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Structure and Dynamics of the Isoprenoid Pathway Network

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ABSTRACT Isoprenoids are functionally and structurally the most diverse group of plant metabolites reported to date. They can function as primary metabolites, participating in essential plant cellular processes, and as secondary metabolites, of which many have substantial commercial, pharmacological, and agricultural value. Isoprenoid end products participate in plants in a wide range of physiological processes acting in them both synergistically, such as chlorophyll and carotenoids during photosynthesis, or antagonistically, such as gibberellic acid and abscisic acid during seed germination. It is therefore expected that fluxes via isoprenoid metabolic network are tightly controlled both temporally and spatially, and that this control occurs at different levels of regulation and in an orchestrated manner over the entire isoprenoid metabolic network. In this review, we summarize our current knowledge of the topology of the plant isoprenoid pathway network and its regulation at the gene expression level following diverse stimuli. We conclude by discussing agronomical and biotechnological applications emerging from the plant isoprenoid metabolism and provide an outlook on future directions in the systems analysis of the plant isoprenoid pathway network.

Key words: isoprenoids; flux; metabolites; network; pathway.

INTRODUCTION

Isoprenoids represent functionally and structurally the most diverse group of metabolites, with at least 50 000 different molecules that have been identified in extant organisms (Thulasiram et al., 2007). Most of the isoprenoids are found in the plant kingdom. As primary metabolites, they have functions in photosynthesis, respiration, membrane fluidity, and regulation of growth and development. As secondary metabolites, they participate in allelopathic and plant–pathogen interactions. Many plant isoprenoids also have substantial commercial, pharmacological, and agricultural value as essential oils, drugs, polymers in rubber, flavors, colorants, and agrochemicals. Understanding the biochemical and molecular regulation of the isoprenoid pathway is therefore of considerable interest to both scientists and industry.

A direct measure of metabolic pathway activity is metabolic flux, which is defined as the flow of molecules through a metabolic network (Ratcliffe and Shachar-Hill, 2006). At first, regulation of isoprenoid pathway flux in plants was expected to be simple. A single factor, such as an environmental or developmental cue, was thought to activate a dedicated branch of the isoprenoid pathway network via one or a few rate-limiting enzymes. Consequently, much focus was placed on the analysis of rate-limiting enzymes and their effect on flux through specific branches of the pathway network. With accumulating knowledge, however, our view on isoprenoid pathway flux regulation has changed. Today, we know that most, if not all, enzymes in the pathway network are subject to regulation. Moreover, environmental and developmental cues not only regulate single pathway network branches, but, depending on the cue, may regulate the entire isoprenoid pathway network. In addition, pathways of primary metabolism that feed into the isoprenoid pathway network are often regulated in concert with isoprenoid synthesis. To fully understand the regulation of isoprenoid synthesis, it is therefore important to understand the complexity of the isoprenoid metabolic network together with its associated pathway networks. Once the network structure is defined, dynamic characteristics of the network can be studied at the level of enzymes and gene expression. Since the 1930s, when Ruzicka proposed the biogenetic isoprene rule that defined isoprenoids as compounds derived from the isoprenoid precursor (Croteau et al., 2000), significant efforts have been made to characterize reaction steps in the isoprenoid pathway network. The development

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of molecular biology techniques and more recently functional genomic tools facilitated this effort and has now considerably expanded our view on enzymes and metabolites in the isoprenoid pathway network.

STRUCTURE OF THE ISOPRENOID PATHWAY NETWORK

C5 Isoprenoids Are Synthesized by Cytosolic and Plastid Pathways

All isoprenoids are derived from the common precursor isopentenyl diphosphate (IPP), which, in plants, is synthesized via two different pathways (Figure 1). The cytoplasmic mevalonate (MVA) pathway begins with the condensation of three units of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and continues by reduction to mevalonate (MVA), followed by two successive phosphorylation steps at C-5 of mevalonate, and a decarboxylation/elimination step leading to IPP (Croteau et al., 2000). IPP derived from this pathway is used for the synthesis of cytosolic and mitochondrial isoprenoids such as sesquiterpenes, sterols, and the side chain of ubiquinone. Plastid isoprenoids are derived from IPP that is synthesized by the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The initial reaction of the MEP pathway is catalyzed by 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS) and involves the condensation of (hydroxyethyl) thiamin derived from pyruvate with the C1 aldehyde group of D-glyceraldehyde 3-phosphate (GA-3P) to produce DXP. In the second step, an intramolecular rearrangement and reduction of DXP by the enzyme DXP reductoisomerase (DXR) yields 2-C-methyl-D-erythritol 4-phosphate (MEP). After conversion of MEP into 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-2,4cPP) in three enzymatic steps, a reduction catalyzed by 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBPP) synthase (HDS) produces HMBPP, which is finally converted into a mixture of IPP and dimethylallyl diphosphate (DMAPP) by the enzyme HMBPP reductase (HDR) (Rodríguez-Concepción, 2006). DMAPP serves as the reactive starter molecule for subsequent condensation reactions with IPP. The equilibrium of IPP and DMAPP is controlled by IPP isomerase (IPPI), which reversibly converts IPP to DMAPP (Nakamura et al., 2001). Synthesis of IPP and DMAPP can be conceptually viewed as the first module of the isoprenoid pathway network. Homologs of all known enzymes for cytoplasmic and plastid IPP synthesis are found in all analyzed plant species such as *Arabidopsis thaliana*, soybean (*Glycine max*), tomato (*Lycopersicon esculentum*), rice (*Oryza sativa*), and maize (*Zea mays*) (Lange et al., 2000), suggesting that both MVA and MEP pathways are highly conserved in plants.

Synthesis of Longer-Chain Isoprenoids

The second order of complexity in isoprenoid synthesis involves condensation of IPP subunits (C5) and generation of linear polymers of defined chain lengths (e.g. C10, C15, C20, . . . Cn). These molecules serve as direct precursors for the synthesis of different classes of isoprenoid compounds (Figure 1). In plants, synthesis of C10 (Burke et al., 1999; Burke and Croteau, 2002; Tholl et al., 2004; Wang and Dixon, 2009), C15 (Cunillera et al., 1996, 1997; Hemmerlin et al., 2003b), C20 (Hefner et al., 1998; Okada et al., 2000; Sitthithaworn et al., 2001), C25 (Hsieh et al., 2011), C30 (Nakashima et al., 1995; Hanley et al., 1996), C35 (Hsieh et al., 2011), C40 (Bartley et al., 1992), C45 (Hirooka et al., 2003, 2005), and polyprenyls in the range of C50–C130 has been reported (Stone et al., 1967; Wellburn et al., 1967; Ibata et al., 1983; Cunillera et al., 2000; Oh et al., 2000; Sakaihara et al., 2000).

DMAPP (C5) is a precursor for the synthesis of cytokinins, isoprene, and other hemiterpenoids. Geranyl diphosphate (GPP; C10) serves as a precursor for monoterpenoids and farnesyl diphosphate (FPP; C15) for sesquiterpenoids. Geranylgeranyl diphosphate (GGPP; C20) is a precursor of diterpenoids, such as gibberellic acid, and of side chains of chlorophyll, phylloquinone, and tocopherols. Squalene (C30) serves as a precursor for sterols, brassinosteroids, and other triterpenoids. Phytoene (C40) is a precursor of abscisic acid (ABA), carotenoids, and other apocarotenoids and solanesyl diphosphate (SPP; C45) is a precursor of side chains of plasto- and ubiquinone. Metabolites synthesized from the other oligoprenyl diphosphates (OPP; C25–C45) and from the polyprenyl diphosphates (PPP; C50) have not been widely studied in plants. A few functionally characterized examples derived from the polyprenyl diphosphates are represented by dolichol phosphate, which has an essential role in protein glycosylation (Cunillera et al., 2000; Zhang et al., 2008) and rubber polymer from *Hevea brasiliensis* (Asawatreratanakul et al., 2003).

Short-chain prenyl diphosphates GPP, FPP, GGPP, medium-chain prenyl diphosphates OPP, and long-chain prenyl diphosphate SPP are synthesized by *trans*- or (*E*)-prenyl diphosphate synthases also called prenyltransferases, which add IPP subunits to the reactive starter molecule by sequential *trans*-condensation (Ogura and Koyama, 1998). Polyprenyl diphosphates (C50) in plants are synthesized by *cis*- or (*Z*)-prenyl diphosphate synthases, which add IPP to the reactive starter molecule by sequential *cis*-condensation (Ogura and Koyama, 1998). In tomato, short chain *cis*- or (*Z*)-prenyl diphosphate synthases were also identified. Prenyl diphosphates in the *cis*-configuration, namely neryl diphosphate (NPP; Schilmiller et al., 2009) and *Z,Z*-FPP (Sallaud et al., 2009), were synthesized by these prenyl diphosphate synthases and represent substrates of terpene synthases in this species.

A substrate for the short-chain prenyl diphosphate synthases is any prenyl allylic diphosphate that is shorter by at least one C5 unit compared to the product (Hefner et al., 1998; Dewick, 2002; Hemmerlin et al., 2003b; Figure 1). For example, GGPP synthase (GGPS) can use DMAPP, GPP, or FPP as reactive starter molecules (Hefner et al., 1998; Wang and Dixon, 2009). A substrate for the medium-chain prenyl diphosphate synthase is GPP, FPP, and GGPP but not DMAPP (Hsieh et al., 2011). The precursor for the synthesis of long-chain prenyl diphosphates is either FPP or GGPP but not DMAPP and GPP (Oh et al., 2000; Hirooka

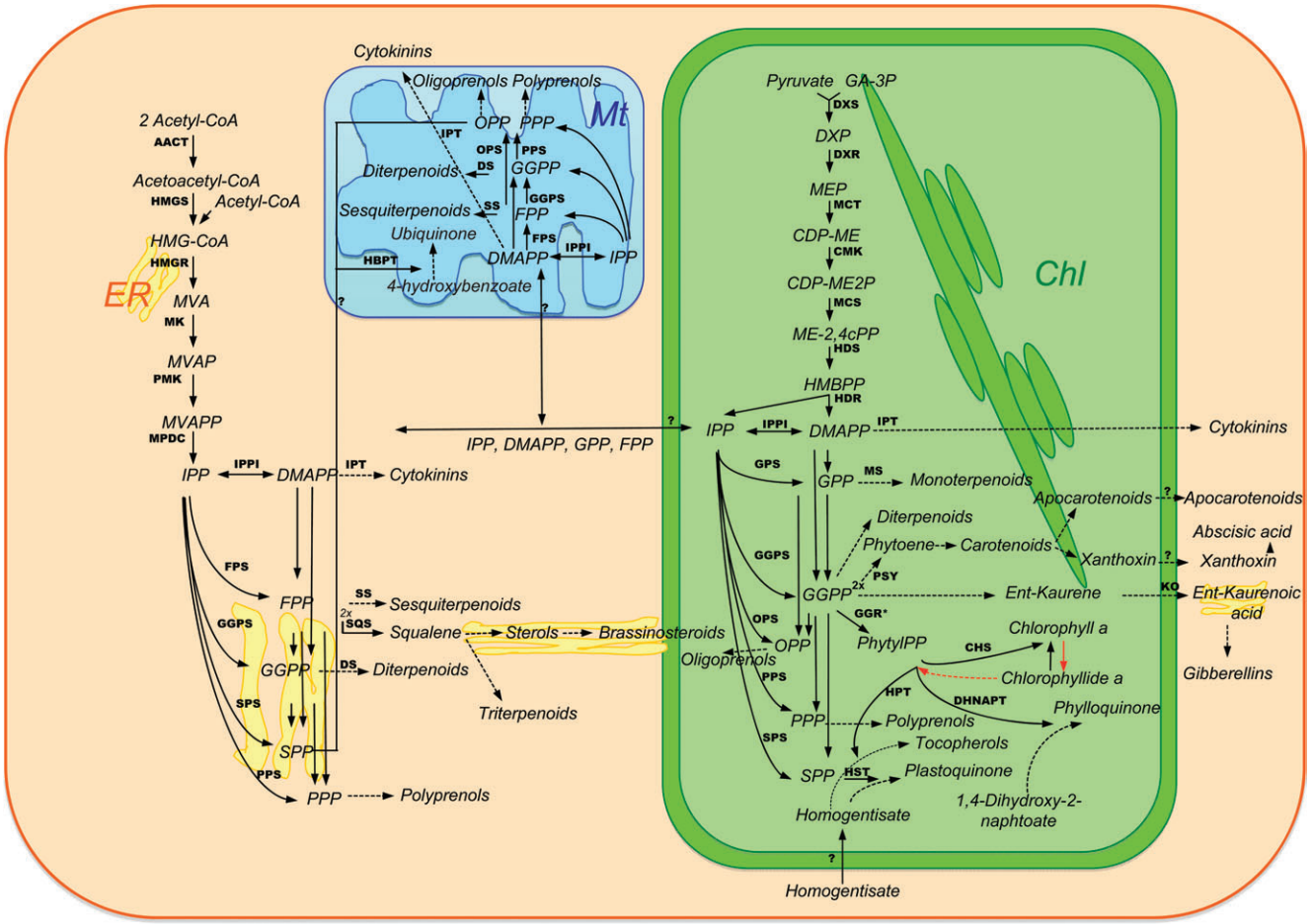

Figure 1. Cellular Topology of the Isoprenoid Metabolic Network in Plant Cells as Deduced from the Compartmentalization of *A. thaliana* Enzymes.

Mt, mitochondria; Chl, chloroplasts; ER, endoplasmic reticulum; AACT, acetoacetyl-CoA thiolase; HMGS, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MVA, mevalonate; MK, mevalonate kinase; MVAP, Mevalonate-5P; PMK, phosphomevalonate kinase; MVAPP, mevalonate-5PP; MPDC, diphosphomevalonate decarboxylase; GA-3P, D-glyceraldehyde 3-phosphate; DXS, 1-deoxy-D-xylulose-5-phosphate (DXP) synthase; DXR, DXP reductoisomerase; MEP, 2-C-methyl-D-erythritol 4-phosphate; MCT, MEP cytidyltransferase; CDP-ME, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol; CMK, CDP-ME kinase; CDP-ME2P, 2-Phospho-4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol; MCS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-2,4cPP) synthase; HDS, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBPP) synthase; HDR, HMBPP reductase; IPPI, isopentenyl diphosphate (IPP,C5) Delta-isomerase; DMAPP (C5), dimethylallyl diphosphate; GPS, geranyl diphosphate (GPP,C10) synthase; FPS, farnesyl diphosphate (FPP,C15) synthase; GGPS, geranylgeranyl diphosphate (GGPP,C20) synthase; OPS, oligoprenyl diphosphate (HOPP, C25-C45) synthase; SPS, solanesyl diphosphate (SPP,C45) synthase; PPS, polyprenyl diphosphate (PPP,C50) synthase; SQS, squalene synthase; PSY, phytoene synthase; IPT, isopentenyl transferase; SS, sesquiterpenoid synthase; MS, monoterpenoid synthase; DS, diterpenoid synthase; HPT, homogentisate phytyl transferase; HST, homogentisate solanesyl transferase; DHNAPT, 1,4-dihydroxy-2-naphtoate phytyl transferase; HBPT, 4-hydroxybenzoate polyprenyltransferase; GGR, geranylgeranyl reductase; CHS, chlorophyll synthase. Compartmentalization of *A. thaliana* enzymes is based on the pathway network constructed by Vranová et al. (2011). Solid lines indicate a single enzymatic step, and dashed lines indicate several enzymatic steps. The salvage pathway for phytol metabolism is indicated by red arrows. (*) GGPP can be reduced to PhytylPP as a free molecule or as attached to the chlorophyll (Keller et al., 1998). For clarity, the attachment of PhytylIPP to chlorophyll is not shown. Most transporters of isoprenoid metabolites are unknown; for e*nt*-kaurene, it was proposed that export from the plastid is linked to its subsequent oxidation by *ent*-kaurene oxidase (KO), which is located on the outer plastid envelope membrane (Helliwell et al., 2001).

et al., 2003; Chen et al., 2005). All the above-described condensation reactions result from head-to-tail condensation of the isoprene (C5) subunit. Squalene (C30) and phytoene (C40) are synthesized by head-to-head joining of two C15 or C20 chains, respectively (Croteau et al., 2000). Condensation of IPP subunits to form linear polymers can be conceptually viewed as the second module of the isoprenoid pathway network.

Cyclization of Linear Isoprenoids Results in Biologically Active Molecules

In subsequent pathway network branches, linear isoprenoid polymers of different lengths undergo a range of cyclization reactions to produce parent skeletons that are then transformed by redox, isomerization, substitution, and conjugation reactions into various isoprenoid end products.

Alternatively, prenyl chains are condensed with chemical moieties synthesized by other pathways to form products of mixed origin. The network structures of these isoprenoid pathway branches are relatively well defined for essential isoprenoid compounds such as sterols, brassinosteroids, cytokinins, quinones, chlorophyll, tocopherols, carotenoids, ABA, and gibberellins (Figure 1) and are further expanding towards other compounds. For example, squalene (C30) serves as a precursor not only for sterols and brassinosteroids, but also for other triterpenoids. More than 100 different triterpene skeletons have been reported from nature (Xu et al., 2004) that are produced by cyclization of oxidosqualene catalyzed by the enzymes oxidosqualene cyclase (OSC) or triterpene synthase. More than 40 OSCs with distinct product specificity have been cloned from higher plants (Phillips et al., 2006). Functional analysis of these OSCs revealed the presence of multiproduct OSCs in addition to single-product OSCs and both of them contribute to the structural diversity of triterpenes found in higher plants. Recently, lanosterol synthase genes were identified among OSC genes from dicotyledonous plant species including *Arabidopsis*, suggesting that, similarly to yeast and mammals, higher plants can also synthesize phytosterols via lanosterol (Ohyama et al., 2009).

The most recent version of a curated isoprenoid pathway gene network for the model plant *Arabidopsis thaliana* is provided by the Arabidopsis thaliana Isoprenoid Pathway Database (AtIPD, www.atipd.ethz.ch; Vranová et al., 2011). For other plant species, information can be extracted from either MetaCyc (http://metacyc.org/) or Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/pathway.html). The pathway network structure is also fully, or at least partially, known for isoprenoid pathway branches that synthesize species-specific secondary metabolites such as artemisinin (Liu et al., 2011), taxol (Zhong and Yue, 2005), some triterpenoid saponins (Lambert et al., 2011), and different sesqui- and monoterpenoids (Yu and Utsumi, 2009; Nagegowda, 2010).

Considering the large number of identified isoprenoid compounds, however, our knowledge of the entire isoprenoid metabolic network structure is still incomplete (Thulasiram et al., 2007). Concerted efforts in genome sequencing, phylogenetics, metabolomics, and mutant analysis will help to identify additional enzymes and intermediates or entirely new branches in the isoprenoid pathway network. An example of the successful application of these approaches was the discovery of volatile terpenoids in *Arabidopsis*. Although *Arabidopsis* was not known to produce mono- and sesquiterpenoid secondary metabolites, its genome analysis revealed 30 mono- and sesquiterpenoid synthase-like genes (Aubourg et al., 2002; Lange and Ghassemian, 2003). This discovery facilitated research on secondary isoprenoid metabolism and added mono- and sesquiterpenoid synthesis branches to the isoprene pathway network (Aharoni et al., 2003; Chen et al., 2003, 2011). Another example is the use of functional genomics data to identify candidate genes involved in the elaboration, hydroxylation, and glycosylation of the triterpene skeleton in the model legume *Medicago truncatula*. Comprehensive gene expression clustering predicted multiple genes involved in triterpene saponin biosynthesis and functional analysis in *E. coli* and in loss-of-function *M. truncatula* confirmed the *in vivo* function of uridine diphosphate glycosyltransferases in saponin biosynthesis (Naoumkina et al., 2010).

Compartmentalization of Enzymes and Metabolites

An important additional element that determines pathway network topology is the subcellular localization of pathway branches. Separation of individual enzymatic reactions by cellular membranes imposes constraints on the isoprenoid metabolic network by disrupting network connections that otherwise would be possible based on reaction mechanisms. If certain isoprenoid metabolites are transported across the membranes, new enzymes such as transporters would therefore have to be added to the network. The currently known subcellular topology of the *Arabidopsis* isoprenoid pathway network is shown in Figure 1 and in detail in AtIPD (www.atipd.ethz.ch). Based on both *in vivo* and *in silico* data, isoprenoid pathway network branches are compartmentalized to the cytosol, endoplasmic reticulum, plastids, and mitochondria (Figure 1). However, some pathway enzymes were either shown *in vivo* or predicted *in vitro* to reside in other locations such as the peroxisome, vacuole, plasma membrane, extracellular space, or nucleus (www.atipd.ethz.ch).

Since the discovery of the MEP pathway, IPP produced in plastids is considered the precursor for isoprenoid biosynthesis initiated in plastids, while IPP produced in the cytosol is used for isoprenoid biosynthesis initiated in the cytosol or mitochondria. This was largely confirmed by several labeling studies, in which labeled substrates specific for either the MVA or MEP pathway were fed to plant cells and end products were analyzed for the incorporation of the label. Isoprenoid compounds synthesized in the cytosol and mitochondria, such as sterols, brassinosteroids, and ubiquinone, predominantly incorporated labeled substrate derived from the MVA pathway, while compounds synthesized in the plastids, such as chlorophylls, carotenoids, and plastoquinone, incorporated substrate derived predominantly from the MEP pathway (Lichtenthaler, 1999). In agreement, plants overexpressing enzymes of the MVA pathway had increased levels of metabolites synthesized in the cytosol, but not in plastids and vice versa, plants overexpressing the MEP pathway enzymes had an increased level of plastidial isoprenoids while the level of cytosolic metabolites was unaffected (Chappell et al., 1995; Enfissi et al., 2005). The autonomy of the two pathways was also demonstrated using genetic approaches. Mutations in genes for enzymes of the MEP pathway produce pale seedlings that generally arrest at the seedling stage (reviewed in Phillips et al., 2008). Mutations in genes for enzymes of the MVA pathway result in male gametophyte lethality (Suzuki et al., 2009; Ishiguro et al., 2010) and later in the pathway (FPP synthesis) in embryo lethality (Closa et al., 2010).

Labeling experiments indicated, however, that there must also be a cross-flow of metabolites between the MVA and

MEP branches in the isoprenoid pathway network. For example, feeding experiments using labeled DXP or 1-deoxy-D-xylulose have shown that intermediates of the MEP pathway can be directed into the biosynthesis of sterols to a certain extent (Arigoni et al., 1997; Hemmerlin et al., 2003a) and can largely contribute to the protein prenylation in the cytosol (Gerber et al., 2009). Mevalonate-derived intermediates can also contribute to the formation of plastidic isoprenoids (Kasahara et al., 2002; Hemmerlin et al., 2003a). Inhibitors and mutants defective in particular steps in the MEP or MVA pathway were used to assess the extent of cross-flow between these two pathways. For example, feeding 1-deoxy-D-xylulose to tobacco BY-2 cells could partially rescue the inhibition of the MVA pathway by the inhibitor mevinolin (Hemmerlin et al., 2003a). Exogenous MVA could partially rescue the phenotype of the *Arabidopsis cla1-1* mutant that is defective in the first enzyme of the MEP pathway (Nagata et al., 2002). Sterol synthesis could be rescued, likely via flow from the MEP pathway, after inhibiting MVA pathway with lovastatin in *Arabidopsis* seedlings (Laule et al., 2003). The contribution of the MEP or MVA branches to isoprenoid synthesis in the network can also differ between plant species and/or organs. For example, the MEP pathway provides IPP for both plastid monoterpene and cytosolic sesquiterpene biosynthesis in the epidermis of snapdragon petals (Dudareva et al., 2005). In carrot leaves and roots, however, monoterpenes are synthesized exclusively via the MEP pathway, whereas sesquiterpenes are derived from both MEP and MVA pathways (Hampel et al., 2005).

Molecular genetics approaches have provided evidence that exchange of metabolites between cellular compartments is a highly regulated process. To identify signals involved in the process, *Arabidopsis* mutants resistant to mevinolin (MEV), which specifically blocks the MVA pathway, were isolated that could grow in the presence of the MEP pathway inhibitors fosmidomycin (FSM) or chlamazone (CHMZ). This screen revealed the *rim1* mutant, which is a new *phyB* allele (Rodríguez-Concepción et al., 2004), and the *loi1* mutant, which is a novel pentatricopeptide repeat (PPR) protein involved in RNA editing of mitochondrial genes related to the respiratory chain (Kobayashi et al., 2007; Tang et al., 2010; Verbitskiy et al., 2010).

IPP and short prenyl diphosphates are likely the metabolites that connect the MVA and MEP branches of the isoprenoid pathway network. The following evidence supports this notion. First, IPP and short prenyl diphosphates are substrates of enzymes in isoprenoid network branches connected to both the MVA and the MEP branches. Second, IPP (C5), DMAPP (C5), GPP (C10), and FPP (C15) can be translocated through the plastid membrane that separates the MVA and MEP branches (Bick and Lange, 2003; Flügge and Gao, 2005). Higher prenyl diphosphates, such as GGPP (C20), are not transported with appreciable efficiency through the plastid membrane and likely do not participate in metabolic cross-flow between connected pathways downstream of the MVA and MEP branches (Bick and Lange, 2003).

Linear isoprenoid polymers are at nodes of the isoprenoid pathway network to which many branches synthesizing isoprenoid end products are connected. These connected branches of the pathway network are often highly specialized and therefore no exchange of metabolites between specialized pathway branches is expected. Thus, the concept of prenyl recycling is widely accepted in isoprenoid research (Dumdei et al., 1989; Thai et al., 1999), but increasing knowledge about the isoprenoid pathway network may reveal new connections. One such example is phytyl diphosphate biosynthesis (Figure 1), which is used for the synthesis of chlorophyll, tocopherols, and phylloquinone. For a long time, it was thought to be generated from GGPP by geranylgeranyl reductase (GGR) until a novel salvage pathway for phytol metabolism was identified in *Arabidopsis*. Phytol is released during chlorophyll degradation and by two subsequent phosphorylation steps converted to phytyl diphosphate (Ischebeck et al., 2006; Valentin et al., 2006). This way, the chlorophyll branch of the isoprenoid pathway network is linked to tocopherol and phylloquinone biosynthesis. Indeed, a mutant of phytol kinase, an enzyme in the salvage pathway, has only 20% of the wild-type tocopherol levels in the seeds (Valentin et al., 2006).

DYNAMICS OF THE ISOPRENOID PATHWAY NETWORK

Regulation of Isoprenoid Pathway Metabolite Flux

Pathway structure and topology set constraints for metabolite flux in the isoprenoid pathway network. There are, however, other factors that determine dynamics of isoprene carbon flow. One of them is co-localization of pathway enzymes. Metabolic enzymes that catalyze a series of sequential reactions can form complexes on membranes or cytoskeletal structures within the same cellular compartment (Newman and Chappell, 1999). Such metabolic enzyme complexes are called 'metabolons', which are thought to function as metabolic channels that facilitate metabolite flux to dedicated end products. Metabolons can 'move' metabolites more efficiently through the pathway and restrict availability of potential common metabolite intermediates to other branches of the network.

HMGR and several other enzymes of the sterol and brassinosteroid biosynthetic pathways localize to the ER or ER-derived vesicles, or they are predicted to be integral membrane proteins (Kribii et al., 1997; Klahre et al., 1998; Lange and Ghassemian, 2003; Leivar et al., 2005; Busquets et al., 2008; Vranová et al., 2011). Thus, synthesis of sterols and/or brassinosteroids may represent defined metabolons within the cytosol-localized isoprenoid pathway.

Coordinating metabolic pathways may also involve dynamic shifts between the assembly and disassembly of metabolons or their interactions with proteins that modulate their output function (Møller, 2010). Such transient metabolons assembling in response to specific metabolic demands or abiotic and biotic challenges has so far not been identified in plant isoprenoid metabolism though.

In addition to metabolons, the temporal and spatial regulation of enzyme activities and the expression of their genes

represent another level of isoprenoid pathway regulation. They are likely primary regulatory mechanisms to ensure dynamic flux distribution of isoprenoid metabolites. Several pieces of evidence suggest that flux control does not converge on a single rate-limiting enzyme, as previously assumed, but involves several pathway enzymes. For example, DXS and DXR, the first enzymes in the MEP pathway, were considered to be the rate-limiting enzymes and the only ones regulating synthesis of plastid isoprenoid compounds (Botella-Pavía et al., 2004). However, overexpression of HDR, the last enzyme in the MEP pathway, also increases production of carotenoids (Carretero-Paulet et al., 2006). Similarly, in the cytosol, increased sesquiterpenoid biosynthesis is associated with the increased activities of both HMGR and sesquiterpene cyclase (Chappell and Vögeli, 1991). Increased sterol production in response to wounding is associated with increased squalene synthase activity (Zook and Kuć, 1991) and increased expression of HMGR gene (Choi at al., 1992). Data from genome-wide gene expression profiling are becoming a rich source of information on gene and metabolic networks. Modeling approaches applied to genome-wide expression data demonstrated that transcriptional control is distributed over the entire isoprenoid pathway network, including associated metabolic pathways (Lois et al., 2000; Botella-Pavía et al., 2004; Wille et al., 2004; Strack and Fester, 2006; Ghassemian et al., 2006; Meier et al., 2011). This suggests the existence of transcriptional regulon(s) that control metabolite flux through the pathway as well as substrate influx into the pathway.

Metabolic gene clusters within dynamic chromosomal regions could provide another mechanism of transcriptional co-regulation. An operon-like gene cluster that is required for the synthesis of the triterpene avenacin has been discovered in diploid oat (*Avena strigosa*) (Qi et al., 2004, 2006; Mugford et al., 2009; Chu et al., 2011). Two gene clusters for the synthesis of different diterpenes (momilactones and phytocassanes) have been characterized in rice (*Oryza sativa*) (Wilderman et al., 2004; Shimura et al., 2007; Swaminathan et al., 2009; Wang et al., 2011) and two gene clusters for the synthesis of different triterpenes (thalianol and marneral) were identified in *Arabidopsis* (Field and Osbourn, 2008; Field et al., 2011).

Environmental and Developmental Factors Affect Isoprenoid Pathway Flux

Considering the synergistic or antagonistic functions of isoprenoid end products, it is likely that the isoprenoid metabolic pathway network is controlled by environmental and developmental cues in a complex way. The dynamics of the isoprenoid pathway network have not been comprehensively studied in plants, mainly because suitable methods are missing for detailed analysis of enzyme activities and isoprenoid compounds.

Light has the most profound effect on the MEP isoprenoid pathway. This is not unexpected considering that metabolites derived from the MEP pathway are part of the pigment–protein complexes in the chloroplasts (chlorophylls, carotenoids, and their oxygenated xanthophylls). Moreover, side chains of phylloquinone and plastoquinone, which function as cofactors in photosystems I and II, respectively, as well as the side chain of tocopherols, which function as lipophilic antioxidants, are derived from the MEP pathway. Dark-grown *Arabidopsis* seedlings irradiated continuously with low-fluency red light initiate photomorphogenesis and etioplasts develop into chloroplasts. This is associated with the accumulation of chlorophyll a and b, xanthophylls, β-carotene, phylloquinone, and α-tocopherol. In this case, enhanced accumulation of metabolites generally correlated with increased expression of the respective pathway genes (Ghassemian et al., 2006). Interestingly, dark-grown *Arabidopsis* seedlings exposed to far-red light, which does not induce chloroplast greening, do not synthesize chlorophylls a and b, β-carotene, and xanthophylls, although they accumulate lutein and α-tocopherol. This difference was not reflected at the level of transcript abundance, which was similar in both red and far-red light-treated seedlings (Ghassemian et al., 2006). This suggests that MEP pathway enzymes are regulated during photomorphogenesis at both transcriptional and post-transcriptional levels. Certain MEP pathway enzymes interact with thioredoxins and therefore their activity could be regulated by light via the ferredoxin–thioredoxin system (Balmer et al., 2003; Lemaire et al., 2004). Alternatively, light could modulate protein levels post-transcriptionally via novel but currently unknown mechanisms (Rodríguez-Villalón et al., 2009).

While light positively regulates MEP pathway activity and many downstream branches of the MEP pathway, MVA pathway activity appears to be regulated negatively by light. Transcripts encoding HMGR, a rate-limiting enzyme in the MVA pathway, are rapidly down-regulated by light (Enjuto et al., 1994; Learned, 1996; Learned and Connolly, 1997). Similarly, HMGR enzyme activity decreases in light (Moore and Oishi, 1993). Consistently with reduced MVA pathway activity, sitosterol and stigmasterol levels are reduced in de-etiolated *Arabidopsis* seedlings (Ghassemian et al., 2006).

Regulation of the Isoprenoid Network by the Circadian Clock and Phytochrome

Phytochrome B (PHYB) is instrumental in orchestrating the light-dependent changes in isoprenoid pathway activities. HMGR mRNA levels and enzyme activity are induced in a *phyB* mutant while accumulation of MEP pathway downstream products are repressed (Rodríguez-Concepción et al., 2004). At the same time, however, the *phyB* mutant is more resistant to the MEP pathway inhibitor fosmidomycin, which suggests that isoprenoid intermediates from the cytosol are transported into plastids. Based on this and other results, Rodríguez-Concepción (2006) and colleagues proposed a model for light-mediated regulation of isoprenoid biosynthesis during the early stages of plant development (Figure 2A). According to this model, seedlings germinating in the dark obtain the precursors for sterol and brassinosteroid synthesis (which are required at higher levels when seedlings grow in search of light) from the MVA pathway. Some of the MVA-derived prenyl diphosphates might be transported to the plastid for the synthesis of carotenoids

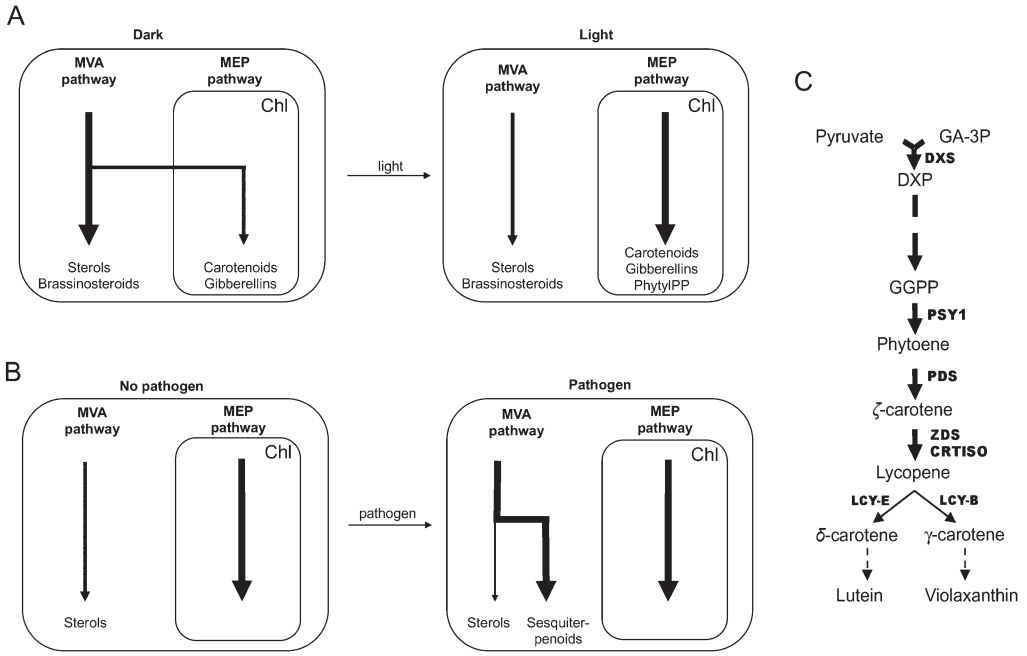

Figure 2. Examples of Environmental and Developmental Factors that Affect the Isoprenoid Pathway Network.

(A) Effect of de-etiolation. Dark-grown seedlings produce mainly MVA-derived isoprenoids that are required for rapid seedling growth. At this stage, the MVA pathway is proposed to provide precursors also for the synthesis of plastid isoprenoids (Rodríguez-Concepción, 2006). Upon illumination, synthesis of plastid isoprenoids is increased, while the synthesis of sterols becomes progressively reduced (Ghassemian et al., 2006).

(B) Effect of pathogens. Pathogens, especially in *Solanacea* species, induce synthesis of sesquiterpenoids, while synthesis of sterols is reduced (Vögeli and Chappell, 1988).

(C) Accumulation of lycopene during tomato fruit ripening is associated with the accumulation of its precursor phytoene and ζ-carotene and decreased synthesis of xanthophylls (Fraser et al., 1994). Flux via MEP pathway is proposed to be enhanced as well (Lois et al., 2000). Transcription of the genes encoding 1-deoxy-D-xylulose-5-phosphate (DXP) synthase (DXS/DXL), phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), and carotene isomerase (CRTISO) is up-regulated, whereas the genes for lycopene β-cyclase (LCY-B) and lycopene ε-cyclase (LCY-E) are not transcribed (Lois et al., 2000; Hirschberg, 2001). Note: Figure 2B and 2C are discussed later in the text.

and gibberellins that are needed during seedling development. Upon illumination, activity of the MEP pathway increases and MVA-derived isoprenoid precursors are no longer required by the plastid.

In addition to PHYB, PHYA regulates light-dependent responses as well. Far-red light induction of Calvin cycle and plastid isoprenoid pathway genes is abolished in *phyA* mutants (Meier et al., 2011). Phytochrome Interacting Factors (PIFs), which are basic helix-loop-helix (bHLH) transcription factors that bind to the promoter of light-induced genes and repress their expression in dark-grown seedlings (Castillon et al., 2007; Leivar et al., 2009), likely coordinate the expression of isoprenoid pathway genes. PIF1 binds to the phytoene synthase (PSY) promoter to repress gene expression (Toledo-Ortiz et al., 2010) and, in *pif-1*, *-3*, *-4*, and *-5* quadruple mutant (*pifq*), expression of several genes for enzymes in carotenoid, ABA, plastoquinone, and GA biosynthesis is enhanced (Meier et al., 2011).

Phytochromes entrain the circadian clock (Castillon et al., 2007) and therefore it is not surprising that the circadian clock also has a profound effect on isoprenoid pathway activities. For example, the emission of volatile terpenoids (Dudareva et al., 2005; Abel et al., 2009) and isoprene (Loivamäki et al., 2007; Wilkinson et al., 2006) follows a diurnal rhythm. Accumulation of ABA, cytokinins, and GAs shows diurnal variation as well (Hedden and Kamiya, 1997; Nováková et al., 2005; Barta and Loreto, 2006). In addition, genes encoding enzymes in the MEP pathway and downstream pathways such as tocopherol, carotenoid, and ABA biosynthesis also have typical circadian expression profiles (Dudareva et al., 2005; Hsieh and Goodman, 2005; Covington et al., 2008). *CCA1* (*CIRCADIAN CLOCK-ASSOCIATED 1*), *LHY* (*LATE ELONGATED HYPOCOTYL*), and *TOC1* (*TIMING OF CAB EXPRESSION 1*) are genes encoding proteins of the circadian oscillator. TOC1 is a member of the pseudo-response regulator (PRR) protein family, which includes PRR9, PRR7, PRR5, PRR3, and TOC1/PRR1 (Matsushika et al., 2000; Nakamichi et al., 2005). The expression of genes and metabolite levels in chlorophyll, carotenoid, ABA, and tocopherol biosynthetic pathways are all increased in the *prr9/prr7/prr5* triple mutant, suggesting that PRR9/PRR7/PRR5 are negative regulators of these pathways (Fukushima et al., 2009).

To understand the extent to which the isoprenoid pathway network is controlled by the circadian clock and to identify candidates that could link circadian regulation to isoprenoid synthesis, we constructed a gene co-expression network using microarray data from 6-week-old *Arabidopsis* shoots grown under 12L/12D condition (James et al., 2008). *LHY* and *CCA1* form modules of many and overlapping edges with isoprenoid pathway genes encoding plastid enzymes (Figure 3). These modules are sparsely connected to the modules formed by the morning loop genes (e.g. *PRR7*, *PRR9*). The circadian clock genes have different degrees of connectivity with MVA and MEP pathway genes. All MEP pathway genes are significantly correlated with circadian clock genes, except for *HDS*. However, only a few significant edges exist between circadian clock and MVA pathway genes (Figure 4). As a general trend, MEP pathway genes are co-expressed with core circadian clock genes from the 'morning loop' (*PRR9*, *CCA1*, *LHY*), whereas MVA pathway genes correlate with circadian clock genes from the 'evening loop' (e.g. *TOC1*). Interestingly, the co-expression network of the circadian clock, MVA, and MEP pathway genes is different in roots (Figure 5A). Here, the MVA pathway genes have a higher connectivity and notably with other clock genes compared with the network in the shoot. In contrast, the MEP pathway genes show no significant correlation with the core circadian clock genes in roots (Figure 5A). Moreover, the transcript levels of MEP and MVA pathway genes that are differentially co-expressed in roots and shoots with 'morning loop' and 'evening loop' genes, respectively, appear to have an antagonistic circadian oscillation (Figure 5B). This is consistent with the model that the circadian clock in roots appears to be a simplified version of the circadian clock in green aerial parts, in which it is controlled by a photosynthesis-related signal (James et al., 2008).

Plant–Microbe Interactions Modulate the Isoprenoid Pathway Network

Many metabolites derived from the MVA pathway, primarily mono- sesqui-, di-, and saponin tri-terpenoids, have potent antifungal, antimicrobial, and repellent properties or serve to attract predators. It is well established that pathogens trigger

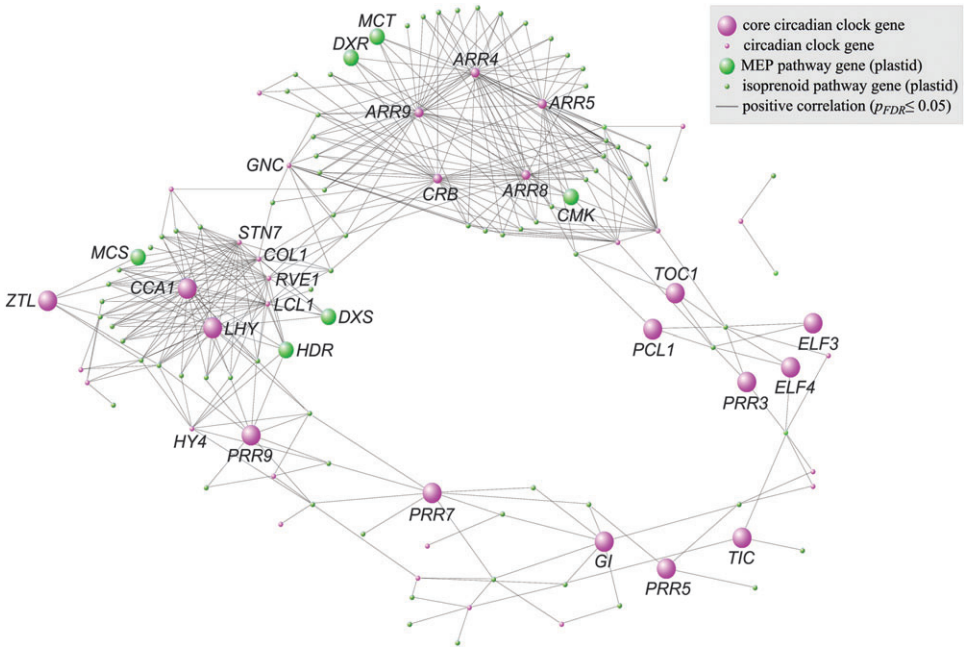

Figure 3. Modular Co-Expression Network of the Circadian Clock Genes and Isoprenoid Pathway Genes Encoding Enzymes Localized in Plastids.

Microarray expression data of 6-week-old *Arabidopsis* shoots grown under 12L/12D conditions (James et al., 2008) were used. Briefly, to generate a gene co-expression network, the pair-wise Pearson correlation coefficients were calculated and the statistical significance was assessed by applying false discovery rate (FDR) family-wise error correction. The gene co-expression network is shown as a graph, with nodes representing genes and edges indicating statistically significant positive correlations (FDR p-value ≤ 0.05). The circadian clock genes are shown in magenta and core circadian clock genes are emphasized by a larger node size. The isoprenoid pathway genes encoding plastid enzymes (Vranová et al., 2011) are shown in green and the MEP pathway genes are labeled. Except for *HDS*, all the MEP pathway genes are co-expressed with circadian clock genes. *ARR*, *type A response regulator*; *CCA1*, *CIRCADIAN CLOCK-ASSOCIATED 1*; *CMK*, *4-(cytidine 5′-phospho)-2-C-methyl-D-erithritol kinase*; *COL1*, *CONSTANS-LIKE 1*; *CRB*, *chloroplast RNA binding*; *DXR*, *DXP reductoisomerase*; *DXS*, *DXP synthase*; *ELF*, *EARLY FLOWERING*; *GI*, *GIGANTEA*; *GNC*, *GATA transcription factor 21*; *HDR*, *HMBPP reductase*; *HY4*, *ELONGATED HYPOCOTYL 4*; *LCL1*, *LHY/CCA1-LIKE 1*; *LHY*, *LATE ELONGATED HYPOCOTYL*; *MCT*, *2-C-methyl-D-erythritol 4-phosphate cytidyltransferase*; *MCS*, *2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase*; *PCL1*, *PHYTOCLOCK 1*; *PRR*, *pseudo-response regulator*; *RVE1*, *REVEILLE 1*; *STN7*, *serine/threonine-protein kinase 7*; *TIC*, *TIME FOR COFFEE*; *TOC1*, *TIMING OF CAB EXPRESSION 1*; *ZTL*, *ZEITLUPE 1*.

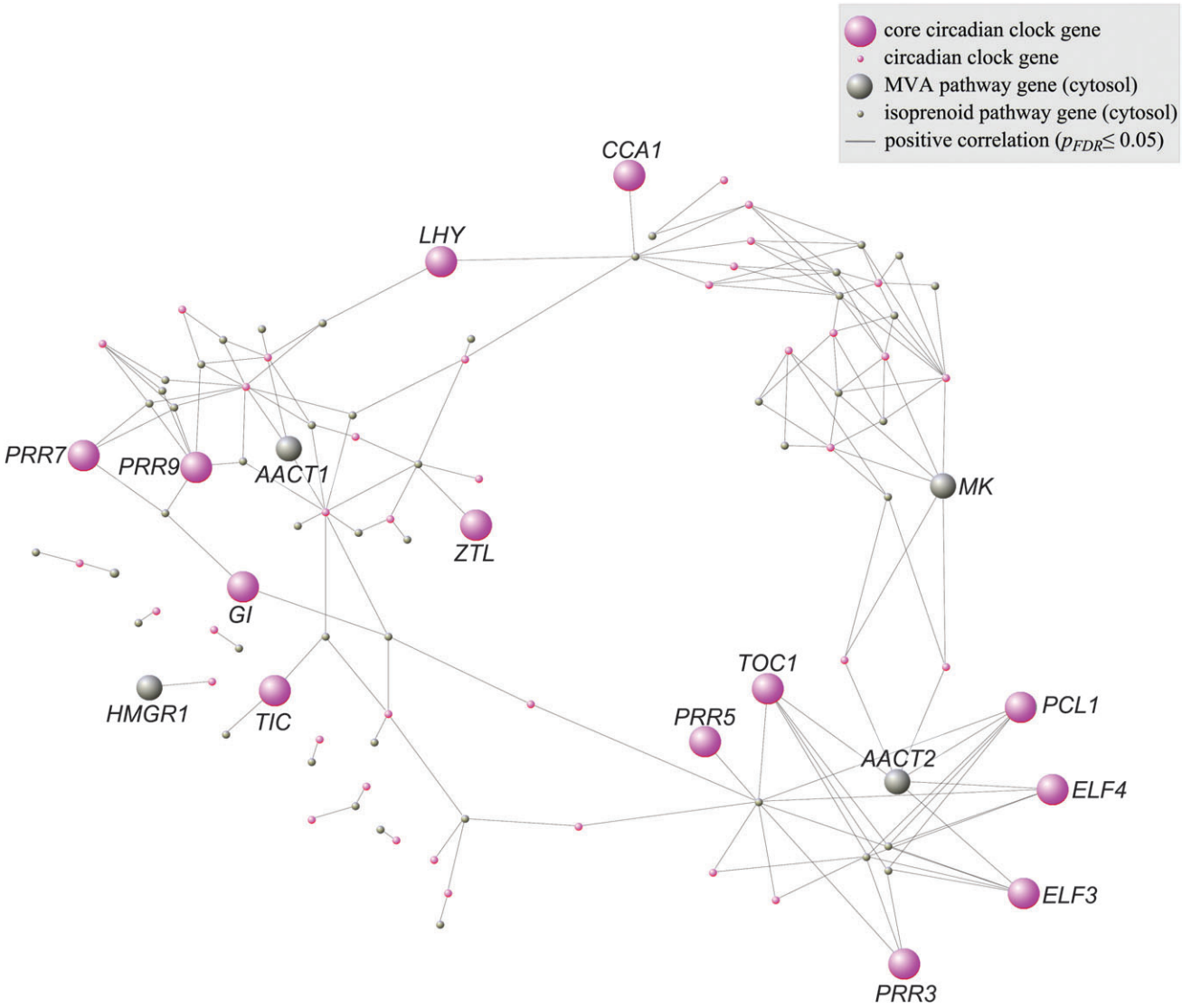

Figure 4. The Co-Expression Network of the Circadian Clock and Isoprenoid Pathway Genes Encoding Enzymes Localized in the Cytosol. The analysis was carried out as described in Figure 3. The circadian clock genes are shown in magenta and core circadian clock genes are emphasized by a larger node size. The isoprenoid pathway genes encoding enzymes localized in cytosol (Vranová et al., 2011) are shown in gray and the MVA pathway genes are labeled. The network connectivity is smaller compared to the co-expression network of circadian clock genes with isoprenoid pathway genes encoding enzymes located in plastids. Only a few significantly co-expression relationships exist between the circadian clock and the MVA pathway genes. In particular, *CCA1* and *LHY* are loosely connected to isoprenoid pathway genes encoding cytosolic enzymes. *MK*, *AACT1*, and particularly *AACT2* have the highest number of significant correlations among the MVA pathway genes. *AACT, acetoacetyl-CoA thiolase*; *HMGR1*, *HMG-CoA reductase 1*; *MK*, *mevalonate kinase*.

the redirection of MVA pathway flux to production of pathogenesis-related compounds. For example, sesquiterpenoids constitute a large fraction of the antimicrobial compounds produced in *Solanaceaeous* species. Early experiments showed that treatment of the cell cultures with either *Phytophthora* cell wall fragments or cellulase induces both HMGR and sesquiterpenoid cyclase activities (Vögeli and Chappell, 1988; Chappell et al., 1991). However, pathogen-related elicitors not only increase sesquiterpenoid biosynthesis (Figure 2B), but can also affect other parts of the isoprenoid pathway network.

The accumulation of apocarotenoids in plant roots colonized by arbuscular mycorrhizal (AM) fungi is another example of coordinated regulation of the isoprenoid pathway network during plant–microbe interactions. Soil-born symbiotic fungi *Glomeromycota* colonize most terrestrial plants to form arbuscular mycorrhizal roots, which stimulates apocarotenoid synthesis. Transcript levels of the first two enzymes of the MEP pathway, DXS and DXR, and of the carotenoid pathway, phytoene desaturase and ζ-carotene desaturase, along with a carotenoid-cleaving dioxygenase, are markedly increased in AM roots (Strack and Fester, 2006; Walter et al., 2010). Although

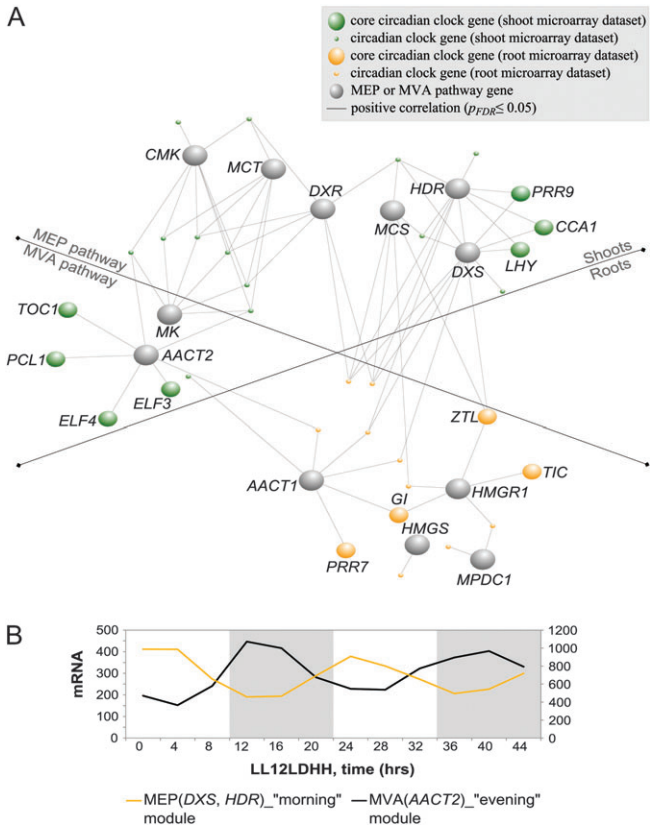

Figure 5. Tissue-Specific Co-Expression Network of the Circadian Clock with the MEP and MVA Pathway Genes.

For this analysis, the root microarray dataset was used in addition to the shoot dataset (James et al., 2008) and the gene co-expression network was constructed as described in Figure 3.

(A) The MEP and MVA pathway genes are shown in gray. The circadian clock genes are shown in green when significant correlations were obtained using the shoot microarray dataset or in orange for significant co-expression using the root microarray dataset. The core circadian clock genes are emphasized by a larger node size and are labeled. In roots, the MVA pathway genes have a higher connectivity and notably with other circadian clock genes compared with the network in the shoots. In roots, however, there was no significant correlation of the MEP pathway genes with the core circadian clock genes. As a general trend, in shoots, the MEP pathway genes are co-expressed with morning and core circadian clock genes (e.g. *PRR9*, *CCA1*, *LHY*), whereas the MVA pathway genes correlate with circadian clock genes from the evening loop (e.g. *TOC1*).

(B) Transcript circadian oscillation of the co-expressed modules formed by circadian clock genes with MVA and MEP pathway genes. The MVA (*AACT2*)_'evening' module consists of *AACT2*, *TOC1*, *PCL1*, *ELF3*, and *ELF4*. The MEP(*DXS*, *HDR*)_ 'morning' module consists of *DXS*, *HDR*, *LHY*, *CCA1*, and *PRR9*. The transcript expression pattern of each module was plotted based on the microarray dataset from Harmer et al. (2000). In brief, the LL12LDHH dataset was generated from plants grown in light/dark cycles of 12 h each for 7 d and then released into constant light. Starting at time point 0 (subjective beginning of the dark period on day 9), the plants were harvested every 4 h over a 48-h period (Harmer et al., 2000). For each co-expressed module, an average mRNA level was calculated and plotted. The MEP (*DXS*, *HDR*)_ 'morning' module co-expressed with core circadian clock genes from the 'morning loop' in shoots shows circadian oscillation and is induced during the light cycles (transcript levels are shown on the right, on the secondary *y*-axis). In contrast, the MVA (*AACT2*)_ 'evening' module co-expressed in shoots with circadian clock genes from the 'evening loop' appears to have an anti-phase oscillation indicated by the expression levels peaking at the dark cycles (transcript levels are shown on the left, on the main *y*-axis). *HMGS*, *3-hydroxy-3-methylglutaryl-CoA synthase*; *MPDC1*, *mevalonate diphosphate decarboxylase 1*.

the function of apocarotenoids is not understood at present, they seem to be associated with the maturation of arbuscules.

Developmental Regulation of the Isoprenoid Pathway Network

The red fruits of the cultivated tomato have high concentrations of lycopene (Sandmann et al., 2006). The accumulation of lycopene during tomato ripening is closely correlated with the accumulation of the precursors phytoene and ζ-carotene and the decrease in xanthophylls (Fraser et al., 1994). The accumulation of lycopene is determined by the differential expression of genes encoding biosynthetic enzymes (Hirschberg, 2001; Figure 2C). Transcription of phytoene synthase (*PSY1*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), and carotene isomerase (*CRTISO*) genes is up-regulated during fruit ripening, whereas expression of lycopene β-cyclase (*LCY-B*) and lycopene ε-cyclase (*LCY-E*) ceases during the breaker stage of ripening. Consequently, the increased flux of carotene in the pathway is arrested at lycopene. However, lycopene synthesis is not only controlled at the carotenoid branch of the isoprenoid pathway. The MEP pathway *DXS* gene is co-regulated with *PSY1* during fruit ripening and injection of 1-deoxy-D-xylulose, the substrate of the MEP pathway, into mature green tomato fruit accelerates carotenoid synthesis (Lois et al., 2000; Figure 2C). This demonstrates that *DXS* is a part of the isoprenoid pathway network required for carotenoid synthesis during tomato fruit ripening. Interestingly, enhanced activity of enzymes in the carotenoid branch of the pathway network is negatively correlated with chlorophyll biosynthesis. Both pathways share the GGPP precursor but it is currently unclear whether competition for the same substrate may be the reason for this regulation during tomato ripening. Unexpectedly, carotenoid synthesis during tomato ripening is correlated with the decreased expression of metabolic genes from associated pathways, such as the Calvin cycle, tricarboxylic acid (TCA) cycle, starch synthesis, and others (Carrari and Fernie, 2006).

BIOTECHNOLOGICAL APPLICATIONS FROM ISOPRENOID RESEARCH

Understanding the plant isoprenoid pathway network and flux regulation can facilitate molecular breeding and genetic engineering of agronomically useful traits. Sterols and phytohormones synthesized by the isoprenoid pathway network represent both building blocks and modulators of growth

and development. Manipulation of their synthesis in a controlled manner can therefore be useful to improve crop yield and nutritional quality (Sakamoto, 2006; Salas Fernandez et al., 2009). Isoprenoids (chlorophylls, carotenoids, phylloquinone, plastoquinone, tocopherols) also play a direct or indirect role in photosynthesis. Photosynthetic capacity and efficiency are other factors that could increase crop productivity, although the underlying molecular mechanisms are currently not well understood. However, there is strong evidence that increasing photosynthesis will increase crop yields, provided that other constraints do not become limiting (Parry et al., 2011).

Isoprenoids also include biologically active substances produced in fruits and vegetables that are an important part of the human diet. For example, lycopene and β-carotene, among others, are effective antioxidants and have cancer-preventive activity. Phytosterols have been shown to lower blood cholesterol levels and therefore can contribute to decreasing the incidence of cardiovascular diseases. Dietary triterpenoid saponins also reduce blood cholesterol, stimulate the immune system, and inhibit the growth of cancer cells *in vitro*. Tocopherols and tocotrienols protect DNA, low-density lipoproteins, and polyunsaturated fatty acids from oxidative damage (reviewed in Poiroux-Gonord et al., 2010). Enhancing the synthesis of biologically active isoprenoid compounds in crops could therefore be an attractive approach to produce food with the added nutritional value. A successful example of such functional food is the so-called 'Golden Rice', which expresses enzymes of the carotenoid pathway in rice endosperm (Ye et al., 2000; Paine et al., 2005). Golden rice was developed to combat vitamin A deficiency, which affects eyesight and weakens the immune system in malnourished children. Although Golden Rice provides the recommended daily allowance (RDA) of β-carotene in one average daily portion of rice (300 g), the nutritionally improved rice variety has not yet been approved and released to farmers for production.

In addition to improved crop yield and food quality, isoprenoids can also contribute to the plant-based production of economically valuable plant terpenoids for biomaterials and pharmaceuticals. Genetic engineering of crops and microbial organisms for production of flavor and aroma compounds derived from mono-, sesqui-, di-, and tetraterpenoids, such as natural rubber, polyterpene polyisoprenoids, artemisinin, and taxol, is currently underway in many laboratories (reviewed in Bohlmann and Keeling, 2008; Schwab et al., 2008; Van Beilen and Poirier, 2008).

CONCLUSIONS AND OUTLOOK

Understanding the complexity of the isoprenoid pathway network in plants and its regulation remains a major challenge in isoprenoid research during the coming years. Dynamic network inference represents the ultimate goal of network analysis. Building a complete dynamic model of isoprenoid pathway regulation first requires identification of all elements of the isoprenoid pathway network and associated regulatory networks. This can be achieved by increasingly sensitive quantitative proteomics and metabolomics technologies and by building phenomenological models using these 'omics' data. Bottom-up modeling approaches can then extend such models to dynamic regulatory and metabolic network models. Initial studies to obtain kinetic models of isoprenoid metabolism (although on a small network scale) have already been initiated (Zimmer et al., 2000; Rios-Estepa et al., 2008, 2010). It is likely that both top-down and bottom-up approaches will evolve simultaneously and ultimately merge to provide a systems understanding of isoprenoid pathway regulation. This would allow molecular breeding and biotechnology to make efficient use of the potential of the isoprenoid pathway network for crop improvement and industrial feedstock applications.

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