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## **Investigating herbicide targets in the very long-chain fatty acid biosynthetic pathway**

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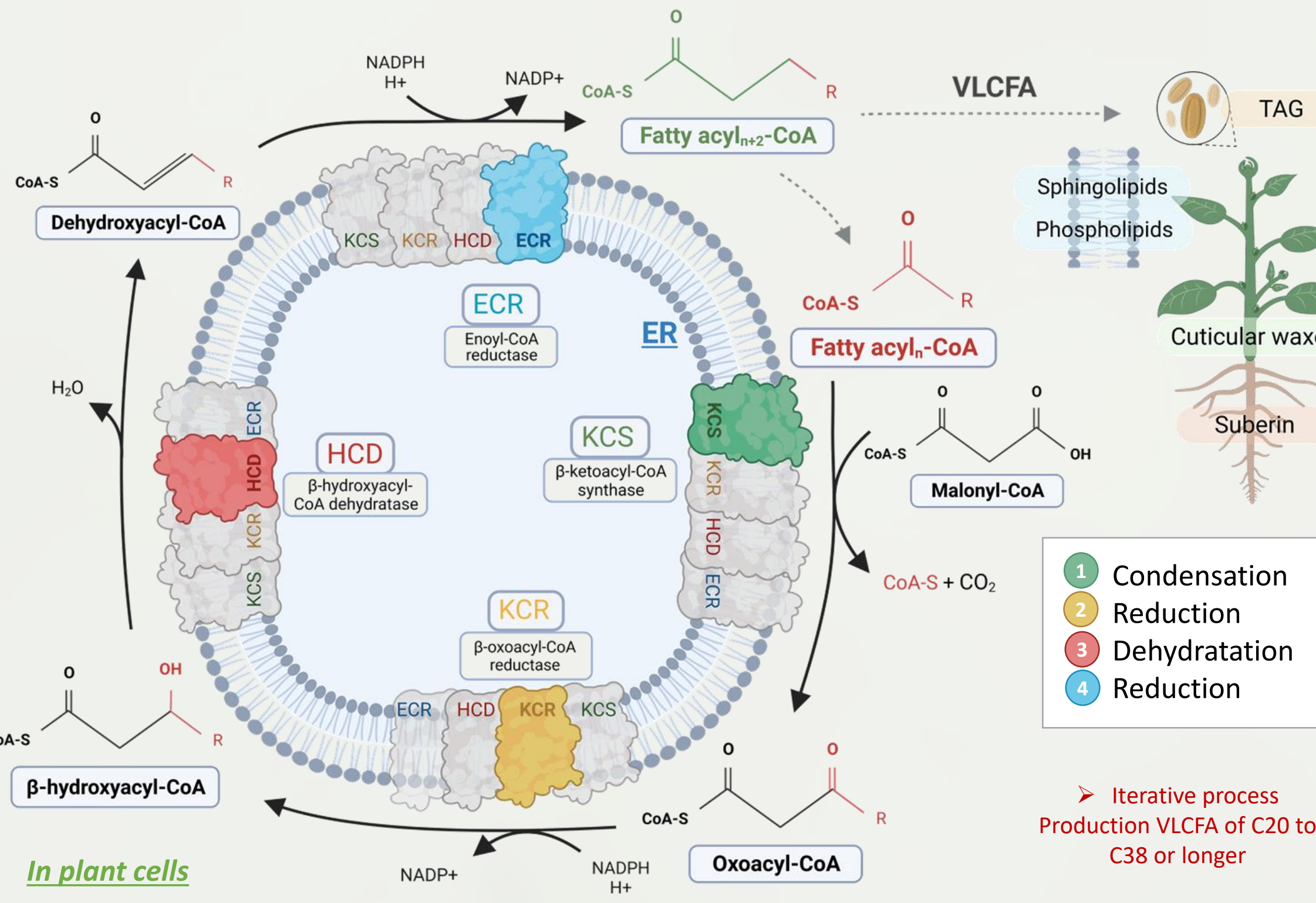
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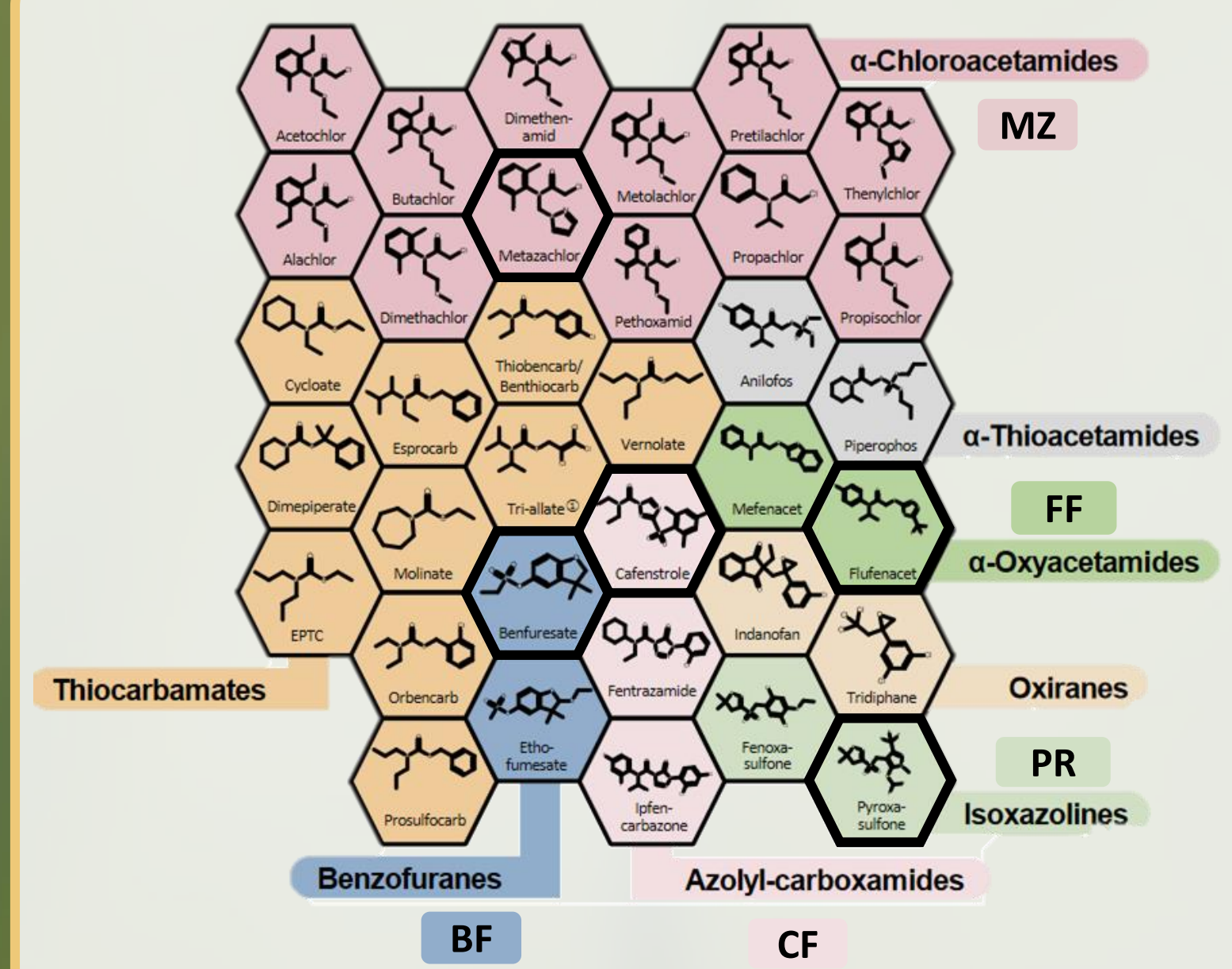
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## 1 Introduction



Very-long-chain fatty acids (fatty acids with more than 18 carbons) have crucial physiological and structural roles in plants. VLCFA are produced by the fatty acid elongase (FAE complex) which is an integral ER membrane enzymatic complex with multiple components. If the biochemical mechanism of elongation is now well characterized, little is known about the specific proteins involved in this process. Especially biochemical and genetic studies in *Arabidopsis* led to the idea that multiple elongase complexes with distinct chain-length specificities perform sequential and parallel reactions to produce the wide range of VLCFA found in plants. Using CRISPR-CAS9 technology, we established an expression platform to reconstitute the different *Arabidopsis* FAE complexes in yeast and characterize the substrate specificity of the 10 *Arabidopsis* KCS candidates. Herbicides inhibiting the synthesis of VLCFA (HRAC group K3), such as flufenacet, pyroxasulfone or metazachlor, play an important role in weed management strategies, especially in cereals. The mode of action of these molecules was first identified based on the typical phenotype observed in treated plants suggesting an inhibition of VLCFA synthesis (Trenkamp *et al.*, 2004). To better understand the mechanism responsible for the selective action of K3 herbicides within the plant kingdom, in particular between dicotyledonous and monocotyledonous plants, we expressed and characterized KCS from *A. thaliana*, *Zea mays*, *Setaria viridis*, *Brassica Napus*, *Raphidocelis subcapitata* in *Saccharomyces cerevisiae* and examined the VLCFAs profiles of plant elongase expressing strains in the presence of various herbicides.

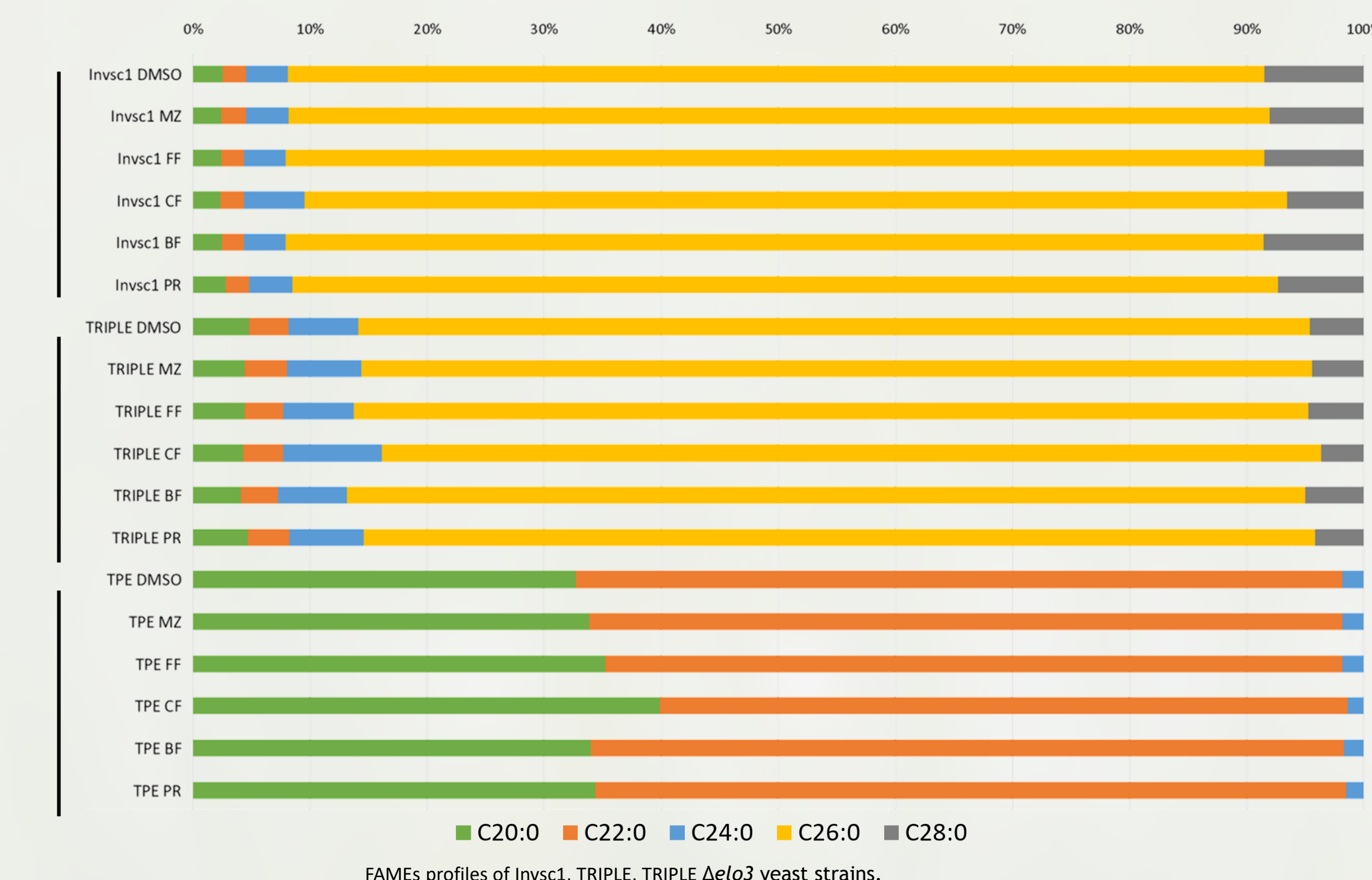
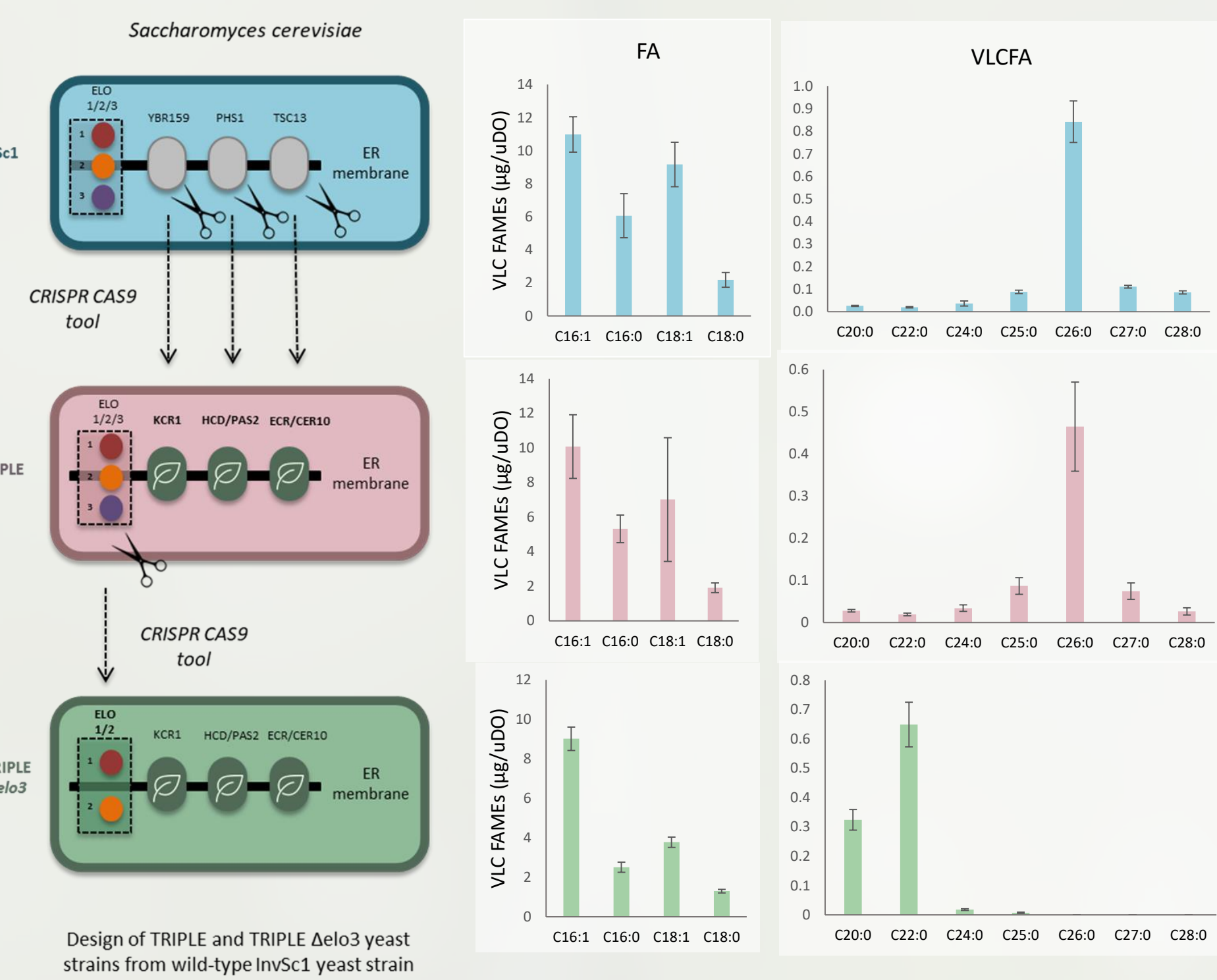
## Inhibitors of VLCFA synthesis



Structures of K3 herbicides (Herbicide Resistance Action Committee (HRAC) Mode of Action Classification 2022). Herbicides circled in bold have been tested here as potential VLCFA inhibitors in plant elongase-expressing yeast strains.

## 2 Context

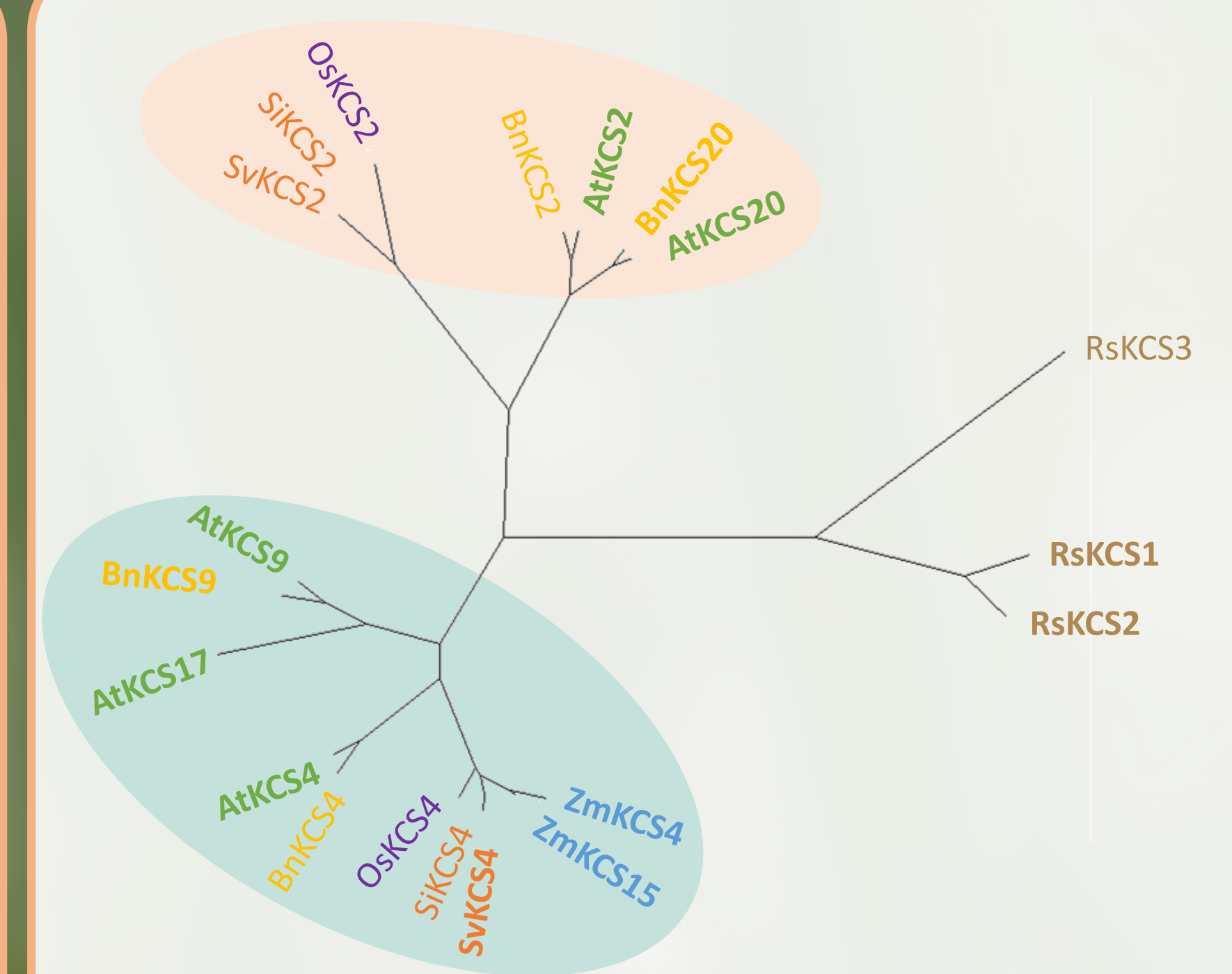
### Yeast engineering - herbicides specifically inhibit the *Arabidopsis* KCS enzymes



CRISPR Cas9 technology was used to replace the yeast last three enzymes involved in fatty acid elongation (YBR159, PH51 and TCS13) by their *Arabidopsis* orthologues (KCR, HCD/PAS2 and ECR/CER10) and to delete the gene of the elongase ELO3 which elongates C22:0 to C24:0 and C26:0.

FAMES profiles of Invsc1, TRIPLE and TRIPLE  $\Delta$ elo3 (TPE) collections treated with different herbicides (10  $\mu$ M). Values are the average of five replicates. "DMSO" corresponds to the negative control treatment.  
➤ Herbicides do not modify the FAMES profiles on Invsc1, TRIPLE and TRIPLE  $\Delta$ elo3 (TPE) collections.  
➤ Herbicides do not inhibit the activity of *Arabidopsis* KCR, HCD and ECR enzymes.

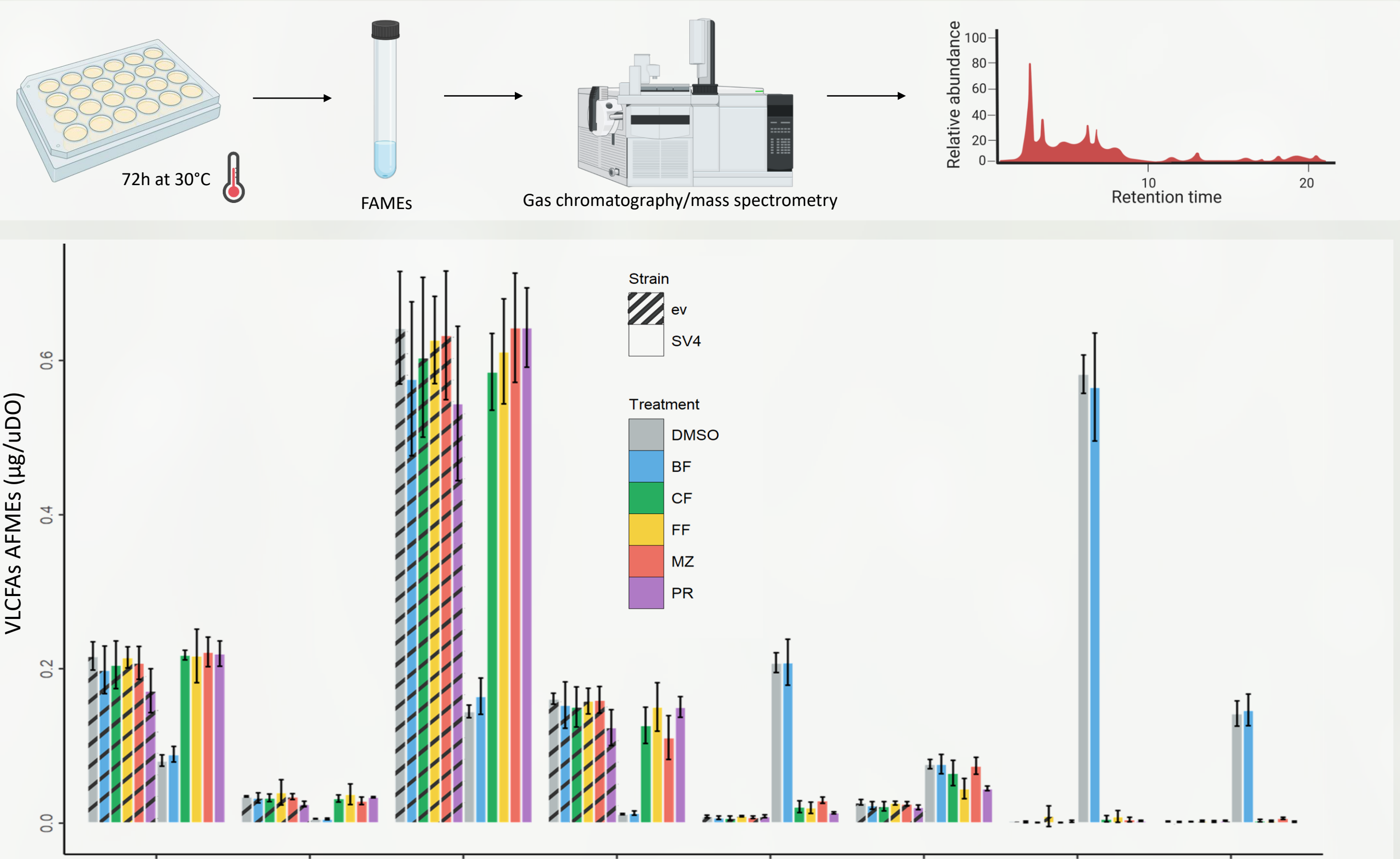
### AtKCS4 orthologs expressed in *S. cerevisiae*



Phylogenetic analysis of the KCS sequences. The protein sequences were compared using the multiple alignment program MUSCLE. The MEGA package was used for the construction of the phylogenetic tree and comparison of parsimony and distance (Neighbor Joining algorithm) methods. The robustness of the tree was assessed by the bootstrap method on 1,000 replicates. Genes in bold are functional in *S. cerevisiae*.

## 3 Results

### in vivo : KCS elongases are inhibited by a set of herbicides



FAMES profiles of SvKCS4 in TRIPLE  $\Delta$ elo3 (TPE) strain treated with different herbicides. Relative quantification based on the internal standard C17:0 (20  $\mu$ g). Values are the average of five replicates. "EV" corresponds to the negative control strain containing the empty vector and "DMSO" corresponds to the negative control treatment.

Treatment	TPE SvKCS4	TPE ZmKCS4	TPE ZmKCS15	TPE BnKCS20	TPE BnKCS9	TPE RsKCS2	TPE RsKCS1
BF 10 $\mu$ M	15 15 15 14 14	-6 27 -16 -17 -18	2 -7 -7 -5 -3	0 -3 -2 -19 -72	4 5 -1 5 14	8 13 11 12 10	2 5 5 9 10
FF 10 $\mu$ M	61 330 542 -85 -100	56 240 442 -94 -99	34 182 371 -92 -100	61 2 -31 -59 -57	14 242 632 -97 -97	78 -45 -56 -98 -96	21 -67 -34 -82 -100
MZ 10 $\mu$ M	67 315 586 -82 -100	26 181 645 -91 -100	27 212 489 -83 -100	31 -33 -58 -55 9	14 212 624 -97 -99	62 -49 -36 -84 -99	14 -58 -31 -81 -100
PR 10 $\mu$ M	79 411 750 -91 -100	27 188 670 -95 -100	35 251 608 -88 -99	44 -23 -48 -62 45	43 296 797 -97 -99	215 -7 -23 -98 -97	10 -70 -34 -80 -100
CF 10 $\mu$ M	42 226 404 -84 -100	52 176 398 -92 -99	57 220 451 -86 -99	67 0 -34 -53 -15	28 230 601 -96 -97	138 -12 -40 -98 -85	38 -60 -16 -77 -99

Treatment	TPE AtKCS2	TPE AtKCS4	TPE AtKCS5	TPE AtKCS6	TPE AtKCS9	TPE AtKCS17	TPE AtKCS20
BF 10 $\mu$ M	-3 9 14 41 20	4 6 6 3 7	7 14 4 57 111	0 -13 -4 0 24	7 -20 -49 39 51	-5 -6 -9 -2 32	-11 -11 -6 -4 -22
FF 10 $\mu$ M	48 12 -59 -90 -10	58 189 626 -94 -100	42 52 254 -72 -98	-8 -8 172 -94 -96	46 191 301 -82 -88	22 63 220 -97 -69	132 80 -39 -96 11
MZ 10 $\mu$ M	49 11 -56 -87 -22	49 161 503 -89 -100	28 35 246 -56 -95	-7 4 256 -87 -95	17 149 241 -92 -100	-4 28 138 -96 -83	120 83 -36 -95 -46
PR 10 $\mu$ M	40 2 -61 -93 10	79 267 851 -92 -100	30 35 241 -76 -98	10 17 258 -93 -96	41 209 357 -96 -100	23 60 230 -97 -65	124 75 -45 -96 -61
CF 10 $\mu$ M	58 28 -52 -91 -66	44 161 597 -95 -100	61 99 377 -70 -97	25 59 424 -89 -100	30 190 276 -92 -92	39 95 280 -96 -100	134 66 -44 -96 -43

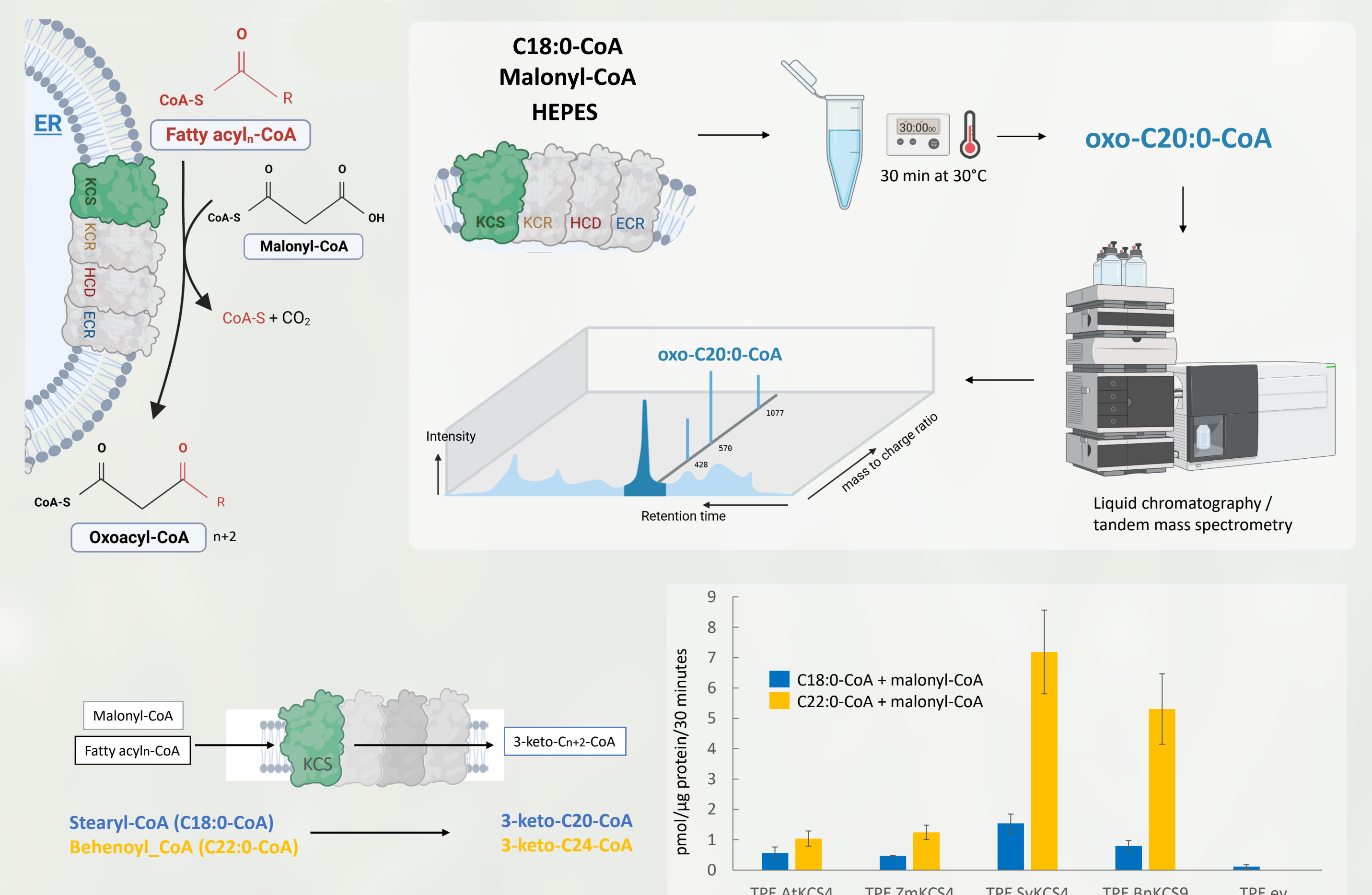
Treatment	TRIPLE AtKCS01	TRIPLE AtKCS18	TRIPLE OsKCS
BF 10 $\mu$ M	-6 3 8 -2 -17	-33 -31 -14 -33 -24	-7 -7 -8 -12 -18
FF 10 $\mu$ M	-44 -63 -66 -66 -10	-26 -85 -64 -64 -14	51 -44 -88 -85 -74
MZ 10 $\mu$ M	-30 -58 -53 -49 10	-18 -76 -68 -51 -15	31 -70 -91 -89 -81
PR 10 $\mu$ M	-38 -71 -74 -68 4	-10 -81 -69 -68 -22	-68 -92 -89 -78
CF 10 $\mu$ M	-31 -62 -70 -71 -3	-14 -84 -69 -74 -9	28 -55 -89 -90 -81

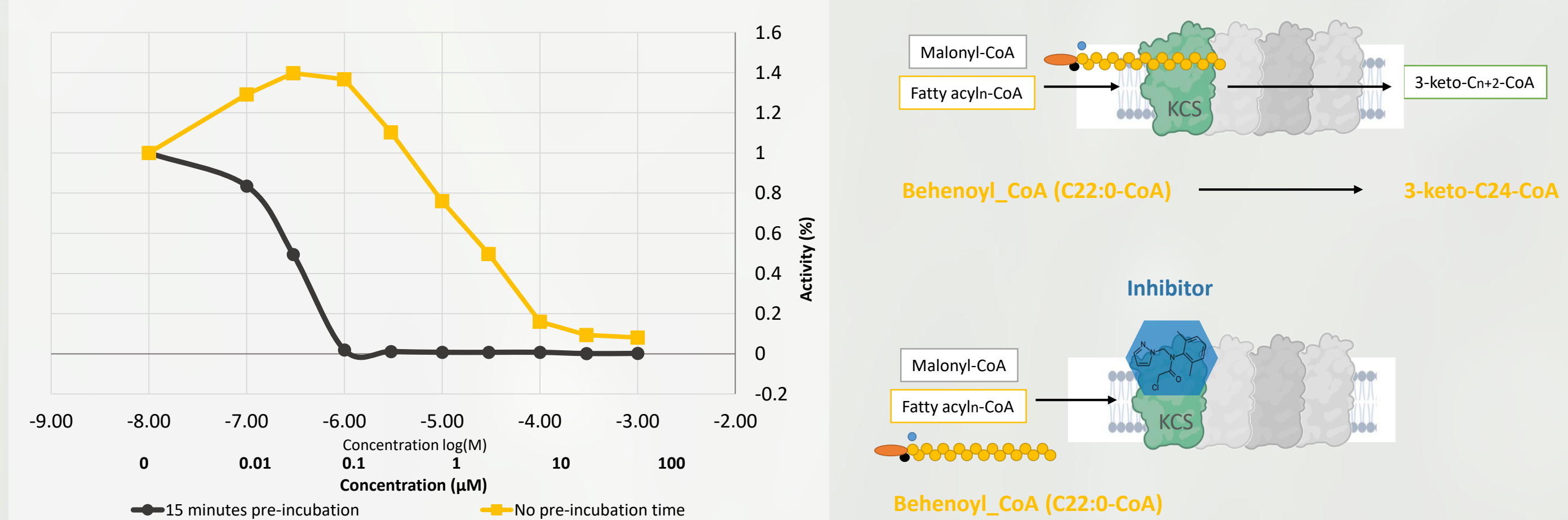
Treatment	C18	C20	C22	C24	C26	% accumulation
BF 10 $\mu$ M						100
FF 10 $\mu$ M						100
MZ 10 $\mu$ M						100
PR 10 $\mu$ M						100
CF 10 $\mu$ M						100

Percentage of fatty acids in *S. cerevisiae* expressing different KCS elongases in function of group K3 herbicides treatment (10  $\mu$ M) compared to control treatment (DMSO). Results of four replicates. TRIPLE  $\Delta$ elo3 (TPE), benfuresate (BF), flufenacet (FF), metazachlor (MZ), pyroxasulfone (PR), cafenstrole (CF).

### in vitro : SvKCS4 is inhibited by the herbicide flufenacet



Substrate specificity assayed with C18:0-CoA or C22:0-CoA for equal amounts of yeast microsomal fraction expressing AtKCS4-like group. Condensation reaction were performed as described above. Oxo-acyl-CoA product were quantified in LC-MS/MS. Values are the average of three replicates. "EV" corresponds to the negative control strain containing the empty vector.



Inhibition by flufenacet on elongation of behenoyl-CoA (C22:0-CoA) by KCS04-like from *Setaria viridis*. Pre-incubation-time (15 minutes) between inhibitor and yeast microsomal fraction.

➤ Irreversible inhibition