KIPEs3: Automatic annotation of biosynthesis pathways

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Abstract

Background: Flavonoids and carotenoids are pigments involved in stress mitigation and numerous other processes. Both pigment classes can contribute to flower and fruit coloration. Carotenoids and flavonoid aglycons are produced by a pathway that is largely conserved across land plants. Glycosylations, acylations, and methylations of the flavonoid aglycones can be species-specific and lead to a plethora of biochemically diverse flavonoids. We previously developed KIPEs for the automatic annotation of biosynthesis pathways and presented an application on the flavonoid aglycone biosynthesis.

Findings: KIPEs3 is an improved version with additional features and the potential to identify not just the core biosynthesis players, but also candidates involved in the decoration steps and in the transport of flavonoids. Functionality of KIPEs3 is demonstrated through the analysis of the flavonoid biosynthesis in *Arabidopsis thaliana* Nd-1, *Capsella grandiflora*, and *Dioscorea dumetorum*. We demonstrate the applicability of KIPEs to other pathways by adding the carotenoid biosynthesis to the repertoire. As a proof of concept, the carotenoid biosynthesis was analyzed in the same species and *Daucus carota*. KIPEs3 is available as an online service to enable access without prior bioinformatics experience.

Conclusion: KIPEs3 facilitates the automatic annotation and analysis of biosynthesis pathways with a consistent and high quality in a large number of plant species. Numerous genome sequencing projects are generating a huge amount of data sets that can be analyzed to identify evolutionary patterns and promising candidate genes for biotechnological and breeding applications.

Findings

Introduction

Carotenoids and flavonoids are two important classes of plant pigments. Colorful flowers are often pigmented by one or both of these pigments. Flavonoids (anthocyanins) are known for blue, purple, red, and orange colors, while carotenoids can provide yellow, orange, and red pigmentation. Biological roles of carotenoids include photoprotection [1,2], light capture [3], coloration of plant structures [4–6], and hormone precursors [7]. Therefore, carotenoids are considered part of the primary metabolism. In contrast, flavonoids are specialized plant metabolites that contribute to stress resilience and play important roles in the reproduction of many plants [8–10].

The biosynthesis of flavonoid aglycones [11,12], the decoration with sugar and acyl groups [13–15], and the intracellular transport of flavonoids [16–18] have been reviewed elsewhere. The core of the flavonoid biosynthesis pathway is well conserved across land plant species, while the aforementioned decorating reactions are often species-specific. Glycosylation and acylation modify the properties of flavonoids, but specific properties depend on the added sugar moiety or acyl group. Glycosylation can enhance the anthocyanin stability [19–21] and be required for intracellular flavonoid transport [17,18]. Acylation can also boost the stability of anthocyanins [22].

Here, we present an updated approach for the automatic annotation of the flavonoid biosynthesis genes in any land plant species of interest based on prior knowledge about the pathway. We also demonstrate that this approach can be applied to other pathways by investigating the carotenoid biosynthesis genes in multiple land plant species. A web server makes this automatic annotation workflow available to the research community.

Extended bait sequence collection

Bait sequences for the identification of candidates in a new data set form the basis of the KIPEs analysis. This collection was extended by adding anthocyanin 3-O glucosyltransferase (UGT79B2 and UGT79B3), anthocyanidin 5-O-glucosyltransferase (UGT75C1), flavonoid 3-O-glucosyltransferase (UGT78D1 and UGT78D2), UGT72L1, UGT80B1/TT15, anthocyanin acyl transferase (AAT), anthocyanin O-methyl transferase (OMT), BAHD acyltransferase, glutathione S-transferase (GST)-like ligandins/TT19, TT10, anthocyanin transporting ABC transporter, anthocyanin and PA precursor transporting MATE, P_{3A}-ATPase AHA10/TT13, and GFS9/TT9 (**Table 1**).

Table 1: New flavonoid biosynthesis steps in the KIPEs3 repertoire. These steps were added during the development of KIPEs3. Steps of the core flavonoid aglycon biosynthesis were part of the initial KIPEs release [12]. Required bait sequences and related information are available via GitHub [23].

Name of the step	Label in KIPEs3	Representative sequence	Reference
anthocyanin 3-O glucosyltransferase (UGT79B2 and UGT79B3)	A3GT	AT4G27560	[24]
anthocyanidin 5-O-glucosyltransferase (UGT75C1)	A5GT	AT4G14090	[13]
flavonoid 3-O-glucosyltransferase (UGT78D2 and UGT78D1)	F3GT	AT5G17050	[13]
UGT72L1	UGT72L1	XP_013443944.2	[25]
UGT80B1/TT15	TT15	AT1G43620	[26]
anthocyanidin 3-O-glucoside coumaroyl CoA transferase	3AT	AT1G03940	[22]
anthocyanin 5-glucoside malonyl CoA acyl transferase	5MAT	AT3G29590	[22]
anthocyanin 3-glucoside malonyl CoA acyl transferase	3MAT	AAO12206.1	[27]
anthocyanin O-methyl transferase	OMT	VIT_01s0010g03510	[28]
glutathione S-transferase (GST)-like ligandins/TT19	TT19	AT5G17220	[29,30]
Flavonoid oxidase LAC15/TT10	TT10	AT5G48100	[31]
anthocyanin transporting ABC transporter	ABCC	AT2G34660	[32]
anthocyanin and PA precursor transporting MATE	MATE	AT3G59030	[33]
P_{3A}-ATPase AHA10/TT13	AHA10	AT1G17260	[34,35]
GFS9/TT9	TT9	AT3G28430	[36]

Major technical updates

The initial Python2 implementation of KIPEs [12] was transferred to Python3. This ensures that more users are able to run KIPEs without installing an outdated Python version.

A central component of KIPEs3 is the identification of orthologs for all bait sequences in the new species of interest (**Figure 1**). This approach is based on the established assumption that orthologs are likely to have the same function. This identification of orthologs is now based on the widely used Python module dendropy [37]. However, KIPEs3 is not restricted to orthologs, but checks a larger set of homologs for their overall sequence similarity and the presence of functionally relevant amino acids.

A new option allows users to provide expression data that are subjected to a co-expression analysis for the identified candidates. It is generally expected that genes associated with following or preceding steps of the same branch in the flavonoid biosynthesis show a similar expression pattern. A strong co-

expression between members of the flavonoid biosynthesis can further boost the reliability of identified candidates.

Many users expressed an interest in phylogenetic trees that place the candidates in the context of previously characterized homologous sequences. KIPEs3 can automatically construct trees of the identified candidates and the corresponding bait sequences for all steps in the pathway. This enables a manual inspection without the need for additional analyses.

Users can define if they are providing peptide, transcript, or genomic DNA sequences. Since this information might not always be accurate, an option was added to check if the provided sequences comprise amino acids or nucleotides. A warning is shown if the result of this analysis contradicts the sequence type set by the user.

Computational resources required for running KIPEs are neglectable. A single core and less than 2GB of memory are already sufficient for the analysis. The entire analysis is completed within a few minutes, but the precise run time depends on the specific settings and size of the analyzed data set.

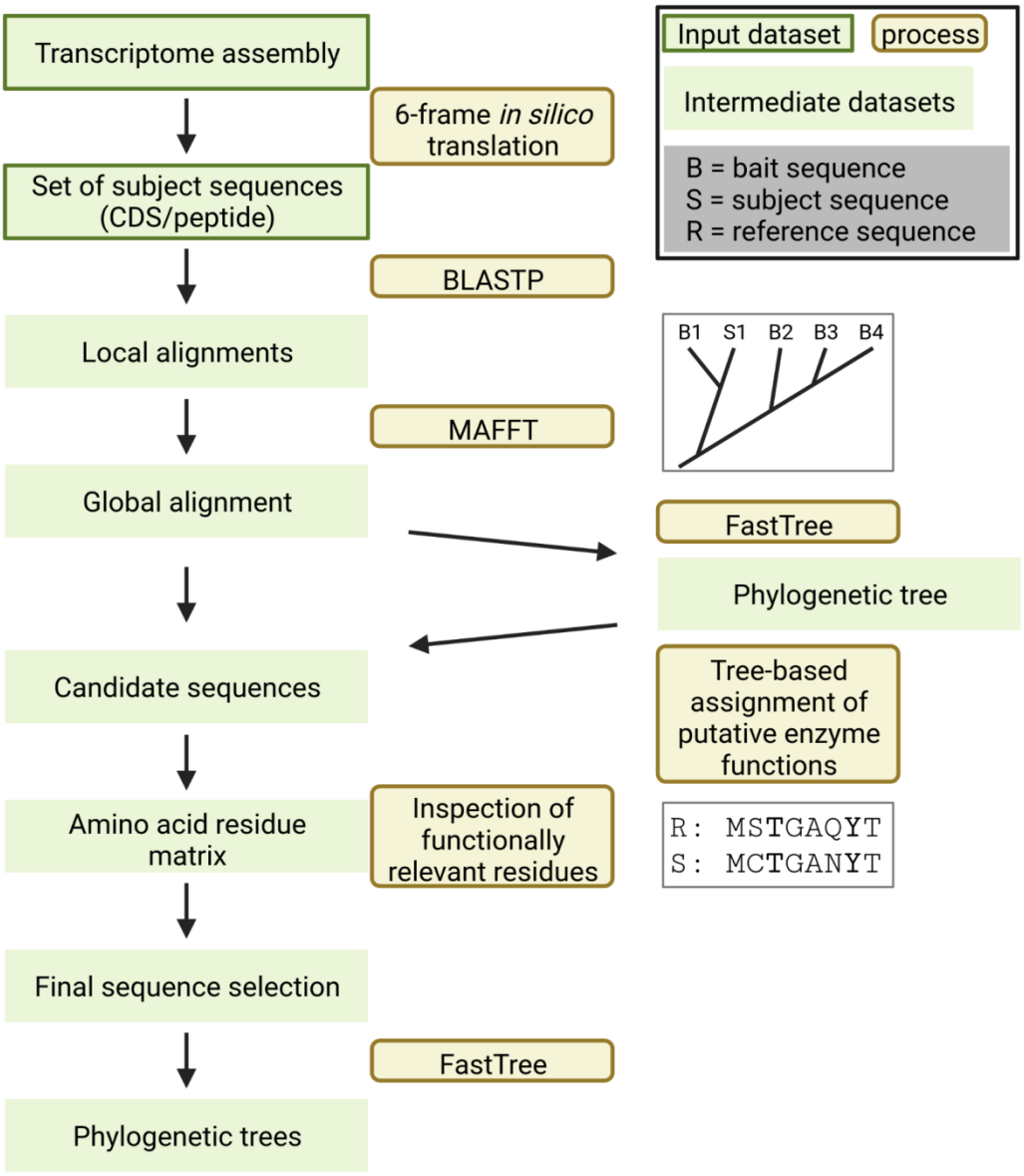

Figure 1: Simplified illustration of central KIPEs3 steps and functions that are included in the standalone and in the online version. Transcriptome or peptide sequences are supplied by the user. Initial candidates are identified by BLAST and checked through global alignments, phylogenetic trees, and the inspection of functionally relevant amino acid residues. Phylogenetic trees are constructed for all final candidates.

KIPEs3 is available as an online service

KIPEs3 is provided as an online service hosted on a webserver at TU Braunschweig [38]. Users can upload their FASTA files containing coding sequences or peptide sequences of a species. Bait sequence sets are stored on the server and can be directly used without the need to be manually uploaded by the user. Optional parameters are pre-configured to allow an automatic analysis with default settings, but

can also be modified by the user through the web interface if necessary. Users may opt-in to receive email notifications once their analysis is finished and the results are available or store a cookie to allow their browser to keep track of the progress. The archive provided for download includes all result files that users would get when running KIPEs3 locally.

Analysis of the flavonoid biosynthesis in *A. thaliana* Nd-1, *Capsella rubella*, and *Dioscorea dumetorum*

A technical check was performed by analyzing the flavonoid biosynthesis in the *A. thaliana* accession Niederzenz-1 (Nd-1). It is expected that orthologs of all characterized genes of the reference accession Columbia-0 (Col-0) are also present in Nd-1, while the sequences are slightly different from the bait sequences. All genes known in Col-0 were also discovered in Nd-1 (Additional file 1). Next, *Capsella grandiflora* was analyzed with KIPEs3 to show that the identification of flavonoid biosynthesis genes works also in species that are not represented in most of the bait sequence sets like *A. thaliana*. Genes present in *A. thaliana* were also discovered in the close relative *C. grandiflora* (Additional file 1). To demonstrate the potential of KIPEs3 to annotate the flavonoid biosynthesis in a distant relative of *A. thaliana*, the flavonoid biosynthesis of *Dioscorea dumetorum,* which belongs to the monocots, was analyzed (Additional file 1).

The new flavonoid biosynthesis pathway steps added to the repertoire of KIPEs3 include the diverse and promiscuous decoration enzymes like glycosyltransferases, acyltransferases, and methyltransferases. Since some of these steps might be species-specific, it is possible that some steps are missing in a given plant species of interest. This requires specific attention when interpreting the KIPEs3 results to avoid false positives. While the aglycon biosynthesis is highly conserved and catalyzed by well characterized enzymes, flavonoid decorating enzymes are less studied and show a high level of promiscuity [39–41]. This poses a challenge for the identification through sequence similarity. Little is known about the functionally relevant amino acids in the flavonoid decorating enzymes. In addition, this extension of the KIPEs3 flavonoid biosynthesis bait sequence sets is no longer restricted to enzymes, but also includes flavonoid transporters. They do not have an active center that could be composed of functionally relevant amino acids. This turns the reliable identification of transport associated proteins into a remaining challenge.

Analysis of the carotenoid biosynthesis

A novel set of bait sequences was compiled to enable the annotation of the carotenoid biosynthesis with KIPEs3 (**Table 2**). The carotenoid biosynthesis is well studied in many plant species including *A. thaliana* [42]. The set of compiled bait and reference sequences was evaluated by running the annotation workflow on *A. thaliana* Nd-1, *Daucus carota*, *C. rubella*, and *D. dumetorum* (Figure 2; Additional file 2). *Daucus carota* carotenoid biosynthesis genes previously described in the literature [43] were recovered by KIPEs3 (Additional file 2). This supports the accuracy of the candidate gene identification.

Table 2: Carotenoid biosynthesis genes. [1]most likely non-functional [2]BCH duplication after monocot-dicot split

Name of the step	**Representative sequence / Label in KIPEs3**	**Reference**
Phytoene synthase (PSY)	AT5G17230	[44]
Phytoene desaturase (PDS)	AT4G14210	[45]
Zeta-carotene desaturase (ZDS)	AT3G04870	[46]
15-cis-zetra-carotene isomerase (ZISO)	AT1G10830	[47]
Carotenoid isomerase 1 (CRTISO1/CCR2)	AT1G06820	[48]
Carotenoid isomerase 2 (CRTISO2)[1]	AT1G57770	-
Lycopene cyclase (LCYB/CRTL-B)	AT3G10230	[49]
Lycopene cyclase (LCYE/CRTL-E)	AT5G57030	[50]
Beta-carotene hydroxylase 1 (BCH1/CHYB1/CHY1)	AT4G25700	[51]
Beta-carotene hydroxylase 2 (BCH2/CHYB2/CHY2)[2]	AT5G52570	[52]
Carotene beta-ring hydroxylase (CYP97A3/LUT5)	AT1G31800	[53]
Cytochrome P450 97B3 (CYP97B3)	AT4G15110	[54]
Carotene epsilon-monooxygenase (CYP97C1/CHYE/LUT1)	AT3G53130	[55]
Zeaxanthin epoxidase (ZEP/ABA1/NPQ2)	AT5G67030	[56]
Violaxanthin de-epoxidase (VDE/NPQ1)	AT1G08550	[56]
Neoxanthin synthase (NSY/ABA4)	AT1G67080	[57]

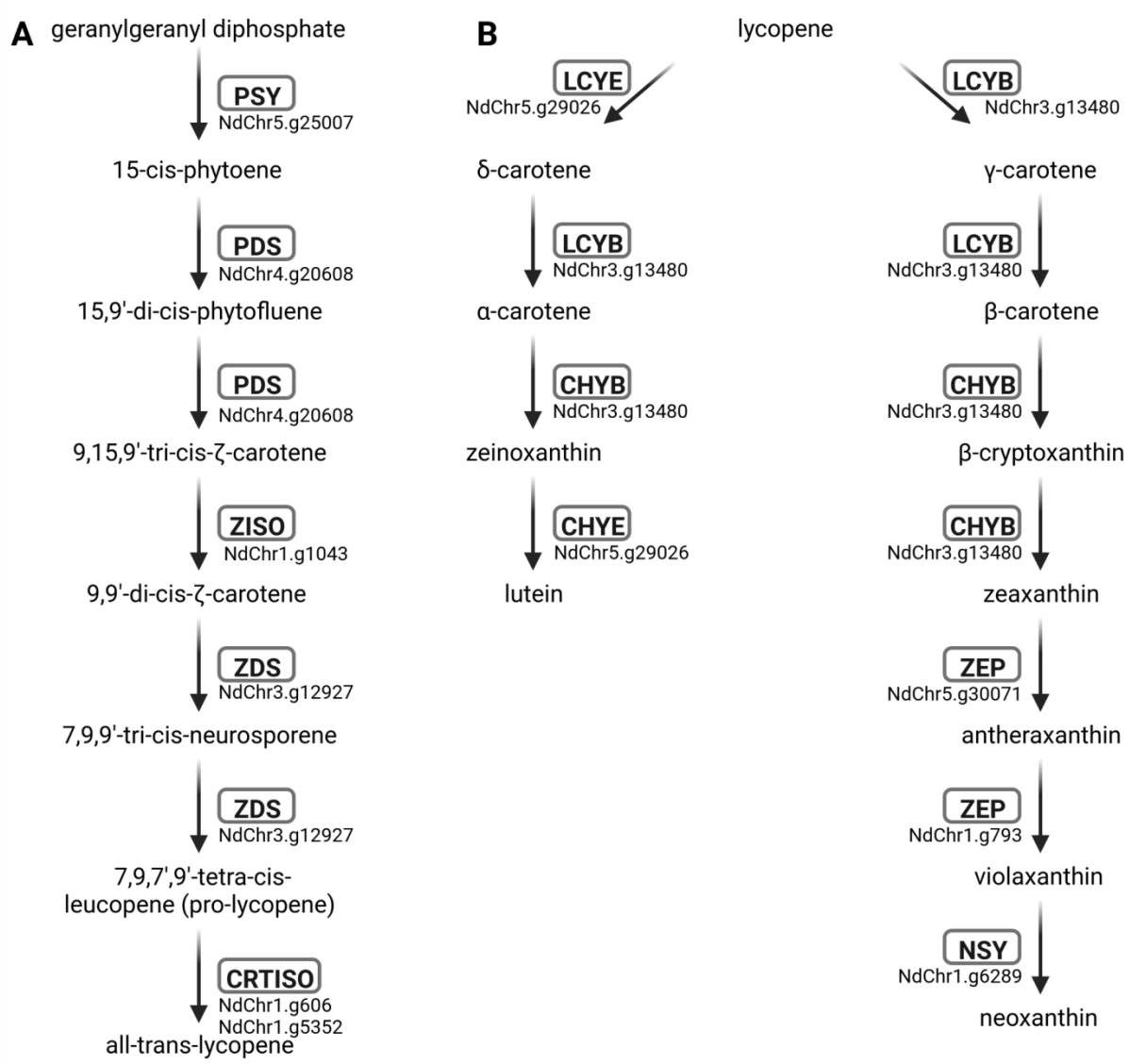

Figure 2: Carotenoid biosynthesis genes in *Arabidopsis thaliana* Nd-1. The carotenoid biosynthesis comprises a linear part (A) and a branching part (B).

Transcriptional regulation of biosynthesis pathways

Many plant biosynthesis pathways are regulated at the transcriptional level by members of large transcription factor families. Examples are the R2R3-MYBs and bHLHs that form complexes involved in the control of the flavonoid biosynthesis [58]. As we demonstrated previously, it is possible to analyze large gene families with KIPEs [12]. However, KIPEs3 is optimized for the analysis of pathways that comprise genes with low copy numbers. Most structural genes in these pathways are part of a small gene family. Ideally, KIPEs should be applied for the annotation of structural genes in the flavonoid biosynthesis, but other tools should be applied for the investigation of the transcriptional regulators.

Members of the MYB gene family can be annotated by a dedicated workflow [59] and general approaches can be deployed to study other involved transcription factor families [60,61].

Outlook

While the generation of high-quality genome sequences is turning into a routine task, the structural and especially the functional annotation of these sequences is an increasing challenge. The ability to annotate biosynthesis pathways beyond the flavonoid biosynthesis establishes KIPEs3 as a versatile platform for functional annotation in projects focused on specialized metabolites. While we focused on plant metabolism in this study, KIPEs3 could also be applied for the investigation of non-plant organisms like fungi or bacteria. The automatic annotation of biosynthesis pathways paves the way for comparative genomic studies that are looking for species-specific differences or intraspecific variation.

Methods

KIPEs3 validation

Validation of KIPEs3 was based on *A. thaliana* Nd-1 [62], *Capsella grandiflora* [63], *Daucus carota* [64], and *Dioscorea dumetorum* [65]. Identified candidates were manually inspected in a phylogenetic tree constructed by FastTree2 [66] based on alignments generated with MAFFT v7 [67].

Web server implementation

The web server is implemented in Python3 using the Django web framework v4 [68]. Whenever a user uploads a file and submits a new job through the form on the website, the form data is sent to the server via an HTTP POST request and the file is temporarily stored on the server machine. The job is placed into a queue and executed as soon as there are enough free resources (disk space, memory and CPU cores) available. KIPEs3 and all its dependencies are installed on the server machine. The user is redirected to a page with a console showing the command that is executed and the program output is displayed in real time using XMLHttpRequests. All user data are deleted from the server 48 hours after the job has been finished and the results have been downloaded.

Availability of supporting code and requirements

Project name: KIPEs3

Project home page: https://github.com/bpucker/KIPEs

Operating system(s): Linux (website is platform independent)

Programming language: Python3

Other requirements: BLAST, MAFFT, FastTree2, dendropy, scipy

License: GNU General Public License v3.0

RRID: SCR_022370

Data Availability

All data sets analyzed in this study are publicly available. Data sets generated as part of this study are shared via GitHub (https://github.com/bpucker/KIPEs). A docker image is available via DockerHub (https://hub.docker.com/r/bpucker/kipes).

Declarations

List of abbreviations

Not applicable

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

AR has no competing interests. BP is head of the technology transfer center Plant Genomics and Applied Bioinformatics at iTUBS.

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Authors' contributions

AR developed the web server framework and contributed to the manuscript. BP designed the research, conducted experiments, wrote the first draft, and revised the manuscript.

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Additional files

Additional file 1: Results of the flavonoid biosynthesis analysis in *Arabidopsis thaliana* Nd-1, *Capsella rubella*, and *Dioscorea dumetorum*.

Additional file 2: Results of the carotenoid biosynthesis analysis in *A. thaliana* Nd-1, *Daucus carota*, *C. rubella*, and *D. dumetorum*.