyl diphosphates inhibited the *E. coli* enzyme *in vitro*, but instead methylerythritol phosphate enhanced its activity. However, apparent stabilization effects of GPP and FPP on methylerythritol cyclodiphosphate synthase activity were identified and discussed [66]. Eventually, the resolution of the crystal structure of the upstream *Arabidopsis* methylerythritol phosphate cytidyltransferase (catalyzing the formation of 4-diphosphocytidyl-2*C*-methyl-D-erythritol) provides evidence for feedback regulation associating a cytidine monophosphate [67]. We should recall that cytidine monophosphate is a 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase enzyme product. The mode of regulation of methylerythritol cyclodiphosphate synthase needs to be further enzymatically confirmed.

Besides prenyl diphosphates, other small molecules have been described as interacting with mevalonate pathway enzymes. Farnesol is a key intermediate in retro-controlling mammalian HMG-CoA reductase activity [68]. A fluorescent analog of farnesol, added to tobacco cells, rapidly associated with different subcellular compartments, including ER, where HMG-CoA reductase is localized [69]. The regulatory outcome in plants was in total contradiction with the observations in animal cells: transcription of *HMGR2*, the stress-responsible tobacco HMG-CoA reductase isoform, is greatly induced and results in an increase of specific enzyme activity [70]. One cannot exclude that the house-keeping isoform *HMGR1* would be at the same time, negatively retro-controlled, which would be masked by the effects on *HMGR2*. In plants, at this point farnesol cannot yet be included as a post-translational regulatory intermediate for isoprenoid biosynthesis in plants based on this limited information. Additionally, farnesol added to the enzyme reaction mixture had no effect on activity [70]. The effect at this level of regulation has not been investigated in a greater detail. This is technically hardly trivial, as activity results from a battery of enzymes being simultaneously expressed in same tissues or cells. Therefore in plants, it seems difficult to resolve this scientific issue. Interactions of HMG-CoA reductase with calcium ions were thought to control enzyme activity [71]. This is probably an indirect interaction *via* HMG-CoA reductase-inactivating kinases, which are Ca^{++}-dependent [72]. A more intricate way to regulate the mevalonate pathway was observed in potato (*Solanum tuberosum*). Potatoes are sprayed with *S*-carvone, a monoterpene-derived compound to avoid sprout growth. A post-translational HMG-CoA reductase inhibition has been proposed [73]. This monoterpene may enhance HMG-CoA reductase proteolysis, in a similar manner to farnesol in mammalian cells [68]. It appears that the stress-induced isoform is specifically inhibited by this compound. The molecular details were not unraveled, but it appears now unlikely that HMG-CoA reductase is the only molecular target of *S*-carvone. The underlying biological process remains therefore mysterious.

4. Structural regulation

From the discussion above, it can be concluded that allosteric regulation of enzyme activity is also the result of structural adjustments. Enzyme structure itself can as well be responsible for differential activity as we will discuss below.

4.1. Variable primary enzyme structures

Analyses and comparisons of some plant amino acid sequences with their prokaryotic homologs suggested the presence of protein domains in addition to the basic catalytical entity. The function of some of them is related to subcellular targeting functions. For instance, all seven plant methylerythritol phosphate pathway enzymes carry N-terminal plastid-targeting sequences [74]. Three enzymes of the mevalonate pathway (phosphomevalonate kinase, diphospho-mevalonate decarboxylase and isopentenyl diphosphate isomerase), when fused to a yellow fluorescent protein, are localized in peroxisomes [75]. These proteins contain putative type 2 peroxisomal targeting signals motifs [75]. Modification of the primary structure of an enzyme may play a significant function in the regulative process. In this matter, eukaryotic HMG-CoA reductases, including those from plants, are membrane-associated due to the presence of an N-terminal membrane domain. This membrane association is clearly responsible for limiting the accumulation of end-of chain products [76–78]. Thus, the additional membrane domain in eukaryotes connected to the catalytical entity negatively regulates HMG-CoA reductase activity (reviewed by [14,79]. Another example refers to plant 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase, which have the feature of containing one internal extra protein domain that is absent in prokaryotic enzymes [80,81]. Despite this structural dissimilarity, this protein is fully active and shares the requirement of an iron-sulfur [4Fe-4S] cluster with its bacteria homologs in its active site [82]. The function of this 30 kDa-central structural domain remains unknown, but it has been speculated that this plant-specific peptide might be related to plastid localization, protein stability or enzyme activity [81]. One can imagine a function in thylakoid-association, which might facilitate the electron transfer from the photosynthetic machinery needed for activity [45]. Some differences arise when tertiary or quaternary structures are compared. Thus resolution of the crystal structures permitted to highlight basic particularities of plant enzymes. In this context, we should refer again to the size difference observed within the cavity of methylerythritol cyclodiphosphate synthase [64,65]. But structural data or even models of plant enzymes are scarce at present.

4.2. Organization in multiprotein complexes

A further mode of regulation, widespread in the plant kingdom, is to contain virtually identical parallel pathways. This requires the production of isozymes with specific functions. Such isoforms can either be produced from a single gene or are more likely to be encoded by gene families. Alternatively, differential translation initiation was as well observed. By this way, in *Arabidopsis* two protein products are produced from the HMG-CoA reductase gene (At1G76490): HMGR1S and a 50 amino acids N-terminal extended HMGR1L [83]. Thus, several isozymes operate in tandem in different tissues or even cell compartments. Some enzymes result from alternative splicing and thus carry a different protein sequence that confers an alternative function to the enzyme. Alternative splicing is classified as a post-transcriptional regulatory process as new primary structures are created after protein translation. Alternative splicing offers a real possibility of producing multiple and even new enzymes with special features starting from one single gene. These enzymes attain new properties and even functions. Alternative spliced variants of the messengers coding for HMG-CoA reductase have been isolated after treatments of human cells with specific inhibitor of HMG-CoA reductase belonging to the family of statins [84]. The newly obtained enzyme is characterized by a reduction in sensitivity to statins [84]. Some genes coding for enzymes participating in the early steps of plant isoprenoids are duplicated, sometimes even more than one time. This organization into protein families allows increase of productivity and permits expression in specific cellular tissues, thanks to a different promoter organization. Relevant variations between regulatory sequence motifs were noticed in promoter regions controlling expression of genes involved in early steps isoprenoid pathways [85,86]. Dedicated functions are also attributed to these enzymes. It becomes increasingly clear that plants evolved in two parallel isoprenoid pathways, the first one providing necessary pools for synthesizing primary metabolites (MVA1 and MEP1), the second being responsible for the biosynthesis of secondary metabolites (MVA2 and MEP2) specifically induced under stress conditions (*cf.* [14]).

Proteins are hardly functional as isolated entities. This is particularly true for enzymes that are part of biosynthesis pathways producing natural compounds [87–89]. It was therefore speculated that methylerythritol phosphate – and mevalonate – pathway enzymes interact closely and may be part of so-called metabolons. Bifunctional bacterial enzymes infer the existence of metabolons, substantiating this statement. Indeed, bacteria such as *Agrobacterium tumefaciens*, *Campylobacter jejuni*, *Mesorhuzobium loti*, *Helicobacter pylori* express a 2*C*-methyl-D-erythritol 4-phosphate cytidyltransferase/2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase fused protein [90–93]. These two non-consecutive proteins catalyzed the third and the fifth reaction in the methylerythritol phosphate pathway, respectively. *In vitro*, this bifunctional enzymes associates with 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase (the fourth enzyme), but substrate channeling was not seen [92]. Bacteria synthesizing isoprenoids *via* the mevalonate pathway use a similar strategy. Indeed, a bifunctional acetoacetyl-CoA thiolase-HMG-CoA reductase enzyme was isolated from the bacterial genus *Enterococcus* [94]. HMG-CoA synthase is necessary to fulfill MVA production, which has to interact closely with the bifunctional enzyme.

Plant HMG-CoA reductase is unique in being directly membrane-bound *via* a trans-membrane domain localized at its N-terminus site. It is also unique in that it appears to be the core enzyme initiating the building of the expected metabolon allowing product channeling [88]. The organization of bacterial genomes in biosynthesis gene clusters would be a further argument for metabolic channeling [95]. This type of organization allows transcriptional co-regulation and equal spatio-temporal protein expression. Gene expression clustering is a commonly used as a tool to the predict formation of multi-enzyme complexes in plants [4]. However at present, there is no convincing experimental proof arguing in favor of isoprenoid biosynthesis channeling involving such metabolons.

Plant HMG-CoA reductase interacts with several proteins unrelated to the metabolic pathway [96,97]. When the N-terminal region of *Arabidopsis* HMGR1 was used as a bait to identify interacting proteins, a B″ subunit of protein phosphatase 2A was identified as a positive hit [96]. This protein interacts specifically with the HMGR1 isoform and modulates its activity. For its part, *Medicago truncatula* HMG-CoA reductase 1 binds to a receptor-like kinase thought to be part of the endosymbiosis signaling pathway, highlighting a substantial function of HMG-CoA reductase in nodule organogenesis [97]. The intricacy of organization and regulation of these metabolic pathways appears to be even more complex as was foreseen.

5. Redox regulation

Two methylerythritol phosphate pathway enzymes (the 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase and the reductase) depend on reducing agents. They require quite specific handling under an argon atmosphere when studied *in vitro* [98]. It is initially quite surprising that these oxygen-sensitive enzymes occur in such an unsuitable cellular environment such as the oxygen generating chloroplasts. These enzymes must be tightly regulated and protected from the effects of oxygen generated in this subcellular compartment. In this context, the plant cell responds to light and temperature increases by activating a chloroplastic network of antioxidant reactions involving metabolites such as β-carotene, tocopherols, ascorbate, glutathione and enzymes such as peroxiredoxins, superoxide dismutase, ascorbate peroxidase, *etc.* [99]. The oxygen sensitive property of these enzymes may play a pivotal role in pathway-regulation. Some plants respond to high light stress by accumulating a methylerythritol phosphate-derived metabolite, namely 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate the 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase substrate [35,100,101]. It is quite questionable whether this accumulation relies on an overall physiological regulation process, which can be generalized to other plant species. Recently, a plastid-to-nucleus retrograde signaling function was assigned to this methylerythritol phosphate-derived metabolite [102]. The molecule acts in *Arabidopsis* as a stress sensor and coordinates expression of stress-responsive nuclear-encoded plastidial proteins, such as *hydroperoxide lyase*. The accumulation of this product induces also salicylic acid production [102,103]. Only an activation of the upstream portion of the pathway and a down-regulation of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase catalytical activity can explain the increase of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate production. Light positively control the expression of methylerythritol phosphate pathway genes (*cf.* [14]). The remaining question is how 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase activity is down-regulated? Corynebacteria and Mycobacteria exposed to oxidative stress accumulate high levels of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate [104]. As a consequence, it has been proposed that plant 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase activity becomes limiting under such stress conditions [35]. The molecular mechanism is unclear, but a model based on the properties of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase can be designed. It was proposed that thioredoxin acts as a co-substrate for 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase and repeatedly reduces a disulfide bond needed for catalysis [105]. Then, ferredoxin reduced by the activity of the photosystem I acts as an

electron-donor needed for reduction of the -hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase iron-sulfur cluster. The efficiency of this methylerythritol phosphate pathway enzyme activity depends on an electron donating system that relies, in case of the plant enzyme, on the electron flow from photosynthesis [45]. While it appears that this enzyme is photosynthesis-controlled, under dark conditions it requires ferredoxin $NADP^+$/ferredoxin oxido-reductase and NADPH as an electron shuttle [45]. Do high-light conditions somehow collapse the redox regulation of this enzyme? Future studies will answer this question.

Activities of several enzymes implied in isoprenoid precursor biosynthesis, besides these oxygen-sensitive proteins, are also redox-regulated. Free thiol groups are essential for HMG-CoA reductase activity *in vitro* [57,58], but also for 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity [105]. These redox regulations may imply *in vivo* thioredoxins, as it is the case for the three enzymes already discussed, which were shown to interact with this redox protein [105,106]. For some years now, the interdependence between chloroplasts and mitochondria has become obvious [107,108]. Loss of function of genes encoding methylerythritol phosphate pathway enzymes alters chloroplast function, which itself affects the expression of nuclear and mitochondrial genes [109]. Loss of editing of mitochondria genes as *nad4*, *cc203* and *cox3* in *Arabidopsis*, results in hyposensitivity to treatments with the methylerythritol phosphate pathway specific inhibitor fosmidomycin and the HMG-CoA reductase specific inhibitor mevinolin [110]. A mitochondrial pentatricopeptide repeat protein is responsible for editing these genes at a post-transcriptional level [110]. In addition, when the function of the mitochondrial respiratory chain complex III was repressed, the activity of HMG-CoA reductase was stimulated at the post-translational level. This suggested a respiratory cytochrome pathway-dependent regulation of HMG-CoA reductase activity [110]. However, the direct link and mode of regulation are still unclear.

6. Post-translational modifications

Last but not least, post-translational modifications play an essential function in regulation of metabolic pathways. These modifications reorganize functionality of proteins in terms of action, interaction with other proteins, protein stability or even subcellular localizations.

6.1. Chemical post-translational modifications

Much recent research has focused on the identification of molecules modifying enzymes that are involved in isoprenoid biosynthesis, as well as on the nature of the modified amino acid. The identification of post-translational modifications of metabolic key enzymes is at the moment still a challenging task. Undeniably, such enzymes are expressed at low levels and only a fraction of these low-abundant enzymes might in fact be modified *in vivo*! These technical difficulties have been partly alleviated by more specialized proteomic analyses of specifically modified peptides [111]. Using such approaches and by consulting various databases, several modifications of enzymes of either the mevalonate, the methylerythritol phosphate pathway or prenyl diphophosphate synthases including phosphorylation, glycosylation or ubiquitination could be identified (Table 1). These amino acids modifications include largely phosphoserines, but also a phosphothreonine, a phosphotyrosine, two N-glycosylation on asparagines and a lysine conjugation of a 76 amino-acids ubiquitin polypeptide (Table 1). Most of these modifications were recognized by systemic proteomic analyses, which means that the functions of such chemical modifications are unknown and that a direct link of such protein maturation with overall regulation of the pathways still remains ambiguous. Nevertheless, phosphorylation and ubiquitin chain conjugation are necessary for proteasome-mediated proteolysis in eukaryotes. Post-translational modifications are thought to promote conformational changes that would directly interfere with enzyme activity, or are involved in subcellular localization of the enzyme or alternatively initiate protein degradation [112]. For that reason, these enzymes modifications may primly function as negative regulators of enzyme activities.

Phosphopeptides, related to the early steps of isoprenoid biosynthesis were identified in several plant species, including *Arabidopsis thaliana*, *M. truncatula*, *Chlamydomonas reinhardtii* and *Ocymum basilicum* (Table 1). Phosphopeptides from 1-deoxy-D-xylulose 5-phosphate synthase were several times identified (Table 1) and some enzymes, such as the *C. reinhardtii* deoxyxylulose phosphate synthase DXS or the *A. thaliana* HMG-CoA reductase HMGR2 isoform, are simultaneously modified by several phosphates. This enzyme seems therefore to be phosphorylated *in vivo*, but the functional meaning of this modification remains unclear. The serine identified in *Arabidopsis* 1-deoxy-D-xylulose 5-phosphate synthase DXS1 as being phosphorylated, is not conserved in the DXS2 amino acid sequence (also referred to as CLA1). The phosphorylation of this serine may indicate a special function of DXS2 controlled by some unknown signaling cascade. The situation of HMG-CoA reductase is entirely different. A phosphorylation of the serine 577 (S577) localized in the active site of *Arabidopsis* HMGR1 (At1G76490) negatively regulates HMG-CoA reductase activity *in vitro* [113]. Because this phosphorylated serine has not been identified in any of the public available *Arabidopsis* phosphoproteome databases, it is worth investigating other plant species. Recently, a phosphopeptide mapping with the *M. truncatula* HMGR5 was isolated (GenBank/EMBL accession number: AJ418369) [116]. The gene coding for this isoform has no specificity of expression and is highly transcribed in all analyzed plant organs [97]. Protein extracts, used to isolate the phosphopeptide, were prepared from roots and a phosphorylated serine, equivalent to the *Arabidopsis* S577, was clearly identified ([116] in Table 1). This would imply that the expressed HMGR5 in *M. truncatula* roots is inactivated through phosphorylation, but this requires independent proof. This holds also true for the physiological function of an analogous inactivation of this specific HMG-CoA reductase isoform. The identity of the molecular switches acting as signals to modify the enzyme activity is even more obscure. SNF1 (sucrose non-fermenting 1)-related protein kinase 1 recognizes plant HMG-CoA reductase as a protein substrate *in vitro* [72,113,119]. These kinases are described as global plant metabolism regulators, controlling energy-conserving processes as well as the remobilization of alternative energy sources [119]. Besides HMG-CoA reductase, sucrose phosphate synthase or nitrate reductase are also substrates for this kinase. All enzymes are inactivated once phosphorylated. This specific type of kinase is responsible for the phosphorylation of the serine at position 577 of the *Arabidopsis* HMG-CoA reductase 1 isoform [113,114]. Such a regulation would only make sense if the modification is reversible, for instance by the action of a protein phosphatase [114]. An *Arabidopsis* protein phosphatase 2A was identified as a post-translational negative regulator of HMG-CoA reductase activity and protein levels [96]. It also functions as a positive transcriptional regulator of *HMG1* [96]. The five-member B″ protein family, which is the regulatory subunit of protein phosphatase 2A, is proposed to have a major role in *Arabidopsis* HMG-CoA reductase multilevel-control [96]. Their findings confirmed that reverse-phosphorylation plays a major function in post-translationally controlling the enzyme activity. In fact, a complex kinase cascade mediates inactivation of HMG-CoA reductase *in vivo* [114,123]. In addition, this type of kinases is AMP-activated [113,120,121], but the plant HMG-CoA reductase kinase lacks this

Table 1
Post-translational modifications of enzymes involved in early steps plant isoprenoid pathways.

Pathway	Identity/locus	Protein	Description	Proteomic isolated peptide sequence or other type of analyses	Reference
MVA pathway					
A.thaliana	AT1G76490	HMGR1	3-Hydroxy-3-methylglutaryl-coenzyme A reductase	analysis: S577: YNRpSSRDISG	[113]
A. thaliana	AT1G76490	HMGR1	3-Hydroxy-3-methylglutaryl-coenzyme A reductase	*In vitro* analysis: S577:	[114]
A. thaliana	AT2G17370	HMGR2	3-Hydroxy-3-methylglutaryl-coenzyme A reductase	GFKAIHLpSGGAFpSVLVK	[115]
M. truncatula	AJ418369	HMGR5	3-Hydroxy-3-methylglutaryl-coenzyme A reductase	YNRpSSRDMSK	[116]
S. lycopersicum	–	HMGR2	3-Hydroxy-3-methylglutaryl-coenzyme A reductase	In *vitro* analysis: EKIRgNSTPLH	[78]
MEP pathway					
A. thaliana	AT3G21500	DXS1	1-Deoxy-D-xylulose-5-phosphate synthase	YHRGNGIGVpSLPPGNK	[115]
A. thaliana	AT4G15560	DXS2	1-Deoxy-D-xylulose-5-phosphate synthase (Cla1)	LQpSNPALR	[117]
A. thaliana	AT4G15560	DXS2	1-Deoxy-D-xylulose-5-phosphate synthase (Cla1)	LQpSNPALR	[115]
C. reinhardtii	CAA07554	DXS	1-Deoxy-D-xylulose-5-phosphate synthase	AGPAGSpYpSGEWDKLpSVEEIDEWR	[118]
C. reinhardtii	CAA07554	DXS	1-Deoxy-D-xylulose-5-phosphate synthase	AMpSYTNYFADALTAEAER	[118]
O. basilicum	Basil-Q6RI20_NICBE	HDS	(E)-4-Hydroxy-3-methylbut-2-enyl diphosphate synthase	ELSGGAQubKLLPEGTRLVVSVR	[40]
O. basilicum	Basil12_0159_02_3	HDS	(E)-4-Hydroxy-3-methylbut-2-enyl diphosphate synthase	VFSMKApSNPVIMVQAYR	[40]
A. thaliana	AT5G16440	IDI2	Isopentenyl diphosphate isomerase	pSQLSVR	[115]
Prenyltransferases					
O. basilicum	Basil12_1770_01_3	ssGDS	Geranyl diphosphate synthase small subunit	ATAVAVpSG	[40]
A. thaliana	AT2G18640	GGDS3	Geranylgeranyl diphosphate synthase	VAGKLpTYPK	[115]
A. thaliana	AT2G18640	GGDS3	Geranylgeranyl diphosphate synthase	EFLDpSSIK	[115]
A. thaliana	AT3G14550	GGDS7	Geranylgeranyl diphosphate synthase	MATTVHLpSSFSLFIQSRGR	[115]
A. thaliana	AT3G29430	GGDS9	Geranylgeranyl diphosphate synthase	YNSLSpSFNNLQK	[115]
A. thaliana	AT3G32040	GGDS10	Geranylgeranyl diphosphate synthase	QTRGRKYgNSILSFNN	[127]
A. thaliana	AT3G32040	GGDS10	Geranylgeranyl diphosphate synthase	YNSILSFgNNLQKRTV	[127]
A. thaliana	AT3G32040	GGDS10	Geranylgeranyl diphosphate synthase	YNSILSFNgNLQKRTV	[127]

property [122]. Other serine residues might be involved in differential catalytical control of the isoforms [123] and/or differential subcellular localization [30,124]. Are all HMG-CoA reductase isoforms substrates of these kinases? Is there any spatio-temporal regulated expression of the protein-substrates and their dedicated kinases? Co-expressions of the enzyme substrate and the protein kinase are essential to control catalytical activities *in vivo*. Sucrose phosphate synthase, nitrate reductase, as well as HMG-CoA reductase are recognized by these kinases, and all their activities seem to be stimulated under high sucrose/low glucose conditions [125]. It may therefore be possible that other enzymes of the pathway are also substrates for these kinases, enlarging the substrate specificity of these enzymes.

Phosphorylation is the major protein modification identified in enzymes of the early steps of plant isoprenoid biosynthesis (Table 1). Other modes of regulation probably occur. Tomato HMG-CoA reductase can be N-glycosylated *in vitro* [78]. This specific post-translational modification acts on the structure and folding of proteins [126]. One HMG-CoA reductase isoform has a function in the biosynthesis of dolichol, a polyprenol essential for N-glycosylation, but only the stress-responsive HMG-CoA reductase (HMGR2) is glycosylated. The N-glycosylation site within the ER luminal occurs also in the sequence of *Arabidopsis* HMGR2. PHOSIDA, a post-translational modification database, repertories phosphorylated, N-glycosylated and acetylated sites identified in *Arabidopsis* [127], but unfortunately none of the mevalonate or methylerythritol phosphate pathways enzymes, including HMGR2 are listed. However, short chain prenyl transferase (AT3G32040-GGDS10) contains N-glycosylated sites (Table 1). The function of this modification is unknown. Finally, the human mitochondrial HMG-CoA synthase, which is involved in isoprenoid biosynthesis but in ketone bodies production, is acetylated, a modification causing allosteric changes [128]. Such a modification has not yet been identified in plant enzymes, but one would expect that acetylation will appear as a regulatory mechanism in the isoprenoid pathway as well.

6.2. Proteolysis

Post-translational regulation controls the decrease of enzyme activity in a cell. The enzyme can either be covalently modified, for instance with a phosphate that inactivates HMG-CoA reductase, or by a degradation program. Proteolysis is recognized as a regulatory mechanism [129], whose activity can be controlled by proteolytic induction through specific metabolites. For example, the degradation of mammalian HMG-CoA reductase controlling the biosynthesis of cholesterol has been intensively reviewed [79]. Phosphorylation of mammalian HMG-CoA reductase induces recruitment of the ubiquitination-dependent proteolytic machinery [79]. Still, while there is a clear induction of HMG-CoA reductase degradation in response to cholesterol and farnesol accumulation in mammals and yeast, little is known about enhanced degradation of this enzyme degradation in plants. The linker region between the membrane domain and the catalytical part of plant HMG-CoA reductase is clearly rich in PEST amino acids [78], but the function of such elements in plant enzymes remains imprecise. Nevertheless, plant HMG-CoA reductase activity declines rapidly (within minutes) when etiolated pea seedlings were briefly irradiated with red-light [130]. It could thus be concluded that phytochrome indirectly controls HMG-CoA reductase activity through a

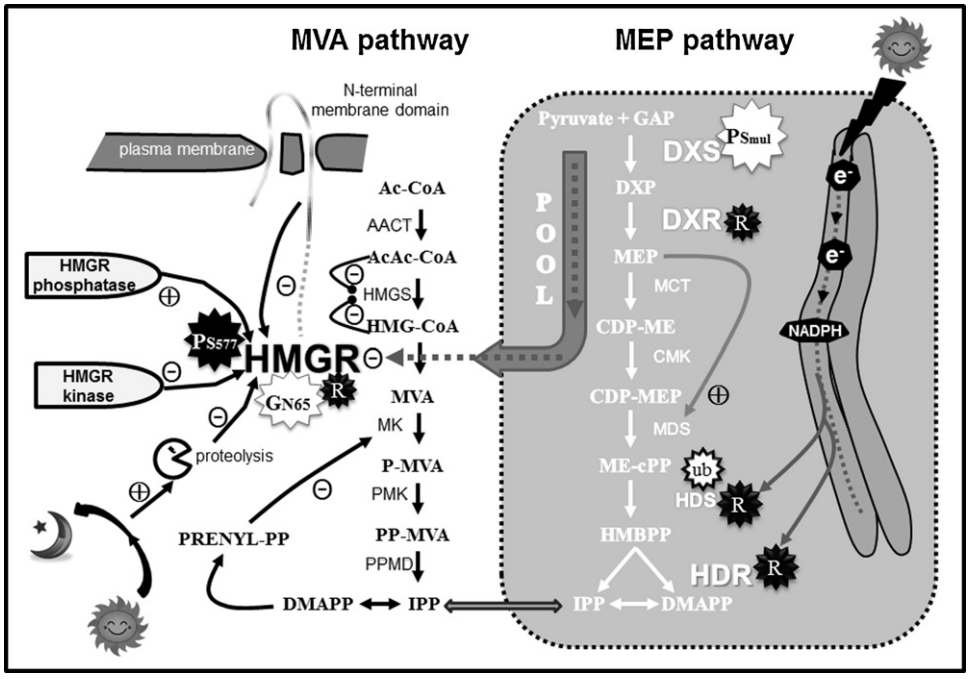

Fig. 3. Post-translational regulation of MVA and MEP pathway-enzymes in plants. Substrates and enzyme-products of the MVA pathway are as follows: Ac-CoA, acetyl-coenzyme A; AcAc-CoA, acetoacetyl-coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MVA, mevalonate; P-MVA, mevalonate 5-phosphate; PP-MVA, mevalonate 5-diphosphate. Enzymes of the MVA pathway are as follows: AACT, AcAc-CoA thiolase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PPMD, diphospho-mevalonate decarboxylase. Substrates and enzyme-products of the MEP pathway are as follows: pyruvate; GAP, D-glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2*C*-methyl-D-erythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-2*C*-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate; ME-cPP, 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate. Enzymes of the MEP pathway are as follows: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT, 2*C*-methyl-D-erythritol 4-phosphate cytidyltransferase; CMK, 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase; MDS, 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; HDR, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase. Modifications proven to affect activity *in vitro* or *in vivo* are indicated in black. PS: phosphorylated serine, the following number correspond to the position of the modified amino acid taking as reference the *Arabidopsis* enzyme, or alternatively "mul" is representative for multiple modifications with unknown functions. GN: N-glycosylation. R: redox regulation. ub: ubiquitination. ⊖ negative feedback-regulation, ⊕ positive feedback-regulation.

post-translational mechanism. Potato HMG-CoA reductase activity is reduced when plants were transferred from light to darkness [31]. While transcript levels were constant, the loss of activity was correlated with protein level diminution. Furthermore, it was not a translational regulation that occurred, as RNA remained constantly bound to polyribosomes. Again, the loss of HMG-CoA reductase activity was post-translationally regulated, presumably through cysteine proteinase-catalyzed degradation [31]. Other molecules, such as sterols, abscisic acid, ubiquinone or 4-hydroxybenzoic acid, inhibited HMG-CoA reductase activity in etiolated tissues, but the mechanism of regulation was not further investigated [131]. Unfortunately, data for enzymes other than HMG-CoA reductase are not available. In yeast and mammalian cells, in response to sterol accumulation, HMG-CoA reductase activity is post-translationally regulated by ubiquitination and proteasome-mediated protein degradation [78]. This mode of regulation has not been detected but is likely to occur in plants as well. An ubiquitinated peptide becoming part of a basil 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase, was identified [40]. It is unusual to find ubiquitinated plastid enzymes as this process is exclusively eukaryotic [132] and that prokaryote-related ATP-dependent proteolytic machinery is responsible for proteolysis in plastids [133].

All methylerythritol phosphate pathway enzymes are encoded by the nuclear genome. For this reason, they are subject to a further regulatory process as they need to be imported into the plastids and undergo subsequent maturation into active stromal entities (*cf.* [134]). Plastid proteolysis is thought to regulate the methylerythritol phosphate pathway. First, polyadenylation-promoted RNA degradation through the action of the chloroplast ribonuclease polynucleotide phosphorylase regulated the methylerythritol phosphate pathway [39]. Second, enzymes of the methylerythritol phosphate pathway are direct substrates of the Clp protease, a stromatal-localized ATP-dependent serine protease [135]. It has been proposed that the supply of isoprenoid pools in the plastid compartment is mediated by the level of methylerythritol phosphate pathway regulatory enzymes, which is adjusted by proteolysis. However, this model explanation is a matter of debate [136]. The reduced formation of the photosynthetic apparatus in *Arabidopsis clp* loss-of-function mutants may explain the connection between plastid translation and methylerythritol phosphate protein levels [137]. With 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase being redox-regulated, a deficiency in photosynthesis capability prevents the methylerythritol phosphate pathway to work suitably.

7. Conclusion and future prospects

Current knowledge regarding post-translational regulation of enzymes involved in the early steps of the plant isoprenoid biosynthesis pathways is depicted in Fig. 3. This knowledge still appears somewhat limited. Plant HMG-CoA reductase remains the leading player in isoprenoid biosynthesis and this both in the plant and the mammalian pathways. All findings are consistent with HMG-CoA reductase activity being controlled by hormonal variations, environmental signals or metabolic needs. Interestingly, with one exception, HMG-CoA reductase activity appears as mostly down-regulated by modulating entities (Fig. 3). This enzyme is not only multilevel-regulated, but also responds to fluctuations and changes within the methylerythritol phosphate pathway. It has to be mentioned that most bibliographic available data refer to HMG-CoA reductase. For that reason, this simple statement considering HMG-CoA reductase as the central control point, may in the future be reconsidered. Data on methylerythritol phosphate pathway enzymes are comparably limited. Post-translational regulations rapidly modify enzyme properties and are often reversible. The plant cell post-translational regulation of HMG-CoA reductase limits production of some isoprenoid products. The physiological function is still unclear: it might ensure energy or metabolic efficiency, but it might also avoid accumulation of toxic metabolites or intermediates.

Scientific approaches for studying regulation of metabolic pathways as a whole, including isoprenoids, have been evolving during the last few of years, which will improve our understanding. In this particular case, the -omics era opened new avenues of exploring regulatory interactions. Major scientific breakthroughs have been achieved using such state-of-the-art tool, there are but best used in combination with some more basic biochemical techniques. Systematic analyses of kinetic properties of purified enzymes from plant extracts and/or recombinant proteins may improve the determination of post-translational regulation of such regulatory enzymes. This task may remain difficult to solve as many enzyme activities are difficult to discriminate from others in plant crude extracts. New plant regulatory events might be identified in next future by means of differences in enzyme characteristics. With the development of methods for the detection of post-translational modified proteins such as gel-free proteomics, enrichment of specific modified peptides or protein microarrays, new information should become available about regulation under *in vivo* conditions.

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