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Production of 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid in the fungus *Aspergillus oryzae*: a step towards heterologous production of pyrethrins in fungi

Abstract

Pyrethrins are natural insecticides, which accumulate to high concentrations in pyrethrum (*Chrysanthemum cinerariaefolium*) flowers. Synthetic pyrethroids are more stable, more efficacious and cheaper, but contemporary requirements for safe and environmentally friendly pesticides encourage a return to the use of natural pyrethrins, and this would be favoured by development of an efficient route to their production by microbial fermentation. The biosynthesis of pyrethrins involves ester linkage between an acid moiety (chrysanthemoyl or pyrethroyl, synthesized via the mevalonic acid pathway from glucose), and an alcohol (pyrethrolone). Pyrethrolone is generated from 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid, which originates from α-linolenic acid via the jasmonic acid biosynthetic cascade. The first four genes in this cascade, encoding lipoxygenase 2, allene-oxide synthase, allene-oxide cyclase 2 and 12-oxophytodienoic acid reductase 3, were amplified from an *Arabidopsis thaliana* cDNA library, cloned in a purpose-built fungal multigene expression vector and expressed in *Aspergillus oryzae*. HPLC-MS analysis of the transgenic fungus homogenate gave good evidence for the presence of 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid.

Keywords

3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid; *Aspergillus oryzae***;** Biosynthesis; Heterologous gene expression; Pyrethrins

Introduction

Pyrethrum (*Chrysanthemum cinerariaefolium*) flowers, and the pyrethrins isolated from them, have been used successfully as household insecticides for the last 150 years. Natural pyrethrins, however, are expensive as well as photosensitive, and their production affects ecosystems by occupying agricultural landscapes (1). It is not surprising, therefore, that the natural compounds have been substantially replaced by synthetic pyrethroids, which are more stable, have greater insecticidal efficacy and are cheaper to produce (2), but recent requirements for safe and environmentally friendly pesticides encourage the re-adoption of natural pyrethrins (3). This is unlikely to happen to any extent if production costs remain high, so development of a cheaper production platform, such as microbial fermentation, would be highly desirable.

Filamentous fungi are seen as attractive heterologous production hosts due to their ability to synthesise large amounts of protein, and to perform post-translational modifications on recombinant proteins. The most commonly used filamentous fungal platforms are *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei* (4, 5). *A. oryzae* has been used in the food and fermentation industry for several hundred years, and has GRAS (Generally Regarded As Safe) status (6). Its use as a heterologous expression system for recombinant protein production dates back to 1988, and more recently it has been used for the heterologous production of secondary metabolites (6-8). A useful feature of *A. oryzae* is that its genome has been sequenced (9, 10), and established methods for its genetic manipulation have been highly refined (11, 12). Over a decade of improvements to the *A. oryzae* expression system have made it a versatile and high yielding platform for protein and secondary metabolite production (6, 8, 12, 13). It is currently used in the production of biopharmaceuticals such as antibodies and antimicrobial peptides (14-16), and as a research tool to elucidate biosynthetic

pathways of secondary metabolites such as polyketides (17, 18). It has the potential to be used for cost-effective production of pyrethrins by fermentation.

Natural pyrethrins include six major compounds: pyrethrin I and II, cinerin I and II, and jasmolin I and II (Figure 1A) (2). Their biosynthesis involves ester linkage between an acid moiety (chrysanthemoyl or pyrethroyl) and an alcohol (pyrethrolone) (Figure 1B) (19, 20). Chrysanthemoyl and pyrethroyl moieties are both synthesized via the mevalonic acid pathway from glucose, while pyrethrolone is generated from 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid (OPC8:0) originating from α-linolenic (ALA) acid via the jasmonic acid biosynthetic cascade (Figure 2) (21).

[Place Figure 1]

[Place Figure 2]

This work reports on the ability of transformed cultures of *A. oryzae* to produce OPC8:0, which represents an important step in production of pyrethrolone, the alcoholic moiety of pyrethrins. Production of this pyrethrolone precursor was achieved using genes from the jasmonate biosynthesis cascade in *Arabidopsis thaliana*.

Materials and Methods

Gene isolation from *Arabidopsis thaliana*

The coding regions of the first four genes in the jasmonic acid biosynthetic cascade were amplified from an *Arabidopsis thaliana* cDNA library using Phusion High Fidelity DNA Polymerase (Thermo Scientific). The genes encoding Lipoxygenase 2 (ATLOX2), Allene-oxide synthase (ATAOS), Allene-oxide cyclase 2 (ATAOC2) and 12-oxophytodienoic acid reductase 3 (ATOPR3) (Genbank accession numbers NM_114383, X92510, NM_113476, NM_126619, respectively) were amplified using the primers shown in Table 1 and directionally cloned in pENTR/D-TOPO (Life Technologies). DNA sequencing confirmed that all cloned PCR products were mutation free.

Table 1. Primers used in this work. Those with names commencing with "FUN" have 5' extensions corresponding to the cut ends of the vector (in green) to facilitate cloning of PCR products by heterologous recombination in yeast. Underlining indicates the start or (reverse complement of) stop codons of the coding regions amplified.

Primer Name	Primer Sequence
ATLOX2For	5’ CACCATGTATTGTAGAGAGTCCTTGTC 3’
ATLOX2Rev	5’ AGATCAAATAGAAATACTATAAGG 3’
ATAOSFor	5’ CACCATGGCTTCTATTTCAACCCCTTTT 3’
ATAOSRev	5’ CTCCTAAAAGCTAGCTTTCCTTAAC 3’
ATAOC2For	5’ CACCATGGCTTCTTCAGCAGTGTC 3’
ATAOC2Rev	5’ GAATTAGTTGGTATAGTTACTTAT 3’
ATOPR3For	CACCATGACGGCGGCACAAGGGAACT
ATOPR3Rev	CAATCAGAGGCGGGAAAAAGGAGCC
FUNATLOX2For	5’ TACCCAAGCGCTTCGATTAGGAAGTAACCCACCATGTATTGTAGAGAGTCCTTGTC 3’
FUNATLOX2Rev	5’ TCAGTGTCGAAAGATCCACTAGAGTAAATCAGATCAAATAGAAATACTATAAGG 3’
FUNATAOSFor	5’ TAACAGCTACCCCGCTTGAGCAGACATCACCACCATGGCTTCTATTTCAACCCCTTTT 3’
FUNATAOSRev	5’ GGGAATGCGGCTCCACAGCTGCAGGCGTAGCTCCTAAAAGCTAGCTTTCCTTAAC 3’
FUNATAOC2For	5’ ATCTCAACTCCATCACATCACAATCGATCCCACCATGGCTTCTTCAGCAGTGTC 3’
FUNATAOC2Rev	5’ ATTTCACACCGCATACGTCAAAGCAACCATGAATTAGTTGGTATAGTTACTTAT 3’

Fungal expression vector construction

This followed the strategy described in (12) essentially as follows. Multigene expression vector pTAYAGS3tgo (Figure 3A) was cut downstream of each of the three promoters using *Asc*I, and three of the cloned coding regions (ATLOX2, ATAOS and ATAOC2) were amplified using primers with 5' extensions corresponding to the cut ends of the vector (Table 1). The digested vector and three PCR products were assembled into a single recombinant plasmid by homologous recombination in yeast strain YPH500 (Agilent Technologies). The resultant pTAYAGS3tgoLSC expression plasmid (Figure 3B) was shuttled to *E. coli* for confirmation and amplification and the ATOPR3 coding region was transferred into the *amyB* expression cassette by the Gateway LR recombination reaction (Life Technologies) to produce pTAYAtgoLSCO (Figure 3C).

[Place Figure 3]

Fungal transformation and fermentation

Transformation of *Aspergillus oryzae* M-2-3 with pTAYA-GS3tgoLSCO was carried out as previously described (13). Transformants were selected and sub-cultured on Czapek Dox Agar then on solid DPY medium (2% dextrin, 1% peptone, 0.5% yeast extract, 0.5% potassium dihydrogen orthophosphate, 0.05% magnesium sulphate, 1.5% agar) for further production of spores, which were used to inoculate 100 mL liquid cultures (DPY with or without supplementation with 1 mg α-linolenic acid). Cultures were shaken for 7 days at 200 rpm and 28 °C.

Metabolite extraction and analysis by LCMS

Liquid cultures were homogenised, acidified with 38% HCl to pH 3 and filtered. The supernatant was extracted twice with 70 ml ethyl acetate, and the organic phase dried with anhydrous $MgSO_4$ before evaporation to dryness under reduced pressure. The crude extract was dissolved in 10% aqueous methanol and defatted by extraction with hexane. The

methanolic layer was evaporated to dryness and the extract dissolved to a concentration of 10 mg/mL in HPLC grade methanol. Metabolites were analysed using a Waters Platform Liquid chromatography-mass spectrometry system comprising a Waters 600 pump system, Waters 996 diode array detector (detecting between 210 and 400 nm), Macromass Platform LC mass spectrometer (detecting between 150 and 600 m/z units ESI^+) and Phenomenex Luna 5 µ C 18 (250 ×4.6 mm, 5 µm) reverse phase column with water + 0.05% TFA (solution A) and acetonitrile (solution B) as the mobile phase. The injection volume was 30-50 µl, and elution was done using a non-linear gradient, water/acetonitrile 95/5 % to 5/95 % (0 min, 5% B; 5 min, 5% B; 45 min, 75% B; 46 min, 95% B; 50 min, 95% B; 55 min, 5% B; 60 min, 5%B). Compounds were identified by reference to the Wiley Registry of Mass Spectral Data (10th edition, April 2013), the NIST 11 Mass Spectral Library (NIST11/2011/EPA/NIH) and literature data (22-24).

Results and Discussion

The coding regions of the first four genes required for the biosynthesis of the pyrethrolone moiety of pyrethrins were amplified from an *A. thaliana* cDNA library and cloned. LOX2, AOS, AOC2 and OPR3 are ubiquitous in all plants as the first four enzymes in jasmonate biosynthesis. The coding sequences were inserted into the four expression cassettes of the fungal multigene expression vector pTAYA-GS3tgoLSCO in a two-step process (Figure 3), and used to transform *A. oryzae*. Seven transformants bearing the 4-gene recombinant expression plasmid were obtained and genetically purified by serial sub-culturing on solid selective medium; two were then put into liquid culture for fermentation and extraction of metabolites. LCMS analysis of transgenic fungal extracts, monitored at ES^+ m/z 316 (M^+ + sodium), indicated the presence of a new peak eluting at 9.4 minutes in extracts of both transformants; the data for one transformant and an untransformed (control) culture extract are shown in Figure 4. Submitting the mass spectrum of the newly identified peak to a library search indicated the production of 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0), the expected product of the combined activities of all four transgenes. This finding was also confirmed by comparing the mass spectrum of the new peak with published literature (23, 24). Figure 4B shows that the transgenic lines were capable of producing OPC-8:0 in the absence of added ALA precursor, but supplementing the culture medium with ALA greatly enhanced production of OPC-8:0 (Figure 4C). Optimisation of the system could therefore include increasing the endogenous ALA content above the maximum reported level of about 3.7 mol % (25). Since linoleic acid accounts for about 60% of the fatty acid pool this could be achieved by over-expression of the native Δ^{15} desaturase or of a foreign enzyme such as that encoded by the *Arabidopsis FAD3* gene (26). No OPC-8:0 was apparent in the extract of the untransformed fungus culture, even on supplementation with ALA (Figure 4A).

[Place Figure 4]

Figure 2 shows that cis-jasmone may also be made by an alternative route from 12-oxophytodienoic acid (OPDA), the immediate precursor of OPC-8:0. This route requires isomeration of OPDA followed by β-oxidation of the iso-OPDA product. While no plant OPDA isomerase enzyme has been identified, an insect glutathione S transferase has been described with this activity (27), and thus the possibility exists of investigating this alternative route by substituting the oxophytodienoic acid reductase (OPR) gene with one for OPDA isomerase. However the expected product would be much more difficult to detect. In contrast our deployment of OPR has yielded the easily detectable OPC-8:0, whose existence testifies to the prior production of the OPDA substrate, and goes a step further along the route to cis-jasmone via the established route.

Successful expression of the four selected genes represents an important step towards heterologous production of pyrethrolone by microbial fermentation. The next step will involve the conversion of OPC-8:0 to *cis*-jasmone or 7-hydroxy-jasmonic acid (19) by simple decarboxylation with β-oxidation resulting in chain shortening (28). However, while production of pyrethrolone from *cis*-jasmone or 7-hydroxy-jasmonic acid should involve simple decarboxylation and terminal unsaturation, the enzyme(s) involved have not been identified. Different natural pyrethrins can be made via esterification between pyrethrolone and chrysanthemyl moieties, and it should be possible to produce a chrysanthemoyl moiety by heterologous expression of the chrysanthemyl diphosphate synthase gene isolated previously from the pyrethrum flower (29).

In summary, current global environmental sentiments favour the re-use of natural pyrethrin insecticides over their synthetic pyrethroid counterparts. Thus the low production level of pyrethrins in the natural host, the pyrethrum flower, encourages the idea of producing them heterologously in a fungal host using DNA recombination and metabolic engineering tools. The first four genes responsible for jasmonic acid biosynthesis in *A. thaliana* were expressed

in *A. oryzae* to OPC-8:0, a precursor of the alcoholic moiety of natural pyrethrins. The production of natural pyrethrins in a fungal platform could provide a commercially viable alternative source of these valuable pesticides and offers an approach to protect the environment and plant ecosystems.

Acknowledgment

Conflict of interests

The author declare no conflict of interests

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List of Figures:

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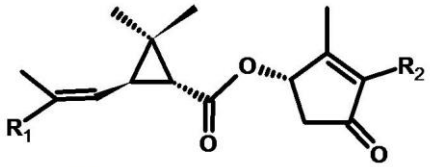
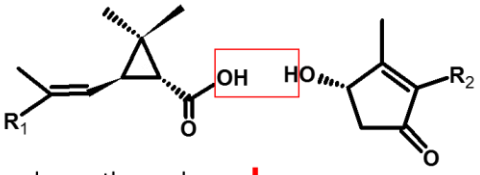


R_1	R_2	Name
CH_3		Pyrethrin I
CH_3		Jasmolin I
CH_3		Cinerin I
CH_3COO-		Pyrethrin II
CH_3COO-		Jasmolin II
CH_3COO-		Cinerin II

B

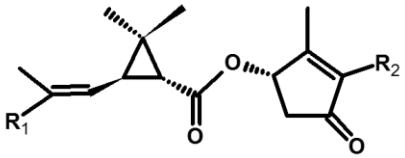


chrysanthemoyl or pyrethroyl (acid moiety)

Pyrethrolone (alcohol)

Synthesized via the mevalonic acid pathway from glucose

Synthesized from 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid, which originates from α-linolenic acid via the jasmonic acid biosynthetic cascade



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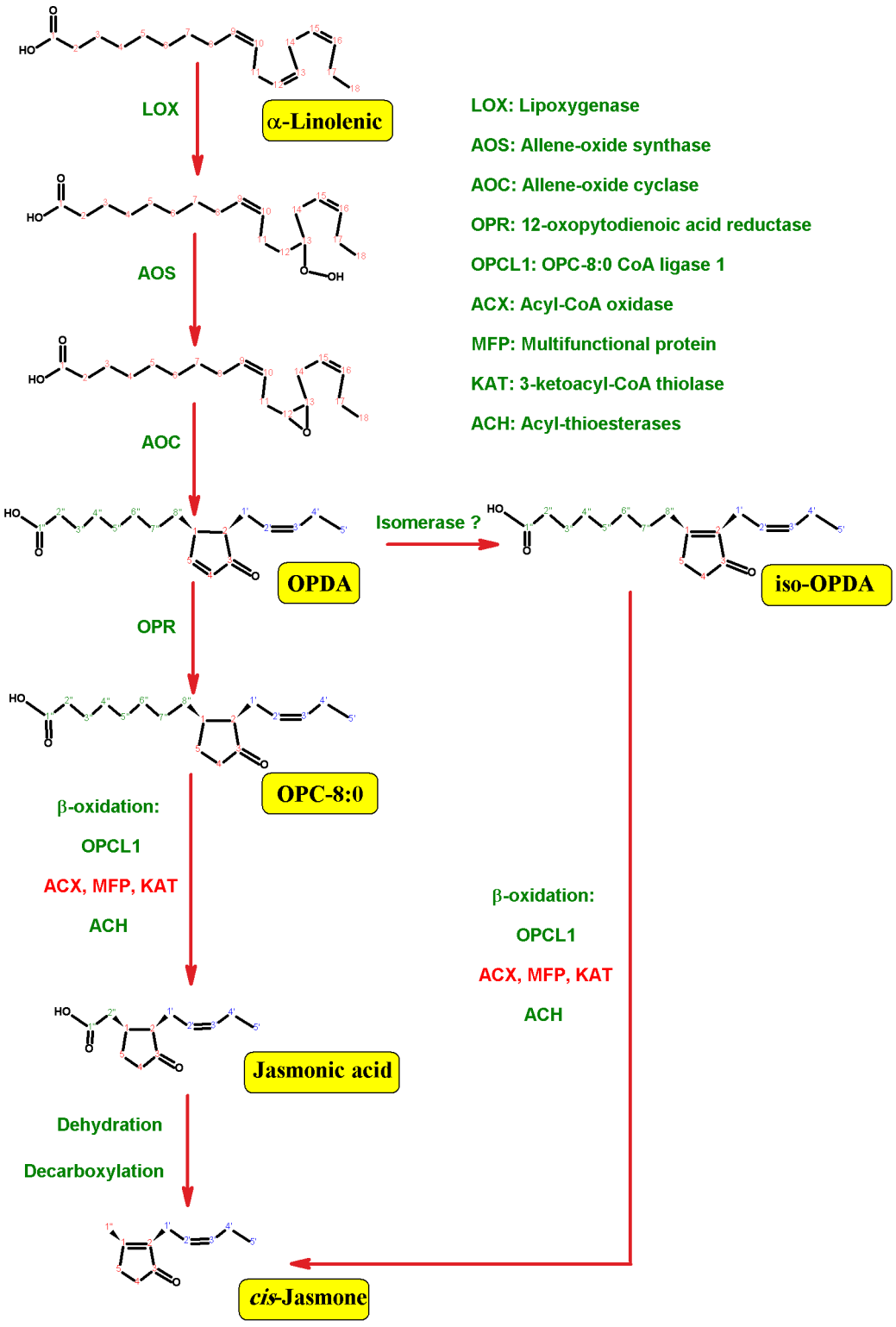
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α-Linolenic
AOS
AOC
OPDA
Isomerase ?
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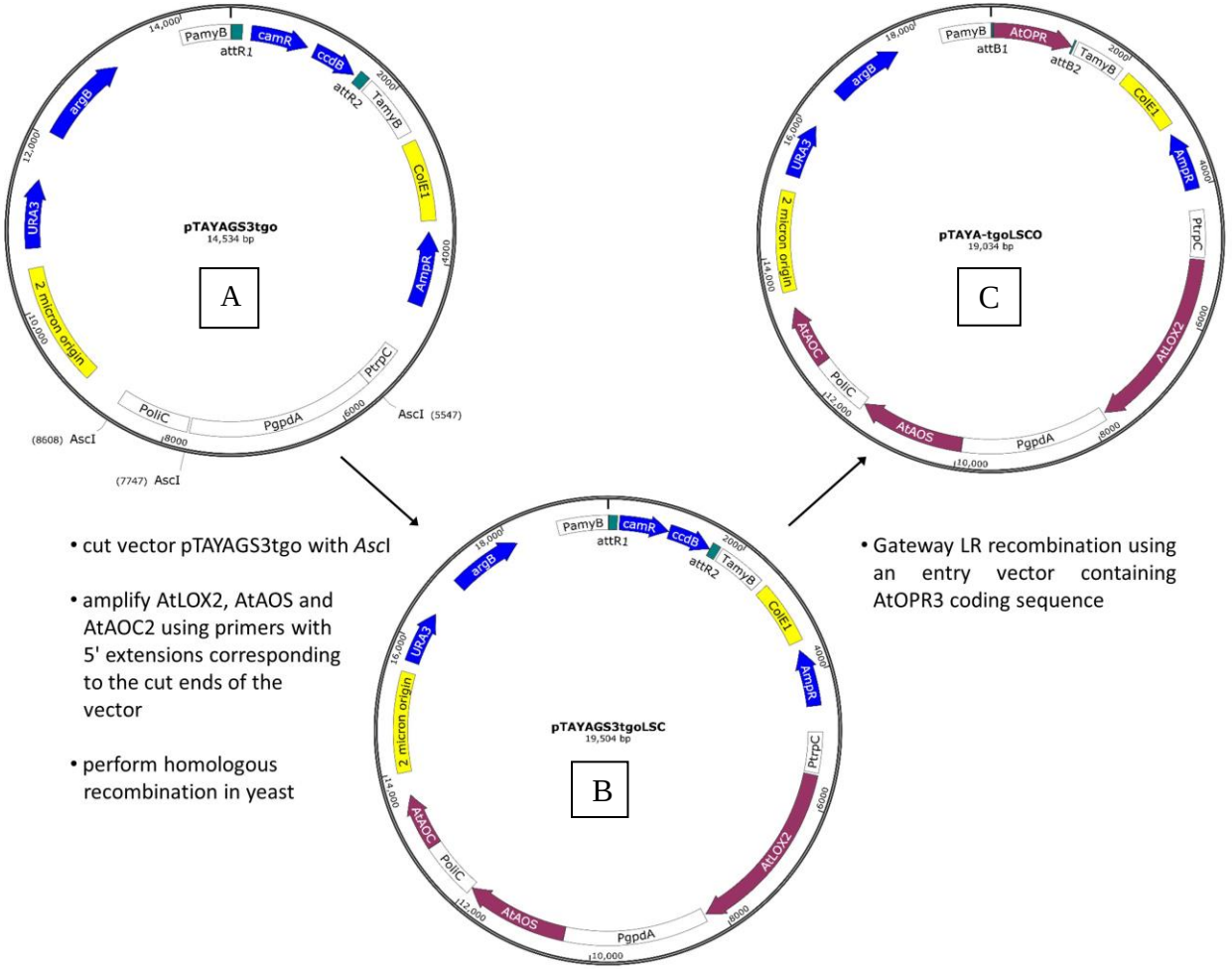
OPR
OPC-8:0
β-oxidation:
OPCL1
ACX, MFP, KAT
ACH
Jasmonic acid
Dehydration
Decarboxylation
cis-Jasmone
β-oxidation:
OPCL1
ACX, MFP, KAT
ACH
LOX: Lipoxygenase
AOS: Allene-oxide synthase
AOC: Allene-oxide cyclase
OPR: 12-oxopytodienoic acid reductase
OPCL1: OPC-8:0 CoA ligase 1
ACX: Acyl-CoA oxidase
MFP: Multifunctional protein
KAT: 3-ketoacyl-CoA thiolase
ACH: Acyl-thioesterases

Figure 2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attB2
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AscI (5547)
• cut vector pTAYAGS3tgo with AscI
• amplify AtLOX2, AtAOS and AtAOC2 using primers with 5' extensions corresponding to the cut ends of the vector
• perform homologous recombination in yeast
• Gateway LR recombination using an entry vector containing AtOPR3 coding sequence

Figure 3

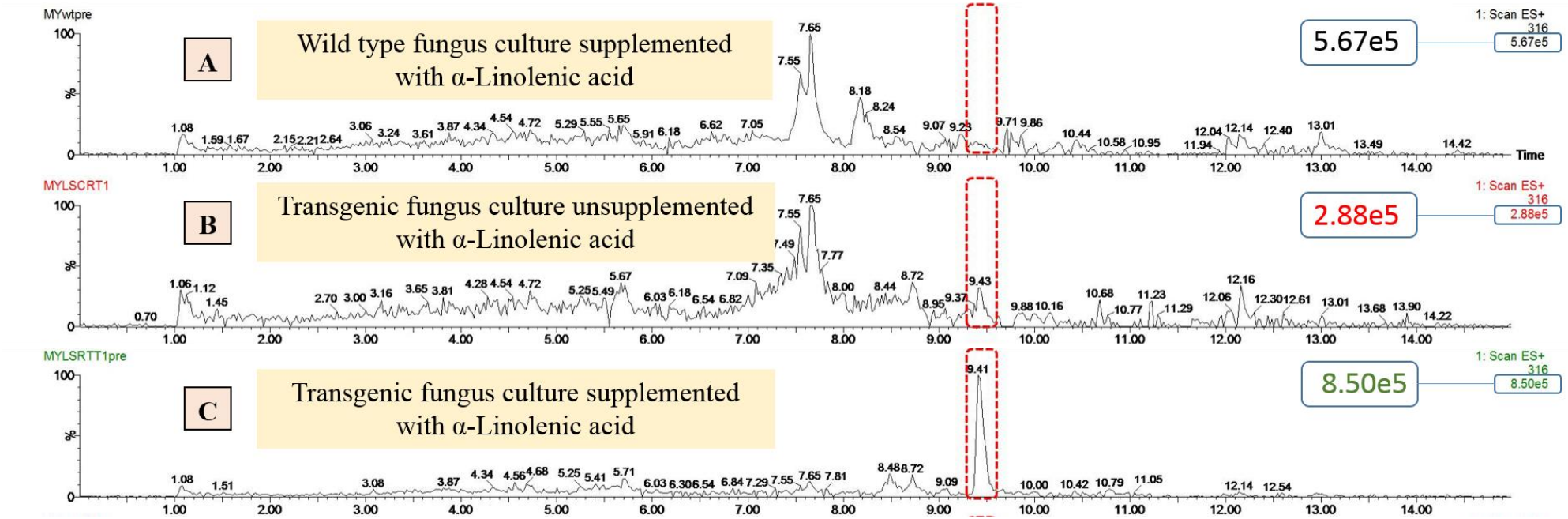
MYwtpre
A
Wild type fungus culture supplemented with α-Linolenic acid
1: Scan ES+ 316
5.67e5
MYLSCRT1
B
Transgenic fungus culture unsupplemented with α-Linolenic acid
1: Scan ES+ 316
2.88e5
MYLSRTT1pre
C
Transgenic fungus culture supplemented with α-Linolenic acid
1: Scan ES+ 316
8.50e5
Time

Figure 4