# Rapid, Whole-cell Engineering of Plant Alkaloid Biosynthesis in Yeast Using Twist Gene Fragments


## ABSTRACT

Plant-derived medicines are prone to shortages because their agriculture-based supply chains are vulnerable to geopolitical and environmental factors. Recreating the biosynthesis of these medicines in a scalable organism like Baker's yeast (*Saccharomyces cerevisiae*) would provide an economical alternative to plant cultivation that is resilient to regional and global disruptions and scalable to meet global demands. This application note showcases the engineering of Baker's yeast to synthesize the tropane alkaloids hyoscyamine and scopolamine, two neuromuscular agents typically cultivated from nightshade plants. Whole-cell engineering, including the incorporation of 34 chromosomal modifications, leveraged Twist Gene Fragments to supply cloning-ready heterologous genes for rapid screening and integration into yeast, removing a critical bottleneck in the engineering workflow.


## INTRODUCTION

Plant-derived pharmacoactive compounds represent a substantial component of modern healthcare, but their existing production methods are inefficient, unsustainable, and prone to environmental disruption. In addition, medicinal plants are difficult to cultivate, requiring precise growth conditions and considerable management to obtain satisfactory yields of the desired compound. Even so, growth can be slow, requiring extensive land use to ensure supply can meet demand.

Scopolamine and its chemical precursor hyoscyamine illustrate the cultivation issue in plant-derived compounds. Due to its general use as anti-nausea and anti-salival medicine, the World Health Organization cites scopolamine on its list of essential medicines (WHO, 2019). Hyoscyamine is prescribed to relieve spasmodic symptoms in lower abdominal disorders. Both compounds are cultivated from *Duboisia* shrubs endemic to Australia, where wildfires occur seasonally, leading to shortages (Ullrich, Hagels, & Kayser, 2017). Chemically synthesizing these medicines is not a viable alternative due to their challenging stereochemistries. Thus, alternative strategies for economically producing plant-derived drugs like scopolamine and hyoscyamine are desperately needed to mitigate the public health risk imposed by vulnerable, agriculture-based supply chains.

Heterologous production of plant-based medicines is an alternative to agriculture cultivation. Heterologous production aims to transfer the plant biosynthetic pathway to a more tractable organism, like *Escherichia coli* and *Saccharomyces cerevisiae*. In particular, *S. cerevisiae* represents an attractive host for economic heterologous production owing to its rapid proliferation during fermentation. Also, compared to other scalable unicellular organisms like *E. coli*, *S. cerevisiae* is more similar to plants in cell structure (e.g., membranous compartmentalization) and metabolism (Kotopka, Li, & Smolke, 2018).

Historically, heterologous production has been handicapped by a lack of biological insight and technological capability. Without access to the DNA synthesis, manipulation, and sequencing technologies available today, yesterday's synthetic biologists resorted mainly to trial and error. The literature documents the laborious and costly nature of this approach. For example, one group invested over 150 person-years to achieve economical production of a plant-derived antimalarial drug in yeast (Ro et al., 2006; Kwok, 2010). Other examples also report substantial efforts: Dupont and Genencor spent 575 person-years to produce 1,3-propanediol and Amyris Biotechnologies between 130 and 575 person-years to make farnesene (Kwok, 2010). The availability of next-generation DNA technologies promises shorter timelines for such engineering feats.

Researchers at Stanford recently utilized a whole-cell engineering approach, recreating a highly complex tropane alkaloid biosynthesis pathway from deadly nightshade *(Atropa belladonna)* in *S. cerevisiae* (hereafter referred to as "yeast") for the synthesis of scopolamine and hyoscyamine (Srinivasan & Smolke, 2020). Recreating a plant's biological process in yeast, including the spatial organization and functionality of the pathway, required 34 chromosomal modifications (26 additions and 8 disruptions) and a combination of functional genomics, protein engineering, and strain optimization. With access to modern methods, this feat took only three person-years to complete.

This application note describes the whole-cell engineering approach for heterologous production of hyoscyamine and scopolamine in yeast and highlights how Twist Gene Fragments contributed to the authors' rapid engineering workflow.

## WORKFLOW

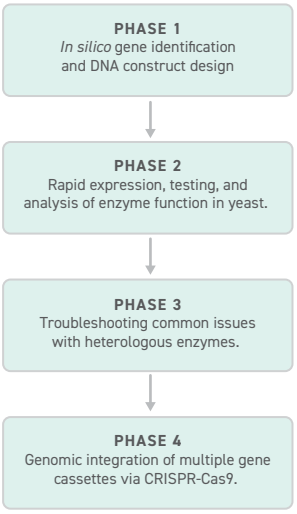
*Refer to Srinivasan & Smolke (2020) for a complete description of the materials and methods used throughout this study*

**Phase 1: *In silico* gene identification and DNA construct design.** Genes were derived from various sources, including the *Saccharomyces* genome database, UniPROT/SwissPROT, and GenBank. For unknown genes, transcriptional mining of plants was required. Once identified, gene sequences were codon-optimized and appended with cloning adapters *in silico*. Backbone vectors were also designed. This involved identifying which genes will need to be expressed together, selecting appropriate backbones for initial testing, and adding compatible ends to enable the insertion of synthesized gene fragments.

**Phase 2: Rapid expression, testing, and analysis of enzyme function in yeast.** Twist Bioscience synthesized the heterologous gene fragments designed in Phase 1. Yeast genes were generated by colony PCR. Gene fragments were subsequently cloned using Gibson assembly, and the resulting vectors were transformed in yeast. Colonies were grown and functionally tested for their ability to produce intermediates or the end-products of the biosynthetic pathway in small-scale fermentations.

**Phase 3: Troubleshooting common issues with heterologous enzymes.** Phase 2 identified heterologous enzymes with low or no activity in yeast. Phase 3 determines the underlying reason using a series of troubleshooting experiments. There are many reasons why a heterologous enzyme may not function in yeast: poor expression, insufficient intrinsic activity in yeast, incorrect subcellular location, and interference from yeast metabolism. Some solutions are simple. For example, poor expression can generally be rectified by switching to a high-copy number plasmid. Others are much less straightforward. For instance, interference from yeast metabolism can only be addressed by physically isolating the enzyme away from interfering factors (e.g. in a different subcellular compartment) or by knocking out disruptive yeast genes.

**Phase 4: Genomic integration of multiple gene cassettes via CRISPR-Cas9.** Once efficacious heterologous enzymes were identified, they were ready to be stably expressed in the yeast strain using CRISPR-Cas9 gene editing. The process involved three basic steps: genomic site selection, integration construct design, and strain verification. For genomic site selection, safe harbor sites in open chromatin were prioritized due to improved genomic stability and transcriptional activity. Alternatively, when yeast genes needed deleting to make a heterologous enzyme functional, direct gene replacement was performed. When designing integration constructs, the number of genes to be integrated and the type of promoter used was considered. Finally, after gene editing, genomic integration was confirmed by colony PCR, and the expressed enzymes were tested for functionality.



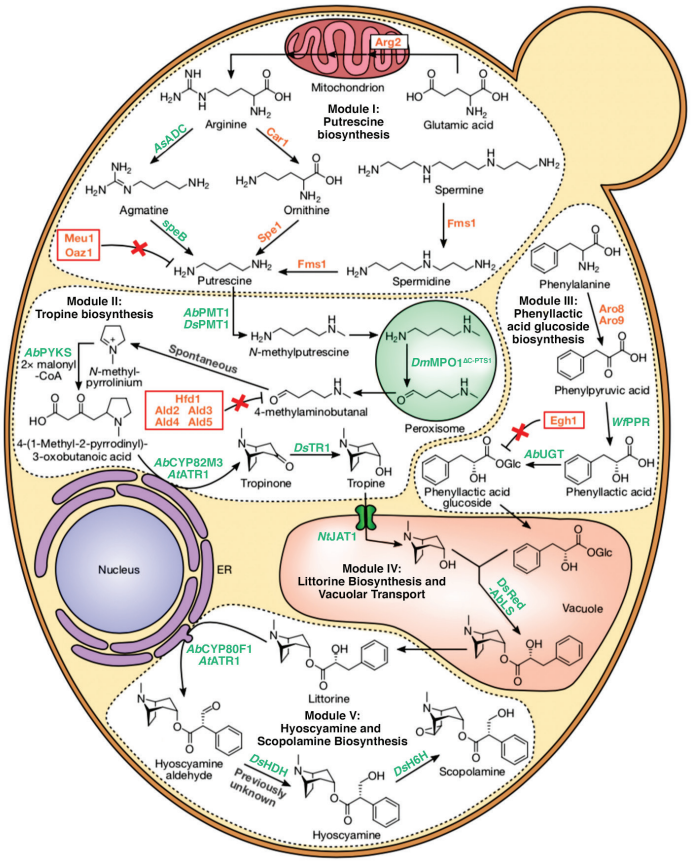




**Figure 1. Modular reconstruction of scopolamine biosynthesis in yeast.** Modules are outlined. Module I: Putrescine biosynthesis. Module II: tropine biosynthesis. Module III: phenyllactic acid glucoside biosynthesis. Module IV: Littorine Biosynthesis and Vacuolar Transport. Module V: Hyoscyamine and Scopolamine Biosynthesis. Color scheme: orange - overexpressed yeast genes; green - heterologous genes synthesized as Gene Fragments. Red boxes - disrupted yeast genes.

## RESULTS

### Pathway Overview

A biosynthetic pathway for hyoscyamine and scopolamine production was developed in yeast using a pathway consisting of five modules outlined in **Figure 1**. Modules I and II synthesize putrescine and tropine, respectively, from glutamic acid and spermine. Module III synthesizes phenyllactic acid (PLA) glucoside from the amino acid phenylalanine. Module IV connects the upstream and downstream components of the pathway with the synthesis of littorine from tropine and PLA glucoside. Finally, in module V, littorine is converted into hyoscyamine and scopolamine. The present work began with a yeast strain already capable of synthesizing tropine (i.e., Modules I & II), as described in a previous paper by the same authors (Srinivasan & Smolke, 2019).

### Module III: PLA Glucoside Biosynthesis

Yeast cells produce only trace levels of PLA, the precursor to PLA glucoside in Module III. To increase PLA production, phenylpyruvate reductases (PPR) from various heterologous organisms were screened for activity in yeast. Most PPRs showed minimal improvements in PLA titer, barring that of *Wickerhamia fluorescens*, which generated 80-fold more PLA than control yeast. The pathway from phenylalanine to PLA glucoside was completed by expressing a heterologous uridine diphosphate glycosyltransferase from *A. belladonna* (*Ab*UGT). In addition, an endogenous glucosidase (EGH1) that has been shown to degrade heterologous glucosides in yeast (like PLA glucoside) was also disrupted.

### Module IV: Littorine Biosynthesis and Vacuolar Transport

In *A. belladonna*, littorine synthase (*Ab*LS) esterifies PLA glucoside with tropine to create littorine (Qiu et al., 2020). However, it is unknown whether this enzyme can function in non-plant hosts due to the extensive post-translational processing and trafficking littorine synthase undergoes in plants. Littorine synthase must pass through the plant secretory pathway to gain functional modifications, but this pathway functions differently in yeast. Indeed, when expressed in yeast, *Ab*LS causes a growth defect as it stalls in the trans-Golgi network (TGN) of the yeast secretory pathway.

To facilitate the routing of A*b*LS through the yeast secretory pathway, *Ab*LS was converted into a transmembrane protein, which is automatically sorted from the TGN to the yeast vacuole (**Figure 2**). To this end, several N-terminal *Ab*LS fusions were generated to mask the N-terminal signal peptide that mediates the release of membrane-bound *Ab*LS in the TGN. These candidates were then screened for functionality in yeast. Interestingly, oligomeric *Ab*LS fusions (e.g., DsRed, which forms tetramers) outperformed monomeric *Ab*LS fusions (e.g., tagBFP). The reason for this performance benefit remains unknown.

The Module II pathway generates tropine in the cytosol, isolated from vacuolar DsRed-*Ab*LS. Therefore, several multidrug and toxin extrusion transporters were screened to identify a way to transport tropine into the vacuole. A jasmonate-inducible alkaloid transporter (JAT1) and a multidrug and toxin extrusion transporter from *Nicotiana tabacum* (*Nt*JAT1 and *Nt*MATE2, respectively)

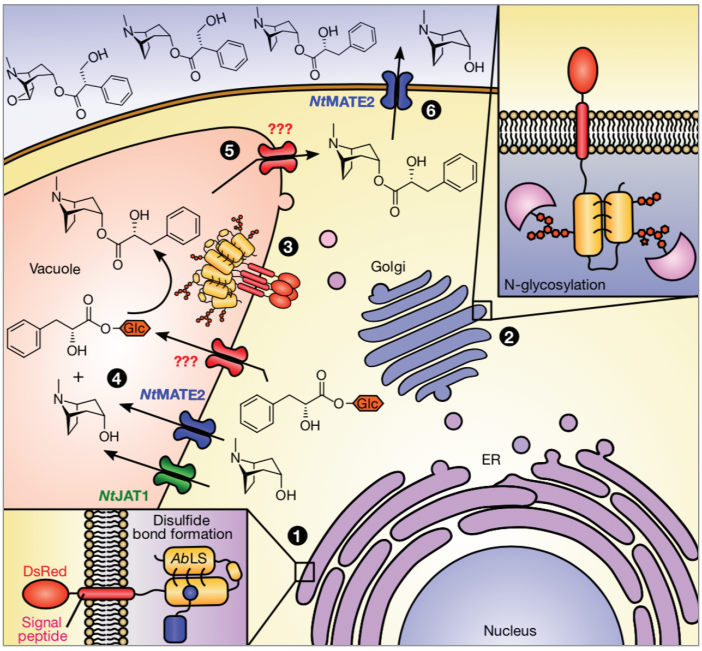



**Figure 2. Substrate transport and proposed DsRed-*Ab*LS trafficking (Module IV).** Numbers denote significant steps for littorine synthesis in Module IV. They include maturation of DsRed-*Ab*LS in the ER lumen (1) and Golgi (2), its trafficking to vacuole membrane (3), vacuolar substrate import (4), and product export (5), and cellular tropane alkaloid export (6).

improved tropane alkaloid production in yeast. However, because *Nt*MATE2 also facilitates tropane alkaloid export from the cell (**Figure 2**), *Nt*JAT1 was chosen for integration into the yeast strain.

### Module V: Hyoscyamine and Scopolamine Biosynthesis

Before this study, the enzyme that reduces hyoscyamine aldehyde to hyoscyamine was unknown. To discover the missing enzyme, existing plant transcriptome datasets were mined for genes that co-express with tropane alkaloid biosynthetic genes and contain the appropriate catalytic domains. Twelve hyoscyamine dehydrogenase (HDH) candidates were identified. These enzymes were screened for their ability to produce hyoscyamine from littorine. Among them, an NADPH- and zinc-dependent dehydrogenase (HDH2) was identified that resulted in the accumulation of hyoscyamine. Subsequent screening of HDH2 orthologs in yeast revealed a more efficient enzyme in *Datura stramonium* (*Ds*HDH). A Hyoscyamine 6 beta-hydroxylase from *D. stramonium* (*Ds*H6H) completes the final reaction converting hyoscyamine to scopolamine.

### Final Yeast Strain Optimization

Once the pathway was complete in yeast, Srinivasan and Smolke took steps to increase tropane alkaloid production. First, rate-limiting enzymes, including *Wf*PPR and *Ds*H6H, were overexpressed by integrating multiple copies of each heterologous gene. Second, 3-isopropylmalate dehydrogenase (LEU2) was expressed in yeast to reduce their dependence on the culture medium for leucine. As a result, the conversion

of hyoscyamine to scopolamine was enhanced more than threefold by increasing the availability of essential cofactors in tropane alkaloid biosynthesis (e.g., $Fe^{2+}$). Finally, a UDP-glucose pyrophosphorylase, which catalyzes the formation of a co-substrate for *Ab*UGT, was also overexpressed.

## CONCLUSIONS

Whole-cell engineering takes a holistic approach to the problem of recreating heterologous pathways in scalable organisms. Instead of merely transferring the parts from one organism to another, the method actively co-opts the cell biology of the new host to achieve its purpose. In this application note, Srinivasan and Smolke undertook whole-cell engineering to recreate the biosynthesis of plant alkaloids in yeast and completed the entire process within three person-years. Such a feat was made possible by the advent of several key technologies, including next-generation sequencing (discovery of new genes), CRISPR/Cas9 gene editing (quick gene integration), and the Twist Silicon DNA Synthesis Platform (rapid, accurate DNA synthesis).

Access to these new technologies significantly accelerates the process of engineering complex biosynthetic pathways into easily scalable organisms like yeast. Expressing, testing, and analyzing enzymes in yeast represents a significant engineering bottleneck in such whole-cell engineering workflows. After vector construction, clones must be screened and sequenced to verify their identities. Twist Gene Fragments reduce the time it takes to screen clones with rapid, high-fidelity gene synthesis. By synthesizing all heterologous enzymes as Twist Gene Fragments, Srinivasan & Smolke could advance from gene identification to an optimal enzyme in 3 weeks on average (Srinivasan, personal communication).

Importantly, in such complex bioengineering pipelines, an iterative improvement process is adopted as it is not possible to know every required modification until results from previous iterations are analyzed. For example, in the study in question, the researchers only identified the need for vacuolar shuttling of key enzymes after observing that plant littorine synthase caused a growth defect in yeast. They were able to solve this issue in the next round of design. Being able to undertake such rapid design-build-test cycles provides considerable flexibility to engineering pipelines, helps avoid unsustainable timelines, and ultimately opens the door for large-scale whole-cell genetic engineering projects like that published by Srinivasan and Smolke (2020).

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