Orthologs of the archaeal isopentenyl phosphate kinase regulate terpenoid production in plants

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Terpenoids, compounds found in all domains of life, represent the largest class of natural products with essential roles in their hosts. All terpenoids originate from the five-carbon building blocks, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which can be derived from the mevalonic acid (MVA) and methylerythritol phosphate (MEP) pathways. The absence of two components of the MVA pathway from archaeal genomes led to the discovery of an alternative MVA pathway with isopentenyl phosphate kinase (IPK) catalyzing the final step, the formation of IPP. Despite the fact that plants contain the complete classical MVA pathway, IPK homologs were identified in every sequenced green plant genome. Here, we show that IPK is indeed a member of the plant terpenoid metabolic network. It is localized in the cytosol and is coexpressed with MVA pathway and downstream terpenoid network genes. *In planta*, IPK acts in parallel with the MVA pathway and plays an important role in regulating the formation of both MVA and MEP pathway-derived terpenoid compounds by controlling the ratio of IP/DMAP to IPP/DMAPP. IP and DMAP can also competitively inhibit farnesyl diphosphate synthase. Moreover, we discovered a metabolically available carbon source for terpenoid formation in plants that is accessible via *IPK* overexpression. This metabolite reactivation approach offers new strategies for metabolic engineering of terpenoid production.

plant terpenoids | isopentenyl diphosphate | isopentenyl phosphate kinase | MVA pathway | MEP pathway

All living organisms produce terpenoids, directing a considerable amount of their available carbon to the biosynthesis of one of the most structurally and functionally diverse classes of primary and secondary metabolites in nature (1). These molecules play essential and specialized roles in their hosts in photosynthesis and respiration (the phytyl side-chain of chlorophyll, quinones), modulating membrane fluidity (sterols), regulating growth and development (hormones), photoprotection and energy transfer (carotenoids), and communication, environmental adaptation, and chemical defense (mono-, sesqui-, and diterpenes) (2, 3). Some compounds, like quinones, chlorophylls, and certain proteins, which require targeting to membranes for their functions, are anchored by terpenoid structures. In addition to their vital biological roles, terpenoids are also widely used by humans as nutritional supplements, flavors, fragrances, biofuels, and pharmaceuticals (4–6). Multiple metabolic pathways operate in parallel, often intersect, and routinely exchange intermediates, leading to one of the most chemically complex groups of natural products in the biosphere. Therefore, achieving a complete understanding at both genetic and biochemical levels of the underlying regulatory networks that coordinate and homeostatically govern these biosynthetic systems is of paramount importance to fully capitalize on the utility of terpenoids to natural ecosystems and humans.

All terpenoids originate from C5 building blocks, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which are synthesized by two independent pathways, the mevalonic acid (MVA) and methylerythritol phosphate (MEP) pathways (Fig. 1). Interestingly, these pathways are not systematically distributed among the three domains of life: eukaryotes, archaea, and bacteria. Although the MEP pathway is found in most bacteria, the MVA pathway resides in the cytosol and peroxisomes of eukaryotic cells. Plants contain both the MEP and MVA pathways, which act independently in plastids and cytosol/peroxisomes, respectively (Fig. 1). Nevertheless, metabolic cross-talk between these two pathways occurs via the exchange of IPP—and to a lesser extent of DMAPP—in both directions (1, 2). IPP and DMAPP are subsequently used in multiple compartments by short-chain prenyltransferases to produce prenyl diphosphate intermediates, including geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15), and geranylgeranyl diphosphate (GGPP, C20). Whereas GPP synthases localize exclusively in plastids and provide precursors for monoterpenes, FPP synthases (FPPS) localize in cytosol and mitochondria and produce FPP for sesquiterpene, homoterpene, triterpene, sterol, brassinosteroid, and polyprenol biosynthesis. GGPP synthases reside in plastids, mitochondria, and the endoplasmic reticulum, producing precursors for gibberellins, homoterpenes, carotenoids, phytyl side-chains for chlorophyll/tocopherols/quinones, polyprenols, oligoprenols, abscisic acid, and strigolactones, among others.

In contrast to plants, archaea rely exclusively on the MVA pathway. Although some archaea have a complete MVA pathway (7), most lack genes encoding the two final enzymes, phosphomevalonate kinase (PMK) and mevalonate 5-diphosphate decarboxylase (MDD), catalyzing phosphorylation and ATP-dependent decarboxylation, respectively (Fig. 1). Instead of PMK, archaea possess isopentenyl phosphate kinase (IPK), catalyzing the conversion of isopentenyl phosphate (IP) to IPP (8, 9). The discovery of

Significance

Terpenoids, one of the largest and most diverse classes of natural products, are found in all living organisms, where they play essential roles in growth and development, respiration and photosynthesis, and interactions with the environment. We discovered that a functional homolog of isopentenyl phosphate kinase (IPK), originally identified in archaebacteria, is unexpectedly present in plants and functions in their terpenoid metabolic network. Cytosolically localized, IPK phosphorylates isopentenyl phosphate (IP) and its isomer dimethylallyl phosphate (DMAP) to their corresponding diphosphates, IPP and DMAPP, the universal C5 building blocks of all natural terpenoids. IPK enhances terpenoid formation by returning IP/DMAP to the terpenoid biosynthetic network. This metabolite reactivation process offers a new approach for metabolic engineering of economically important terpenoids.

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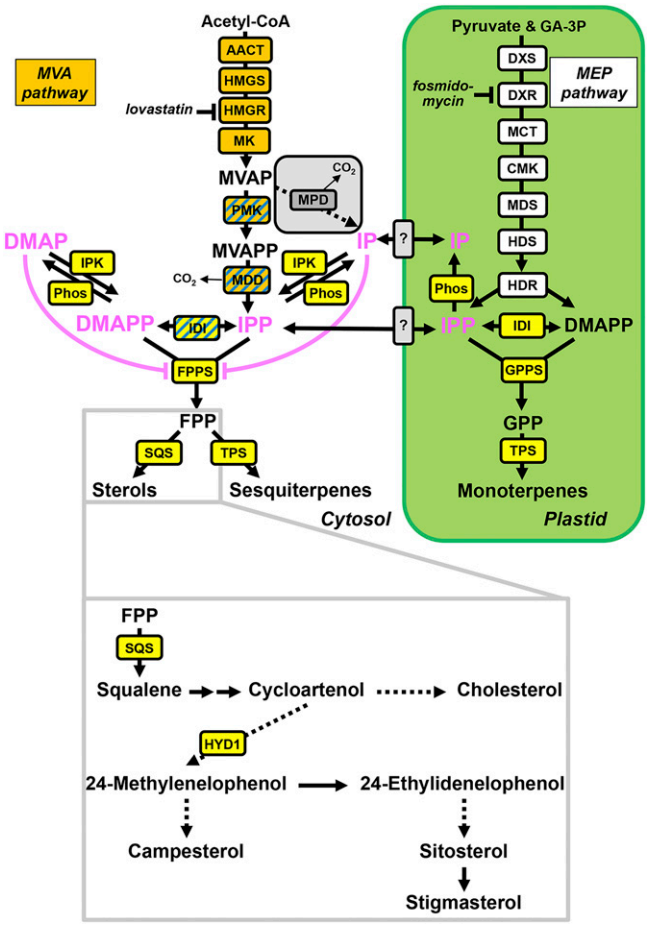

Fig. 1. Position and potential role of IPK in the plant terpenoid metabolic network. Cytosolic and plastidial (highlighted in green) terpenoid metabolic pathways involved in the biosynthesis of sterols, sesquiterpenes, and monoterpenes in plants with individual enzymes depicted as boxes. The MVA pathway enzymes are highlighted in orange, the MEP pathway enzymes are highlighted in white, and enzymes involved in downstream terpenoid formation are highlighted in yellow. The enzymes with peroxisomal localization are depicted on a striped background. The unknown transporters involved in IPP and IP exchange across the plastid envelope membranes are shown in gray. Site of action of the MVA and MEP pathway-specific inhibitors (lovastatin and fosmidomycin, respectively), as well as feed-forward inhibition of FPPS by IP/DMAP, are indicated. The recently discovered MPD acting in the alternative MVA pathway in *R. castenholzii* and *H. volcanii* is shown on a gray background. Dashed lines in sterol biosynthesis represent multiple enzymatic steps. Abbreviations: AACT, aceto-acetyl-CoA thiolase; CMK, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GA-3P, D-glyceraldehyde 3-phosphate; HDR, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; HDS, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HYD1, C-8,7 sterol isomerase; MCT, 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase; MDS, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MK, mevalonate kinase; Phos, phosphatase(s); SQS, squalene synthase; TPS, terpene synthases (including monoterpene synthases and sesquiterpene synthases).

IPK suggested the existence of a modified MVA pathway in archaea, with the last two enzymatic steps hypothesized to occur in reverse order: initial ATP-dependent decarboxylation of mevalonate 5-phosphate (MVAP) by a phosphomevalonate decarboxylase (MPD) to form IP, followed by phosphorylation of IP to IPP by IPK (Fig. 1). To date, MPD activity has been identified in the Chloroflexi bacterium *Roseiflexus castenholzii* and the archaeal extremophile *Haloferax volcanii*, thus providing evolutionary precedence for an alternative MVA metabolic pathway (10, 11).

Unexpectedly, functional IPK homologs were found in every sequenced plant genome (10), despite the fact that plants contain a complete classic MVA pathway. The ubiquitous presence of an IPP-generating kinase in the green plant lineage suggests that the presence of IP, and its phosphorylation to IPP, may play a critical role in terpenoid metabolism and homeostasis. Here, we show that IPK is indeed a member of the plant terpenoid metabolic network. It is localized in the cytosol and plays an important role in modulating the formation of FPP-derived terpenoid compounds by controlling the ratio of IP/dimethylallyl phosphate (DMAP) to IPP/DMAPP. IP and DMAP can also serve as competitive inhibitors of FPPS. In addition, *IPK* overexpression leads not only to increased production of FPP-derived terpenoids in the cytosol, but also of GPP-derived compounds in plastids.

Results and Discussion

***IPK* Is Coexpressed with the MVA Pathway and Downstream Terpenoid Network Genes and the Encoded Protein Is Localized in the Cytosol.** In plants, the MVA and MEP pathways provide IPP and DMAPP precursors for short-chain prenyltransferases and ultimately for cellular terpenoid production. Therefore, to examine whether IPK is part of the cytosolic or plastidial terpenoid biosynthetic network, we searched for genes coexpressing with *Arabidopsis thaliana IPK* (*AtIPK*, At1g26640) using the ATTED-II CoExSearch tool (12). The resulting list (Dataset S1) containing the top 0.5% of genes coexpressed with *AtIPK* includes *acetoacetyl-CoA thiolase* (*AtAACT2*/At5g48230), *MDD* (*AtMDD1*/At2g38700), *FPPSs* (*AtFPPS1*/At5g47770 and *AtFPPS2*/At4g17190), *squalene synthase 1* (*AtSQS1*/At4g34640), and *C-8,7 sterol isomerase* (*AtHYD1*/At1g20050), suggesting that IPK may be functionally connected to the MVA pathway and biosynthesis of downstream terpenoids, including sesquiterpenes, triterpenes, and sterols (Fig. 1). This finding raised the question of whether plants harbor both the classic and an alternative MVA pathway in which IPK catalyzes the final step in the formation of IPP. Because functional MPDs and MDDs (Fig. 1) share high sequence similarity, as previously noted (10), we searched the *A. thaliana* protein database for potential MPD candidates using *R. castenholzii* MPD as the query sequence. The top hits included two annotated MDD homologs, AtMDD1 (At2g38700) and AtMDD2 (At3g54250), which shared 25% sequence identity with *R. castenholzii* MPD with e-values of $1e^{-4}$. Additional sequences retrieved gave e-values greater than 1.3 with little to no homology noted. Thus, we tested substrate specificities of AtMDD1 and AtMDD2, as well as their ability to catalyze the penultimate step in such an alternative pathway, the formation of IP from MVAP (Fig. 1). AtMDD1 and AtMDD2 steady-state kinetics using either mevalonate 5-diphosphate (MVAPP) or MVAP as substrates at saturating concentrations of ATP revealed that both MDDs have a 10,000-fold preference for MVAPP compared with MVAP (Table 1). Given their strong substrate preference for MVAPP, it seems unlikely that in *Arabidopsis* IPK is part of an alternative MVA pathway. Rather, in response to metabolic demands, IPK is likely to function in reactivating a pool of IP generated by endogenous plant phosphatases and as part of a homeostatic mechanism to balance the levels of IPP (10, 13–15).

The last two MVA pathway enzymes, PMK and MDD, as well as isopentenyl diphosphate isomerase (IDI), catalyzing the interconversion of IPP and DMAPP, are localized in peroxisomes (16, 17). To determine the site of IPK action, we established its subcellular localization. Analysis of the amino acid sequence of AtIPK using multiple subcellular prediction programs (WoLF PSORT, Predotar, and TargetP) did not reveal any known intracellular targeting sequences. To experimentally demonstrate AtIPK subcellular localization, GFP was fused to the N or C terminus of AtIPK and transiently expressed in tobacco. With both constructs, GFP signals were only detected in the cytosol (Fig. S1 *A* and *B*) and did not overlap with chlorophyll autofluorescence (Fig. S1 *A* and *B*), peroxisomal (Fig. S1 *D* and *E*), or

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Table 1. Steady-state kinetic parameters for *A. thaliana* MDDs with MVAPP and MVAP

Enzyme	Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}\cdot M^{-1}$)
AtMDD1	(*R*)-MVAPP	26 ± 3	3.2 ± 0.1	$1.2 \times 10^5 \pm 0.1$
	(*R*)-MVAP	2,800 ± 800	0.033 ± 0.004	$1.2 \times 10^1 \pm 0.3$
AtMDD2	(*R*)-MVAPP	25 ± 3	3.2 ± 0.1	$1.3 \times 10^5 \pm 0.2$
	(*R*)-MVAP	4,600 ± 1100	0.034 ± 0.005	7.4 ± 2

Data are means ± SD (n = 3 independent experiments).

mitochondrial (Fig. S1*F*) markers, indicating that—in contrast to PMK, MDD, and IDI—IPK is localized in the cytosol.

IPK *in Planta* Acts in Parallel with the MVA Pathway and Regulates the Formation of FPP-Derived Terpenoids. *A. thaliana* IPK was previously identified and kinetically characterized in vitro using IP as a substrate (10). Because thermophilic archaeal IPKs are promiscuous with regard to substrate use (18), we analyzed whether AtIPK can also use DMAP and geranyl phosphate (GP) as substrates. Steady-state kinetics with these alternative substrates revealed that, like archaeal IPKs, AtIPK can phosphorylate DMAP and GP. The catalytic efficiency with DMAP was similar to that of IP, whereas three orders-of-magnitude lower catalytic efficiency was observed for the 10-carbon GP molecule, where high enzyme concentrations were required to detect even negligible turnover (Table 2).

To further characterize IPK's in vivo role, we used reverse genetics and obtained two *Arabidopsis* T-DNA insertion lines (*ipk1*, *ipk2*) (Fig. S2*A*). Quantitative RT-PCR (qRT-PCR) with two sets of gene-specific primers, one located upstream of T-DNA insertions and the other downstream near the 3′ end of the gene (Fig. S2*A*), were performed to analyze *AtIPK* mRNA levels in mutants. No *AtIPK* transcripts were detected in *ipk1*, whereas transcript levels were reduced by 83% in *ipk2*, with remaining expression likely a result of residual splicing despite the intron-localized T-DNA insertion (Fig. 2*A*). Sterol levels were analyzed in these mutant plants because these cyclic terpenoids represent the key metabolites to which a significant portion of the MVA pathway isoprenoid flux is directed (1). Both *ipk1* and *ipk2* seedlings showed a significant decrease in campesterol and sitosterol content (50% and 37% of wild-type, respectively), while the stigmasterol levels were unchanged (Fig. 2*D*), indicating that *AtIPK* knockout or knockdown affects formation of two of the three predominant *Arabidopsis* sterols. Similar sterol levels in knockout (*ipk1*) and knockdown (*ipk2*) mutants indicate that remaining *AtIPK* expression in *ipk2* supports only marginal flux toward IPP/DMAPP formation without increasing downstream terpenoid production relative to the null *ipk* knockout. The lack of reduction in stigmasterol levels suggests that the reduced pool of sitosterol, the immediate precursor of stigmasterol (Fig. 1), is still sufficient to sustain unaltered production of the latter. In addition, emission of β-caryophyllene, the most abundant sesquiterpene compound in *Arabidopsis* (19, 20), was reduced by 25–31% in flowers of *ipk* mutants compared with wild-type (Fig. 2*E*). These results suggest that in plants IPK indeed plays a role in modulating the formation and pool sizes of FPP-derived terpenoids.

To examine the genetic interactions of IPK with the classic MVA pathway, we generated *ipk*/*mdd* double-mutants. MDD is the last enzyme of the classic MVA pathway and, like IPK, responsible for IPP formation (Fig. 1). In *Arabidopsis*, MDD is encoded by two genes (1), with *AtMDD1* displaying the highest transcript levels (Fig. S3) and coexpressing with *AtIPK* (Dataset S1). *AtMDD1* knockout lines, *mdd1-1* and *mdd1-2* (Fig. 2*B* and Fig. S2*B*), showed decreases in campesterol and sitosterol contents (by 45–58% and 27–37%, respectively, relative to wild-type) as well as decreases in β-caryophyllene emission (by 31–38% compared with wild-type), similar to that observed in *ipk* mutants (Fig. 2 *D* and *E*). Even more profound effects were observed in *ipk1*/*mdd1-1* and *ipk2*/*mdd1-1* double-mutants (Fig. 2 *C*–*E*). The levels of sitosterol, the most abundant sterol in *Arabidopsis*, were further reduced compared with the single-mutant lines (by 58–82% relative to wild-type) (Fig. 2*D*), but there were no changes in the other two less-abundant sterols, campesterol and stigmasterol. The absence of further effect on stigmasterol level could be because of the sitosterol precursor pool being sufficient to sustain stigmasterol formation. Similar to sitosterol, emissions of β-caryophyllene were substantially decreased in *ipk*/*mdd1* double-mutant plants relative to *ipk* and *mdd1* single-mutant plants (Fig. 2*E*). Lesser effects on terpenoid formation observed in *ipk2*-knockdown/*mdd1-1*-knockout double-mutants (Fig. 2 *D* and *E*) suggest that some IPP/DMAPP flux is still achieved through IPK in this *ipk* knockdown line. Residual formation of terpenoids in the *ipk1*/*mdd1-1* knockout double-mutant plants suggests that low-expressing *AtMDD2* and the MEP pathway likely provide precursors for their biosynthesis (Fig. 1). Taken together, the additive effect on sitosterol and β-caryophyllene observed in double-mutant plants suggests that IPK acts in parallel with the MVA pathway in generating IPP/DMAPP and subsequent terpenoid products.

IPK Contributes to Terpenoid Formation via Increasing IPP/DMAPP Formation and Relaxing FPPS Inhibition by IP/DMAP. Considering the role of IPK in IPP/DMAPP formation, we examined whether a pool of IP/DMAP exists in plants and if IPK is able to access it to increase precursor pool, and thus ultimately terpenoid production. Twelve independent tobacco lines overexpressing *AtIPK* under control of the cauliflower mosaic virus (CaMV) 35S promoter were generated. Three lines, with different levels of *AtIPK* expression, *IPK-1*, *IPK-4*, and *IPK-19* (Fig. 3*A*), were analyzed for terpenoid formation. All these lines exhibited substantial increases in terpenoid production. Although increases in the levels of the most abundant tobacco sterols were positively correlated with *AtIPK* expression levels, comparable increases in emitted sesquiterpenes were observed irrespective of *AtIPK* transcript abundance (Fig. 3 *B* and *C*). Whereas all lines show increases in sterol formation, in the *IPK*-4 line with the highest *AtIPK* transcript level, cholesterol, stigmasterol, sitosterol, and campesterol were, respectively, 2.0-, 3.0-, 2.2-, and 2.5-fold higher relative to transgenic control plants containing empty vector, the latter plants indistinguishable from untransformed wild-type (Fig. 3*B*). Leaves of all tobacco lines emitted up to 3.4-fold more β-caryophyllene and 5-*epi*-aristolochene sesquiterpenes relative to wild-type or transgenic control plants (Fig. 3*C*). Because IP/DMAP is a cosubstrate for cytosolic IPK, the observed increase in terpenoid production indicates that there is a substantial pool of metabolically accessible IP/DMAP likely formed by the presence and activity of phosphatases acting on IPP/DMAPP in plants (13–15).

Previously it was shown that prenyltransferase activity is inhibited in vitro by a series of C5-monophosphates, including IP and DMAP, although the physiological relevance of this observation was unknown (21). The existence of a cytosolic IP/DMAP pool suggests that IP and DMAP may serve as FPPS inhibitors in vivo, thus controlling carbon flux toward downstream products. *AtFPPS1* is one of two *Arabidopsis FPPS* genes that is highly expressed in vegetative tissue (22) and shows coexpression with *AtIPK* (Dataset S1).

Table 2. Steady-state kinetic parameters for *A. thaliana* IPK

Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}\cdot M^{-1}$)
IP	32 ± 10	3.7 ± 0.4	$10.1 \times 10^4 \pm 0.1$
DMAP	33 ± 9	2.7 ± 0.2	$8.5 \times 10^4 \pm 2$
GP	220 ± 120	0.0039 ± 0.0006	$1.8 \times 10^1 \pm 1$

Data are means ± SD (n = 3 independent experiments).

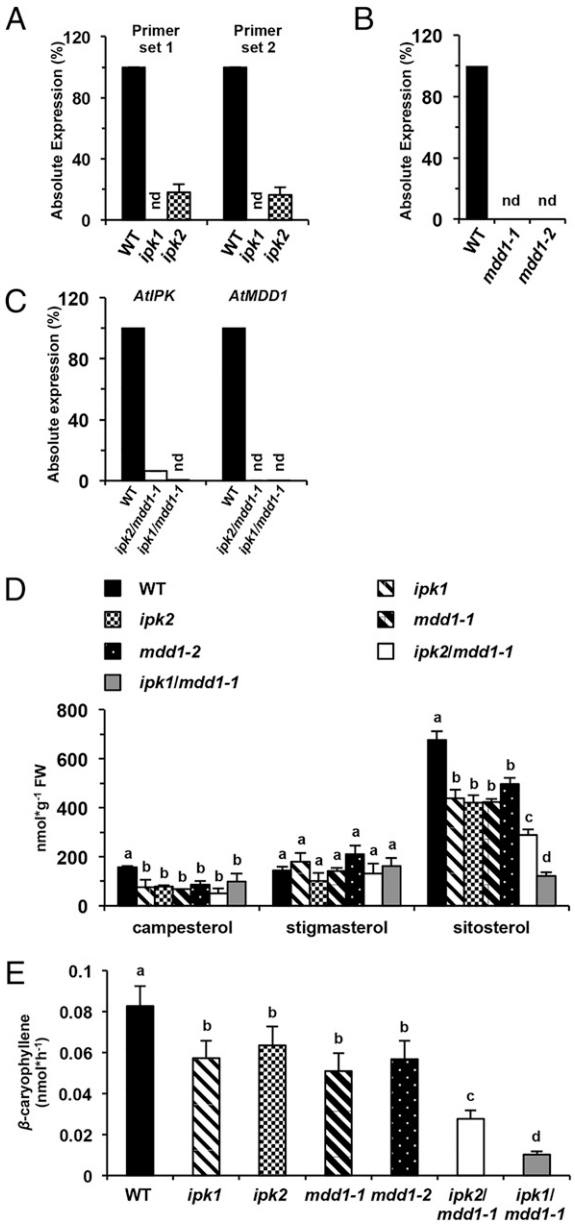

Fig. 2. Effect of *IPK* knockout on sterol and sesquiterpene production in the wild-type (ecotype Col-0) and *mdd1 Arabidopsis* background. (*A*) *AtIPK* transcript levels in wild-type and *ipk* mutants determined by qRT-PCR (means ± SEM, $n = 3$ biological replicates) using primer sets 1 and 2 located 5′ and 3′, respectively, of the T-DNA insertions (Fig. S2). (*B*) *AtMDD1* transcript levels in wild-type and *mdd1* mutants (means ± SEM, $n = 3$ biological replicates). (*C*) *AtIPK* and *AtMDD1* transcript levels in wild-type and *ipk*/*mdd1* double mutants determined by qRT-PCR (means ± SEM, $n = 3$ biological replicates). (*D*) Sterol levels in 8-d-old seedlings of wild-type, *ipk*, *mdd1*, and *ipk*/*mdd1* mutant lines. (*E*) Emission of β-caryophyllene from flowers of wild-type, *ipk*, *mdd1*, and *ipk*/*mdd1* mutant lines. Floral volatiles were collected from 50 inflorescences and analyzed by GC-MS. Data are means ± SEM ($n \geq 5$). Letters indicate (a) no and (b–d) statistically significant differences in mutant lines relative to wild-type (b, $P < 0.05$; c, $P < 0.001$; d, $P < 0.0005$, Student's *t* test); nd, not detected.

Analysis of recombinant FPPS activity in the presence of increasing concentrations of IP and DMAP revealed that AtFPPS1 is indeed inhibited by IP, with a relative IC_{50} value of 99 ± 2 μM (Fig. 4*A*) and DMAP with a relative IC_{50} value of 94 ± 2 μM (Fig. 4*D*). Further analysis revealed that AtFPPS1 inhibition by IP is competitive with respect to IPP and noncompetitive with respect to DMAPP (Fig. 4 *B* and *C*), whereas inhibition by DMAP is competitive with respect to DMAPP and noncompetitive with respect to IPP (Fig. 4 *E* and *F*). Thus, the observed decrease in the MVA-derived terpenoids in *ipk* mutant plants (Fig. 2 *D* and *E*) may result from the inability to convert IP/DMAP to their corresponding diphosphates and inhibition of AtFPPS1 activity by IP/DMAP. This decrease in terpenoids may then, in part, be accounted for by feed-forward regulation of FPPS activity by IP/DMAP inhibition of AtFPPS1. It should be noted that IP and DMAP could work independently because they interact with different binding sites in the active site of FPPS (23). Taken together, these results suggest that *in planta* IPK may not only be responsible for IP/DMAP recycling, but also for controlling the ratio of IP to IPP and DMAP to DMAPP, thus

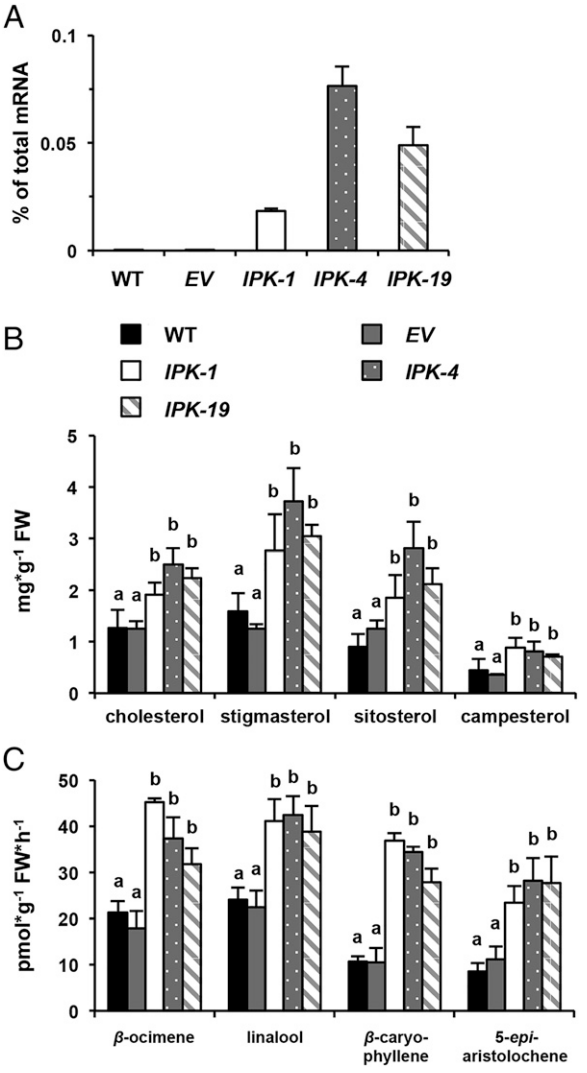

Fig. 3. Effect of *AtIPK* overexpression on terpenoid formation in tobacco. (*A*) *AtIPK* transcript levels in wild-type and transgenic tobacco lines (empty vector control *EV*, *IPK-1*, *IPK-4*, and *IPK-19*) determined by qRT-PCR (means ± SEM, $n = 3$ biological replicates). (*B*) Levels of sterols in young leaves of tobacco wild-type, *EV*, and *AtIPK* overexpressing lines. (*C*) Emission of monoterpenes (β-ocimene and linalool) and sesquiterpenes (β-caryophyllene and 5-*epi*-aristolochene) from leaves of wild-type, *EV*, and *AtIPK* overexpressing lines. Data are means ± SEM ($n \geq 3$). Letters indicate (a) no and (b) statistically significant differences in *AtIPK* overexpressing lines relative to tobacco *EV* transgenic plants ($P < 0.05$, Student's *t* test).

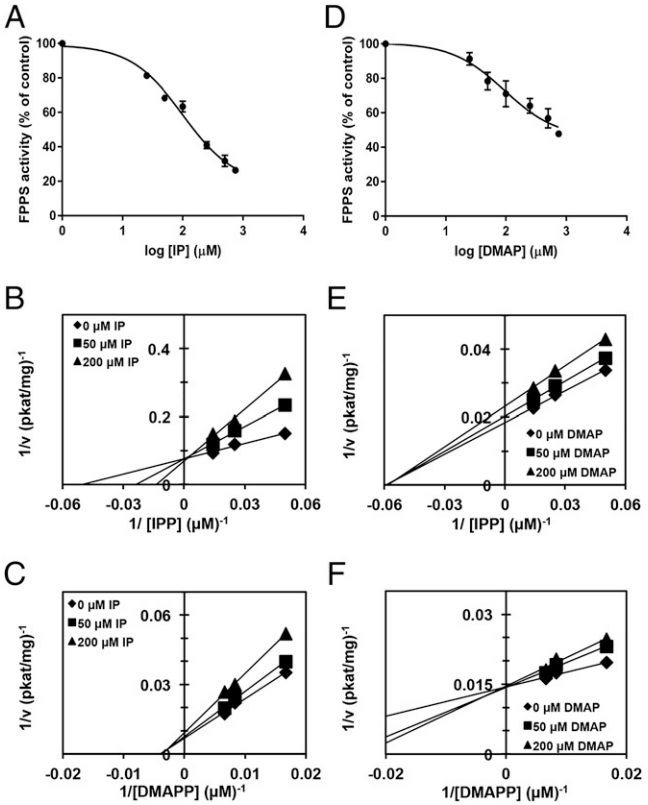

Fig. 4. Inhibitory effect of IP and DMAP on FPPS prenyltransferase activity. IC_{50} determination for inhibition of recombinant AtFPPS1 (measured with IPP and DMAPP substrates) in the presence of increasing concentrations of IP (*A*) and DMAP (*D*). Assays were performed in triplicate and data are shown as means ± SD. (*B* and *C*) Kinetic analysis of the inhibition of AtFPPS1 by IP ($n = 5$). Shown are double-reciprocal plots of initial velocities in the presence of different concentrations of IP, with varied IPP and fixed DMAPP concentrations (*B*), or fixed IPP and varied DMAPP concentrations (*C*). (*E* and *F*) Kinetic analysis of the inhibition of AtFPPS1 by DMAP ($n = 5$). Shown are double-reciprocal plots of initial velocities in the presence of different concentrations of DMAP, with varied IPP and fixed DMAPP concentrations (*E*), or fixed IPP and varied DMAPP concentrations (*F*).

playing a central role in regulating carbon flux toward FPP-derived terpenoid products.

IPK also Contributes to Formation of GPP-Derived Terpenoids in Plastids. Unexpectedly, *AtIPK* overexpression in tobacco enhanced not only emission of sesquiterpenes but also led to almost twofold increases in plastid-synthesized monoterpenes, including β-ocimene and linalool (Fig. 3*C*). Previously, it had been shown that although in plants the MVA and MEP pathways act in parallel in separate subcellular compartments (Fig. 1), there is metabolic cross-talk between these pathways that allows IPP, and to lesser extent DMAPP, exchange from the cytosol to the plastid, and vice versa (1, 2, 24). To determine the contribution of the cytosolic MVA and plastidic MEP pathways to formation of sesquiterpenes and monoterpenes, tobacco leaves were treated with lovastatin and fosmidomycin, specific inhibitors of the key pathway enzymes 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) of the MVA pathway and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) of the MEP pathway, respectively (Fig. 1). In wild-type, sesquiterpene formation was primarily supported by the MVA pathway and monoterpene formation by the MEP pathway, with the respective parallel pathway contributing to a lesser extent (Fig. 5). Surprisingly, despite its cytosolic localization, *AtIPK* overexpression increased the contribution not only of the MVA pathway to both monoterpene and sesquiterpene formation, but also of the plastidic MEP pathway to monoterpenes and sesquiterpene levels. Thus, *AtIPK* overexpression increases formation of terpenes irrespective of the metabolic route providing the C5-IPP/DMAPP precursors necessary for their biosynthesis. Our results also suggest that both the MVA and MEP pathways contribute to the IP/DMAP pool, likely formed by phosphatase-dependent IPP/DMAPP catabolism. IPP and DMAPP formed by the MEP pathway can be either exported from plastids via unknown plastidial transporters (24–26)

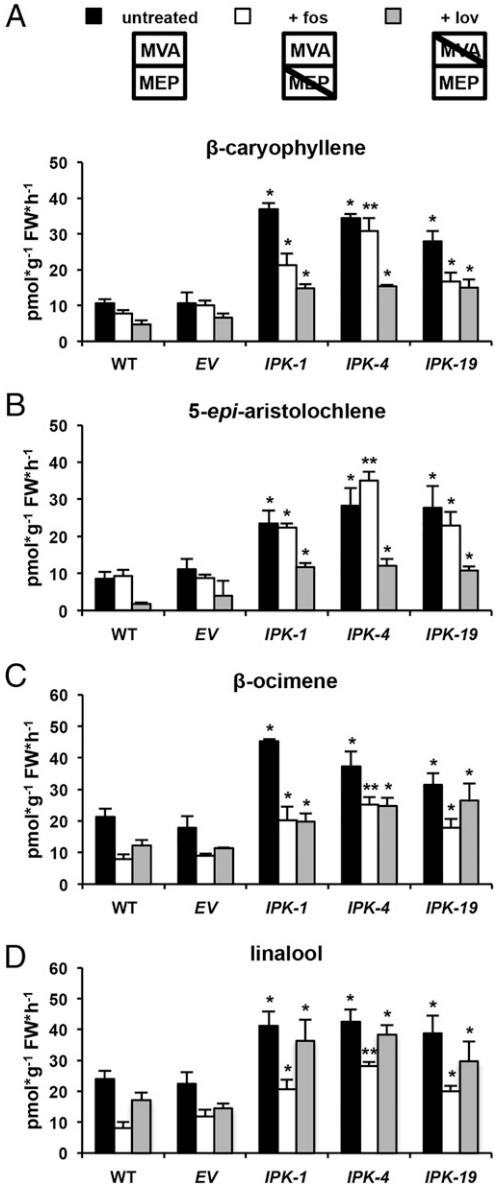

Fig. 5. Effect of MVA and MEP pathway-specific inhibitors on mono- and sesquiterpene formation in *AtIPK* overexpressing tobacco lines. Emission of sesquiterpenes β-caryophyllene (*A*) and 5-*epi*-aristolochene (*B*), and monoterpenes β-ocimene (*C*) and linalool (*D*) from leaves of tobacco wild-type, and transgenic tobacco lines (empty vector control *EV*, *IPK-1*, *IPK-4*, and *IPK-19*) in the presence of the MEP pathway inhibitor fosmidomycin (+fos) or the MVA pathway inhibitor lovastatin (+lov). Volatiles were collected from leaves by a closed-loop stripping method and analyzed by GC-MS. Data are means ± SEM ($n \geq 5$). Statistically significant differences in *AtIPK* overexpressing lines relative to the corresponding transgenic empty vector control, *EV*, across treatments are shown (*$P < 0.01$ and **$P < 0.005$, Student's *t* test).

and then subjected to cytosolic dephosphorylation, or dephosphorylated in plastids (13) and then exported to the cytosol (Fig. 1). In the cytosol, IPK phosphorylates IP/DMAP back to IPP/DMAPP, thus providing an IP/DMAP reactivation mechanism by returning it to an active IPP/DMAPP pool for utilization in the cytosol for sesquiterpenes formation and via cross-talk for monoterpene production in plastids.

Concluding Remarks

Our results show that IPK is a new member of the terpenoid metabolic network in plants and it supports terpenoid biosynthesis by increasing the pool of IPP/DMAPP through IP/DMAP recycling and by relaxing FPPS inhibition by IP/DMAP. IPK provides access to a new metabolically available and previously unknown source of terpenoid-based carbon, thus contributing to the biosynthesis of downstream terpenoid products. The work presented here further suggests that IP/DMAP formation is likely an adaptive trait for regulating carbon flow through the MVA and MEP biosynthetic pathways and not just the unintended consequence of general phosphatase activity. Interestingly, *AtIPK* overexpression in tobacco resulted in similar enhancing effects on sterol biosynthesis (Fig. 3*B*), as did the constitutive expression of *HMGR*, catalyzing a rate-limiting step in the MVA pathway (27, 28). Although increasing carbon flux through the MVA pathway by *HMGR* overexpression had no effect on sesquiterpene formation (27), accessing an existing IP/DMAP pool via *IPK* overexpression increased not only sesquiterpene but also monoterpene levels (Fig. 3*C*). Whereas the role of metabolite repair in metabolism and its potential implementation in metabolic engineering was recently proposed (29), our results present, to our knowledge, the first successful example of using an endogenous metabolite recycling strategy for increasing terpenoid formation. Such an approach is especially attractive considering the limitations of using the cytosolic MVA pathway for high yield terpenoid production in plants (30–32). Thus, our results open a new target for metabolic engineering that, in combination with efforts to increase fluxes through upstream and downstream terpenoid metabolic pathways, will result in high production of economically important terpenoid compounds.

Materials and Methods

A. thaliana ipk1 (SALK_099454), *ipk2* (WiscDsLox461-462B), *mdd1-1* (SAIL_191_A07), and *mdd1-2* (SAIL_807_F03) T-DNA insertion lines were obtained from the *Arabidopsis* Biological Resource Center. Isolation of homozygous *Arabidopsis AtIPK* and *AtMDD1* mutants and generation of their double-mutants, as well as transgenic *Nicotiana tabacum* cv. Xanthi plants overexpressing *AtIPK*, were performed as described in *SI Materials and Methods*. Transcript level determinations and targeted metabolic profiling of transgenics were carried out according to protocols described in *SI Materials and Methods*. Activities of AtMDD1 and AtMDD2 were analyzed by the lactate dehydrogenase-pyruvate kinase coupled assay (*SI Materials and Methods*). To measure FPPS1 inhibition by IP and DMAP, prenyltransferase assays were used (*SI Materials and Methods*). Subcellular AtIPK localization was performed by infiltrating 3-wk-old *N. tabacum* leaves with *Agrobacterium* (strain LBA 4404) containing AtIPK GFP constructs and peroxisomal or mitochondrial markers (*SI Materials and Methods*). See Table S1 for primers used.

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