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# An endoplasmic reticulum-engineered yeast platform for overproduction of triterpenoids

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ABSTRACT

Saponins are a structurally diverse family of triterpenes that are widely found as main constituents in many traditional plant-based medicines and often have bioactivities of industrial interest. The heterologous production of triterpene saponins in microbes remains challenging and only limited successful pathway engineering endeavors have been reported. To improve the production capacities of a *Saccharomyces cerevisiae* saponin production platform, we assessed the effects of several hitherto unexplored gene knockout targets on the heterologous production of triterpenoids. Here, we show that the disruption of the phosphatidic acid phosphatase-encoding *PAH1* through CRISPR/Cas9 results in a dramatic expansion of the endoplasmic reticulum (ER), which stimulated the production of recombinant triterpene biosynthesis enzymes and ultimately boosted triterpenoid and triterpene saponin accumulation. Compared to the wild-type starter strain, accumulation of the oleanane-type sapogenin β-amyrin, of its oxidized derivative medicagenic acid, and its glucosylated version medicagenic-28-*O*-glucoside was respectively increased by eight-, six- and 16-fold in the *pah1* strain. A positive effect of *pah1* could also be observed for the production of other terpenoids depending on ER-associated enzymes for their biosynthesis, such as the sesquiterpenoid artemisinic acid, which increased by twofold relative to the wild-type strain. Hence, this report demonstrates that pathway engineering in yeast through transforming the subcellular morphology rather than altering metabolic fluxes is a powerful strategy to increase yields of bioactive plant-derived products in heterologous hosts.

## 1. Introduction

Saponins are members of the triterpene subfamily of terpenoids and are synthesized in plants via the mevalonic acid (MVA) pathway. Analogous to the biosynthesis of the membrane steroids cholesterol and ergosterol, the first committed step in the biosynthesis of saponins is the cyclization of 2,3-oxidosqualene by oxidosqualene cyclases (OSCs) to a variety of tri-, tetra-, or pentacyclic structures. These triterpene skeletons subsequently undergo various functionalizations such as cytochrome P450-mediated oxygenation, which serve as anchor points for subsequent conjugations such as glycosylation through UDP-dependent glycosyltransferases (UGTs), thereby rendering the highly apolar compounds amphipathic (Seki et al., 2015; Thimmappa et al., 2014).

Many saponins and their aglycones, sapogenins, exhibit valuable pharmacological properties but in many cases they accumulate only in little amounts in their natural hosts. Their purification is challenging due to the complex plant metabolome with hundreds of compounds with similar chemical properties. Furthermore, due to the complex structure and chirality of many triterpenoids, their synthesis by chemical means is not trivial. An attractive alternative is the production in heterologous microbial hosts as these are easy to engineer, inexpen-

*Abbreviations:* AaADS, *Artemisia annua* amorpha-4,11-diene synthase; AaCPR, *Artemisia annua* cytochrome P450 reductase; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; ERG, ergosterol; FPP, farnesyl diphosphate; GgbAS, *Glycyrrhiza glabra* β-amyrin synthase; gRNA, guide RNA; HMGR, HMG-CoA reductase; HR, homologous recombination; HRD, HMG-CoA reductase degradation; KO, knockout; MtCPR, *Medicago truncatula* cytochrome P450 reductase; MVA, mevalonic acid; PAH, phosphatidic acid phosphatase; PRB, proteinase B; OSC, oxidosqualene cyclase; RFLP, restriction fragment length polymorphism; SD medium, synthetic defined medium; TEM, transmission electron microscopy; UGT, UDP-dependent glycosyltransferase; WT, wild type

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sive to grow and generally display a simpler metabolome complexity. Especially the budding yeast *Saccharomyces cerevisiae* has emerged as the work horse for terpenoid engineering and a semi-synthetic yeast platform for the synthesis of the important anti-malarial drug artemisinin is currently the flag ship of metabolic engineering (Paddon et al., 2013; Peplow, 2013; Ro et al., 2006). *S. cerevisiae* is especially interesting for the synthesis of triterpenoids because, as a eukaryote, it possesses an endoplasmic reticulum (ER) that allows the heterologous expression of membrane-localized cytochrome P450 enzymes (P450s).

Classical metabolic engineering efforts for terpenoid-producing yeasts generally focus on boosting the flux through the MVA pathway. This can be achieved by deregulation of the MVA gate keeper, HMG-CoA reductase (HMGR), which is subject to regulation on multiple levels (Burg and Espenshade, 2011). In case of yeast, the regulation of the two HMGR isoforms can be overcome by overexpression of a truncated form of Hmg1p (*tHMG1*) or a mutated form of Hmg2p (*HMG2K6R*). Likewise, the overexpression of a mutated version of the sterol transcription factor *UPC2* (*upc2-1*) leads to the upregulation of most *ERG* (ergosterol) genes and as such increases the metabolic flux through the MVA pathway (Davies et al., 2005; Ro et al., 2006; Shiba et al., 2007; Westfall et al., 2012). Finally, endogenous promoters of competing pathway branches, such as squalene synthase (*ERG9*) for sesquiterpenoids or lanosterol synthase (*ERG7*) for triterpenes, can be replaced with repressible promoters such as the methionine-regulated $P_{MET3}$ (Kirby et al., 2008; Moses et al., 2014; Ro et al., 2006).

Despite these universal engineering strategies, the microbial production of most terpenoids does not reach the optimal theoretical yields, and hence, new strategies and tools, such as new targets for gene knockout (KO) are desired for metabolic engineering programs. Such new targets can be identified through the screening of carotenoid-producing yeast KO collections in which general carotenoid overproducers can be identified through a change of the color spectrum. However, the selection of such novel gene KOs may be biased to the production of carotenoids while only having little effect on the production of other terpenoid classes as shown for the sesquiterpenoid bisabolene (Özaydın et al., 2013; Trikka et al., 2015). Here, we identified new, rationally selected gene KO targets that specifically increase the capacity of a yeast triterpene production platform. As a first target for gene KO, we selected the E3 ligase-encoding *HRD1* of the HMG-CoA reductase degradation (HRD) pathway, which is involved in the endoplasmic reticulum-associated degradation (ERAD) of *HMGR* (Brodsky and Skach, 2011; Hampton and Garza, 2009; Ignea et al., 2012). As a second target, we chose the *PEP4*-encoded proteinase A which is responsible for the maturation of various vacuolar peptidases (Parr et al., 2007). A KO of *PEP4* has been reported to have a positive effect on the stability of heterologous proteins in yeast in a number of studies (Liao et al., 2005; Oka et al., 2007). As a third target we chose *PAH1*, which encodes phosphatidic acid phosphatase that generates neutral triglycerides from phosphatidic acid in yeast (Han et al., 2006). KOs of *PAH1* display severe morphological changes such as the drastic proliferation of the outer nuclear membrane and the ER, which have been successfully employed for the functional overproduction of ER-localized proteins (Guerfal et al., 2013; Santos-Rosa et al., 2005). Here we show that when the *PAH1* KO was combined with heterologous expression of plant terpenoid biosynthesis enzymes, the production of all tri- and sesquiterpenoid molecules tested was boosted.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals and solvents were purchased from Sigma-Aldrich unless specified otherwise. The artemisinic acid standard was a kind gift from Dieter Deforce (Department of Pharmaceutics, Ghent University).

### 2.2. Construction of the advanced CRISPR vector pCASmGG

To facilitate the cloning of protospacer sequences for CRISPR/Cas9 experiments, we adapted the vector pCAS-ccdB (Miettinen et al., 2017) for GoldenGate cloning. To this end, the three *Sap*I recognition sites in the vector backbone were mutagenized by PCR-amplification of pCAS1 using primer pairs P1+P2, P3+P4, and P5+P6 (Table 1), each designed to mutagenize the *Sap*I recognition sites. The three fragments were *Sap*I-treated, gel-purified, and ligated, thereby yielding pCASm. Subsequently, pCASm-ccdB was generated by cloning the Gateway™ ccdB cassette into pCASm as described previously (Miettinen et al., 2017). The gRNA-GoldenGate cassette was generated by PCR-amplifying SNR52p and crRNA-SUP4t from p426-SNR52p-gRNA.CAN1.Y-SUP4t (DiCarlo et al., 2013) using primer pairs P7+P9 and P10+P8, respectively. A lacZ cassette was PCR-amplified from pICH47751 (Addgene #48002) using primers P11+P12. Then, the three fragments were joined by overlap extension PCR, subcloned into pDONR221, and finally recombined into pCASm-ccdB, thereby creating pCASmGG.

### 2.3. Cloning of protospacer sequences into pCAS-ccdB and pCASmGG

Single guide RNA (gRNA) cassettes were generated using the generic primer pair P7+P8 in combination with primer pairs P13+P14 and P15+P16 for generation of *PEP4* and *HRD1* KO cassettes, respectively, as described previously (Miettinen et al., 2017). Functional CRISPR plasmids based on the advanced CRISPR vector pCASmGG were generated by GoldenGate cloning of short fragments comprising the protospacer sequences with a 5′ ATC and a 3′ TAA overhang. Fragments were made by heating a solution of two oligonucleotides at a concentration of 20 μM in 1× buffer C (Promega) in a boiling water bath for 5 min and slowly cooling down to room temperature. The solution was diluted 100-fold in 1× buffer C and 1 μM was used for a standard GoldenGate reaction with 100 ng pCASmGG, T4 ligase (Thermo Fisher Scientific), and the type IIS restriction enzyme *Sap*I (New England Biolabs). The reaction mixture was incubated at 20 °C and 37 °C for 5 min each for 30 cycles with final incubations at 50 °C and 80 °C for 10 min each. The reaction mixture was used to transform *E. coli* cells that were subsequently plated on LB plates containing appropriate amounts of carbenicillin, IPTG, and X-Gal. Positive colonies were identified by blue-white selection and selected plasmids were analyzed by control digest and Sanger sequencing. Oligonucleotides used for cloning are listed in Table 1.

### 2.4. Construction of expression vectors

Expression vectors (Table 2) were mostly generated by Gateway™-recombination of available in-house entry clones with destination vectors (Alberti et al., 2007) (Addgene Kit #1000000011). For the construction of the vector expressing the *M. truncatula* medicagenic acid genes as self-splicing polyproteins, CYP72A67 and CYP72A68 were first PCR-amplified from plasmid DNA using primer pairs P31+P32 and P33+P34. The fragments were joined by overlap extension PCR using primers P31+P34. Both the fragment and pESC-LEU (Agilent) were cut with *Not*I and *Bgl*II (Promega) and ligated using T4 ligase (Thermo Fisher), thereby generating pESC-LEU[*GAL1/CYP72A67-E2A-CYP72A68*]. Then, MtCPR1 and CYP716A12 were amplified from plasmid DNA using primer combinations P35+P36 and P37+P38, respectively. Fragments were joined using primers P35+P38, cut with *Sal*I and *Xho*I (Promega) and cloned into the intermediate vector. The resulting vector, pESC-LEU[*GAL1/MtCPR1-T2A-CYP716A12; GAL10/CYP72A67-E2A-CYP72A68*], was verified by Sanger sequencing and control digest. To generate expression vectors for C-terminal HA-peptide tagged proteins, *CYP716A12* and *GgbAS* were PCR-amplified without stop codon using the primer pairs P39+P40 and P41+P42, respectively, and Gateway™ recombined into the donor vector pDONR221. The sequence-verified entry clones were