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A Scaffoldomics Platform for Modular In Vivo Enzyme Colocalisation and Its Application to Naringenin Biosynthesis

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ABSTRACT

Insufficient product yield remains a major bottleneck in the development of microbial cell factories for fine chemical production. In vivo enzyme colocalisation on synthetic scaffolds has emerged as a promising strategy to enhance metabolic efficiency, yet current approaches are often labour-intensive and inefficient. Here, we present the development of a ‘scaffoldomics’ platform, a generic synthetic framework designed to enable combinatorial scaffolding of biosynthetic pathways for the generation of diverse multi-enzyme complexes. This system enables the assembly of up to four pathway enzymes onto a protein scaffold. As a proof of concept, the platform was applied to the biosynthesis of naringenin, a key flavonoid intermediate. Integration of a chromosomally encoded naringenin biosensor in *Escherichia coli* allowed for real-time detection and pathway evaluation. The biosensor response curve was established and confirmed functional naringenin production. Moreover, comparative experiments demonstrated that the addition of a scaffold to docking enzymes significantly enhanced yield up to a ~19-fold, indicating a positive colocalisation effect. These results highlight the utility of the scaffoldomics platform as a powerful tool for further efficient combinatorial design, construction, and optimisation of biosynthetic pathways in synthetic biology.

1 | Introduction

The increasing demand for sustainable alternatives to petrochemical-based production processes and the extraction of valuable compounds from plants and other natural sources has driven the development of microbial cell factories (MCFs) as versatile platforms for the biosynthesis of a wide range of products. These include, but are not limited to, biofuels, pharmaceuticals, bioplastics, food additives, and fine chemicals. Engineered microorganisms offer the ability to convert inexpensive, renewable substrates into complex molecules under mild, environmentally friendly conditions. As such, they are central to the ongoing transition towards a circular and bio-based economy (Katsimpouras and Stephanopoulos 2021). Despite substantial advances in synthetic biology, strain engineering, and metabolic

pathway optimisation, one of the main remaining challenges in microbial bioproduction is to attain product yields and productivities that meet industrial standards to be economically competitive. However, this hurdle is less critical for the production of fine chemicals, such as flavours, fragrances, or intermediates for pharmaceuticals, where the high market value of the products may enable a faster recovery of production costs, making biosynthetic approaches more readily competitive compared to conventional chemical synthesis or extraction (Schaerlaekens et al. 2015). Nevertheless, even in this context, further optimisation remains crucial to improve efficiency, minimise resource use, and enhance robustness.

One promising strategy to boost pathway performance is the spatial colocalisation of enzymes that operate sequentially in a

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metabolic cascade. Positioning these enzymes in close proximity may enhance substrate channelling, reduce diffusion losses and intermediate degradation, and increase local concentrations of reaction intermediates (Dueber et al. 2009). Collectively, these effects can accelerate flux through the pathway and improve the overall conversion rate. This principle, known as scaffolded enzyme assembly, mimics naturally occurring multi-enzyme complexes. To achieve enzyme colocalisation, several types of scaffolding systems have been developed, including nucleic acid-based (Delebecque et al. 2011; Wilner et al. 2009) and protein-based scaffolds (Vanderstraeten and Briers 2020). Among these, modular synthetic protein scaffolds stand out for their ability to control enzyme stoichiometry and spatial arrangement with high precision (Gad and Ayakar 2021). A widely used strategy for assembling multi-enzyme complexes on protein scaffolds involves cohesin-dockerin interactions, originally derived from cellulosomes (Bayer et al. 1994). In this approach, enzymes are genetically fused to dockerin domains, forming docking enzymes (DE) that specifically bind to matching cohesin modules in the scaffold, thereby enabling targeted and modular complex formation.

Our group introduced the VersaTile technique, a technique suitable for the efficient assembling of modular proteins at the DNA level. This modular DNA assembly method, based on Type II restriction enzymes, allows for the streamlined shuffling and combination of genetic parts encoding protein modules (Gerstmanns et al. 2020). VersaTile implies two steps: (1) the construction of a repository of all modules or so-called 'tiles' that are cloned in a specific entry vector (VersaTile Cloning); and (2) the subsequent assembly of a freely chosen selection of these tiles in a predesigned order in a VersaTile-compatible destination vector (VersaTile Shuffling). A tile represents a coding sequence encoding a specific protein module, designed to be compatible with the VersaTile system. Vanderstraeten et al. (2022) utilised the VersaTile system to efficiently construct diverse DEs and scaffolds, each assembled separately in dedicated VersaTile destination vectors (pVTDs). Recently, this tailored VersaTile system was used to colocalise the first three glycolytic enzymes on a cellulosome-based synthetic protein scaffold (Elias et al. 2025). Additionally, Lamote and colleagues used the VersaTile system to generate scaffoldin and docking enzyme constructs for a self-assembling *S. cerevisiae* display platform in consolidated bioprocessing (Lamote et al. 2025).

While inspired by natural multi-enzyme assemblies, the practical implementation of this approach in engineered systems remains technically challenging. This research was therefore motivated by the need for a standardised, modular, and flexible solution for constructing such multi-enzyme assemblies. To address this, we developed a dedicated 'scaffoldomics' platform that enables the systematic design and assembly of multi-enzyme complexes in MCFs. Its modular and standardised architecture enables combinatorial high-throughput design and optimisation of biosynthetic pathways, supporting synthetic scaffolding at the 'omics' scale. The generic design of the platform allows its convenient application to other biosynthetic cascades.

An interesting class of fine chemicals that has attracted the attention of the industry are plant metabolites such as flavonoids. These flavonoids have been used as pigments and flavouring

agents. They have also garnered ample interest from industry due to the rising demand for healthy food and pharmaceutical innovations, for example, they are reported to serve as antioxidants (Watson and Oliveira 1999). Besides, flavonoids are known to prevent cardiac diseases, cancer, gastrointestinal diseases and inflammation (García-Mediavilla et al. 2007; Goris et al. 2021; Miesan and Mohamed 2001; Watson and Oliveira 1999; Zu et al. 2006). A key backbone of flavonoids is the flavanone naringenin from which flavones are derived with apigenin and luteolin as the most prominent compounds (Vries et al. 1997). Naringenin detection can be achieved using transcriptional biosensors, based on natural transcriptional regulatory systems. By reprogramming these natural mechanisms, it is possible to create transcriptional biosensors capable of detecting small molecules such as naringenin and linking them to an easily measurable output signal, such as fluorescence. A first method for the industrial production of flavonoids is extraction from plant material. However, since the starting material contains only trace amounts (several ppm to tens of ppm), and significant losses occur during extraction, conversion, and purification, this method is highly inefficient (WO2006/093368; WO/2001/051482). In addition to plant extraction, flavonoids can be synthesised chemically or produced via plant cell cultures. However, both methods suffer from low production efficiencies and high costs. Hence, there is a market need for alternative, scalable and economically viable production technologies (Bourgau et al. 2001; Rao and Ravishankar 2002; Verpoorte et al. 2002).

The first part of this study describes the development of the scaffoldomics platform. Next, as proof-of concept, this scaffoldomics platform was applied to naringenin biosynthesis. A validated plasmid-based naringenin-responsive biosensor was incorporated into the genome of *E. coli*, resulting in a strain with a chromosomally encoded naringenin biosensor. Finally, naringenin production and a potential colocalisation effect were investigated.

2 | Results and Discussion

2.1 | Hierarchical Assembly of Multi-Enzyme Complexes

The proposed multi-enzyme complexes are composed of two types of modular proteins: the scaffold and the DEs. The scaffold acts as the structural backbone, consisting of multiple cohesin domains, while the DEs are each linked to a specific dockerin module via a flexible linker, allowing them to bind to the corresponding cohesin domains on the scaffold upon co-expression. Here, a new hierarchical assembly system (Figure 1) was developed to integrate the coding sequences of up to four pathway enzymes together with a scaffold into a single destination vector (pVTD). This led to the development of a modular 'scaffoldomics' platform for constructing multi-enzyme complexes in *Escherichia coli*, enabling efficient in vivo colocalisation of pathway enzymes. By applying enzyme scaffolding in a combinatorial and high-throughput manner, the platform can reach the 'omics' level. This system builds upon a modified version of the VersaTile approach previously described by Vanderstraeten et al. (2022), where DEs and scaffolds are assembled separately in

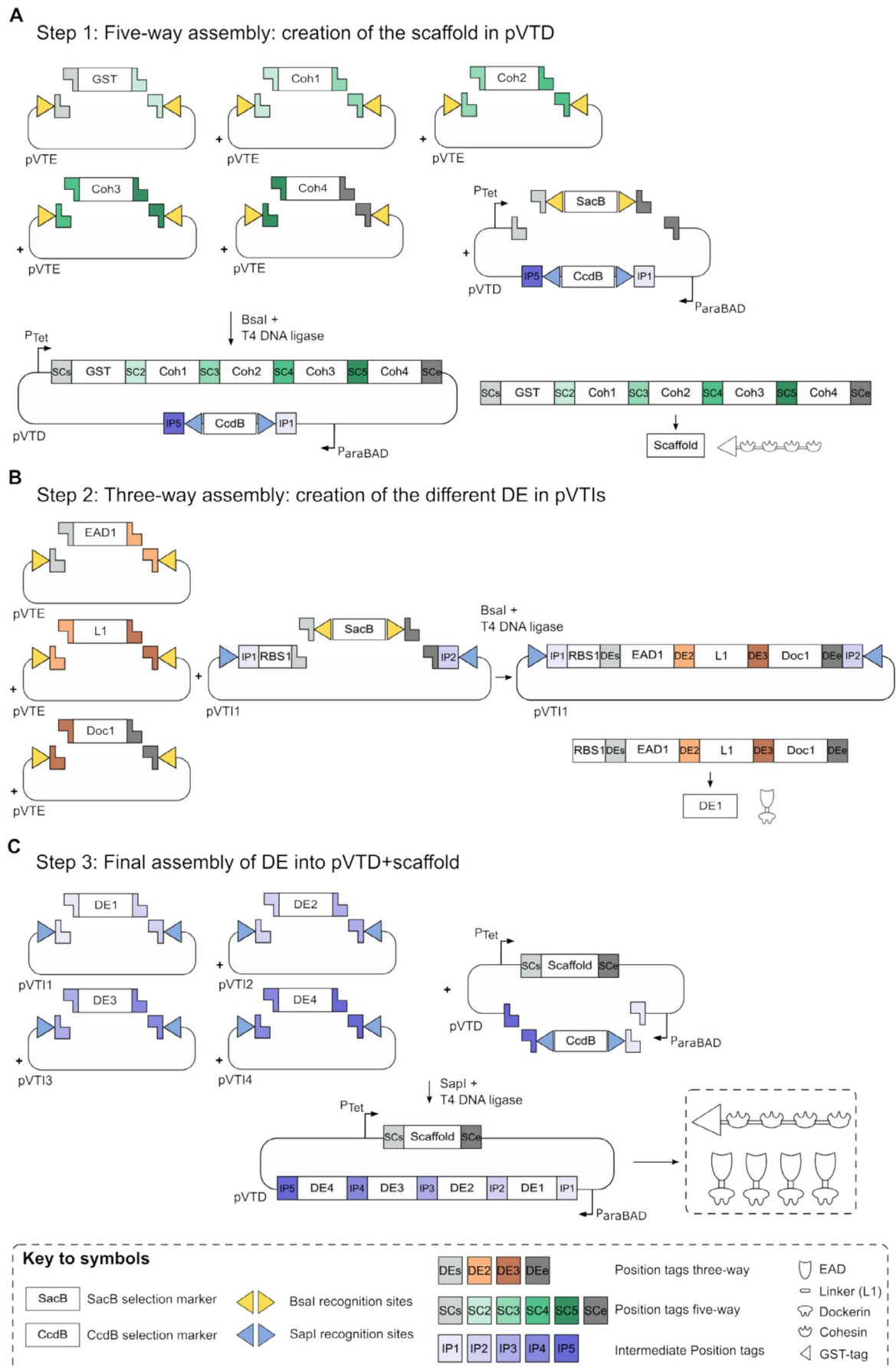


FIGURE 1 | Legend on next page.

FIGURE 1 | Hierarchical assembly of docking enzymes (DEs) and scaffold in pVTD. Stepwise construction of the expression plasmid pVTD (Figure S1) bearing all components of a scaffolded biosynthetic pathway with four enzymes. (A) Step 1: The scaffold sequence is assembled into the VersaTile Destination vector pVTD using BsaI-mediated cleavage and the five-way position system, creating complementary overhangs for seamless integration. (B) Step 2: DE coding sequences are first inserted into four VersaTile Intermediate vectors (pVTIs), each carrying unique ribosome binding site (RBS) and Intermediate Position (IP) tags. BsaI digestion enables precise orientation generating DEs as intermediate constructs. The assembly process for docking enzyme 1 (DE1) is shown; the other three DEs are assembled in a similar manner. EAD represents the enzymatically active domain. (C) Step 3: Final assembly of the four DEs from the four pVTIs into the pVTD vector, which already contains the scaffold. This is achieved through SapI digestion and the four-way position system, ensuring correct positioning of the RBS-DE cassettes. The scaffold is regulated by an inducible P_{Tet} promoter, whereas the DEs are driven by an inducible P_{araBAD} promoter. The yellow and blue arrowheads indicate the orientation of the BsaI and SapI recognition sites, respectively, which determine how each tag is cut. Importantly, the orientation of these sites differs between pVTD and pVTE, pVTE and pVTI, and pVTI and pVTD, allowing for correct assembly.

dedicated pVTDs. To implement this system, all building blocks, such as enzymatically active domains (EADs), linkers, dockerins, and cohesins, are converted into 'tiles' using the VersaTile Cloning method (Gerstmans et al. 2020). A tile refers to a coding sequence designed for a specific protein module, optimised for compatibility with the VersaTile technique. This sequence is flanked by position tags and BsaI recognition sites and subsequently cloned into the VersaTile Entry vector pVTE. Upon BsaI digestion, the position tags generate distinct single-stranded overhangs. To create the new hierarchical assembly system the five-way and a three-way assembly system for the construction of scaffolds and DEs, respectively, was implemented in a consecutive way. The five-way system enabled the construction of scaffolds by fusing five modules, each linked to distinct pairs of position tags (e.g., SCs-SC2, SC2-SC3, SC3-SC4, SC4-SC5, SC5-SCe, Table S1). Similarly, the three-way system was used to assemble docking enzymes by fusing three modules, each associated with a specific pair of position tags (e.g., DEs-DE2, DE2-DE3, DE3-DEe, Table S2). Both the five-way and three-way position tags were introduced in (Vanderstraeten et al. 2022). Once a tile repository is available, both rational, semi-random and random assembly of tiles is highly convenient and efficient. At any time point during an optimisation process, the tiles can be re-used to make and test new assemblies, or tiles can be added to increase the possible variation. During the hierarchical assembly process, the appropriate tiles are selected, mixed, and concatenated to generate the modular coding sequences for the DEs and the scaffold. An overview of the different steps is provided in Figure 1.

The *first* step involves assembling the scaffold directly into the pVTD vector (LMBP 14352, available at BCCM/GeneCorner, Figure 1A). The pVTD plasmid map is shown in Figure S1. During this process, the pVTD and position tags from the five-way shuffling system (Scs, Sc1, Sc2, Sc3, Sc4, and Sc5) are cleaved by BsaI, creating complementary overhangs that allow the assembly of the scaffold sequence into pVTD.

In a *second* step, the DEs are created in four intermediate vectors (pVTIs; Figure 1B). These pVTI vectors (pVTI1: LMBP 14348; pVTI2: LMBP 14349; pVTI3: LMBP 14350; pVTI4: LMBP 14351, all available at BCCM/GeneCorner) are identical except for their intermediate position (IP) tags and ribosome binding site (RBS) sequences and are used solely as an intermediate step to create DEs for each pathway enzyme (plasmid map is shown in Figure S2). The pVTI and position tags of the three-way DE shuffling system (DEs, DE2, DE3, and DEe) are cleaved by BsaI,

creating complementary overhangs that allow the assembly of a DE sequence into pVTI. Four separate assembly reactions are performed in parallel, resulting in four distinct intermediate vectors. After successful assembly, the SacB cassette in the intermediate vector is replaced with the DE coding sequence. Additionally, each intermediate vector incorporates a pair of position tags from a newly introduced set (IP1, IP2, IP3, IP4, and IP5, Table S3), which determine the orientation of the cassettes in the third assembly reaction (Step 3). Thus, these four vectors carry RBS1_DE1, RBS2_DE2, RBS3_DE3, and RBS4_DE4 flanked by position tags IP1 and IP2, IP2 and IP3, IP3 and IP4, and IP4 and IP5, respectively.

In the *third* step, at the second hierarchical level, the four DEs (encoded by four pVTIs) and one scaffold (pVTD + scaffold) are assembled into a single plasmid (Figure 1C). In this step, the RBSx_DEx cassettes are excised from the intermediate vectors and inserted into the scaffold-containing expression vector pVTD. This is accomplished by SapI digestion of pVTD and the position tags, which generates overhangs allowing the cassettes to be inserted in the correct order. Once the assembly is complete, the CcdB cassette in pVTD is replaced with the final set of DE coding sequences. Cassettes RBS1_DE1, RBS2_DE2, RBS3_DE3, and RBS4_DE4 will be placed in the first, second, third, and fourth positions, respectively, allowing up to four DE cassettes to be concatenated into a single plasmid that already comprised the sequence of the modular scaffoldin.

The assembled scaffold coding sequence in the pVTD is provided with an upstream inducible P_{Tet} promoter and a downstream terminator. The final DE coding sequences in pVTD are equipped with an upstream inducible P_{araBAD} promoter and a downstream T7 terminator. This configuration leads to the transcription of a single polycistronic mRNA encoding the four DEs. Since each DE is preceded by its own RBS and contains a distinct start codon, all four DEs will be translated independently from the same transcript. To balance expression, RBS strengths were varied from weak (first DE) to strong (last DE) (Table S4). This is important to compensate for translational polarity, a phenomenon in polycistronic mRNAs where downstream genes are translated less efficiently due to ribosome drop-off after upstream ORFs (Chemla et al. 2020), 5' → 3' mRNA degradation (Hui et al. 2014), and obstructive mRNA secondary structures near RBS sites (De Smit and Van Duin 1990). However, translation efficiency and functionality remain difficult to predict, as RBS effectiveness is highly dependent on the downstream protein sequence (Srivastava and Kumar 2024).

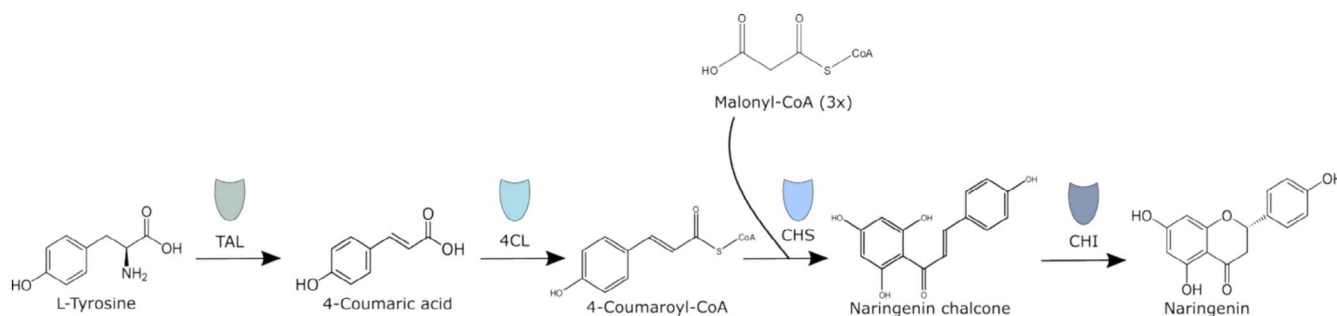


FIGURE 2 | Biosynthetic pathway of naringenin. Tyrosine ammonia lyase (TAL) catalyses the deamination of L-tyrosine, removing the amino group ($-\text{NH}_2$) to form 4-Coumaric acid. Next, 4-coumaric acid is converted into its CoA derivative, 4-coumaroyl-CoA, by the enzyme 4-coumarate-CoA ligase (4CL). This reaction requires ATP and coenzyme A (CoA). Chalcone synthase (CHS) catalyses the condensation of p-coumaroyl-CoA with another CoA derivative, malonyl-CoA, to form chalcone. The final enzyme, chalcone isomerase (CHI), catalyses the ring closure of chalcone, leading to the formation of naringenin. This is an isomerisation of the chalcone structure into a flavonoid structure, with the hydroxyl group positioned correctly, resulting in naringenin, a flavonoid.

TABLE 1 | Composition of scaffold and stuffer constructs assembled by the five-way assembly system in the pVTD.

P1 (Scs_Sc2)	P2 (Sc2_Sc3)	P3 (Sc3_Sc4)	P4 (Sc4_Sc5)	P5 (Sc5_Sce)	Vector (Scs_Sce)	Abbreviation
GST-tag 	Coh-Ctl 	Coh-CtlI 	Coh-Ac 	Coh-Af 	pVTD	pVTD+Scaff
stuffer 2					pVTD	pVTD+S2

Note: Columns P1–P5 correspond to the five available positions, with the indicated position tags (between brackets) (SCs (s = start), SC2, SC3, SC4, SC5, SCe (e = end)). The ‘pVTD + Scaff’ row shows the full scaffold with four cohesins (Coh) originating from different organisms (Table S8) and one glutathione-S-transferase (GST) tag, whereas the ‘pVTD + S2’ row is the stuffer control, in which all positions are occupied by stuffer 2. The stuffer is a non-coding tile sequence that contains stop codons in all reading frames.

Abbreviations: Ac, *Acetivibrio cellulolyticus*; Af, *Archaeoglobus fulgidus*; Ct, *Clostridium thermocellum*.

2.2 | In Vivo Biosynthesis and Detection of Naringenin Using Enzyme-Scaffold Complexes

As mentioned earlier, low product yield is a major obstacle in developing MCFs for the production of fine chemicals. The co-localisation of enzymes on a shared protein scaffold has been shown to significantly improve yields (Dueber et al. 2009). Using the previously described shuffling system, complexes can be constructed with up to four pathway enzymes assembled onto a scaffold. While the approach is generic, naringenin production in *E. coli* was selected to elaborate the scaffoldomics framework, as naringenin serves as a key intermediate in the biosynthesis of flavonoids, a diverse class of specialised plant metabolites with numerous essential applications (Bourgaud et al. 2001; Hussain et al. 2012; Pollier et al. 2011; Verpoorte et al. 2002; Wang et al. 2011). To tailor the scaffoldomics platform in *E. coli* for the naringenin biosynthetic pathway, four non-native enzymatic reactions need to be introduced. These reactions start with precursor molecules naturally found in *E. coli*, namely L-tyrosine, coenzyme A (CoA) and malonyl-CoA. The conversion is facilitated by four key enzymes: tyrosine ammonia-lyase (TAL, EC 4.3.1.23), 4-coumarate-CoA ligase (4CL, EC 6.2.1.12), chalcone synthase (CHS, EC 2.3.1.74), and chalcone isomerase (CHI, EC

5.5.1.6). An overview of the enzyme cascade can be found in Figure 2.

2.2.1 | Overview of Rational Constructs for the Scaffolded Pathway

Various constructs were rationally designed with the scaffoldomics platform. As interaction pairs, cohesin-dockerin pairs that have been rigorously validated and were known to exhibit no cross-binding (Vanderstraeten and Briers 2020) were employed (Table S8). First, either a complete scaffold or a stuffer sequence (S2) was inserted into pVTD, resulting in the constructs ‘pVTD + Scaff’ and ‘pVTD + S2’ (Table 1). The S2 sequence (shown in Table S5) served as a negative control to distinguish scaffold-specific effects in downstream assays. A glutathione-S-transferase (GST)-tag was fused to the scaffold for verification of DE assembly on the scaffold, using a GST pull-down assay.

Next, a set of docking and free enzymes (DEs and FEs) was generated in pVTIs (Table 2). The naringenin pathway enzymes are derived from various organisms (Table S9) and have been shown

TABLE 2 | Composition of docking and free enzymes constructs assembled by the three-way assembly system in the VersaTile Intermediate vectors (pVTIs).

P1 (DEs_DE2)	P2 (DE2_DE3)	P3 (DE3_DEe)	Vector (DEs_DEe)	DE	Abbreviation
<i>Fj</i> -TAL 	Li-E 	Doc-Ctl 	pVTI1	<i>Fj</i> -TAL_Li-E_Doc-Ctl 	DE1
<i>Pc</i> -4CL	Li-F	Doc-CtII	pVTI2	<i>Pc</i> -4CL_Li-F_Doc-CtII	DE2
<i>Gh</i> -CHS	Li-G	Doc-Ac	pVTI3	<i>Gh</i> -CHS_Li-G_Doc-Ac	DE3
<i>Ph</i> -CHI	Li-H	Doc-Af	pVTI4	<i>Ph</i> -CHI_Li-H_Doc-Af	DE4
P1 (DEs_DE2)	P2-P3 (DE2_DEe)	Vector (DEs_DEe)		FE	Abbreviation
<i>Fj</i> -TAL 	His-tag -His ₆	pVTI1		<i>Fj</i> -TAL_His-tag 	FE1
<i>Pc</i> -4CL	His-tag	pVTI2		<i>Pc</i> -4CL_His-tag	FE2
<i>Gh</i> -CHS	His-tag	pVTI3		<i>Gh</i> -CHS_His-tag	FE3
<i>Ph</i> -CHI	His-tag	pVTI4		<i>Ph</i> -CHI_His-tag	FE4

Note: Columns P1-P3 correspond to the three available positions, with the indicated position tags between brackets (e.g., DEs (s = start), DE2, DE3, DEe (e = end)). Each construct contains either a docking domain (Doc) (for scaffold recruitment) or a His-tag (for purification). The final columns display the abbreviation. Abbreviations: 4CL, 4-coumarate-CoA ligase; Ac, *Acetivibrio cellulolyticus*; Af, *Archaeoglobus fulgidus*; CHI, chalcone isomerase; CHS, chalcone synthase; Ct, *Clostridium thermocellum*; Fj, *Flavobacterium johnsoniae*; Gh, *Gerbera hybrida*; Li, linker; Pc, *Petroselinum crispum*; Ph, *Petunia hybrida*; TAL, tyrosine ammonia lyase.

to successfully produce naringenin in *E. coli* (Van Brempt 2019). The DEs contain dockerins that can bind to their cognate cohesin partners in the scaffold. A dockerin module can be fused to either the N- or C-terminus of an enzyme, and the enzymatic activity can be strongly influenced by the fusion site and the specific dockerin used, due to factors such as steric hindrance. In most cases within the field of designer cellulosomes, dockerins are fused to the C-terminus of enzymes, reflecting their most common natural position (Vanderstraeten et al. 2022). Therefore, in this study, we chose to fuse the dockerin to the enzyme's C-terminus to align with this commonly observed configuration and to minimise potential structural interference. The four different linker variants are provided in Table S10.

Next, these DEs and FEs were combined in 'pVTD + Scaff' and 'pVTD + S2' (Table 3). Additionally, another stuffer (S1) (shown in Table S5), replacing the enzymes, was combined with 'pVTD + S2' and 'pVTD + Scaff' to generate a negative control plasmid and a plasmid that solely harbours the scaffold, respectively.

The expression systems of the scaffoldomics platform, regulating scaffold and DE production via the P_{Tet} and P_{araBAD} promoters, respectively, were evaluated using the rational constructs for controlled induction using anhydrotetracycline (aTc) and L-arabinose (L-ara). Successful expression and purification of scaffold and FE constructs confirmed the functionality of the genetic designs (Figures S3 and S4). Furthermore, GST pull-down and shotgun proteomics analysis demonstrated effective recruitment of docking enzymes onto the scaffold, with a higher abundance for the first and third docking enzyme compared to the second and fourth docking enzyme (Data S1: Evaluation of effective assembly of docking enzymes on the scaffold; Figures S5 and S6).

2.2.2 | Chromosomal Naringenin Biosensor for Naringenin Detection In Vivo

To evaluate naringenin production in vivo, a reliable and sensitive detection method was required. For this purpose, a chromosomally integrated biosensor was constructed, enabling real-time monitoring of intracellular naringenin levels. This biosensor uses a transcriptional regulator that responds to naringenin by activating expression of a fluorescent reporter gene, allowing straightforward detection via fluorescence measurements (Figure 3). The design of this biosensor was inspired by the pSynSens construct reported by De Paepe et al. (2018). Genomic integration of the biosensor minimises variability from plasmid-based expression and reduces plasmid burden, which is important given that an additional large plasmid will be required for the scaffolded pathway.

Upon successful integration of the biosensor into the genome, we assessed its functionality. Therefore, naringenin was externally supplied at varying concentrations, and a response curve was generated (Figure 4). Increasing concentrations of naringenin in the growth medium lead to a corresponding rise in fluorescence, indicating enhanced transcriptional activation and supporting the functionality of the biosensor. By fitting the Hill function to the response curve, a more detailed analysis of this biosensor can be conducted, with the predicted Hill parameters provided in Figure 4.

The shape of the biosensor's response curve is characterised by a Hill coefficient of $n = 1.48 \pm 0.17$, which is greater than 1, suggesting positive cooperativity between the ligand and transcription factor. This is consistent with the results of previous studies that used similar plasmid-based biosensors for naringenin

TABLE 3 | Final plasmid constructs combining scaffolds and enzymes and their corresponding icons. This table outlines the final constructs resulting from the combination of docking enzymes (DEs), free enzymes (FEs), or a stuffer control (S1) with either the full scaffold ('pVTD + Scaff') or the stuffer-only control ('pVTD + S2'). Positions IP1–IP4 represent the four intermediate positions used in the final assembly, and constructs are abbreviated accordingly. Constructs with stuffer 1 (S1), which is a non-coding tile sequence that contains stop codons in all reading frames, serve as negative controls or scaffold-only references.

P1 (IP1_IP2)	P2 (IP2_IP3)	P3 (IP3_IP4)	P4 (IP4_IP5)	Vector (IP1_IP5)	Abbreviation
DE1 	DE2 	DE3 	DE4 	pVTD+Scaff 	Scaff+DE
FE1 	FE2 	FE3 	FE4 	pVTD+Scaff 	Scaff+FE
DE1 	DE2 	DE3 	DE4 	pVTD+S2	S2+DE
FE1 	FE2 	FE3 	FE4 	pVTD+S2	S2+FE
P1-P4 (IP1_IP5)				Vector (IP1_IP5)	Abbr.
stuffer 1				pVTD+S2	S2+S1
stuffer 1				pVTD+Scaff 	Scaff+S1

detection (De Paepe et al. 2018). The maximum fluorescent signal, M , is quantified as 8968 ± 1431 a.u. The operational range extends from 5.00 to 55.20 mg/L of naringenin, within which a maximum relative error of 30% in the predicted concentration is guaranteed for any given biosensor output. Within this operational range, the half-maximal concentration (K_M) is estimated to be 35.33 ± 8.15 mg/L naringenin.

2.2.3 | Validation of Naringenin Production and Detection

Having established the detection method, the next step was to confirm actual naringenin production. To this end, a mL-scale production experiment was performed, comparing a condition

containing only stuffer sequences, lacking both scaffold and enzymes ($S2 + S1'$), to a setup expressing all four pathway enzymes in their free form ($S2 + FE'$). This allowed us to assess whether naringenin was effectively produced and detectable (Figure 5).

An increase in $(FP/OD)_{cor}$ naringenin production was observed in ' $S2 + FE'$ ' ($35.7 \text{ a.u.} \pm 4.8$) vs. ' $S2 + S1'$ ' ($0.3 \text{ a.u.} \pm 0.4$), confirming that naringenin is indeed synthesised when all four pathway enzymes are present. To further assess the impact of the fusion of a dockerin to the enzyme on its catalytic activity and naringenin biosynthesis, we also compared in Figure 5 the free enzymes ' $S2 + FE'$ ' ($35.7 \text{ a.u.} \pm 4.8$) with the docking enzymes ' $S2 + DE'$ ' ($108.4 \text{ a.u.} \pm 6.6$), and we found that docking enzymes produced roughly three times more naringenin. The reported fold-increases should be considered cautiously, as the measured fluorescence values fall below

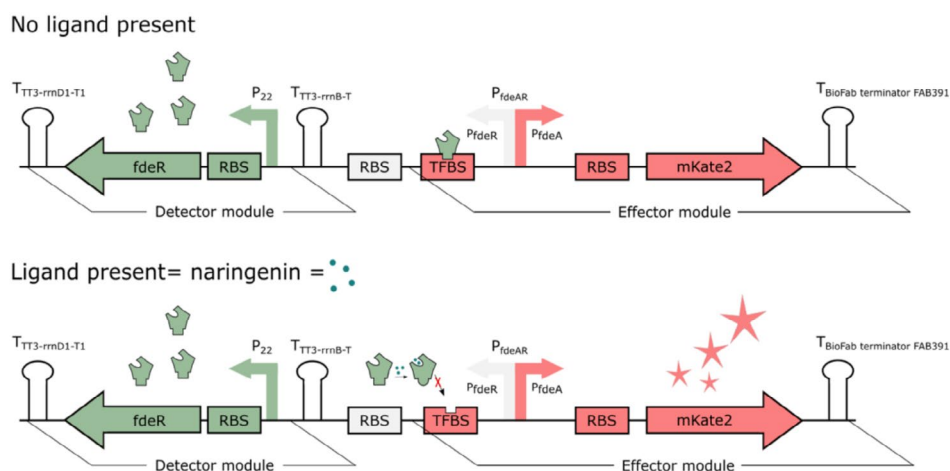


FIGURE 3 | Schematic representation of the naringenin biosensor, showcasing its detector and effector modules along with their genetic components. In the detector module of both panels, the transcription factor FdeR is continuously expressed under the control of a synthetic P_{22} promoter. In the upper panel, in the absence of any ligand molecule, transcription factor FdeR represses the transcriptional activation of the effector module. As a result, no desired output signal, such as a FP, is generated. In contrast, the lower panel demonstrates that the ligand molecule naringenin binds to the transcription factor FdeR, leading to the derepression of the effector module. This initiates the transcriptional activation of the desired output signal, more specifically, the fluorescent protein mKate2. Figure based on De Paepe et al. (2018).

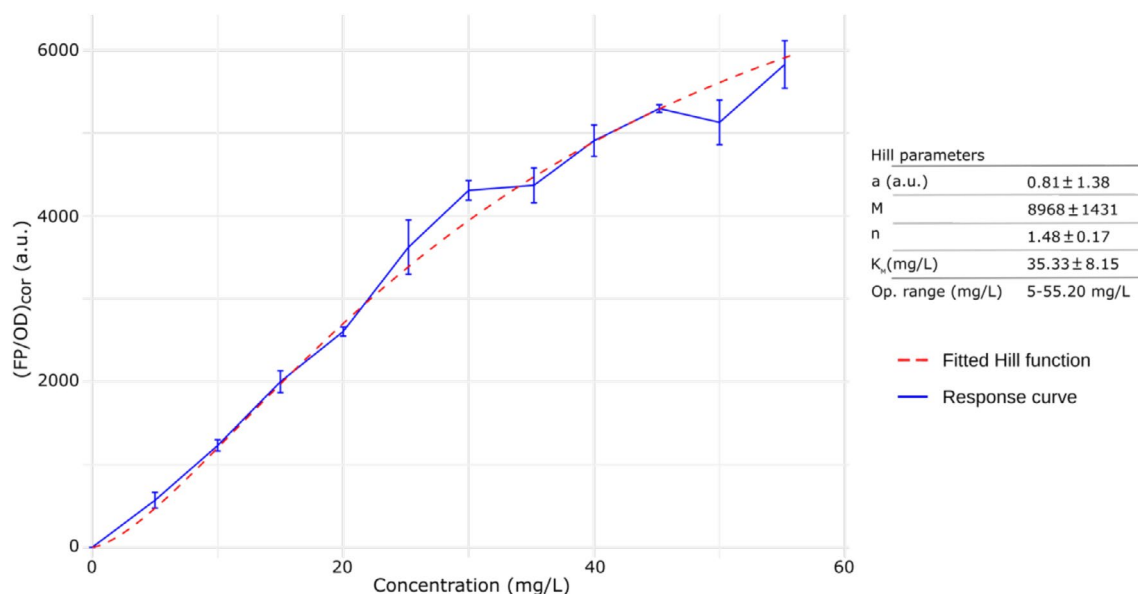


FIGURE 4 | Response curve of cells with the chromosomally integrated naringenin-responsive biosensor and fitted Hill function for a concentration range of 0–55.2 mg/L naringenin. In addition, the corresponding biosensor Hill parameters and the operational range are listed; with a: The basal normalised fluorescent signal (leaky expression) (a.u., arbitrary units), M: The maximum normalised fluorescent signal (a.u.), n: Hill coefficient (cooperativity) and K_M : The Hill constant (Transcription factor-ligand affinity) (half-maximal naringenin concentration, mg/L). Error bars represent standard error of the mean of four biological replicates ($n=4$).

the operational range of the biosensor (5–55 mg/L). The fitted Hill function indicates that positive cooperativity is most pronounced at low naringenin concentrations, so small increases in naringenin may produce disproportionately large changes in fluorescence. The reported improvements should therefore be interpreted as relative trends, rather than absolute titers.

The observation that docking enzymes outperformed the free enzymes in naringenin production indicates that fusion with the docking module does not introduce steric hindrance but rather enhances catalytic efficiency or expression level, or alternatively, that DEs are more efficiently produced or cause

less metabolic burden compared to their equivalent FEs. Additionally, it is important to note that our measurements reflect only the final product, naringenin, leaving it uncertain which specific enzyme(s) in the pathway is (are) affected by the fusion of the His-tag or dockerin module. Further investigation is needed to analyse intermediate products to pinpoint the exact enzymatic steps influenced by the His-tag or dockerin fusion.

The main objective was to investigate whether the addition of a scaffold would positively influence naringenin production through a colocalisation effect. To this end, a μ L-scale naringenin production experiment was performed, comparing the

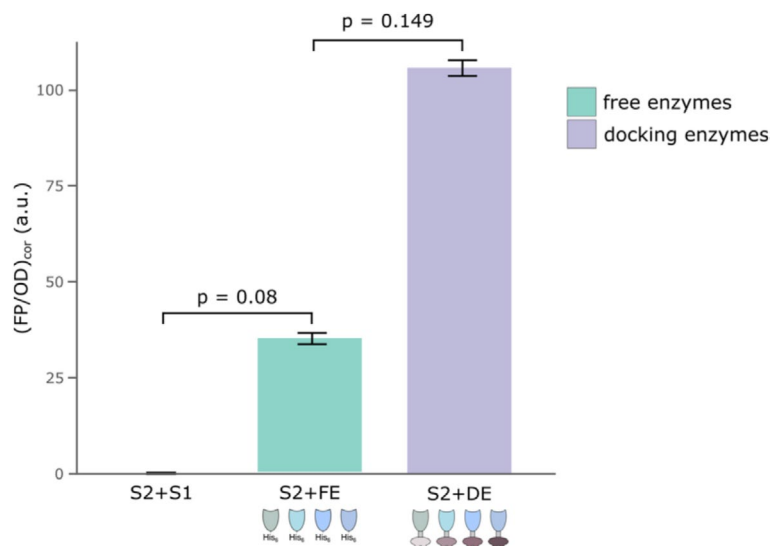


FIGURE 5 | $(FP/OD)_{cor}$ (a.u.) for both free and docking enzymes. Each bar represents the mean $\pm$ SE of biological replicates. Since three biological replicates for conditions 'S2 + S1' and 'S2 + FE' were included, and two replicates for the 'S2 + DE' condition, the statistical power of classical tests such as the Shapiro–Wilk test for normality and Levene's test for equality of variances is limited. Therefore, non-parametric Wilcoxon rank-sum tests were applied. No p -value reached statistical significance ($\alpha = 0.05$), and the p -value was higher when the 'S2 + DE' condition was involved.

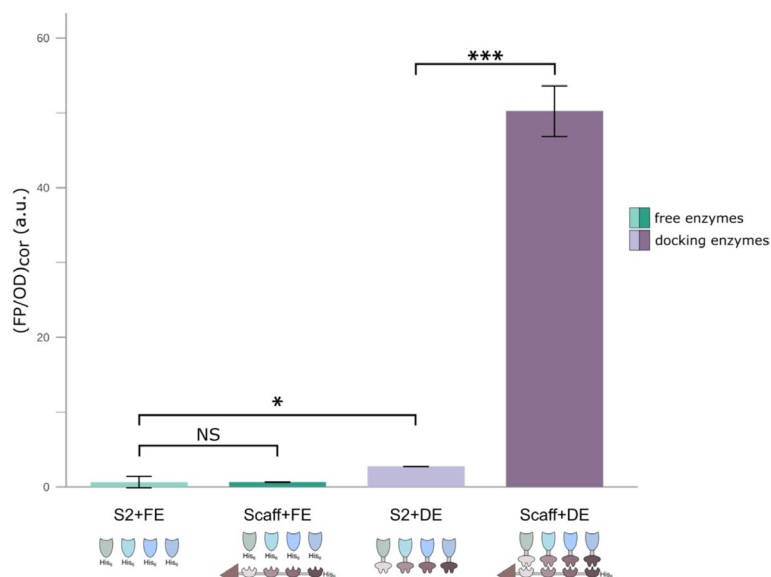


FIGURE 6 | $(FP/OD)_{cor}$ (a.u.) for both free (FEs) and docking enzymes (DEs) with or without scaffold. Each bar represents the mean $\pm$ standard error of five biological replicates. All conditions followed a normal distribution (Shapiro–Wilk test, $p > 0.05$), but did not exhibit equal variance (Levene's test, $p < 0.05$). Therefore, pairwise Welch's t -tests were performed. Significance levels are indicated as follows: * $p < 0.05$, *** $p < 0.001$ (Welch's t -test).

performance of free and docking enzymes both with and without the scaffold ('S2 + FE', 'S2 + DE', 'Scaff + FE', and 'Scaff + DE'). The results are shown in Figure 6.

Docking enzymes without the scaffold ('S2 + DE', 2.62 a.u. $\pm$ 0.01) produced significantly more naringenin ($p < 0.05$) than free enzymes without the scaffold that did not show a significant production ('S2 + FE', 0.68 a.u. $\pm$ 0.68), which aligns with the trend observed in the previous mL-scale experiment. The comparison between the mL-scale and μ L-scale experiments revealed a more than 40-fold difference in $(FP/OD)_{cor}$ values (108.4 a.u. vs. 2.62 a.u.), indicating that scaling up substantially enhances naringenin production. No significant difference was observed between 'SE +

FE' and 'Scaff + FE' (0.50 a.u. $\pm$ 0.03), likely because the 'S2 + FE' value was already minimal. However, the addition of the scaffold to the docking enzymes ('Scaff + DE', 49.48 a.u. $\pm$ 3.36 vs. 'S2 + DE', 2.62 a.u. $\pm$ 0.01) led to a significant increase (~19-fold compared to 'S2 + DE') in naringenin production ($p < 0.001$), indicating a colocalisation effect. This result supports the hypothesis that scaffold-assisted colocalisation of pathway enzymes can enhance metabolic flux towards the target product, as shown in similar biosynthesis studies (Elias et al. 2025).

While the current system supports the colocalisation of five enzymes, comprising four distinct docking enzymes and a scaffold with up to five binding sites, its capacity is currently limited to

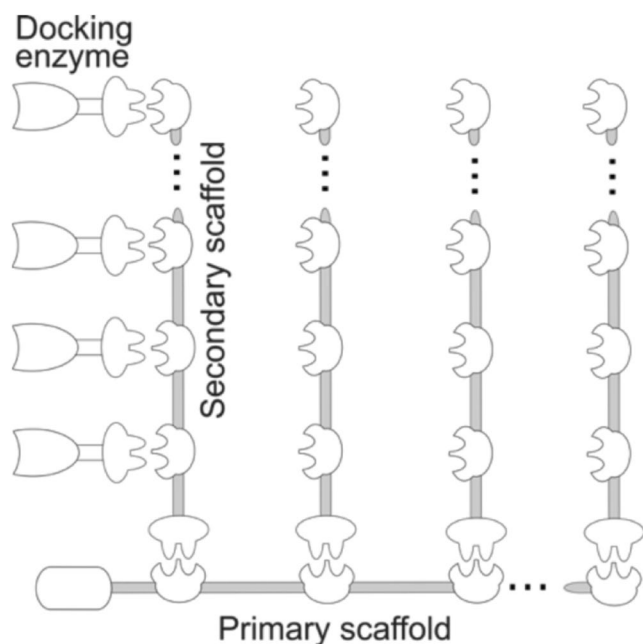


FIGURE 7 | Secondary scaffolds. Implementation of additional scaffold layers to adjust enzyme ratios and support the construction of more advanced metabolic pathways.

pathways involving a maximum of four enzymatic steps, which may restrict its applicability to more complex biosynthetic routes. To address this limitation, future developments could focus on expanding the platform's capacity by introducing additional docking enzyme positions on the pVTD or by incorporating secondary scaffolds (Figure 7). These approaches would not only enable the colocalisation of pathways involving more than four enzymes, but also provide greater flexibility in controlling enzyme stoichiometry, thereby broadening the platform's applicability to more complex and finely tuned biosynthetic systems. However, increasing the number of docking enzyme positions or scaffold elements on a single plasmid introduces practical limitations, as large plasmids can reduce transformation efficiency, stability and increase the metabolic burden on the host organism.

To address this, an alternative and more modular solution would be to introduce an additional plasmid encoding the secondary scaffolds. This secondary plasmid should carry a replication origin compatible with that of the primary plasmid, ensuring stable coexistence within the same bacterial host. By distributing the genetic load across two plasmids, the overall size per construct remains manageable, while still enabling advanced pathway design. Furthermore, placing each scaffold on a separate plasmid under orthogonal inducible promoters would offer the flexibility to independently tune expression levels. This would support balanced assembly and minimise metabolic burden during pathway optimisation.

The ultimate goal of the scaffoldomics platform is to enable the generation and screening of fully combinatorial libraries of multi-enzyme complexes. These libraries could encompass variations in enzyme orientation (N- versus C-terminal), linker type and length, cohesin-dockerin pair selection, scaffold architecture such as the number and order of cohesin domains and

linkers, and the inclusion of alternative interaction domains than cohesin-dockerin pairs.

Such libraries can be expressed *in vivo* and analysed at single-cell resolution using FACS. Cells exhibiting the highest fluorescence, corresponding to the highest product output via the integrated biosensor, can then be isolated and characterised. To mitigate the effects of clonal variability and confirm true performance, follow-up validation of selected hits can be performed in bulk using microtiter plate assays. Notably, the best-performing variants *in vitro* are not necessarily those with the most optimal scaffold architecture *in vivo*; high fluorescence may also result from constructs that balance efficient enzyme colocalisation with minimal metabolic burden. This screening strategy therefore enables selection of consensus designs that perform best under physiological conditions. Additionally, sequencing of selected constructs will link genotype to phenotype and support further design refinement. As more data are collected, it may become possible to deduce general design rules for scaffolded enzyme colocalisation (Figure 8). These may include optimal positions, preferred linker lengths, or specific interaction pair combinations that consistently improve pathway efficiency. These rules can then inform the construction of a second-generation, more focused library for deeper screening as part of a new iterative Design-Build-Test-Learn cycle.

3 | Conclusion

A hierarchical assembly system was established in which four docking enzymes and a scaffold are co-assembled on a single plasmid, thereby establishing a 'scaffoldomics' platform for the optimisation of multi-enzyme biosynthetic pathways. The platform's potential was validated for the production of naringenin as a representative of fine chemicals that can be produced by microbial cell factories, relying on a validated biosensor. Naringenin was produced by four consecutive pathway enzymes. In this case fusion of dockerins had a positive impact since the set of docking enzymes resulted in roughly 3-fold higher production compared to the free enzymes. A systematic analysis including stuffer fragments could demonstrate a proof-of-concept with an up to 19-fold increased production of naringenin, suggesting that colocalisation enhances naringenin biosynthesis.

While here demonstrated for a rationally assembled scaffolded pathway, the scaffoldomics platform is generic and can be ultimately used in a combinatorial manner as well. Through random shuffling of scaffold and DE components, such a combinatorial library can be generated and transformed into *E. coli*, creating a diverse population of cells, each expressing a unique multi-enzyme complex. These complexes may differ, on the one hand, in docking enzyme composition, by varying linker sequences, dockerin domains, or by introducing different enzymes with identical biochemical functions or engineered variants thereof. On the other hand, differences in scaffold design, such as the number and spatial arrangement of docking enzyme binding sites, also contribute to the structural and functional diversity of these assemblies. Given that each complex may exhibit distinct catalytic performance, this approach enables the exploration of a broad design space. This combinatorial library can be screened

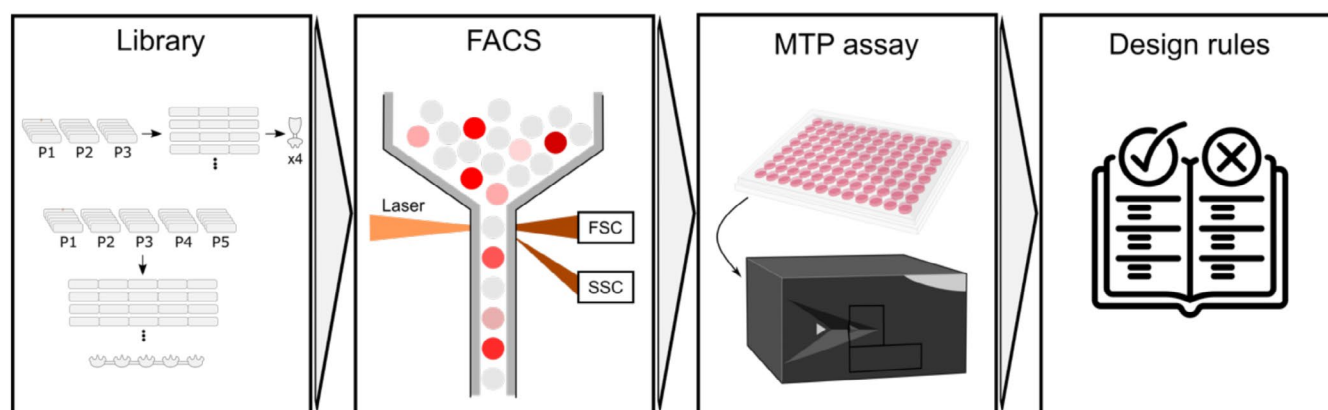


FIGURE 8 | Workflow for high-throughput, combinatorial optimisation of scaffolded pathways. Combinatorial libraries of multi-enzyme complexes are first generated and expressed *in vivo*. These are subjected to FACS analysis to rapidly identify top-performing variants at the single-cell level. Selected candidates are subsequently validated using microtiter plate (MTP) assays. The resulting data are then used to derive design rules that guide the construction of optimised multi-enzyme complexes for future pathway engineering.

for fine chemical production, for instance using a biosensor, allowing rapid identification of the most effective multi-enzyme configurations for the target pathway. To conclude, this work aligns with the ongoing transition towards a sustainable bioeconomy, in which biotechnological processes are developed to replace both fossil-based production routes and the inefficient extraction of compounds from natural sources. Efficient pathway engineering, through strategies such as enzyme colocalisation and modular design, can support this shift by enabling more controllable and scalable microbial production systems. By establishing and evaluating the scaffoldomics approach *in vivo*, this work offers a potential basis for further exploration in microbial biosynthesis.

4 | Experimental Procedures

4.1 | Bacterial Strains and Growth Media

E. coli TOP10 and ET10_N cells were used for plasmid storage and protein expression, respectively. These strains were grown at 30°C in lysogeny broth (LB) (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) with shaking (Inova 44 shaking incubator, 180 rpm) or on LB agar (1.5% (w/v) agar). For proper selection, LB was supplemented with 100 µg/mL ampicillin, 50 µg/mL kanamycin, 50 µg/mL spectomycin, 25 µg/mL chloramphenicol, and/or 5% (w/v) sucrose.

4.2 | Construction of Vectors

To generate VersaTile Intermediate vectors (pVTIs, pVTI1: LMBP 14348; pVTI2: LMBP 14349; pVTI3: LMBP 14350; pVTI4: LMBP 14351, all available at BCCM/GeneCorner), three DNA fragments were assembled using Golden Gate cloning with the Type IIs restriction enzyme BsmBI. The fragments were PCR-amplified using Phusion DNA polymerase (1 U) and primers containing 5' BsmBI recognition sites. As templates, three synthetic DNA fragments obtained from TWIST Bioscience were used (Table S6). The primers were designed to introduce sequence variations distinguishing the different pVTI constructs

(Table S7), specifically the ribosome binding sites (RBS1–RBS4) and the four-way position tags (IP1–IP2, IP2–IP3, IP3–IP4, and IP4–IP5). The VersaTile Destination vector, pVTD38, LMBP 14352, available at BCCM/GeneCorner, was synthesised by GENEWIZ (Azenta Life Sciences).

4.3 | Expanding the Tile Repository via VersaTile Cloning

Linker, cohesin and dockerin tiles were already constructed before (Tables S8 and S10) (Vanderstraeten et al. 2022). Cohesin tiles prepared to be fitted at the first, second, third and fourth position include the cohesin-encoding domain followed by a sequence encoding a C-terminal linker. Cohesin tiles prepared to be fitted at the fifth position do not include this linker sequence. In the case of Coh-CtI, Coh-CtII and Coh-Ac, part of the natural linker was included. The linker sequences have lengths of 41, 7 and 6 amino acids, respectively. The sequence added to the Coh-Af tiles encodes VVPST, this sequence is found in the natural cellulosomal scaffoldin (Vazana et al. 2013).

The DNA encoding the EADs of the naringenin biosynthetic pathway was converted into tiles compatible with positions one and three of the three-way assembly system, following the procedure described by Vanderstraeten et al. (2022). These tiles were generated using primers from Integrated DNA Technologies (Table S9).

To construct appropriate control plasmids, two non-coding stuffer tiles, S1 and S2, were created. Stuffer S1 replaces the *ccdB* counter-selection marker and serves as a substitute for the docking enzyme tiles, whereas S2 replaces the *sacB* gene and acts as a scaffold substitute. Both stuffers consist of non-coding sequences containing stop codons in all reading frames (Table S5) and were generated via DNA hybridisation. Complementary forward and reverse primers (ssDNA; IDT DNA oligos) were mixed at equimolar concentrations (10 µM). The hybridisation reaction was performed by heating to 95°C for 5 min, followed by gradual cooling to 20°C, yielding double-stranded DNA fragments flanked by position tags and type IIS restriction sites.

4.4 | Hierarchical Assembly for a Complete Scaffolded Naringenin Biosynthetic Pathway

The expression plasmid pVTD, designed to encode a scaffold and four docking enzymes (DEs), was constructed through a three-step hierarchical cloning strategy using type IIS restriction enzymes and negative selection markers. In the first step, the scaffold sequence was inserted into the pVTD backbone by replacing a SacB counter-selection cassette using the following protocol: 1 μ L of pVTD plasmid (100 ng/ μ L), 1 μ L of each DNA tile (50 ng/ μ L), 1 μ L of BsaI (10 U/ μ L), 3 μ L of T4 DNA ligase (1 U/ μ L), and 2 μ L of 10 $\times$ T4 DNA ligase buffer in a final volume of 20 μ L. The reaction mixture was subjected to 30 cycles of 2 min at 37°C and 3 min at 22°C, followed by 5 min at 50°C and a final inactivation step at 80°C for 5 min. The ligation mixture was transformed into *E. coli* and plated on LB agar containing 25 μ g/mL chloramphenicol and 5% (w/v) sucrose to select for correct assemblies. In the second step, the docking enzymes were constructed in pVTIs using the same BsaI-based cloning approach. Each DE sequence was assembled by replacing a SacB cassette. Transformants were selected on LB agar supplemented with 50 μ g/mL kanamycin and 5% (w/v) sucrose. In the final step, the four DEs were simultaneously inserted into the scaffold-containing pVTD plasmid through an assembly reaction using SapI instead of BsaI, following the same restriction-ligation protocol described above. Proper assembly resulted in excision of the *ccdB* toxin gene, enabling selection of successful transformants on LB agar containing 25 μ g/mL chloramphenicol.

4.5 | Chromosomal Naringenin Biosensor for Naringenin Detection In Vivo

To integrate the naringenin biosensor into the *E. coli* TOP10 genome, a protocol was adapted from Jiang et al. (2015). Using the CRISPR/Cas9 system in combination with λ -Red recombinases requires two plasmids: pCas (Addgene plasmid #62225) and pTarget (Addgene plasmid #62226). pTarget was modified to include the regions homologous to *E. coli* TOP10 genomic DNA (H1 and H2) and the amplified biosensor sequence. The CRISPR-Cas9 target locus was chosen within the *yjcS* gene based on the genomic sequence of *E. coli* strain K12 substr. DH10B (Data S1: Integration locus of biosensor). The selected target site, defined by the 20-nucleotide (N20) sequence 5'-GTGTAGATAACGGCAACAAT-3' and the adjacent protospacer adjacent motif (PAM) (5'-CGG-3'), was flanked by upstream and downstream homologous regions H1 (980 bp) and H2 (1001 bp). The homologous regions (H1 and H2) and the biosensor cassette were amplified with Phusion DNA polymerase (1U) (Thermo Fischer Scientific) following the manufacturer's instructions, using genomic DNA of *Escherichia coli* TOP10 or the biosensor plasmid as template (Data S1: DNA sequence naringenin biosensor). The pTarget plasmid was assembled in two steps. First, the target-specific N20 sequence was inserted into a linearised version of the pTarget plasmid. Linearisation was performed using PrimeSTAR HS DNA Polymerase (Takara Bio), and the insertion was carried out via circular polymerase extension cloning (CPEC) with Q5 High-Fidelity DNA Polymerase (New England Biolabs), following the manufacturer's instructions. In the second step, homologous regions H1 and H2 were incorporated

using CPEC, while the biosensor cassette was inserted via Golden Gate assembly. The latter method was chosen because internal homologous sequences within the biosensor fragment rendered it incompatible with CPEC. After the knock-in and plasmid curing following the protocol of Jiang et al. (2015), the resulting cells with the chromosomally integrated naringenin-responsive biosensor are referred to as ET10_N.

4.6 | Naringenin Response Curve

Strains, with four biological replicates ($n=4$ for each naringenin concentration), were inoculated in 150 μ L of LB and grown for 18 h on a microtiter plate shaker (Heidolph Titramax 1000 Microplate Shaker, VWR) at 900 rpm and 30°C, with an air-permeable sticker (BREATHseal, Greiner). The next day, these cultures were diluted 1:300 into 150 μ L of fresh LB containing varying concentrations of the ligand naringenin (Sigma-Aldrich) (0, 5, 10, 15, 20, 25.2, 30, 35.2, 40, 45.2, 50, 55.2 mg/L). Naringenin was prepared in ethanol, added to each well, and the solvent was evaporated prior to inoculation. The cultures were then grown in a Tecan Infinite 200 PRO plate reader at 30°C with orbital shaking at an amplitude of 2. Every 10 min, mKate2 fluorescence was measured at excitation and emission wavelengths of 588 and 633 nm, respectively, while optical density was recorded at 600 nm.

4.7 | mL-Scale Naringenin Production and Detection

Single colonies of ET10_N cells, whether transformed with a plasmid or not, were grown for 18 h at 30°C, shaking (Inova 44 shaking incubator, 180 rpm) in 10 mL LB supplemented with Cam²⁵ in a 50 mL Falcon tube. The OD₆₀₀ of the grown culture was measured to determine the required volume for downstream processing. The cells were harvested and resuspended in 1 mL LB supplemented with 10 mM L-arabinose to induce the FEs or DEs, adjusting the suspension to a final concentration of 1 OD₆₀₀/mL. The resuspended culture was incubated at 30°C for 22 h with shaking at 700 rpm (Heidolph Titramax 1000 Microplate Shaker, VWR). After incubation, fluorescence and optical density were measured using a Tecan Infinite 200 PRO plate reader. mKate2 fluorescence was recorded at excitation and emission wavelengths of 588 and 633 nm, respectively, with a gain setting of 100. Optical density was measured at 600 nm.

4.8 | μ L-Scale Naringenin Production and Detection

Single colonies of ET10_N cells, with or without plasmid, were inoculated into 150 μ L LB in a preculture 96-well plate and incubated for 18 h on a microtiter plate shaker (Heidolph Titramax 1000 Microplate Shaker, VWR) at 900 rpm and 30°C. The following day, the cultures were diluted 1:300 into 150 μ L of fresh LB supplemented with 10 mM L-arabinose and 100 ng/mL anhydrotetracycline. These cultures were subsequently grown in a Tecan Infinite 200 PRO plate reader at 30°C with orbital shaking at an amplitude of 2. Every 10 min, mKate2 fluorescence was measured at excitation and emission wavelengths of 588

and 633 nm, respectively, while optical density was recorded at 600 nm.

4.9 | Data Processing and Statistical Analysis

For fluorescence measurements, LB without bacterial culture was used to correct for background fluorescence and the optical density at 600 nm of the medium (FP_{med} and OD_{med} , respectively) at each tested naringenin concentration. To account for background fluorescence and optical density of the cell culture itself (FP_{blank} and OD_{blank} , respectively), *E. coli* TOP10 cells without a plasmid were used. The fluorescence, normalised to optical density, was then calculated as described by De Paepe et al. (2018):

$$\left(\frac{FP}{OD}\right)_{cor} = \frac{FP - FP_{med}}{OD - OD_{med}} - \frac{FP_{blank} - FP_{med}}{OD_{blank} - OD_{med}}$$

The resulting mean corrected fluorescence per OD $\left(\frac{FP}{OD}\right)_{cor}$ ($n=5$) for each concentration of the ligand molecule, naringenin, is expressed in arbitrary units (a.u.) and was fitted using the following Hill function:

$$\left(\frac{FP}{OD}\right)_{cor} = f(C) = a + k \frac{C^n}{C^n + K_M^n}$$

C =The concentration of naringenin in the growth medium (mg/L), a =The basal normalised fluorescent signal (leaky expression, a.u.), k =The relative maximum normalised fluorescent signal (a.u.), $M=a+k$ =The maximum normalised fluorescent signal (a.u.), n =The Hill coefficient (cooperativity), K_M =The Hill constant (half-maximal naringenin concentration, mg/L).

All data processing and statistical analysis were performed using RStudio as the programming environment. Normality was assessed using the Shapiro–Wilk test, and equality of variances was evaluated with Levene's test. The specific statistical tests used are indicated in the figure legends, with p -values <0.05 considered statistically significant ($*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$). Because of the small sample sizes in the large-scale expression (three replicates per condition, two for 'S2 + DE'), normality and variance tests lacked power; therefore, non-parametric Wilcoxon rank-sum tests were used.

Author Contributions

Marte Elias: conceptualization, formal analysis, funding acquisition, investigation, data curation, methodology, project administration, visualization, validation, writing – original draft, writing – review and editing. **Brecht De Paepe:** conceptualization, formal analysis, writing – review and editing. **Julie Vanderstraeten:** methodology. **Babette Lamote:** methodology. **Marjan De Mey:** conceptualization, funding acquisition, supervision, writing – review and editing. **Yves Briers:** conceptualization, funding acquisition, validation, supervision, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in Data S1 of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** Supporting information.