KIPEs3: Automatic annotation of biosynthesis pathways

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Abstract

Flavonoids and carotenoids are pigments involved in stress mitigation and numerous other processes. Both pigment classes can contribute to flower and fruit coloration. Flavonoid aglycones and carotenoids 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.

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 technical 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.

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.

Background

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, whereas the 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]. Applications of anthocyanins as food colorants rely on a high stability.

Biotechnological applications involving flavonoids are generally facilitated by knowledge about the genes involved in their biosynthesis. For example, such insights are crucial for the production of flavonoids in heterologous systems [23]. The flavonoid biosynthesis pathway is well studied in several model organisms and crops like *Arabidopsis thaliana* [18, 20], *Petunia hybrida* [11, 24, 25], *Antirrhinum majus* [26, 27], *Vitis vinifera* [28–30], and *Zea mays* [20, 31]. An ongoing challenge is the efficient transfer of this knowledge to other plant species. In fact, the functional annotation transfer challenge goes far beyond knowledge about the flavonoid biosynthesis. Plant genome sequences are becoming available with an increasing pace [32, 33], but the functional annotation is lacking behind. Automatic approaches for biological interpretation of these data sets are crucial to exploit the full value of genomic resources. There are several comprehensive databases collecting and sharing knowledge about plant metabolism, e.g. The Plant Metabolic Network [34], Plant Reactome [35], and MetaCyc [36]. Tools like InterProScan5 [37], Blast2GO [38], KEGG Automatic Annotation Server [39], and Mercator4 [40] allow the assignment of certain annotation terms to input sequences. While these tools are able to annotate genome-scale data sets, they are less specific with respect to the assigned annotation. Researchers looking for a specific pathway could benefit from tools focusing on their pathway of interest.

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.

Materials and Methods

Validation of KIPEs3 results through manual inspection

A validation of KIPEs3 was based on *Arabidopsis thaliana* Nd-1 v1.0 [41], *Capsella grandiflora* v1.1 [42], *Daucus carota* v2.0 [43], and *Dioscorea dumetorum* v1.0 [44]. Identified candidates were manually inspected in a phylogenetic tree constructed by FastTree2 [45] based on alignments generated with MAFFT v7 [46].

Run time analysis of KIPEs3

The average run time of KIPEs3 per data set was assessed using a previously defined set of 121 plant species [47]. All these sequence files were retrieved from Phytozome [48]. The analysis was performed on coding sequences to represent transcriptome assemblies. Performance of KIPEs3 with FastTree v2.1.10 [45] was analyzed using default parameters. Run time and the number of sequences in the analyzed data sets were documented. Run times were measured for analyses with the construction of phylogenetic trees for all final candidate genes.

Comparison of KIPEs3 predictions against annotation databases

The accuracy of KIPEs3 predictions was compared against MetaCyc [49], Plant Reactome [35], and Plant Metabolic Network (PMN) [34]. *FLS* was selected as a representative gene of the flavonoid biosynthesis and *LYC-b* was selected as a representative gene of the carotenoid biosynthesis. The compared species were determined by overlap between the databases with respect to the covered species. The usage of the same genome sequence and annotation for a species across different databases was important. *FLS* annotations in *Arabidopsis thaliana*, *Cicer arietinum*, and *Citrus sinensis* were compared. The *LYC-b* annotations in *A. thaliana*, *Solanum lycopersicum*, and *Daucus carota* were compared. The candidates were searched using the Enzyme Commission numbers, EC 1.14.20.6 for *FLS* and EC 5.5.1.19 for *LYC-b* in Metacyc and PMN. In Plant Reactome, the genes were searched using the terms “flavonol synthase” and “lycopene beta cyclase” in the search bar. KIPEs3 v0.35 was run as a command-line tool providing flavonoid biosynthesis baits, residues, and the predicted peptide sequences of the species to identify *FLS* candidates in each species. The *LYC-b* candidates were annotated in another run based on the carotenoid biosynthesis bait sequences, residues, and the predicted peptide sequences of the respective species. The *FLS* and *LYC-b* candidates listed in the three databases and identified by KIPEs3 were aligned using MAFFT v7 [46] to discriminate functional proteins and the products of pseudogenes by identifying *FLS* and *LYC-b* specific motifs and residues, respectively. A phylogenetic tree of the *LYC-b* candidates was generated using FastTree v2.1 [45].

Gene expression analysis

Gene expression was investigated in all plant species that were used for comparison of KIPEs3 against MetaCyc, Plant Reactome, and Plant Metabolic Network. As basis for the gene expression served the *A. thaliana* TAIR10 sequence combined with the Araport11 annotation [50], *Cicer arietinum* v1.0 (phytozome 492) [51], *Citrus sinensis* v1.1 (phytozome 154) [52], *Solanum lycopersicum* (phytozome 514 ITAG3.2) [53], and *Daucus carota* v2.0 (phytozome 388) [43]. All available RNA-seq data sets were retrieved from the Sequence Read Archive (SRA) via fastq-dump [54] (Additional File 1).

Results and Discussion

Extended bait sequence collection

The initial KIPEs version was exclusively focused on annotating the biosynthesis of flavonoid aglycones [12]. Now, KIPEs3 is able to identify additional candidates that are relevant for the decoration of flavonoid aglycones. The basis for the identification of candidates in new data sets are bait sequences that are previously characterized sequences from other species. The initial KIPEs bait sequence collection was extended by adding anthocyanidin 3-O glucosyltransferase (UGT79B2 and UGT79B3), anthocyanin 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 aglycone biosynthesis were part of the initial KIPEs release [12]. Required bait sequences and related information are available via GitHub [55].

Name of the step	Label in KIPEs3	Representative sequence	Reference
anthocyanidin 3-O glucosyltransferase (UGT79B2 and UGT79B3)	A3GT	AT4G27560	[24]
anthocyanin 5-O-glucosyltransferase (UGT75C1)	A5GT	AT4G14090	[13]
flavonoid 3-O-glycosyltransferase (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]
anthocyanidin 5-glucoside malonyl CoA acyl transferase	5MAT	AT3G29590	[22]
anthocyanidin 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 converted into a Python3 implementation. Syntax changes and the replacement of modules were an integral part of this transformation process. This technical upgrade ensures that more users are able to run KIPEs without installing an outdated Python version. It is also important for long term preservation of KIPEs and to avoid security issues. While upgrading the code to the latest Python version, we also added new features.

The established KIPEs components form the basis of KIPEs3 starting with the identification of orthologs for all bait sequences in the new species of interest (**Figure 1**). Each step in the pathway (enzyme/gene) is represented by one FASTA file containing numerous bait sequences from different plant species. Each collection of bait sequences is used to identify orthologs, i.e. most likely candidates, in the species of interest. This approach is based on the established assumption that orthologs are likely to have the same function (see [12] for details). This identification of orthologs is now based on the widely used Python module dendropy [56]. However, KIPEs3 is not restricted to finding orthologs, but checks a larger set of homologs for their overall sequence similarity and the presence of functionally relevant amino acids. The function of an enzyme is not determined by overall sequence similarity, but can depend on a few essential amino acid residues.

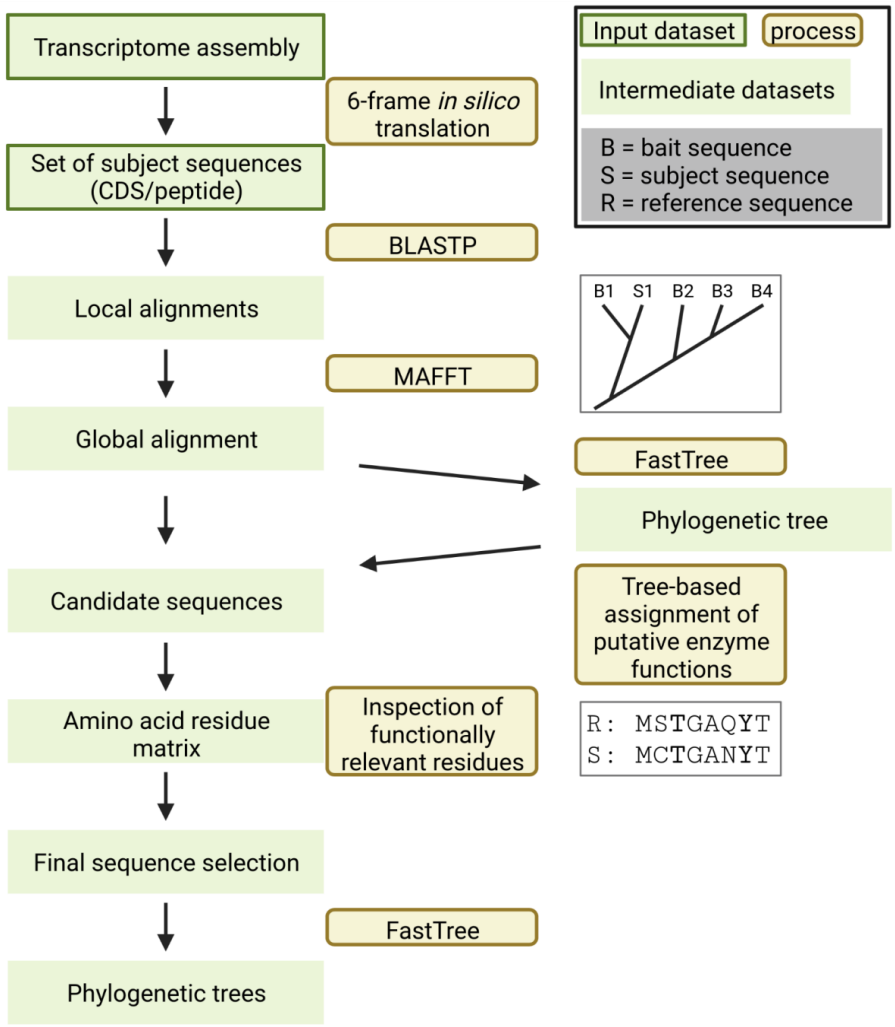

Figure 1: Simplified illustration of central KIPEs3 steps and functions that are included in the standalone and in the online version. Transcriptome or peptide sequences are supplied by the user. Initial candidates are identified by BLAST and checked through global alignments, phylogenetic trees, and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

A new option allows users to provide expression data that are subjected to an all-vs-all co-expression analysis among the identified candidates. It is generally expected that genes associated with following or preceding steps of the same branch in a biosynthesis pathway show a similar expression pattern, i.e. co-expression with other genes in this pathway branch. A strong co-expression value can further boost the reliability of identified candidates. KIPEs3 will produce a file that can serve as Cytoscape [57] input to visualize the gene expression network (**Figure 2**). This co-expression feature can also help to identify the most important candidate if multiple close paralogs exist for a given function.

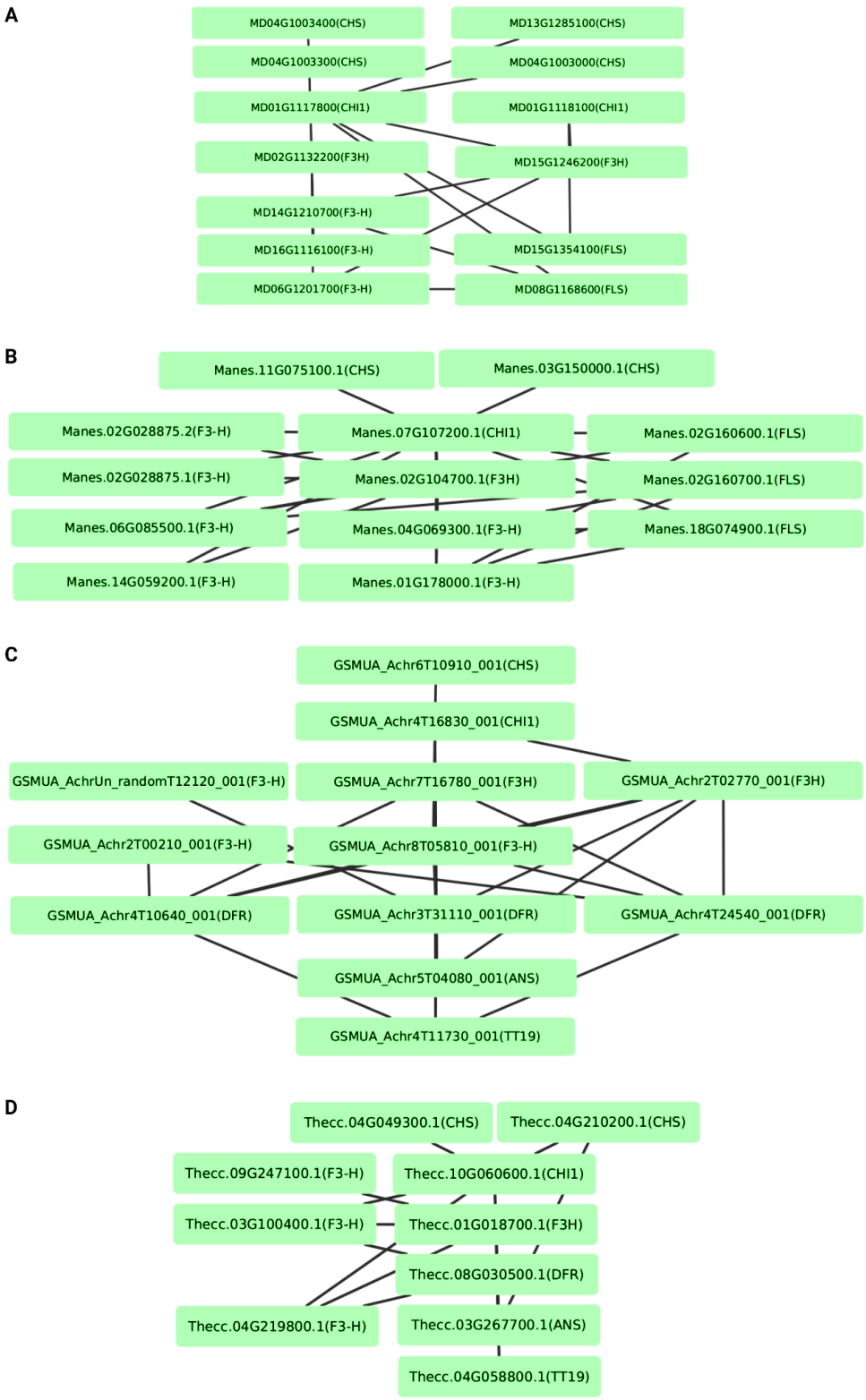

Figure 2: Visualization of co-expression networks via Cytoscape. Flavonol biosynthesis genes in *Malus domestica* (A) and *Manihot esculenta* (B) were analyzed based on co-expression. The anthocyanin biosynthesis genes were studied in *Musa acuminata* (C) and *Theobroma cacao* (D).

156

157 Many KIPEs users expressed an interest in phylogenetic trees that place the identified candidates in the
158 context of previously characterized homologous sequences. These trees can help to explore the
159 relationships of multiple candidates to previously characterized bait or reference sequences. KIPEs3 can
160 automatically construct trees of the identified candidates and the corresponding bait sequences for all
161 steps in the pathway. This enables a manual inspection without the need for additional phylogenetic
162 analyses. KIPEs3 generates files in the Newick format which can be visualized and modified for
163 publication with established tools like FigTree or iTOL [58].

164 KIPEs is able to start with coding sequences or with peptide sequences. It is even possible to provide a
165 genome sequence, but performing the gene prediction with dedicated tools is recommended for most
166 applications. Generally, it is best to provide peptide sequences as this speeds up the analysis. Users
167 define the type of input sequences that they are providing. Since this information might not always be
168 accurate, an option was added to check if the provided sequences comprise amino acids or nucleotides.
169 A warning is shown if the result of this analysis contradicts the sequence type set by the user. However,
170 it remains unfeasible to distinguish a collection of coding sequences from a highly fragmented genome
171 sequence assembly based on short reads. Therefore, it remains the users' responsibility to set the
172 correct sequence type.

173 Given the power of modern hardware, the computational resources required for running KIPEs are
174 neglectable. A single core and less than 2GB of memory are already sufficient for the analysis. The entire
175 analysis is completed within minutes, but the precise run time depends on the specific settings and size
176 of the analyzed data set (**Figure 3**). Especially large data sets can cause run times of about one hour, but
177 most plant species can be analyzed in less than 20 minutes (**Figure 3A**). This run time analysis was
178 performed on Phytozome data sets of a wide range of plant species to simulate a realistic application of
179 KIPEs3.

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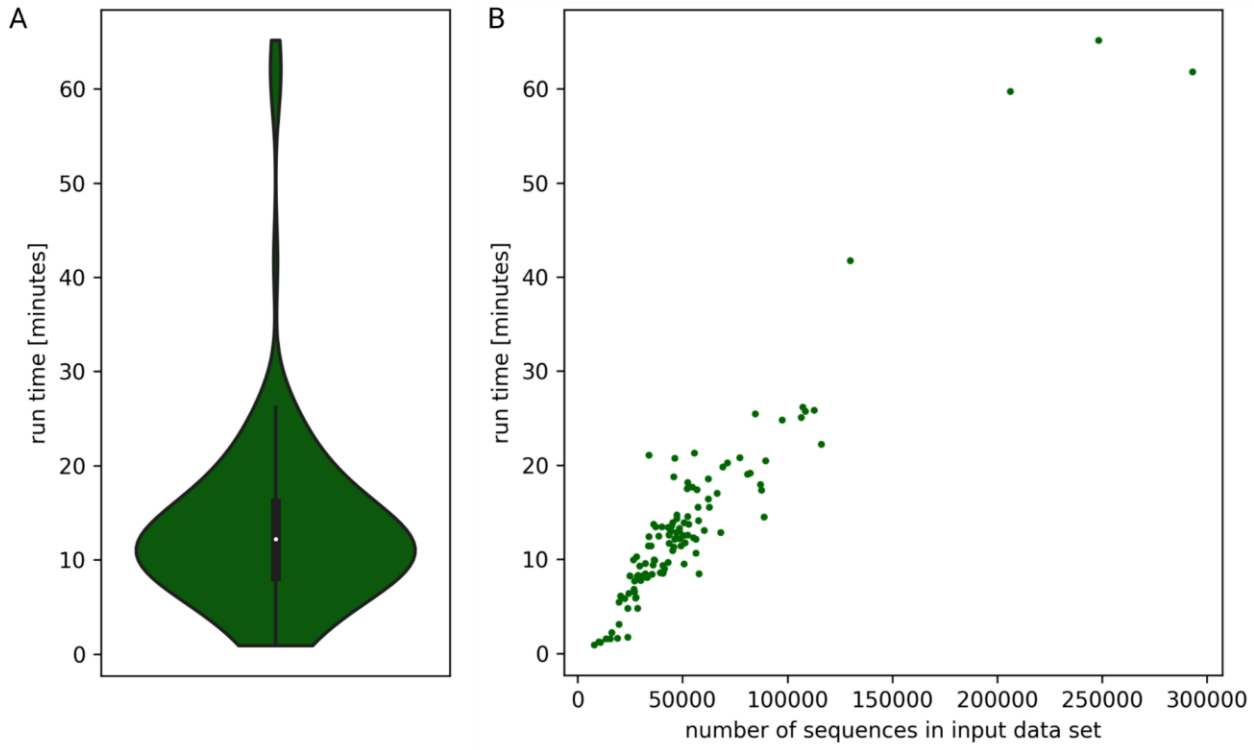


Figure 3: KIPEs3 run time analysis based on 121 Phytozome data sets (A) revealed that the run time is depending on the number of sequences in the input data set (B).

Comparison of KIPEs3 to other databases

MetaCyc, Plant Metabolic Network (PMN), and Plant Reactome are comprehensive databases describing the pathways, enzymes, and substrate compounds. While MetaCyc contains information about all domains of life, PMN and Plant Reactome are specific to plants and green algae. KIPEs3 is a specialized tool which provides individual gene level annotation for a user defined biosynthesis pathway in any species. MetaCyc contains only genes with experimentally determined functions in the represented pathways. PMN comprises 126 species-specific datasets and houses PlantCyc which covers over 500 additional plant species. PlantCyc harbors experimentally supported and predicted gene functions. The predicted enzymes are classified using the Ensemble Enzyme Prediction Pipeline (E2P2) software and RPSD v4.2 [59]. E2P2 uses BLAST [60] and PRIAM [61] to assign functions using sequence similarity to previously-known genes sourced from MetaCyc, BRENDA [62], and SwissProt [63] among others. The genome sequences used for this annotation are selected on the basis of at least 75% BUSCO (Benchmarking Universal Single-Copy Orthologs) completeness [34]. Plant Reactome uses *Oryza sativa* as a reference species to predict enzymes in 120 other species currently available in the database. Enzyme prediction in a species is based on orthologs of that enzyme in rice. Enzyme prediction in KIPEs3 is more stringent as it starts with ortholog identification based on multiple experimentally identified genes defined in literature (bait sequences). Ortholog identification is followed by an in-depth sequence similarity analysis including a check of the presence of functionally relevant amino acids. Only those

203 candidates which pass all these steps are reported as most likely functional. Moreover, KIPEs3 can also
204 identify similar candidates that do not fulfill all selection criteria. Contrary to most databases,
205 researchers can use their own assembled dataset when running KIPEs3. This option enables the
206 functional annotation of new genome sequences to identify a particular pathway of interest, given some
207 prior knowledge about the pathway. Some features of these databases compared to KIPEs are
208 summarized in **Table 2**.

209

210 **Table 2**: Comparison of KIPEs3 against established pathway databases.

Parameters	KIPEs3	PMN	MetaCyc	Plant Reactome
Web server available	✔	✔	✔	✔
Works with user supplied datasets	✔	✖	✖	✖
Cross-linking with other databases	✖	✔	✔	✔
Basis of Enzyme prediction	Identification of orthologs using reference datasets, conserved functional residues, phylogenetic trees, co-expression analysis	Ensemble Enzyme Prediction Pipeline v4.0 and RPSD v4.2	Scientific experimental literature	Reference species *Oryza sativa*
Annotation extent	Functional gene annotation in specific pathways	Annotate metabolites, reactions and multiple pathways	Describes metabolic pathways, chemical compounds, reaction and enzymes	Database of plant metabolic and regulatory pathways

211

212 Functional annotation performance of KIPEs3 compared to other databases

213 We compared the annotation results for FLS and LYC-b in terms of correctness of the annotation.
214 Functional annotation correctness of a candidate can be assessed (a) in terms of correct amino acid
215 residues required for a functional enzyme, (b) in terms of its position in a phylogenetic tree with
216 characterized orthologous sequences, and (c) in terms of expression pattern. A higher gene expression is
217 usually suggesting functional relevance of a given candidate. Co-expression with other members of the
218 same biosynthesis pathways is also helpful in identifying the most promising candidates. We followed a
219 combined approach for LYC-b and FLS annotation. The annotation correctness was assessed by the co-
220 expression of a given candidate with other genes of the flavonoid or carotenoid biosynthesis,
221 respectively. The presence of functionally relevant amino acid residues served as the final and deciding
222 factor for declaring a candidate gene as “functional”.

223

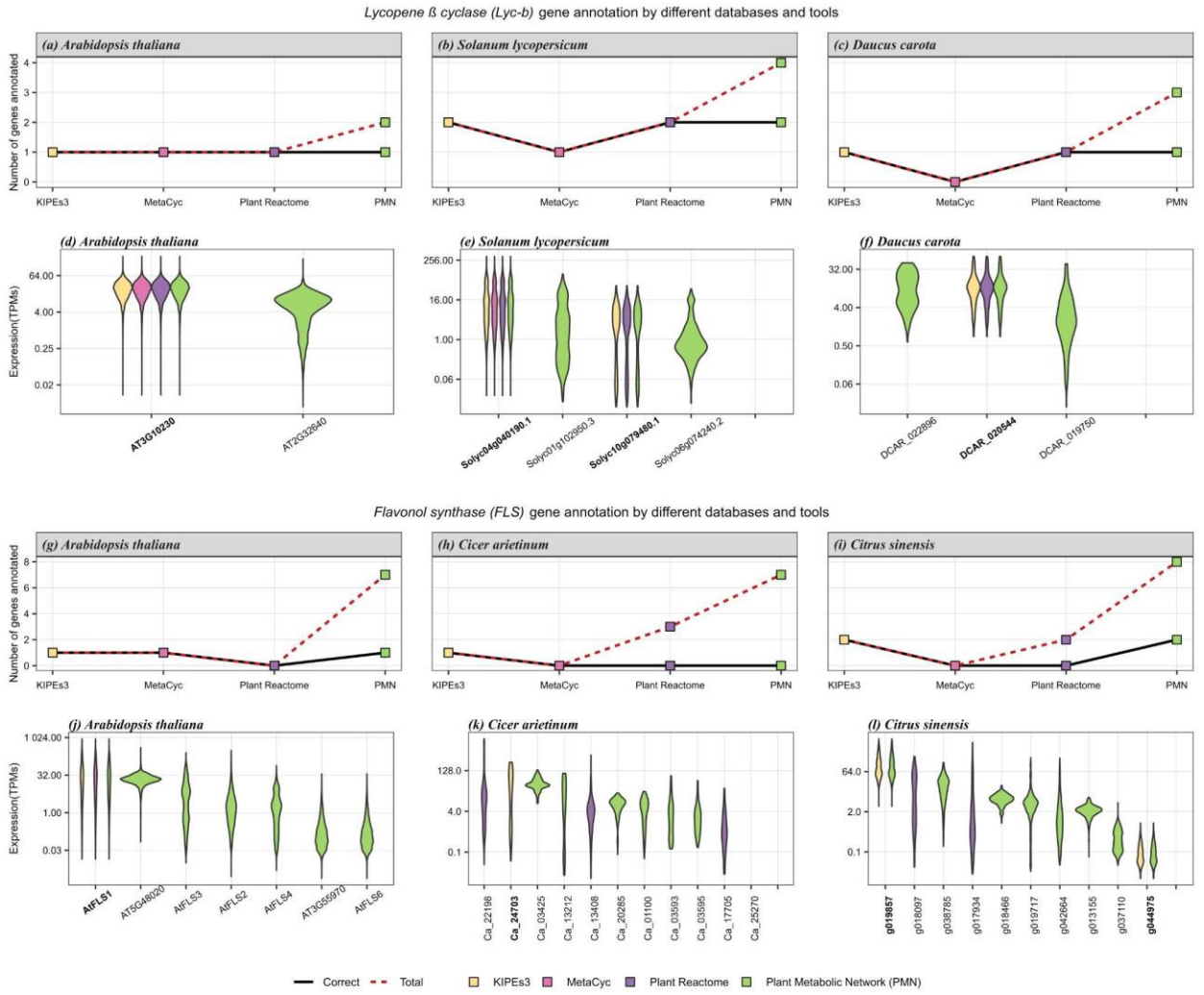


Figure 4: Functional annotation tool comparison using the *LYC-b* gene from the carotenoid biosynthesis pathway and the *FLS* gene from the flavonoid biosynthesis pathway. Comparison of LYC-b annotation **(a-f)** between KIPEs and three databases. The number of annotated (red dashed line) and correctly annotated (black solid line) LYC-b genes is displayed per tool/database for *Arabidopsis thaliana* **(a)**, *Solanum lycopersicum* **(b)**, and *Daucus carota* **(c)**. The corresponding gene expression (TPM) of all candidate genes was investigated to differentiate between genes and potential pseudogenes **(d-f)** for the three species, respectively. Functional gene IDs are written in *boldface*. Comparison of FLS annotation **(g-l)** between KIPEs and three databases. The number of annotated (red dashed line) and correctly annotated (black solid line) FLS genes is displayed per tool/database for *Arabidopsis thaliana* **(g)**, *Cicer arietinum* **(h)**, and *Citrus sinensis* **(i)**. The corresponding gene expression (TPM) of all candidate genes was investigated to differentiate between genes and potential pseudogenes **(j-l)** for the three species, respectively. Functional gene IDs are written in *boldface.*

Lycopene β cyclase (LYC-b) is one of the key enzymes in the carotenoid biosynthesis pathway and catalyzes the β-cyclization of both ends of lycopene to produce β-lycopene. LYC-b is known to have

240 conserved domains such as “dinucleotide binding motif”, “Cyclase motif I and II” , “Conserved region β-
241 LYC”, and a “β cyclase motif” [64]. Since studies about the amino acid residues responsible for catalytic
242 sites are still lacking, a phylogenetic tree of LYC-b candidates was constructed to identify likely
243 functional orthologs. The carotenoid genes, neoxanthin synthase (NSY) and lycopene ε-cyclase (LYC-ε),
244 were taken as an outgroup due to their high structure similarity to LYC-b [65] (Additional File 2,
245 Additional File 3).

246 FLS is a key enzyme of the flavonol biosynthesis pathway and catalyzes the oxidation of dihydroflavonols
247 to flavonols. FLS belongs to the 2-oxoglutarate dependent dioxygenase (2-ODD) superfamily. They are
248 characterized by conserved 2-oxoglutarate binding residues (Arg287 and Ser289) and conserved ferrous
249 iron-binding residues (His221, Asp223, and His277) [66]. A functional FLS also possesses residues
250 responsible for proper folding of 2-ODD polypeptide (Gly68, His75, Pro207, and Gly261) [67]. FLS specific
251 motifs, “PxxxIRxxxEQP” and “SxxTxLVP”, distinguish FLS from other plant 2-ODDs like F3H, ANS, and FNS
252 II (Additional File 4).

253 We used two metrics (*specificity* and *accuracy*) to characterize the annotation performance of the
254 different tools and databases. *Specificity* describes how well the database/tool can annotate a functional
255 enzyme. It is calculated as the ratio of the number of correct candidates predicted by the database/tool
256 to the total number of functional candidates present in that species. *Accuracy* denotes the ability of a
257 database/tool to discriminate between functional and non-functional candidates. It is calculated as the
258 ratio of correct predictions to the total predictions made by a database/tool for a given species and a
259 gene in a pathway. Finally, the annotation performance is calculated as the average sum of s*pecificity*
260 and *accuracy*. For LYC-b annotation, we found that the performance of Plant Reactome and KIPEs was
261 100% in all three species, *Arabidopsis*, tomato, and carrot. MetaCyc showed 100% annotation
262 performance for *Arabidopsis*, 75% for tomato, and dropped to 0% for carrot as it did not annotate any
263 LYC-b gene in carrot. Despite the high specificity (100%) of Plant Metabolic Network, the performance
264 was between 65-75% for all three species due to low accuracy. (**Figure 4** (a-f)). KIPEs3 maintained its
265 100% performance in all three species, *Arabidopsis*, chickpea, and orange, used for FLS annotation as
266 well. Like KIPEs3, MetaCyc provides a correct *Arabidopsis* FLS annotation. However, MetaCyc does not
267 report any FLS in chickpea and orange, while KIPEs3 identified candidates in both species. Plant
268 Reactome was neither specific nor accurate in FLS annotation and failed to annotate any candidate in
269 any of the above species. Plant Metabolic Network showed low performance (~0-60%) again due to low
270 accuracy (**Figure 4** (g-i)).

271 Plant Metabolic Network exhibited the least accuracy which is likely due to the function prediction
272 approach using sequence similarity alone which can lead to inaccurate prediction. Highly similar
273 enzymes belonging to the same family might have similar sequences, but may have different functions *in*
274 *vivo*. The performance of Plant Reactome was also low in case of FLS annotation emphasizing the
275 downfall of using only rice in curation and prediction, as genes in species distant from rice will be
276 predicted less reliably and genes missing in rice cannot be predicted in other species. MetaCyc contains
277 only experimentally evidenced genes, hence has high accuracy but low specificity. This also leads to
278 annotation of a limited number of genes and species.

279 In summary, KIPEs3 showed the best performance for functional annotation of genes having both high
280 accuracy as well as specificity. This makes KIPEs3 a good tool for annotating novel genome sequences
281 with focus on a particular pathway with a substantial amount of prior knowledge about the involved
282 genes in other species.

283

284 KIPEs3 is available as an online service

285 KIPEs3 is provided as an online service hosted on a webserver at TU Braunschweig [68]. Users can
286 upload their FASTA files containing coding sequences or peptide sequences of a species. Bait sequence
287 sets are stored on the server and can be directly used without the need to be manually uploaded by the
288 user. Optional parameters are pre-configured to allow an automatic analysis with default settings, but
289 can also be modified by the user through the web interface if necessary. Users may opt-in to receive
290 email notifications once their analysis is finished and the results are available. Alternatively, users may
291 choose to store a cookie to allow their browser to keep track of the progress. The archive provided for
292 download includes all result files that users would get when running KIPEs3 locally.

293 The web server is implemented in Python3 using the Django web framework v4 [69]. Whenever a user
294 uploads a file and submits a new job through the form on the website, the form data is sent to the
295 server via an HTTP POST request and the file is temporarily stored on the server machine. The job is
296 placed into a queue and executed as soon as there are enough free resources (disk space, memory, and
297 CPU cores) available. KIPEs3 and all its dependencies are installed on the server machine. The user is
298 redirected to a page with a console showing the command that is executed, and the program output is
299 displayed in real time using XMLHttpRequests. All user supplied data are deleted from the server 48
300 hours after the job has been finished and the results have been downloaded.

301

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

304 First, a technical check was performed by analyzing the flavonoid biosynthesis in the *A. thaliana*
305 accession Niederzenz-1 (Nd-1). It is expected that orthologs of all characterized genes of the reference
306 accession Columbia-0 (Col-0) are also present in Nd-1, while the sequences are slightly different from
307 the bait sequences. All genes known in Col-0 were also discovered in Nd-1 (Additional File 2). Next,
308 *Capsella grandiflora* was analyzed with KIPEs3 to show that the identification of flavonoid biosynthesis
309 genes works also in species that are not represented in most of the bait sequence sets like *A. thaliana*.
310 Genes present in *A. thaliana* were also discovered in the close relative *C. grandiflora* (Additional File 3).
311 To demonstrate the potential of KIPEs3 to annotate the flavonoid biosynthesis in a distant relative of *A.*
312 *thaliana*, the flavonoid biosynthesis of *Dioscorea dumetorum,* which belongs to the monocots, was
313 analyzed (Additional File 5).

314 The new flavonoid biosynthesis pathway steps added to the repertoire of KIPEs3 include the diverse and
315 promiscuous decoration enzymes like glycosyltransferases, acyltransferases, and methyltransferases.
316 Since some of these steps might be species-specific, it is possible that some steps are missing in a given

317 plant species of interest. This requires specific attention when interpreting the KIPEs3 results to avoid
318 false positives. While the aglycon biosynthesis is highly conserved and catalyzed by well characterized
319 enzymes, flavonoid decorating enzymes are less studied and show a high level of promiscuity [70–72].
320 This poses a challenge for the identification through sequence similarity. Little is known about the
321 functionally relevant amino acids in the flavonoid decorating enzymes. In addition, this extension of the
322 KIPEs3 flavonoid biosynthesis bait sequence sets is no longer restricted to enzymes, but also includes
323 flavonoid transporters. They do not have an active center that could be composed of functionally
324 relevant amino acids. This turns the reliable identification of transport-associated proteins into a
325 remaining challenge.

326

327 Analysis of the carotenoid biosynthesis

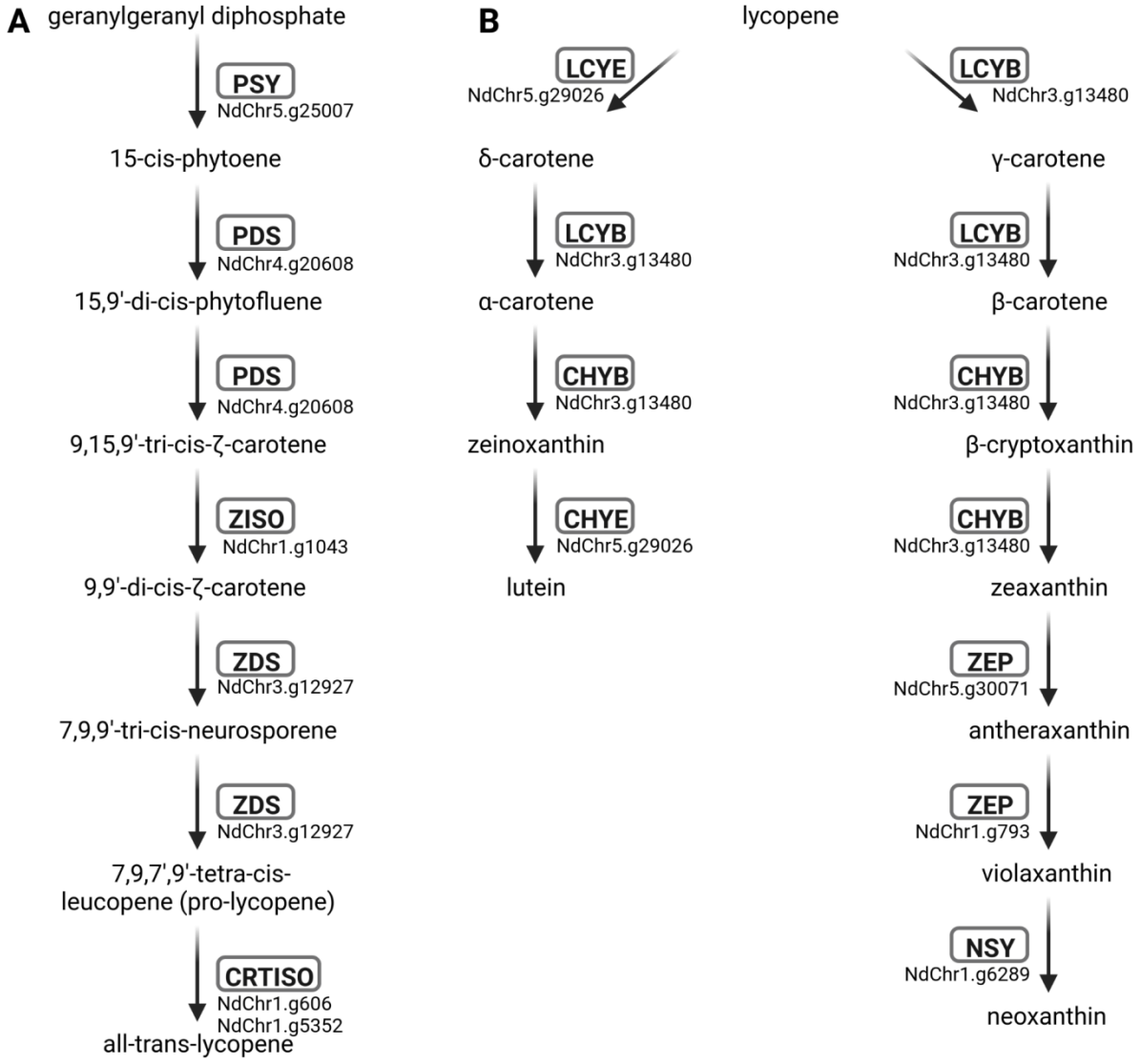
328 A novel set of bait sequences was compiled to enable the annotation of the carotenoid biosynthesis
329 with KIPEs3 (**Table 3**). The carotenoid biosynthesis is well studied in many plant species including *A.*
330 *thaliana* [73]. The set of compiled bait and reference sequences was evaluated by running the
331 annotation workflow on *A. thaliana* Nd-1, *Daucus carota*, *C. rubella*, and *D. dumetorum* (**Figure 5**;
332 Additional File 3). *Daucus carota* carotenoid biosynthesis genes previously described in the literature
333 [74] were recovered by KIPEs3 (Additional File 6). This supports the accuracy of the candidate gene
334 identification.

335

336 **Table 3**: Carotenoid biosynthesis genes. [1] most likely non-functional [2] BCH duplication after monocot-
337 dicot split

EC Number	Name of the step	Representative sequence / Label in KIPEs3	Reference
EC:2.5.1.32	Phytoene synthase (PSY)	AT5G17230	[75]
EC:1.3.5.5	Phytoene desaturase (PDS)	AT4G14210	[76]
EC:1.3.5.6	Zeta-carotene desaturase (ZDS)	AT3G04870	[77]
EC:5.2.1.12	15-cis-zeta-carotene isomerase (ZISO)	AT1G10830	[78]
EC:5.2.1.13	Carotenoid isomerase 1 (CRTISO1/CCR2)	AT1G06820	[79]
--------	Carotenoid isomerase 2 (CRTISO2)[1]	AT1G57770	-
EC:5.5.1.19	Lycopene cyclase (LCYB/CRTL-B)	AT3G10230	[80]
EC:5.5.1.18	Lycopene cyclase (LCYE/CRTL-E)	AT5G57030	[81]
EC:1.14.15.24	Beta-carotene hydroxylase 1 (BCH1/CHYB1/CHY1)	AT4G25700	[82]
EC:1.14.15.24	Beta-carotene hydroxylase 2	AT5G52570	[83]

	(BCH2/CHYB2/CHY2)[2]		
EC:1.14.-.-	Carotene beta-ring hydroxylase (CYP97A3/LUT5)	AT1G31800	[84]
EC:1.14.-.-	Cytochrome P450 97B3 (CYP97B3)	AT4G15110	[85]
EC 1.14.14.158	Carotene epsilon-monooxygenase (CYP97C1/CHYE/LUT1)	AT3G53130	[86]
EC 1.14.15.21	Zeaxanthin epoxidase (ZEP/ABA1/NPQ2)	AT5G67030	[87]
EC 1.23.5.1	Violaxanthin de-epoxidase (VDE/NPQ1)	AT1G08550	[87]
EC 5.3.99.9	Neoxanthin synthase (NSY/ABA4)	AT1G67080	[88]



340 **Figure 5**: Carotenoid biosynthesis genes in *Arabidopsis thaliana* Nd-1. The carotenoid biosynthesis
341 comprises a linear part (A) and a branching part (B).

342

343 Transcriptional regulation of biosynthesis pathways

344 Many plant biosynthesis pathways are regulated at the transcriptional level by members of large
345 transcription factor families. Examples are the R2R3-MYBs and bHLHs that form complexes involved in
346 the control of the flavonoid biosynthesis [89]. As we demonstrated previously, it is possible to analyze
347 large gene families like MYBs and bHLHs with KIPEs [12]. However, KIPEs3 is optimized for the analysis of
348 pathways that comprise genes with low copy numbers. Most structural genes in these pathways are part
349 of a small gene family. Large gene families like the P450 cytochromes (CYPs) or 2-oxoglutarate
350 dioxygenases (2-ODDs) comprise multiple smaller subfamilies with distinct biological functions. Only the
351 individual subfamilies are relevant for the analysis with KIPEs3. Ideally, the application of KIPEs3 should
352 be restricted to the annotation of structural genes in a biosynthesis. While it is possible to investigate
353 large transcription factor families, dedicated tools will perform better. Members of the MYB and bHLH
354 gene families can be annotated by specific tools [47]. General phylogeny-based approaches can be
355 deployed to study other involved transcription factor families [90, 91].

356

357 Conclusions

358 While the generation of high-quality genome sequences is turning into a routine task, the structural and
359 especially the functional annotation of these sequences is an increasing challenge. The ability to
360 annotate biosynthesis pathways beyond the flavonoid biosynthesis positions KIPEs3 as a versatile
361 platform for functional annotation in projects focused on specialized metabolites. We demonstrated the
362 capabilities of KIPEs3 by comparing the annotation of *FLS* and *LYC-b* in three species against the results
363 of other popular tools and databases. We showed that the use of broad taxonomic range bait sequences
364 and functionally relevant amino acids allows KIPEs3 to recover more correct annotations per pathway
365 than other tools that rely on general sequence similarity. KIPEs3 can easily annotate novel genome
366 sequences with focus on a particular pathway while this is not possible with other annotation tools.
367 Although we focused on plant metabolism in this study, KIPEs3 could also be applied for the
368 investigation of non-plant organisms like fungi or bacteria. A sufficient amount of prior knowledge about
369 the pathway in other species is the only requirement. The automatic annotation of biosynthesis
370 pathways paves the way for comparative genomic studies that are looking for species-specific
371 differences or intraspecific variation.

Availability 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

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

400

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404

405 **Authors' contributions**

406 AR developed the web server framework and contributed to the manuscript. NC performed
407 benchmarking of KIPEs3 and improved the carotenoid biosynthesis annotation. BP designed the
408 research, conducted experiments, wrote the first draft, and revised the manuscript.

409

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415

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619

620 ## Additional files

621 **Additional file 1:** Accession numbers of the analyzed RNA-seq datasets.

622 **Additional file 2:** Multiple sequence alignment of the LYC-b candidates in three species (*Arabidopsis*
623 *thaliana*, *Solanum lycopersicum*, and *Daucus carota*) annotated by KIPEs3, MetaCyc, Plant Reactome,
624 and Plant Metabolic Network. The alignment was generated using MAFFTv7. Characteristic regions of
625 plant LCY-bs are indicated above the sequences: Dinucleotide binding site, Cyclase motifs (CM) I and II,
626 and Charged region. Domains described as essential for LCY-b activity are shown as LCY-b CAD (Catalytic
627 Activity Domain). The functional LYC-b on the basis of presence of conserved amino acid residues are
628 highlighted in blue color.

629 **Additional file 3:** Phylogenetic tree of the LYC-b candidates in the three species (*Arabidopsis thaliana*,
630 *Solanum lycopersicum*, and *Dacus carota*). The highly similar sequences of Neoxanthin synthase (NSY)
631 and Lycopene-e-cylase (LYC-e) were included as outgroup (highlighted in gray). The functional LYC-b
632 clade was highlighted in the phylogenetic tree.

633 **Additional file 4:** Multiple sequence alignment of the FLS in three species (*Arabidopsis thaliana*, *Cicer*
634 *arietinum*, and *Citrus sinensis*) annotated by KIPEs3, MetaCyc, Plant Reactome, and Plant Metabolic
635 Network. The alignment was generated using MAFFTv7. The two black boxes highlight the FLS-specific
636 motifs "PxxxIRxxxEQP" and "SxxTxLVP." Amino acid residues responsible for binding ferrous iron (H221,
637 D223, and H277) and 2-oxoglutarate (R287 and S289) are marked with black and gray arrows,
638 respectively. The residues known to be involved in the proper folding of the 2-ODD polypeptide are
639 marked with asterisks (G68, H75, P207, and G261). The functional FLS on the basis of the presence of
640 conserved amino acid residues are highlighted in red-yellow color.

641 **Additional file 5**: Results of the flavonoid biosynthesis analysis in *Arabidopsis thaliana* Nd-1, *Capsella*
642 *rubella*, and *Dioscorea dumetorum*.

643 **Additional file 6**: Results of the carotenoid biosynthesis analysis in *A. thaliana* Nd-1, *Daucus carota*, *C.*
644 *rubella*, and *D. dumetorum*.