

Applied Microbiology and Biotechnology

Supplementary material

Step-by-step optimization of a heterologous pathway for *de novo* naringenin production in *Escherichia coli*

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Table S1. Sequence of all the genes used in this study.

Gene	Sequence
<i>RgTAL</i>	ATGGCTCCGCGTCCGACCTCGCAATCCCAAGCTCGCACCTGCCCGACCACCCAAGTTACC CAAGTTGACATCGTTGAAAAAATGCTGGCGGCGCCGACCGATTTCGACGCTGGAAGTGGAC GGCTATAGCCTGAACCTGGGTGATGTGGTTTCTGCAGCACGTAAAGGTCGTCCGGTGCGT GTTAAAGATTGACAGCAAATTCGCTCGAAAAATCGATAAAAGCGTGGAATTTCTGCGTAGC CAGCTGAGCATGTCTGTTTACGGCGTCACCACGGGTTTCGGCGGTTTCAGCCGATACCCGC ACGGAAGACGCCATTTTCGCTGCAGAAAGCACTGCTGGAACATCAACTGTGCGGCGTGCTG CCGAGCTCTTTTGATAGCTTCCGCCTGGGCCGTGGTCTGGAAAACTCTCTGCCGCTGGAAG TCGTGCGTGGTGCAATGACCATCCGTGTTAATTCCCTGACGCGCGGTCATTCAGCTGTCCG TCTGGTTGTCTTGGAAAGCGCTGACCAACTTTCTGAATCACGGTATTACGCCGATCGTGCCG CTGCGTGGTACCATTAGTGATCCGGTGATCTGAGCCCGCTGTCTTATATTGCAGCTGCGA TCTCTGGCCACCCGGACAGTAAAGTTCATGTGGTTCACGAGGGTAAAGAAAAAATCCTGT ACGCCCCGTGAAGCTATGGCGCTGTTCAACCTGGAACCGGTCGTGCTGGGCGGAAAGAA GCCTGGGTCTGGTGAATGGTACGGCTGTTTCAGCGTCGATGGCCACCCTGGCACTGCATG ATGCCACATGCTGAGCCTGCTGAGCCAGTCTCTGACCGCGATGACGGTCGAAGCGATGG TGGGCCATGCAGGTAGCTTTCATCCGTTCTGACGATGTGACCCGTCCGCACCCGACGC AGATTGAAGTTGCAGGCAACATCCGCAAACCTGCTGGAAGGTAGCCGTTTTCGCGTGATC ACGAAGAAGAAGTGAAAGTGAAAGATGACGAAGGCATTCTGCGCCAGGATCGTTATCCG CTGCGTACCAGTCCGCAATGGCTGGGTCCGCTGGTCTCCGACCTGATTATGCCACGCA TGGCGACCATCGAAGCGGGTCAGAGTACCGGATAACCCGCTGATTGACGTGGAATA TAAAACTCTCATCACGGCGGTAACCTTCAAGCCGACGTGTTGCCAATACGATGGAATA AACGCGCCTGGGCCTGGCACAGATCGGTAACTGAATTTACCCAACTGACGGAAATGCT GAACGCAGGCATGAATCGTGGTCTGCCGAGCTGCCTGGCAGCAGAAGATCCGAGTCTGTC CTATCATTGTAAAGGCCTGGACATTGCAGCTGCGGCCTACACCTCTGAACTGGGTATCT GGCGAACCCGTTACCACGCACGTCCAGCCGGCTGAAATGGCGAACCAAGCCGTGAATT CCCTGGCACTGATCTCAGCTCGTCGCACCACGGAATCGAATGATGTCCTGAGCCTGCTGC TGGCGACCATCTGTATTGTGTTCTGCAGGCTATTGACCTGCGCGCGATCGAATTTGAATT CAAAAAACAGTTTGGCCCGCTATTGTGAGCCTGATCGATCAACACTTCGGCTCTGCCAT GACCGGTAGTAACCTGCGTGACGAACTGGTGGAATAAGTTAATAAACGCTGGCCAAAC GCCTGGAACAGACCAACAGTTACGATCTGGTGCCGCGTTGGCATGACGCATTTTCTTCG CAGCTGGTACGGTTGTCAAGTTCTGAGTTCACCTCACTGTCGCTGGCGGCCGTCAATGC CTGGAAGTGGCAGCTGCGGAAAGTGCAATTTCCCTGACCCGCCAAGTGCGTGAAACGTT TTGGTCAGCAGCATCGACGTCATCGCCGGCACTGAGCTATCTGTCTCCGCGCACCCAAAT TCTGTACGCTTTTGTTCGTGAAGAACTGGGCGTCAAAGCGCGTCGCGCGCATGTTTTCTG GGTAAACAGGAAGTGACCATCGGTAGTAATGTTTCCAAAATCTATGAAGCTATCAAAAGC GGTCGTATCAATAATGTGCTGCTGAAAATGCTGGCATAA
<i>FjTAL</i>	ATGAACACCATCAACGAATATCTGAGCCTGGAAGAATTTGAAGCCATTATCTTTGGCAAT CAGAAAGTGACCATTAGTGATGTTGTTGTGAATCGCGTTAACGAGAGCTTTAACTTTCTG AAAGAATTTAGCGGCAACAAAGTGATCTATGGTGTGAATACCGGTTTGGTCCGATGGCA CAGTATCGTATTAAGAAAGCGATCAGATTCAGCTGCAGTATAATCTGATTCTGAGCCAT AGCAGCGGCACCGGTAAACCGCTGAGTCCGTTTGTGCAAAAGCAGCAATTCGCGCACGT CTGAATACCCCTGAGTCTGGGTAATAGCGGTGTTTCATCCGAGCGTTATTAATCTGATGAGC GAACTGATCAACAAAGATATCACACCGCTGATTTTGAACATGGTGGTGTGGTGCAAGC GGTGATCTGGTTCAGCTGAGCCATCTGGCACTGGTTCTGATTGGTGAAGGTGAAGTTTCT ATAAAGGTGAACGTCGTCCGACACCGGAAGTTTGAATTTGAAGGTCTGAAACCGATCC AGGTGGAAATTCGCGAAGGTCTGGCCCTGATTAATGGCACCAGCGTTATGACCGGTATTG GTGTTGTTAATGTGTACCATGCAAAAAAACTGCTGGATTGGAGCCTGAAAAGCAGCTGTG CAATTAATGAAGTGGTTCAGGCATATGATGATCACTTTAGCGCAGAACTGAATCAGACCA AACGTCATAAAGGTGAGCAAGAAATTTGCACTGAAAATGCGTCAGAATCTGAGCGATAGC ACCTGATTTCGCAAACGTGAAGATCATCTGTATAGCGGTGAAAACACCGAAGAAATCTTC AAAGAAAAAGTGCAAGAGTATTATAGCCTGCGTTGTGTTCCGCAGATTCTGGGTCCGGTT CTGGAAACCATTAACAATGTTGCAAGCATTCTGGAAGATGAATTTAACAGCGCAAAACGAT AACCCGATCATCGATGTTAAAAACAGCATGTTTATCACGGTGGCAATTTTCATGGTGATT ATATCAGCCTGGAATGGATAAACTGAAATCGTGATTACCAAACCTGACCATGCTGGCAG AACGTCAGTGAATTATCTGCTGAATAGCAAAATTAACGAACTGCTGCCTCCGTTTGTTA ATCTGGGCACCCCTGGGTTTTAACTTTGGTATGCAGGGTGTTCAGTTTACCGCAACCAGCAC CACCGCAGAAAGCCAGATGCTGAGCAATCCGATGTATGTTTCATAGCATTCCGAACAATAA TGATAACCAGGATATTGTTAGCATGGGCACCAATAGCGCAGTTATTACCAGCAAAGTTAT CGAAAATGCCTTTGAAGTTCTGGCCATTGAAATGATTACCATGTTTCAGGCGATTGATTAT CTGGGCCAGAAAGATAAAATCAGCAGCGTTAGCAAAAAATGGTATGATGAAATCCGCAA CATCATCCCGACCTTTAAAGAAGATCAGGTGATGTATCCGTTTCGTGCAGAAAGTAAAGA CCACCTGATTAACAATTAA

At4CL

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Gm4CL

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Vv4CL

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AtCHS

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CmCHI ATGAATCCGTCGCCGTCTGTTACCGAACTGCAAGTGGAATAATGTCACCTTTACGCCGAGT
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MsCHI

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Table S2. List of primers used in this study for cloning of the desired genes, colony PCR and sequencing.

Primer	Sequence¹	Restriction Enzyme
<i>FjTAL_BamHI_Fwd</i>	AAAAGGATCCCATGAACACCATCAACGAATATCTG	<i>Bam</i> HI
<i>FjTAL_HindIII_Rev</i>	AACCCAAGCTTTTAATTGTTAATCAGGTGGTC	<i>Hind</i> III
<i>Gm4CL_BamHI_Fwd</i>	AAAAAGGATCCCATGGCGCCTTCGCCG	<i>Bam</i> HI
<i>Gm4CL_EcoRI_Rev</i>	AAAAAGAATTCTAATTGGCCACAACCAGACC	<i>Eco</i> RI
<i>Vv4CL_HindIII_Fwd</i>	AAAAAAAGCTTATGATTAGTATTGAAACGCAA	<i>Hind</i> III
<i>Vv4CL_NotI_Rev</i>	AAAAAAAGCTTATGATTAGTATTGAAACGCAA	<i>Not</i> I
<i>Pc4CL_EcoRI_Fwd</i>	AAAAAGAATTCTATGGGAGACTGTGTAGCA	<i>Eco</i> RI
<i>Pc4CL_NotI_Rev</i>	AAAAAGCGGCCGCTTATTTGGGAAGATCACCGG	<i>Not</i> I
<i>AtCHS_NdeI_Fwd</i>	AAAAACATATGGTGATGGCTGGTGCT	<i>Nde</i> I
<i>AtCHS_XhoI_Rev</i>	AAAAACTCGAGTTAGAGAGGAACGCTGTGC	<i>Xho</i> I
<i>AtCHI_NdeI_Fwd</i>	AAAAACATATGTCTTCATCCAACGCCTG	<i>Nde</i> I
<i>AtCHI_XhoI_Rev</i>	AAAAACTCGAGTCAGTTCTCTTTGGCTAGTTT	<i>Xho</i> I
<i>CmCHI_NdeI_Fwd</i>	AAAAAACATATGAATCCGTCGCCG	<i>Nde</i> I
<i>CmCHI_XhoI_Rev</i>	AAAAACTCGAGTCACACCAGACT	<i>Xho</i> I
<i>MsCHI_NdeI_Fwd</i>	AAAAAACATATGGCTGCATCAATCA	<i>Nde</i> I
<i>MsCHI_XhoI_Rev</i>	AAAAACTCGAGTCAGTTTCCAATCTTGAAAGC	<i>Xho</i> I
<i>At4CL_NdeI_Fwd</i>	AAAAACATATGATGGCGCCACAAGAACA	<i>Nde</i> I
<i>At4CL_XhoI_Rev</i>	AAAAACTCGAGTCACAATCCATTGCTAGTTTGC	<i>Xho</i> I
MCS1_Fwd	GGATCTCGACGCTCTCCCTT	-
MCS1_Rev	CGATTATGCGGCCGTGTAC	-
MCS2_Fwd	GTACACGGCCGCATAATCG	-
T7term_Rev	CTAGTTATTGCTCAGCGGT	-

¹ Start and stop codons in *italic*; restriction sites in **bold**. To maintain the sequence in frame, one base was occasionally added between the restriction site and the gene start codon.

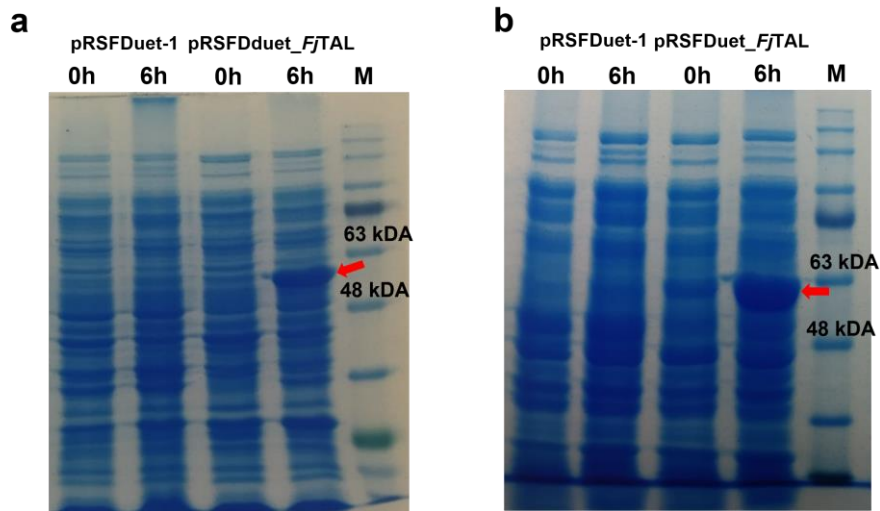


Fig. S1 Protein SDS-PAGE gels of soluble (a) and insoluble (b) protein fractions showing tyrosine ammonia-lyase from *Flavobacterium johnsoniae* (FjTAL) expression in *E. coli* M-PAR-121 strain at time zero (0 h) of induction and after 6 h of induction. FjTAL is expected around 58.26 kDa. Red arrows indicate the bands of interest. M: marker (NZYColour Protein Marker II – NZYTech).

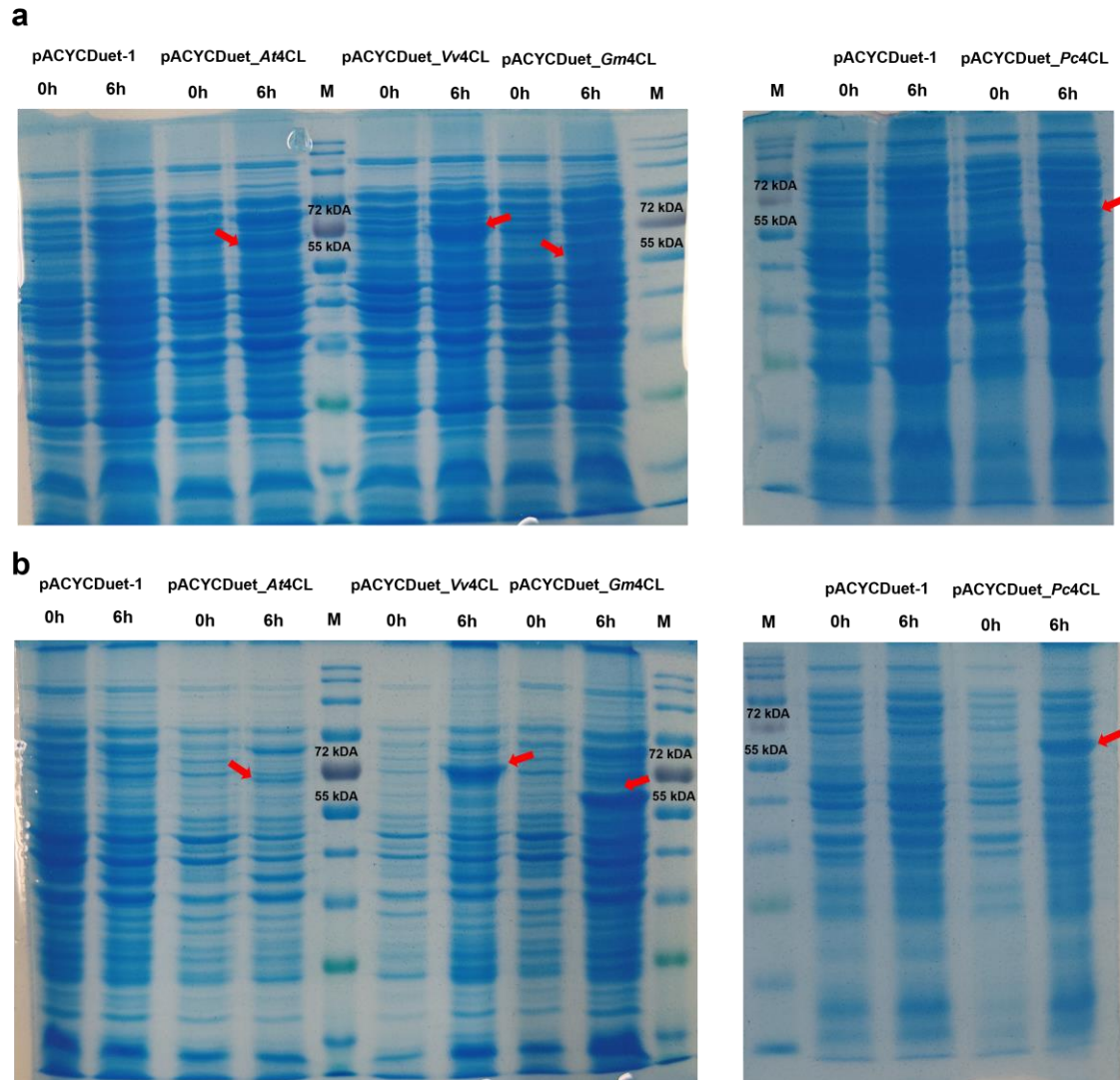


Fig. S2 Protein SDS-PAGE gels of soluble (a) and insoluble (b) protein fractions showing 4-coumarate-CoA ligase (4CL) from *Arabidopsis thaliana* (At4CL), 4CL from *Vitis vinifera* (Vv4CL), 4CL from *Glycine max* (Gm4CL), and 4CL from *Petroselinum crispum* (Pc4CL) expression in *E. coli* M-PAR-121 strain at time zero (0 h) of induction and after 6 h of induction. At4CL, Vv4CL, Gm4CL, Pc4CL are expected around 62.67 kDa, 69.25 kDa, 60.97 kDa, and 61.61 kDa respectively. Red arrows indicate the bands of interest. M: marker (Color Pre-stained Protein Standard, Broad Range (10-250 kDa) – NEB).

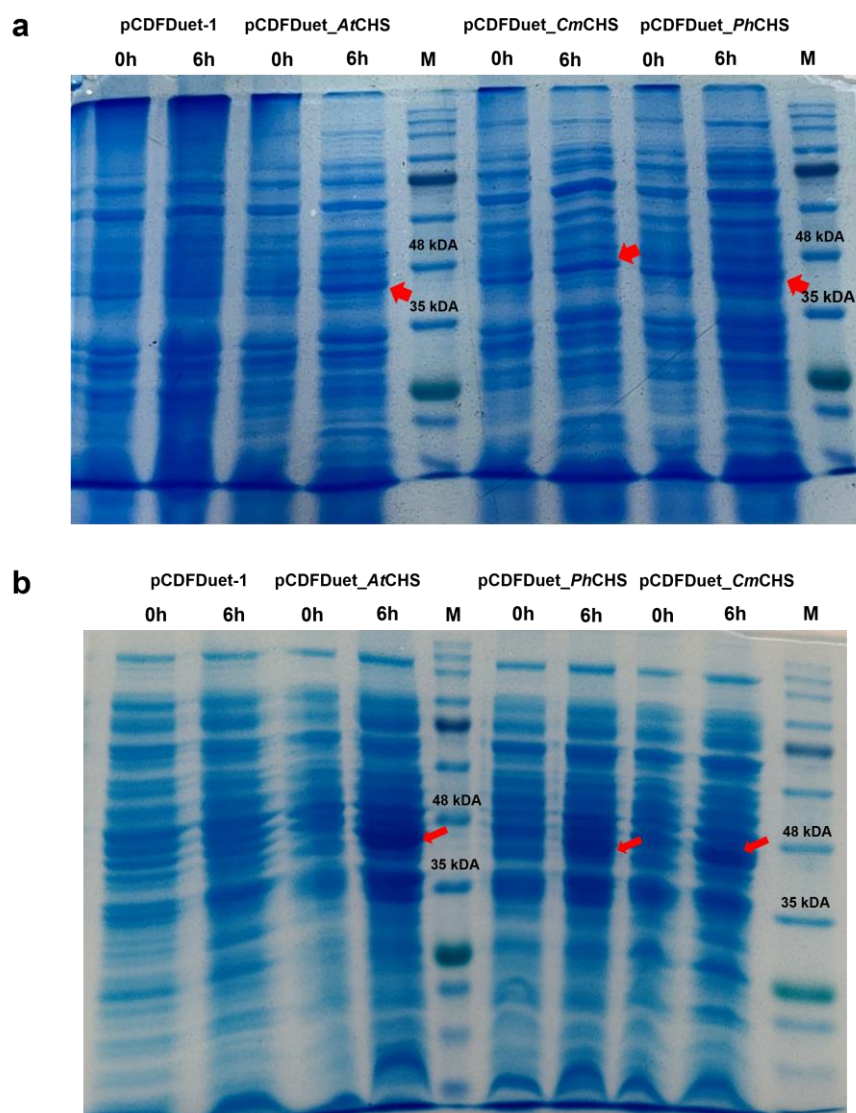


Fig. S3 Protein SDS-PAGE gels of soluble (a) and insoluble (b) protein fractions showing chalcone synthase (CHS) from *Arabidopsis thaliana* (AtCHS), CHS from *Petunia hybrida* (PhCHS), and CHS from *Cucurbita maxima* (CmCHS) expression in *E. coli* M-PAR-121 strain at time zero (0 h) of induction and after 6 h of induction. AtCHS, PhCHS, and CmCHS are expected around 43.12 kDa, 42.50 kDa and 44.81 kDa, respectively. Red arrows indicate the bands of interest. M: marker (NZYColour Protein Marker II – NZYTech).

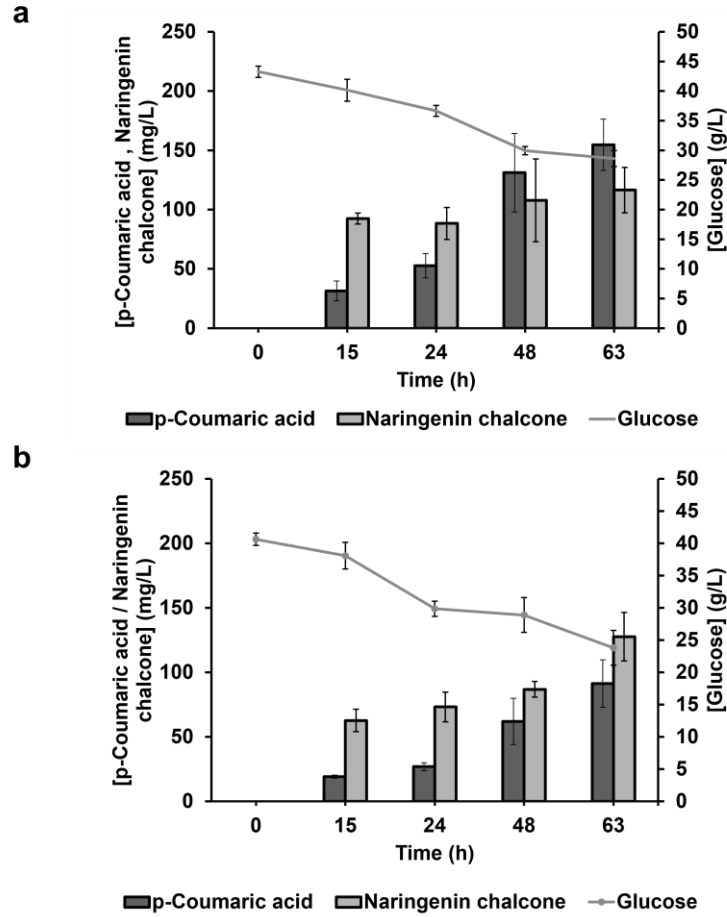


Fig. S4 Evaluation of the effect of metabolic burden reduction in the production of naringenin chalcone. **a.** Profile of naringenin chalcone and *p*-coumaric acid production and glucose consumption for the *E. coli* M-PAR-121 strain expressing pACYCDuet_FjTAL and pRSFDuet_At4CL_CmCHS. **b.** Profile of naringenin chalcone and *p*-coumaric acid production and glucose consumption for the *E. coli* M-PAR-121 strain expressing pACYCDuet_FjTAL_At4CL and pRSFDuet_CmCHS. Results correspond to the average of three independent experiments $\pm$ standard deviation.

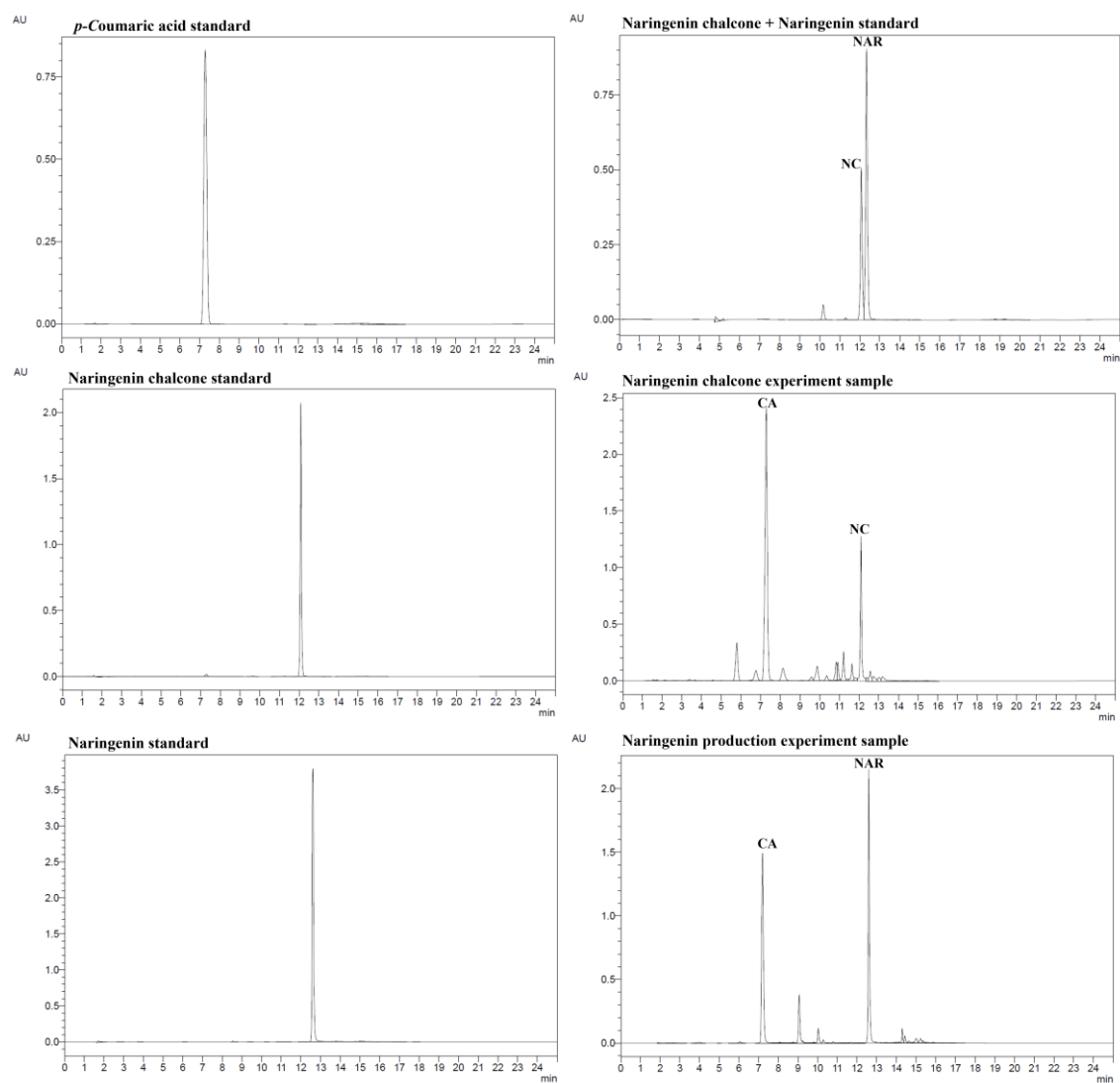


Fig. S5 Representative chromatograms of analytical standards (*p*-coumaric acid, naringenin chalcone, naringenin and the mixture of naringenin chalcone and naringenin standards) and samples from naringenin chalcone and naringenin production experiments with higher production levels. CA corresponds to *p*-coumaric acid. NC corresponds to naringenin chalcone. NAR corresponds to naringenin.

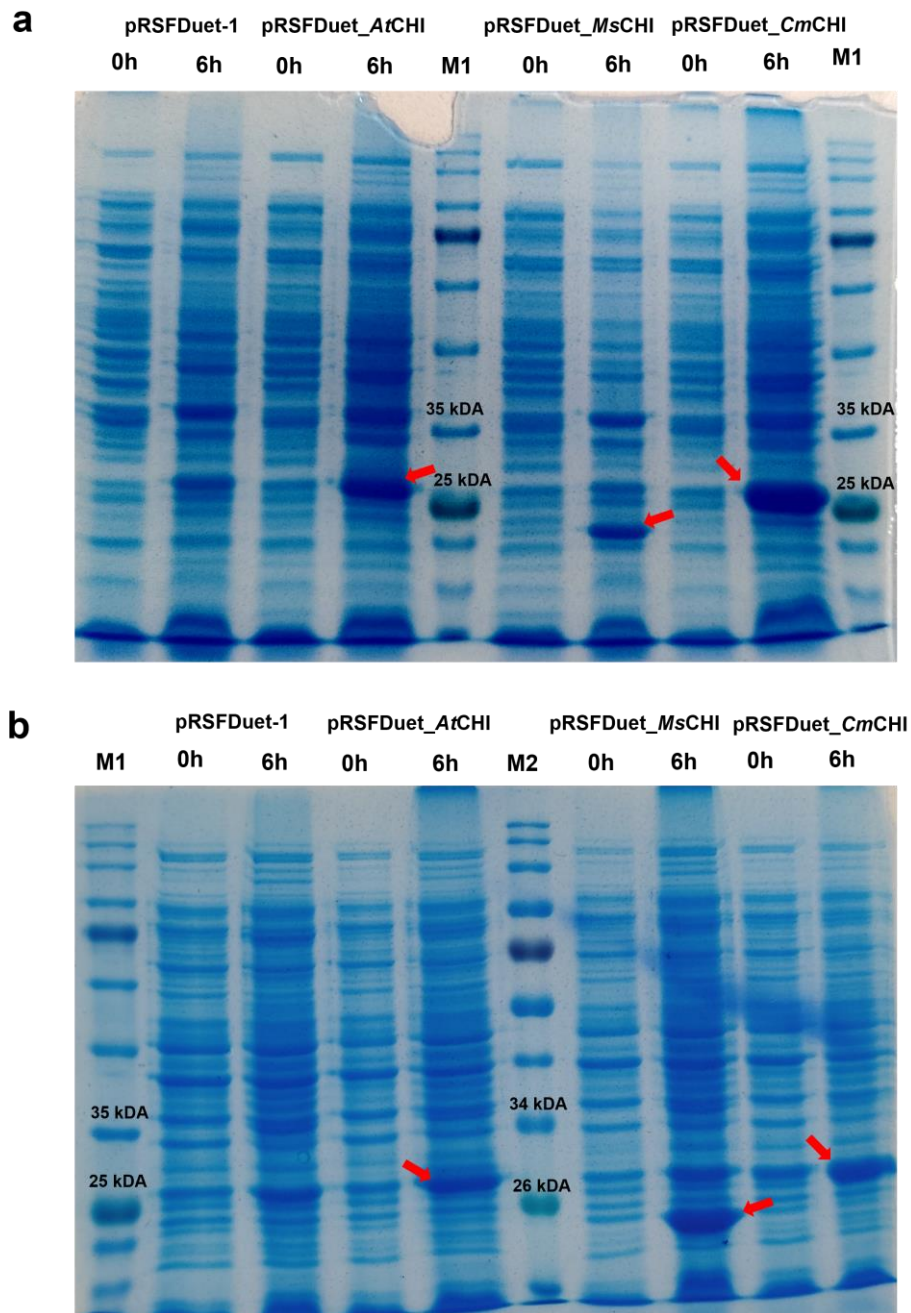


Fig. S6 Protein SDS-PAGE gels of soluble (a) and insoluble (b) protein fractions showing chalcone isomerase (CHI) from *Arabidopsis thaliana* (AtCHI), CHI from *Medicago sativa* (MsCHI), and CHI from *Cucurbita maxima* (CmCHI) expression in *E. coli* M-PAR-121 strain at time zero (0 h) of induction and after 6 h of induction. AtCHI, MsCHI, and CmCHI are expected around 26.60 kDa, 23.83 kDa and 25.60 kDa, respectively. Red arrows indicate the bands of interest. M1: marker 1 (NZYColour Protein Marker II – NZYTech); M2: marker 2 (Color Pre-stained Protein Standard, Broad Range (10-250 kDa) – NEB).

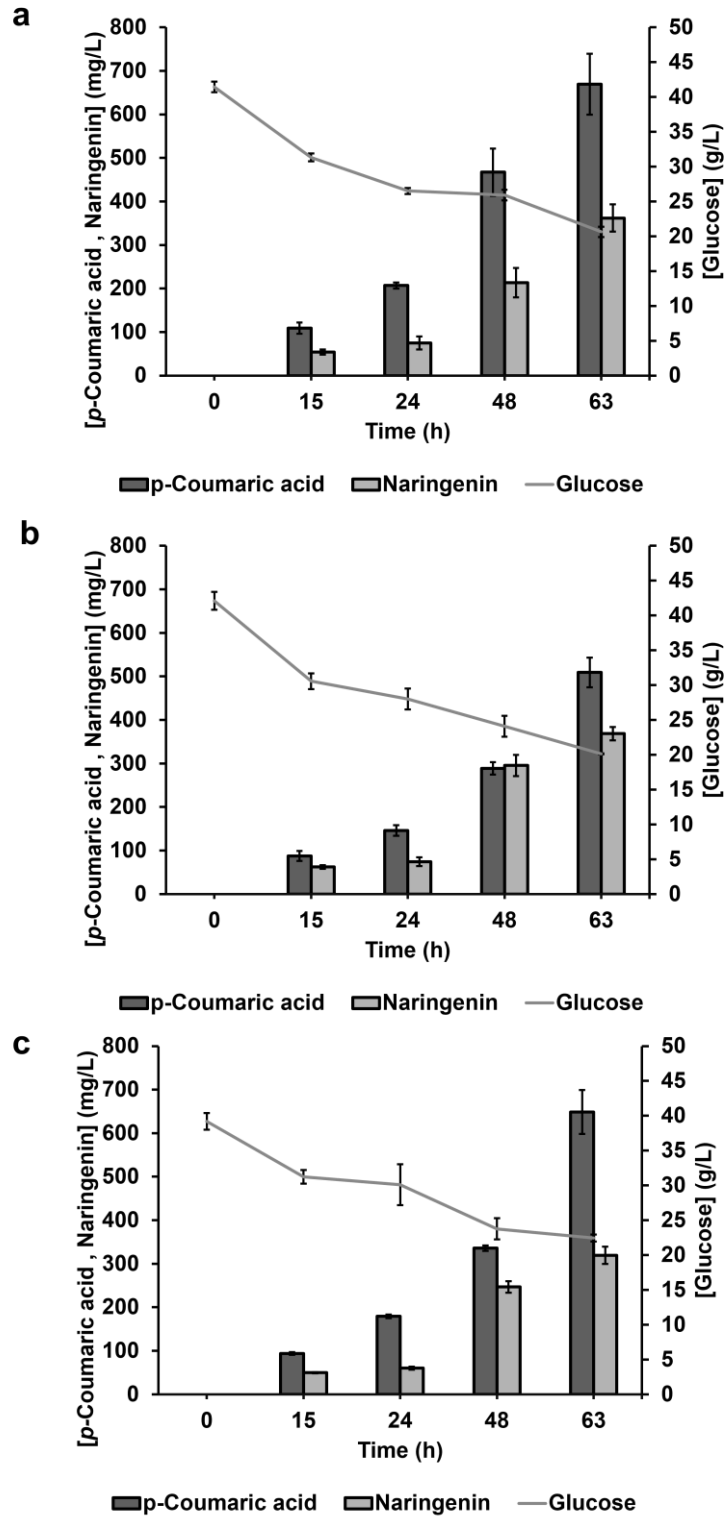


Fig. S7 Naringenin production by *Escherichia coli* M-PAR-121 expressing three different biosynthetic pathways after 63 h of fermentation. **a.** Profile of naringenin and *p*-coumaric acid production, and glucose consumption for the *E. coli* M-PAR-121 strain expressing pRSFDuet_FjTAL_CmCHS and pACYCDuet_At4CL_AtCHI. **b.** Profile of naringenin and *p*-coumaric acid production and glucose consumption, for the *E. coli* M-PAR-121 strain expressing pRSFDuet_FjTAL_CmCHS and pACYCDuet_At4CL_CmCHI. **c.** Profile of naringenin and *p*-coumaric acid production and glucose consumption, for the *E. coli* M-PAR-121 strain expressing pRSFDuet_FjTAL_CmCHS and pACYCDuet_At4CL_MsCHI. Results correspond to the average of three independent experiments $\pm$ standard deviation.

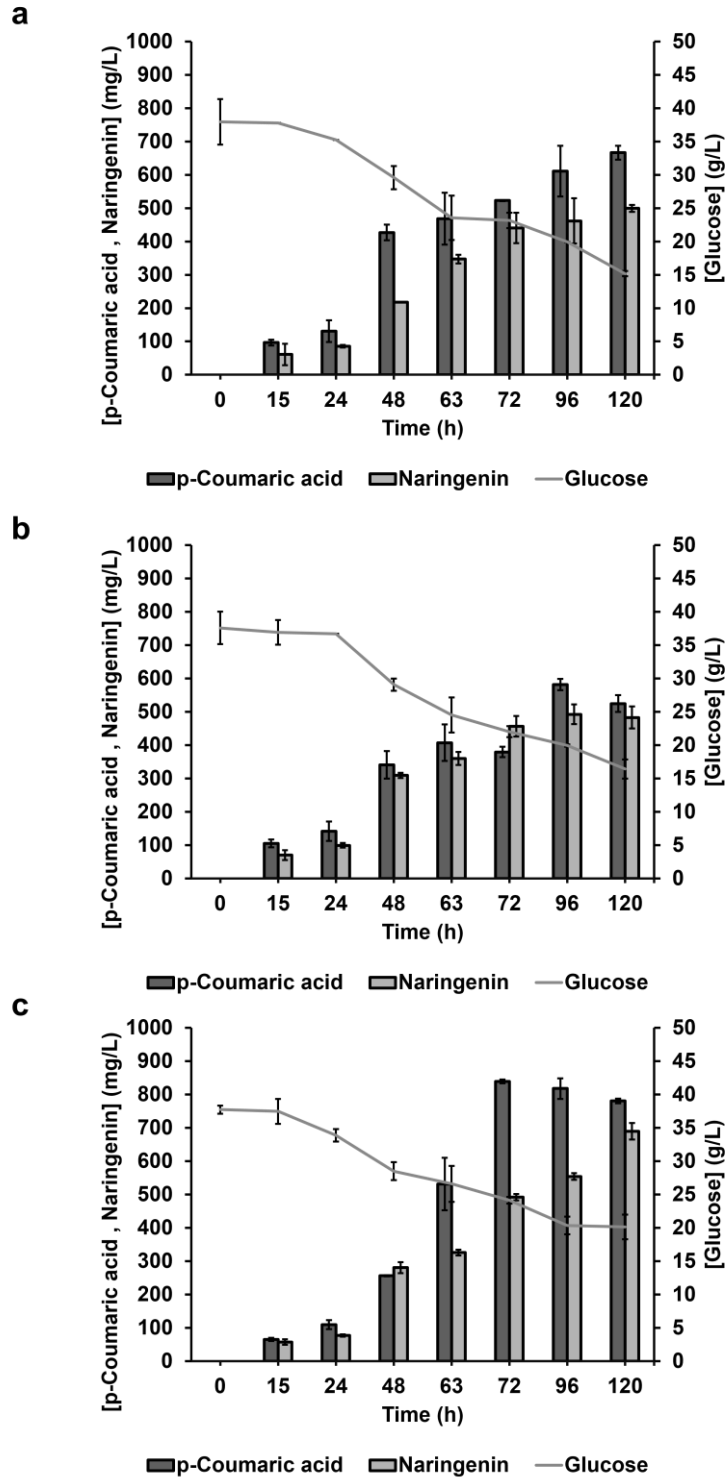


Fig. S8 Evaluation of fermentation time increase to 120 h in the production of naringenin. **a.** Profile of naringenin and *p*-coumaric acid production, and glucose consumption for the *E. coli* M-PAR-121 strain expressing pRSFDuet_FjTAL_CmCHS and pACYCDuet_At4CL_AtCHI. **b.** Profile of naringenin and *p*-coumaric acid production and glucose consumption, for the *E. coli* M-PAR-121 strain expressing pRSFDuet_FjTAL_CmCHS and pACYCDuet_At4CL_CmCHI. **c.** Profile of naringenin and *p*-coumaric acid production and glucose consumption, for the *E. coli* M-PAR-121 strain expressing pRSFDuet_FjTAL_CmCHS and pACYCDuet_At4CL_MsCHI. Results correspond to the average of three independent experiments $\pm$ standard deviation.