# Compartmentalized metabolic engineering of plant for artemisinin biosynthesis

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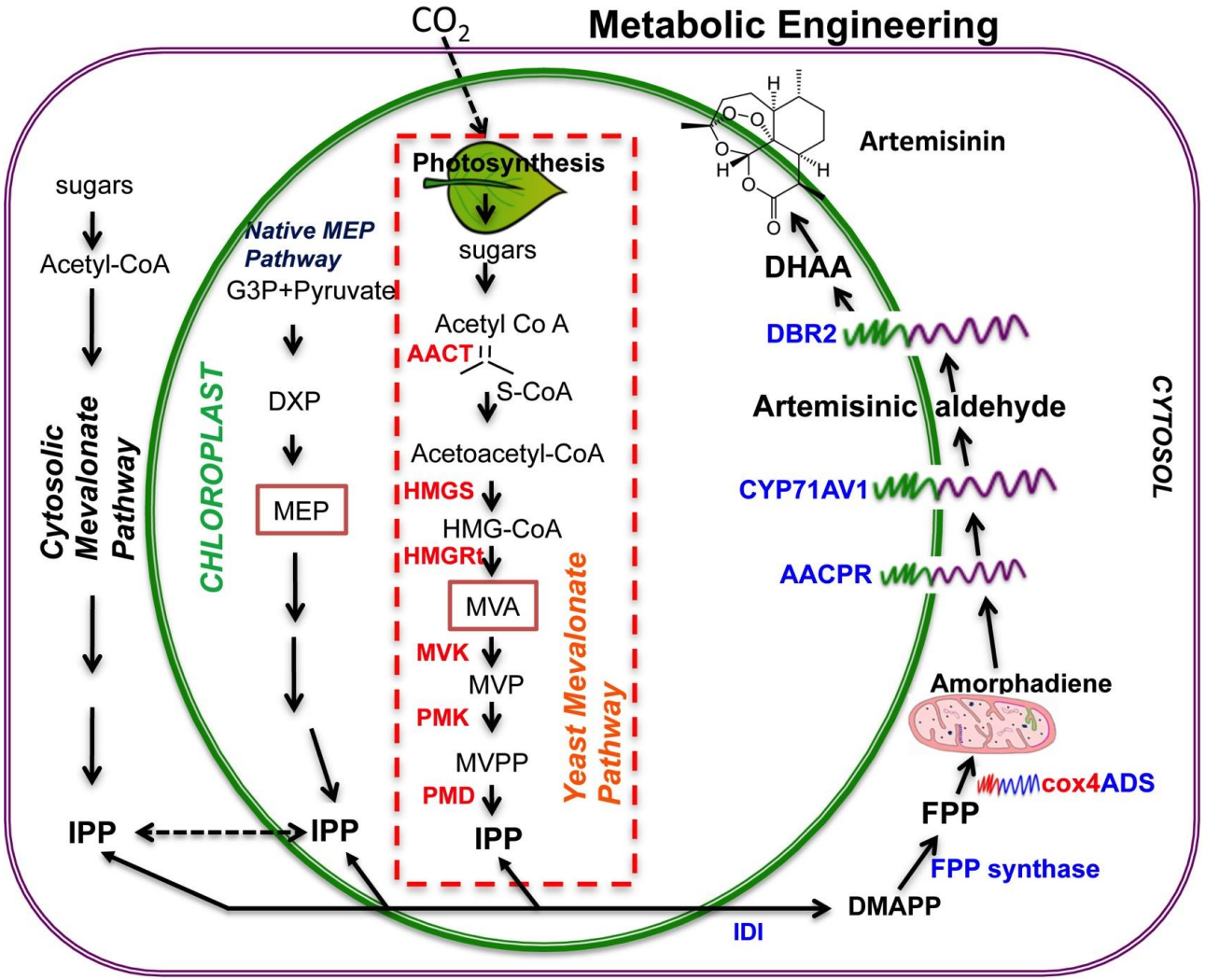
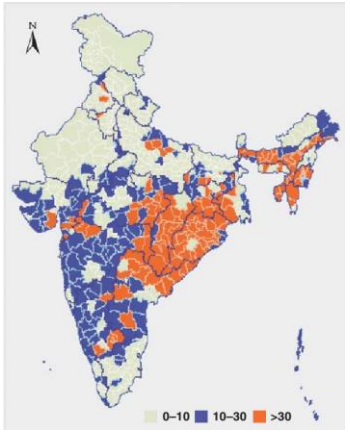
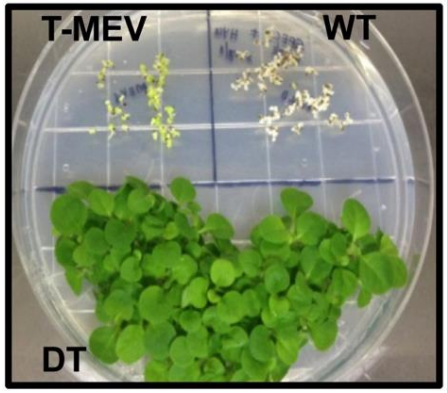
# **Compartmentalized MBE** (chloroplast, mitochondria and nuclear organelles) **for artemisinin biosynthesis**



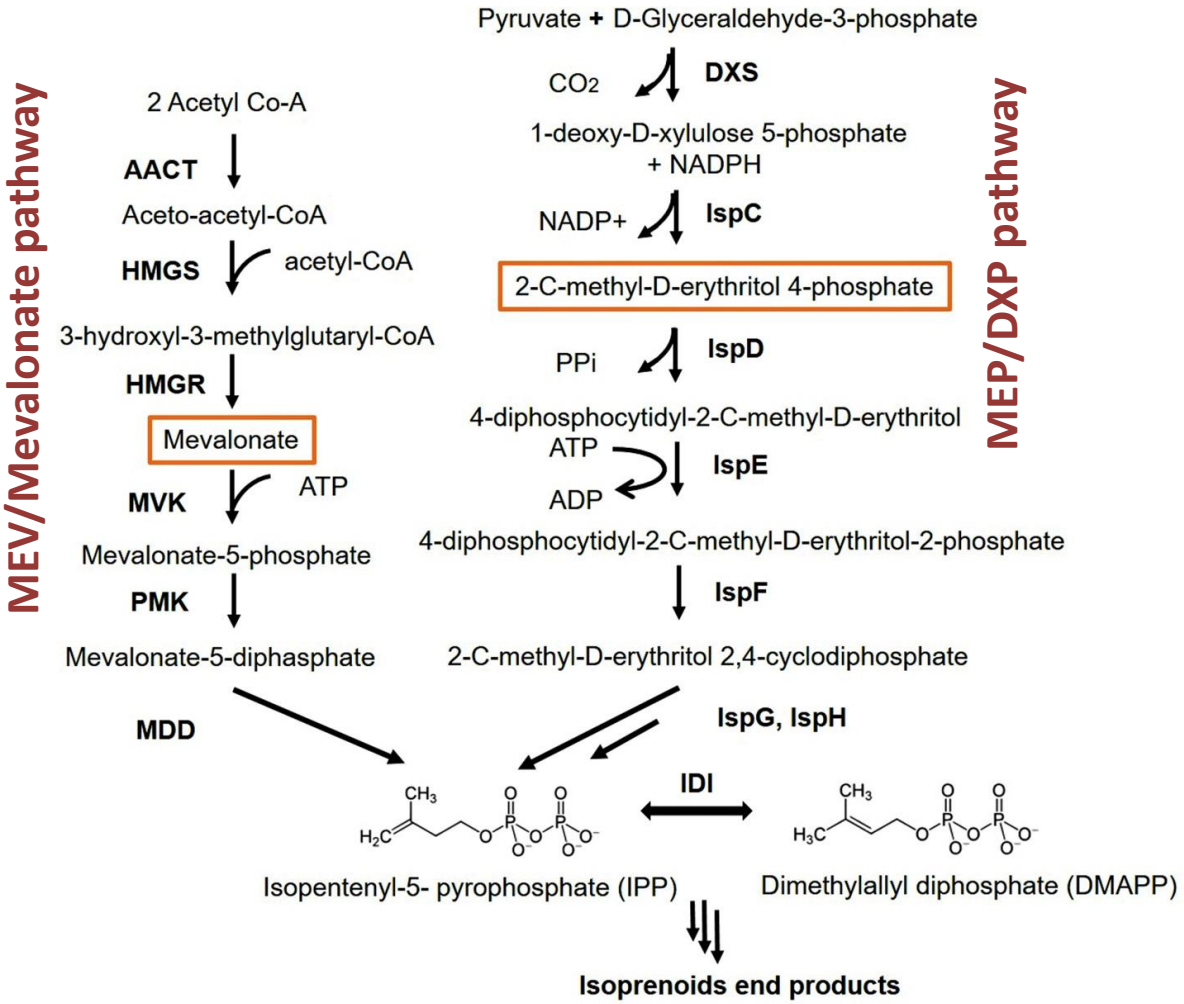




***P. falciparum***




Artemisinin biosynthesis by sequential metabolic engineering. Six genes AACT, HMGS, HMGRt, MVK, PMK and PMD (in red font) encoding yeast MEV pathway were integrated into chloroplast genome to generate a high IPP pool. Homoplastomic plant's nuclear genome was transformed with six genes (ADS, CYP71AV1, AACPR, DBR2, IDI and FPP) of artemisinin biosynthetic pathway. Subcellular targeting of DBR2, AACPR and CYP71AV1 were done by chloroplast transit peptide. Dihydroartemisinic acid (DHAA) converts itself to artemisinin via self photochemical-oxidation, a non-enzymatic reaction.

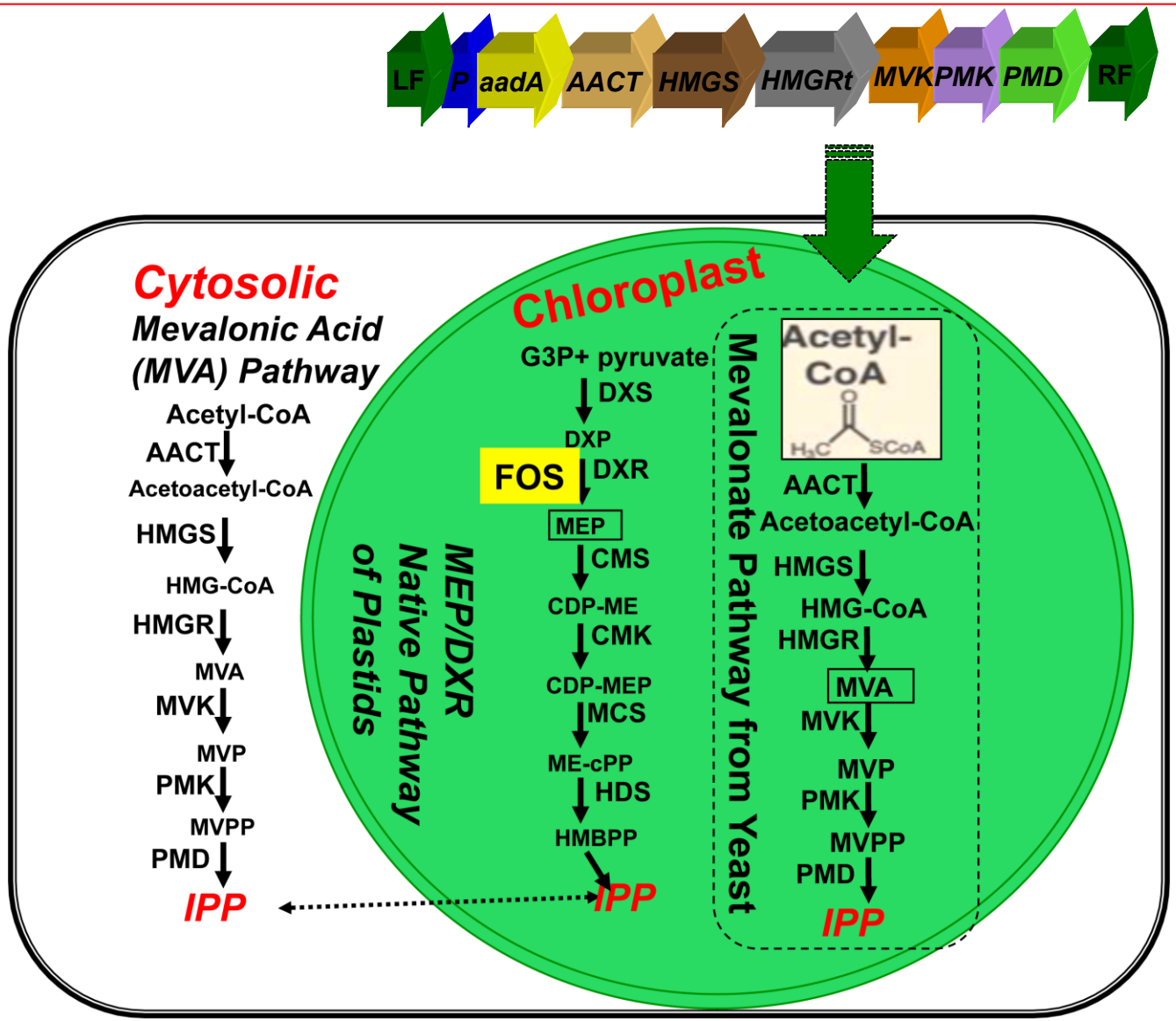
# IPP



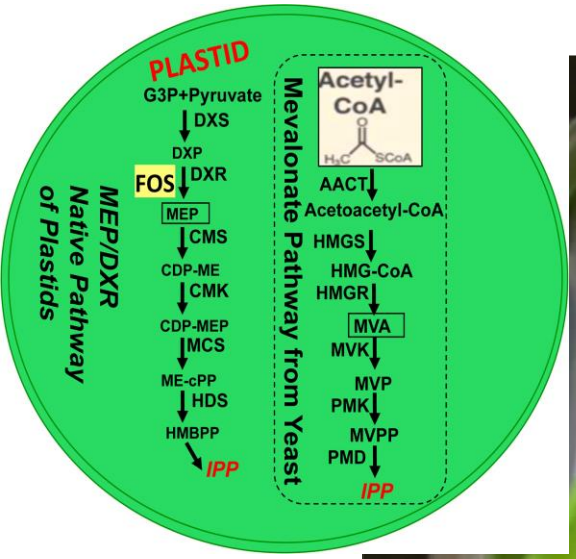

Bacteria, and malaria parasites have MEP while higher plants have both MEP (plastids) and MEV (cytosol)

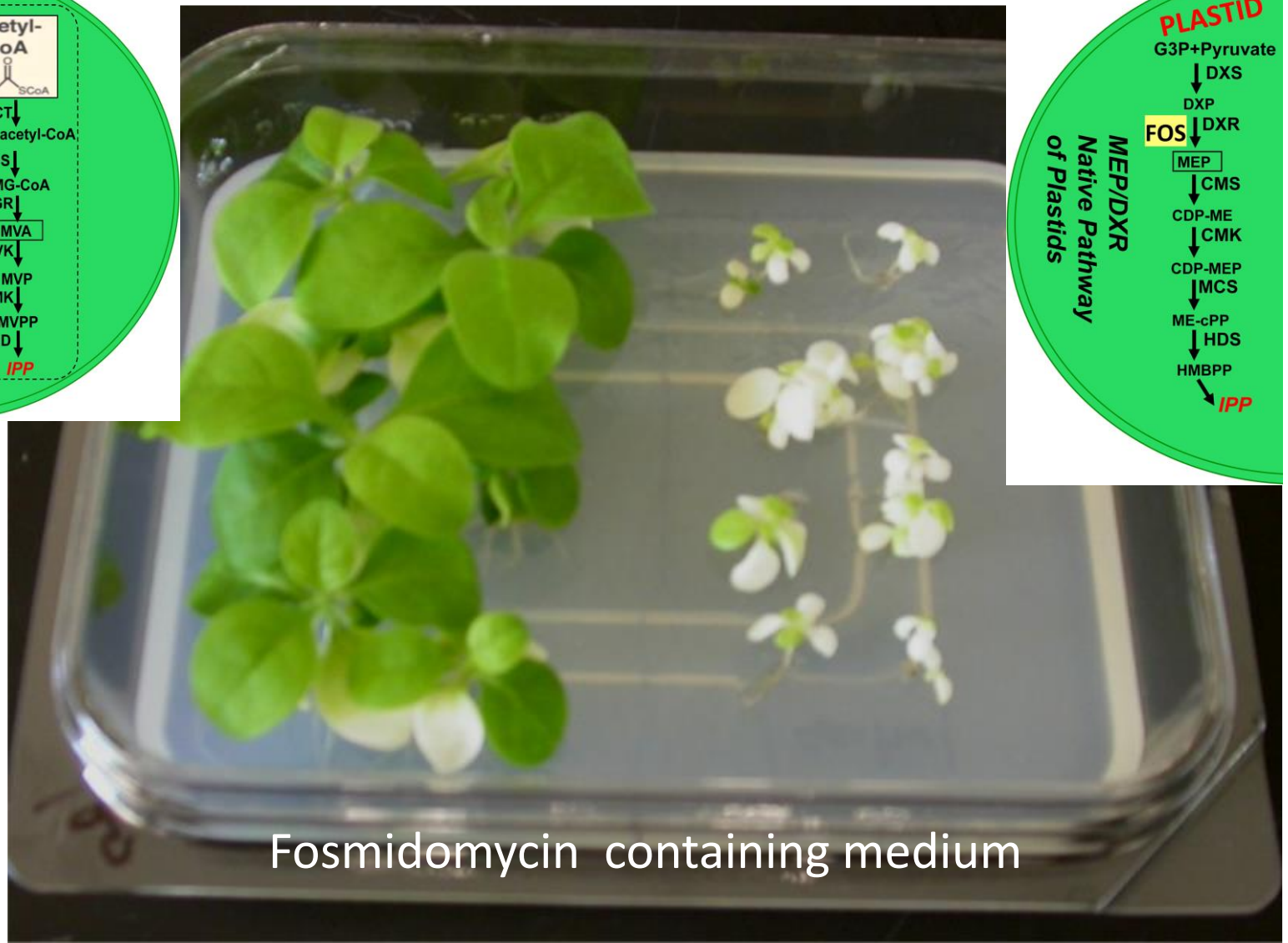
Pathways produces two five-carbon building blocks called isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are used to make a diverse class of over **50,000 known biomolecules** such as cholesterol, heme, vitamin K, coenzyme Q10, and all steroid hormones, metabolic drugs, latex, squalene etc.

# MBE of yeast Mev pathway into chloroplasts to enhance IPP



# Functionality of Yeast MEV (IPP) pathway in chloroplast



Metabolic Engineering

Volume 14, Issue 1, January 2012, Pages 19–28

# Remodeling the isoprenoid pathway in tobacco by expressing the cytoplasmic mevalonate pathway in chloroplasts

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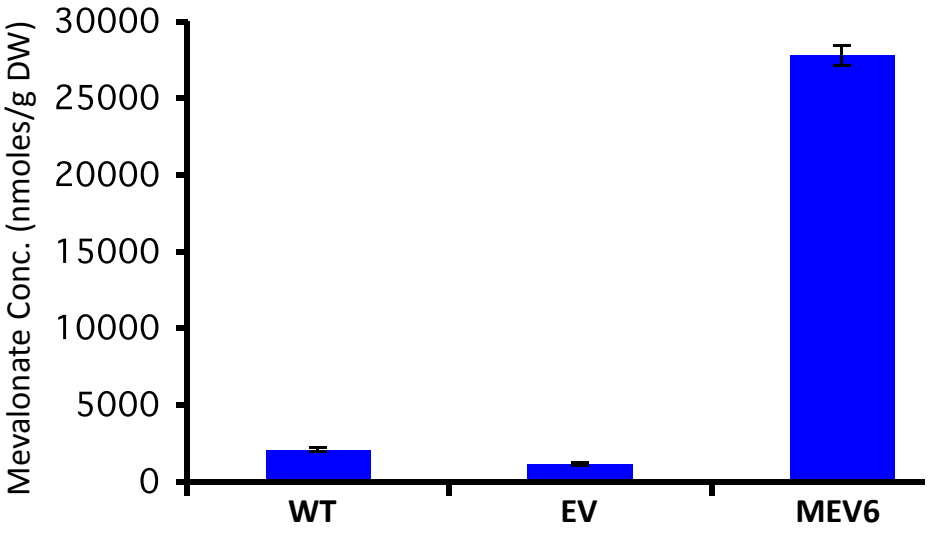
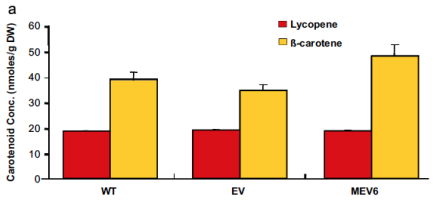
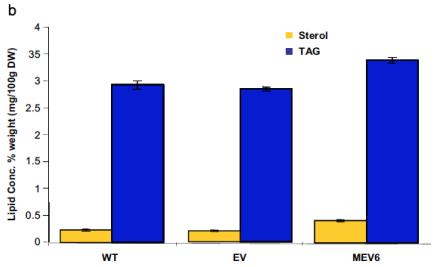
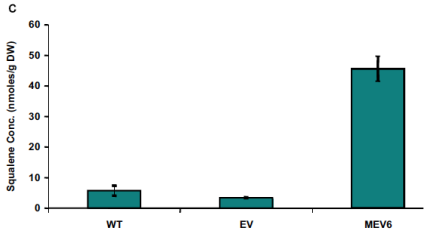



Carotenoid




Lipid

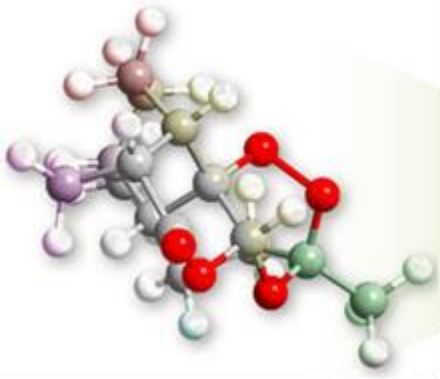


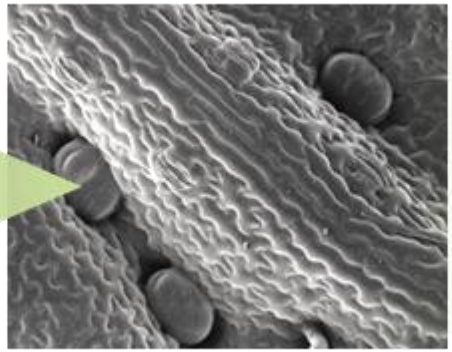

Squalene

# Use of excess flux of IPP to produce artemisinic acid

## (precursor to artemisinin)

Artemisinin is produced by trichomes on *Artemisia annua* leaves





**artemisinin...**

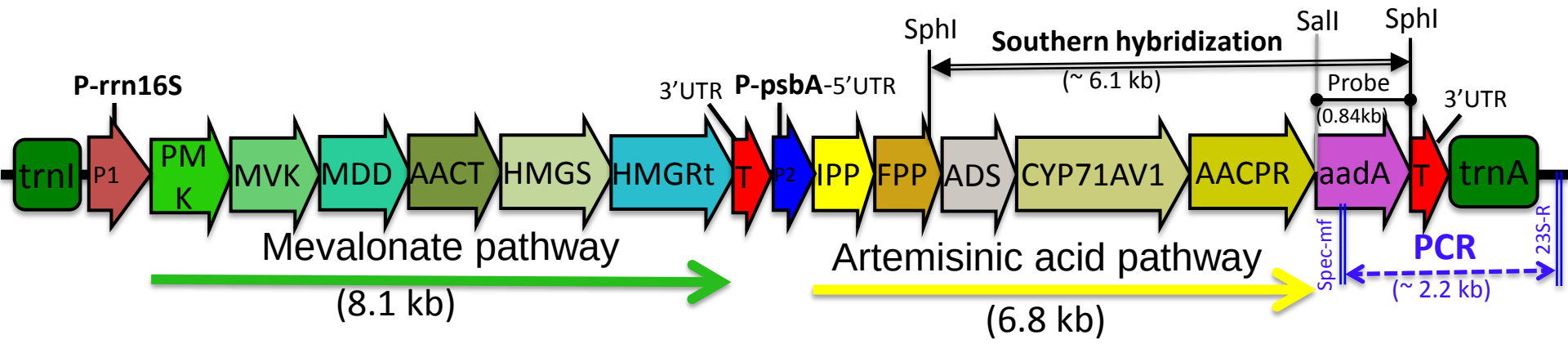
Antimalarial and
Anticancer drug

**...is produced by trichomes...**

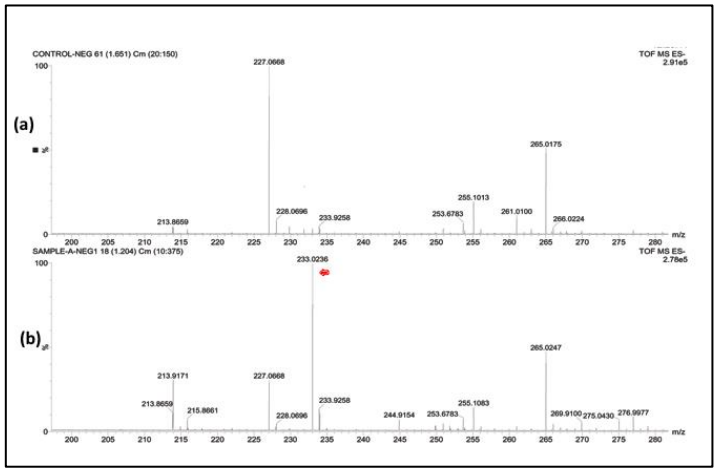
**...found on *Artemisia annua* leaves...**

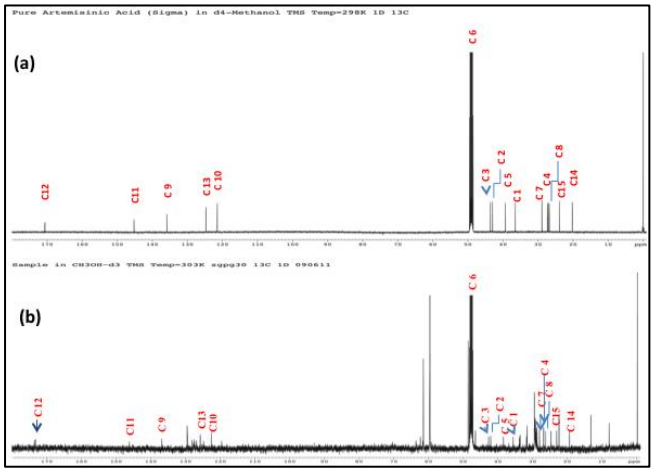
Low artemisinin is produced by trichomes on *Artemisia annua* leaves

# Artemisinic acid biosynthesis in chloroplast




Negative Electrospray Ionization (ESI) Mass spectrometric (MS) m/z spectra of artemisinic acid




$^{13}C$ NMR Spectra of artemisinic acid (a) control

*J Biosci. 2014 Mar;39(1):33-41*

**Metabolic engineering of chloroplasts for artemisinic acid biosynthesis and impact on plant growth**

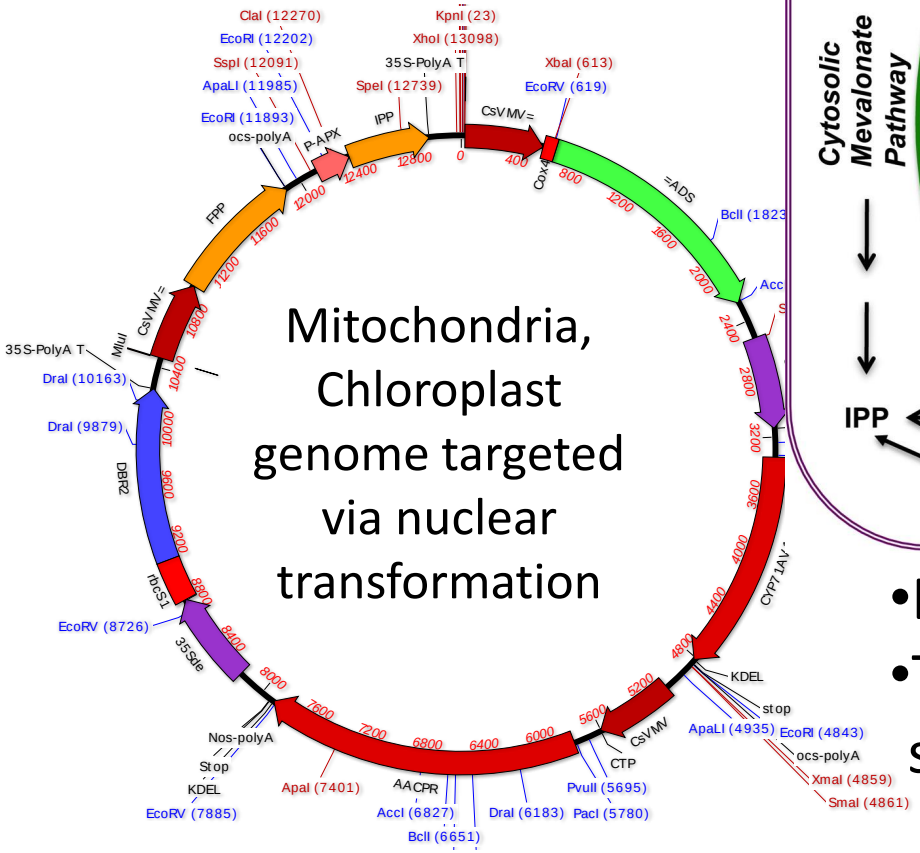
Saxena B, Subramaniyan M, Malhotra K, Bhavesh NS, Kumar S

Plan B

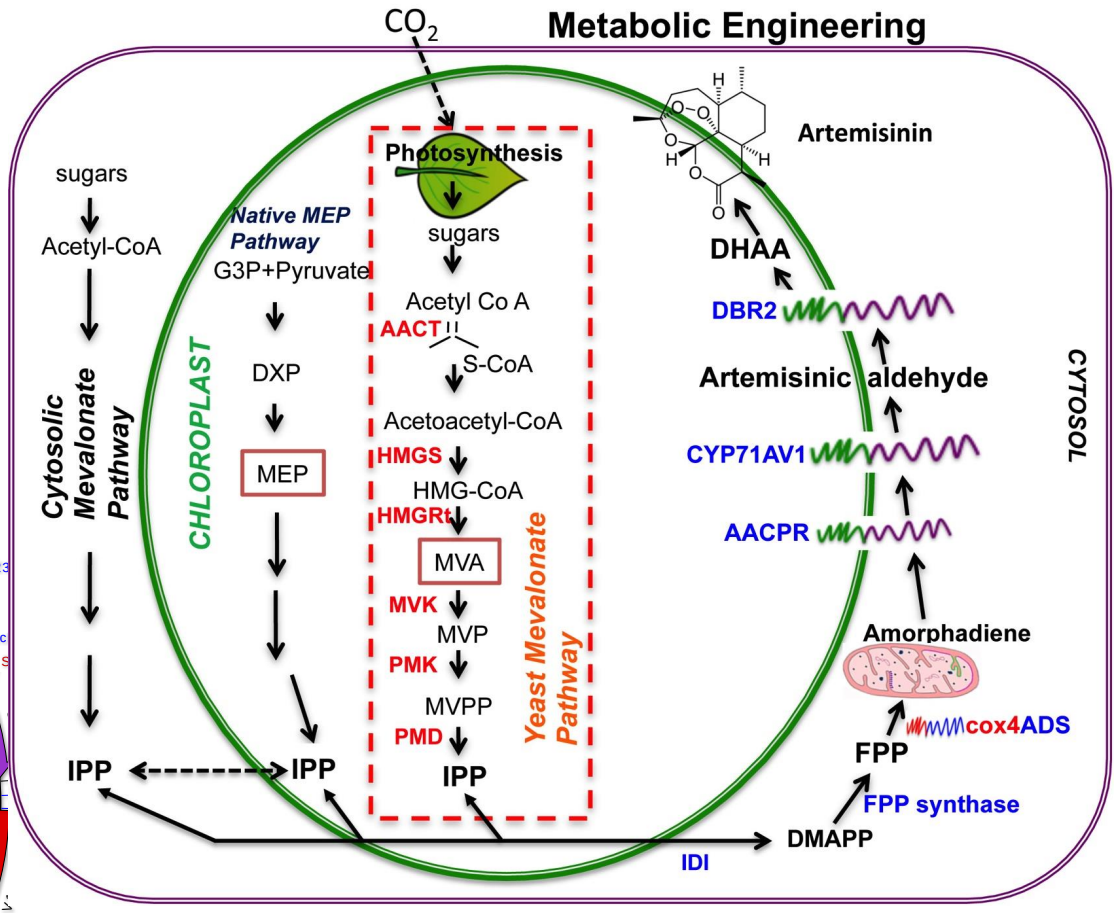
# Rationalized MBE for Artemisinin via dihydroartemisinic acid (DHAA) pathway

T-MEV6 with additional **IPP**

+



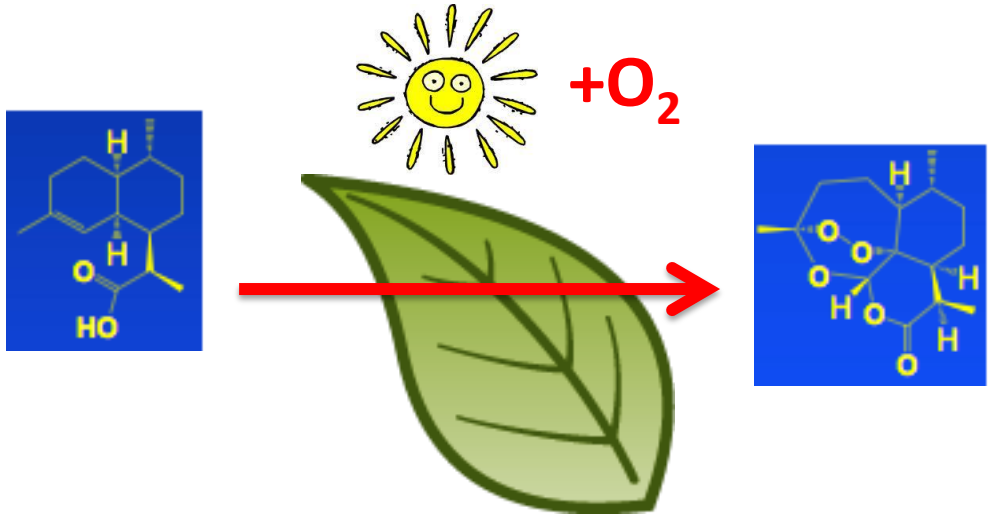

Mitochondria, Chloroplast genome targeted via nuclear transformation




- Dual transformation
- Targeted enzymes to three different subcellular compartments

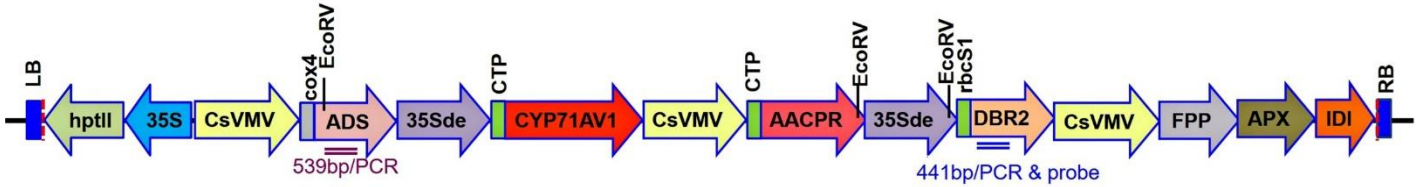
# Advantage of expressing dihydroartemisinic acid (DHAA) biosynthesis pathway

## (Spontaneous Conversion of DHAA to ART)




- Conversion of DHAA to artemisinin is **a spontaneous photo-oxidative process** and does not require additional enzymes and energy
- The exposure of light after harvesting the plant favored this bioconversion (Farhi et al., 2011). The maximum bioconversion of DHAA to artemisinin was achieved when *A. annua* plants were sun-dried (Ferreira and Luthria, 2010).
- To minimize adverse effects and negative impact of artemisinin on growth of transgenic lines (Toxic)

# Nuclear genome transformation of homoplastomic plant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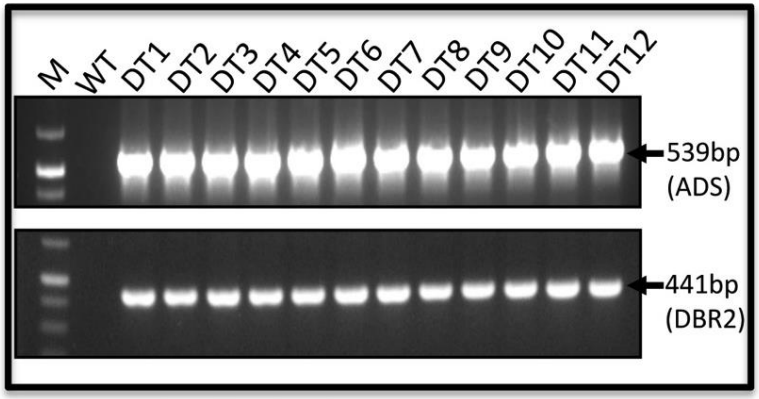
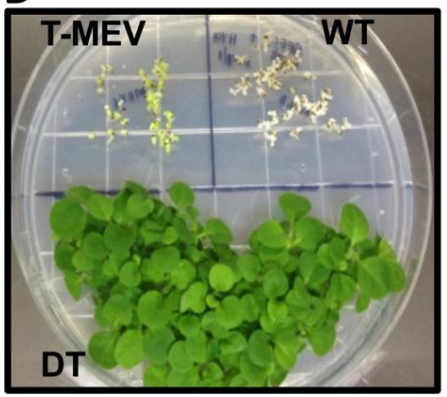


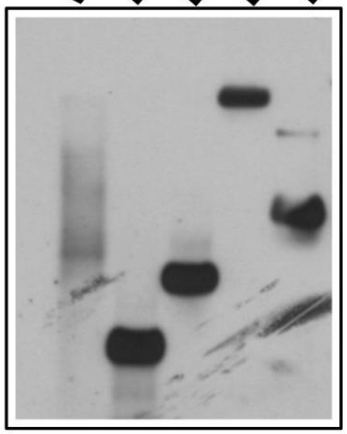

C

D




Hygro+Spec

E

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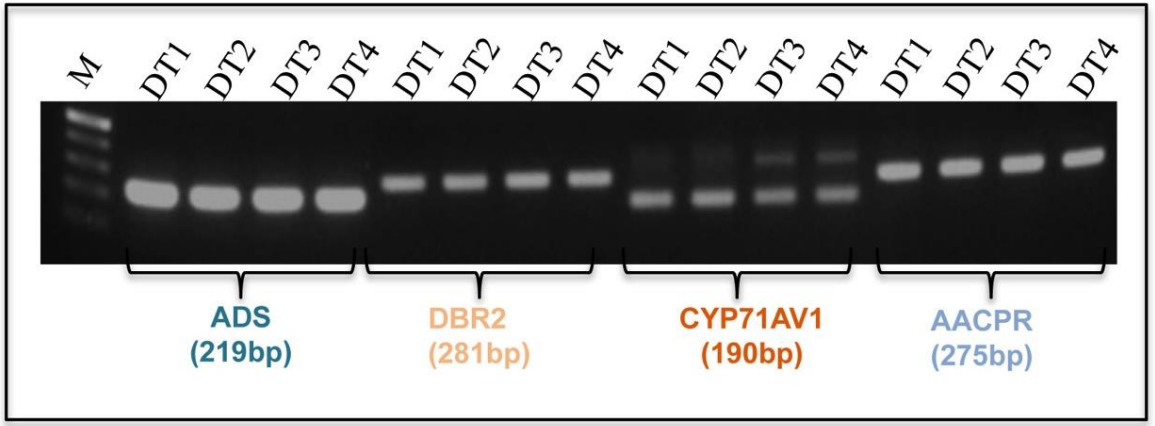


# Identification of pathway-metabolites in DT

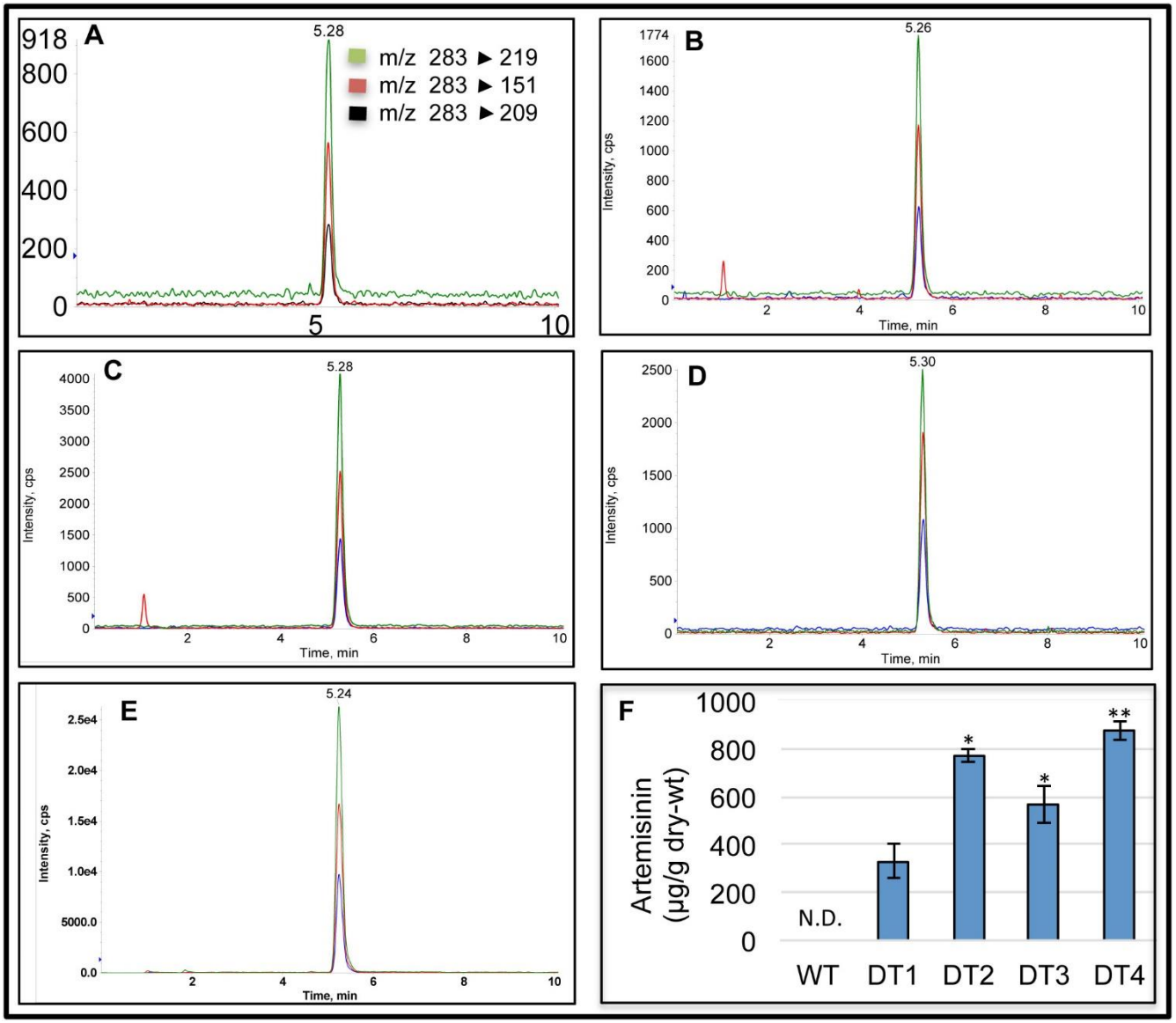

Artemisinin (LC-MS/MS)

Dihydroartemisinic acid (LC-MS/MS)

Isopentenyl diphosphate (LC-MS/MS)

Amorphadiene (GC-MS)

# Artemisinin biosynthesis in alternative plant

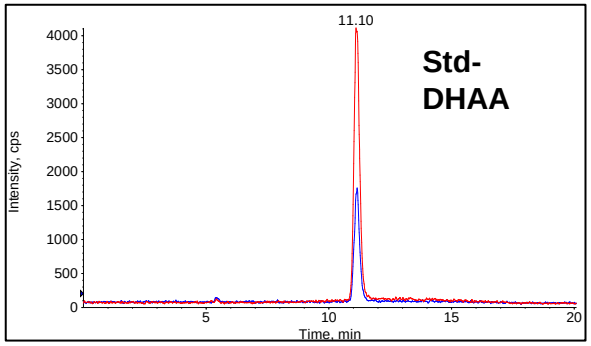


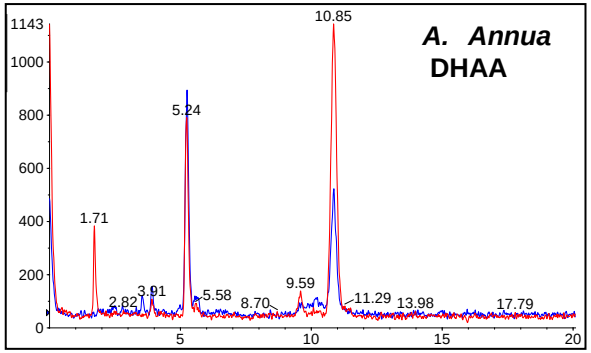

Bioconversion of DHAA to artemisinin spontaneously in presence of light and oxygen

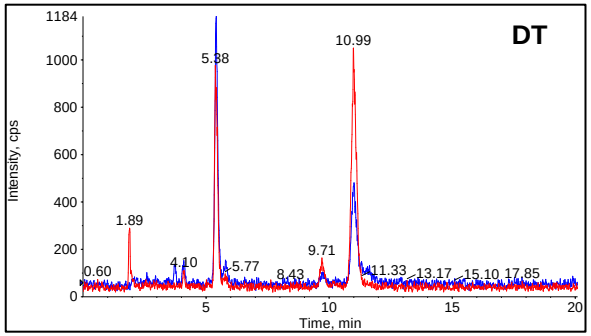
Maximum in DT4 transgenic line

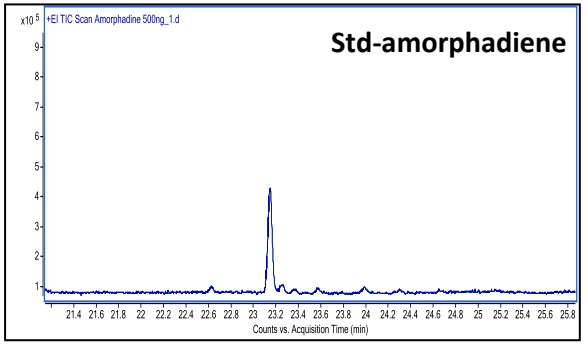
Maximum titer 0.8µg/g DW (~0.08%)

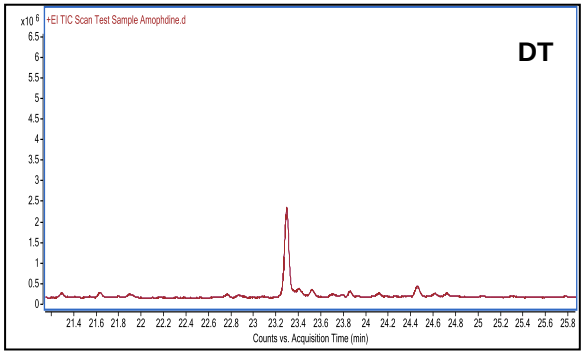
# DHAA analysis by LC-MS & Amorphadiene by GC-MS of DT plants

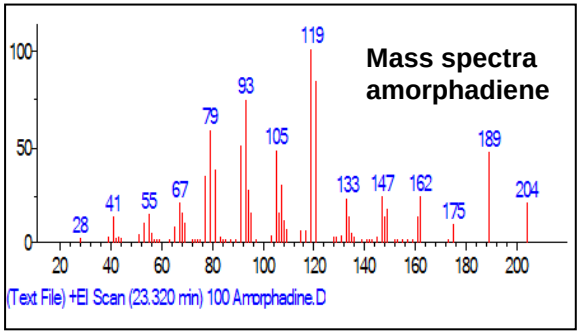


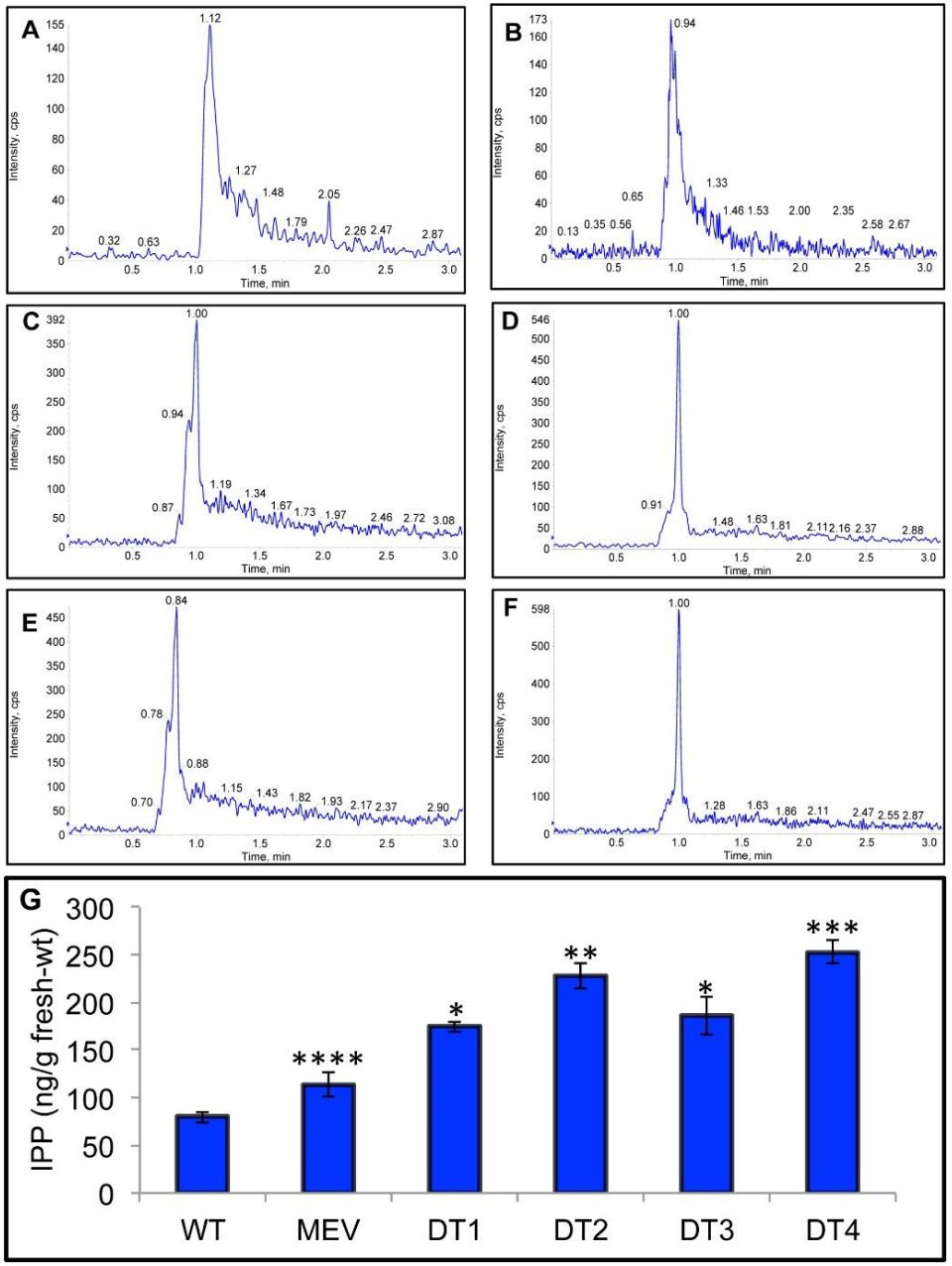



# Enhanced accumulation of isoprenoid IPP



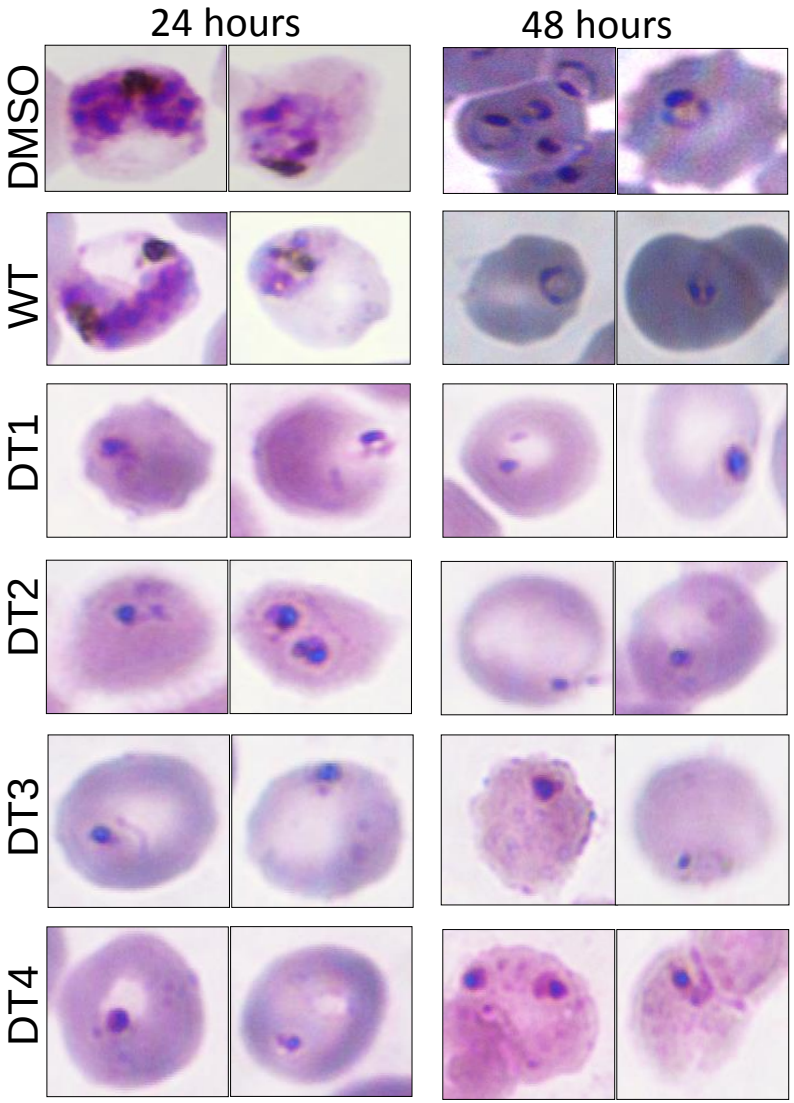

IPP/DMAPP are universal isoprenoid precursors

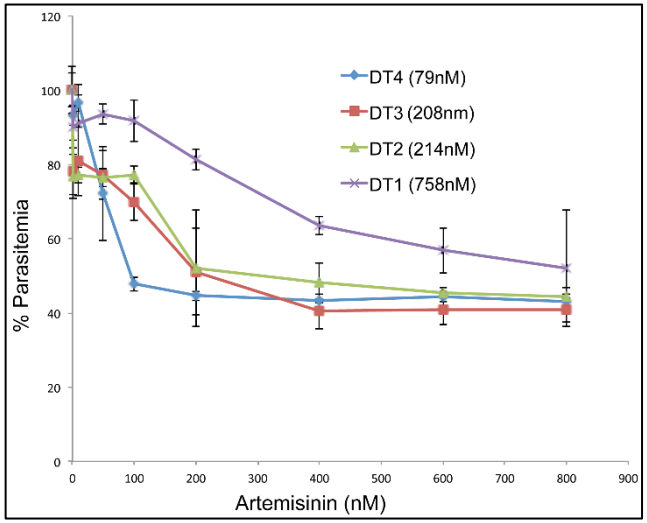
IID gene introduction in nuclear genome for maintaining equilibrium ratio between IPP and DMAPP

Nearly ~3-fold enhancement in IPP

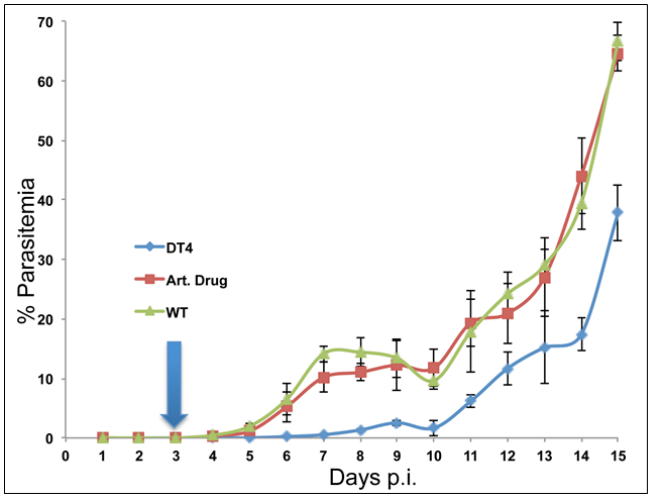
Sufficient IPP is essential for high artemisinin biosynthesis

# Functionality of Artemisinin



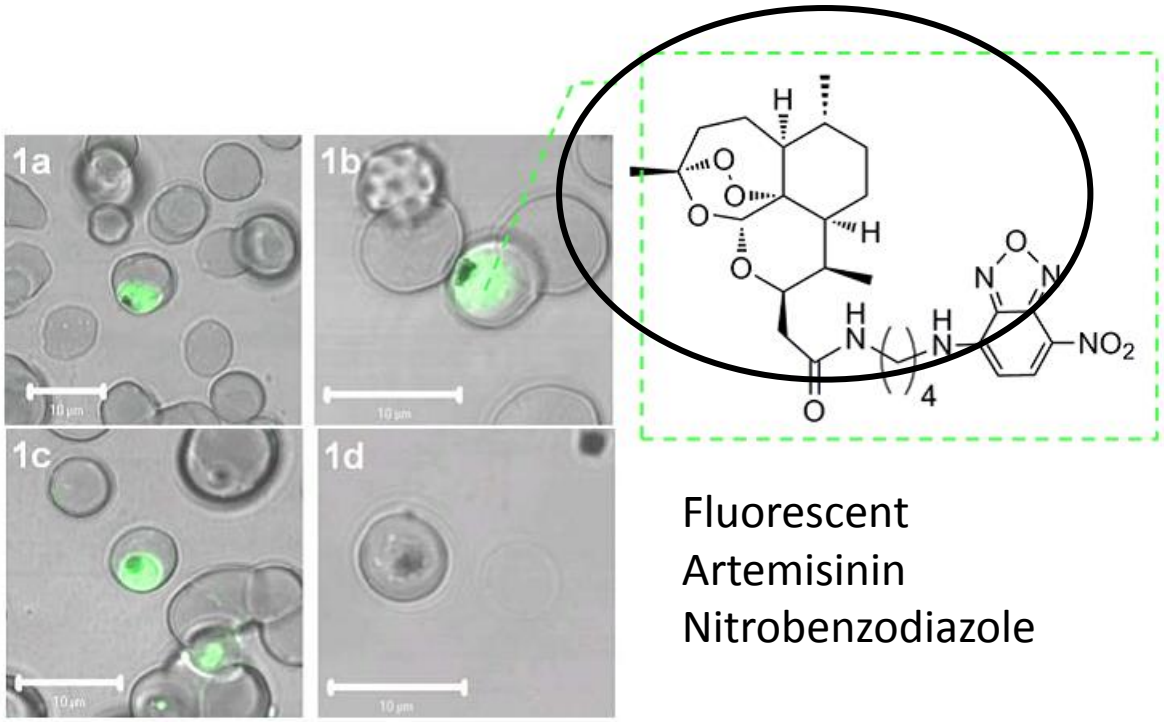




Extracts from DT1-DT4 inhibited parasite Growth in 24-48h assay




Oral feeding of intact whole plant DT4 in Balb/C mice reduced the parasitemia levels.

# Mechanism of action of artemisinin

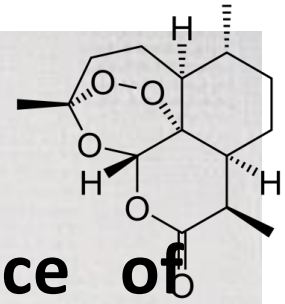



Fluorescent
Artemisinin
Nitrobenzodiazole

**Figure - Confocal images of parasite infected RBC incubated with Fluorescent Artemisinin Nitrobenzodiazole conjugate without iron chelator DFO before (1a) and after wash (1b), and with DFO (100 µM) before (1c) and after wash (1d)**

**In 1991, Meshnick and collaborators showed that artemisinin interacted with intraparasitic heme, and suggested that intraparasitic heme or iron might function to activate artemisinin inside the parasite into toxic free radicals (Meshnick et al., 1991). The malaria parasite is rich in heme-iron, derived from the proteolysis of host cell hemoglobin (Rosenthal and Meshnick, 1996). This could explain why artemisinin is selectively toxic to parasites.**

# Conclusions



- ART is frontline treatment for rapid clearance of malarial parasitemia
- Lengthy biosynthesis period (18 months) and low yield of ART from native plant. Higher cost of chemical synthesis limits the ART supply for malarial treatment.
- Low supply from the natural sources, and the non-availability of an antimalarial vaccine, has necessitated producing this drug with an alternative method.
- Chloroplast and nuclear genome transformation has produced the complete artemisinin, stop the growth of *Plasmodium falciparum*
- Higher biomass and rapid growth of tobacco plant may provide sufficient supply of ART for endemic regions

# Acknowledgments

MBE Group

Collaborators

H Daniell
UPenn

M Abdin
JHU

J Keasling
UC Berkeley

Katrina Cornish
OSU

Pawan Malhotra

Asif Mohmad

Neel S Bhavesh

Funding

DBT

Department of Biotechnology Govt. of India

Department of Science and Technology

NSF

ICGEB

International Centre for Genetic Engineering and Biotechnology colleagues, friends and ICGEB Director Prof. Dinakar Salunke