Elucidation of gene clusters underlying withanolide biosynthesis in ashwagandha

By Erin E. Reynolds

Withanolides are medicinally important steroidal lactones produced by *Withania somnifera* (ashwagandha) amongst other Solanaceae family plants. *W. somnifera* has been used for over three thousand years in Indian Ayurvedic traditional medicine to treat ailments ranging from tuberculosis to infertility. Preliminary clinical trials have indicated that *W. somnifera* extracts may be effective in treating depression and anxiety, as well as enhancing cardiorespiratory endurance, memory, and cognitive function. Withanolides are the main bioactive components of *W. somnifera* and possess a range of pharmacological properties including anti-cancer, anti-inflammatory, neurological, and immunomodulatory activities. There is significant interest in the development of withanolide-derived drugs, however, the biosynthetic pathway to withanolides is largely unknown, preventing scale-up and hindering pharmaceutical applications.

Prior to the work presented in this thesis, only the first step of withanolide biosynthesis was known: conversion of phytosterol pathway intermediate 24-methylenecholesterol to 24-methyldesmosterol by sterol Δ^{24}-isomerase (*24ISO*). Putative biosynthetic gene clusters around *24ISO* were identified in the genomes of several Solanaceae family plants, suggesting that the genome of *W. somnifera* may contain a *24ISO*-associated biosynthetic gene cluster involved in withanolide biosynthesis. To investigate this, I sequenced and annotated the genome of *W. somnifera*, producing a chromosome-scale assembly suitable for genome mining and evolutionary analysis.

Mining the *W. somnifera* genome revealed two withanolide biosynthetic gene clusters, each subdivided into a root tissue-expressed and leaf tissue-expressed subcluster. These clusters are some of the largest and most complex identified in plants to date, contributing to fundamental understanding of biosynthetic gene clustering in plants. We characterized the cluster genes using heterologous expression in yeast and tobacco, in conjunction with enzyme assays, leading to the discovery of two cytochromes P450 (CYP87G1 and CYP749B2) and a short-chain dehydrogenase (SDH2) responsible for formation of the lactone ring on the sterol side chain of 24-methyldesmosterol, a defining chemical feature of withanolides. Two additional cytochromes P450 (CYP88C7 and CYP88C10) and a sulfotransferase (SULF1) were found to generate the characteristic A-ring structure of withanolides, featuring a C_1 ketone and C_2-C_3 unsaturation.

In this thesis we report optimized yeast strains capable of producing milligram per liter levels of intermediates containing all key chemical features of withanolides, opening new avenues for the sustainable production of withanolides through biomanufacturing and enabling future drug development leveraging the withanolide scaffold. In addition, the discovery of SULF1 as a core withanolide pathway enzyme challenges the conventional view of sulfotransferases as tailoring enzymes in secondary metabolism and suggests a wider role for this enzyme family than previously appreciated.

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