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MINI-REVIEW

Complex interplays between phytosterols and plastid development

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ABSTRACT

Isoprenoids comprise the largest class of natural compounds and are found in all kinds of organisms. In plants, they participate in both primary and specialized metabolism, playing essential roles in nearly all aspects of growth and development. The enormous diversity of this family of compounds is extensively exploited for biotechnological and biomedical applications as biomaterials, biofuels or drugs. Despite their variety of structures, all isoprenoids derive from the common C_5 precursor isopentenyl diphosphate (IPP). Plants synthesize IPP through two different metabolic pathways, the mevalonic acid (MVA) and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathways that operate in the cytosol-RE and plastids, respectively. MEP-derived isoprenoids include important compounds for chloroplast function and as such, knock-out mutant plants affected in different steps of this pathway display important alterations in plastid structure. These alterations often lead to albino phenotypes and lethality at seedling stage. MVA knock-out mutant plants show, on the contrary, lethal phenotypes already exhibited at the gametophyte or embryo developmental stage. However, the recent characterization of conditional knock-down mutant plants of farnesyl diphosphate synthase (FPS), a central enzyme in cytosolic and mitochondrial isoprenoid biosynthesis, revealed an unexpected role of this pathway in chloroplast development and plastidial isoprenoid metabolism in post-embryonic stages. Upon FPS silencing, chloroplast structure is severely altered, together with a strong reduction in the levels of MEP pathway-derived major end products. This phenotype is associated to misregulation of genes involved in stress responses predominantly belonging to JA and Fe homeostasis pathways. Transcriptomic experiments and analysis of recent literature indicate that sterols are the cause of the observed alterations through an as yet undiscovered mechanism.

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Isoprenoids are the most diverse class of natural compounds with more than 55000 members identified.[1] Some isoprenoids act as primary metabolites playing fundamental roles in basic cell processes such as photosynthesis, respiration, signaling and maintenance of membrane architecture. Others are specialized metabolites serving essential functions in important biological processes such as reproduction or defense. The huge variety of this family of compounds is, moreover, extensively exploited in many biotechnological and biomedical applications as biofuels, biomaterials and drugs. Despite this enormous variety of structures and functions, they are all derived from a common C_5 building block, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Plants synthesize IPP through two different pathways, the classical mevalonic acid (MVA) pathway that operates in the cytosol-RE and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway that is localized in the plastids.[2] The sequential condensation of IPP with DMAPP and the resulting allylic diphosphate substrates through the action of prenyltransferases, generate isoprenoid linear chains of increasing length. These can be further cyclized, decorated and/or conjugated to produce the thousands of different isoprenoids compounds found in the plant kingdom.[3]

MEP pathway-derived isoprenoids include essential compounds for the proper function of chloroplast biogenesis and physiology. For example, some plastidial isoprenoids are crucial in the photosynthetic process (chlorophylls and carotenoids), in electron chain transport (plastoquinone and phylloquinone), and have functions as antioxidants (tocopherols and plastochromanol-8). In addition, some important plant hormones (ABA, GAs, and strigolactones) are synthesized through the MEP pathway as well as many specialized metabolites (monoterpenes and diterpenes). Not surprisingly, mutant plants affected in enzymes of the MEP biosynthetic pathway display important alterations in plastid structure and development that lead to albino phenotypes and eventually to lethality at seedling stage.[2,4,5] In addition, MEP-derived metabolites have been proposed as signaling molecules in the retrograde signaling pathway that regulate nuclear gene expression according to the physiological status of the chloroplast. Some examples include the methylerythritol cyclodiphosphate (MEcPP), cleavage product(s) of phytofluene and/or δ-carotene, and

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β-cyclocitral (β-CC), which are involved both in biogenic and operational control networks.[6]

Cytosolic and mitochondrial isoprenoids are synthesized through the MVA pathway and include essential compounds such as sterols (membrane architecture and signaling), ubiquinone and heme groups (electron transfer), dolichols and polyprenols (protein glycosylation), sesquiterpenes (defense) and isoprenyl diphosphates involved in post-translational protein modification (farnesyl diphosphate and geranylgeranyl diphosphate). Cytosolic isoprenoids are also involved in the biosynthesis of important plant hormones such as cytokinins and sterol-derived brassinosteroids. In agreement with such essential functions, knock-out mutant plants affected in the biosynthesis of the precursors of these important compounds have a lethal phenotype often displayed at the embryo or gametophyte development stage.[2] For example, while no major developmental and metabolic defects are observed in farnesyl diphosphate synthase (FPS) *fps1* and *fps2* single knockout mutants, *fps1/fps2* double knock-out mutant embryos show arrested development at the pre-globular stage.[7]

MVA-derived isoprenoids are involved in plastid development

Intriguingly, in addition to the embryo lethal phenotypes, mutant plants carrying weak alleles of MVA pathway genes display specific defects in plastidial physiology and/or structure (Table 1). For example, characterization of *flaky pollen1*-1 (*fkp1*-1), a knock-down mutation in HMG-CoA synthase (HMGS), revealed a requirement of the MVA pathway for the development of tapetum-specific elaioplasts (a type of plastid rich in plastoglobuli containing sterol-esters).[8] Similarly, HMG-CoA reductase (HMGR) mutant plants (*hmg1*-1) display pollen defects associated to abnormal sterol distribution in tapetum cells, as well as dwarf and chlorotic phenotypes attributed to sterol depletion.[9] Moreover, a phosphoproteome characterization of *hmg1*-1 mutant plants revealed 31 proteins in a differential phosphorylation state when compared to wild type plants. Interestingly, these include important proteins for plastid function such as TOC159 (chloroplast protein importer necessary for chloroplast biogenesis), AtOEP7-like (outer chloroplast envelope protein) and PSIID2 (Photosystem II D2 protein, a chlorophyll binding protein important for the homeostasis of photosystem II), as well as different factors associated with light signaling and photomorphogenesis.[10] These data suggest a close link between MVA pathway and chloroplast function. Furthermore, exogenous addition of lovastatin, a specific inhibitor of HMGR, induces further depigmentation and inhibition of plastid development in Arabidopsis *cla1*-1 (affected in the first-step of the MEP pathway) mutant seedlings.[11] Likewise, IPP1/IPP2 double mutant plants (*idi1*- 2/*ippi2*) show a pale green phenotype due to reduced pigmentation when grown under continuous light.[12] More recently, characterization of FPS conditional knock-down mutant plants has revealed a strong effect of cytosolic isoprenoids on plastid development.[13] FPS belongs to the family of prenyltransferases and catalyzes the synthesis of farnesyl diphosphate (FPP) from IPP and DMAPP. FPP is a central metabolite in isoprenoid metabolism as precursor of a wide range of essential compounds including sterols and brassinosteroids, ubiquinones, dolichols and polyprenols, sesquiterpenes and the prenyl moiety of farnesylated proteins and heme groups. Upon FPS silencing, plants show a dramatic reduction in size and display a chlorotic phenotype that correlates with a strong decrease in the levels of chlorophylls, carotenoids and other plastidial isoprenoids, together with important alterations of chloroplast ultrastructure. These alterations include formation of irregular outer membranes, dismantled thylakoid system, increased plastoglobuli number and massive accumulation of starch granules. In addition, transcriptomic analysis of FPS silenced plants show altered expression of genes related to stress pathways, most notably the induction of Jasmonic acid (JA) pathway and misregulation of genes related to Fe deficiency.[13] Collectively, these data show a requirement of MVA-derived compounds for the proper development and physiology of chloroplasts.

A simple mechanism to explain the involvement of cytosolic isoprenoids on plastid development and physiology is the possibility that MVA precursors are directly used on the biosynthesis of one or more plastidial isoprenoid end products. However, although there is a certain exchange of metabolites

Table 1. MVA and sterol-related biosynthetic mutant plants affected in plastid development or physiology. HMGS: HMG-CoA synthase. HMGR: 3-hydroxy-3-methylglutaryl coenzyme A reductase. IPPI: IPP isomerase. FPS: farnesyl diphosphate synthase. SQE1: squalene epoxidase 1. CAS1: cycloartenol synthase 1. CYP51A2: obtusifoliol 14a-demethylase. PSAT: phospholipid sterol acyltransferase.

Gene	AGI	Mutagen	Allele	Phenotype	Reference
HMGS	*At4g11820*	T-DNA	*fkp1*-1	Deffective elaioplast development	Ishiguro et al., 2010
HMGR	*At1g76490*	T-DNA	*hmg1-1*	Chlorosis/altered phosphorylation of chloroplast proteins	Suzuki et al., 2004; Heintz et al., 2012
IPPI1/IPPI2	*At3g02780/ At5g16440*	T-DNA	*idi1- 2/ippi2*	Pale Green, reduced chlorophyll and carotenoid levels under continuous light	Okada et al., 2008
FPS1/FPS2	*At5g47770/ At4g17190*	amiRNA inducible	*amiFPSa/amiFPSb*	Chlorosis/altered chloroplast development/reduced plastidial isoprenoid levels	Manzano et al., 2016
SQE1	*At1G58440*	EMS	*dry2/sqe1-5*	Chlorosis/reduced chlorophyll levels	Posé et al., 2009
CAS1	*At2g07050*	T-DNA	*cas1-1*	Albino stems and flowers/altered chloroplast development/ reduced chlorophyll and carotenoid levels	Babiychuk et al., 2008
		CRE/loxP inducible	*cas1-2*	Albino leaves	
CYP51A2		T-DNA	*cyp51A2-3*	Altered chloroplast development/Transcriptional and translational repression of photosynthesis-related genes	Kim et al., 2010
PSAT1	*At1g04010*	T-DNA	*psat1-1*	Chlorosis	Bouvier-Navé et al., 2010
GAME1	*Solyc07g043490*	RNAi	*GAME1i*	Altered chloroplast development	Itkin et al., 2011

GAME1: tomatidine galactosyltransferase.

derived from MEP and MVA pathway, this seems to be limited to certain compounds, species and/or specific tissues and stages of development. Indeed, lethal mutations in the MEP pathway cannot be rescued by the MVA pathway and vice versa, indicating that this exchange is highly restricted.[4] Therefore, considering the strong compartmentalization of isoprenoid biosynthesis in plants, it is unlikely that the observed defects in plastid development displayed by some MVA pathway mutants are caused by a direct effect of cytosolic precursors into the biosynthesis of plastidial isoprenoids. Rather, the observed phenotypes must be a consequence of the depletion of MVA pathway end products.

Complex interplays between phytosterol homeostasis and chloroplast development

In this regard, several lines of evidence point towards sterol depletion and/or sterol profile misbalance as the cause of the observed plastidial phenotypes. Indeed, changes in the sterol profile of tobacco plants expressing an *Actinomyces* 3-hydroxisteroid oxidase gene results in altered rates of chloroplast photosynthetic whole chain electron transport.[14] Furthermore, the analysis of some biosynthetic mutant plants reveals a requirement of sterols in proper chloroplast development or physiology (Table 1). For example mutations in squalene epoxidase (*dry-2*/*sqe1*-5) lead to a pale green phenotype and a reduction of chlorophyll levels.[15] Similarly, mutations in cycloartenol synthase (*cas1*-1 and conditional *cas1*-2 mutant plants) cause an albino phenotype with important alterations in chloroplast development and a strong reduction in chlorophyll and carotenoid levels.[16] Likewise, mutations in obtusifoliol 14α-demethylase (*cyp51A2*-3) provoke severe alterations in chloroplast development together with the transcriptional and translational repression of photosynthesis-related genes.[17] Moreover, mutations in phospholipid sterol acyltransferase1 (*psat1*-1) affected in sterol esters formation also display a chlorotic phenotype.[18] Interestingly, similar effects on chloroplast ultrastructure have been observed in tomato, where downregulation of the GLYCOALKALOID METABOLISM1 (GAME1) gene involved in the glycosylation of steroidal alkaloids leads to misbalanced sterol levels and major ultrastructural chloroplast alterations, despite no bleaching is observed,[19] and in the microalga *Nannochloropsis oceanica* where the specific inhibition of the sterol biosynthetic pathway cause a strong alteration of chloroplasts structure and depressed photosynthetic efficiency.[20] In agreement with these observations, specific inhibition of sterol biosynthetic pathway (either by genetic or chemical blockage) mimics the transcriptomic responses observed in FPS silenced plants, suggesting that at least at the molecular level, sterols are the primary cause of the observed phenotypes upon FPS suppression.[13] Conversely, mutant plants affected in the biosynthesis of other FPP-derived metabolites different from sterols (such as those affecting dolichol, ubiquinone and brassinosteroids biosynthesis as well as protein farnesylation) are not affected in plastid development nor show chlorotic phenotypes.[21–26]

The molecular mechanism behind the sterol requirement for chloroplast development remains to be established. One possibility is the existence of a direct mechanism involving a structural role of sterols in chloroplast architecture. Although still controversial, several works report the presence of sterols in plastidial outer membrane in different plant species.[27–29] In addition, some reports suggest the possibility of an exchange of sterols across plastidial envelope. For example, the expression of an sterol oxidase in tobacco chloroplasts lead to a metabolization of sterol major end products in the cytosol, reducing the total pool of free sterols within the cell. The authors proposed the existence of two distinct sterol biosynthetic pathways in cytosol and chloroplasts or the existence of a highly regulated exchange of sterols between these two compartments and that a substantial portion of the cellular sterol must at some point be exposed to the interior of the chloroplast.[30]

A second possibility is that the sterols found in plastidial membrane are not structural but rather temporarily allocated by interaction with RE-derived membranes. Indeed, specific contact sites between RE and plastid membranes have been described[31,32] and more recently transorganellar complementation experiments lead to a model where hemifusion of plastid-RE membranes facilitates interorganellar exchange of some non-polar compounds including different metabolites of tocopherol and carotenoid pathways.[33] These contact sites also allow for an exchange of other metabolites, including lipids [34–36] and in fact, lipid metabolism plays a relevant role in plastid biogenesis and thylakoid development.[36,37] Collectively, a plausible explanation is that disturbing sterol biosynthesis or homeostasis may alter RE function ultimately affecting chloroplast-RE contact sites and or lipid exchange between these two compartments. This would ultimately lead to alterations in chloroplast biogenesis and physiology. The fact that only some sterol biosynthetic mutants display specific alterations of plastid development may reflect differences in the homeostasis and/or the ratio of particular sterol intermediates and final end products. Certainly, many sterol knock-out mutant plants display a lethal phenotype but plants carrying weak alleles of the sterol pathway display altered ratios between particular types of sterols and are predominately affected in one specific physiological or developmental process, but not in others.[38] Altogether, these data reflects the importance of MVA pathway in the proper development of plastids, likely through the particular effect of sterol biosynthesis and/or homeostasis. However, the specific mechanisms (and the putative sterol-derived signals) regulating chloroplast development and physiology are still to be identified.

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