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HIGHLIGHT

Heterologous biosynthesis of saponin adjuvants from *Quillaja saponaria*: A symbolic achievement in metabolic engineering

KEY WORDS

QS-21;
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Plant natural products (PNPs);
Heterologous biosynthesis

Heterologous production of pharmaceutically important plant natural products (PNPs) in heterologous hosts is an attractive strategy for the sustainable use of the resources bestowed by nature. Over the past two decades, a plethora of structurally divergent valuable PNPs including alkaloids, terpenoids, and phenylpropanoids have been synthesized in heterologous hosts *via* manipulating endogenous and/or heterologous genes that range from single digit to dozens[1,2]. Recently, the groundbreaking studies conducted by Anne Osbourn and Jay D. Keasling's research groups[3,4,5] unveiled a remarkable achievement in the field of PNP biosynthesis. Their works, published in *Science*[3], *Nature Chemical Biology*[4], and *Nature*[5], showcase the successful production of triterpene glycoside (saponins) adjuvants derived from the bark of *Quillaja saponaria* in *Saccharomyces cerevisiae* and *Nicotiana benthamiana*. QS-21 and QS-7 (21st and 7th chromatographic fraction, respectively) are the only clinically approved saponin-based human vaccine adjuvants that have been employed in the formulation of vaccines for shingles, malaria, and COVID-19[6]. QS-21, a fraction of aqueous extract that accounts for 0.0032% biomass of a 30 to 50-year-old tree, is mainly comprised of two structurally similar isoforms, QS-21-Api and QS-21-Xyl[5]. Both comprise a triterpene core quillaic acid (QA), a branched trisaccharide group at C-3, a linear tetrasaccharide group at C-28, and an L-arabinofuranose capped C_{18} acyl chain that connects with the first D-fucopyranose of the C-28 sugar chain. The D-apiofuranose or D-xylopyranose at the terminal of the C-28 sugar chain defines QS-21-Api or QS-21-Xyl, respectively[3,4]. The C_{18} acyl chain is specific to the *Quillaja* species and is indispensable to stimulate cytotoxic T cell proliferation[7]. The QS-7 is similar to QS-21-Api but has an acetyl group rather than an L-arabinofuranose-capped C_{18} acyl chain and two additional sugar groups on the C-28 sugar chain.

The biosynthetic pathways of the two saponins (Fig. 1) were dissected through transient expression assays in *N. benthamiana* by Anne Osbourn's group[3,4] which resulted in two papers published in *Science* and *Nature Chemical Biology*. The core structure is the QA, which is derived from six steps of oxidation mediated by three cytochrome P450 monooxygenases at C-16α, C-23, and C-28 positions of β-amyrin, one of the core skeletons of triterpenoids that is derived from cyclization of 2,3- oxidosqualene by β-amyrin synthases (BAS). For the addition of the C-3 sugar chain, two cellulose synthase-like proteins, CSLM1 and CSLM2, are capable of adding glucuronic acid at C-3 of QA to yield QA-Mono. UGT73CU3 and UGT73CX1 carry out, respectively, the addition of the second galactose and the third D-xylose moieties to give QA-TriX. The C-28 linear sugar chain is added by sequential catalysis of UGT74BX1, UGT91AR1, and UGT91AQ1, along with the help of a short-chain dehydrogenase/reductase that facilitates D-fucosylation by reduction of the added 4-keto-6-deoxy-D-glucose at C-28, to produce QA-TriX-FRX. D-xylopyranose or D-apiofuranose is added, respectively, by UGT73CY2 or UGT73CY3, to finally produce QA-TriX-FRXX (intermediate of QS-21-Xyl) or QA-TriX-FRXA (intermediate of QS-21-Api and QS-7)[3]. Co-expression of *QsACT1*, *UGT73B44*, and *UGT91AP1* with QA-TriX-FRXA biosynthetic genes produces the QS-7 at 7.9 μg/g dry leaf weight[3]. The addition of the L-arabinofuranose-capped C_{18} acyl chain needs at

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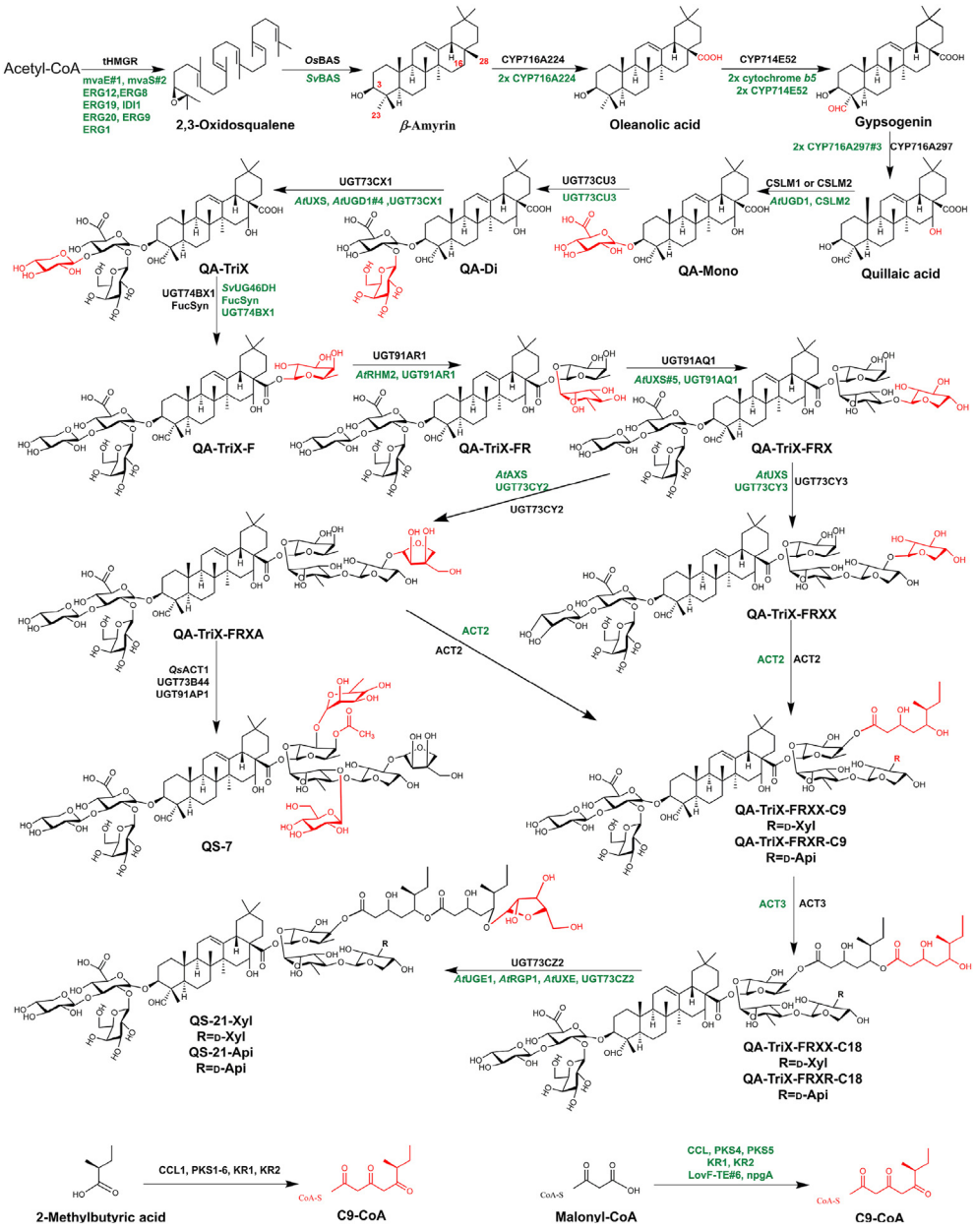

Figure 1 Biosynthetic route of QS-21 and QS-7. Genes in black are synthetic genes used for QS-21 or QS-7 production in tobacco by Anne Osbourn's group. Genes in green are engineered genes for the biosynthesis of QS-21 in yeast. Two copies of P450s redox partner gene *AtATR1* and two copies of membrane steroid-binding protein gene *Sv*MSBP1 were used in QS-21 producing strain but are not shown in the figure. *Sv*: *Saponaria vaccaria*. *At*: *Arabidopsis thaliana*. BAS: β-amyrin synthase. 2 × : two copies of gene insertion. *At*UGD: UDP-glucose dehydrogenase. CSLM2: 3-UDP-glucuronic acid transferase 2. *At*UXS: UDP-xylose synthase. FucSyn: short-chain dehydrogenase/reductase. *Sv*UG46DH: UDP-glucose 4,6-dehydratase. *At*RHM2: trifunctional UDP-glucose 4,6-dehydratase/UDP-4-keto-6-deoxy-D-glucose 3,5-epimerase/UDP-4-keto-L-rhamnose-reductase 2. *At*AXS: UDP-D-apiose/UDP-D-xylose synthase. ACT 2/3: Acyl transferase 2/3. *At*UGE1: Bifunctional UDP-D-glucose/UDP-D-galactose 4-epimerase 1. *At*RCP1: reversibly glycosylated polypeptide 1. CCL1: short-chain fatty acid CoA ligase 1. PKS: chalcone-synthase-like enzymes. KR: keto reductases. npgA: 4'-phospho-pantetheinyl transferase from *Aspergillus nidulans FGSC A4*. #1: codon optimized ERG10. #2: mvaS with an A110G mutation. #3: a 22-amino-acid transmembrane domain was fused at the N terminus of CYP716A297. #4: *At*UGD1 with a A101L mutation to reduce feedback inhibition of UDP-D-xylose. #5: a second copy of *At*UXS is necessary for providing enough UDP-D-xylose. #6: fusion protein comprised of truncated LovF from *Aspergillus terreus* and M6 thioesterase.

least the action of seven enzymes. Carboxyl-CoA ligase 1 (CCL1) converts 2-methylbutyric acid to (*S*)-2-methylbutyryl-CoA (2-MB-CoA); Type III polyketide synthases (PKS1-PKS6) and ketoreductases (KR1 and KR2) are evidenced in the formation of dimeric C_9 acyl chains from 2-MB-CoA and malonyl-CoA. Then, BAHD acyl transferases (ATC2 and ATC3) and UGT73CZ2 complete the addition of L-arabinofuranose-capped C_{18} acyl chain[4]. Therefore, transient co-expression of 19 *Q. saponaria* genes along with expression of a 3-hydroxy-3-methylglutaryl-CoA reductase to boost 2,3-oxidosqualene

supply and a mutated threonine deaminase to enhance L-isoleucine level enables producing one of the QS-21 isoforms in *N. benthamiana* at 8.6 μg/g dry leaf weight[4].

Based on the structural genes described above, Jay D. Keasling's group[5] performed extensive build and test works to construct QS-21-producing yeast (Fig. 1). First, aside from boosting the upstream 2,3-oxidosqualene biosynthetic pathway by overexpressing yeast endogenous genes, BASs from different plant species were tested, in which *Sv*BAS from *Saponaria vaccaria* gave the highest titer of 899.0 mg/L of *β*-amyrin. Second, insertion two copies of CYP450s, CYP450 redox partner, cytochrome *b5* reductase, and membrane steroid-binding protein 1 (MSBP1), coupled with directing CYP716A297 to endoplasmic reticulum membrane significantly enhanced the production of QA. Notably, the strategy of using MSBPs as a scaffold for promoting P450s activities increased QA production by four-fold. Third, glycosylation of QA and downstream intermediates requires a total of seven UDP-sugars as donors. Whereas, yeast only produces UDP-D-glucose and UDP-D-galactose that need to be converted into other UDP-sugars by expression of nucleotide sugar synthases from *Arabidopsis thaliana*. Moreover, the A101L mutation of UDP-glucose dehydrogenase effectively reduced the feedback inhibition of UDP-D-xylose. Fourth, due to the lack of a post-translational modification mechanism in yeast, complicated production of 2-MB-CoA was performed by expressing phosphopantetheinyl transferase and a fusion protein comprised of truncated type I PKS protein F and M6 thioesterase. At length, the incorporation of 38 heterologous genes, the longest known synthetic pathway expressed in yeast, enables the production of QS-21 at a yield of 0.0012% (*w*/*w*) from galactose, which is considerably faster than its production in *Q. saponaria*[5].

Producing pharmaceutically important PNPs in microbial cells is no doubt a promising strategy for sustainable and green production. With the accumulation of big bio-data, more PNP biosynthetic pathways will soon be revealed and, of course, will be tested in heterologous hosts. However, rather than demonstrating their biosynthetic abilities in heterologous hosts, scaled and economically viable production is the ultimate objective. The reasons engineered strains fail to bridge the "Valley of Death" could be due to many aspects[8], economic advantages between the cost of microbial production and natural extraction or chemical synthesis, input in constructing strains, and yield of the end products are three key factors. Taking QS-21 as an example, aside from trace presence and their chemical complexity, there are over 100 structurally similar saponins in the *Q. saponaria*, making its purification laborious and low-yielding. Consequently, the market price of QS-21 is over several million US dollars per kilogram. However, one liter of engineered yeast strain can produce about 100 mg QS-21 in a few days, the cost is cheaper than its purification from plant and chemical synthesis, presenting a viable opportunity for commercialization. Input in constructing the QS-21-producing strain includes the labor and intellectual costs in gene mining, functional characterization of synthetic genes, reconstruction of the synthetic pathway in yeast cells, and the licenses of technologies like the CRISPR/Cas9 that are used for integration of the heterologous genes. With respect to the yield, although the QS-21 rate of production in yeast is a thousand times faster than in its native plants, optimizing downstream fermentation processes, augmenting the pool of acetyl-CoA, enzyme engineering for directing metabolic flux toward final products, employment of transporting proteins to secret PNPs to extracellular spaces and even testing new hosts for QS-21 biosynthesis are all possible strategies to further enhance production of QS-21 in microbial cells. Alternatively, as QS-21 could be readily synthesized in tobacco cells through transient expression[4], engineering the pathway in stably transformed plants is also triable. For what it's worth, the work on QS saponins stands as a milestone in the field of metabolic engineering.

Author contributions

Weiqiang Chen: Writing − original draft, Conceptualization. Zhichao Xu: Writing − review & editing, Conceptualization. Wei Sun: Writing − review & editing, Conceptualization.

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