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A translational synthetic biology platform for rapid access to gram-scale quantities of novel drug-like molecules

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ABSTRACT

Plants are an excellent source of drug leads. However availability is limited by access to source species, low abundance and recalcitrance to chemical synthesis. Although plant genomics is yielding a wealth of genes for natural product biosynthesis, the translation of this genetic information into small molecules for evaluation as drug leads represents a major bottleneck. For example, the yeast platform for artemisinic acid production is estimated to have taken > 150 person years to develop. Here we demonstrate the power of plant transient transfection technology for rapid, scalable biosynthesis and isolation of triterpenes, one of the largest and most structurally diverse families of plant natural products. Using pathway engineering and improved agro-infiltration methodology we are able to generate gram-scale quantities of purified triterpene in just a few weeks. In contrast to heterologous expression in microbes, this system does not depend on re-engineering of the host. We next exploit agro-infection for quick and easy combinatorial biosynthesis without the need for generation of multi-gene constructs, so affording an easy entrée to suites of molecules, some new-to-nature, that are recalcitrant to chemical synthesis. We use this platform to purify a suite of bespoke triterpene analogs and demonstrate differences in anti-proliferative and anti-inflammatory activity in bioassays, providing proof of concept of this system for accessing and evaluating medicinally important bioactives. Together with new genome mining algorithms for plant pathway discovery and advances in plant synthetic biology, this advance provides new routes to synthesize and access previously inaccessible natural products and analogs and has the potential to reinvigorate drug discovery pipelines.

1. Introduction

The Plant Kingdom harbors an enormous reservoir of diverse chemicals. Accessing these molecules promises to reinvigorate drug discovery pipelines and provide novel routes to synthesize complex compounds that are beyond the reach of synthetic chemistry Anarat-Cappillino and Sattely, 2014). Although breakthroughs in DNA sequencing technology and bioinformatics have accelerated discovery of candidate genes for new natural product pathways, effective harnessing of plant metabolic diversity will require: i) a quick and easy method to rapidly screen combinations of enzyme-encoding genes for production of novel structures by expression in a suitable heterologous host, and ii) the capability to purify suites of structural variants of these compounds in sufficient quantities for evaluation in bioactivity assays.

Microorganisms, particularly yeasts, have proven to be useful heterologous hosts for expression of several plant natural product pathways, including the artemisinin precursor artemisinic acid (Paddon et al., 2013), opioids (DeLoache et al., 2015; Galanie et al., 2015; Li and Smolke, 2016) and monoterpene indole alkaloids (Brown et al., 2015; Qu et al., 2015), and can be used for industrial-scale production of high-

Abbreviations: CPMV, cowpea mosaic virus; CYP, cytochrome P450; ELISA, Enzyme-Linked ImmunoSorbent Assay; EpHβA, 12,13β-epoxy,16β-hydroxy-β-amyrin; FPP, farnesyl diphosphate; FPS, farnesyl diphosphate synthase; GFP, green fluorescent protein; HMGR, 3-hydroxy,3-methylglutaryl-CoA reductase; HT, hyper-translatable; LPS, lipopolysaccharide; Nrf2, nuclear factor erythroid 2 related factor 2; SAD1, *Avena strigosa* β-amyrin synthase; SQE, squalene epoxidase; SQS, squalene synthase; tHMGR, truncated HMGR; TIC, total ion chromatogram; TNFα, tumor necrosis factor alpha

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value plant products. However, despite some successes, many metabolites have only been produced at low levels in yeasts and optimization is far from trivial. For example, the development of a yeast platform for the production of artemisinic acid is estimated to have taken more than 150 person years (Kwok, 2010). Making microbes into efficient factories for the production of heterologous metabolites is hindered by lack of fundamental knowledge about cellular processes (Nielsen and Keasling, 2016).

Plants have a number of advantages as heterologous hosts for metabolic engineering. They require only simple inorganic nutrients, water, carbon dioxide and sunlight for efficient growth. They are also more amenable to expression of genes of plant origin than microbes since they support correct mRNA and protein processing, protein localisation and metabolic compartmentalisation, and already have many of the necessary metabolic precursors and co-enzymes. Species of tobacco are relatively easy and fast to transform by the integration of new genes into the genome but high yields can also be achieved in just a few days through transient expression following infiltration into the leaves of a culture of *Agrobacterium tumefaciens* carrying genes of interest on a binary plasmid vector, a process commonly known as agro-infiltration. *Nicotiana benthamiana*, a wild relative of tobacco, is particularly amenable to agro-infiltration-mediated transient expression and is currently being used for commercial production of flu vaccines (Marsian and Lomonossoff, 2016). The expression process is rapid, taking only 5–6 days from infiltration of leaves with *A. tumefaciens* strains harboring expression constructs to extraction of plant tissue. Importantly, combinations of genes can be co-expressed without the need for making multi-gene vectors simply by co-infiltrating multiple *A. tumefaciens* strains containing different expression constructs. Although various plant natural product pathways have previously been engineered by transient expression in this plant (Andersen-Ranberg et al., 2016; Geisler et al., 2013; Geu-Flores et al., 2009; Khakimov et al., 2015; Miettinen et al., 2014; Polturak et al., 2016; Rajniak et al., 2015; Wang et al., 2016), the levels achieved are usually in the microgram range. Here, we have optimized the *N. benthamiana* platform for production of up to gram-scale amounts of purified triterpene, and have demonstrated the power of this transient expression system for rapid and facile combinatorial biosynthesis by generating over 40 different types of oxygenated triterpene analogs. We further show the potential of this strategy for investigating structure-activity relationships in bioassays. This overall strategy will greatly accelerate systematic investigation of the vast array of triterpene diversity found in the Plant Kingdom by enabling the outputs of plant genomics to be translated into purified compounds for evaluation as drug leads and for other applications.

2. Results and discussion

2.1. Pathway engineering for increased triterpene yield in N. benthamiana

The triterpenes are one of the largest classes of plant natural products (> 20,000 reported to date) and are of considerable interest as potential drug leads (Hill and Connolly, 2012; Moses et al., 2014a). However few hypotheses exist regarding links between structure and activity because of the lack of available suites of structural analogs for bioactivity evaluation. These compounds are synthesized from the mevalonate pathway by cyclisation of the isoprenoid precursor 2,3-oxidosqualene to diverse triterpene scaffolds, the most common of which is β-amyrin (Thimmappa et al., 2014) (Fig. 1a). These scaffolds are then further modified by cytochromes P450 (CYPs) and other tailoring enzymes. Although yeast has been used as a heterologous expression host to make and modify triterpene scaffolds (Fukushima et al., 2013; Moses et al., 2014b; Thimmappa et al., 2014) the potential of *in planta* production has not been fully explored. We previously showed that transient expression of the oat β-amyrin synthase SAD1 in *N. benthamiana* leads to accumulation of β-amyrin in infiltrated leaf tissue (Geisler et al., 2013). Since precursor availability may be limiting, we co-expressed SAD1 with different upstream mevalonate pathway genes to determine the effects on triterpene production. Farnesyl diphosphate synthase (FPS) and squalene epoxidase (SQE) had little or no effect on β-amyrin content, while squalene synthase (SQS) gave a modest increase (Fig. 1b). The largest effect was seen with a feedback-insensitive version of HMG-CoA reductase (tHMGR), which gave a four-fold increase (Fig. 1b, c; Fig S1). CYP51H10 oxygenates β-amyrin at two positions to give 12,13-epoxy, 16-hydroxy-β-amyrin (EpHβA) (Fig. 1d) (Geisler et al., 2013). We used this CYP to evaluate the effects of co-expression with tHMGR on production of oxidized triterpene scaffolds by expressing SAD1 and CYP51H10 in *N. benthamiana* leaves with or without tHMGR, and showed that inclusion of tHMGR led to an increase in EpHβA production of ~10-fold (Fig. 1e,f; Fig S1). Thus, co-expression with tHMGR proved sufficient to significantly increase triterpene production in this transient plant expression system.

2.2. Gram-scale triterpene production using vacuum infiltration

We next used *tHMGR* in combination with *Sad1* to investigate the potential of the transient plant expression platform for preparative-scale triterpene production. Our experiments up to this point had relied on infiltration of *N. benthamiana* leaves with *A. tumefaciens* cells using a syringe without a needle. To increase our capacity for effective leaf infiltration we designed a device that would enable efficient vacuum infiltration of 4–6 plants simultaneously (Fig. 2a). Preliminary experiments using a reporter construct showed that this method resulted in very good GFP expression in infiltrated leaves (Fig. 2b). We then carried out batch-wise infiltration of ~460 *N. benthamiana* plants with a mixture of two *A. tumefaciens* strains carrying CPMV-*HT* expression constructs for the *tHMGR* and *Sad1* genes, respectively, and purified > 800 mg of β-amyrin as white needles (Fig. 2c). The crystals were estimated to have a purity of > 98% by HPLC-CAD (Fig S2a). Given that the mother liquor still contained > 150 mg of β-amyrin (Fig S2b), we estimate the total amount of β-amyrin recovered to be in the region of one gram, which corresponds to 0.4% dry leaf weight. These results demonstrate the potential of the *N. benthamiana* transient expression system for preparative-scale biosynthesis, with the opportunity for further scale-up, for instance by using larger vacuum infiltration devices.

2.3. Combinatorial biosynthesis using agro-infiltration provides rapid access to novel molecular diversity

Selective functionalization of triterpene scaffolds using synthetic chemistry is limited by lack of available reactive groups. Within the last few years considerable advances have been made in identifying and characterizing triterpene biosynthetic enzymes from plants (Biazzi et al., 2015; Boutanaev et al., 2015; Carelli et al., 2011; Fukushima et al., 2011, 2013; Geisler et al., 2013; Itkin et al., 2016; Khakimov et al., 2015; Miettinen et al., 2017; Moses et al., 2014b, 2014c, 2015a, 2015b; Salmon et al., 2016; Seki et al., 2008, 2015; Shang et al., 2014; Shibuya et al., 2006; Thimmappa et al., 2014; Yasumoto et al., 2016; Zhang et al., 2016), opening up opportunities to harness enzymes from nature for systematic investigation of a much broader sector of biologically relevant chemical space than can be achieved using conventional chemistry.

Since agro-infiltration enables co-expression of genes of interest without the need to make multi-gene constructs through co-infiltration of *A. tumefaciens* strains harboring different expression constructs, we evaluated the potential of the transient *N. benthamiana* expression system for combinatorial biosynthesis. We selected five CYPs from diverse plant species (oat, licorice, soy bean, barrelclover) that had previously been shown to oxidase β-amyrin at different scaffold positions (Carelli et al., 2011; Fukushima et al., 2011; Geisler et al., 2013; Moses et al., 2014c; Seki et al., 2008; Shibuya et al., 2006) and co-expressed