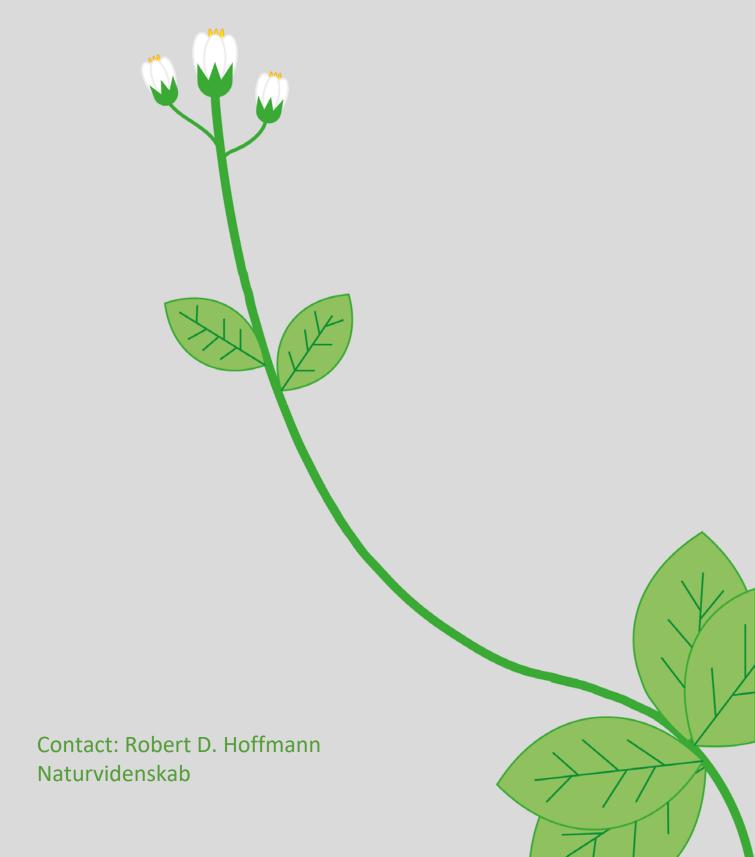
# VITAMIN D3 IN ARABIDOPSIS THALIANA

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## 1 Introduction

NASA has plans for inhabiting Mars. With the prospect of NASA sending humans to Mars in the 2030's, this future moves closer and closer. An important part of this project is supplying the astronauts with a nutritious diet and this includes vitamins. NASA will supply their astronauts through dietary supplements, which require energy to produce compared with a product that could grow on Mars [5]. Therefore, a genetically modified plant that produced the sufficient amount of vitamin D<sub>3</sub> without using up scarce resources would be beneficial. The plant *Arabidopsis thaliana* is an excellent choice because of its well-known genome, its ability to self-pollinate, and its relatively short growth cycle at five to eight weeks [18].

When in space, astronauts will not get enough sunlight to produce the necessary amount of vitamin  $D_3$ . The major role of vitamin  $D_3$  in humans is maintaining calcium homeostasis. It is therefore very important for the development of a healthy skeleton [12]. A lack of vitamin D has been shown to lead to increased risk of hypertension, autoimmune diseases, diabetes and cancer [9]. Dietary supplements are therefore needed to ensure a healthy skeleton.

Vitamin  $D_3$  is synthesised in our skin from the precursor 7-dehydrocholesterol, also known as provitamin  $D_3$ . The photosynthesis of previtamin  $D_3$  from provitamin  $D_3$  takes place in the dermis with the use of solar ultraviolet light at wavelengths between 295-300 nm [11][16]. This undergoes thermal isomerization to become vitamin  $D_3$  and is then - by a vitamin  $D_3$  binding protein - transported to the circulatory system [10].

#### 1.1 Aims

This project could eventually supply permanent inhabitants on Mars with an easy supply of vitamin D<sub>3</sub>. My goal is to utilize plants' ability to build complex organic molecules from water and carbon dioxide, and to gain information on how this could be used in the space industry.

# 2 Background

## 2.1 Synthesis in humans to insert into A. thaliana

The precursor for most human sterols is lanosterol and the pathway from lanosterol to vitamin D<sub>3</sub> has seven steps and is done by nine different enzymes. The synthesis from lanosterol to

provitamin  $D_3$  involves the removal of three methyl groups, the migration of a double bond and the reduction of two double bonds [20]. Afterwards, UVB light and a thermal isomerization changes it to vitamin  $D_3$  [xyxz]. This pathway can be seen in A1. There can be variations in the pathway, as the reduction of carbon-24 can happen between any of the other steps [27]. Vitamin  $D_3$  is biologically inactive and must undergo two hydroxylations before it becomes active. The first is in the liver, where it is hydroxylated into 25OHD<sub>3</sub>, which is the most common form found in the circulatory system in vertebrates. Then it is hydroxylated in the kidneys to  $1\alpha,25(OH)_2D_3$  [12].

#### 2.2 Synthesis of 7-dehydrocholesterol in *A. thaliana*

Higher plants do not synthesise 7-dehydrocholesterol. Sterol synthesis in higher plants start with cycloartenol while in animals and fungi, it starts with lanosterol [4]. To make use of the human pathway in *A. thaliana* and produce 7-dehydrocholesterol, we must first mutate it so it can produce lanosterol. A point mutation in the gene oxidosqualene-cycloartenol synthase (*CAS1*) on chromosome 2 changes the production from 100% cycloartenol to 24% lanosterol, 20% parkeol and 56% cycloartenol. The mutation is called I481V and will change the 481th amino acid from valine to isoleucine [28]. The sequence for valine is ATC and the most common for isoleucine in *A. thaliana* is GTT [19]. This is the mutation we will use.

## 3 Methods

#### 3.1 PCR Mutagenesis to create lanosterol

First DNA must be extracted from the plant. To do this, the cell walls must be broken, then the cell membrane must be disrupted. Afterwards, the DNA must be separated from other tissue and proteins. The procedure from [22] will be followed for this. Then *CAS1* (Arabidopsis gene identifier AT2G07050) will be amplified using PCR. For this, we need nucleotides, DNA polymerase and primers. The primers used will be: 5'atgtggaaac tgaagatcgc'3 and 5'tcattctcct tgttgcaata ata'3. This is based on the gene sequence seen in A2 [25].

When the gene has been amplified it is ligated into the directional pENTR vector and in a second step transferred into the vector pMDC32 by LR clonase (Invitrogen). To introduce the mutation site-directed PCR mutagenesis will be used. The primers 5'ca cggttggccc gtttctgact gcac'3 and

5'gtgc agtcagaac gggccaaccg tg'3 will mutate valine to isoleucine [14]. The vector contains the strong constitutive promoter CaMV35S which works well in eudicots [6], the kanamycin resistance gene for selection in *E. coli* and *A. tumefaciens*, and the hygromycin resistance gene for selection in Arabidopsis.

The vector is then inserted into the *E. coli* TOP 10 strain by heat-shock transformation. If this method does not work as this vector is over 10Kb, the electropolation transformation method will be used [1]. Then transformed bacteria are selected on agar plates containing ampicillin as a selection marker. On the next day, 10 positive colonies will be re-streaked on fresh selective plates and grown over night. The plasmids are then extracted using a mini-prep kit. To control that the vectors are correct, they will be sent to sequencing.

To transform the plants, we will first insert the vectors by electropolation into *Agrobacterium tumefaciens* strain GV3101::pMP90 [13][7]. These are selected with the use of kanamycin. Flowering *A. thaliana* plants are dipped into a solution of *A. tumefaciens* according to the protocol described in [3]. This will make about 1% of the plants transformed [3]. These are selected with hygromycin and the F1 generation can be screened for being homozygous for the T-DNA insert.

This method has been tested before as cited earlier and should therefore work if executed correctly. This method has many steps, and if anything is done wrong at any point in the procedure it will take longer to complete, especially if something goes wrong in *A. thaliana*.

#### 3.2 Pathway insertion

A. thaliana already possesses a C4-demethylation complex [17][21] and can therefore omit to insert the genes for the enzymes involved in this process. The genes will be inserted into a vector as in 3.1, only this time there will need to be inserted around 1200 nucleotides per enzyme. There are six enzymes in total, and these can be viewed in A4.

There are many factors where this project can run into problems. First, the genes might not be transcribed. This is avoided by using a strong constitutive promoter. Second, they might not get translated into a protein. Translation is the subject of much research, but is such a complex system that it is not entirely understood yet. It is thought that micro-RNA's play a big part in the role of translation because they break down a lot of mRNA [26]. Third, the enzymes' place in the cell is often given by the ER and this might lead to the enzymes getting either broken down or not being

in the same area. Lastly, the product vitamin  $D_3$  might get broken down by the cell. This is however not a big problem as CAS1 resides in the vacuole where the point mutation will be [15]. As the vacuole is the storage area in the cell this might mean the cell will not pay attention to unneeded products.

The method described in 3.1 will be used to complete this part of the project as well.

## 3.3 Analytical methods

Vitamin D<sub>3</sub> is the first product to analyse. This will be done by a mix of high performance liquid chromatography (HPLC) at 265 nm which is one of the more used analytical methods [12][2], mass spectrophotometry (MS), and nuclear magnetic resonance (NMR). NMR lacks the sensitivity of MS and HPLC, but is a valuable tool for structure elucidation [12]. The procedures that will be followed are [24] for HPLC, and [8] for MS.

If vitamin  $D_3$  cannot be found in larger quantities than normal [12] then this analysis will be done on the other products as well. If no products can be found the activity of the inserted genes will be examined with a northern blot made according to this protocol [23].

## 4 Plan

#### 4.1 Stage one

Month	Week	Day	Tasks
December			Order the needed gene sequences, vectors, and organisms, and
			start growing a 1.000 A. thaliana.
January	1		Prepare E. coli.
	2	Mon	Extract and purify PCR product.
		Tue	Insert gene into vector, make point mutation and insert into E.
			coli.
		Wed	Extract vectors and send to sequencing.
		Fri	Insert vector into A. tumefaciens.
	3	Mon	Floral dip
	4		Select transformed A. thaliana (around 10) and pollinate.

February	ry Plant a 1.000 seeds	
March		Grow A. thaliana
April		Select A. thaliana (if selected correctly before all of them)

## 4.2 Stage two

Month	Week	Day	Tasks
Мау			Order the needed gene sequences, vectors, and organisms, and
			start growing a 1.000 A. thaliana.
June	1		Prepare E. coli.
	2	Mon	Extract and purify PCR product.
		Tue	Insert gene into vector and insert into E. coli.
		Wed	Extract vectors and send to sequencing.
		Fri	Insert vector into A. tumefaciens.
	3	Mon	Floral dip
	4		Select transformed A. thaliana (around 10) and pollinate.
July			Plant a 1.000 seeds
August			Grow A. thaliana
September			Select A. thaliana (if selected correctly before all of them)

# 4.3 Stage three

As it cannot be predicted whether the occurrence of vitamin  $D_3$  will be present, I cannot predict the timeframe for how long this stage will take. Therefore, I have chosen the pessimistic way of doing things and made this schedule presuming there was never any signs of products or expression.

Month	Week	Day	Tasks
September	1		Prepare samples for analysis.
	2		NMR for all products in succession
	3		HPLC for all products in succession

	4	MS for all products in succession
October	1	Northern Blotting to test for gene expression.

## 4.4 Budget

Materials	Price
Primers	200 kr.
Polymerase and dNTPs for PCR	1000 kr.
A. thaliana, E. coli and A. tumefaciens	Free
Growing plants in climate chamber	600 kr.
Sequencing	3500 kr.
Mini prep kit	1500 kr.
Gateway Cloning Kit	4000 kr.
Use of NMR, HPLC, and MS apparatus'	4000 kr.
General chemicals, media, and	5000 kr.
consumables	
Sum	19.800 kr.

## Conclusion

This project's completion could be able to supply astronauts in space with a producing organism that does not require organic molecules to create them. We might be able to use this thought process to make plants synthesise expensive and time-consuming molecules without damaging their ability to grow. If this works it could make way for a new way to synthesise not only dietary supplements, but other molecules as well. Even a step on the way to the utilization of plants in this way would be a success. Maybe it could be synthesised so eating one *A. thaliana* plant would provide you with half your daily vitamin intake. This is of course still future speculation, but bacteria and fungi are being used very competently. I believe we are closer to using plants in this way as well.

Acknowledgements

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# A Attachments

# A1 Vitamin D<sub>3</sub> pathway

A1 shows the Kandutsch-Russell pathway. [20] This is the pathway inserted into A. thaliana to convert lanosterol to provitamin  $D_3$ .

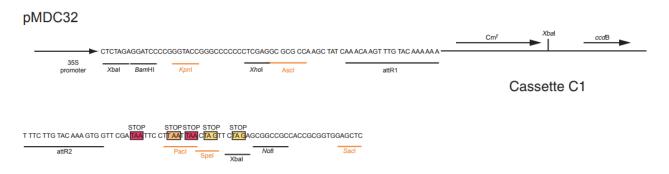
# A2 CAS1 gene

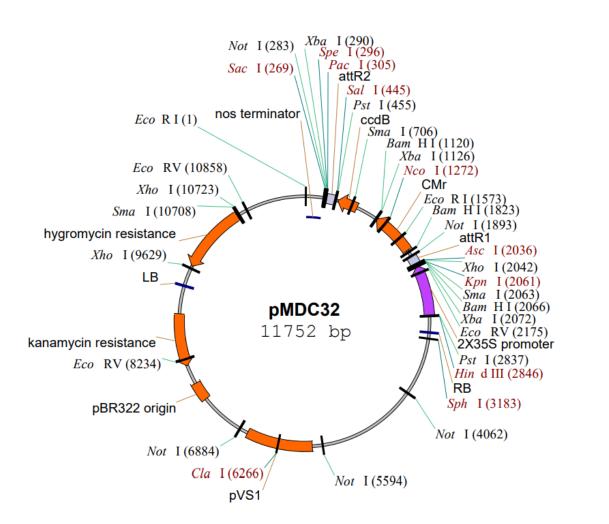
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gcacattcat ttgtcaagaa ttcccaggtg ttagaagact gccctggaga tctgaattac tggtatcgcc acatttctaa aggggcttgg cctttctcaa ctgcagatca cggttggccc atctctgact gcaccgcaga aggactgaaa gctgctcttt tgctatccaa agttcccaag gcgattgttg gtgaaccaat agatgcaaaa cggttatatg aagctgtaa tgttatcatt tctttacaga atgcagatgg aggcctcgca acatatgagc tcaccaggtc atacccttgg ttagagctaa tcaacccagc agaaaccttt ggcgatattg ttattgatta tccttacgtg gaatgtacat cagctgctat ccaagctttg atatcatttc gaaagctgta tcctggtcat cgaaagaagg aagtagatga gtgcattgag aaggcggtta agttcattga atccattcaa gcagcagatg gctcatggta tggatcatgg gctgtttgct tcacgtatgg tacgtggtt ggagtgaaag ggctggtagc tgttggaaaa acattgaaaa actctccaca tgttgctaaa gcttgtgaat ttctattgtc gaaaccacaa ccttcgggcg gctgggaga aagctatctt	gctgaagatg	gaatgaagat	gcagggttat	aacggaagcc	agctatggga	tacaggtttt	1260
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gcgattgttg gtgaaccaat agatgcaaaa cggttatatg aagctgttaa tgttatcatt tctttacaga atgcagatgg aggcctcgca acatatgagc tcaccaggtc ataccettgg ttagagctaa tcaacccagc agaaaccttt ggcgatattg ttattgatta tccttacgtg gaatgtacat cagctgctat ccaagctttg atatcatttc gaaagctgta tcctggtcat cgaaagaagg aagtagatga gtgcattgag aaggcggtta agttcattga atccattcaa gcagcagatg gctcatggta tggatcatgg gctgtttgct tcacgtatgg tacgtggttt ggagtgaaag ggctggtagc tgttggaaaa acattgaaaa actctccaca tgttgctaaa gcttgtgaat ttctattgtc gaaacaacaa ccttcgggcg gctggggaga aagctatctt	tggtatcgcc	acatttctaa	aggggcttgg	cctttctcaa	ctgcagatca	cggttggccc	1440
tetttacaga atgeagatgg aggeetegea acatatgage teaceaggte ataccettgg ttagagetaa teaaceeage agaaacettt ggegatattg ttattgatta teettaegtg gaatgtacat eagetgetat eeaagetttg atateattte gaaagetgta teetggteat egaaagaagg aagtagatga gtgeattgag aaggeggtta agtteattga ateeatteaa geageagatg geteatggta tggateatgg getgtttget teaegtatgg taegtggttt ggagtgaaag ggetggtage tgttggaaaa acattgaaaa acteteeaca tgttgetaaa gettgtgaat ttetattgte gaaacaacaa eettegggeg getgggaga aagetatett  1	atctctgact	gcaccgcaga	aggactgaaa	gctgctcttt	tgctatccaa	agttcccaag	1500
ttagagctaa tcaacccagc agaaaccttt ggcgatattg ttattgatta tccttacgtg gaatgtacat cagctgctat ccaagctttg atatcatttc gaaagctgta tcctggtcat cgaaagaagg aagtagatga gtgcattgag aaggcggtta agttcattga atccattcaa gcagcagatg gctcatggta tggatcatgg gctgtttgct tcacgtatgg tacgtggttt ggagtgaaag ggctggtagc tgttggaaaa acattgaaaa actctccaca tgttgctaaa gcttgtgaat ttctattgtc gaaacaacaa ccttcgggcg gctggggaga aagctatctt 1	gcgattgttg	gtgaaccaat	agatgcaaaa	cggttatatg	aagctgttaa	tgttatcatt	1560
gaatgtacat cagctgctat ccaagctttg atatcatttc gaaagctgta tcctggtcat cgaaagaagg aagtagatga gtgcattgag aaggcggtta agttcattga atccattcaa gcagcagatg gctcatggta tggatcatgg gctgtttgct tcacgtatgg tacgtggttt ggagtgaaag ggctggtagc tgttggaaaa acattgaaaa actctccaca tgttgctaaa gcttgtgaat ttctattgtc gaaacaacaa ccttcgggcg gctggggaga aagctatctt 1	tctttacaga	atgcagatgg	aggcctcgca	acatatgagc	tcaccaggtc	atacccttgg	1620
cgaaagaagg aagtagatga gtgcattgag aaggcggtta agttcattga atccattcaa 1 gcagcagatg gctcatggta tggatcatgg gctgtttgct tcacgtatgg tacgtggttt 1 ggagtgaaag ggctggtagc tgttggaaaa acattgaaaa actctccaca tgttgctaaa 1 gcttgtgaat ttctattgtc gaaacaacaa ccttcgggcg gctggggaga aagctatctt 1	ttagagctaa	tcaacccagc	agaaaccttt	ggcgatattg	ttattgatta	tccttacgtg	1680
gcagcagatg gctcatggta tggatcatgg gctgtttgct tcacgtatgg tacgtggttt ggagtgaaag ggctggtagc tgttggaaaa acattgaaaa actctccaca tgttgctaaa gcttgtgaat ttctattgtc gaaacaacaa ccttcgggcg gctggggaga aagctatctt 1	gaatgtacat	cagctgctat	ccaagctttg	atatcatttc	gaaagctgta	tcctggtcat	1740
ggagtgaaag ggctggtagc tgttggaaaa acattgaaaa actctccaca tgttgctaaa 1 gcttgtgaat ttctattgtc gaaacaacaa ccttcgggcg gctggggaga aagctatctt 1	cgaaagaagg	aagtagatga	gtgcattgag	aaggcggtta	agttcattga	atccattcaa	1800
gcttgtgaat ttctattgtc gaaacaacaa ccttcgggcg gctggggaga aagctatctt 1	gcagcagatg	gctcatggta	tggatcatgg	gctgtttgct	tcacgtatgg	tacgtggttt	1860
	ggagtgaaag	ggctggtagc	tgttggaaaa	acattgaaaa	actctccaca	tgttgctaaa	1920
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tcatgtcaag acaaggtcta ttcaaacctt gatggcaaca gatctcacgt cgtgaataca 2	tcatgtcaag	acaaggtcta	ttcaaacctt	gatggcaaca	gatctcacgt	cgtgaataca	2040
gcatgggcta tgctcgcact cattggtgct gggcaagctg aggtagaccg gaaaccacta 2	gcatgggcta	tgctcgcact	cattggtgct	gggcaagctg	aggtagaccg	gaaaccacta	2100

caccgggctg	caagatactt	gattaatgct	caaatggaga	atggtgattt	tccacaacag	2160
gaaataatgg	gagtcttcaa	taggaactgc	atgataacat	atgccgcgta	tcgaaacatt	2220
tttccgatat	gggctttggg	ggagtaccgt	tgtcaggtat	tattgcaaca	aggagaatga	2280

A2 shows the mRNA for the *CAS1* gene. The location of the mutation I481V is highlighted with green.

## A3 Vector pMDC32





A3 shows the vector mentioned in 3.1.

A4 Pathway enzyme structure

#### DHCR24:

LEFVLIHQRWVFVCLFLLPLSLIFDIYYYVRAWVVFKLSSAPRLHEQRVRDIQKQVREWKEQGSKTFMCTGRPGW
LTVSLRVGKYKKTHKNIMINLMDILEVDTKKQIVRVEPLVTMGQVTALLTSIGWTLPVLPELDDLTVGGLIMGTGI
ESSSHKYGLFQHICTAYELVLADGSFVRCTPSENSDLFYAVPWSCGTLGFLVAAEIRIIPAKKYVKLRFEPVRGLEAI
CAKFTHESQRQENHFVEGLLYSLDEAVIMTGVMTDEAEPSKLNSIGNYYKPWFFKHVENYLKTNREGLEYIPLRH
YYHRHTRSIFWELQDIIPFGNNPIFRYLFGWMVPPKISLLKLTQGETLRKLYEQHHVVQDMLVPMKCLQQALHT
FQNDIHVYPIWLCPFILPSQPGLVHPKGNEAELYIDIGAYGEPRVKHFEARSCMRQLEKFVRSVHGFQMLYADCY
MNREEFWEMFDGSLYHKLREKLGCQDAFPEVYDKICKAARH

#### **CYP51A1:**

MLLLGLLQAGGSVLGQAMEKVTGGNLLSMLLIACAFTLSLVYLIRLAAGHLVQLPAGVKSPPYIFSPIPFLGHAIAF GKSPIEFLENAYEKYGPVFSFTMVGKTFTYLLGSDAAALLFNSKNEDLNAEDVYSRLTTPVFGKGVAYDVPNPVFL EQKKMLKSGLNIAHFKQHVSIIEKETKEYFESWGESGEKNVFEALSELIILTASHCLHGKEIRSQLNEKVAQLYADL DGGFSHAAWLLPGWLPLPSFRRRDRAHREIKDIFYKAIQKRRQSQEKIDDILQTLLDATYKDGRPLTDDEVAGML IGLLLAGQHTSSTTSAWMGFFLARDKTLQKKCYLEQKTVCGENLPPLTYDQLKDLNLLDRCIKETLRLRPPIMIM MRMARTPQTVAGYTIPPGHQVCVSPTVNQRLKDSWVERLDFNPDRYLQDNPASGEKFAYVPFGAGRHRCIGE NFAYVQIKTIWSTMLRLYEFDLIDGYFPTVNYTTMIHTPENPVIRYKRRSK

#### DHCR14:

MAPTQGPRAPLEFGGPLGAAALLLLLPATMFHLLLAARSGPARLLGPPASLPGLEVLWSPRALLLWLAWLGLQA ALYLLPARKVAEGQELKDKSRLRYPINGFQALVLTALLVGLGMSAGLPLGALPEMLLPLAFVATLTAFIFSLFLYMK AQVAPVSALAPGGNSGNPIYDFFLGRELNPRICFFDFKYFCELRPGLIGWVLINLALLMKEAELRGSPSLAMWLV NGFQLLYVGDALWHEEAVLTTMDITHDGFGFMLAFGDMAWVPFTYSLQAQFLLHHPQPLGLPMASVICLINA TGYYIFRGANSQKNTFRKNPSDPRVAGLETISTATGRKLLVSGWWGMVRHPNYLGDLIMALAWSLPCGVSHLL PYFYLLYFTALLVHREARDERQCLQKYGLAWQEYCRRVPYRIMPYIY

#### $\Delta$ **7**, $\Delta$ **8**-isomerase:

MTTNAGPLHPYWPQHLRLDNFVPNDRPTWHILAGLFSVTGVLVVTTWLLSGRAAVVPLGTWRRLSLCWFAVC GFIHLVIEGWFVLYYEDLLGDQAFLSQLWKEYAKGDSRYILGDNFTVCMETITACLWGPLSLWVVIAFLRQHPLR

FILQLVVSVGQIYGDVLYFLTEHRDGFQHGELGHPLYFWFYFVFMNALWLVLPGVLVLDAVKHLTHAQSTLDAK ATKAKSKKN

#### 5-desaturase:

MDLVLRVADYYFFTPYVYPATWPEDDIFRQAISLLIVTNVGAYILYFFCATLSYYFVFDHALMKHPQFLKNQVRREI KFTVQALPWISILTVALFLLEIRGYSKLHDDLGEFPYGLFELVVSIISFLFFTDMFIYWIHRGLHHRLVYKRLHKPHHI WKIPTPFASHAFHPIDGFLQSLPYHIYPFIFPLHKVVYLSLYILVNIWTISIHDGDFRVPQILQPFINGSAHHTDHHM FFDYNYGQYFTLWDRIGGSFKNPSSFEGKGPLSYVKEMTEGKRSSHSGNGCKNEKLFNGEFTKTE