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Review Article

Jasmonates are signals in the biosynthesis of secondary metabolites — Pathways, transcription factors and applied aspects — A brief review

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ABSTRACT

Jasmonates (JAs) are signals in plant stress responses and development. One of the first observed and prominent responses to JAs is the induction of biosynthesis of different groups of secondary compounds. Among them are nicotine, isoquinolines, glucosinolates, anthocyanins, benzophenanthridine alkaloids, artemisinin, and terpenoid indole alkaloids (TIAs), such as vinblastine. This brief review describes modes of action of JAs in the biosynthesis of anthocyanins, nicotine, TIAs, glucosinolates and artemisinin. After introducing JA biosynthesis, the central role of the SCF^{COI1}-JAZ co-receptor complex in JA perception and MYB-type and MYC-type transcription factors is described. Brief comments are provided on primary metabolites as precursors of secondary compounds. Pathways for the biosynthesis of anthocyanin, nicotine, TIAs, glucosinolates and artemisinin are described with an emphasis on JA-dependent transcription factors, which activate or repress the expression of essential genes encoding enzymes in the biosynthesis of these secondary compounds. Applied aspects are discussed using the biotechnological formation of artemisinin as an example of JA-induced biosynthesis of secondary compounds in plant cell factories.

Introduction

Jasmonates (JAs) have been identified as essential signals in numerous stress responses and distinct developmental processes of plants. The first detected components of JA signaling pathways were changes in gene expression manifested by the strong accumulation of JA-induced proteins in barley (rev. by [1] and formation of defense proteins, such as proteinase inhibitors (PINs), in wounded tomato leaves [2]. Another early observed JA-induced process was the formation of secondary compounds, such as benzophenanthridine alkaloids upon elicitation of cell suspension cultures of *Eschscholtzia californica* or *Rauvolfia canescens* [3]. In this paper [3], a link between an endogenous rise of JA levels and JA-induced formation of secondary compounds was shown for the first time. Since these early days of JA research, there has been a rapid increase in publications dealing with JA-related aspects (see review in [1]), preferentially in aspects of biosynthesis, accumulation and biotechnological application of secondary compounds. Nearly all biosynthetic pathways leading to secondary metabolites, such as anthocyanins, nicotine, terpenoid indole alkaloids (TIA), glucosinolates (GS), benzophenanthridine alkaloids or flavonoids, were found to be induced by applied JA or processes triggering an endogenous increase in JA. Pathway analysis was carried out by cloning involved genes, including analysis of the corresponding promoters, and studying regulatory aspects, involved transcription factors (TFs), as well as cell and tissue-specificity . This research has been extensively reviewed, e.g., [4–7] including aspects of jasmonates [8–11] or synthetic biology [12].

In the following text, we briefly review the formation of a few secondary metabolites, such as anthocyanin, nicotine, TIA, artemisinin and GS, with an emphasis on the role of JA, describe the TFs involved and discuss some applied aspects using artemisinin as an example. Secondary metabolites are formed using primary metabolites as building blocks. Thus, the role of JA is discussed in terms of (i) JA perception and the core signaling complex, (ii) its role in reprogramming primary metabolism, and (iii) its role in the synthesis of secondary metabolites. Usually, these signaling cascades occur in a tissue- and cell-specific manner because some secondary metabolites are formed exclusively in specialized cells, such as trichomes, or under specific conditions of growth in cell suspension cultures. The following review will be a brief overview for some JA-inducible secondary compounds. Owing to space limitations, only key references are included in this brief review.

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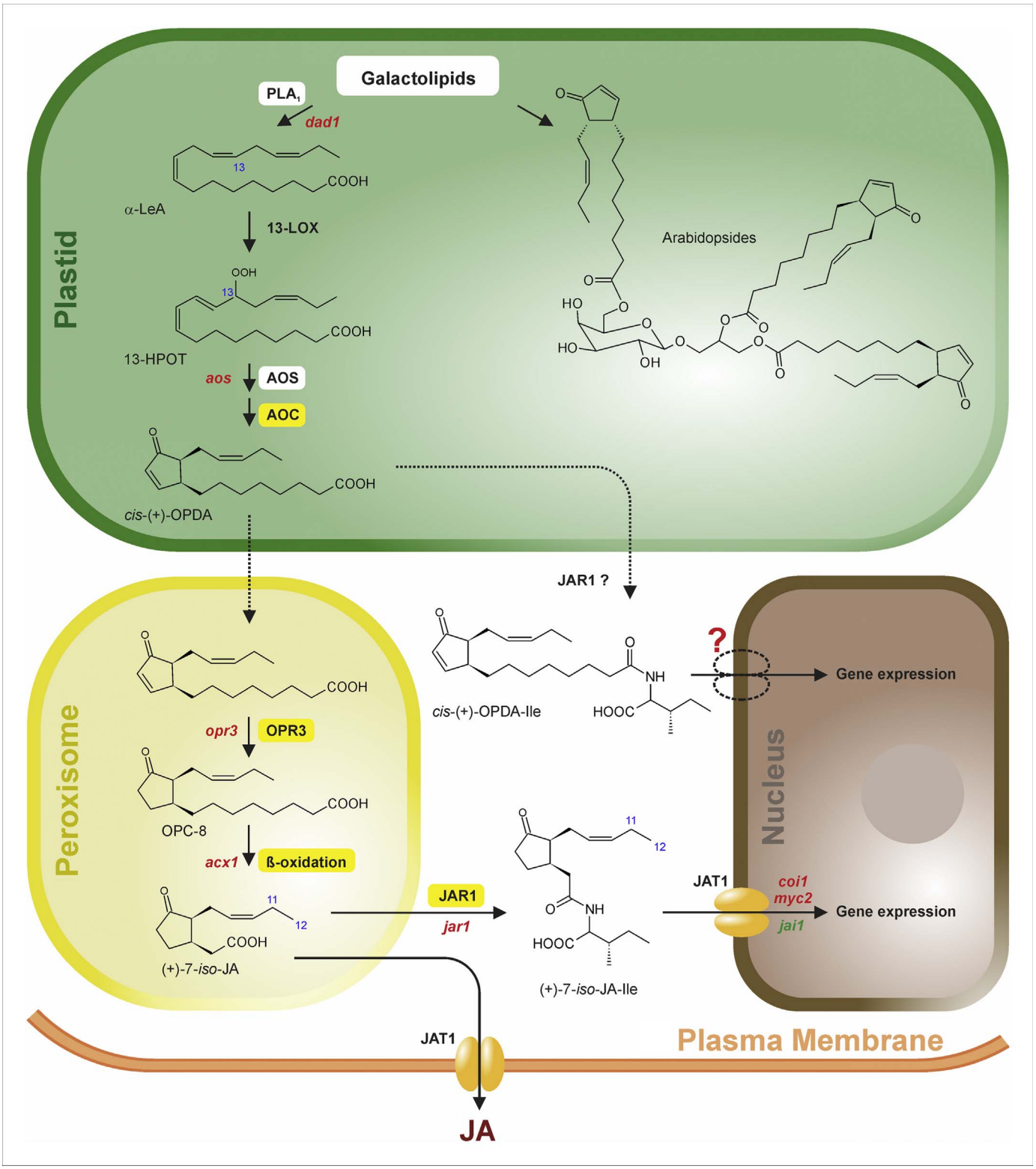

Fig. 1. Synthesis of JA/JA-Ile from α-linolenic acid generated from galactolipids and their intracellular transport.
Mutants known for Arabidopsis (red) or tomato (green) are indicated.
Abbreviations for compounds: α-LeA – α-linolenic acid, 13-HPOT – (13S)-hydroperoxyoctadecatrienoic acid, *cis*-(+)-OPDA – *cis*-(+)-12-oxophytodienoic acid; OPDA-Ile - *cis*-(+)-12-oxophytodienoic acid isoleucine conjugate; JA - jasmonic acid; JA-Ile - Jasmonic acid isoleucine conjugate; OPC-8 - 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid
Abbreviations for enzymes/proteins: *acx1, acyl CoA-oxidase1*; AOC – allene oxide cyclase; AOS – allene oxide synthase; *coi1, coronatine insensitive1*; *dad1, delayed anther dehiscence1*, *jai1–jasmonic acid insensitive1;* JAR1 – JA-amino acid synthetase; JAT1–jasmonic acid transporter1, 13-LOX – 13-lipoxygenase, *myc2, bHLHzip transcription factor MYC2*; OPR3 – OPDA reductase3; PLA_1–phospholipase A1. (redrawn based on [11] with permission) (colour in print).

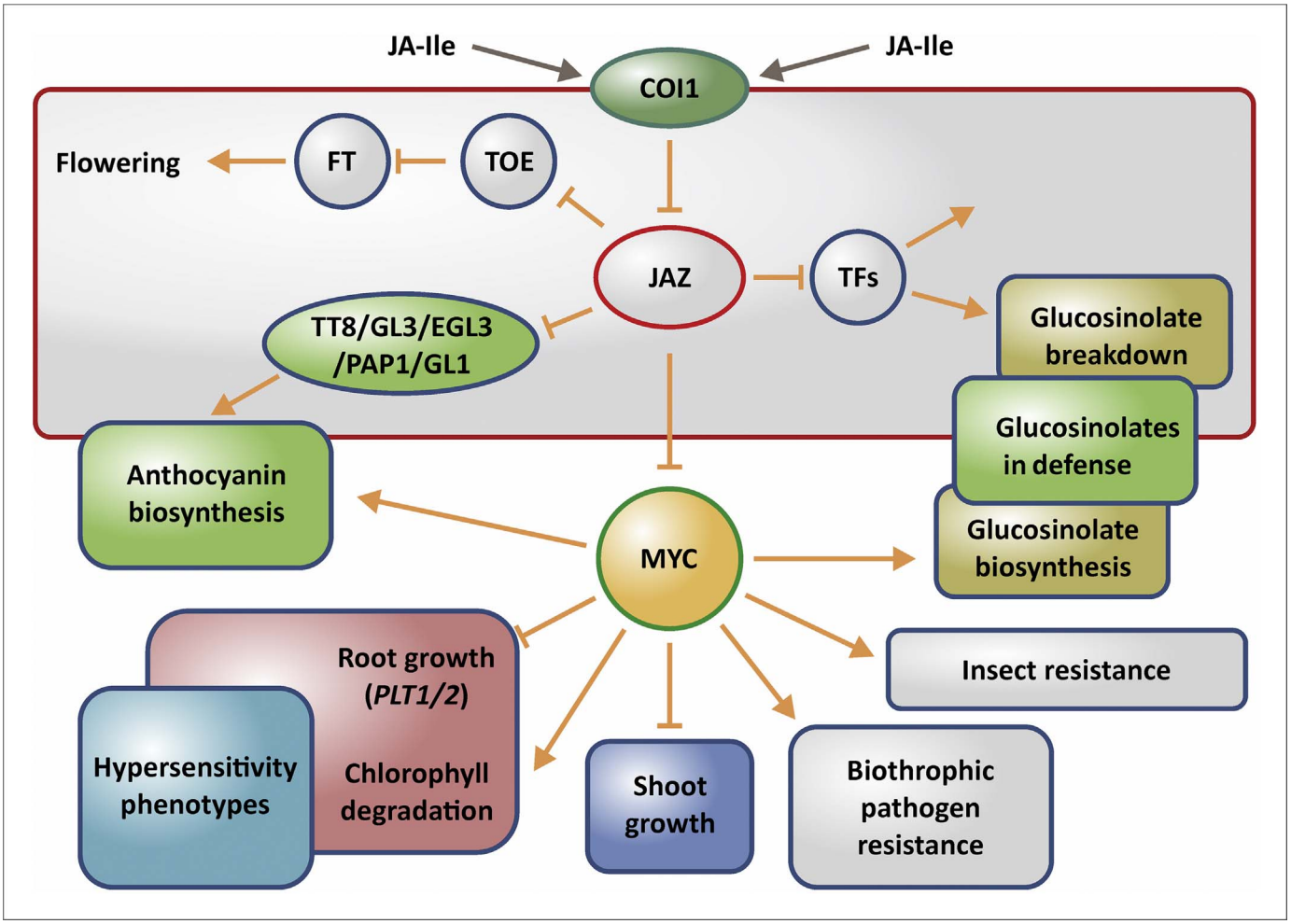

Fig. 2. Model of JA signaling in synthesis and degradation of glucosinolates and synthesis of anthocyanins as well as other JA-Ile dependent processes showing the modular action of JAZs with diverse TFs in a MYC independent manner (framed) or their action via MYCs leading to negative and positive regulation by MYCs in (redrawn from [33] open access under CC-BY license).

JA biosynthesis and perception

An endogenous rise in JA may be triggered by numerous environmental cues, such as abiotic or biotic stresses, including wounding by herbivores or attack by pathogens, but also following developmental signals. A rapid increase of JA levels upon wounding of leaves occurs within minutes owing to the presence of preexisting enzymes of JA biosynthesis, followed by expression of genes encoding enzymes of JA biosynthesis within 2–6 h. An early and transient rise in JA and its precursor *cis*-(+)-12-oxo-phytodienoic acid (OPDA) also takes place upon elicitation of cell suspension cultures leading to accumulation of alkaloids [3,13]. JA biosynthesis is initiated by the oxygenation of α-linolenic acid released from chloroplast membranes by the phospholipase A1 (PLA1), followed by sequential action of an allene oxide synthase (AOS) and an allene oxide cyclase (AOC), leading to formation of *cis*-(+)-OPDA within the chloroplast (Fig. 1). In the AOC-catalyzed step, the specific stereo-isomeric form of OPDA is synthesized, leading to the naturally occurring JA ((+)-*7*-JA-iso-JA; (*3R,7S*)-JA). Upon transport of OPDA into peroxisomes via an ion trapping mechanism and/or the ABC transporter COMATOSE, reduction of the cyclopentenone ring by an OPDA reductase3 (OPR3) to OPC8 (3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid) takes place. Subsequently, OPC8 is shortened in the carboxylic acid side chain by three rounds of the fatty acid ß-oxidation machinery, which includes acyl-CoA oxidase1 (ACX1). (+)-*7*-JA-iso-JA is transported by an unknown mechanism into the cytosol, where its conjugation with amino acids, preferentially isoleucine, takes place catalyzed by the jasmonoyl amino acid conjugate synthase (JAR1). (+)-*7*-JA-iso-JA-Ile is the ligand of the JA-receptor complex (see below) [14,15]. Transport of the JAR1 product into the nucleus takes place by the JA transporter1 (JAT1), a member of the ABC transporter family [16,17]. In the nucleus, JA-Ile is perceived by the SCF^{COI1}-JAZ co-receptor complex, causing expression of JA-Ile-inducible genes (see below). JAT1 has dual specificity as it also mediates the export of (+)-*7*-JA-iso-JA via the plasma membrane [16]. The intercellular transport of JA or JA-Ile has been suggested to occur by the multifunctional glucosinolate transporter GTR1, which has also been shown to transport JA-Ile and gibberellin [18,19]. Minor activity of JAR1 may lead to OPDA-Ile (Fig. 1), a naturally occurring OPDA derivative [20] which exhibits biological activity similar to OPDA in a JA- and COI1-independent manner [21].

Besides transport mechanisms, the homeostasis of JA and JA-Ile is sustained by metabolic conversion, including conjugation with other amino acids, hydroxylation, carboxylation, decarboxylation, methylation or sulfation (for details cf. [11,22]. Depending on stress responses or developmental cues, a pattern of active, partially active or inactive JA compounds is generated.

In 2007, JASMONATE ZIM DOMAIN (JAZ) proteins were identified as a family of 12 members [23–25]. They are active as negative regulators of JA-induced gene expression (26, and for review see [27,28]. Subsequently, the core signaling module was found to consist of the ubiquitin-proteasome degradation machinery with the Skp1/Cullin/F-box (SCF^{COI1}) complex active as E3 ubiquitin ligase interacting with a

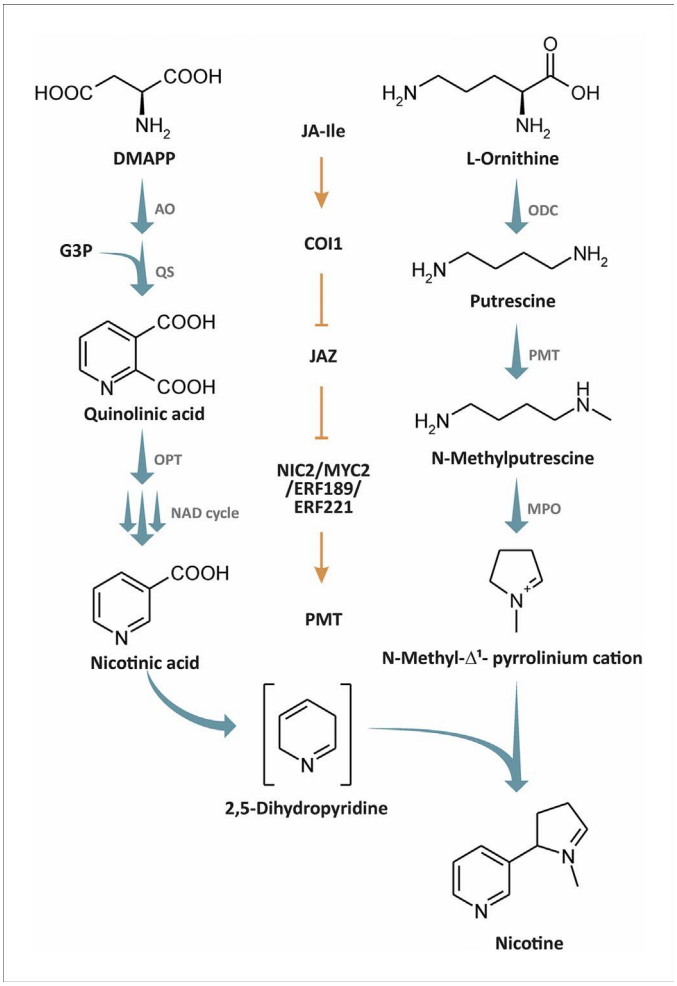

Fig. 3. Biosynthesis of nicotine (A) takes place in two branches with enzymes (B). The pyrrolidine ring is formed from L-ornithine by ornithine decarboxylase (ODC), *N*-putrescine methyltransferase (PMT) and *N*-methylputrescine oxidase (MPO). The nicotinic acid/pyridine ring is formed from dimethylallylpyrophosphate (DMAPP) via aspartate oxidase (AO), quinolinate synthase (QS) and quinolinate phosphoribosyl transferase (QPT) followed by enzymes of the NAD cycle.

JAZ protein as a target [15,27]. The interaction takes place via COI1, a JA-specific F-box protein [29]. JAZ proteins are repressors of positively acting transcriptions factors (TFs) of the basic helix loop helix (bHLH) IIIe subgroup, such as MYC2, which bind to JA responsive elements (G-boxes) of JA-inducible genes. Repression of MYC activity by JAZ can take place directly by JAZ-mediated inhibition of interaction of MYC with MEDIATOR25 (MED25), a component of the co-activator complex [30]. Additional repression can take place by the co-repressor TOPLESS (TPL) and adaptor proteins such as NOVEL INTERACTORS OF JAZ (NINJA) [26] via histone decacetylase 6 and 19 (HDA6,HDA19), which bind directly to DNA leading to chromatin remodeling (for review see [9,11,31,32].

Reprogramming of primary metabolism

Terpenoids are composed of carbon units, such isopentenyl pyrophosphate generated via photosynthetic activities and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway as well as the mevalonate (MVA) pathway, whereas nicotine, anthocyanins, glucosinolates and TIA are synthesized from proteinogenic amino acids. Many of the reactions needed for formation of these precursor molecules are JA-inducible. Initially, involvement of JA was proved by expression analyses of genes involved in precursor formation as well as in the complete biosynthetic pathway. Recent data support a hierarchy of JA-mediated transcriptional regulation of JA-regulated genes, including genes involved in the formation of secondary compounds [33]. Here, activators, repressors and transcription factors (TFs) interact and function as modules, allowing specific output by seemingly redundant signaling components, such as repressors like JAZs or TFs like MYCs. Recently, generation of an octuple mutant *jazQmycT* allowed dissection of such modules [33]. In that study, the genes *JAZ1/3/4/9/10* and *MYC2,3,4,* were mutated and resulting mutant plants were inspected by transcriptomic, proteomic and metabolomic analyses of glucosinolate biosynthesis and degradation, anthocyanin formation and other JA-Ile dependent processes (Fig. 2). The data indicated a dominant role of a JAZ-repressible transcriptional hierarchy of distinct modules of JA-Ile signaling components, which links primary metabolism occurring during photosynthesis and growth with the formation of defense compounds, such as the secondary metabolites anthocyanins and glucosinolates. The link between primary and secondary metabolism has been frequently analyzed by metabolic flux analyses, e.g., [34].

Synthesis of secondary metabolites

Nicotine

Nicotine, the main alkaloid of *Nicotiana* species, is formed from ornithine and putrescine as well as from quinolinic acid via nicotinic acid (Fig. 3). The JA-induced nicotine biosynthesis pathway takes place exclusively in roots, and nicotine is subsquently transported into leaves, where it accumulates in glandular trichomes. The enzymatic steps of nicotine biosynthesis is known since the 1990s [35]. Since then, most genes encoding enzymes in nicotine biosynthesis have been shown to be regulated transcriptionally by JA in a COI1-JAZ −dependent manner [36] including the TFs NbbHLH1 and NbbHLH2, which are close homologues of the master regulator MYC2 [37] (Fig. 3) (Table 1). NtMYC2 and NtAP2/ERFs are members of the *NIC2* locus [38], including ERF189 and ORC1/ERF221. All structural genes active in

Table 1
JA-induced TFs and activated genes involved in biosyntheses of nicotine, glucosinolates (GS), terpenoid indole alkaloids (TIA), artemisinin and anthocyanins.

JA-induced metabolite	JA-induced TF involved	activated gene (s)	plant species
Nicotine	AP2/ERF		
	bHLH1, bHLH2	most	*N. benthamiana*
	ORC1/ERF221	*PMT*	*N. tabacum*
	JAP1/ERF10	*NIC1*	*N. tabacum*
	ERF189, ERF1		*N. tabacum*
	ERF179, ERF32	*NIC2*	*N. tabacum*
Glucosinolates (GS)	bHLH IIIe		
	MYC2, MYC3, MYC4	*CYP79B3, BCAT4*	*A. thaliana*
	R2R3-MYB		
	MYB34, MYB51, MYB122	indole GS	*A. thaliana*
	MYB28, MYB29, MYB76	aliphatic GS	*A. thaliana*
Terpenoid indole alkaloids (TIA)	bHLH1, bHLH2	*PMT, MPO*	*C. roseus*
	MYC2a, MYC2b	*TDC*	*C. roseus*
	BIS1, BIS2	iridoid pathway	*C. roseus*
	ORCA3,4,5, ORCA2	*STR*	*C. roseus*
Artemisinin	AP2/ERF		
	ERF1, ERF2	*ADS*	*A. annua*
Anthocyanins	bHLH IIIf		
	TT8, GL3, EGL3		*A. thaliana*
	bHLH IIIe		
	MYC2		*A. thaliana*
	bHLH IIId		
	JAM1, JAM2, JAM3		*A. thaliana*
	MYB		
	GL1, MYBL2		*A. thaliana*
	MYB115, MYB134		*Populus spp.*

nicotine biosynthesis are JA-inducible via the *NIC2*-encoded TFs [39]. ERF189 can bind to a GCC box element within the promoter of the putrescine *N*-methyltransferase, the first committed step in nicotine biosynthesis. Its over-expression leads to elevated nicotine levels in transgenic hairy roots [5,38]. ORC1 is a close homologue of ORCA3 of *Catharanthus roseus* active in TIA biosynthesis (see below). It is interesting to note, that (i) these homologue TFs of two different pathways (nicotine biosynthesis and TIA biosynthesis) evolved independently [5,38], and (ii) the regularly occurring induction of biosynthesis of secondary compounds by JA seems to indicate an evolutionary advantage given by an early established regulatory module [5]. Recently, such an evolutionary advantage has been shown by analysis of gene duplication and insertion of transposable elements in nicotine biosynthesis genes during the evolution of *Nicotiana* adaptive traits. Here, species such as *N. attenuata* have to produce a large amount of nicotine to defend attacker in nature, seeming to be the reason for an adaptive evolution of nicotine biosynthesis genes leading to high yield species [40]. Genomic insights into evolution of the nicotine biosynthesis pathway have revealed large chromosomal deletion in the *NIC2* locus, which can be used for breeding of cultivars with low-nicotine content [41].

Terpenoid indole alkaloids (TIA)

TIAs such as vincolin, vinblastin or vincristin occur in species of *Catharanthus* (*Vinca*) and are used as anti-cancer agents owing to their effects on cell division, there is much biotechnological interest in their production. TIA biosynthesis has been preferentially studied in cell suspension cultures of *C. roseus* and consists of two branches containing three pathways. Two of these pathways are the MEP pathway leading to isopentenyl pyrophosphate followed by the iridoid pathway leading to loganic acid (Fig. 4). Enzymes of both pathways are located in the internal phloem-associated parenchyma cells leading to loganic acid accumulation in epidermal cells [9]. The third pathway, the shikimate pathway, located in epidermal cells and forming tryptamine, converges with the iridoid branch through formation of strictosidine, which is converted to catharanthine and vinblastine/vincristine (Fig. 4).

The first JA-responsive TFs active in TIA biosynthesis were identified during the formation of vinblastin, a TIA of *C. roseus*. These TFs were called OCTADECANOID RESPONSIVE CATHARANTHUS ALKALOID2 (ORCA2) and ORCA3 and were characterized as members of the ERF subfamily within the AP2/ERF TF superfamily [8]. Initially, interaction was shown of ORCA2 and ORCA3 with specific sequences including GCC-boxes of target promoters of genes encoding enzymes of all branches of the TIA pathways (Fig. 4). Later, an *ORCA* gene cluster, CrORCA3,4,5, was identified [42]. Expression of the *ORCA* genes is controlled by CrMYC2, a IIIe subgroup bHLH TF and master regulator [43]. A major role of CrORCA3 was proposed based on the fact that all branches of the TIA pathways are under the control of CrORCA3. However, over-expression lines of *CrORCA3* did not show elevated TIA synthesis [44], suggesting the involvement of additional TFs. Meanwhile, an MYC2-regulated *CrORCA* gene cluster *CrORCA,3,4,5* has been identified [42]. *CrORCA4* shows functional overlap with *CrORCA3* [42]. Over-expression of *CrORCA4* led to a dramatic increase of TIA accumulation in *C. roseus* hairy roots. *CrORCAs* and *CrMYC2* are co-regulated leading to differential activation of the different branches of the TIA pathways: (i) CrMYC2/CrORCA3/4 activate the TRYPTOPHAN DECARBOXYLASE (TDC), (ii) CrORCA4 together with BIS1 (see below) activates some steps of the iridoid pathway, and (iii) CrORCA3/4/5 co-regulate together with a G-box-binding factor the strictosidine synthase (STR) gene (Fig. 4). All these TFs, CrMYC2, and CrORCA3/4/5 have to be phosphorylated for their activity, which is mediated by CrMAPK3/6 downstream of CrMAPKK1. Consequently, over-expression of CrMAPKK1 has been shown to increase expression of genes involved in the TIA pathway [42] (Fig. 4). Finally, TIA biosynthesis is regulated by two JA-inducible bHLH TFs of the subclade IVa, bHLH IRIDOID SYNTHESIS1 and 2 (BIS 1, BIS2) [45,46]. Both of them are specifically involved in the iridoid branch (Fig. 4) and are controlled by an amplification loop. Over-expression of *CrBIS1* or *CrBIS2* leads to increased expression of iridoid pathway genes and the preceding MEP pathway genes as well as increased accumulation of TIAs [45,46]. Interestingly, artemisinin (see below) has been shown to be an inducer of the expression of *ORCA3* and biosynthetic genes such as *TDC* and *STR* without affecting JA biosynthesis [47].

Anthocyanin

Anthocyanins accumulate in flowers and are involved in attraction of pollinators. In leaves, anthocyanins act as antioxidants against reactive oxygen species following abiotic stress. Any stress-induced formation of JA leads to visible anthocyanin accumulation in epidermal cells of leaves and shoots, the most obvious phenotype of JA action. This strong link between JA and secondary metabolite formation has prompted research into strategies to enhance production of secondary metabolites using JA [48].

Anthocyanin biosynthesis is part of the general phenylpropanoid pathway leading to flavonons such as naringenin *O*-glycosylated flavonons and *C*-glycosylated flavonons [49].This pathway takes place via the shikimate pathway (Fig. 5) to generate phenylalanine, which is converted via coumarate, naringenin and pelargonidin leading to anthocyanins accumulation in vacuoles. These steps include methylation and glycosylation. Most steps in anthocyanin formation are JA inducible. The key players are phenyl alanine ammonium lyase (PAL), one of the first detected JA-inducible enzymes in secondary compound formation and most sensitive to any stress, and chalcone synthase (CHS), which forms together with the chalcone isomerase flavonones from *p*-coumaric acid-CoA and three molecules of malonyl-CoA (Fig. 5). Also, many other phenylpropanoid pathways, e.g., tashinone

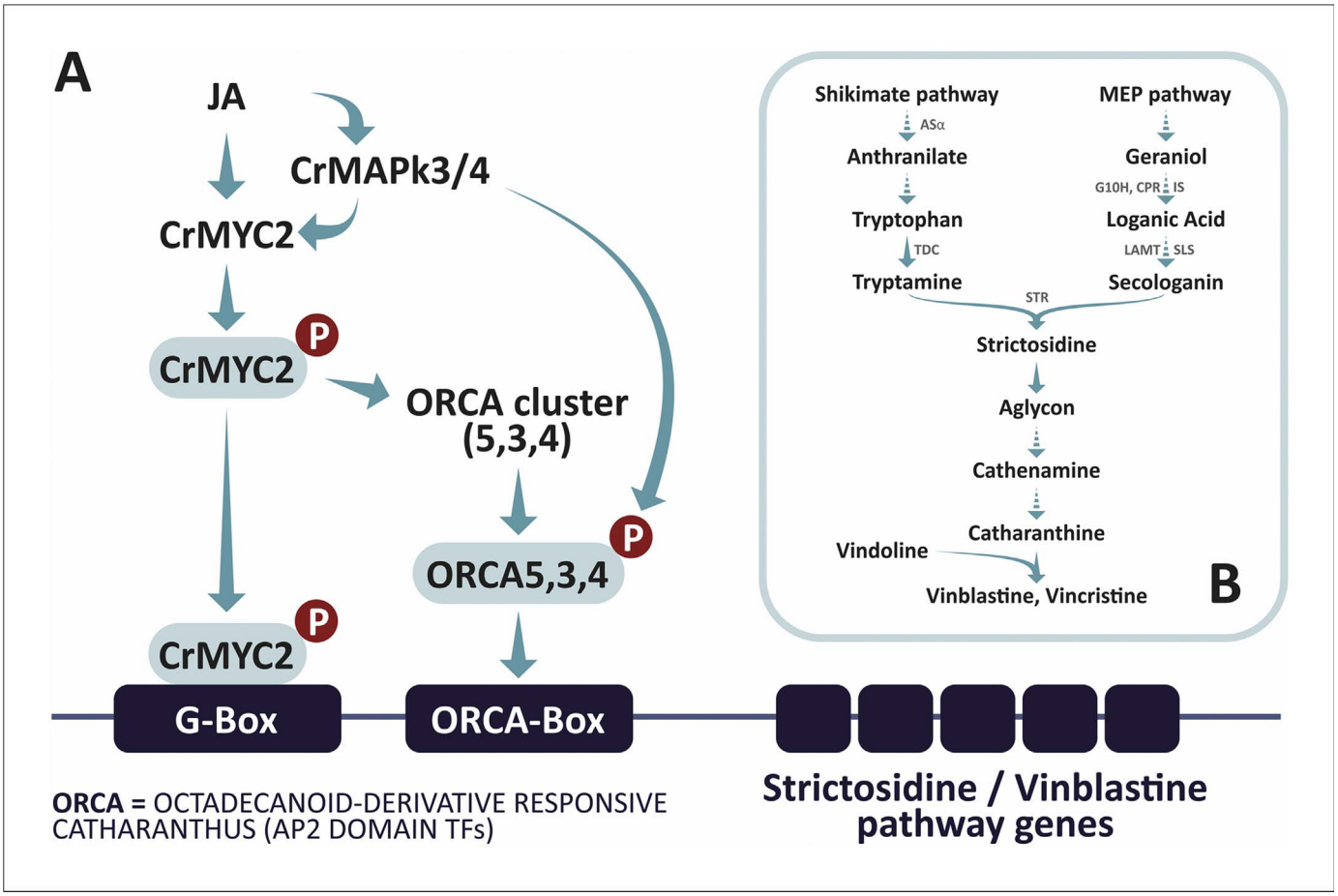

Fig. 4. Biosynthesis of terpenoid indole alkaloids (TIA) in *Catharanthus roseus* cells (A) and transcriptional and posttranscriptional regulation of the ORCA (OCTADECANOID-DERIVATIVE RESPONSIVE CATHARANTHUS ALKALOID) gene cluster and TIA pathway genes (B). ORCA5, ORCA3 and ORCA4 encoded by the ORCA gene cluster and CrMYC2 are phosphorylated by the MAP kinase cascade consisting of CrMAPKK1, CrMAPK3 and CrMAPK6. Both types of TFs activate cumulatively transcription of different members of the shikimate pathway, MEP pathway as well as the iridoid pathway leading to TIAs such as vinblastine and vincristine. Abbreviations of enzymes: AS – anthranilate synthase; TDC – tryptophan decarboxylase, CPR – cytochrome P450 reductase; IS – iridoid synthase; LAMT – loganic acid methyl transferase; SLS – secologanin synthase; STR – strictosidine synthase.

biosynthesis of *Salvia* originating from the MEP and MVA pathways, are regulated by JA in a COI1-dependent manner via MYC2 and JAZ3/JAZ9 [50,51].

Anthocyanin biosynthesis is mainly regulated by WD-repeat/bHLH/MYB transcriptional complexes [9]. Members of such complexes include the group III, subgroup f bHLH TFs, TRANSPARENT TESTA8 (TT8), GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), WD-repeat TF TRANSPARENT TESTA GLABRA1 (TTG1) and MYB TF GLABRA1 (GL1). The latter all TFs act as positive regulators in different steps of anthocyanin biosynthesis and are targets of JAZ proteins [52] (Fig. 5). Usually, the interaction of TFs with JAZ proteins takes place at the N-terminal JAZ-interacting domain (JID) as shown for two subgroups, bHLHd and bHLHe. However, in the case of subgroup bHLHf, the interaction takes place at the C-terminal part including the bHLH domain despite the presence of the JID domain [9,52].

The subgroups bHLHe and bHLHd are also involved in regulation of anthocyanin formation. Members of the former, such as MYC2, act as positive regulators, acting via interaction with MED25 [9,53], whereas members of the latter subgroup act as negative regulators, e.g., JA-ASSOCIATED MYC2-LIKE (JAM) TFs. JAM1, JAM2, and JAM3 carry the JID domain for interaction with JAZ but lack the canonical activation domain. JAMs and MYCs are competitors in binding to *cis*-regulator elements leading to a balance of activation and suppression of anthocyanin biosynthesis and other JA dependent processes, such as senescence [9,54].

Due to the direct interaction of JAZ proteins with bHLH TFs and MYB TFs within the WD-repeat/bHLH/MYB transcriptional complex, the assembly and function of the complex reach a dynamic balance [54]. MYBL2 represses JA-inducible TFs such as TT8 which leads to a negative regulation of anthocyanin formation [55]. A stress-induced rise in JA levels leads to cumulative de-repression of these TFs, followed by activation of expression of biosynthetic genes and strong anthocyanin accumulation.

Transcriptional regulation of anthocyanins and other members of the general flavonoid pathway via the WD-repeat/bHLH/MYB transcriptional complex are widespread among plant species. In some species, additional TFs have been detected, e.g., MYB115 and MYB134 were identified from *Populus* [56].

Further evidence for a direct link between JA and anthocyanin formation has been obtained with the thylakoid formation mutant *thf1* [57]. This mutant showed elevated levels of α-LeA, OPDA and JA, COI1-dependent up-regulation of anthocyanin biosynthetic and regulatory genes, and elevated anthocyanin accumulation.

Besides detailed verification of transcriptional regulation in the biosynthesis of anthocyanins, there are examples of post-transcriptional regulation. Recently, a proteolytic regulator of CHS, called the Kelch domain (repeat)-containing F-box (KFB) protein of CHS (KFB^{CHS}), has been characterized [58]. This KELCH domain-containing F-box protein interacts with CHS causing its ubiquitination and degradation in the JA-regulated response to UV-B stress.

It has to be mentioned here that anthocyanin formation and trichome formation are co-regulated [54,59]. Similar co-regulation has

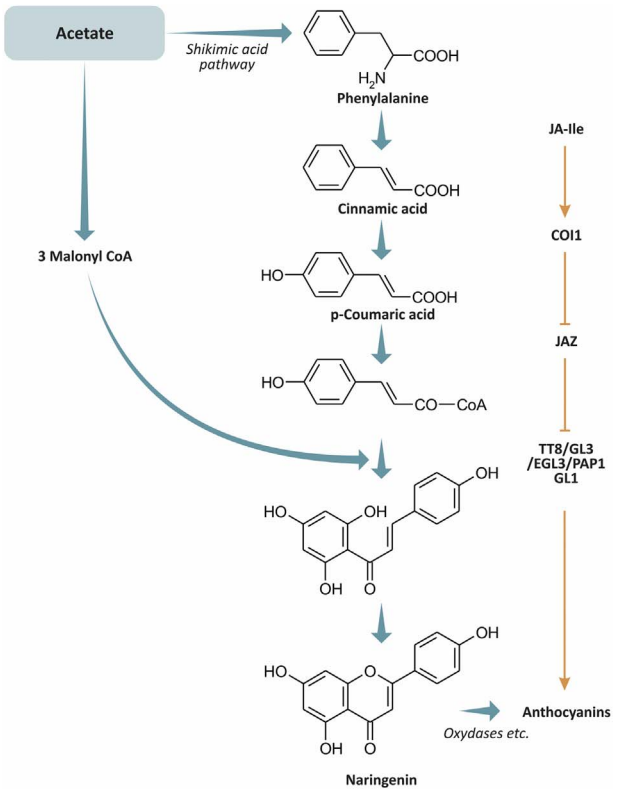

Fig. 5. Biosynthesis of anthocyanins, starting from the shikimate pathway, involves the general phenylpropanoid pathway and three moieties of malonyl-CoA to form naringenin. Perception of JA-Ile by COI1 leads to proteasomal degradation of the repressor JAZ, thereby allowing activity of anthocyanin specific transcription factors, such as TT8; GL3; EGL3; PAP1; GL1 [52].

been found for other secondary metabolites and used for metabolic engineering, e.g., terpene biosynthesis by a trichome-specific TF [60]. Glandular trichomes have been regarded as a cellular factory for secondary compounds [61].

Glucosinolates (GS)

GS occur in species of the order Brassicales, such as cabbage, horseradish, broccoli and mustard. They are secondary compounds responsible for the flavor and aroma of cruciferous vegetables with activity in plant defense against insects and pathogens, and auxin homeostasis, as well as anticancer activity in humans [62]. They are formed from the aliphatic amino acid methionine and aromatic compounds chorismate and tryptophan (Fig. 6). Both pathways converge by aldoxime formation, which is converted subsequently via S-alkyl-thiohydroximate, thiohydroximate and desulfo-glucosinolate to sulfated glucosinolates. Most of the genes encoding enzymes in glucosinolate biosynthesis are JA-inducible, including ASA1 (anthranilate synthase), the cytochome P450 enzymes CYP79F1/2, CYP79B2/3, the C-S-lyase SUR1, the glucosyl transferase UGT74B1 as well as the sulfotransferases SOT16/17/18 describing JA-regulation of GS biosynthesis via MYC2, MYC3 and MYC4 . Consequently, MYB type TFs, which are active in glucosinolate biosynthesis, are targets of JA-Ile-mediated COI1/MYC2/JAZ signaling modules. These MYB type TFs are MYB28 and MYB29, which activate MAM1-3, CYP79F1/2, C-S lyase (SUR1), SOT17/18, whereas MYB51, MYB34, and MYB122 activate *TSB1, CYP79B2/3, CYP83B1, UGT74B1* and *SOT16* (Fig. 6). Upon formation of methionine and tryptophan within the chloroplast, many of the oxidative reactions of the glucosinolate core pathway, downstream of aldoxime, take place in the ER-cytosol interface, with subsequent sequestration in the vacuole or transport within the phloem [62]. Among the final products, the aliphatic glucosinolates (AG) and indole glucosinolates (IG), are the phytoalexins camalexin, brassinin, and cyclobrassinin. Owing to their role in plant defense and human health, as well as potential development of anticancer strategies, metabolic engineering of plants has been performed. In the case of the cabbage phytoalexin brassinin, a minimal set of genes encoding enzymes required for its biosynthesis has been heterologuously expressed and shown to induce brassinin formation in *N. benthamiana* [63]. Interestingly, methyl JA can elevate levels of IG compared to those of AG in a cultivar-specific manner, reducing goitrogenic compounds in pak choi [64].

Applied aspects – artemisinin production

Artemisinin is a sesquiterpene that accumulates at high level in glandular trichomes of *Artemisia annua*. Its synthesis takes place by the chloroplast MEP pathway and cytosolic MVA pathway via farnesyl pyrophosphate and amorphadiene, generating artemisinic acid and dihydroartemisinic acid (Fig. 7). In the formation of dihydroartemisinic acid, CYP71AV1 is involved. Its encoding gene is activated JA-dependently via AaMYC2 and the TF GLANDULAR TRICHOME-SPECIFIC WRKY1 (AaGSW1), a positive regulator of artemisinin biosynthesis [5], [65a,b]. Finally, dihydroartemisinic acid is photo-oxidatively converted to artemisinin in the trichome subcuticular space. Recently, transcriptomic analysis of JA-treated *A. annua* revealed light- and JA-dependent expression of genes involved in artemisinin biosynthesis [66]. Increased artemisinin formation has been found upon over-expression of *AOC* in *A. annua* [67].

A. annua has been sequenced and much effort has been applied to developing biotechnology strategies for artemisinin production. This effort was largely prompted by its high activity against malaria. Nearly 200 million people are infected each year by malaria. Following its initial isolation and identification as an anti-malaria agent by the Chinese scientist Youyou Tu in the 1970s (Nobel Prize in 2015 together with W.C. Campbell and S. Omura), the main source of artemisinin was from *Artemisia annua* grown in the tropical regions of East Africa and

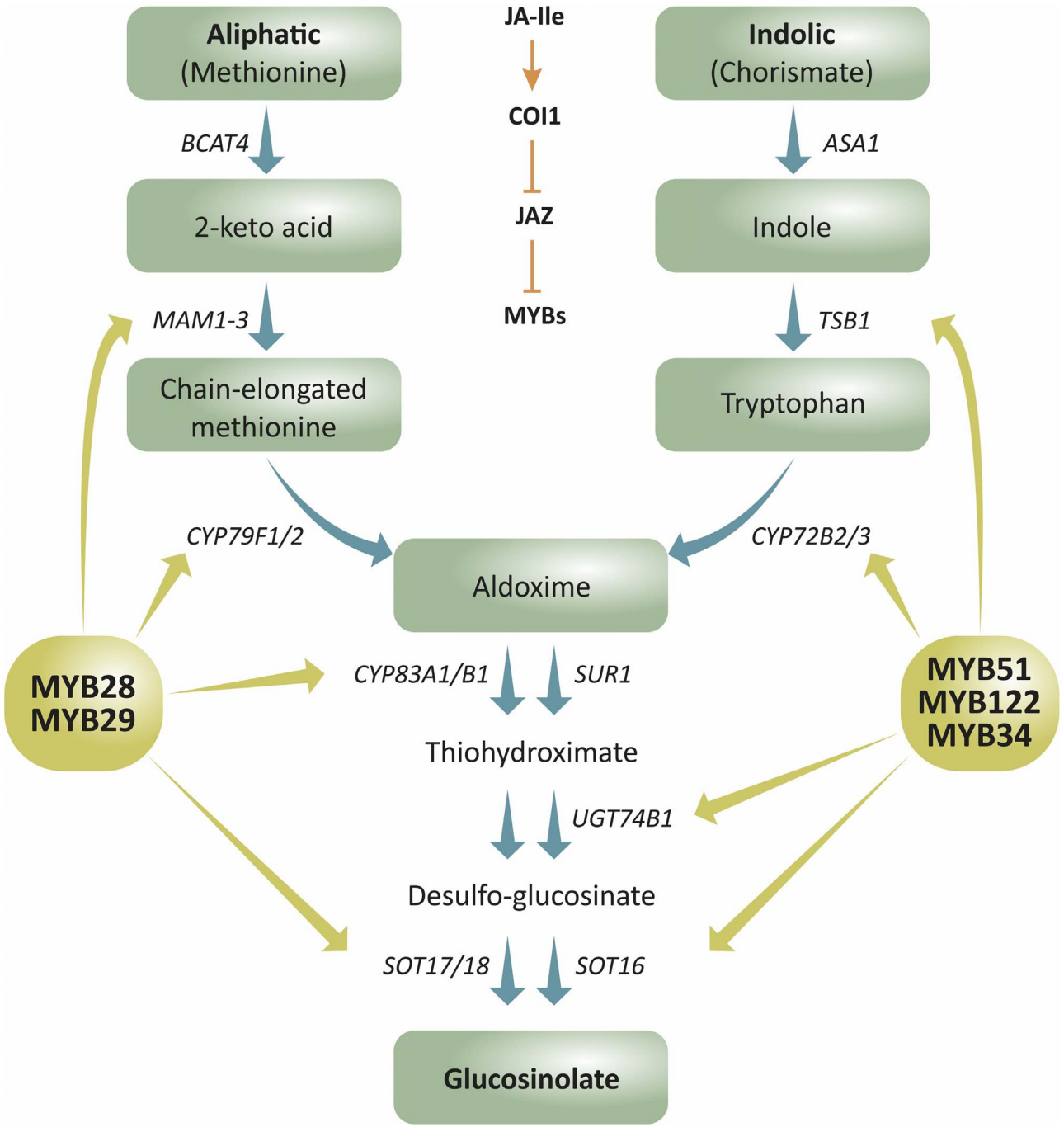

Fig. 6. Biosynthesis of glucosinolates (GS) originates from methionine (aliphatic GS) and chorismate (indolic GA). In the aliphatic GS branch are BCAT4 (branched-chain amino acid amino transferase4), MAM1-3 (methylthioalkylmalate synthase), and CYP79F11/2 (aldoxime synthase) which is also active in the indolic GS branch originating from ASA1 (anthranilate synthase), TSB1 (tryptophan synthase) and CYP79B2/3. The core pathway of GS synthesis takes place downstream of aldoxime and involves oxidation by CYP83A1/B1, a C-S-lyase step (SUR1), glucosyltransferase (UGT74B1) and sulfotransferase (SOT16/17/18). The TFs MYB28 and MYB29 regulate genes of the aliphatic GS branch and the core pathway, whereas MYB51, MYB34 and MYb122 regulate genes of the indolic GS branch and the core pathway.

East Asia. Therefore, early effort was put into developing strategies for synthetic routes of artemisinin formation in genetically engineered yeast. In 2006, artemisinic acid, which can be easily converted chemically and photo-oxidatively into artemisinin, was produced in yeast [68]. Two years later, the pharmaceutical company SANOFI was producing 39 tons of artemisinic acid, a source for 40 million treatments, but then followed dramatic turbulences in the market [69] owing to a strong decrease in production triggered by the lowered price of agriculturally produced artemisinin [70]. Nevertheless, efforts were still made to find new strategies of synthesis. Examples include the successfully performed continuous synthesis of artemisinin by hydrogenation of yeast-formed artemisinic acid followed by photo-oxidative conversion [71] (Fig. 7) and transgenic formation in engineered tobacco plants. An interesting redirection of intermediates in artemisinin

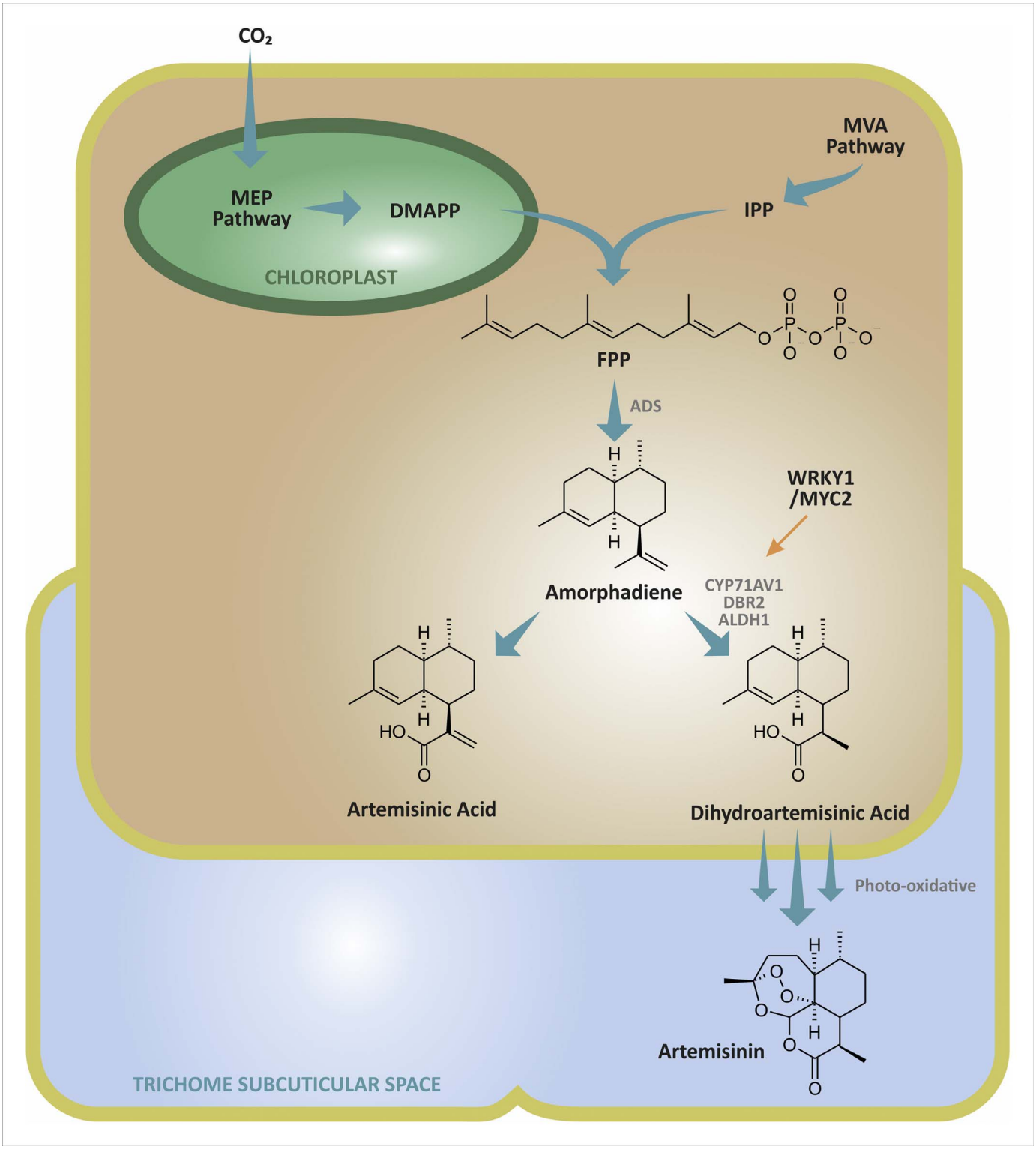

Fig. 7. Artemisinin biosynthesis.
Artemisinin synthesis is initiated by the cytosolic MVA pathway and the plastid-located MEP pathway to generate farnesyl pyrophosphate and amorpha-4,11-diene. The latter compound is converted in three steps by CYP71AV1 monooxygenase and a CYP450 reductase to artemisinic aldehyde, from which artemisinic acid is generated by a CYPB5/reductase-catalyzed step. Alternatively, the aldehyde can be converted to dihydroartemisinic aldehyde and its acid, which finally leads to artemisinin in a final spontaneous photo-oxidative conversion.

biosynthesis was found by analysis of a point mutation in the CYP71AV1 gene encoding amorpha-4,11-diene(A-4,11-D)-C12-oxidase (Fig. 7) [72]. This mutant showed high-flux plasticity to novel and alternative sesquiterpenes, such as arteannuin X, indicating that glandular trichomes, where artemisinin biosynthesis takes place, can be used for production of novel natural products.

Future engineering of secondary metabolites

Besides the above examples of artemisinin, an increasing number of methods are being developed to engineer formation of secondary compounds. Among them are combinatorial biochemistry with gene transfer of pathways, e.g., overproduction of triterpenoids has been

found in ER-engineered yeast [73]. Dihydrosanguinarine formation was detected in yeast upon pathway reconstitution [74]. Upon screening, different photosynthetic host organisms were found for heterologuous production of terpenoids in a synthetic biology approach [12]. Chloroplast transformation can be used for different aspects of plant biotechnology [75], and specific cell types, such as glandular trichomes, have been used to establish trichome-specific expressed sequence tag libraries for biosynthesis pathways of secondary compounds [61].

Technical improvement of production has been achieved for many secondary compounds by ultrasonification [76]. In a promising recently performed synthetic biology approach, the core pathway of artemisinic acid, the precursor of artemisinin, was first introduced into the chloroplast genome of tobacco, followed by transformation with a cassette for all additional pathway genes [77].

Concluding remarks

The present approaches for production of secondary compounds are based preferentially on metabolic engineering methods, which include direct modification of any pathway. Synthetic biology approaches can be applied to modify biological systems for novel properties and applications, including genome modification, finely tuned multigene expression and optimization [78]. Unicellular organisms such *Synechococcus sp*, *Synechocystis*, *Chlamydomonas reinharddtii*, or *Chlorella* have been tested is this respect for production of plant metabolites, including secondary compounds [78]. The increased capacity for large scale sequencing programs has already accelerated discovery of pathways of secondary compounds, which will be useful for the establishment of heterologous systems to produce secondary compounds of medicinal interest [79]. Further, integrated omics analysis of genomic, transcriptomic, proteomic and metabolomic data should enable identification of new biosynthetic pathways and strategies for production of bioactive metabolites [80]. Even single cells, such as glandular trichomes, can be used as a factory of biotechnological interest [61]. At the same time, knowledge is increasing of JA biosynthesis, metabolism and signaling, facilitating advancements in JA-induced biosynthesis of secondary metabolites.

Contributions

C.W. wrote the main part of the review, including the first draft of the figures. M.S. completed the text and was responsible for preparation of the figures.

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