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Expression and role of CYP505A1 in pathogenicity of *Fusarium oxysporum* f. sp. *lactucae*

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Abstract

Background

Cytochrome P450 enzymes (CYPs) are monooxygenases present in every domain of life. In fungi CYPs are involved in virulence. Fusarium wilt of lettuce, caused by *Fusarium oxysporum* f. sp. *lactucae*, is the most serious disease of lettuce. *Fusarium oxysporum* f.sp. *lactucae* MSA35 is an antagonistic fungus. Pathogenic *formae specialis* of *F. oxysporum* possess a CYP belonging to the new family CYP505. This enzyme hydroxylates saturated fatty acids that play a role in plant defence.

Methods

Molecular tools were adopted to search for CYP505 gene in MSA35 genome. *cyp505* gene expression analysis in pathogenic and antagonistic *Fusarium* was performed. The enzyme was expressed in its recombinant form and used for catalytic reactions with fatty acids, the products of which were characterized by mass spectrometry analysis.

Results

A novel MSA35 self-sufficient CYP505 is differentially expressed in antagonistic and pathogenic *F. oxysporum*. Its expression is induced by the host plant lettuce in both pathogenesis and antagonism during the early phase of the interaction, while it is silenced during the late phase only in antagonistic *Fusarium*. Mass-spectrometry investigations proved that CYP505A1 mono-hydroxylates lauric, palmitic and stearic acids.

Conclusions

The ability of CYP505A1 to oxidize fatty acids present in the cortical cell membranes together with its differential expression in its *Fusarium* antagonistic form point out to the possibility that this enzyme is associated with *Fusarium* pathogenicity in lettuce.

General significance

The CYP505 clan is present in pathogenic fungal phyla, making CYP505A1 enzyme a putative

candidate as a new target for the development of novel antifungal molecules.

Keywords: self sufficient cytochrome P450, *Fusarium oxysporum*, wilt of lettuce, fungal pathogens, fungal antagonists new antifungal target.

1. Introduction

Cytochrome P450 enzymes (P450s) are haem-containing monooxygenases present in every domain of life with very diversified biological functions. They are characterized by a strong absorption band at 450 nm by their haem iron when reduced and complexed with carbon monoxide. In fungi, P450s contribute to their fecundity and fitness in different ecological niches, being involved in a plethora of physiological reactions. Secondary metabolites with industrial, biomedical and agricultural significance, are synthesized in filamentous fungi by cytochromes P450 [1]. In addition to very specific functions, P450s play a housekeeping role in fungi; for example, CYP51 is involved in the biosynthesis of sterol and it is a very efficient target for fighting diseases in human as well as fungal diseases of crops [2]. The evolution of fungal pathogenicity has been associated to the expansion and functional diversification of the P450 families [3,4]. Several P450s are involved in pathogen virulence because they neutralize the antibiotic phytoalexins produced by host plants [4]. The best known P450 degrading plant defence compounds is represented by the pisatin demethylase CYP57A1 of *Nectria haematococca*, a pea pathogen. This enzyme acts on pisatin, a fungistatic isoflavonoid produced by pea when attacked by the pathogenic [5].

Fusarium wilt of lettuce, caused by *Fusarium oxysporum* f. sp. *lactucae*, is at present the most serious disease of lettuce, causing significant losses worldwide [6]. *Fusarium oxysporum* MSA35 is an antagonistic fungus that lives in association with a consortium of ectosymbiotic bacteria isolated from a soil suppressive to *Fusarium* wilt [7]. MSA35 produces a different spectrum of microbial volatile organic compounds, the sesquiterpene α -caryophyllene, that reduces the mycelial growth and inhibits the expression of virulence genes in different *formae specialis* of pathogenic *F. oxysporum* [8].

Interestingly, different pathogenic *formae specialis* of *F. oxysporum* possess a cytochrome P450 (P450foxy) belonging to the new family CYP505 and to class VIII [9]. This is a flavocytochrome composed of a P450 hydroxylase with a N-terminal domain naturally fused to the FAD/FMN-

containing cytochrome P450 reductase C-terminal domain [10]. This enzyme is able to hydroxylate saturated fatty acids [11] whose role in inducing plant defences is a recent discovery [12].

In the present study we investigate the presence of CYP505 gene in pathogenic *Fusarium oxysporum* f. sp. *lactucae*. Given the key role played by P450 in fungal pathogenesis, the possibility that this enzyme can be involved in virulence in lettuce was investigated. Its expression profile during both pathogenic and antagonistic *Fusarium* interaction with the host lettuce plant was studied as well as the correlation, if any, with lettuce costunolide synthase gene expression, phytoalexin and fatty acid metabolism.

2. Materials and methods

2.1 Fungal and plant cultures

Fusarium oxysporum MSA 35 and *Fusarium oxysporum* f. sp. *lactucae* Fuslat10 were maintained on *Fusarium*-selective Komada medium [13] and on potato dextrose agar medium (PDA) [14] as described previously [7].

2.2 Plant material

Seeds of *Lactuca sativa* and seedlings were treated as described in Minerdi et al. [7]. Germinated seedlings were transferred after 2 days to plates for the experimental uses described below.

2.3 Co-culturing of *F. oxysporum*-*L. sativa*

The expression of *F. oxysporum* CYP505 gene in the presence of the host plant was performed on plastic Petri dishes with Murashige and Skoog salt (MS) medium (Duchefa Biochemie) containing 0.8% agar and 1.5% sucrose [15] using the same protocol described in Minerdi et al. [7]. Experiments were carried out three times with three replicates each and arranged in a completely randomized manner.

2.4 Bacterial strain and culture media

Escherichia coli TOP10 and DH5 α strains (Invitrogen, Karlsruhe, Germany) were used for vector

propagation and heterologous expression of the fungal recombinant cytochrome P450 haem domain (FOP) and the human recombinant cytochrome P450 reductase (hCPR), respectively. Bacteria were grown at 37°C in LB medium containing 100 µg/ml ampicillin as a selection marker.

2.5 DNA extraction and PCR amplifications on genomic DNA

Genomic DNA was extracted from lettuce seedlings and *F. oxysporum* MSA35 mycelium using the Nucleospin Plant kit (Macherey Nagel) according to the manufacturer's instructions. To amplify the genomic sequence of fungal P450 gene belonging to 505 family, primers CYP505For (5'-TTGCGCCCAACTTTCTCTAT-3') and CYP505Rev (5'-ATGCCATGTCGTAGGGTAGC-3') were designed on the basis of the conserved regions of CYP505 gene sequence of *Fusarium oxysporum* f.sp. *dianthii* (AB030037.1) and *Fusarium oxysporum* f. sp. *lycopersici* (AFQF01002607.1), available in databases. PCR reaction was carried out in a final volume of 20 µl and containing 0.5 M of each primer, 2.0 µl of 10X buffer (Qiagen, Hilden, Germany), 2.5 mM MgCl₂, 250 mM of each dNTP, 2 µl of DNA and 1U of *Taq* DNA polymerase (Qiagen, Hilden, Germany), The PCR cycling conditions were as follows: denaturation at 95°C for 4 min; 30 cycles at 94°C for 45 s, 50°C for 45 s and 72°C for 1 min; and a final extension at 72°C for 7 min using a Techne TC-312 thermal cycler. To obtain the full sequence of the gene, a genome walking strategy was adopted using primers specifically designed on the basis of *F. oxysporum* f.sp. *lycopersici* CYP505 flanking gene sequences.

To amplify the genomic sequence of *F. oxysporum* MSA35 primers NS1/NS2 [16] were used. The experimental conditions used were previously described in Minerdi et al. [17]. *F. oxysporum* CYP505 was amplified by using the specific primers CYP505RT-FOR (5'-AAAGGATTTGCTCGCTCGCTA-3') and CYP505RT-REV (5'-CATCCGCTCAGGAATGAACT-3') designed on the basis of the coding sequence obtained in this work. PCR reactions were carried out in a 20 µl final volume containing 0.5 µM of each primer, 2.0 µl of 10X buffer (Invitrogen), 2.5 mM MgCl₂, 250 µM each dNTP, 500 ng of DNA preparation and

1U of Platinum Taq DNA polymerase (Invitrogen). The PCR cycling conditions were as follows: denaturation at 95 °C for 3 min; 30 cycles at 94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s; and a final extension at 72 °C for 7 min using a Techne TC-312 thermal cycler.

2.6 PCR product purification and sequencing

The PCR product of 800 bp obtained using primers CYP505For/CYP505Rev was excised from the gel and purified using the QIAquick PCR purification kit (Qiagen). The purified product was directly sequenced by using the PCR primers described above using an ABI model 3730 DNA sequencer (Eurofins MWG operon sequencing service, Ebersberg, Germany).

2.7 Semi-quantitative reverse transcription RT-PCR

Total RNA for RT-PCR was extracted from: antagonistic MSA35 and pathogenic *Fuslat10* mycelium grown on PDA and on MS plate close to the lettuce seedlings. RNeasy Plant Mini Kit (Qiagen) was used for RNA extractions according to the manufacturer's instructions. cDNA was synthesized in a two-step process using Superscript II (Invitrogen). The primers used in RT-PCR reactions were the fungal CYP505RTFOR/CYP505RTREV pair. Single-strand cDNAs were obtained with the specific antisense primers CYP505RTREV using total RNA as a template. RNA samples were denatured at 65 °C for 5 min, then reverse transcribed at 55°C for 1 h in a final volume of 20 µl containing 500 ng of total RNA, 1 mM of each specific primers, 0.5 mM dNTPs, 10U RNase inhibitor, 4 µl of 5X buffer, 2 µl 0.1M dithiothreitol and 1 µl of Superscript II (Invitrogen). Control PCR reactions were carried out to assess the absence of undigested genomic DNA by using Platinum Taq DNA polymerase ((Invitrogen) and the 18S rRNA gene primers NS1/NS2 using nine total RNAs (500 ng each) as templates and the PCR conditions described above. CYP505 specific primers were then used to amplify the corresponding gene fragment. The entire RT reactions were used for PCR amplification with the sense and antisense primers using the same PCR conditions as those described above. RT-PCR experiments were conducted using three replicates on three independent samples. The amplified products were analysed by 1.8% agarose gel electrophoresis in a TAE running buffer [18] and the

nucleotide sequences were determined via direct sequencing of the amplified fragments using the specific primer pairs described above.

2.8 Sequence retrieval and alignment

GenBank database was used to retrieve amino acid sequences from completely sequenced fungal genomes. The BLASTP option of BLAST [19] was used to probe the database. Conserved domains in the MSA35 P450 sequence were searched by using Reverse Position-Specific BLAST algorithm. The ClustalW [20] program was used to perform pairwise and multiple amino acid sequence alignments. Alignments were manually checked and misaligned regions were removed.

2.9 Phylogenetic analysis

Phylogenetic analysis was performed using MEGA version 4 [21]. Distances according to the Kimura two-parameter model [22] and clustering with the neighbour-joining method [23] were determined using bootstrap values based on 1,000 replications.

2.10 Nucleotide sequence accession number

The cytochrome P450 coding sequence obtained from *F. oxysporum* MSA35 was submitted to the GenBank database and assigned the Accession No. KT176089.

2.11 Retro-transcription and amplification of P450 haem domain (FOP) coding sequence and cloning in expression vector

The sequence coding for the haem domain of MSA35CYP505 (called FOP: *F. oxysporum* P450 domain) was retro-transcribed from total RNA extracted from lettuce roots inoculated with *Fuslat10* after 8 days of infections and showing clear disease symptoms. Single-strand cDNA was obtained with the specific antisense primers HREV (5'-TTAATCTAACTCGGTTGGGGTCAT-3') designed at the 3' of the haem domain sequence of MSA35CYP505 using the same conditions described above. The entire RT reactions was used for PCR amplification with the sense primer (5'-TAAAGGAGATATACATACCCATG) designed at the 5' of the haem domain coding sequence and antisense primers using the same PCR conditions as those described above. The PCR cycling protocol

was as follows: denaturation at 95 °C for 3 min; 30 cycles at 93 °C for 45 s, 60 °C for 45 s and 72 °C for 90 s; and a final extension at 72 °C for 7 min using a Techne TC-312 thermal cycler.

An amplified fragment of about 1,500 bp was obtained and cloned into pBAD-TOPO (Invitrogen) expression vector, and subsequently *E. coli* TOP10 (Invitrogen) were transformed with this plasmid.

2.12 Heterologous expression of FOP and human cytochrome P450 reductase (hCPR) in *E. coli* cells

Expression of FOP and hCPR was achieved starting from a colony of *E. coli* TOP10 and DH5 α transformed with the pBAD-FOP and pCW-Lic-hCPR plasmids, respectively. Bacteria were grown overnight in 5 ml of LB with 100 μ g/ml of ampicillin at 37°C. This was used as the inoculum of 500 ml of Terrific broth (TB), containing 100 μ g/ml of ampicillin. The cultures were grown at 37°C, until an optical density at 600 nm of 0.8 was achieved. At this point, protein production was induced by the addition of 0.2% L-arabinose and 0.5 mM δ -aminolevulinic acid for FOP and 20 mg/ml riboflavin, 1 mM IPTG in the case of hCPR. The FOP and hCRP- induced cells were then grown for 48 h at 24°C and 24 h at 28°C, respectively. After these times, the cells were harvested by centrifugation at 4,000 rpm for 20 min at 4°C and resuspended in lysis buffer (50 mM potassium phosphate pH 7.4 and 0.1 mM phenylmethyl sulfonyl fluoride). Lysozyme was added to both suspensions at a final concentration of 1 mg/ml and the suspensions were stirred for 30 min at 4°C. Subsequently, the formation of the reduced and carbon monoxide bound form of the P450 was obtained from the difference spectrum at 450 nm using a Hewlett-Packard 8453 diode array spectrophotometer.

2.13 FOP and hCPR purification and spectroscopic analysis

The bacterial lysate from transformed TOP10 cells was centrifuged at 40,000 rpm for 20 min at 4°C, the supernatant was loaded on a DEAE column pre-equilibrated with buffer A (30 mM Potassium Phosphate Buffer pH 7.4, 0.1 mM EDTA, 2 mM DTT). FOP was eluted using a gradient of 50-250 mM NaCl. Eluted fractions that contained the protein (peak absorbance at 418 nm) were pooled and loaded on a Q-sepharose column pre-equilibrated with buffer A. The column was washed extensively

with the same buffer and FOP was eluted using a gradient of 50-250 mM NaCl. Fractions containing FOP were pooled and buffer exchanged to buffer A by Amicon Ultra centrifugal filters (Millipore, Watford, UK) and stored at -80°C. The enzyme was reduced by the addition of sodium dithionite and its UV-vis spectrum recorded with an Agilent 8453E spectrophotometer (Agilent Technologies). Then carbon monoxide was bubbled into the protein solution for a few minutes, and the resulting spectrum was recorded. The binding of CO to the reduced form of the protein causes the appearance of the characteristic 450 nm absorbance peaks. Enzyme concentration was calculated using an extinction coefficient of $91,000 \text{ cm}^{-1} \text{ M}^{-1}$ at 450 nm from the differential spectra of dithionite reduced minus CO-bound (Omura and Sato 1964).

E. coli membranes expressing the recombinant human P450 reductase were resuspended in 500 mM phosphate buffer containing 20% glycerol, 0.02 mM EDTA, 0.2 mM DTT, 1% IGEPAL, 0.5 mM PMSF, solubilized by using a homogenizer, stirred at 4°C for 2 hours and ultracentrifuged at 41,000 g for 1 hour. The supernatant was loaded on a DEAE-Sepharose Fast Flow column (GE Healthcare) pre-equilibrated with 50 mM phosphate buffer pH 7.7 containing 20% glycerol, 0.02 mM EDTA, 0.1% IGEPAL and 0.2 mM DTT. The column was washed with 10 column volumes of the same buffer and the bound proteins were eluted with a KCl gradient of 0 to 300 mM. Fractions containing hCPR were pooled and loaded onto a 5 ml 2'-5'ADP-Sepharose column (Sigma) equilibrated with 50 mM phosphate buffer pH 7.7 containing 20% glycerol, 0.02 mM EDTA, 0.1% IGEPAL, a 0.05 mM DTT and 300 mM KCl. The column was washed with 50 ml of the same buffer plus 5 mM adenosine. The reductase was eluted with the same buffer replacing adenosine with 5 mM 2'-3' AMP. The presence of the characteristic absorption peaks of FAD/FMN at 374 and 455 nm were checked with an Agilent 8453E spectrophotometer (Agilent Technologies), fractions were pooled and elution buffer was exchanged to storage buffer (50 mM potassium phosphate buffer pH 7.7, 20% glycerol, 0.02 mM EDTA and 0.05 mM DTT) by Amicon membranes (Millipore) and stored in small aliquots at -80 °C. The hCPR concentration was calculated assuming a molecular mass of 78 kDa, a molar

content equal to that of FAD, and an extinction coefficient of $11,300 \text{ M}^{-1} \text{ cm}^{-1}$ at 450 nm [24].

After the purification, both FOP and hCPR were visualized in a 10% SDS PAGE gel stained with Coomassie Blue to verify their purity.

To check hCPR functionality, an assay for NADPH-cytochrome *c* reduction was carried out in 50 mM phosphate buffer pH 7.7, 10% glycerol, 5 μM cytochrome C, 75 μM NADPH, 0.2 μM hCPR at room temperature as described previously [25].

2.14 FOP substrate binding assay by UV-vis spectroscopy

Prior to substrate binding assay, the storage buffer was exchanged with 0.1 M phosphate buffer pH 8.0. Spin-state shifts upon substrate binding were analysed under aerobic conditions at room temperature using a UV-vis spectrophotometer Agilent 8453E spectrophotometer (Agilent Technologies) following the characteristic low-to-high spin transition, as a shift in the main Soret absorption band from 419 to 394 nm [26]. Substrate titrations were performed by adding small aliquots of an appropriate stock of costunolide, lauric, stearic and palmitic acids dissolved in ethanol and in 50 mM K_2CO_3 in the reaction mixture, respectively. The reaction mixture contained 1 μM concentration of FOP in 100 mM phosphate buffer pH 8.0 and spectra were recorded after each substrate addition to the sample cuvette. The dissociation constant, K_D , was determined as described in Ferrero et al. [27].

2.15 P450 505A1 catalytic activity

In order to verify the products of the enzymatic reaction of CYP505A1 with lauric, palmitic and stearic acids, GC-MS experiments were performed. Each fatty acid (500 μM) was incubated in phosphate buffer (100 mM, pH 7.7) with 1 μM FOP, 1 μM hCPR and 1.5 mM NADPH at 30°C for 2 hours in 5 mL glass vials. The reaction mixture (500 μL) was acidified with 50 μL of 12 M HCl and extracted twice with 1 mL of diethylether. The organic phase was collected and dried with a nitrogen flush. The samples were silylated with 100 μL of BSTFA+TMCS 99:1 (Supelco) and 2 μL were analyzed by GC-MS1. The molecular weight and the position of OH were deduced on the basis

of derivatization and MS fragmentation. Each OH moiety (both carboxylic and alcoholic) becomes a trimethylsilyl group in the derivatization procedure and allows oxygen α -cleavage to give diagnostic R-O⁺-Si-(CH₃)₃ fragments.

3. Results

3.1 Screening for cytochrome CYP505 genes in *F. oxysporum* f. sp. *lactucae*

The presence of a cytochrome P450 belonging to the 505 family in *F. oxysporum* MSA35 genome was investigated by PCR using primers designed on conserved regions of *CYP505* gene of *F. oxysporum* f. sp. *dianthii* and *F. oxysporum* f. sp. *lycopersici*. An amplicon of the expected size was obtained and analysis of its sequence showed the typical signature of a cytochrome P450 coding gene. The sequence of the full length gene was obtained following a genome-walking strategy. A complete coding sequence of 3,201 bp (ORF 3201) was deduced from the gene sequence of 3,443 bp showing the presence of six introns (data not shown SEQUENCE DEPOSITED? COORDINATES?). The ORF3201 encodes a protein of 1,066 amino acids, with a predicted molecular weight of 117 kDa and low E-values when aligned with 505 family of fungal P450s. This new MSA35 enzyme was named CYP505A1 (<http://drnelson.uthsc.edu>).

3.2 Phylogenetic and amino acid sequence analysis

Neighbor-joining tree of the complete amino acid sequence and analysis of its haem domain (FOP) placed MSA35 CYP505 within the self-sufficient class VIII P450 monooxygenases [9] (Figure 1A). The intimate relationship between FOP and cytochrome P450 fatty acid hydroxylase from *Aspergillus kawachii* is indicated in the phylogenetic tree of the P450 superfamily (Figure 1B) that was constructed between P450 members from various organisms. Hydrophobic clustering analysis suggested that the primary structure of CYP505A1 contained neither a membrane anchor sequence in its amino terminus nor a hydrophobic stretch in the internal regions suggesting that it is a cytosolic protein. The primary structure of CYP505A1 is divided into a haem domain

and a reductase domain with a linker connecting the two. The N-terminal 477 amino acid residues form the haem domain that contains the sequence ⁴⁰⁰FGNGKRACIG (Figure 2A, open triangles) that matches the consensus motif ([F/W][S/G/N/H]X[G/D]X[R/H/P/T]XC[L/I/V/M/F/A/P][G/A/D]) typical of cytochromes P450 [28] with cysteine 406 corresponding to the haem-iron fifth ligand. The consensus motif ²⁶⁶AGHETT (Figure 1A, open circles) corresponds to the putative oxygen binding sequence [28]. The C-terminal sequence from amino acid 497 to 1,066 of CYP505A1 is homologous to reductase proteins. Alignment of this sequence (FOR) with those of *Bacillus megaterium* CYP102A1 reductase (BMR) and reductase domain of *F. oxysporum* f.sp. *cubense* CYP505 (FOXR) is shown in Figure 2B. As can be seen in figure 2B, as expected in this C-terminal region a FMN-binding domain (single underline), a FAD binding domain (double underline) and a NADPH-binding domain (asterisks) are all present. All these data indicate that CYP505A1 belongs to the catalytically self-sufficient cytochrome P450 class VIII with an N-terminal haem domain (residues 1-477) and a C-terminal reductase domain (residues 491-1,066) that includes the cofactors FMN and FAD.

3.3 CYP505A1 gene expression

In order to understand if the CYP505A1 can be involved in the interaction between lettuce and pathogenic *Fusarium*, semi-quantitative RT-PCR experiments were carried out with CYP505A1 specific primers on total RNA extracted from lettuce roots infected by antagonistic MSA35 and the pathogenic *Fusarium oxysporum* *Fuslat10* and the same fungi grown on PDA medium. Results showed that the gene is not expressed when *Fuslat10* and MSA35 antagonistic fungi grow on PDA medium (Figure 3A, 3B). On the contrary, the gene is expressed during the early phase (after 4 days) of the interaction with lettuce both in antagonistic and pathogenic fungi as well as when *Fuslat10* mycelium grows very close to the lettuce seedling roots. Surprisingly, antagonistic hyphae grown

close to lettuce roots do not express the CYP505A1 gene (Figure 3B). During the late phase of interaction (after 8 days), when the lettuce seedlings show visible disease symptoms, the CYP505A1 gene expression is silenced in the antagonistic MSA35 (Figure 3B).

3.4 FOP and hCPR expression and purification

To find the possible substrates of CYP505A1 and to analyze their reaction products, the recombinant haem domain of the fungal CYP505A1 (FOP) was expressed and purified. Since it was not possible to retrotranscribe the full-length CYP505A1 mRNA, FOP coding sequence was retro-transcribed from the meta RNA extracted from lettuce roots infected by *Fuslat10* showing disease symptoms (after 8 days of interaction), cloned in the expression vector pBAD and expressed in *E. coli* TOP10 cells. After purification by ion exchange chromatography (Figure 4A), the UV-vis spectra of the 53 kDa purified protein showed the characteristic low spin iron Soret peak at 418 nm (Figure 4B). When reduced and bubbled with carbon monoxide the peak shifted from 418 to 450 nm as expected, showing the presence of a correctly folded P450 enzyme (Figure 4B).

Since it is known that CYP52 from *Aspergillus oryzae* is able to perform its catalytic activity in the presence of human cytochrome P450 reductase (hCPR) (Uno et al. 2016), this reductase was expressed and successfully purified using DEAE and 5'ADP-Sepharose column (Figure 4C) to be used as FOP redox partner.

3.5 Substrate binding studies by UV-vis spectroscopy

Fatty acids were checked as possible substrates of CYP505A1 since FOP is phylogenetically very closely related to P450 fatty acid hydroxylase from *Aspergillus kawachii* and because the importance these molecules play in plant defence during pathogens attack. Therefore UV-vis spectroscopy was used to study the binding of lauric, palmitic and stearic acids to the active site of purified FOP.

The addition of increasing amounts of lauric acid (0-300 μ M), palmitic acid (0-7 μ M) and stearic acid (0-0.6 μ M) to FOP caused a shift in the main Soret peak from 419 to 394 nm (Figures 5A, 5B, 5C, respectively), indicating that the three fatty acids behave as putative substrates. The apparent

dissociation constant values (K_D) were calculated to be 1.02 ± 0.10 mM for lauric acid, while for palmitic and stearic acid are 1.87 ± 0.17 μ M and 0.17 ± 0.02 μ M, respectively.

3.6 Identification of fatty acids hydroxylation products

Following incubation of CYP505A1 with lauric, palmitic and stearic acids in the presence of hCPR and NADPH, the reaction products were analysed by GC–MS. The results show that a mono-hydroxylation takes place in all of the studied C12, C16 and C18 fatty acids giving rise to three main hydroxy-derivatives. The hydroxylation site is in ω -1, ω -2 and ω -3 position respectively, independently from the chain length, leading to 9-, 10- and 11-hydroxylauric acid, 13-, 14- and 15-hydroxypalmitic acid and 15-, 16- and 17-hydroxystearic acid (Figure 6). The elution order was proportional to the distance of the hydroxyl-group from the terminal methyl group. In all cases the abundance of the isomers was similar with the most abundant isomer being ω -1.

4. Discussion

Fusarium oxysporum causes severe vascular wilt disease [29, 6]. Signals released by host plants induce the germination of soil spores differentiating infection hyphae, which adhere to and penetrate the plant roots invading the cortex and crossing the endodermis [30]. Once they reach the xylem vessels they colonize the plant host and adapt to the hostile plant environment secreting virulence determinants [31]. Several pathogenicity associated genes have been identified in *F. oxysporum*, including those encoding for G-proteins [32], transcription factor responsive to environmental pH [33], arginosuccinate lyase [34], a class V chitinase [35], a mitogen-activated protein kinase [36], a F-box protein [37], a mitochondrial carrier protein [38] and a small secreted protein found in xylem [32, 39].

F. oxysporum strains isolated from healthy roots in suppressive soils are called “antagonistic” since they have the ability to protect plants from pathogenic fungi and several of them are used as biological control agents in sustainable agriculture [40, 41, 42]. Antagonistic and pathogenic

fungi can share the same set of genes, but some genes can be differentially regulated. The possibility to use an antagonistic and a pathogenic strain of *Fusarium oxysporum* allowed us to evaluate if the CYP505A1 can be considered as a pathogenicity related gene in *Fusarium oxysporum* f.sp. *lactucae*. After 4 days of *Fusarium* interaction with lettuce, CYP505A1 is expressed in both antagonistic and pathogenic fungi while during the late phase only the antagonistic *Fusarium* stops the expression of the gene. The plant signal(s) that regulate the *cyp505A1* gene expression is unknown, but it can be assumed that it is a diffusable molecule since the P450 expression is switched on only when hyphae grow on agar medium in close contact with the lettuce roots.

There is increasing evidence that P450 enzymes exhibit important and different roles in the infections of host plants by pathogenic fungi by contributing to the synthesis of secondary metabolites [43]. Fungal P450s are up-regulated during host-pathogen interactions as in the case of *Heterobasidion annosum* [44], *Moniliophthora perniciosa* [4] and *Botrytis cinerea* [45]. The differential expression of P450 coding genes in pathogenic and non pathogenic *Fusarium* strains has already been reported. The gene coding for CYP55, a cytochrome P450 with nitric oxide reductase activity [46] in *F. oxysporum* f.sp. *cubense* has been found to be up-regulated in pathogenic strains following the infection with banana but not in *F. oxysporum* isolates non-pathogenic to banana [47]. This enzyme regulates the nitrogen response pathway, that is essential for pathogenicity [48]. *F. oxysporum* f.sp. *vasinfectum* expresses the *cyp55* gene when it invades cotton plant roots [48]. *Verticillium dahlia*, during the first five days of infection in cotton, up-regulates the expression of a cytochrome P450 that affects the metabolism of sulfacetamide, a secondary metabolite involved in fungal pathogenesis [49]. *Fusarium graminearum* induces the expression of a putative benzoate 4-monooxygenase cytochrome P450 gene during wheat coleoptiles infection, and was speculated to be involved in the pathogenic process by participating in the secondary metabolism [50].

It seems that during the early stage of the interaction, the lettuce plant perceives both antagonistic and pathogenic *Fusarium* as pathogens. These data are in agreement with what was found by Moretti et al. [51] where a comparative time-course analysis of the proteomic profile of MSA35 and pathogenic *Fuslat10* in contact with lettuce was performed. These researchers found that during the early stages of the interaction between the fungal strains (MSA35 and *Fuslat 10*) and the host plant, proteins involved in stress defence, energy metabolism and virulence were equally induced in antagonistic and pathogenic strains with only the pathogenic *Fuslat 10* continuing the production of virulence- and energy-related proteins in the late phase of the inter-kingdom interaction [51].

In the case of our study of CYP505A1 at the beginning of the interaction, both fungi are perceived by the plant as pathogenic but cytochrome P450 is not expressed in antagonistic hyphae grown close to lettuce roots. This data could mean that the presence of the bacterial consortium attached to the MSA35 hyphae [7, 8] modify the micro-environment in such a way that the putative signal(s) is no longer active or cannot reach the fungal target or is not produced. Fungal pathogens present in the soil sense the presence of the host plant through root exudates and respond by changing their gene expression leading to host recognition and penetration, host defence breakdown, proliferation within the host tissue and disease establishment [31]. CYP505 members are fatty acid hydroxylases that carry out the subterminal omega hydroxylation of fatty acids, a step required for the use of these molecules as an energy source [11]. Mass spectroscopy analysis of the reaction products between C12, C16 and C18 fatty acids and CYP505A1 showed that also the fungal cytochrome P450 is able to hydroxylate fatty acids in the subterminal positions.

Moreover, oxidized fatty acids are a group of endogenous signal molecules [53] that play a role in the interaction between plants and fungi. Recent discoveries show a direct role for fatty acids and their breakdown products in inducing plant defence. Both 16- and 18-carbon fatty acids participate in defence to modulate basal, effector-triggered, and systemic immunity in plants [12]. For example, two C18 derivatives have been shown to have a strong capacity to elicit plant defence and H₂O₂

production in cucumber [54]. In *Vicia sativa*, 18-hydroxy-9,10-epoxystearic acid and 9,10,18-trihydroxystearic acids have been described as potential messengers in plant-pathogen interactions [55]. We can speculate that CYP505A1 of pathogenic *Fuslat10*, having entered the lettuce cortical cells, hydroxylates the C12, C16 and C18 fatty acids present in the cells membrane. These hydroxylated compounds might function as signal molecules in activating plant defence system. On the contrary, the non pathogenic MSA35 hyphae are impaired and cannot enter the plant roots [7], they do not express CYP505A1.

In conclusion, this study shows a correlation between the expression of CYP505A1 gene and *Fusarium Fuslat10* pathogenicity in lettuce. Future research is needed to understand if CYP505A1 can represent a new pathogenicity associated gene that is active during the early stage of pathogen invasion in different pathogenic *formae speciales* of *Fusarium oxysporum*.

General significance

Fungal pathogens of important crops cause severe economic loss each year. Resistance to antifungal molecules is rapidly increasing as well as the need for new targets and technologies for a rapid discovery of novel natural and synthetic agrochemicals. MSA35 CYP505A1 is a promising candidate as a new target for antifungal drugs development since it is expressed only in the pathogenic *Fusarium oxysporum* once it enters the roots of the lettuce host plant. Furthermore, the enzyme is widespread in all the four phylum of fungi comprising the most dangerous economic important cultures like *Aspergillus nidulans*, *Aspergillus oryzae*, *Fusarium graminearum*, *Fusarium oxysporum*, *Fusarium verticillioides*, *Gibberella moniliformis* and *Magnaporthe grisea*. Natural and synthetic agrochemicals could be screened for their inhibitory capability of the P450 activity,

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Figures legend

Figure 1

Phylogenetic analysis of the full length sequence (A) and N-terminal domain of cytochrome P450 of *F. oxysporum* MSA35 (B). Neighbor-joining tree was built using pairwise deletion with p-distance options. The numbers at the nodes are the bootstrap confidence values obtained after 1000 replicates.

The scale bar indicates distance in substitutions per nucleotide. The species and proteins are (A): *Fusarium oxysporum* f.sp. *pisi*= CYP505 from *Fusarium oxysporum* f.sp. *pisi* (EXA43448.1); *Fusarium oxysporum* f.sp. *raphani*= CYP505 from *Fusarium oxysporum* f.sp. *raphani* (EXK91707.1); *Fusarium oxysporum* MSA35= CYP505 from *Fusarium oxysporum* MSA35 (KT176089); *Fusarium oxysporum* f.sp. *melonis*= CYP505 from *Fusarium oxysporum* f.sp. *melonis* (EXK442861); *Fusarium fujikuroi*= CYP505 from *Fusarium fujikuroi* (KLO80461.1); *Fusarium oxysporum* f.sp. *verticilloides*= CYP505 from *Fusarium oxysporum* f.sp. *verticilloides* (EWG47019.1); *Fusarium oxysporum* f.sp. *avenaceum*= CYP505 from *Fusarium oxysporum* f.sp. *avenaceum* (KIL90643.1); *Metarhizium acridum*= CYP505 from *Metarhizium acridum* (XP_007813119.1); *Trichoderma reesei*= CYP505 from *Trichoderma reesei* (XP_006965911.1); *Trichoderma virens*= CYP505 from *Trichoderma virens* (EHK20283.1); *Colletotrichum graminicola*= CYP505 from *Colletotrichum graminicola* (XP_008096088.1); *Verticillium alfalfae*= CYP505 from *Verticillium alfalfae* (XP_003004414.1); *Magnaporthe oryzae*= CYP505 from *Magnaporthe oryzae* (XP_003719368.1); *Aspergillus fumigatus*= CYP505 from *Aspergillus fumigatus* (XP_754698.1); *Aspergillus oryzae*= CYP505 from *Aspergillus oryzae* (EIT73160.1); *Bacillus megaterium*= CYP102A1 from *Bacillus megaterium* (KFN05180.1).

B) PDA *Fusarium oxysporum* f. sp. *melonis* = PDA from *Fusarium oxysporum* f. sp. *melonis* (EXK49748.1); PDA *Fusarium oxysporum* f. sp. *lycopersici* = PDA from *Fusarium oxysporum* f. sp. *lycopersici* (EXL41305.1); PDA *Fusarium oxysporum* f. sp. *conglutinans*= PDA from *Fusarium oxysporum* f. sp. *conglutinans* (EXL79015.1); PDA *Fusarium oxysporum* f. sp. *raphani* = PDA from

Fusarium oxysporum f. sp. *raphani* (EXK79131); PDA *Fusarium oxysporum* f. sp. *pisi* = PDA from *Fusarium oxysporum* f. sp. *pisi* (AAR32716.1); *Fusarium oxysporum* f. sp. *cubense* PDA= PDA from *Fusarium* f. sp. *cubense* (ENH75868.1); CYP86A8 *Arabidopsis lyrata*= CYP86A8 from *Arabidopsis lyrata* (XP_002882043); CYP655C2 *Fusarium oxysporum*= CYP655C2 from *Fusarium oxysporum* f. sp. *lycopersici* (XP_018254178); CYP539B1 *Magnaporthe oryzae*= CYP539B1 from *Magnaporthe oryzae* (XP_003709721); CYP52E2 *Candida apicola*= CYP52E2 from *Candida apicola* (Q12573.1); CYP52A13 *Candida tropicalis*= CYP52A13 from *Candida tropicalis* (AAO73953); CYP4A10 *Rattus norvegicus*= CYP4A10 from *Rattus norvegicus* (XP_006238774.1); CYP4A11 *Homo sapiens* = CYP4A11 from *Homo sapiens* (AAB29502.1); CYP97B3 *Arabidopsis thaliana*= CYP97B3 from *Arabidopsis thaliana* (AEE83557.1); FOP= haem domain of CYP505A1 from *F. oxysporum* MSA 35 (KT176089); FAH *Aspergillus kawachii*= Fatty Acid Hydroxylase from *Aspergillus kawachii* (GAA84899); FUM6p *Fusarium verticillioides*= fumonisyn synthase from *Fusarium verticillioides* (ADQ38980); BMP *Bacillus megaterium*= haem domain of CYP102A1 from *Bacillus megaterium* (KFN05180.1); CYP102A5 *Bacillus cereus*= CYP102A5 from *Bacillus cereus* (ADL27534); BMP *Bacillus megaterium*= haem domain of CYP102A1 from *Bacillus megaterium* (KFN05180.1); CYP51 *Fusarium graminearum*= CYP51 from *Fusarium graminearum* (JN416622.1).

Figure 2

A. Amino acid sequence alignment of the N-terminal portion (residues 1-477) of CYP505A1 from *Fusarium oxysporum* MSA35 (KT176089). The conserved oxygen and haem binding sites are marked with open circles and triangles, respectively. The conserved cysteine residue that provides the fifth ligand to the haem iron is marked by an asterisk. BMP (haem domain of CYP102A1 from *Bacillus megaterium*) (KFN05180.1); FOXP (cytochrome P450 from *Fusarium oxysporum* f.sp. *cubense*) (EXM025981); FOP (haem domain of CYP505A1).

B. Amino acid sequence alignment of the C-terminal portion (residues 497-1,066) of CYP505A1 with two different dioxygenase reductase subunits. The residues corresponding to the putative FMN, FAD and NADPH-binding domains are underlined, double underlined and marked with asterisks, respectively. FOXR (CYP505 reductase domain from *F. oxysporum* f. sp. *cubense*) (EXM02598.1); BMR (CYP102A1 reductase domain from *B. megaterium*) (KFN05180.1); FOR (CYP505 reductase domain from *Fusarium oxysporum* MSA35) (KT176089).

Figure 3

A. Agarose gel of RT-PCR products amplified by using primers specific for CYP505A1. RNA extracted from MSA35 mycelium grown on PDA medium (lane 1); RNA extracted from lettuce seedlings infected by *Fuslat10* after 4 days (lane 2); RNA extracted from lettuce seedlings infected by *Fuslat10* after 8 days (lane 3); RNA extracted from lettuce seedlings infected by MSA35 after 8 days (lane 4); RNA extracted from lettuce seedlings infected by MSA35 after 4 days (lane 5).

B: RNA extracted from *Fuslat10* mycelium grown close to lettuce roots (lane 6); RNA extracted from MSA35 mycelium grown close to lettuce roots (lane 7); RNA extracted from *Fuslat10* mycelium grown on PDA medium (lane 8).

Figure 4

A. SDS-PAGE of the purified haem domain of CYP505A1 **B.** Absorption spectra of FOP. Solid line oxidized form; dotted line dithionite reduced form, dashed line dithionite-reduced form plus carbon monoxide. **C.** SDS-PAGE of the purified human P450 reductase. DOESNT EXIST

Figure 5

Spectral perturbation in FOP caused by fatty acids. **A.** Absorption spectra of FOP in the presence of **1:** 100, 150, 200, 250, 300 μ M lauric acid; **2:** 1, 2, 3, 4, 5, 6, 7 μ M palmitic acid; **3:** 0.1, 0.2, 0.3, 0.4,

0.5, 0.6 μ M stearic acid. **B.** Difference spectra. Each difference spectrum was obtained by subtracting line 1 from each of the other lines.

C missing

Figure 6

GC-MS chromatograms of C12 (A), C16 (C) and C18 (E) fatty acid enzymatic transformation mixtures and fragmentation pathways of TMS-fatty acids (C12-Fig.B), (C16 -Fig.D) and (C18 -Fig.F) and their monohydroxylated metabolites.

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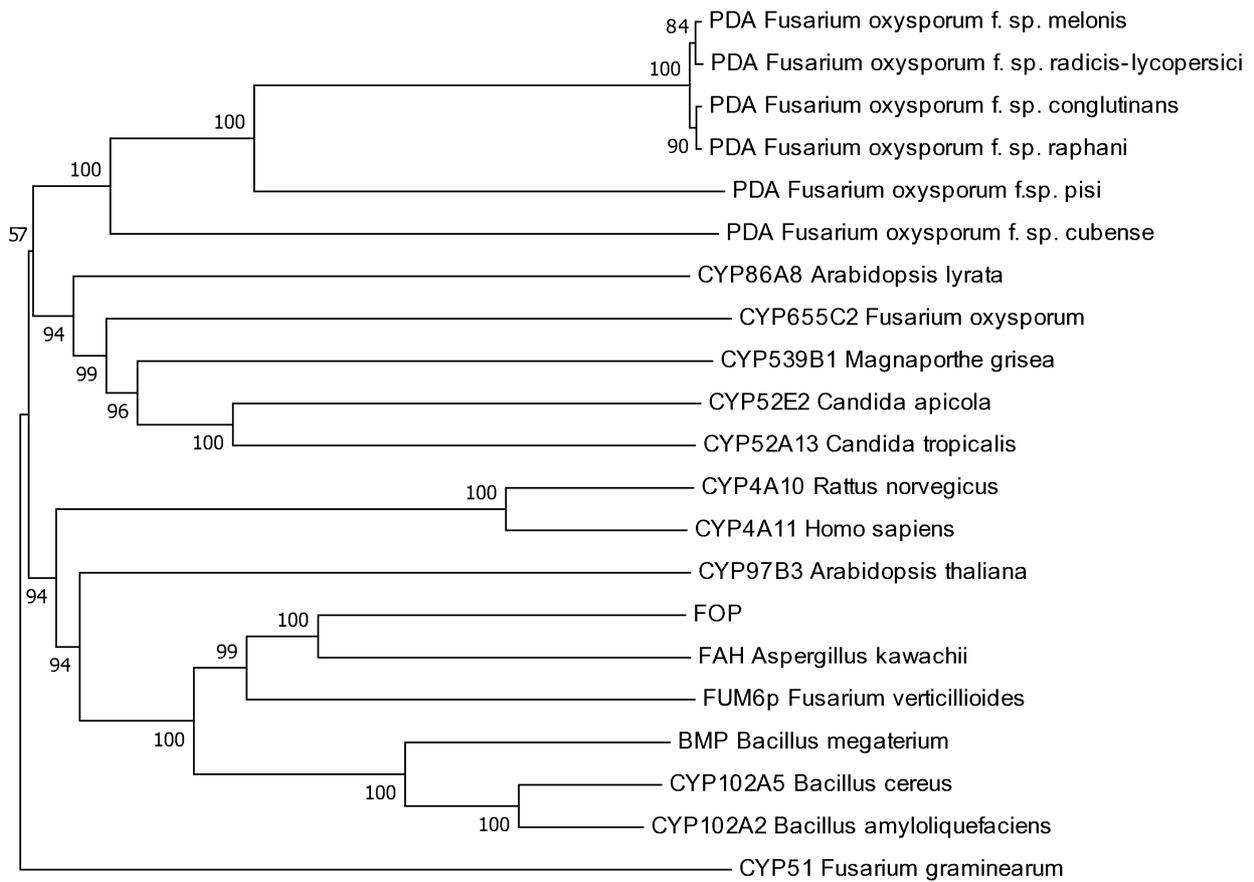


Figure 1B

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      10      20      30      40      50      60
BMP      |      |      |      |      |      |
FOXP     M-TIKEMPQPKTFGELKNLPLLN'NDKPVQALMKIADELGEI'FKFEAPGRVTRYLSSQRLI
FOP      MAESVPIPEPPGYPLIGNLGEFTSN-PLSDLNRLADTYGPIFRLRLGAKAPIFVSSNSLI
          MAESVPIPEPPGYPLIGNLGEFTSN-PLSDLNRLADTYGPIFRLRLGAKAPIFVSSNSLI

      70      80      90      100     110     120
BMP      |      |      |      |      |      |
FOXP     KEACDESRFDKNLSQALKFVRDFAGDGLFTSWTHEKNWKAHNILLPSFSQQAMKGYHAM
FOP      NEVCDEKRFKKTLSVLSQVREGVHDGLFTA'FEDEPNWGAHRIILVPAFGPLSIRGMFPE
          NEVCDEKRFKKTLSVLSQVREGVHDGLFTA'FEDEPNWGAHRIILVPAFGPLSIRGMFPE

      130     140     150     160     170     180
BMP      |      |      |      |      |      |
FOXP     MVDIAVQVLVQKWERLNADEHEI'VEPDMTRLTLDTIGL'CGFNVRFN'SFYRDQPHPFITSMV
FOP      MHD'IATQLCMKFARHGPRTPIDTSDNFTR'LALD'TLALC'AMDFRFYSYK'EE'LPFIEAMG
          MHD'IATQLCMKFARHGPRTPIDTSDNFTR'LALD'TLALC'AMDFRFNYSYK'EE'LPFIEAMG

      190     200     210     220     230     240
BMP      |      |      |      |      |      |
FOXP     RALDEAMNKLQR-ANPDDPAYDENKRQFQEDIKVMNDLVDKIIADR'KASGEQSDDLLTHM
FOP      DFLTESGNRRNRRPPFAPN'FLYRAANEK'FYGDIALMK'VADEVVAARKASPSDRKDLLAAM
          DFLTESGNRRNRRPPFAPN'FLYRAANEK'FYGDIALMK'VADEVVAARKASPSDRKDLLAAM

      250     260     270     280     290     300
BMP      |      |      |      |      |      |
FOXP     LNGKDPETGEPLDDENIRYQIITFLIAGHETTSGLLS'FALYFLVKNPHVLQKAAEEAARV
FOP      LNGVDPQ'GTEKLSDENITNQLITFLIAGHETTSGT'LSFAMYQLLKNPEAYSKVQKEVDEV
          LNGVDPQ'GTEKLSDENITNQLITFLIAGHETTSGT'LSFAMYQLLKNPDAYS'KVQKEVDEV
                        OOOOOO

      310     320     330     340     350     360
BMP      |      |      |      |      |      |
FOXP     LVDPVPSYQVQKQKLVGMV'LNEALRLWPTAF'AFSLYAKEDTVL'GGEYPLEKGDELMVLI
FOP      VGRGPV'LVEHLTKLPYISAVLRE'TLRLNSPI'AFGLEAIDDT'FLGGKYL'VKKGEIVTALL
          VGRGPV'LVEHLTKLPYISAVLRE'TLRLNSPI'AFGLEAIDDT'FLGGKYL'VKKGEIVTALL

      370     380     390     400     410     420
BMP      |      |      |      |      |      |
FOXP     PQLHRDKTIWGDVVEE'FRPE----RFENPSAI'PQHAFK'PFGNGQRACI'GQQFALHEATL
FOP      SRGHVDPV'VYGNDADKFI'PERMLDDE'FARLNKEY'PNCWK'PFGNGKRACI'GRPF'AWQESLL
          SRGHVDPV'VYGNDADKFI'PERMLDDE'FARLNKEY'PNCWK'PFGNGKRACI'GRPF'AWQESLL
                        ▲▲▲▲▲ *▲▲

      430     440     450     460     470     480
BMP      |      |      |      |      |      |
FOXP     VLGMLLKHFD'FEDHT-NYELD IKETLTKPEGFV'VKAKSKKI'PLGGIPLINKERS'PSTEQ
FOP      AMVVL'FQNFNFTMTD'PNYALEIKQTLTIKPDH'FYINATLR---HGMP'TELEHVL'AGNG
          AMVVL'FQNFNFTMTD'PNYALEIKQTLTIKPDH'FYINATLR---HGMP'TELEHVL'AGNG

BMP      SAKKVRKK
FOXP     AT-----
FOP      AT-----

```

Figure 2A

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      500      510      520      530      540      550
FOXR  DAKAGSGKPMAlFYGSNSGTCEALANRLASDAPSHGFSATTVGPLDQAKQNLPEDRPVVI
BMR   -----HNTPLLVLYGSNMGTAEGTARDLADlAMSKGFAP-QVATLDSHAGNLPREGAVLI
FOR   -----SGKPMAlFYGSNSGTCEALANRLASDAPSHGFSATTVGPLDQAKQNLPEDRPVVI

      560      570      580      590      600      610
FOXR  VTASyEGQPpSNAAHFIKWMedLDGNDMEKVSyAVFACGHHDWVETfHRIPKLVdSTLEK
BMR   VTASyNGHPpDnAQfVDWLDQASADDVKGVRySVFGCGDKNWATTyQKVPafIDETLlAA
FOR   ATASyEGQPpSNAAHFIKWMedLNGNEMEKVSyAVFACGHHDWVETfHRIPKLVdSTLEK

      620      630      640      650      660      670
FOXR  RGGTRLVpMGSADAATSDMFSDFEAWEDlVlWpGLKEKYKlSDEESGGQK---GLLVEVS
BMR   RGAENlADrGEADAS-DDfEGTYEeWR-EHMWSDVAAYfNLDlENSEDNKStLSlQfVDS
FOR   RGGTRLVpMGSADAATSDMFSDFEAWEDlVlWpGLKEKYKlSDEESGGQK---GLLVEVS

      680      690      700      710      720      730
FOXR  TPRKtSLRQDVEEAlVVAEKtLTKSGP--AKKHIElQLpSAmTyKAGDYlAlLpLNpKSt
BMR   AdMPLAKMHGAFsANvVASKELQQLGSErSTRHLElAlPKEAStYQEGDHLGvIPrNyEGl
FOR   TPRKtSLRQDVEEAlVVAEKtLTKSGP--AKKHIElQLpSMPtYKAGDYlAlLpLNpKSt

      740      750      760      770      780      790
FOXR  VARVFRrFLSawDSFlKlQSEGP--TTLpTNVAISAFDVFSAyVELSQpATKRNIlAlAE
BMR   VNRVTARfGLDASQqIRLEAEeEKLAHLpLGKtVSVeELl-QYVELQDPVTRtQLRAMAA
FOR   VARVFRrFLSawDSFlKlQSEGP--TTLpTNVAICAFDVFSAyVELSQpATKRNIlAlAE

      800      810      820      830      840      850
FOXR  ATEDKDtIQELeRLAG-DAYQAEISPKRvSVLDLLEKfPAVALpISSYLAmLPPMRvRQY
BMR   KtVCPpHKVELEAlLEKQAYKEQVLAKRlTMLELLEKYPACEMEFSEfIAlLPSIRPr-Y
FOR   ATEDKAtIQELeRLAG-DAYQAEISPKRvSVLDLLEKfPAVALpISSYLAmLPPMRvRQ-

      860      870      880      890      900      910
FOXR  SIssSPfADpSKLTLTYsLLDAPSLSGQGRHvGVATnFLSHlTAGDKLHVSVrASSEAFH
BMR   SIssSPHvDEKQASITVSVVSGEAWSGYGEYKGIASNYLANlQEGDtlTICfVSTpQSGfT
FOR   SIssSPfADpSKLTLTYsLLDAPSLSGQGRHvGVATnFLSHlTAGDKLHVSVrASSEAFH
*****

      920      930      940      950      960      970
FOXR  LPsDAEKtPIICVAAGtGLAPLRGfIQERAAmlAAGRTlAPAlLFFGCRNPEIDDLyAEe
BMR   LPKdSE-TPLIMVGPtGVAPFRGFVQARKQLKEQGQSLGEAHLYFGCRSPHEDLYQEE
FOR   LPsDAEKtPIICVAAGtGLAPFRGfIQERAAmlAAGRTlAPAlLFFGCRNPEIDDLyAEe
*****

      980      990      1000      1010      1020      1030
FOXR  FERWEKMGAVDvRRAYSrATDKSEgCKYVQDRVYHDrADvFKVWDQgAKVfICGSREIGK
BMR   LENAQNegiITLHTAFsRvPNQPK--TYVQHVMERDgKKLIeLLDQGAHFYICGDG---
FOR   FERWEKMGAVDvRRAYSrATDKSEgCKYVQDRVYHDrADvFKVWDQgAKVfICGSREIGK
*****

      1040      1050      1060      1070
FOXR  AVEDVCvRLAIEKAQqNGRDvTEEMARAWfERSRNE-RFATDvFD-
BMR   SQMAPDVEATLMKSYADvYEVSEADARLWLQLEEKGRYAKDVWAG
FOR   AVEDVCvRLAIEKAQqNGRDvTEEMARAWfERSRNE-RFATDvFD-

```

Figure 2B

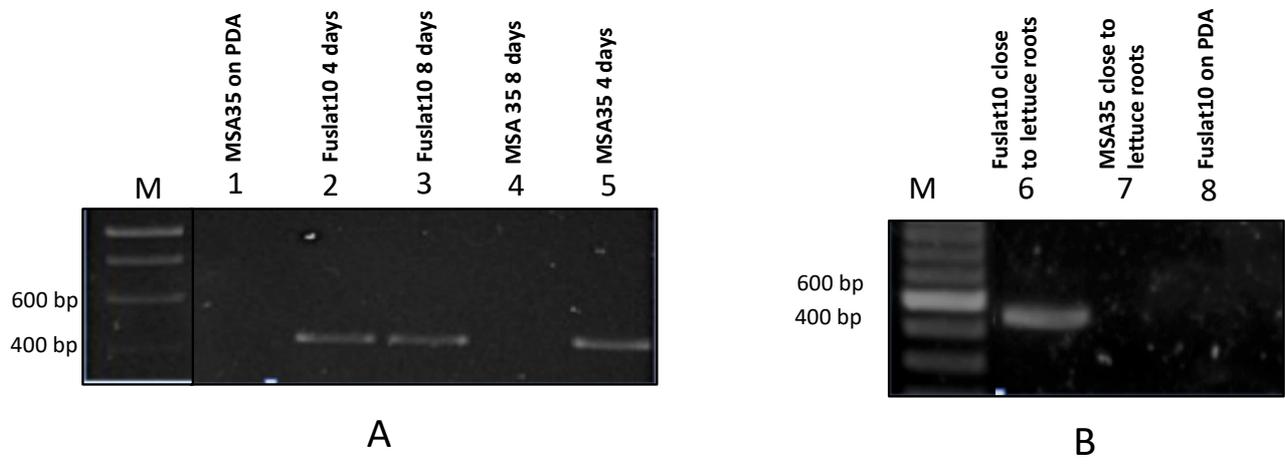
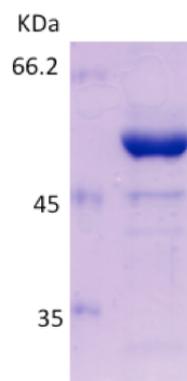
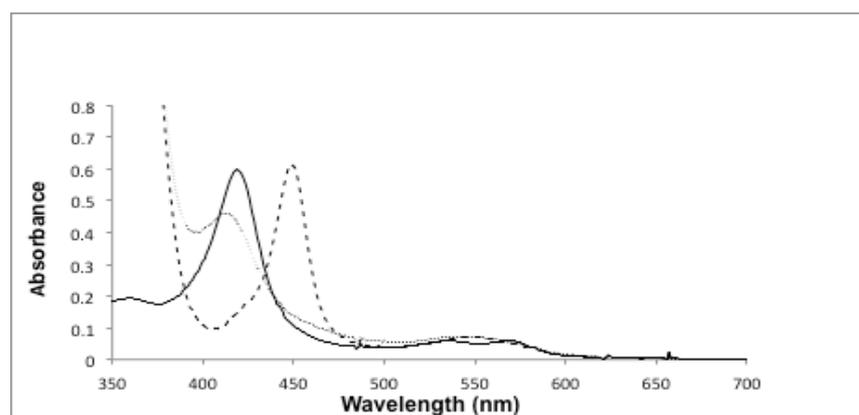


Figure 3

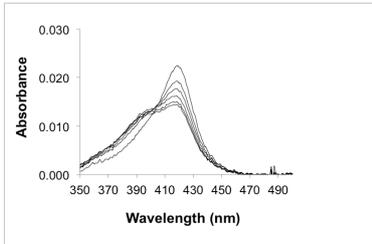
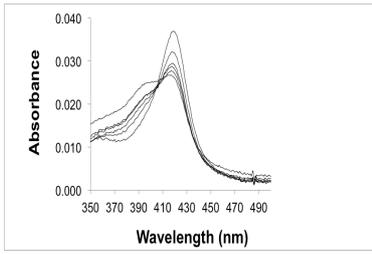
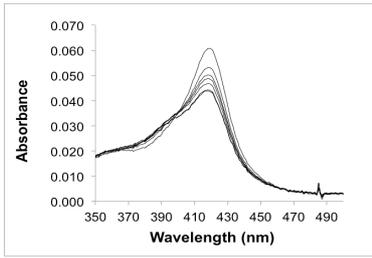
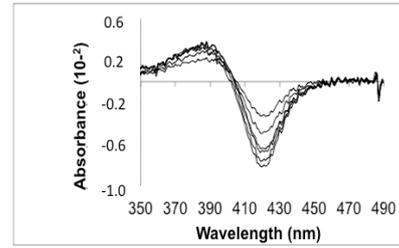
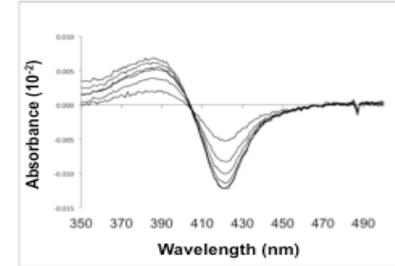
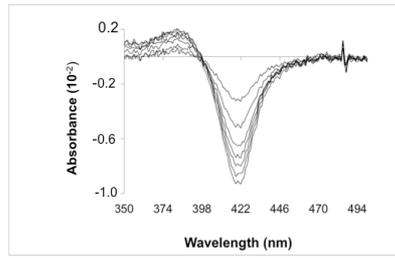
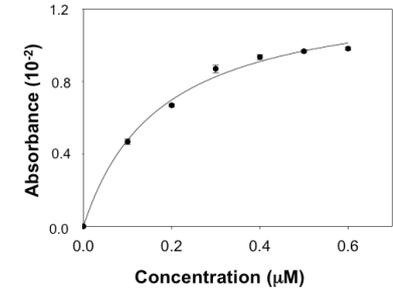
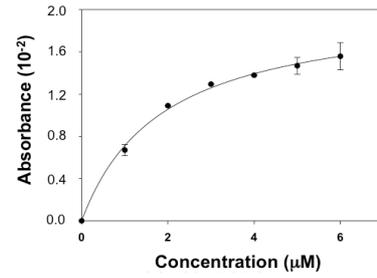
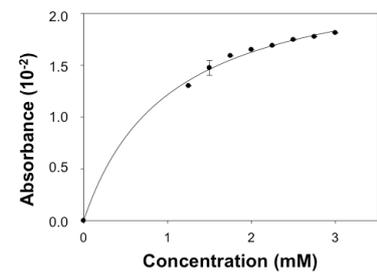


A



B

Figure 4

A**B****C**

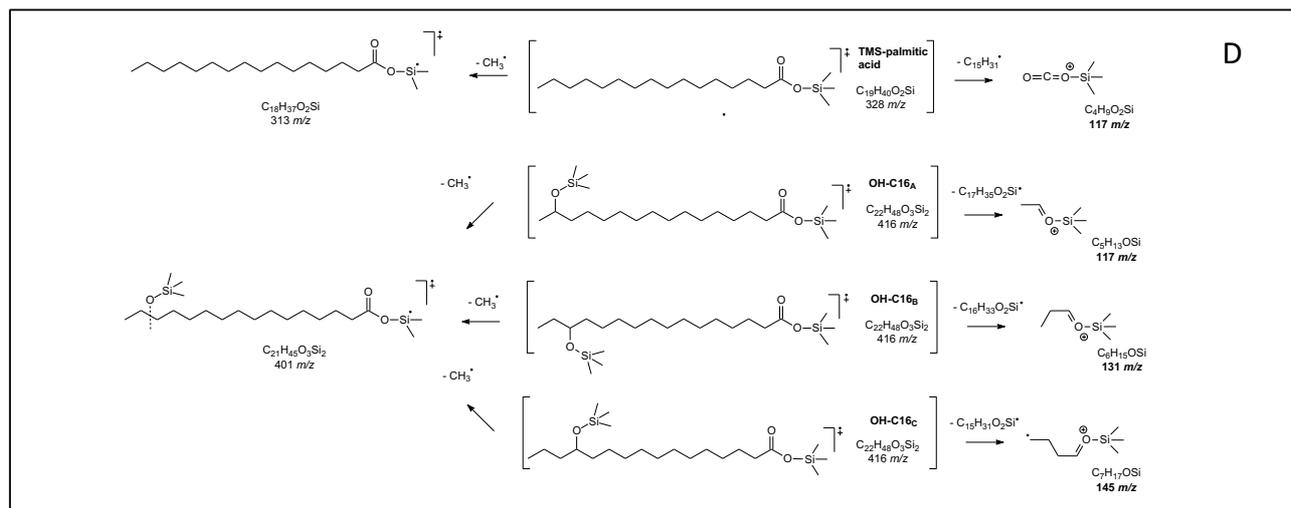
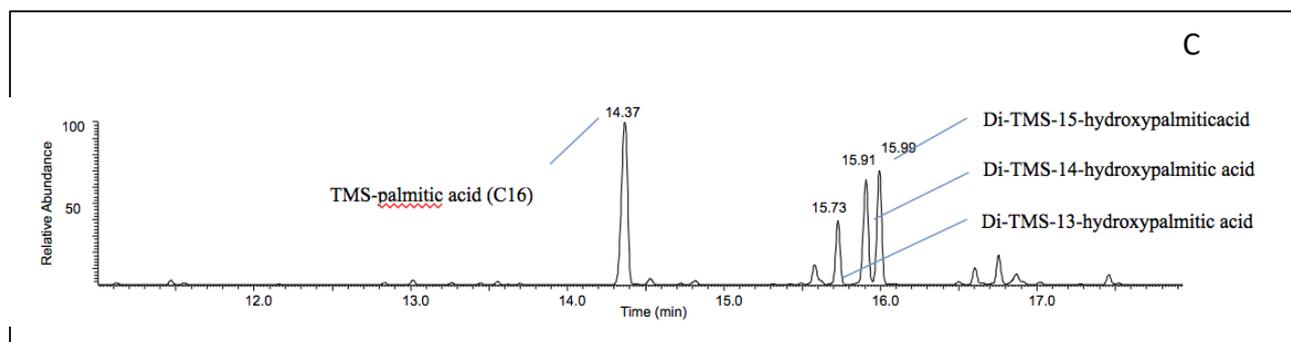


Figure 6

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