Generation of the potent anti-malarial drug artemisinin in tobacco

To the Editor:

The emergence of multidrug-resistant strains of *Plasmodium* spp., the etiological agent of malaria, constitutes a major threat to controlling the disease[1,2]. Artemisinin, a natural compound from *Artemisia annua* (sweet wormwood) plants, is highly effective against drug-resistant malaria. Even so, low-cost artemisinin-based drugs are lacking because of the high cost of obtaining natural or chemically synthesized artemisinin[1,2]. Martin *et al.*[3] were the first to report the generation of an artemisinin precursor in a microbial system. They engineered *Escherichia coli* with a synthetic mevalonate pathway from *Saccharomyces cerevisiae*. Expression of amorphadiene synthase (*ADS*) from *A. annua* in this strain allowed production of amorpha-4,11-diene, the sesquiterpene olefin precursor to artemisinin. However, despite extensive effort invested in the past decade in metabolic engineering of artemisinin and its precursors in both microbial and heterologous plant systems[2–6], production of artemisinin itself has never been achieved. Here we report the metabolic engineering of tobacco to produce artemisinin, generating transgenic plants that express five plant- and yeast-derived genes involved in the mevalonate and artemisinin pathways, all expressed from a single vector. Our experiments demonstrate that artemisinin can be fully biosynthesized in a heterologous (that is, other than *A. annua*) plant system, such as tobacco. Although the artemisinin levels we have generated in transgenic tobacco are currently lower than those in *A. annua*, our experimental platform should lead to the design of new routes for the drug's commercial production in heterologous plant systems.

The World Health Organisation (WHO; Geneva) promotes the use of artemisinin as a first-line treatment for malaria, and it is heavily involved in facilitating the development of artemisinin-based anti-malaria drugs[1]. Artemisinin is biosynthesized from terpenoid backbones generated by the mevalonate and methyl-erythritol phosphate (MEP) pathways[7–9] (**Fig. 1a**). Although detailed knowledge of the artemisinin-biosynthesis pathway is still lacking, it initiates with the cyclization of farnesyl diphosphate by ADS to form amorpha-4,11-diene, which is then oxidized by the cytochrome P450 CYP71AV1, reduced by artemisinic aldehyde reductase (DBR2) and possibly reoxidized by aldehyde dehydrogenase to yield dihydroartemisinic acid—the presumed precursor of artemisinin in plants[6,7,10]. Normally, dihydroartemisinic acid accumulates in *A. annua* and slowly converts to artemisinin, a process that can be stimulated after harvest by drying in the sun. The transformation of dihydroartemisinic acid to artemisinin in *A. annua* has been proposed to be nonenzymatic, requiring only the presence of light and molecular oxygen[6,7]; a singlet oxygen formed as a consequence of exposure to UV/visible light may react with dihydroartemisinic acid to form a keto-enol, which can then react with ground-state oxygen to form a second hydroperoxide that spontaneously forms artemisinin.

Despite the great interest in enhancing yields of artemisinin in its host *A. annua*, classic breeding and genetic engineering strategies have met with only limited success[6,11]. Moreover, recent attempts to produce the artemisinin precursors artemisinic and dihydroartemisinic acids in heterologous plants[4,5] did not lead to their accumulation due to internal glycosylation and insufficient oxidation toward the acids. Harnessing *E. coli* and *S. cerevisiae* has allowed the production of high titers of amorpha-4,11-diene and artemisinic acid[2,3], but not of the active artemisinin drug itself.

To reconstruct the artemisinin-producing pathway in *Nicotiana tabacum*, we first generated a mega-vector carrying cytochrome P450 reductase (*CPR*) from *A. annua* to prevent the accumulation of inactive oxidized P450, as well as *ADS*, *CYP71AV1* and *DBR2* (**Fig. 1b** and **Supplementary Methods**). The mega-vector also contained a truncated and deregulated 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*tHMG*) from yeast to increase the supply of precursor from the mevalonate pathway for artemisinin production. To ensure genetic stability and

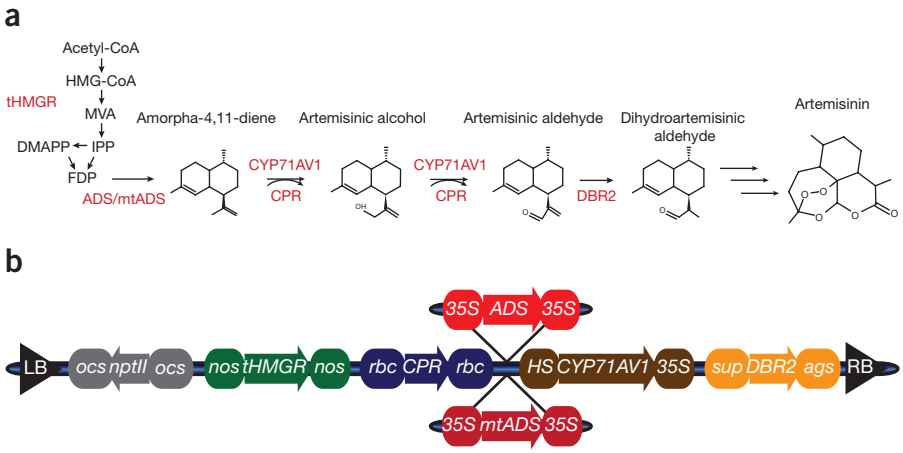

Figure 1 The pathway and constructs for engineering artemisinin production in tobacco. (**a**) Schematic outline of the mevalonate and artemisinin pathways (engineered genes are in red). (**b**) Gene constructs assembled to engineer artemisinin production. Arrows indicate genes, boxes indicate promoters and terminators; constructs with either *ADS* or *mtADS* are shown. ocs, octopine synthase; nos, nopaline synthase; rbc, rubisco; 35S, cauliflower mosaic virus (CaMV) 35S; HS, hps18.1 promoter; sup, super-promoter; ags, agrocinopine synthase.

expression in plants, all five genes and an antibiotic-resistance selection gene were placed under the control of different promoter and terminator sequences (**Supplementary Methods**). To enhance production yields of foreign terpenes in plants, we tested the possibility of targeting ADS to the mitochondria using a mega-vector carrying *ADS* fused to *COX4* signal peptide[12,13] (*mtADS*). Shunting terpenoid biosynthesis via the mitochondria has recently been shown to be greatly advantageous for the metabolic engineering of plant terpenoids[12,13]. Targeting of mtADS and ADS to the mitochondria and cytosol, respectively, was confirmed using protoplasts and fluorescent protein tags (**Supplementary Fig. 1a**).

Tobacco plants were stably transformed with the mega-vector harboring either *ADS* or *mtADS* (ADS and mtADS plants, respectively), and expression of all five genes in transgenic plants was confirmed by reverse transcriptase PCR analysis (**Supplementary Fig. 1b**). Plants transgenic for all of these genes but lacking *ADS* or *mtADS*, plants expressing GFP and wild-type tobacco were used as controls. No phenotypic differences were observed between artemisinin-producing ADS and mtADS plants and control lines. Artemisinin production was monitored in independent transgenic tobacco lines (four mtADS and four ADS lines) using ultra-high performance liquid chromatography coupled with high-resolution accurate mass spectrometry (UPLC-HR-MS). Artemisinin was identified in extracts from *ADS*- and *mtADS*-carrying transgenic lines, based on both an authentic standard and internal deuterium-labeled artemisinin (**Fig. 2a**). To further validate artemisinin identification, extracts were analyzed using UPLC coupled to a triple-quadrupole mass spectrometer (UPLC-MS/MS) operated in multiple reaction monitoring (MRM) mode (**Fig. 2b**). MRM traces identical to those of an artemisinin standard were detected in transgenic tobacco extracts that had shown artemisinin accumulation in the UPLC-HR-MS analysis. Analyses of artemisinin yield in transgenic tobacco lines using a deuterium-labeled artemisinin standard revealed that harnessing the mitochondria for amorpha-4,11-diene production yields higher levels of artemisinin: independent transgenic tobacco lines with cytosolic ADS produced 0.75, 0.88, 0.94 and 0.48 μg artemisinin/g dry weight, whereas tobacco lines with mtADS generated 5.0, 5.9, 6.3 and 6.8 μg artemisinin/g dry weight. Amorpha-4,11-diene production levels in the mtADS transgenic tobacco plants were also higher compared with ADS plants and

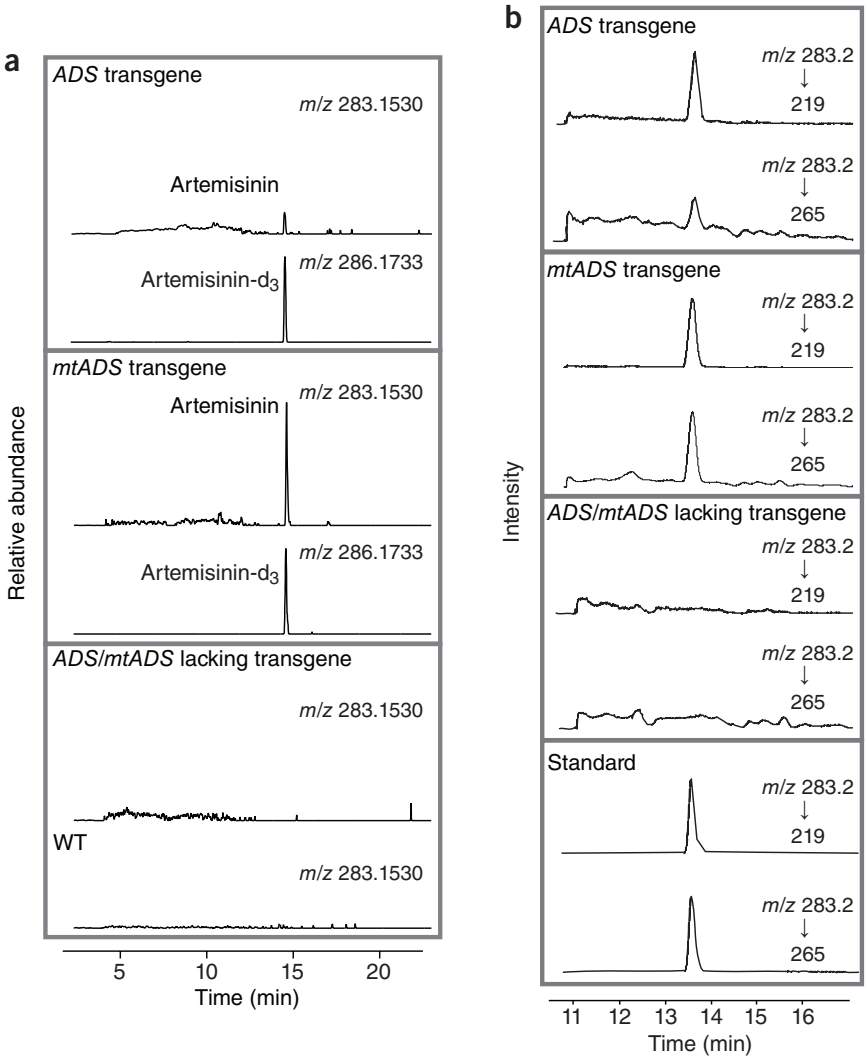

Figure 2 Production of artemisinin in engineered tobacco plants. (**a**) Extracted ion accurate mass chromatograms (UPLC-HR-MS) for artemisinin (*m/z* 283.1530) and artemisinin-d_3 (*m/z* 286.1733) in extracts of plants transformed with the *ADS* (upper panel) or *mtADS* (middle panel) construct. Lower panel shows artemisinin ion in extracts from *ADS*/*mtADS*-lacking transgenic (top chromatogram) and wild-type (bottom chromatogram) control plants. (**b**) Artemisinin production in tobacco extracts analyzed by UPLC-MS/MS operated in MRM mode. Extracts from *ADS*, *mtADS* and *ADS*/*mtADS*-lacking transgenic plants are shown in the upper three panels, respectively. Artemisinin standard is shown in the lower panel. Chromatograms in each panel show artemisinin-specific MRM traces of *m/z* 283.2–219 and *m/z* 283.2–265.

similar to levels obtained in previous attempts to generate artemisinin in heterologous hosts[4,5]. In transgenic plants generated with the ADS construct, we observed amorpha-4,11-diene levels of 26–72 ng/g fresh weight, whereas in plants generated with the mtADS construct, amorpha-4,11-diene accumulated to about tenfold higher levels, 137–827 ng/g fresh weight (as measured by gas chromatography-MS analysis; **Supplementary Methods**). Amorpha-4,11-diene levels in *A. annua* plants were 5.2–10.8 μg/g fresh weight, which is comparable to previous reports[14]. The observation that tobacco and *A. annua* plants accumulated lower levels of amorpha-4,11-diene compared with artemisinin raises the possibility that the olefin's levels are a limiting factor in the drug's production.

Our ability to successfully produce artemisinin in tobacco is most likely due to a combination of factors: expression of *CPR* concomitant with *CYP71AV1*, expression of *tHMG* to enhance precursor availability, the use of intense light conditions during plant cultivation and single-vector-based transformation. Although it is well established that the oxidizing activity of CYP71AV1 is

coupled to that of CPR[2], the effect of elevated reductase levels on oxygenation rates by cytochrome P450s, including CYP71AV1, in the context of metabolic engineering has never been studied in plants. In this respect, Zhang *et al.*[5] suggested that their lack of success in producing dihydroartemisinic acid in a heterologous plant system might be due to low oxidation rates. There is evidence that in the presence of light and oxygen, conversion of dihydroartemisinic acid to artemisinin in *A. annua* is spontaneous rather than enzymatically catalyzed[6,7]. Thus, the use of intense light conditions during transgenic tobacco cultivation may promote this conversion and lead to accumulation of artemisinin in the metabolically engineered tobacco. The integration of five genes encoding a metabolic pathway into a single vector, while employing a range of promoters, has also been shown to be instrumental in supporting robust metabolic engineering of, for example, carotenoids, vitamin E and polyunsaturated fatty acids in plants[15,16]. HMG-R is a putative rate-limiting step in the mevalonate pathway; enhancing carbon flux through the mevalonate pathway by expressing a truncated and deregulated HMG-R has been shown to increase the accumulation of both native and foreign terpenes[12,17]. The use of mitochondria as an alternative or additional cellular compartment recently has been shown to enhance the production of olefin terpenoids[12,13]. Indeed, tobacco plants expressing the catalytic domain of HMG-R (tHMG) and producing the sesquiterpene backbone of artemisinin in mitochondria generated the highest levels of artemisinin. Simultaneous expression of *tHMG* and *mtADS* yielded levels of artemisinin approximately eightfold higher than cytosol-driven drug production.

Although the generated levels of artemisinin in tobacco were lower than in *A. annua*, co-expression of additional enzymes (e.g., aldehyde dehydrogenase 1 and farnesyl diphosphate synthase[4,5,17]), pyramiding different intracellular compartments for drug production (e.g., cytosol with mitochondria and/or chloroplasts[12,13,17]), improving the interaction between consecutive enzymatic steps as well as the use of alternative plant systems should further optimize the yield of metabolically engineered artemisinin. The use, for example, of established industrial crops with well-developed large-scale agricultural practice and extraction methods may substantially reduce the cost of drug production.

The nonprofit drug company OneWorld Health (S. San Francisco, CA, USA) declared 2011 a target year for the development of artemisinin precursor in yeast and its subsequent chemical conversion to a semisynthetic drug. Reaching this important goal should lead to a 30–60% cost reduction in artemisinin-based malaria therapy[18]. Compared with the use of microbial systems for the production of artemisinin-related compounds[2,3], the plant-based platform presented here enables complete synthesis of artemisinin. Moreover, in light of tobacco's high biomass and rapid growth, coupled with the feasibility of generating commercially viable plant cell cultures, our results pave the way for the development of a sustainable plant-based platform for the production of an anti-malarial drug.

Note: Supplementary information is available on the Nature Biotechnology website.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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AUTHOR CONTRIBUTIONS
M.F. and A.V. conceived the work, designed the experiments and wrote the manuscript. M.F., E.M., A.A.-D., Z.L., V.Z., O.E. and J.B.-A. performed experiments and analyzed data. B.S.-R., H.A. and B.S. provided conceptual guidance for selected experiments. T.T. managed cloning and localization experiments. All of the authors discussed, commented on and approved the manuscript.

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Relative potential of biosynthetic pathways for biofuels and bio-based products

To the Editor:

Concerns about the sustainability of fossil fuels and global warming[1], along with energy security[2], have provided a strong incentive recently toward research and development of carbon-neutral alternatives including next-generation biofuels. Currently up to 10% ethanol blends are being used in gasoline with higher biofuel blending targets being used or contemplated by various governments[3]. The compatibility of higher ethanol blends with existing infrastructure still needs to be validated, hence calls for development of fuel molecules beyond ethanol. Second-generation biofuel molecules are likely to resemble those present in gasoline (that is, C_4-C_{10} branched chain hydrocarbons or derivatives thereof including alcohols) and expected to be compatible with existing infrastructure. Another characteristic of second-generation biofuels is that they are expected to entail lower energy input in downstream purification to fuel-grade specifications compared with ethanol, thus engendering the possibility of biofuels with higher return on energy input compared with ethanol.

In view of the considerable interest in