# Supplemental data for

## Acylphloroglucinols biosynthesis in strawberry fruit

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**Table S1.** List of primers used in this study

| Name | Forward primer | Purpose |
|---|---|---|
| Gene21343F | TCTTGCTACGAAATGCGATG | RT-qPCR |
| Gene21343R | TTTCACACAAGCAACTCTTCTGA | RT-qPCR |
| Gene10776F | GGTCCGAACACATCCTGAGT | RT-qPCR |
| Gene10776R | ATGATGGGGTATACCGATGG | RT-qPCR |
| Gene33865F | GATACAGAACATTCGGTGGGTTT | RT-qPCR |
| Gene33865R | CAAGCATATGACTACTGGCATGAT | RT-qPCR |
| Gene00897F | CACACCCACAGCTAGATAAGAGG | RT-qPCR |
| Gene00897R | AAACAGATGCACAGTTGCTTTCT | RT-qPCR |
| Gene23054F | AAAACGGGAACCAACATCAA | RT-qPCR |
| Gene23054R | CTACTGCTAGGGACAAAGATTGC | RT-qPCR |
| Gene35152F | TTGGTTGGGCTATGGATTGT | RT-qPCR |
| Gene35152R | AATCCCGCGATACCTCTACC | RT-qPCR |
| Gene03515F | ATTCAACTACGATATGCTCATGG | RT-qPCR |
| Gene03515R | GTTCAGCAATGGTGTATCAACAG | RT-qPCR |
| Gene27098F | CTGCATGGGTAAATGATAAGAGG | RT-qPCR |
| Gene27098R | CCAGTAACAGCATTAAGGAGAGC | RT-qPCR |
| Gene19724F | TTGCAATCTCTCTTGGATCCTT | RT-qPCR |
| Gene19724R | CTCTCTGCGCTAACATGAAGAAC | RT-qPCR |
| Gene22502F | GTTGGACTTGGTCAAAGATTGAG | RT-qPCR |
| Gene22502R | AAGTGTGTAGATCAAACGCCTTC | RT-qPCR |
| Gene30399F | TTGGGTTAAGGGGATAGGTAGAG | RT-qPCR |
| Gene30399R | TGGTTAGGATGCTGATTCAAAAC | RT-qPCR |
| Gene03472F | CCAGGTTGATTTCTCTTCGTAAA | RT-qPCR |
| Gene03472R | GGCGGTATCCATCTTAGAGAAGT | RT-qPCR |
| Gene21343Fo | CGC GGATCCTCTTGCTACGAAATGCGATG | RNAi |
| Gene21343Re | CCC AAGCTT TTTCAC ACA AGC AACTCT TCTGA | RNAi |
| Gene33865Fo | CGCGGATCCCGACCG GTCCACCTT ATG | RNAi |
| Gene33865Re | CCC AAGCTTGCCCTTCCGTCAATTCCT | RNAi |
| Gene10776Fo | CGCGGATCCATGGCTGCAGTTGCTCAAGCG | Over expression(OE) |
| Gene10776Re | CGAGCTCCTAAGTTCGGATGTGATC | Over expression(OE) |
| Gene00897Fo | CGCGGATCCATGGAGAGTTTCATGCGT | Over expression(OE) |
| Gene00897Re | CGAGCTCTTAACAGTGTTTGGTGCAGAA | Over expression(OE) |
| Gene21343Fo-RNAi | AGA GGC ACC TAT GCC GAC TA | RT-qPCR(RNAi) |
| Gene21343Re-RNAi | CAT CGC ATT TCG TAG CAA GA | RT-qPCR(RNAi) |
| Gene33865Fo-RNAi | TCG ACG TGT GAT TCT AAG CG | RT-qPCR(RNAi) |
| Gene33865Re-RNAi | CAA GCA TAT GAC TAC TGG CAT GA | RT-qPCR(RNAi) |
| Gene10776Fo-OE | ATGATGGGGTATACCGATGG | RT-qPCR(OE) |
| Gene10776Re-OE | GGTCCGAACACATCCTGAGT | RT-qPCR(OE) |
| Gene00897Fo-OE | CACACCCACAGCTAGATAAGAGG | RT-qPCR(OE) |
| Gene00897Re-OE | AAACAGATGCACAGTTGCTTTCT | RT-qPCR(OE) |
| CHS2.1F(Gene26825) | CGCGGATCCATGGTGACCGTTGAGGAA | Whole Length CHS |
| CHS2.1R(Gene26825) | CCGGAATTCTCAAGCAGATACACTGTG | Whole Length CHS |
| CHS2.2F(Gene26826) | CGCGGATCCATGGTGACCGTCGAGGAA | Whole Length CHS |
| CHS2.2R(Gene26826) | CCGGAATTCTCAAGCAGCCACACTGTG | Whole Length CHS |
| CHS2.3-F | CGCGGATCCATGGTGACCGTCGAGGAA | Whole Length CHS |
| CHS2.3-R | CCGGAATTCTCAAGCAGCCACACTGTG | Whole Length CHS |

**Table S2.** Metabolites (*m/z* negative ionization mode and retention time) that were found to be differentially accumulated in strawberry fruit after down-regulation of *CHS* (*CHS*i), gene21343 (*Expansin-A8-like*), and gene33865 (*Ephrin-A1-like*), and overexpression of gene10776 (*SRG1-like*) and gene00897 (*Defensin-like*) when compared with levels in the pBI-Intron-*GUS* control fruit (pBI)[a]

| m/z | retention times | CHSi vs pBI | | gene21343 vs pBI | | gene33865 vs pBI | | gene10776 vs pBI | | gene00897 vs pBI | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Fold change | p-value | Fold change | p-value | Fold change | p-value | Fold change | p-value | Fold change | p-value |
| 357[b] | 34.4 | -17.1 | 0.00005 | -6.2 | 0.00001 | -5.0 | 0.00014 | -5.4 | 0.00003 | -5.9 | 0.00001 |
| 323 | 33.1 | -28.1 | 0.00049 | -118.8 | 0.00046 | -9.1 | 0.00034 | -9.5 | 0.00011 | -109 | 0.00045 |
| 188 | 17.9 | -54.6 | 0.00238 | -81.7 | 0.00229 | -24.7 | 0.00252 | -27.6 | 0.00229 | -85.8 | 0.00231 |
| 321 | 36.8 | -184.2 | 0.00261 | -62.2 | 0.00263 | -6.2 | 0.00401 | -5.3 | 0.00315 | -109.2 | 0.00262 |
| 664 | 18.0 | -65.5 | 0.00275 | -55.3 | 0.00272 | -5.5 | 0.00273 | -20.9 | 0.00264 | -255.8 | 0.00264 |
| 313 | 33.1 | -31.1 | 0.00281 | -77.9 | 0.00262 | -16.9 | 0.00302 | -21.5 | 0.00283 | -88.3 | 0.00262 |
| 455 | 31.7 | -33.8 | 0.00397 | -43.3 | 0.00387 | -7.3 | 0.00547 | -8.6 | 0.00307 | -59.9 | 0.00379 |
| 771 | 40.2 | -86.8 | 0.00608 | -38.1 | 0.00637 | -20.2 | 0.00685 | -360.4 | 0.0059 | -72.4 | 0.00612 |
| 496 | 18.1 | -15.3 | 0.00764 | -17.1 | 0.00701 | -5.1 | 0.00959 | -7.7 | 0.00726 | -32.3 | 0.00675 |

[a] Fold change and p-values are shown as calculated by XCMS for the pairwise comparison.
[b] This metabolite was identified as M2

**Table S3.** Metabolites (*m/z* negative ionization and retention time) that were found to be differentially accumulated in strawberry fruit after down-regulation of *CHS* (*CHS*i) when compared with levels in the pBI-Intron-*GUS* control fruit (pBI)

| *m/z* | retention time | CHSi vs PBI | p-value |
|---|---|---|---|
| 715 | 34.28 | -17.1 | 5.00E-05 |
| 435 | 37.17 | -5.3 | 8.00E-05 |
| 309 | 32.95 | -5.8 | 0.00036 |
| 565 | 34.30 | 5.4 | 0.00042 |
| 709 | 32.49 | 23.9 | 5.00E-04 |
| 355 | 32.93 | -6.7 | 0.00062 |
| 356 | 32.96 | -6.7 | 0.00109 |
| 661 | 34.25 | -16.7 | 0.00173 |
| 347 | 33.00 | -8.1 | 0.00193 |
| 345 | 32.96 | -5.5 | 0.0028 |
| 371 [a] | 38.27 | -5.6 | 0.00305 |
| 389 | 32.43 | 7.5 | 0.00308 |
| 399 | 32.42 | 15 | 0.00404 |
| 709 | 32.47 | 26.9 | 0.00771 |
| 664 | 18.05 | -23.3 | 0.00926 |
| 371 [b] | 37.10 | -6.4 | 0.01726 |

[a, b] Metabolites were identified as M1 and M3, respectively

**Table S4.** $^{1}H$- and $^{13}C$-NMR data derived from HSQC and HMBC data of isolated metabolite M1 and data from literature

| | | | M1 | | | |
|---|---|---|---|---|---|---|
| Atom | δH | Splitting | Bohr et al., 2005 | δC | HMBC | Bohr et al., 2005 |
| 1 | | | | 162.9 | | 161.8 |
| 2 | | | | 107.0 | | 106.8 |
| 3 | | | | 167.3 | | 167.4 |
| 4 | 5.98 | d | 5.94 d 1H | 99.3 | C-6,C-5,C-3 | 98.3 |
| 5 | | | | 165.6 | | 165.6 |
| 6 | 6.20 | d | 6.17 d 1H | 96.4 | C-2,C-1,C-5 | 95.3 |
| 1' | | | | 211.8 | C-2',C-3';C-5' | 211.8 |
| 2' | 3.93 | m | 3.90 m 1H | 47.0 | C-3',C-4;C-5' | 47.0 |
| 3' | 1.81, 1.40 | m | 1.80, 1.38 m 1H | 28.3 | C-4;C-5' | 28.3 |
| 4' | 0.90 | t | 0.87 t 3H | 12.0 | C-2',C-3;C-5' | 12.0 |
| 5' | 1.15 | d | 1.12 d 3H | 16.8 | | 16.8 |
| 1'' | 5.01 | d | 5.03 d 1H | 101.7 | C-2'' | 101.7 |
| 2''-5'' | 3.5-4.0 | m | 3.38 - 3.91 m | 70-78 | | 71.2 – 78.7 |
| 6'' | 3.70 | dd | 3.71 | 62.5 | | 62.5 |

M1

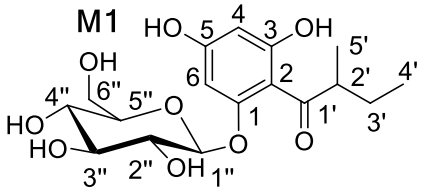
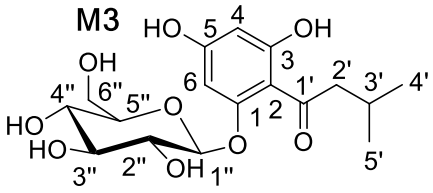



**Table S5.** $^{1}H$-NMR data derived from HSQC and HMBC data of isolated metabolite M3, and $^{1}H$-NMR data from literature (refer also to Aziz-ur-Rehman et al., 2005 and Gao et al., 2004)

| | | M3 | | |
|---|---|---|---|---|
| H-Atom | Chemical shifts δH | Splitting, | Bohr et al., 2005 | Tsukamoto et al., 2004 |
| 4 | 5.98 | 1H d | 5.94 d 1H | 5.93 d 1H |
| 6 | 6.20 | 1H d | 6.17 d 1H | 6.15 d 1H |
| 2' | 3.14 | 2H 2dd | 2.88 dd 1H, 3.17 dd 1H | 2.87 dd 1H, 3.16 dd 1H |
| 3' | 1.82 | 1H sept | 2.24 sept 1H | 2.24 sept 1H |
| 4' u.5' | 0.91 | 6H dd | 0.93 d 3H, 0.96 d 3H | 0.92 d 3H, 0.96 d 3H |
| 1'' | 5.05 | 1H d | 5.01 d 1H | 5.01 d 1H |
| 2''-6'' | 3.5 - 4.0 | m | 3.39 - 3.91 m | 3.39 – 3.91 m |

M3




**Bohr, G., Gerhäuser, C., Knauft, J., Zapp, J., and Becker, H.** (2005) Anti-inflammatory acylphloroglucinol derivatives from hops (*Humulus lupulus*). J. Nat. Prod. **68**: 1545–1548.

**Tsukamoto, S., Tomise, K., Aburatani, M., Onuki, H., Hirorta, H., Ishiharajima, E., and Ohta, T.** (2004) Isolation of cytochrome P450 inhibitors from strawberry fruit, Fragaria ananassa. J. Nat. Prod. **67**: 1839–1841.

**Aziz-Ur-Rehman, M., Riaz N., Ahmad H., Nawaz S.A., and Choudhary M.I.** (2005) Lipoxygenase inhibiting constituents from *Indigofera hetrantha*. Chem. Pharm. Bull. **53**: 263-266.

**Gao, S., Feng, N., Yu, S., Yu De, Wang X.** (2004) Vasodilator constituents from the roots of *Lysidice rhodostega*. Planta Med. **70**: 1128-1134.

# Figures

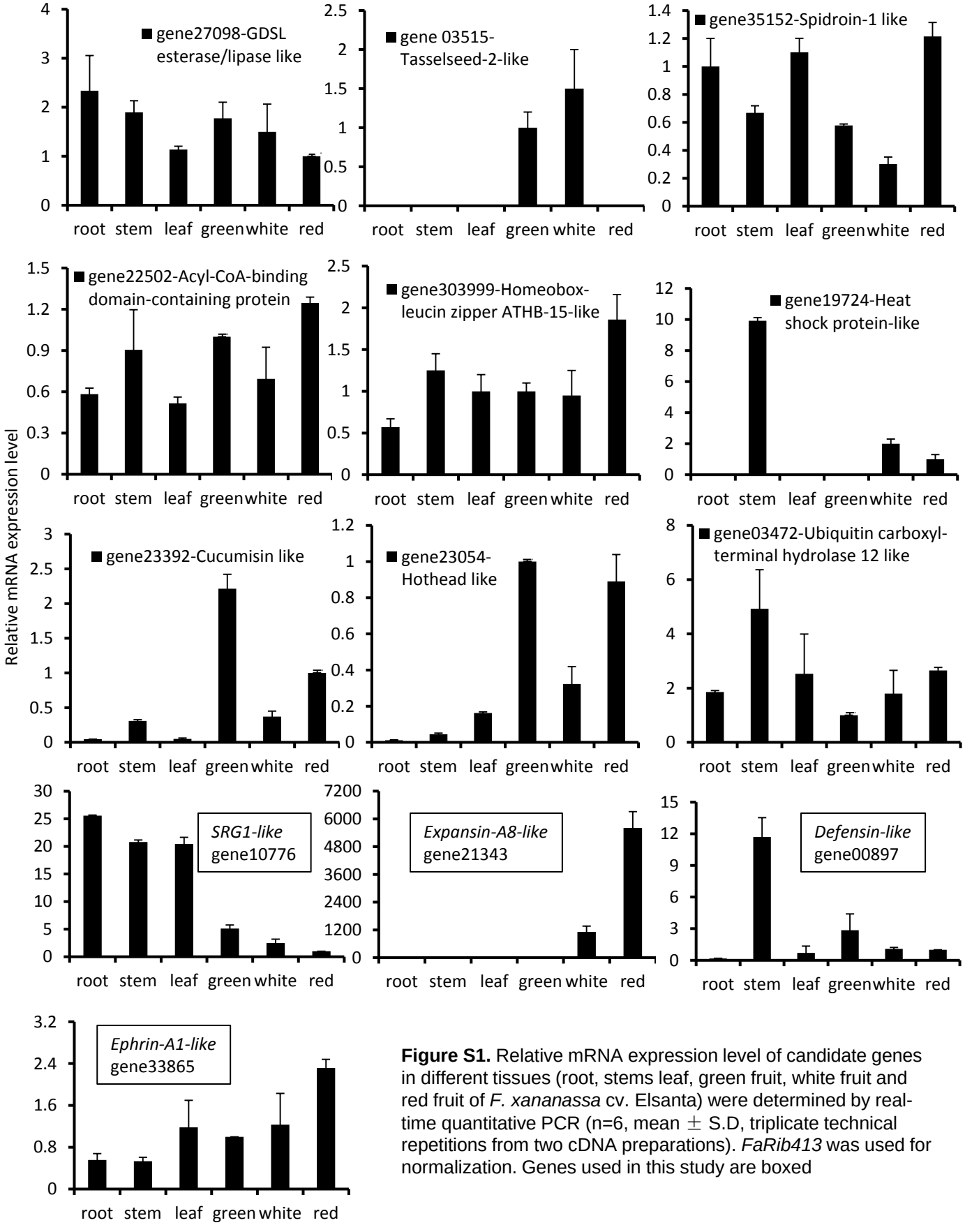
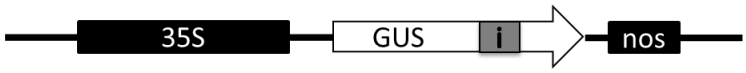
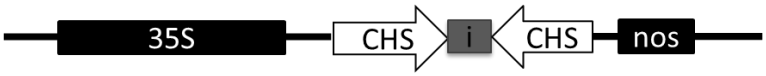



**Figure S1.** Relative mRNA expression level of candidate genes in different tissues (root, stems leaf, green fruit, white fruit and red fruit of *F. xananassa* cv. Elsanta) were determined by real-time quantitative PCR (n=6, mean ± S.D, triplicate technical repetitions from two cDNA preparations). *FaRib413* was used for normalization. Genes used in this study are boxed

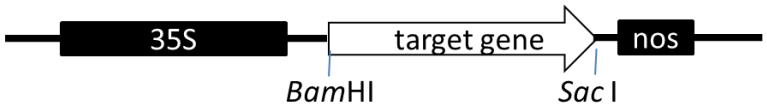
pBI-Intron: used for negative control




pBI-CHSi: used for positive control




pBI-target gene: used for over expression




P9U10-target gene: used for silencing

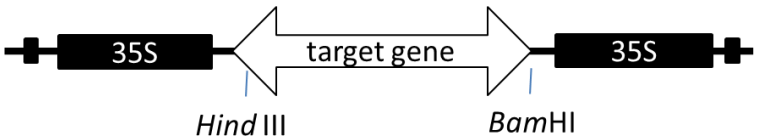



**Figure S2.** Constructs used for this study

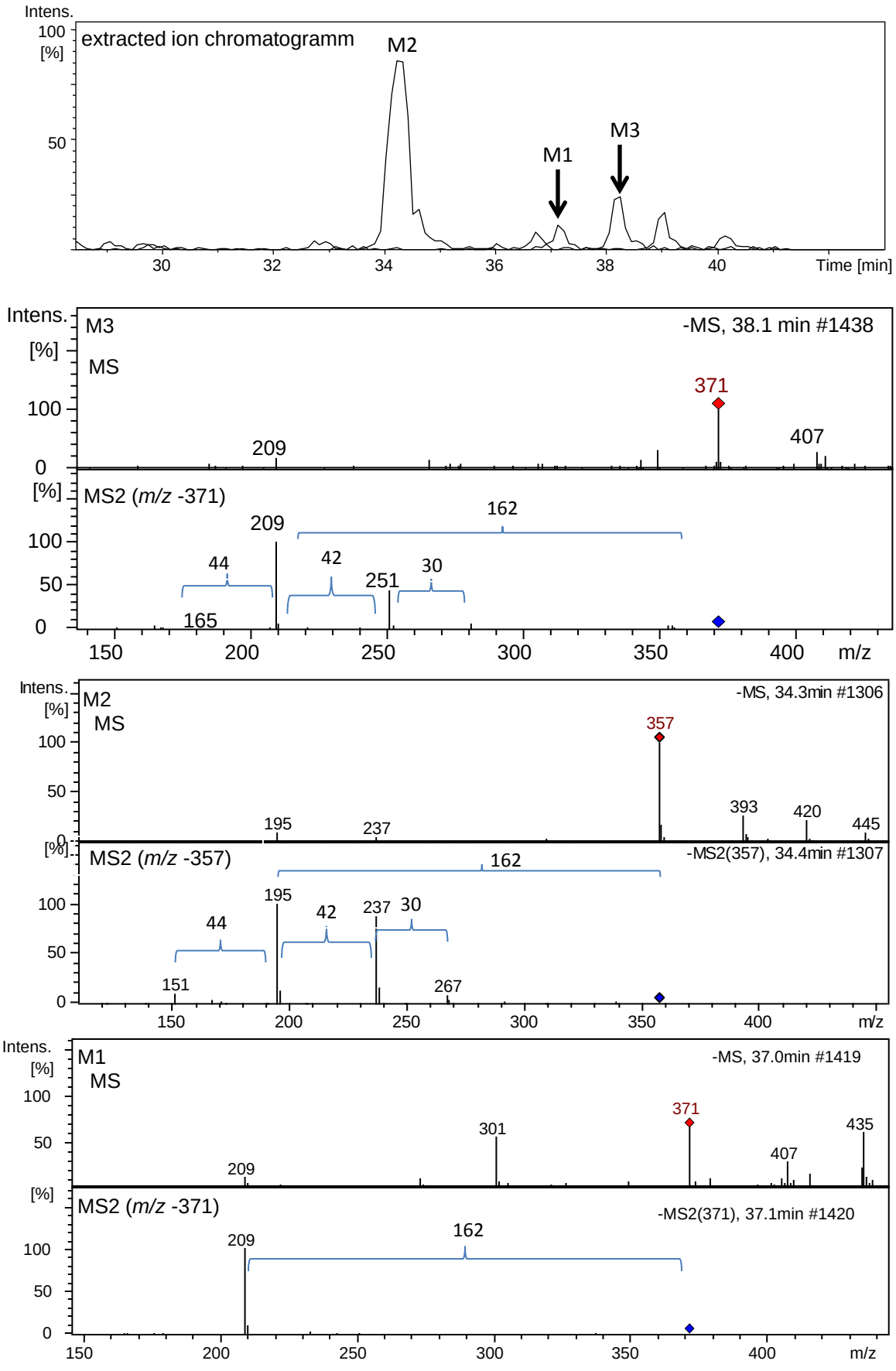



**Figure S3.** Extracted ion chromatogram (*m/z* 357 and 371, superimposed), MS and MS2 spectra of metabolites M1, M2 and M3.

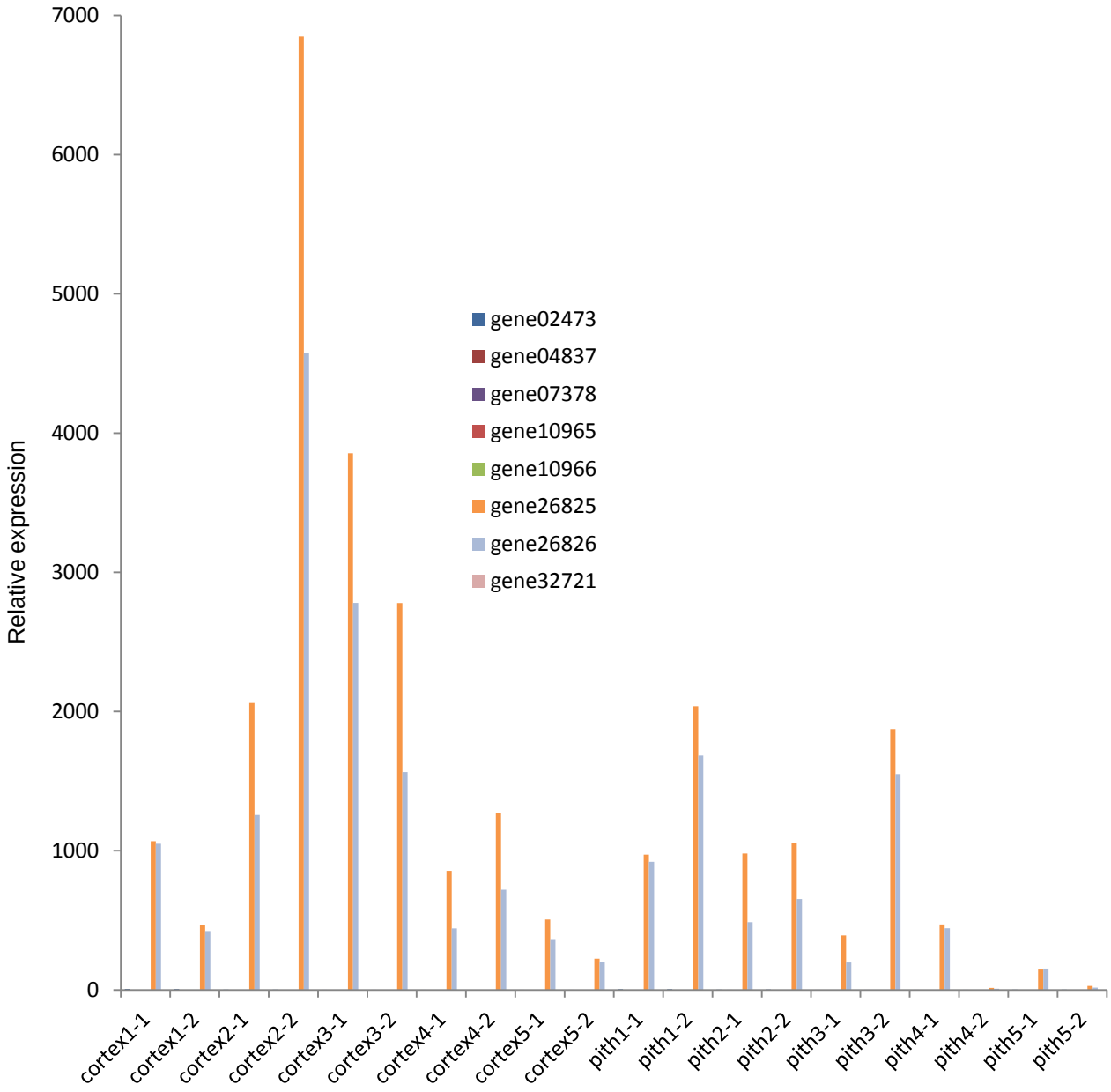



**Figure S4.** Relative expression levels of eight putative chalcone synthase genes in strawberry (*F. vesca* Hawaii-4) fruit tissue (cortex and pith) during early stages of fruit development (Kang et al., 2013). Only gene 26825 and 26826 is expressed. Transcripts of the other six genes were not detected in any of the materials tested. “Cortex1-1” represents “cortex-stage1-replicate1”. Stage 1: prefertilization stage, stage 2: 2 to 4 d postanthesis, stage 3: complete loss of anthers, and a heart stage embryo inside each seed, stage 4: embryos adopt torpedo or walking stick morphology, stage 5: embryos and achenes maturation. For details refer to http://bioinformatics.towson.edu/strawberry/newpage/Search_By_Gene_Desc.aspx

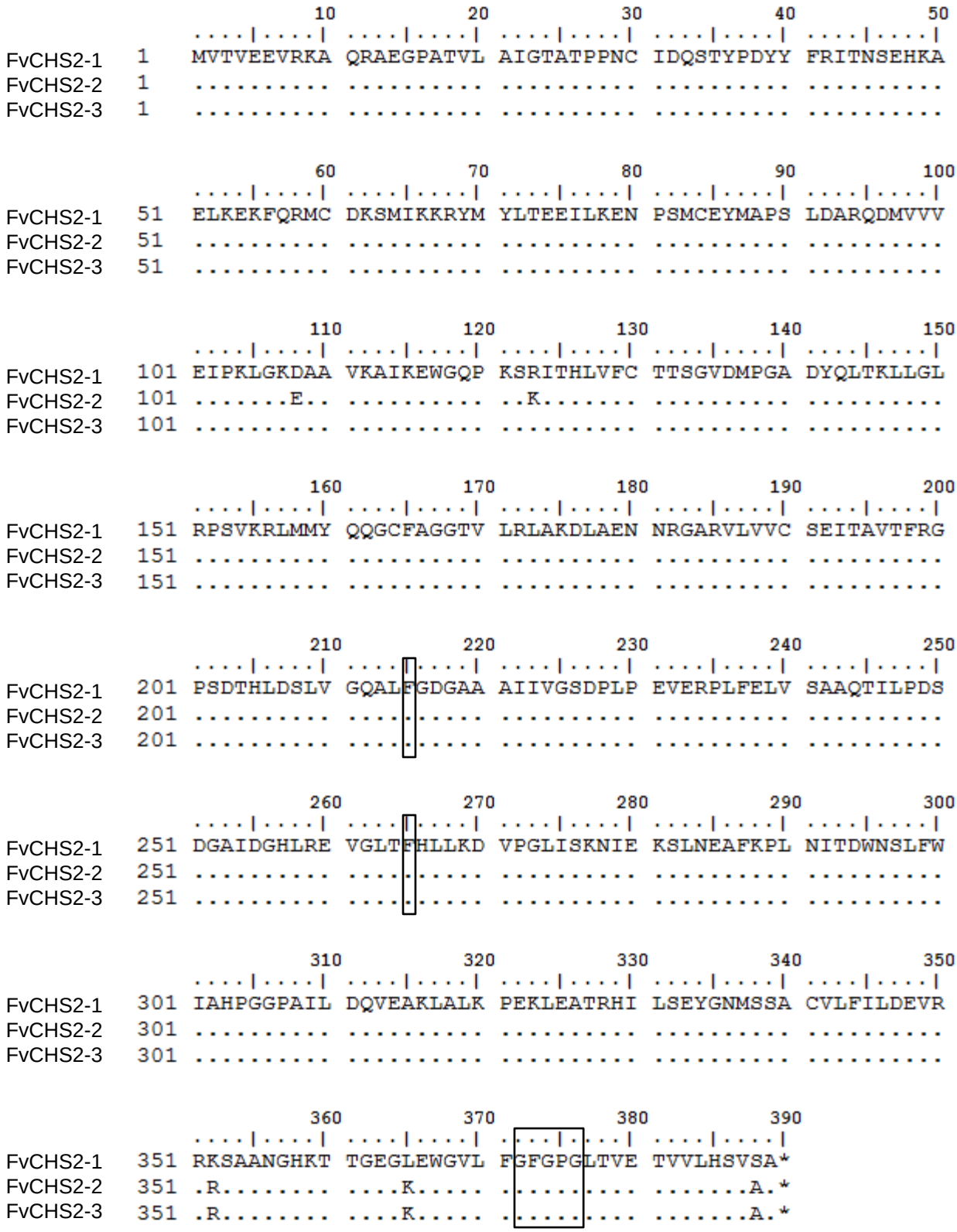



**Figure S5.** Comparison of the deduced amino acid sequences of CHS enzymes from *F. vesca*. FvCHS2-1 (gene26825) and FvCHS2-2 (gene26826) are chalcone synthases from *F. vesca*. FvCHS2-3 is a third protein whose corresponding gene was cloned from *F. vesca* fruit. The sequences were aligned using the ClustalW program. The highly conserved active site loop of CHS enzymes, $G^{372}FGPG$ and two Phe residues (Phe215 and Phe265), important in determining the substrate specificity of CHS are boxed.

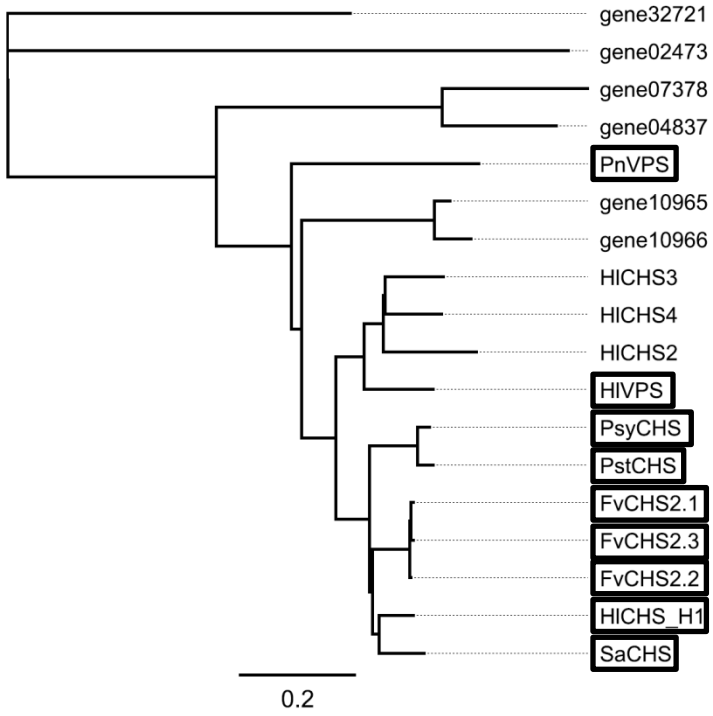



**Figure S6.** Phylogenetic tree of CHS/VPS enzymes and homologues from *F. vesca* and *Humulus lupulus* as well as functionally characterized CHS proteins with VPS promiscuous catalytic activity, generated by the Geneious (Pro 5.5.4) Tree Builder (Jukes Cantor genetic distance model and neighbor-joining method). The scale bar indicates the average number of amino acid substitutions per site. FvCHS2-1 *F. vesca* chalcone synthase 2-1 (XM_004306495.1; gene 26825), FvCHS2.2 *F. vesca* chalcone synthase 2.2 (XM_004306494.1; gene26826), FvCHS2-3 *F. vesca* chalcone synthase 2-3 (chimera of 2-1 and 2-2), PsyCHS *Pinus sylvestris* chalcone synthase (P30079), PstCHS *Pinus strobus* chalcone synthase (O65872), SaCHS *Sinapis alba* chalcone synthase (P13416), PnVPS *Psilotum nudum* valerophenone synthase (Q9SLX9), HlCHS2-4 *Humulus lupulus* chalcone synthase 2-4 (AB061020, AB061022, CAD23044), HlVPS *Humulus lupulus* valerophenone synthase (O80400), HlCHS_H1 *Humulus lupulus* chalcone synthase_H1 (CAC19808), gene02473, 04837, 07378, 10965, 10966, and 32721 are translated protein sequences of putative *F. vesca* chalcone synthases (Shulaev et al., 2011). Proteins with biochemically verified VPS activity are boxed.

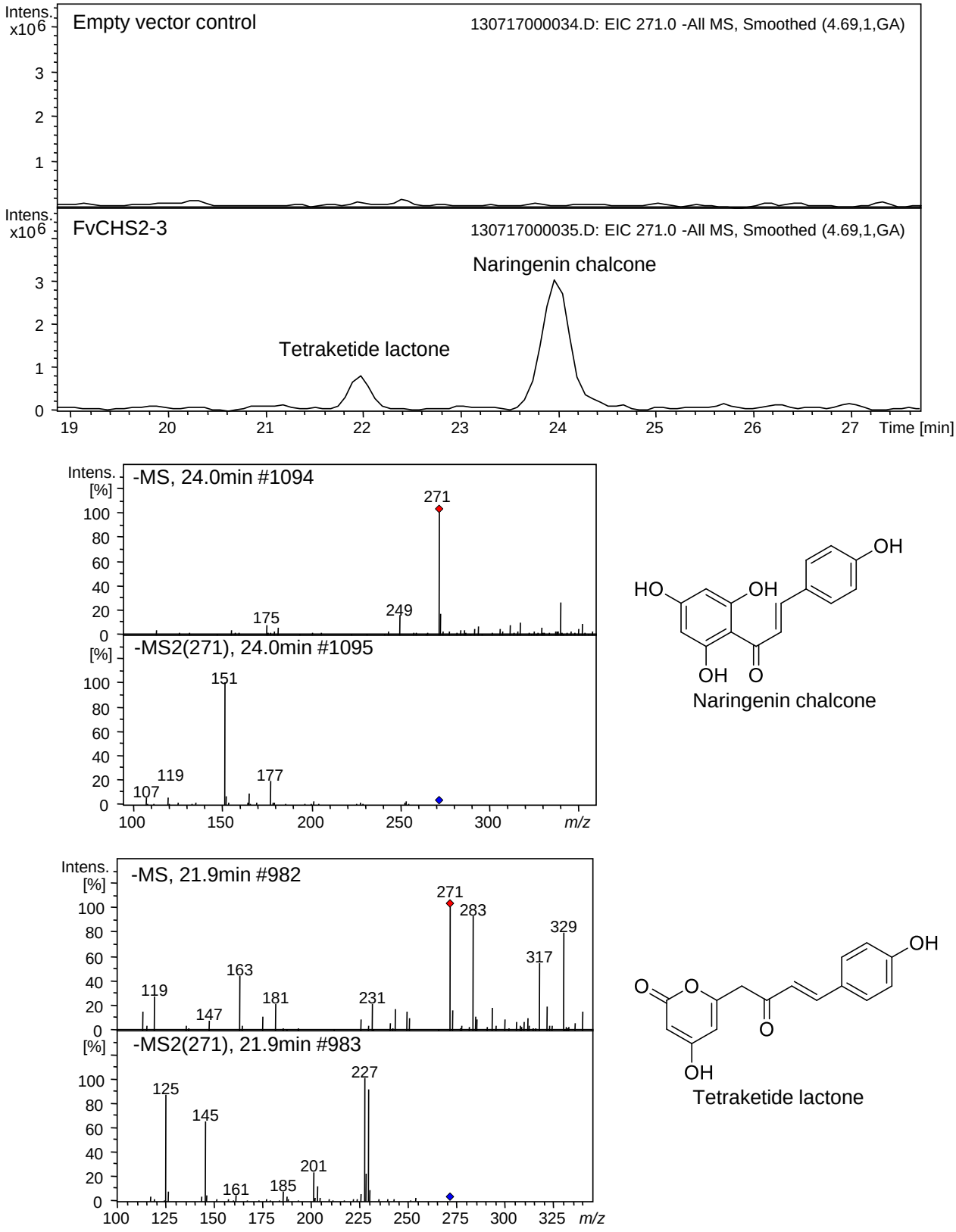



**Figure S7.** LC-MS analysis of products formed by the empty vector control and FvCHS2-3 from the starter molecule 4-coumaroyl-CoA. MS and MS2 spectra of naringenin chalcone and tetraketide lactone.

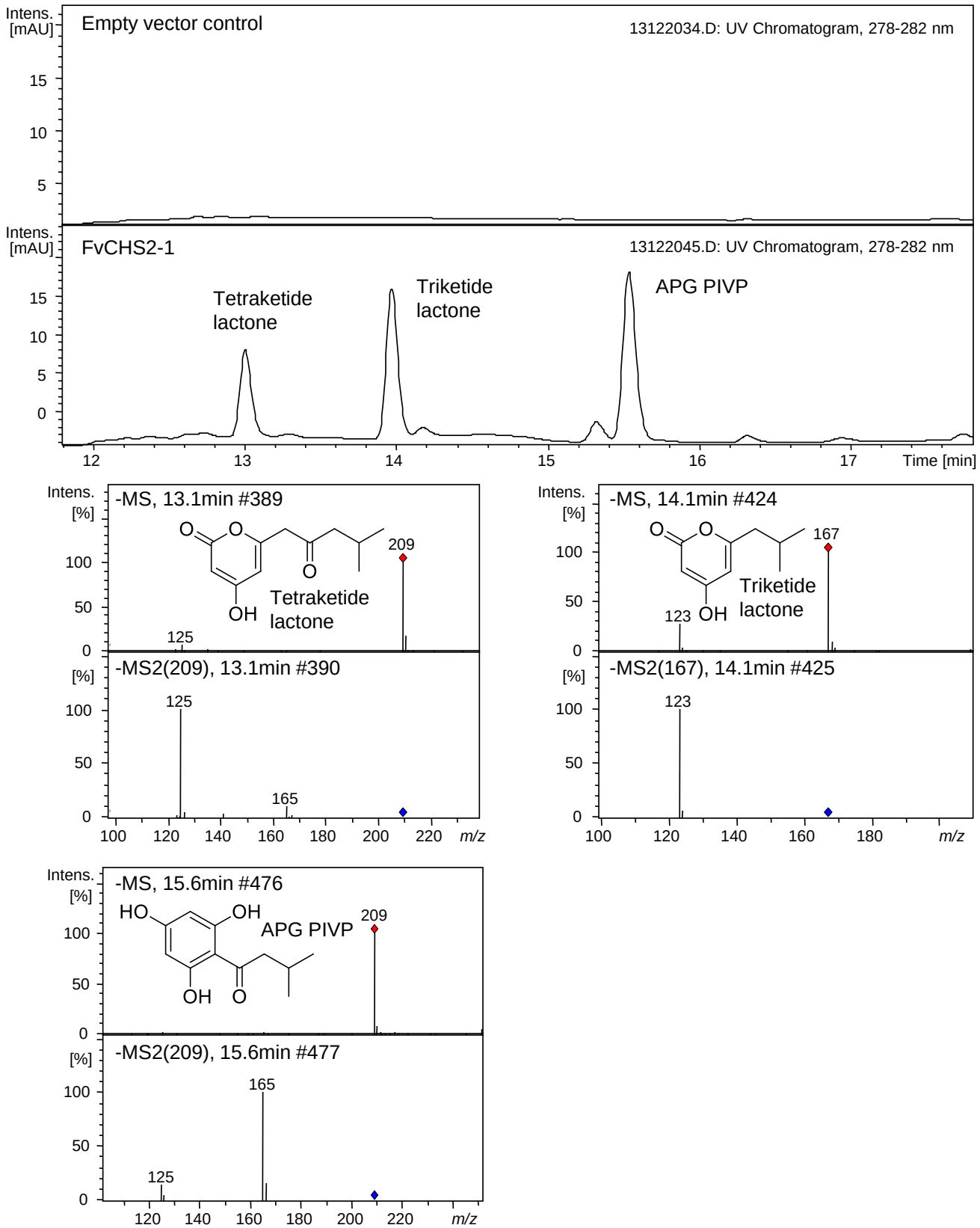



**Figure S8.** LC-MS analysis of products formed by the empty vector control and FvCHS2-1 from the starter molecule isovaleryl-CoA (UV trace). MS and MS2 spectra of tetraketide lactone, triketide lactone and APG (acylphloroglucinol). PIVP phlorisovalerophenone.

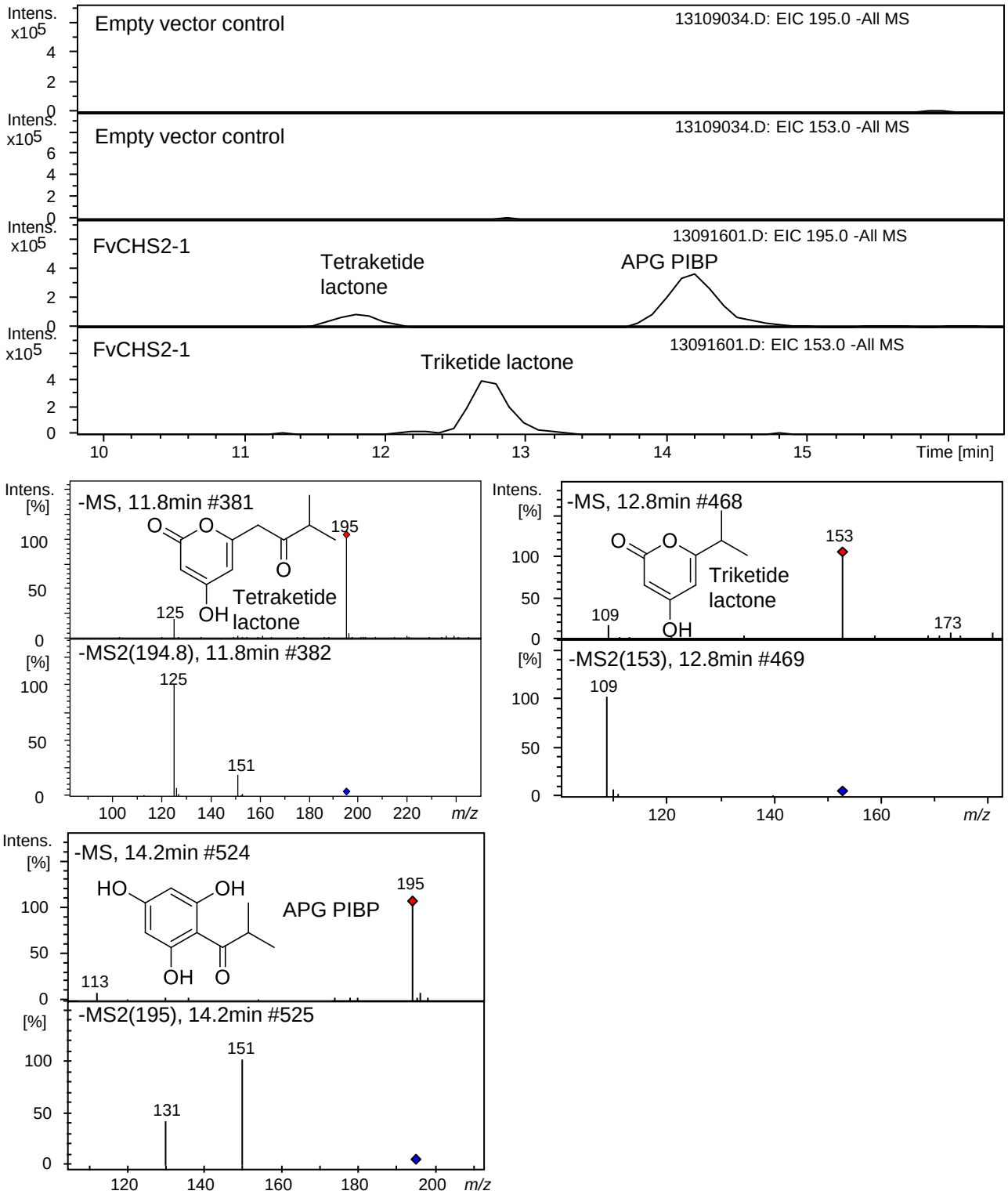



**Figure S9.** LC-MS analysis of products formed by the empty vector control and FvCHS2-1 from the starter molecule isobutyryl-CoA. MS and MS2 spectra of tetraketide lactone, triketide lactone and APG (acylphloroglucinol). PIBP phlorisobutyrophenone.

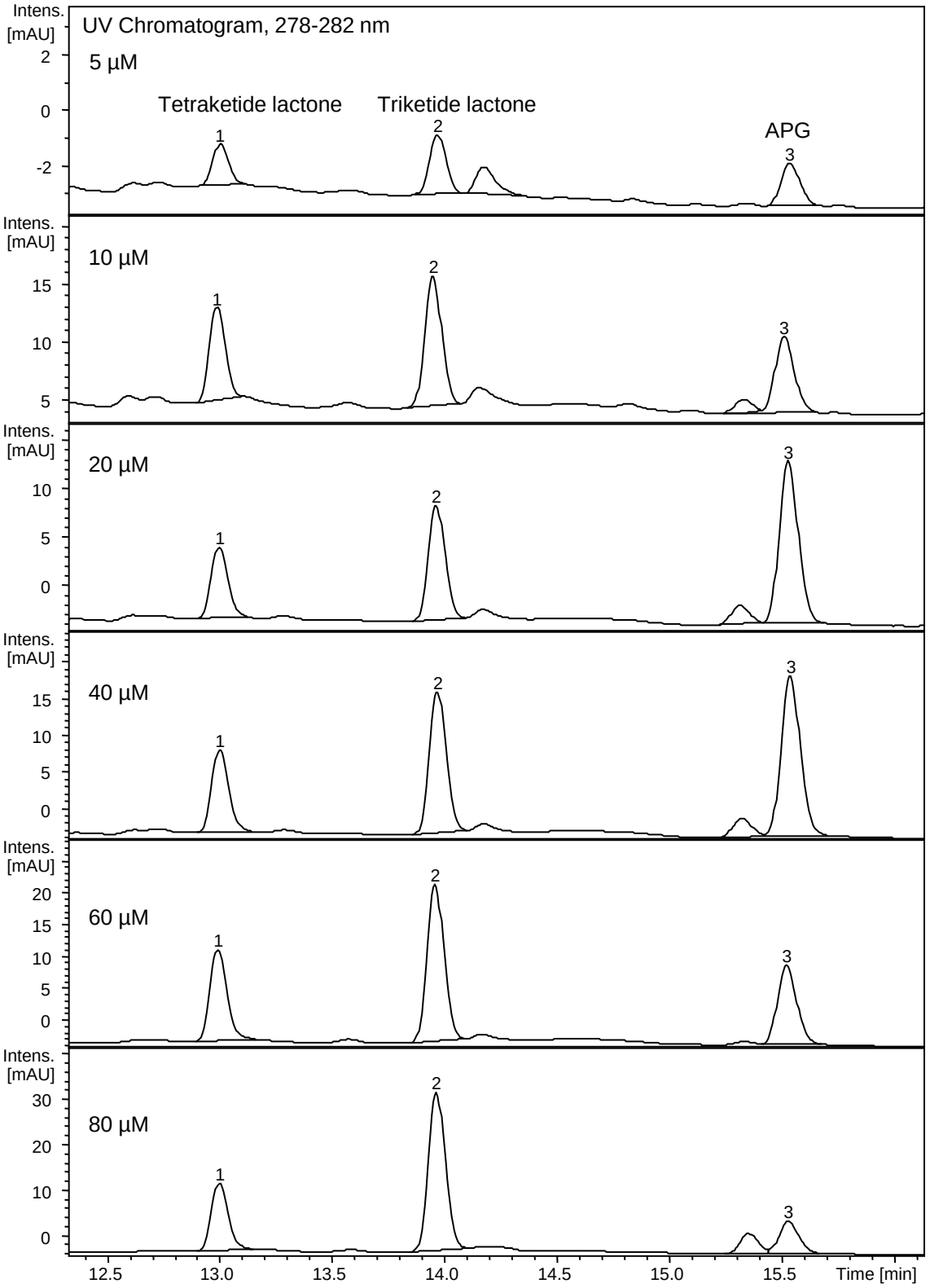



**Figure S10.** Products (tetraketide lactone 1, triketide lactone 2 and APG 3; signals at 14.3 and 15.4 min are unknowns) formed by FvCHS2-1 using 300 µM malonyl-CoA and different concentrations of isovaleryl-CoA (5, 10, 20, 40, 60, and 80 µM). The ratio of APG 3 is highest when the concentration of isovaleryl-CoA is 20 - 40 µM.

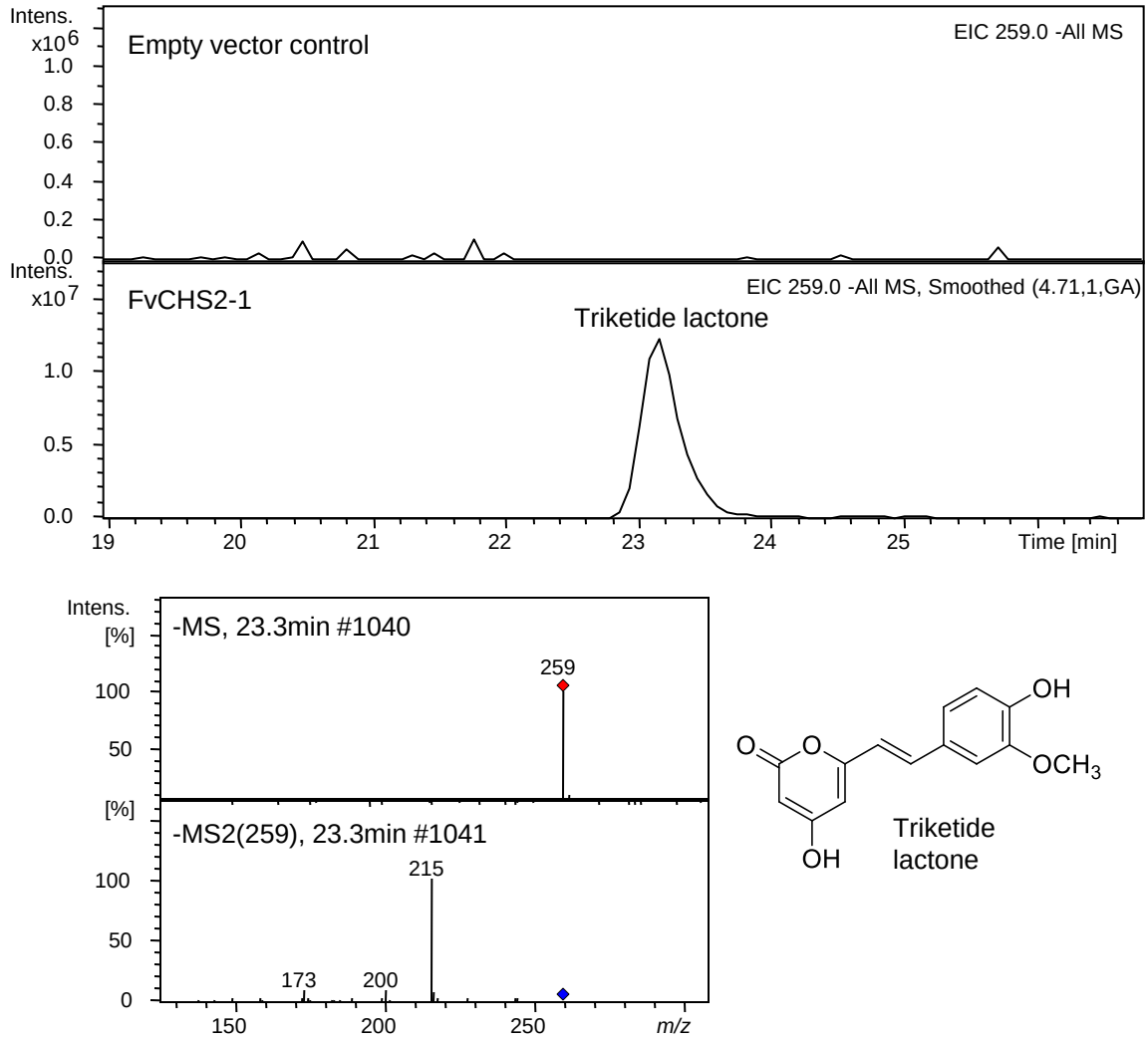



**Figure S11.** LC-MS analysis of the product formed by the empty vector control and FvCHS2-1 from the starter molecule feruloyl-CoA. MS and MS2 spectra of the triketide lactone.

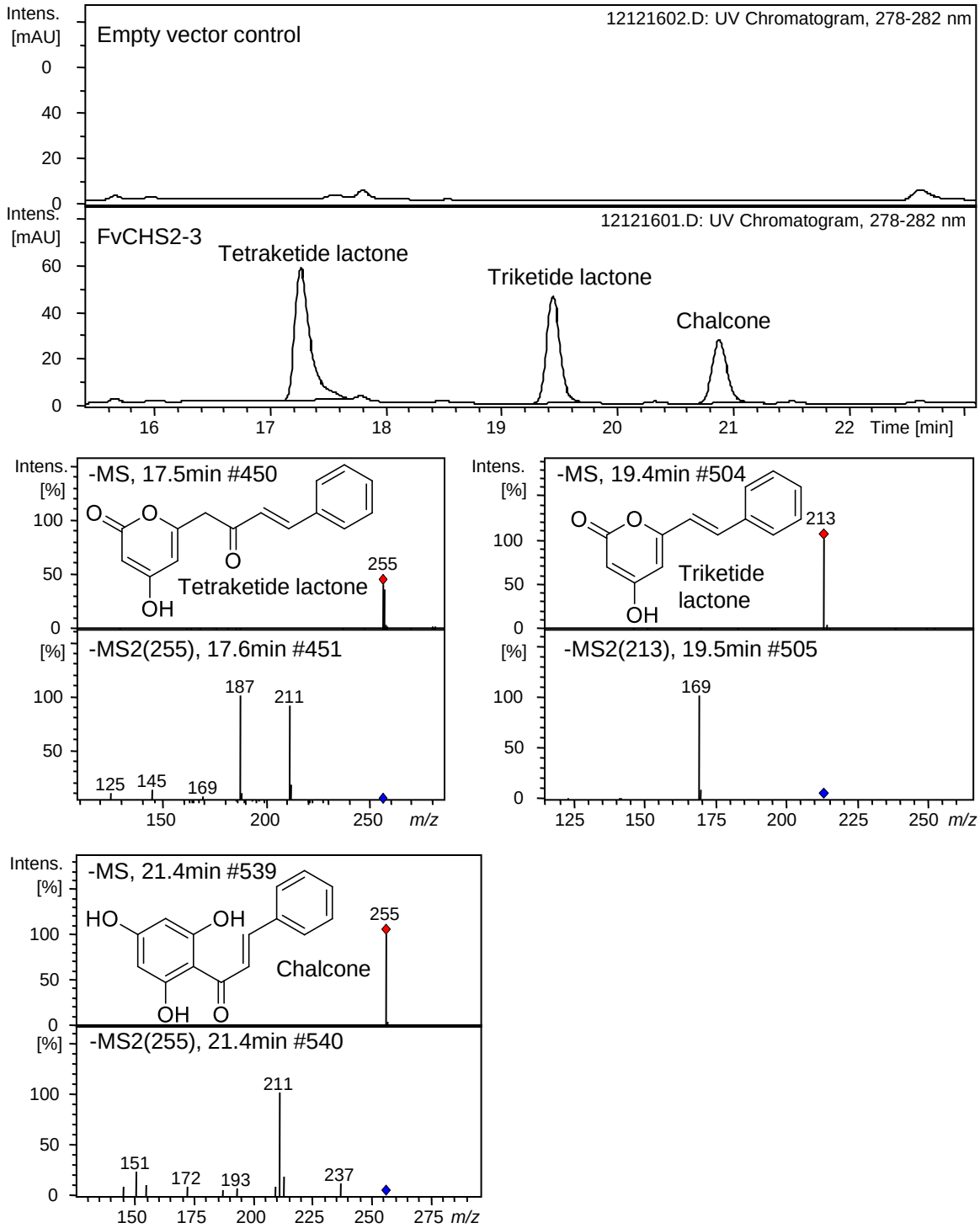



**Figure S12.** LC-MS analysis of products formed by the empty vector control and FvCHS2-3 from the starter molecule cinnamoyl-CoA. MS and MS2 spectra of tetraketide lactone, triketide lactone and chalcone.

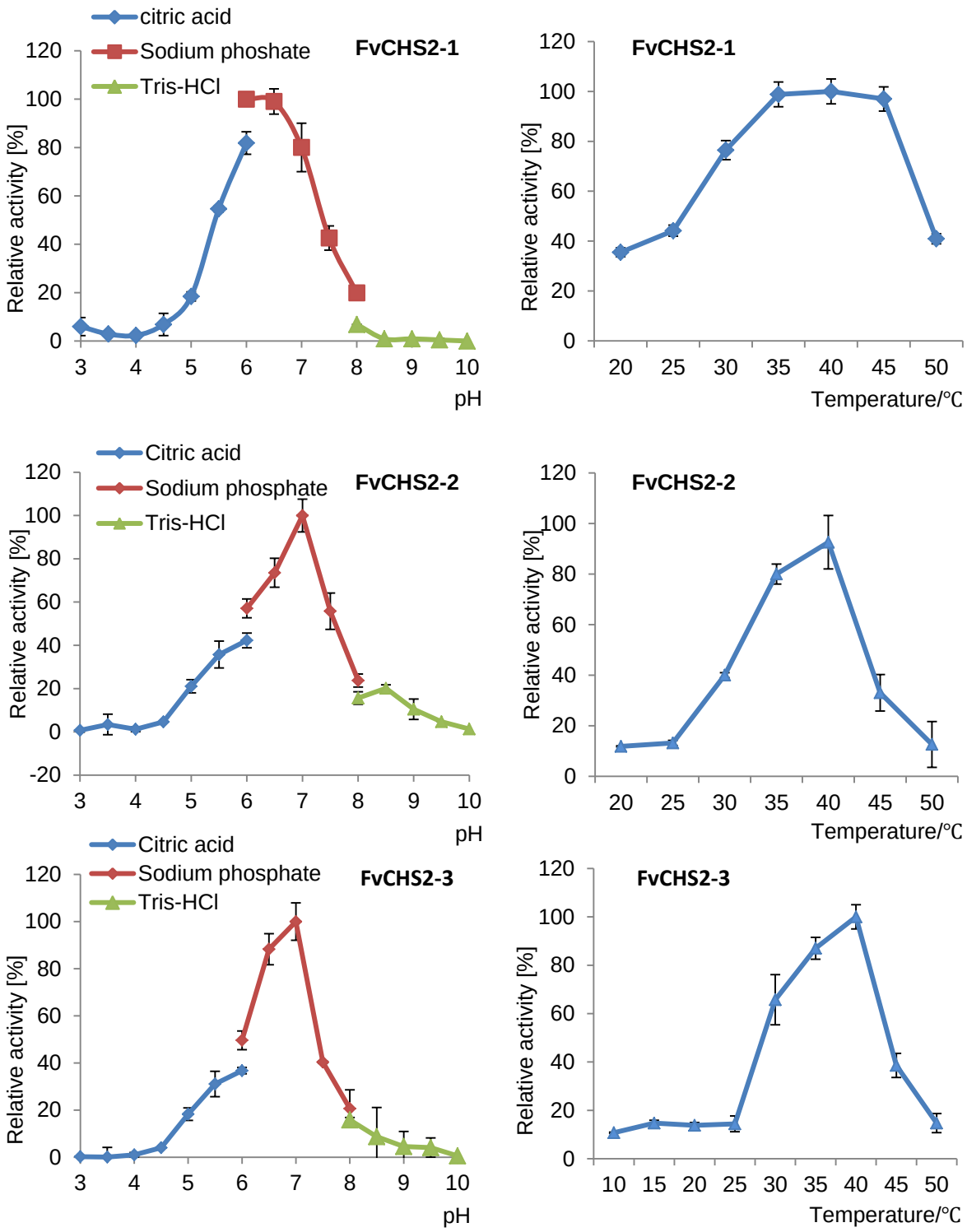



**Figure S13.** The pH and temperature optima. The purified enzymes (FvCHS2-1 – 2-3) were incubated with 100 µM isovaleryl-CoA at 30 °C and different pH values (left column) and at pH 7.0 and different temperature values (right column). Citric acid buffer was used for pH 3 to 6, sodium phosphate buffer was used for pH 6 to 8, Tris-HCl buffer was used for pH 8 to pH 10. Temperature optima were determined in the range of 10 to 50 °C and was carried out at pH 7.0 for 10 min. Product formation was quantified by LC–MS analysis allowing the calculation of the Michaelis–Menten equation by hyperbolic regression and quantified as activity (pmol $mg^{-1}$ $s^{-1}$).

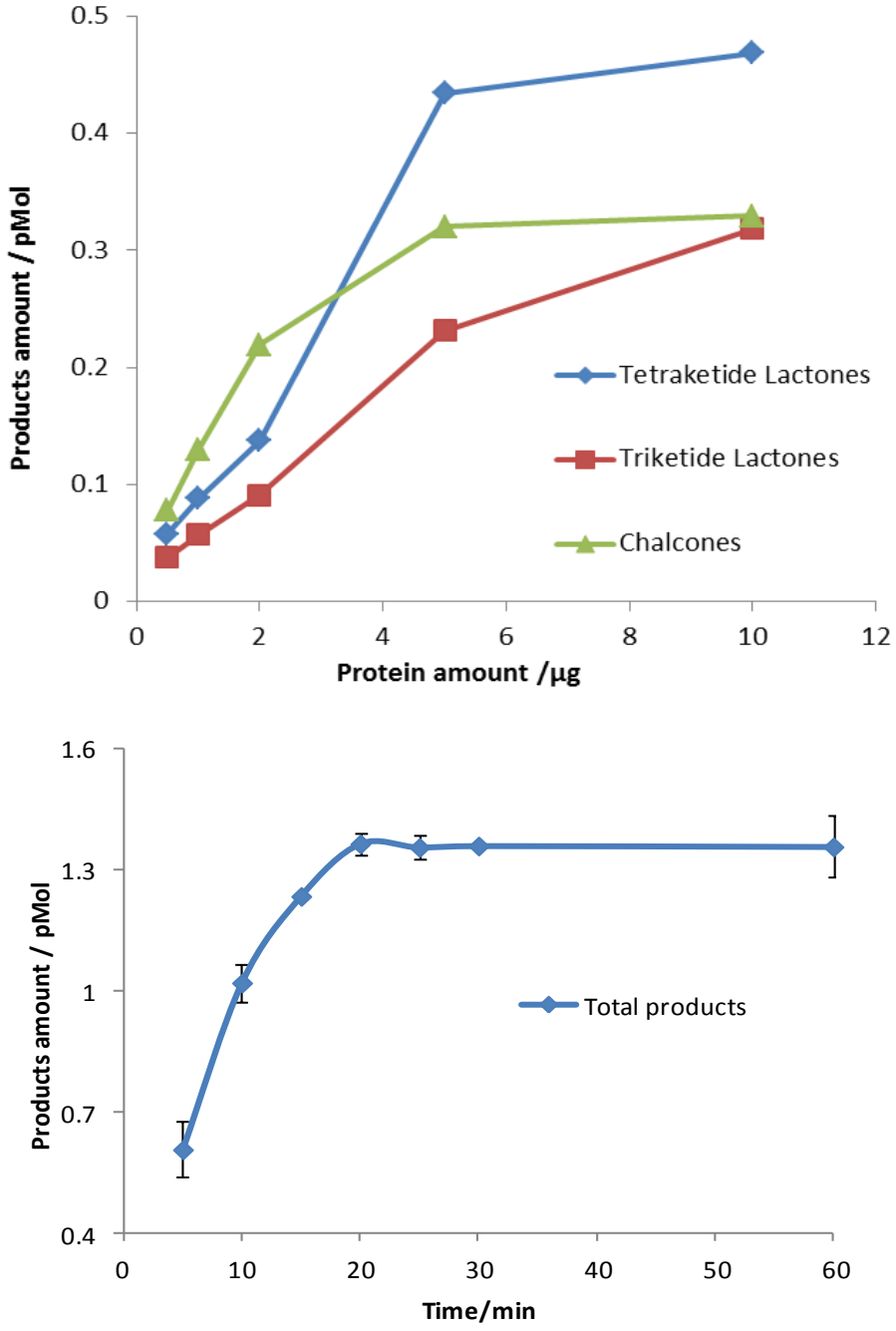
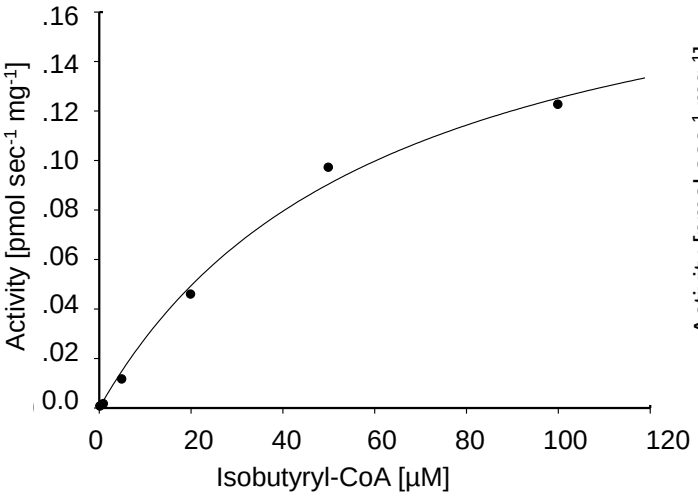
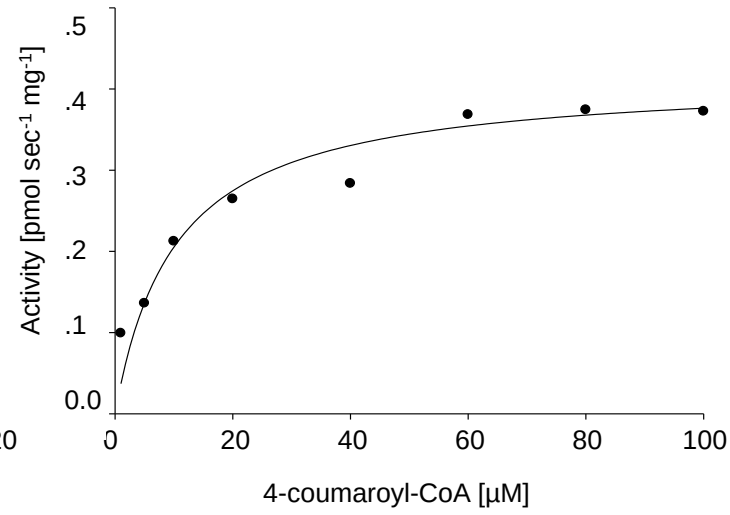
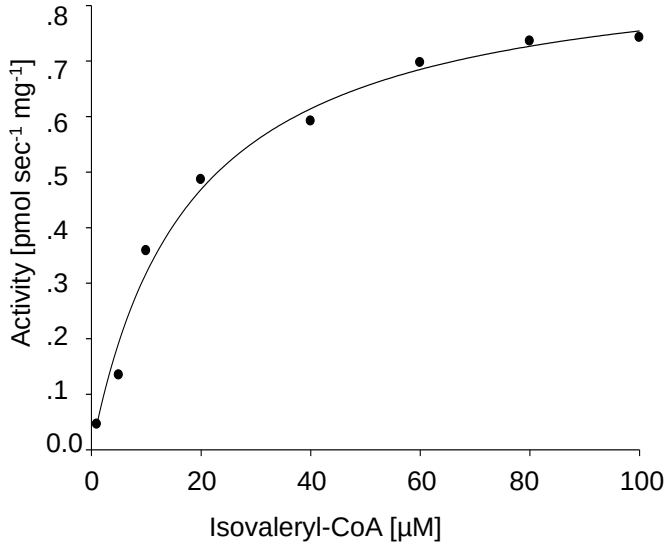
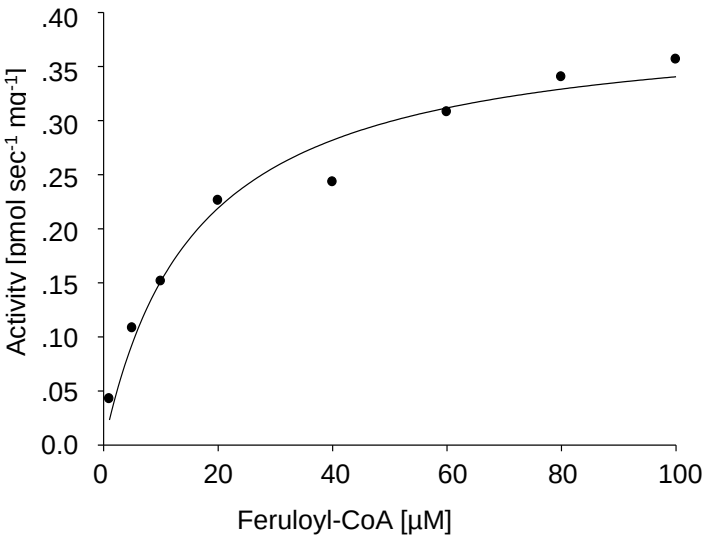



**Figure S14.** The effect of different amounts of protein (0.5-10 µg) and incubation time (5 – 60 min) on the product formation of FvCHS2-3 using cinnamoyl-CoA as the starter substrate. Naringenin (10 µg/ml) was used as an internal standard for quantification by LC-MS.



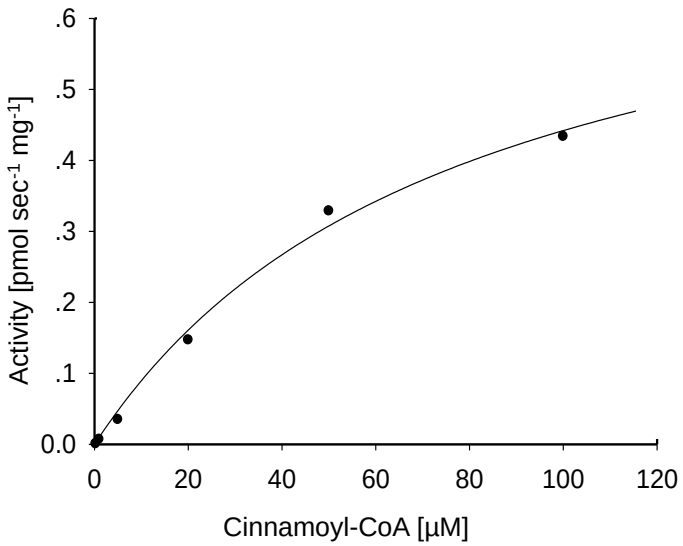




**Figure S15.** Michaelis-Menten plots of purified FvCHS2-3 with the substrate isobutyryl-CoA, 4-coumaroyl-CoA, isovaleryl-CoA, feruloyl-CoA and cinnamoyl-CoA. Activity (pmol $s^{-1}$ $mg^{-1}$) was used to draw the plots and calculate the kinetic parameters.

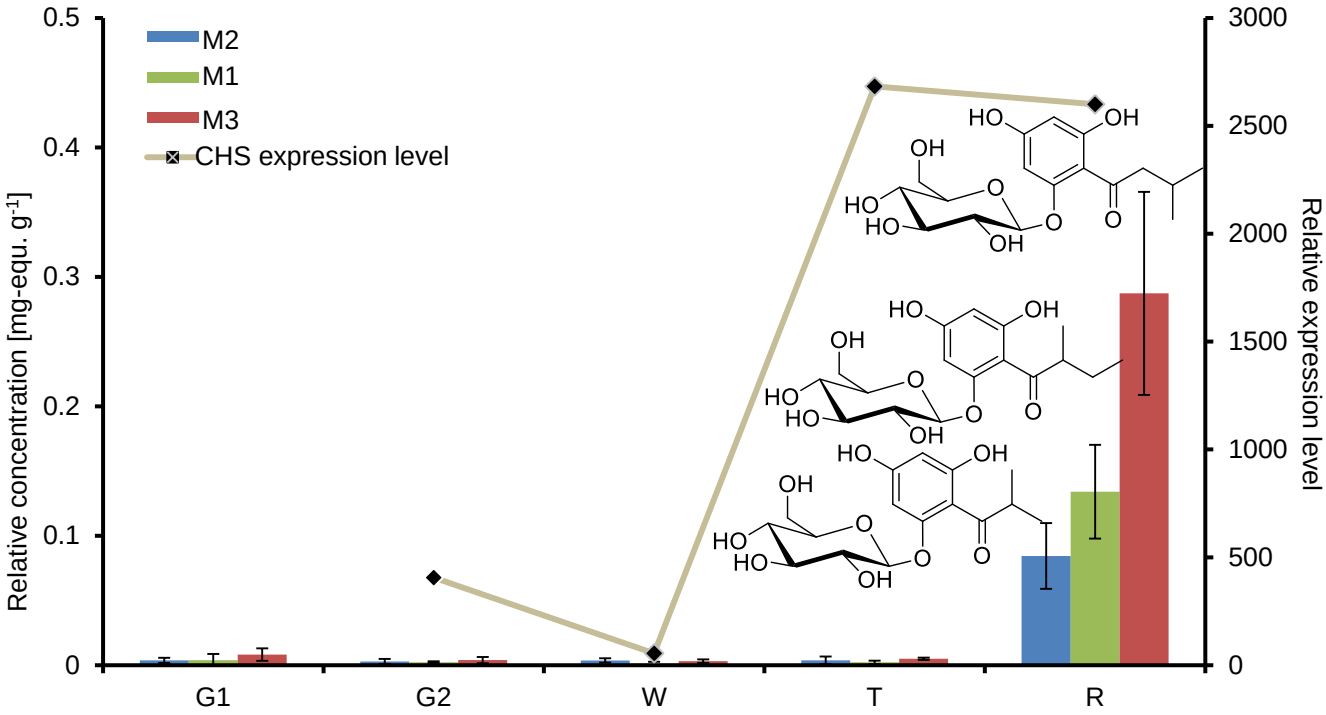



**Figure S16.** Relative content of M1, M2 and M3 measured by LC-MS (n=3-5) and relative *CHS* expression (gene 26825 and gene 26826) determined by quantitative PCR during strawberry fruit ripening (small green G1, large green G2, white W, turning T, red R of *F. x ananassa* cv. Mara des Bois).

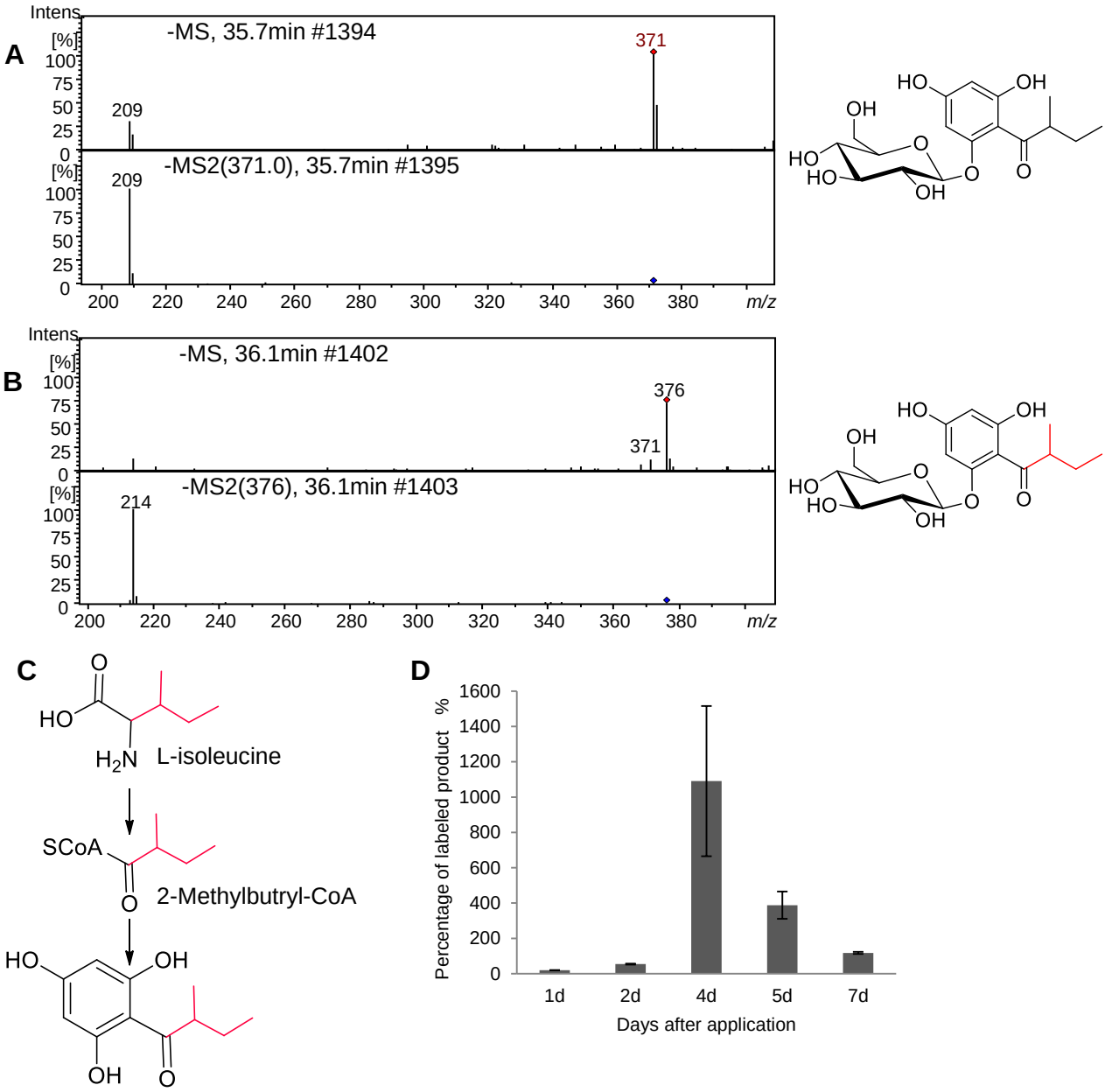



**Figure S17.** Mass spectrum (MS) and MS2 of unlabeled M1 (**A**) and isotopically labeled M1 (**B**) after application of L-isoleucine-$^{13}C_6$, proposed pathway (**C**), as well as the effect of the incubation period on the accumulation of labeled product (**D**) (percentage of labeled product was calculated from the integrated peak area of the pseudomolecular ion of isotopically labeled product *m/z* 376; integrated peak area of pseudomolecular ion *m/z* 371 of unlabeled product was set to 100%).

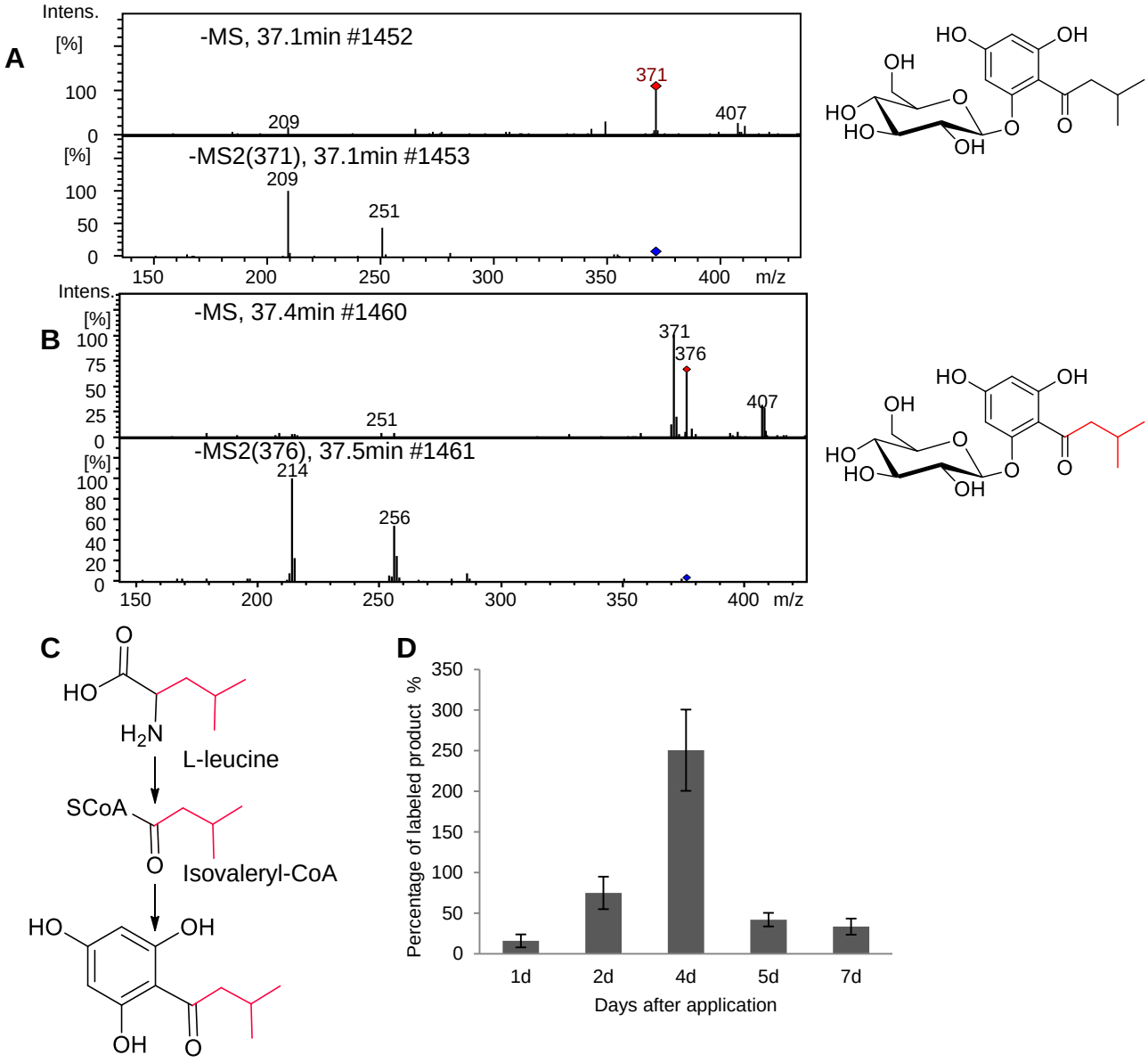



**Figure S18.** Mass spectrum (MS) and MS2 of unlabeled M3 (**A**) and isotopically labeled M3 (**B**) after application of L-leucine-$^{13}C_6$, proposed pathway (**C**), as well as the effect of the incubation period on the accumulation of labeled product (**D**) (percentage of labeled product was calculated from the integrated peak area of the pseudomolecular ion of isotopically labeled product *m/z* 376; integrated peak area of pseudomolecular ion *m/z* 371 of unlabeled product was set to 100%).

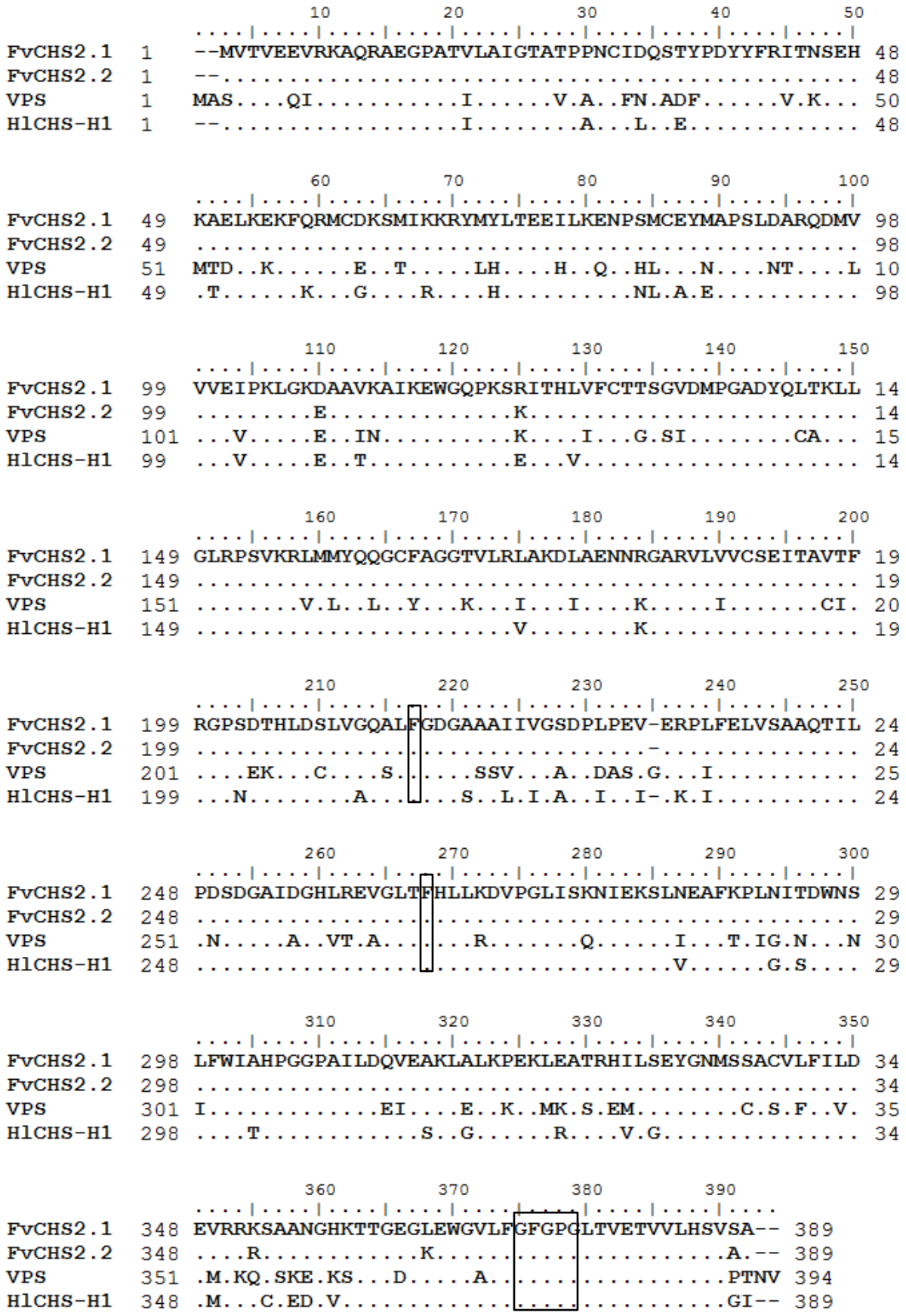



**Figure S19.** Comparison of the deduced amino acid sequences of CHS enzymes from *F. vesca*, FvCHS2-1 and FvCHS2-2, as well as VPS and HICHSH1 from hop. The sequences were aligned using the ClustalW program. The highly conserved active site loop of CHS enzymes, G372FGPG and two Phe residues (Phe215 and Phe265), important in determining the substrate specificity of CHS are boxed.