Review

Natural products – modifying metabolite pathways in plants

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The diversity of plant natural product (PNP) molecular structures is reflected in the variety of biochemical and genetic pathways that lead to their formation and accumulation. Plant secondary metabolites are important commodities, and include fragrances, colorants, and medicines. Increasing the extractable amount of PNPs through plant breeding, or more recently by means of metabolic engineering, is a priority. The prerequisite for any attempt at metabolic engineering is a detailed knowledge of the underlying biosynthetic and regulatory pathways in plants. Over the past few decades, an enormous body of information about the biochemistry and genetics of biosynthetic pathways involved in PNP production has been generated. In this review, we focus on the three large classes of plant secondary metabolites: terpenoids (or isoprenoids), phenylpropanoids, and alkaloids. All three provide excellent examples of the tremendous efforts undertaken to boost our understanding of biosynthetic pathways, resulting in the first successes in plant metabolic engineering. We further consider what essential information is still missing, and how future research directions could help achieve the rational design of plants as chemical factories for high-value products.

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1 Introduction

Plants produce a plethora of secondary metabolites, diverse both in structure and their biosynthetic origins. For any attempt at targeted metabolic engineering of plant natural product (PNP) biosynthesis, it is of utmost importance to understand the biogenesis of PNPs from ubiquitous primary metabolites. The vast majority of PNPs can be categorized into three large classes: isoprenoids, phenylpropanoids, and alkaloids.

Isoprenoids represent by far the largest PNP-family, with an estimated 40 000 compounds [1]. Their value is well established, as they impart flavor and fragrance to foods and cosmetics, are colorants and anti-oxidants, and a rich source of many pharmaceuticals. They are, moreover, biologically important and have been shown to affect many physiological processes in plants, such as respiration, signal transduction, cell division, membrane architecture, photosynthesis, and growth. In addition, isoprenoids have ecological significance, as they play an important role in the attraction of and defense against insects, microorganisms, and other assailants (e.g. parasitic plants). The application of isoprenoids in foods, cos-

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Abbreviations: BBE, berberine bridge enzyme; **BIA**, benzylisoquinoline alkaloid; **DHAA**, dihydroartemisinic acid; **DMADP**, dimethyl allyl diphosphate; **FDP**, farnesyl diphosphate; **FLS**, flavonol synthase; **GDP,** geranyl diphosphate; **GGDP,** geranylgeranyl diphosphate; **GES**, geraniol synthase; **IDP**, isopentenyl diphosphate; **MEP**, methylerythritol phosphate; **MIA**, monoterpenoid indole alkaloid; **MVA**, mevalonic acid; **PNP**, plant natural product; **TPS**, terpene synthase

metics, and drugs makes them interesting commercial targets for biotechnological production. Recently, the biosynthesis and metabolic engineering of plant volatile terpenoids in an ecological context was reviewed by Dudareva et al. [2]. In this review, we focus on the application of modern metabolic engineering techniques for efficient recovery of high-value isoprenoid compounds.

Phenolic secondary metabolites and their intermediates, stemming from general phenylpropanoid metabolism via the shikimate biosynthetic pathway, are ubiquitous in terrestrial plants. These metabolites play important roles in both abiotic and biotic defense. Furthermore, due to their potent antioxidant activity, phenolic metabolites are increasingly considered to be important bioactive compounds from nutritional and medicinal points of view [3, 4]. Through the enzymatic activities of various classes of proteins, including polyketide synthases, hydroxylases, oxygenases, ligases, and oxidoreductases, several different phenolic core structures are built up. The final enormous diversity of phenolic metabolites is achieved through catalytic involvement of several "decorating" enzymes, belonging mainly to the transferase superfamilies, e.g. glycosyl-, acyl-, and methyltransferases. All these proteins are encoded by diverse structural genes, and are concurrently controlled by regulatory genes belonging to several superfamilies. Enzymatic steps within the general phenylpropanoid pathway and the final stages of specific biosynthetic routes, including their evolution, were recently comprehensively summarized [3, 4]. Here, we focus on engineering attempts to modulate phenylpropanoid biosynthesis.

Alkaloids constitute a diverse group of low-molecular-weight nitrogenous compounds found in many plant genera. More than 21 000 different alkaloid structures are currently known [5], and more are likely to be discovered. Biosynthetically, most alkaloids are derived from amino acids, such as phenylalanine, ornithine, arginine, tyrosine, and tryptophan; some, however, originate from alternative precursors (e.g. purine-derived caffeine) [6]. Contrary to the long-abiding belief, alkaloids are not merely end-products of metabolic pathways, but represent a group of specialized components with pronounced eco-chemical defense functions against plant pathogens and herbivores. As deduced from their chemical structure, many alkaloids mimic, block, or modulate neurotransmitter activity or interfere with basic neurological functions; hence their potent biological effects, be they toxic or remedial. Thus, not only do alkaloids boast a famed ethno-pharmacological tradition, but continue to maintain palpable significance in modern-day clinical practice [7]; the latter being the main driving force of metabolic engineering efforts.

The vast biodiversity of PNPs precludes their reductionist classification. Thus, by virtue of their unique properties, some secondary metabolites (e.g. glucosinolates) do not fall neatly into any of the three major groups introduced above. Nonetheless, we will use the proposed traditional systematization to discuss the current state of PNP metabolic engineering in the following sections.

2 Isoprenoids

2.1 Biosynthesis of isoprenoids

Although isoprenoids are extraordinarily diverse, they all originate from the condensation of the universal five-carbon precursors, i.e. isopentenyl diphosphate (IDP) and dimethyl allyl diphosphate (DMADP). In higher plants, two independent pathways, located in separate intracellular compartments, are involved in the biosynthesis of IDP and DMADP (Fig. 1). In the cytosol, these basic precursors are derived from the classic mevalonic acid (MVA) pathway starting from acetyl-CoA [8], whereas in plastids, they are formed from pyruvate and glyceraldehyde 3-phosphate via the methylerythritol phosphate (MEP or non-mevalonate) pathway [9, 10]. Recently, it was shown that final steps of the MVA pathway were further localized to the peroxisomes [11]. Cytosolic IDP and DMADP are converted by farnesyl diphosphate synthase to (*E,E*)-farnesyl diphosphate (FDP, C15), which serves as a precursor for sesquiterpenoids, triterpenoids, and sterols. The plastidial pool of IDP and DMADP is converted by geranyl diphosphate synthase to geranyl diphosphate (GDP, C10) and geranylgeranyl diphosphate (GGDP, C20), which serve as precursors for monoterpenes, and diterpenes and tetraterpenes (carotenoids), respectively [12]. Recently, a plastidically localised (*Z,Z*)-farnesyl diphosphate synthase [13] and a synthase catalyzing the biosynthesis of neryl diphoshate, the (*Z*)-isomer of GDP [14], have been reported as well. All the enumerated prenyl diphosphates, including isomers of IDP, DMADP, GDP, FDP, and GGDP, together with squalene epoxide, constitute the set of precursors for the enzymatically catalyzed production of all 40 000 isoprenoids (Fig. 1). The first step in this process usually consists of dephosphorylation, often (but not always) followed by cyclization of the prenyl substrate. The resulting, most basic, isoprenoid structures are then modified by hydroxylation (usually catalyzed by cytochrome P450 enzymes), further oxidation (catalyzed by the same cytochrome P450s or by alcohol dehydrogenases), reduction (catalyzed by reductases), and/or methylation, acetylation, or glycosylation (catalyzed by acyl-transferases, methyl-transferases, or glycosyl-transferases, respectively). Together, these reactions lead to the vast structural diversity of isoprenoids.

2.2 Metabolic engineering of isoprenoids

2.2.1 Strategies for isoprenoid gene identification and characterization

Many plants providing valuable terpenoids produce them in low quantities, as their biosynthesis is limited to spe-

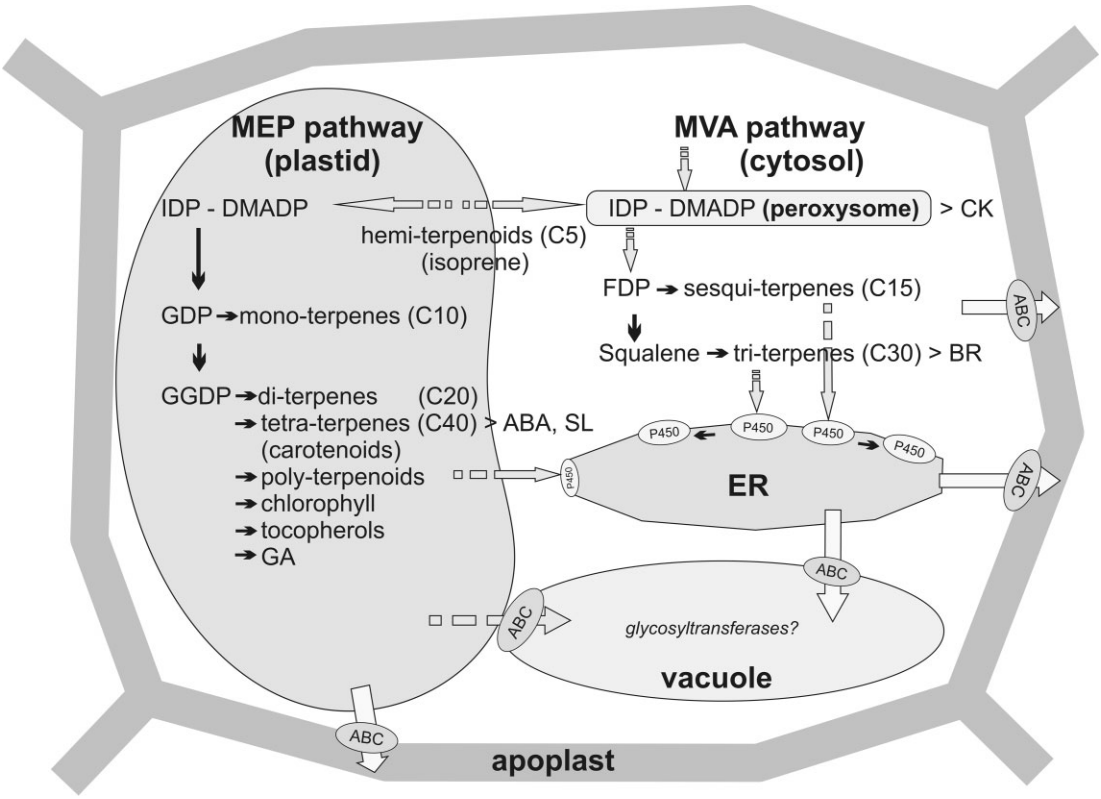

Figure 1. Compartmentation of isoprenoid biosynthesis in the plant cell. Two independent pathways, the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathway, form the C5-units, IDP and DMADP, in the cytosolic and plastidic compartments, respectively. The biosynthesis of FDP and sesquiterpenoids, as well as squalene epoxide and triterpenoids occurs primarily in the cytosol, whereas isoprene and the basic skeletons of monoterpenoids, diterpenoids, and tetraterpenoids (carotenoids) are mostly formed in the plastids. IDP, isopentenyl diphosphate; DMADP, dimethylallyl diphosphate; FDP, farnesyl diphosphate; GDP, geranyl diphosphate; GGDP, geranylgeranyl diphosphate; ABA, abscisic acid; GA, gibberellins; SL, strigolactones; BR, brassinosteroids; CK, cytokinins. Black arrows, enzymatic steps in the pathway; white arrows, putative ABC transporter activities; grey and dashed arrows, enzymatic steps in the pathway involving transport between subcellular compartments; > denotes derivatives of the preceding compound. Modified from Aharoni et al., 2005 [101] and Tholl, 2006 [12].

cialized plant cells such as laticifers and glandular trichomes. Although the production in these dedicated compartments may be high, they usually constitute only a small fraction of all the cells in a tissue (leaf, stem, or ovary). As many terpenoid synthesizers are difficult to cultivate and often not amenable to genetic transformation or conventional breeding, options to optimize the production of valuable isoprenoids in these plant species are usually limited. Spurred by the prospective elucidation of important terpenoid biosynthetic pathways in their entirety, new economically feasible possibilities to produce compounds of value in heterologous platforms are expected to emerge.

Nowadays, the isolation of isoprenoid biosynthetic genes follows a well established course, starting usually with a detailed analysis of product accumulation in specific tissues or cells over plant developmental diversification. The product-accumulation profiles thus rendered are subsequently transposed against the expression profiles of candidate genes in the compound-amassing cells/tissues at corresponding developmental stages. The terpene synthase (TPS) genes can be recognized through comparison to known *TPS* sequences. Based on protein homology, the TPSs can be divided into subfamilies. For instance, the Norway spruce TPSs fall into seven classes, defined by a minimum of 40% identity between members, and are designated TPS-a through TPS-g [15]. All TPSs share a common aspartate-rich DDxxD motif thought to be involved in the coordination of divalent metal ions for substrate binding [16]. Once candidate *TPS* sequences have been thus identified and partially delineated, selective RACE PCR (rapid amplification of cDNA ends with polymerase chain reaction) provides the full-length sequence information. TPS genes are then expressed in *Escherichia coli*, *Saccharomyces cerevisiae,* or various plant species for functional analysis. For introduction into non-plant hosts, signal peptide sequences are often removed to improve expression efficiency and/or protein stability. Functionality of the produced TPS enzymes can be subsequently ascertained by feeding with the appro-

priate precursors (see section 2.1). Many different TPSs have been identified, cloned, and functionally characterized over the past two decades. Analogous strategies have been used to recognize and annotate cytochrome P450 monooxygenase, alcohol dehydrogenase, double bond reductase, and conjugating enzyme candidate genes. Expression profiling of the proposed genes over different tissues and developmental stages can then be applied to effectively target most likely candidates. Because of their localization to the endoplasmic reticulum (ER), cytochrome P450 coding sequences are preferentially heterologously expressed in *S. cerevisiae*. However, especially for candidate genes involved in complex biosynthetic pathways, functional characterization in the aforementioned host may prove difficult when confronted with the lack of specific substrates. Transient expression in *Nicotiana benthamiana* provides a viable alternative, effectively circumventing this bottleneck. Selection of candidates for yet unidentified genes of a biosynthetic pathway may be aided by comparison with expression profiles of an array of contending coding sequences in different tissues, or at diverse developmental stages of a given tissue, with those of known pathway genes. In addition, the tissue-specific expression profiles thus obtained may be further correlated with the expected product accumulation profiles of the tissue in question. In this manner, we have recently identified and characterized the genes of the costunolide [17] and artemisinin biosynthetic pathways [18, 19].

2.2.2 Metabolic engineering efforts

About ten years ago, identification of genes involved in terpenoid formation fueled the first attempts to introduce and modify their biosynthesis within heterologous plant systems. These initial studies, utilizing tobacco as a host and the natural subcellular targeting of the investigated sesquiterpene synthase enzymes, proved rather unsuccessful. Only minute amounts of desired products were detected [20]. In the following sections, we discuss the progress in the metabolic engineering of mono- and sesquiterpenoids as proxies for other plastidic and cytosolic terpenoids, respectively.

2.2.2.1 Monoterpenoids

The use of monoterpene synthases in genetic transformation marks a breakthrough in metabolic engineering of terpenoids. Since monoterpenoids are volatile, the headspace of transgenic plants is normally analyzed to confirm product formation. Surprisingly, however, despite strongly expressing the introduced linalool synthase gene, transgenic petunia plants did not emit linalool [21]. Yet, these plants did accumulate substantial amounts of glycosylated linalool and linalool derivatives, demonstrably indicating that heterologously introduced natural products are further metabolized by endogenous host enzymes [21]. Studies with olefinic monoterpenes, which are less prone to further metabolism due to absence of an alcohol group, demonstrated that heterologous introduction of monoterpene synthases led to substantial volatile product emission [22]. Interestingly, addition of limonene hydroxylase activity into limonene synthase gene-expressing tobacco resulted in decreased emission of limonene as a consequence of iso-piperitenol formation, pointing to effective competition of the introduced heterologous biosynthetic enzyme, localized to the endoplasmic reticulum, with volatile emission [23]. Other studies on linalool synthase gene expression in heterologous hosts demonstrated gaseous linalool production [24–27]. Another monoterpene alcohol synthase gene, geraniol synthase, was successfully expressed in tomato [28] and maize [29]; again, substantial downstream metabolism occurred. In most of the studies on the production of monoterpene alcohol synthases in plants, authors did not evaluate whether non-volatile conjugated products, such as glycosides, were simultaneously formed [24, 25]. With present-day knowledge at our disposal, however, it is safe to assume this phenomenon indeed occurred. Thus, it is crucial to use non-targeted metabolite analysis (metabolomics) to evaluate the sometimes unexpected consequences of metabolic engineering.

Expression of a biosynthetic pathway in a heterologous host may be adversely affected by competing endogenous enzyme activities influencing the ectopically produced target metabolite. For instance, in the effort to introduce the monoterpenoid indole alkaloid (MIA, see section 4.2) biosynthetic pathway from *Catharanthus roseus* into tobacco, the first heterologous step resulted in formation of multiple product derivatives (Bouwmeester and van der Krol – our unpublished data). The MIA pathway consists of a monoterpene iridoid branch and an indole branch [30]; geraniol synthase (GES) catalyzes the first step in the former. Expression of GES in tobacco not only resulted in the volatile geraniol production, but also in the formation of further oxidized and double bond-reduced soluble geraniol derivatives and their glycosides. However, when the ectopic expression of GES was combined with that of the geraniol 10-hydroxylase gene, encoding for the subsequent enzymatic step in the monoterpene iridoid branch of the pathway, the ectopically produced P450 monooxygenase effectively competed with the endogenous tobacco enzymes acting on geraniol (Bouwmeester and van der Krol – our unpublished data). Nevertheless, for multiple-step terpenoid pathway reconstruction in heterologous plant hosts, elimination of endogenous enzyme activities may be important for efficient metabolite channeling towards the desired end-product(s).

2.2.2.2 Sesquiterpenoids

The successful introduction of monoterpene synthases into heterologous systems fueled the application of alternative strategies for the expression of their sesquiter-

penoid counterparts. Chappell and coworkers [31] engineered tobacco to target FDP synthase and a sesquiterpene synthase to the plastids, and showed that these enzymes can indeed draw upon the pool of plastidic IDP, while Bouwmeester's group [32] expressed a sesquiterpene synthase gene with mitochondrial targeting, and demonstrated an increase in product formation as compared to the natural cytosolic localization of the enzyme. Later, several other studies showed that overexpression of sesquiterpene synthase genes resulted in the fair production of the C15 metabolites of interest, also without artificial targeting [33, 34]. Clearly, we do not yet understand the mechanisms that determine whether FDP, in principle ubiquitous in the cytosol, is available to the engineered enzymes. Possible solutions to this problem will be discussed below.

As mentioned in section 2.2.1, the majority (if not all) of artemisinin biosynthetic genes from *Artemisia annua* have been cloned, and the artemisinic acid pathway reconstituted in microorganisms. The pathway genes were also stably transformed into tobacco, resulting in trace artemisinin detection [34]. Furthermore, transient expression of all known artemisinin biosynthetic genes in *N. benthamiana* showed that changes in the relative activity of the individual steps can improve the flux to the dihydroartemisinic acid (DHAA) segment of the pathway, ultimately leading to improved artemisinin production [18]. As the conversion mechanism of dihydroartemisinic acid to artemisinin *in planta* remains elusive, the full potential for production of the valuable antimalarial in heterologous plant systems has not yet been satisfactorily explored.

2.2.3 *Pleiotropic effects of metabolic engineering in plants*

Terpenoid overexpression occasionally perturbs plant development. For example, slower growth and lighter leaf color were reported as a result of linalool synthase transposition into *Arabidopsis thaliana* and potato [26, 27]. Moreover, upon geraniol synthase gene expression, tomato fruits failed to develop their natural red color, as the lycopene (a C40 plastidial terpenoid) levels decreased by about 50% [28]. However, overexpression of monoterpenoids does not always lead to altered phenotypes. No changes in plant development were observed in the studies of limonene synthase gene-expressing tobacco [22] or lavender [35], linalool-producing petunia [21] or tomato [25], as well as geraniol synthase gene-expressing maize or tobacco [29].

2.2.4 *Engineering through transcriptional control*

A study in *A. thaliana* showed that expression of genes of the MEP pathway strongly correlated with the circadian clock core components, exhibiting peak expression during the day. Only a few of their MVA-pathway counterparts showed an equivalent correlation, with expression reaching its apex in the course of the night [36]. Furthermore, transcription factors have been implicated in the control of terpenoid biosynthetic genes in several plant species. The ERF/AP2-type transcription factor, ORCA, bears upon the regulation of MIA formation in *C. roseus* [37]. In cotton, a WRKY transcription factor controls expression of gossypol biosynthetic genes [38]. Moreover, two *A. annua* methyl jasmonate-responsive transcription factors, regulating expression of the first two artemisinin biosynthetic genes, were recently identified [39], as well as an *A. thaliana* MYC factor, involved in transcriptional control of two C15-TPS genes [40]. Further reports established a tight correlation between the *A. annua* ERF/AP2 transcription factor *AaORA* and the expression of three artemisinin pathway genes [41].

Understanding transcriptional regulation of terpenoid biosynthesis has considerable implications for pathway manipulation in cell or tissue cultures of medicinal plants. Overexpression of *ORCA3* resulted in the enhanced expression of several MIA biosynthetic genes and, consequently, in increased accumulation of alkaloids in *C. roseus* cell lines [42]. To date, however, the results of transcription factor overexpression in periwinkle hairy roots or cell lines remain inconclusive [43, 44]. Concurrently, enhanced expression of *AaORA* in *A. annua* resulted in an approximately 50% increase in artemisinin accumulation, and a 35% boost in DHAA (dihydroartemisinic acid) content, respectively [41]. This limited augmentation of product levels suggests involvement of additional factors. Indeed, it is likely that the ectopic overexpression of *AaORA* enhances transcription only in the specialized trichome cells. On the other hand, indiscriminate activation of the pathway might cause toxic effects in the cells that normally do not have to cope with the production of potentially detrimental compounds.

In summary, plants have been successfully engineered to produce valuable terpenoids. As yet, however, little control over the transgenic terpenoid levels can be effectively exercised. Further studies on regulatory features, such as transcription factors, multiple gene clusters, and tissue-specific synthesis and storage, are necessary to ultimately bolster the success of isoprenoid metabolic engineering in plants [45].

3 Phenylpropanoids

3.1 Biosynthesis of phenylpropanoids

The biosynthesis of phenylpropanoids has been investigated extensively and much is known about its underlying mechanisms (Fig. 2). However, several steps, especially rare hydroxylations of the A- or B-ring of flavonoids and the polymerization of stilbenes and proanthocyanidins (PAs), and some minor biosynthetic branches (e.g. gallo- and ellagitannins), are still poorly understood on

both biochemical and molecular levels. Although PAs are associated with numerous biological activities, it is still not clear whether the formation of oligomers and polymers is an enzymatic or non-enzymatic process *in planta*. Moreover, only preliminary and scarce evidence concerning 8-hydroxylation, glucuronylation, prenylation, and C-glycosylation of flavonoids is available on the enzymatic and molecular level ([46] and references therein). While the biosynthesis of phenylpropanoids with a C6-C3-C6 skeleton, e.g. flavonoids, is well established, the formation of C6-C1 structures, exemplified by benzoic and salicylic acid, is still under investigation and the co-existence of several pathways is predicted [46].

The variation in substrate specificity of corresponding enzymes from different plant species is a well described feature (e.g. dihydroflavonol 4-reductase), and can have a serious impact on plant pigmentation and synthesis of distinct metabolite patterns [47]. Side-activities, as found for chalcone synthases (CHS; Fig. 2), or multifunctional properties of some proteins involved in flavonoid biosynthesis, have become apparent from in vitro experiments. Recently, the relevance of these "side reactions" was corroborated *in planta*, as anthocyanidin synthase (ANS) was clearly responsible for residual flavonol formation in an *A. thaliana* flavonol synthase (FLS) mutant line [48]. However, all the remaining side reactions described in vitro for ANS, FLS, and other enzymes need yet to be verified as significant for the synthesis of specific compounds *in planta* [49]. Furthermore, this finding clearly shows that the annotation of gene function, based on sequence comparison and in vitro enzyme assays with generated recombinants, may prove insufficient to determine the exact *in planta* activity. Therefore, the extent to which such "leaky" proteins could be involved in the biosynthesis of unexpected minor metabolites, often of limited distribution within the plant kingdom and in small amounts across various plant species and tissues, needs to be further investigated. Representative studies have been reported for stilbenes and dihydrochalcones [50, 51].

Regulatory elements within the different branches of the general phenylpropanoid metabolism, e.g. flavonol and anthocyanidin biosynthesis, were identified in diverse plant species and have been reviewed extensively [52, 53].

3.2 Metabolon formation and metabolic channeling

Formation of protein complexes and the precise channeling of intermediates of secondary metabolism have been discussed for at least 40 years [54]. In contrast to synthesis via metabolic grids, the presence of metabolons and the channeling of metabolites thus implied would enable plants to perform highly effective and coordinated biosyntheses of specific natural products. Furthermore, the presence of metabolons would entail reduction or complete abrogation of metabolic interference and the accumulation of toxic intermediates. Metabolons can be localized on the cytoplasmic surface of the endoplasmic reticulum (ER), in other cell membranes, or in elements of the cytoskeleton. However, in spite of the recent technical progress in plant molecular biology, very little information on this interesting aspect and its relevance to phenylpropanoid biosynthetic pathway is available [54].

3.3 Metabolic engineering

Due to the ease of visualization of changes, mutations, or genetic imbalances after manipulation, anthocyanin biosynthesis (Fig. 2) has become an attractive model for first engineering approaches. The "orange *Petunia*" project spurred further research, partly driven by the flower industry, to fill the natural lack of blue, red, orange, or yellow flower colors in ornamental species such as rose, carnation, gerbera, cyclamen, and pelargonium [55, 56]. Moreover, due to recent findings suggesting certain health benefits of anthocyanins and other dietary polyphenolics (e.g. stilbenes, proanthocyanidins), manipulation of the anthocyanin biosynthetic pathway in tomatoes and apples has been successfully accomplished [57-60].

The increasing number of available plant whole genome sequences, together with the development of functional genomic tools and genetic mapping, will enable rapid annotation of novel genes and encoded proteins, thus fostering elucidation of the remaining unknown biosynthetic steps and their regulation in model as well as crop plants. Additionally, silencing and overexpression of candidate genes in these plants, combined with the application of sensitive analytical tools, gene expression profiling, complementation, and reverse genetics, will further increase our understanding of in vivo enzymatic functions. Molecular mechanisms of transport, formation of protein complexes and channeling of metabolites, as well as elucidation of minor pathways in medicinal and exotic plants should be emphasized in prospective research programs, to enable future establishment of more powerful metabolic engineering strategies targeted at specific compounds.

4 Alkaloids

To underscore the notable achievements in delineating alkaloid biosynthetic routes in context of the reinforced importance of plant metabolic engineering [61], we herein focus on benzylisoquinoline and monoterpene indole alkaloids.

4.1 Benzylisoquinoline alkaloids (BIAs)

Found principally in the Papaveraceae, Berberidaceae, Menispermaceae, and Ranunculaceae families, benzylisoquinoline alkaloids are a group of varied secondary

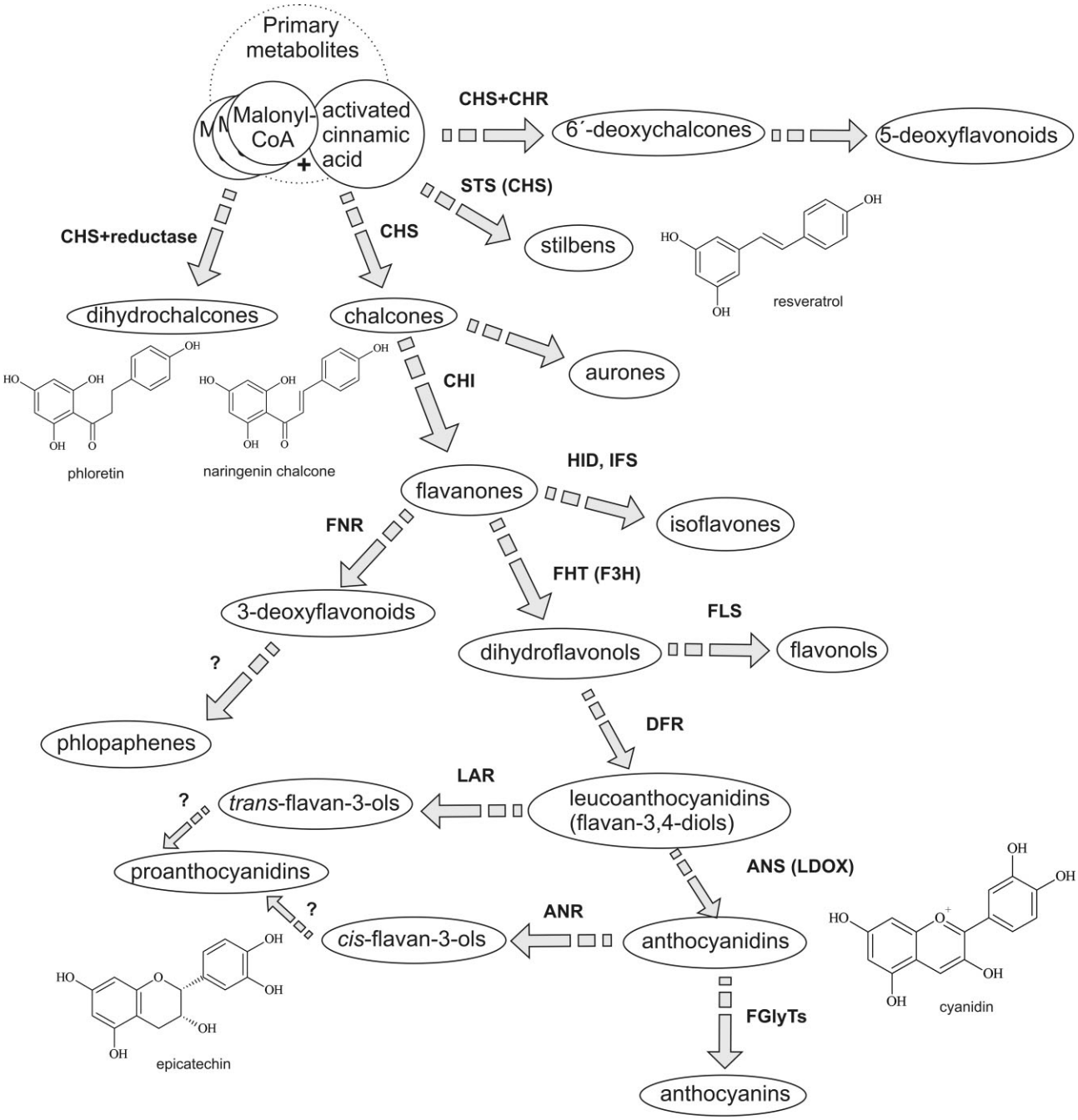

Figure 2. General outline of phenylpropanoid biosynthetic pathway encompassing major subclasses. Arrows might represent more than one enzyme. CHS, chalcone synthase; STS, stilbene synthase; CHR, chalcone reductase; AS, aurone synthase; CHI, chalcone isomerase; FNR, flavanone 4-reductase; HID, 2-hydroxyisoflavanone dehydratase; FHT (F3H), flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; ANS (LDOX), anthocyanidin synthase; ANR, anthocyanindin reductase; FGlyTs, flavonoid glycosyltransferases.

metabolites of well established pharmaceutical importance, as exemplified by narcotic and analgesic morphine, cough-suppressant codeine, antimicrobial sanguinarine and berberine, muscle-relaxant papaverine, and antineoplastic noscapine [62] (Fig. 3). Fundamentally, their biosynthetic origins can be traced back to the Pictet-Spengler condensation of tyrosine-derived dopamine and 4-hydroxyphenylacetaldehyde. The resulting parent intermediate, (*S*)-norcoclaurine, is then divergently modified, yielding various configurational scaffolds. Further functionalization accounts for the myriad documented BIA structures [63].

4.1.1 *Identification of biosynthetic catalysts: Bolstering the engineering toolbox*

The dynamic progress in functional genomics proved invaluable for recent breakthroughs pertaining to the delineation of BIA biosynthetic pathways. Coordinated transcriptional induction and co-expression analysis, augmented by virus-induced gene silencing [64], were the driving force for the discovery of functionally unique enzymes, marking the conclusion of morphine [65] and sanguinarine [66, 67] biosynthetic route elucidation. Application of the latter technique helped shed light on the controversy surrounding our understanding of papaverine formation, more than a century after the pathway was first proposed [68]. It was further utilized to confirm functional annotation of catalytic entities encoded in a putative 10-gene cluster, identified through coordinated genomic and transcriptomic data analysis as acquired via molecular breeding of commercial poppy varieties. This tremendous effort resulted in the discovery of the very first instance of alkaloid biosynthetic gene clustering and proposal of a novel pathway leading to the cough-suppressing and antineoplastic noscapine [69]. Thus, enumerated studies not only accentuate the formidable progress in BIA biosynthetic pathway elucidation, but also lend support to the application of innovative and sophisticated metabolic engineering techniques for overproduction of these valuable compounds.

4.1.2 *Engineering: From native hosts to heterologous pathway reconstitution*

Berberine bridge enzyme (BBE) marks a branching point for the formation of alternative BIA scaffolds. Early reports on the corresponding gene overexpression related elevated yields of downstream alkaloids and diminished amino acid pools; interestingly, the levels of the BIA amino acid parent, tyrosine were unaltered [70]. Conversely, antisense suppression of *BBE* expression resulted in effective silencing of alkaloid production and increased cellular amino acid levels; again, the shift in tyrosine content was non-significant [71]. While the aforementioned studies underscore how tinkering with the BIA biosynthetic machinery implies influence on primary metabolism, a more recent investigation, employing RNAi silencing of *BBE* in cultured California poppy cells, pointed to marked accumulation of (*S*)-reticuline rather than the primary precursors [72]. An alternative pioneering report on transformation of *Papaver somniferum* with antisense *BBE* via somatic embryogenesis demonstrated altered alkaloid profiles in latex, but not in the roots of the plant [73]. Furthermore, an early attempt at engineering opium poppy, utilizing RNAi to silence expression of the penultimate gene of morphine biosynthesis, *codeinone reductase* (*COR*), led to the unanticipated accumulation of the non-narcotic upstream intermediate, reticuline. Verification of the proposed feedback mechanism yielded ambiguous results [74]. In contrast, *COR* overexpression in *P. somniferum* [75] and *Papaver bracteatum* [76] resulted in the predicted increase of morphinan-alkaloid production. The persisting inconsistencies, merely touched upon above, constitute a clear incentive for further in-depth research, especially in the domain of metabolic regulation.

In concert with the impressive boost of our grasp on BIA biosynthetic routes, the current interest in alternative production platforms has spurred coordinated combinatorial efforts [77]. A noteworthy accomplishment, as reported by Hawkins and Smolke [78], involved successful fine-tuning of the expression profiles of BIA biosynthetic genes from various plant sources and human coding sequence candidates in a heterologous host, *S. cerevisiae*.

4.2 Monoterpenoid indole alkaloids (MIAs)

Pictet-Spengler condensation of tryptamine and secologanin, catalyzed by strictosidine synthase (STR), constitutes the fundamental step of monoterpenoid indole alkaloid formation [79]. The functional parallels between the committed enzymes of both BIA and MIA biosynthesis notwithstanding, they do not exhibit any evolutionary relationship, as indicated by structural data analysis [80]. While taxonomic distribution of MIAs involves the botanical families of Rubiaceae, Loganiaceae, and Apocynaceae [62], two representatives of the latter have garnered considerable interest over the past few decades. On one hand, *Rauvolfia serpentina* has become a blueprint for systematic pathway delineation (antiarrhythmic ajmaline, Fig. 3) in concert with structural data acquisition and rational enzyme engineering [80, 81]. On the other, despite patchy knowledge of biosynthetic networks [79, 82], accumulation of valuable metabolites strictly dependent on plant growth conditions and developmental stage, and the persistent shortage of efficient transformation protocols, the demand for antitumor alkaloids, vinblastine and vincristine [83] (Fig. 3), drives a tremendous research effort focused on their producer, *C. roseus*, dubbed the "model non-model system for alkaloid biosynthesis investigation" [62, 84].

4.2.1 *Rationally designed combinatorial biosynthesis of "new-to-nature" molecules*

While combinatorial biosynthesis – the manipulation of pathways to produce unnatural variants of natural products – in prokaryotic systems has become a success story, its equivalent in plants is still in its infancy [85]. Herein, we wish to highlight the pioneering work of O'Connor et al., which afforded successful hijacking of microbial biosynthetic machinery to generate chemical alkaloid analogs in Madagascar periwinkle, as a touchstone of plant metabolic engineering.

Initiated by precursor-directed feeding studies [86], through efficient application of mutasynthesis – arresting

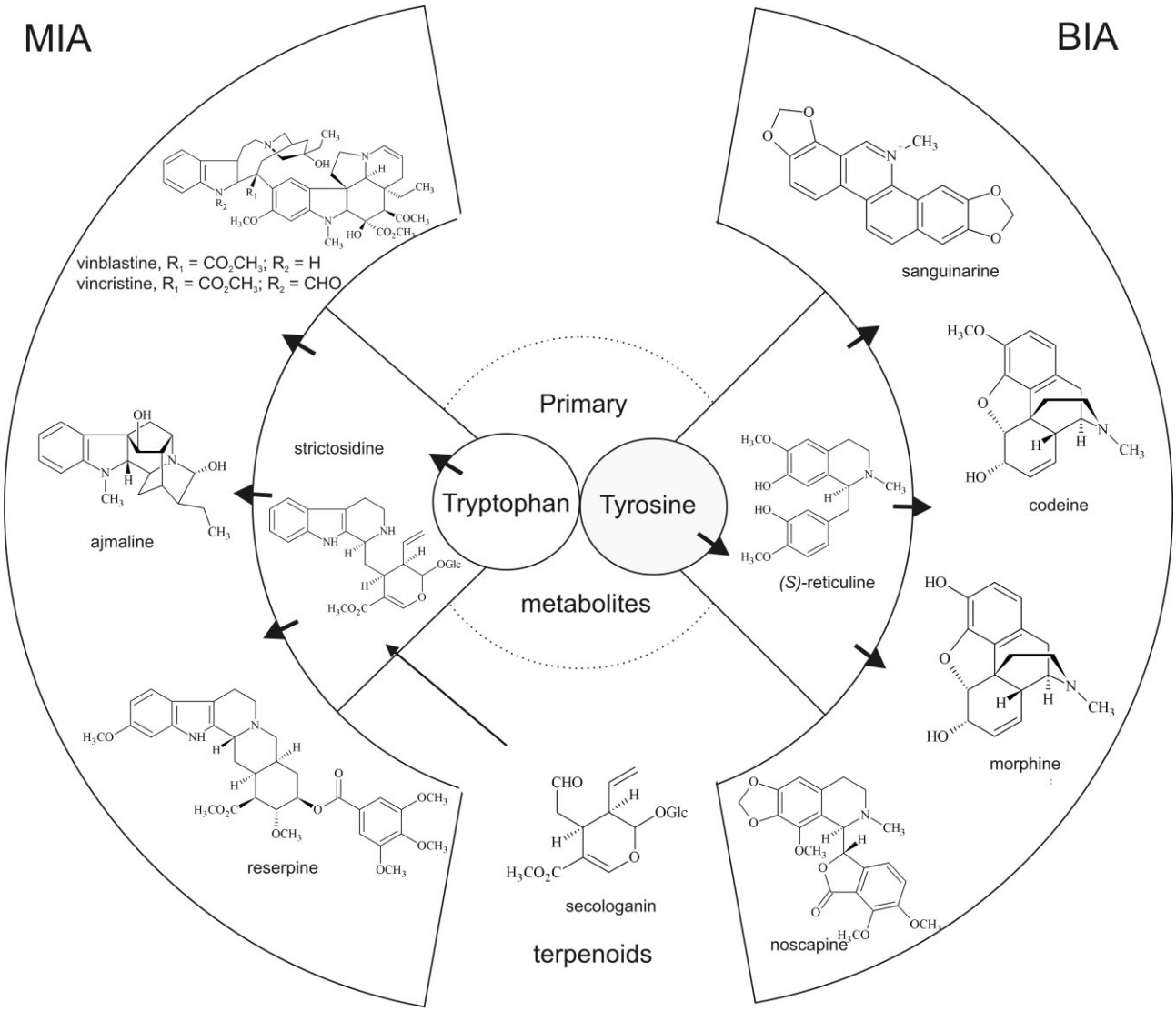

Figure 3. Schematic representation of monoterpenoid indole alkaloid (MIA, left) and benzylisoquinoline alkaloid (BIA, right) biosynthesis, starting from aromatic amino acids. Arrows represent multiple enzymatic and non-enzymatic reaction steps.

biosynthesis by genetic silencing of the natural precursor and restoring it by unnatural structural analogs supplementation [87], to structure-based engineering of STR stringent substrate specificity [88, 89], plant production of an array of new-to-nature halogenated alkaloid derivatives was attained. Groundwork so established fueled the ultimate breakthrough: successful introduction of indole ring chlorination catalysts from soil bacteria (RebH and PyrH) into *C. roseus* hairy root cultures. Chlorotryptophan, thus generated within the context of the plant cell, further integrated with the MIA metabolic flux, yielding substituted alkaloids [90]. Subsequent structural redesign of the actinomycetes halogenase, RebH, helped overcome the in vivo bottleneck of chlorinated tryptophan decarboxylation by streamlining the halogenation onto tryptamine – the direct precursor of monoterpenoid indole alkaloids [91].

This unprecedented example of bona fide combinatorial biosynthesis *in planta* sets the stage for further MIA engineering efforts and reinforces the significance of plants as a viable platform for synthetic biology.

Plant metabolic engineering of alkaloid biosynthetic pathways has been perceived a feasible and attractive venture for over two decades [92]. Over the years, the scope of success in the considered domain of plant natural product research has been relatively limited, rendering its progress to be rather steady than spectacular [93].The recent endeavors outlined here lead us to contend that metabolic engineering of plant alkaloids will become an increasingly rewarding enterprise.

Biotechnol. J. 2013, 8, 1159–1171

5 Limitations and future directions for the engineering of plant-derived natural products

This article illustrates how the rudimentary molecular and biochemical knowledge underlying the synthesis of natural plant products has been determined. This information has enabled the routine modulation of pathways; however, despite these significant advances in the field, fundamental gaps in our knowledge still exist. It is clear that, underpinning the application of innovative technologies, a need for more in-depth fundamental knowledge is paramount at all levels, from gene to small molecule.

It is important to remember that most valuable plant-derived products are classified as secondary metabolites, thus implying evolution of specialized biosynthetic processes in specific cell-types at precise developmental stages. Often, the plant species capable of synthesizing the compounds of interest are not model species and their genetic resources are limited. Owing to the considerable advances in DNA/RNA sequencing techniques, it is now possible to analyze transcriptomes and acquire significant genomic data rapidly [69]. These means, coupled to functional transient expression systems [19], will facilitate identification of numerous candidate genes associated with biosynthetic enzymes in the investigated pathways, as well as transcriptional regulators involved therein. Systems biology approaches, generating gene regulatory networks, will further enable the scientific community to identify modulators of the pathways, their global limitations, and potential strategies for precursor and reductant supply, without unintended effects.

The epigenetic regulation of pathways by methylation [94], as well as small molecules acting as riboswitches [95] and retrograde signals [96, 97] are important findings that should impact the prospective synthetic strategies, especially as it is well documented that environmental factors can have a bearing on levels of valuable secondary metabolites. Besides these new areas of regulation, pertinent to the directed formation of PNPs, classical in-depth studies are also important. For example, the organization of biosynthetic enzymes into metabolons, involving both pathway-specific catalysts and stability-augmenting proteins, is still poorly understood [98]. What is more, the lack of product/intermediate stability upon engineering has become apparent; thus, addressing synthesis concurrently with product accumulation/sequestration is an important aspect that can no longer be ignored. Transport of products and intermediates within the cell and its organelles, as well as the ability of metabolites to alter cellular structures, require elucidation [99].

Considering the chemical diversity in plants, it is surprising that most of our efforts have de facto focused on a relatively small set of compounds from a limited number of species. With the dwindling supplies of fossil fuels and the shift from chemical- to biorefining, it is paramount that more plant-derived resources be discovered and developed. This will require a broader and more comprehensive exploitation of natural diversity. To achieve these goals, the limitations associated with the convention on biodiversity (CBD) need to be overcome to facilitate proof-of-concept studies that will capture the true significance of biochemical diversity (CBD Secretariat, 2002 and Nagoya protocol, CBD, Secretariat, 2010). Linked to the full utilization of the diversity collections are improved metabolite profiling procedures, focusing on specific classes of natural products and their derivatives present in minute abundance, and bioassays. The latter should enable addressing prevalent disease states, be high-throughput, and provide conclusive information based on analysis of small amounts of material.

A common request from various consortia-based programs involved in PNP research is the need for improved databases and repositories. The Solanaceae Genome Network is a good example of a community resource that has augmented progress in the field [100]. More frequently, however, at the completion of a project, access to the metadata, data, and outputs generated is lost to the community.

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In summary, it is clear that the scientific community has been able to convert the euros invested by stakeholders into significant advances, delivering the strategies, tools, and know-how to elucidate and manipulate natural product pathways. Opportunities are now required to perform feasibility studies to show the potential of the products/systems, evolve metabolic engineering into synthetic biology by improving the control and tuning of the systems, and create a community infrastructure that will facilitate knowledge and resource sharing.

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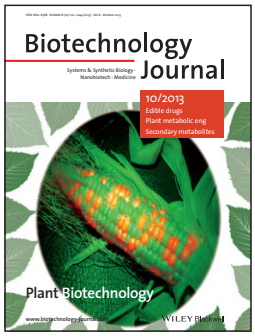

This Special Issue of *Biotechnology Journal* is edited by Prof. Eva Stoger and covers the latest breakthroughs in plant biotechnology. The cover image shows genetically modified corn producing an anti-HIV antibody along with DsRed as a visual marker. Image courtesy of Dr. Thomas Rademacher (RWTH and Fraunhofer IME, Aachen, Germany). Border around image: © boulemon – Fotolia.com.

Biotechnology Journal – list of articles published in the October 2013 issue.

Editorial: From plant biotechnology to bio-based products
Eva Stöger
http://dx.doi.org/10.1002/biot.201300392

Forum Article
Resistance to agricultural biotechnology: The importance of distinguishing between weak and strong public attitudes
Philipp Aerni
http://dx.doi.org/10.1002/biot.201300188

Review
The use of rice seeds to produce human pharmaceuticals for oral therapy
Yuhya Wakasa and Fumio Takaiwa
http://dx.doi.org/10.1002/biot.201300065

Review
Metabolic engineering of aroma components in fruits
Irene Aragüez and Victoriano Valpuesta
http://dx.doi.org/10.1002/biot.201300113

Review
Natural products – modifying metabolite pathways in plants
Agata Staniek, Harro Bouwmeester, Paul D. Fraser, Oliver Kayser, Stefan Martens, Alain Tissier, Sander van der Krol, Ludger Wessjohann and Heribert Warzecha
http://dx.doi.org/10.1002/biot.201300224

Review
***Jatropha curcas*, a biofuel crop: Functional genomics for understanding metabolic pathways and genetic improvement**
Fatemeh Maghuly and Margit Laimer
http://dx.doi.org/10.1002/biot.201300231

Review
Spider silks from plants – a challenge to create native-sized spidroins
Valeska Hauptmann, Nicola Weichert, Marziya Rakhimova and Udo Conrad
http://dx.doi.org/10.1002/biot.201300204

Review
Plant-derived pharmaceuticals for the developing world
Kathleen Hefferon
http://dx.doi.org/10.1002/biot.201300162

Research Article
Efficient recovery of recombinant proteins from cereal endosperm is affected by interaction with endogenous storage proteins
Jenny Peters, Maite Sabalza, Koreen Ramessar, Paul Christou, Teresa Capell, Eva Stöger and Elsa Arcalís
http://dx.doi.org/10.1002/biot.201300068

Research Article
Bactericidal properties of the antimicrobial peptide Ib-AMP4 from *Impatiens balsamina* produced as a recombinant fusion-protein in *Escherichia coli*
Xiaobo Fan, Holger Schäfer, Jürgen Reichling and Michael Wink
http://dx.doi.org/10.1002/biot.201300121

Research Article
Ascorbic acid synthesis and metabolism in maize are subject to complex and genotype-dependent feedback regulation during endosperm development
Georgina Sanahuja, Gemma Farré, Ludovic Bassie, Changfu Zhu, Paul Christou and Teresa Capell
http://dx.doi.org/10.1002/biot.201300064

Research Article
The in-line measurement of plant cell biomass using radio frequency impedance spectroscopy as a component of process analytical technology
Tanja Holland, Daniel Blessing, Stephan Hellwig and Markus Sack
http://dx.doi.org/10.1002/biot.201300125