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SIMB 39th Symposium on Biotechnology for Fuels and Chemicals

High performance terpenoid biofuel production by oleaginous yeast Rhodosporidium toruloides

Xun Zhuang

Sandia National Laboratories

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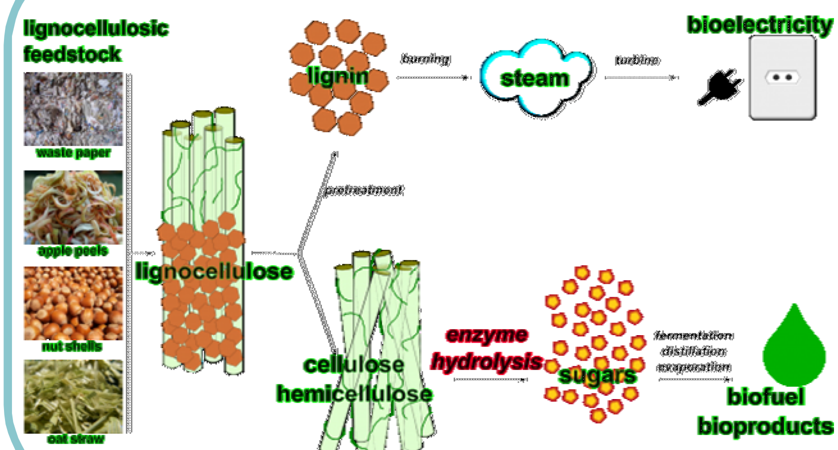
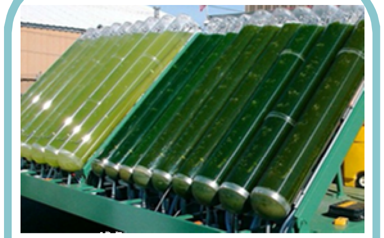
Biofuel Generations

Biofuel products

- 1st generation
- 2nd generation
- 3rd generation

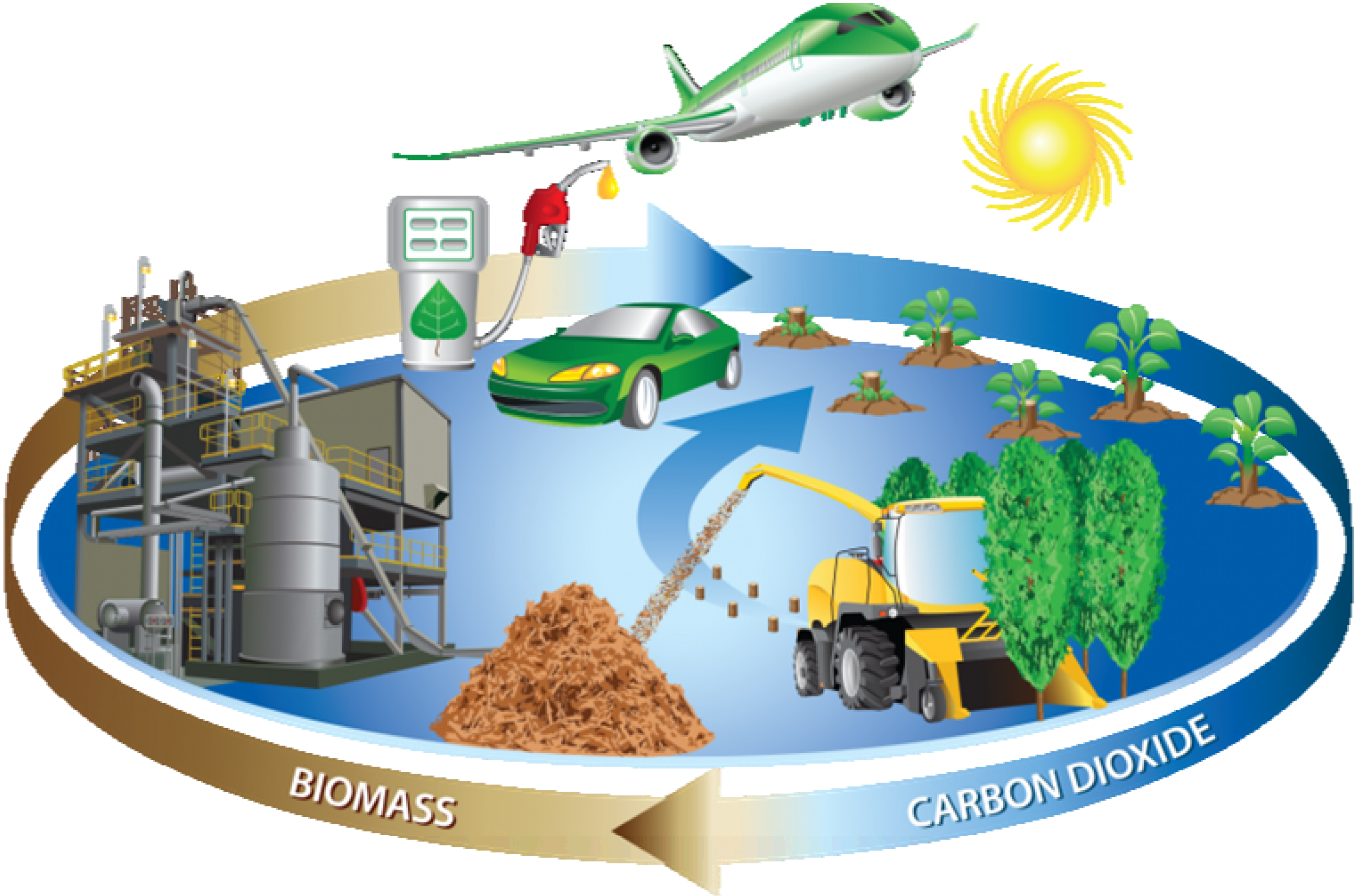




Algae

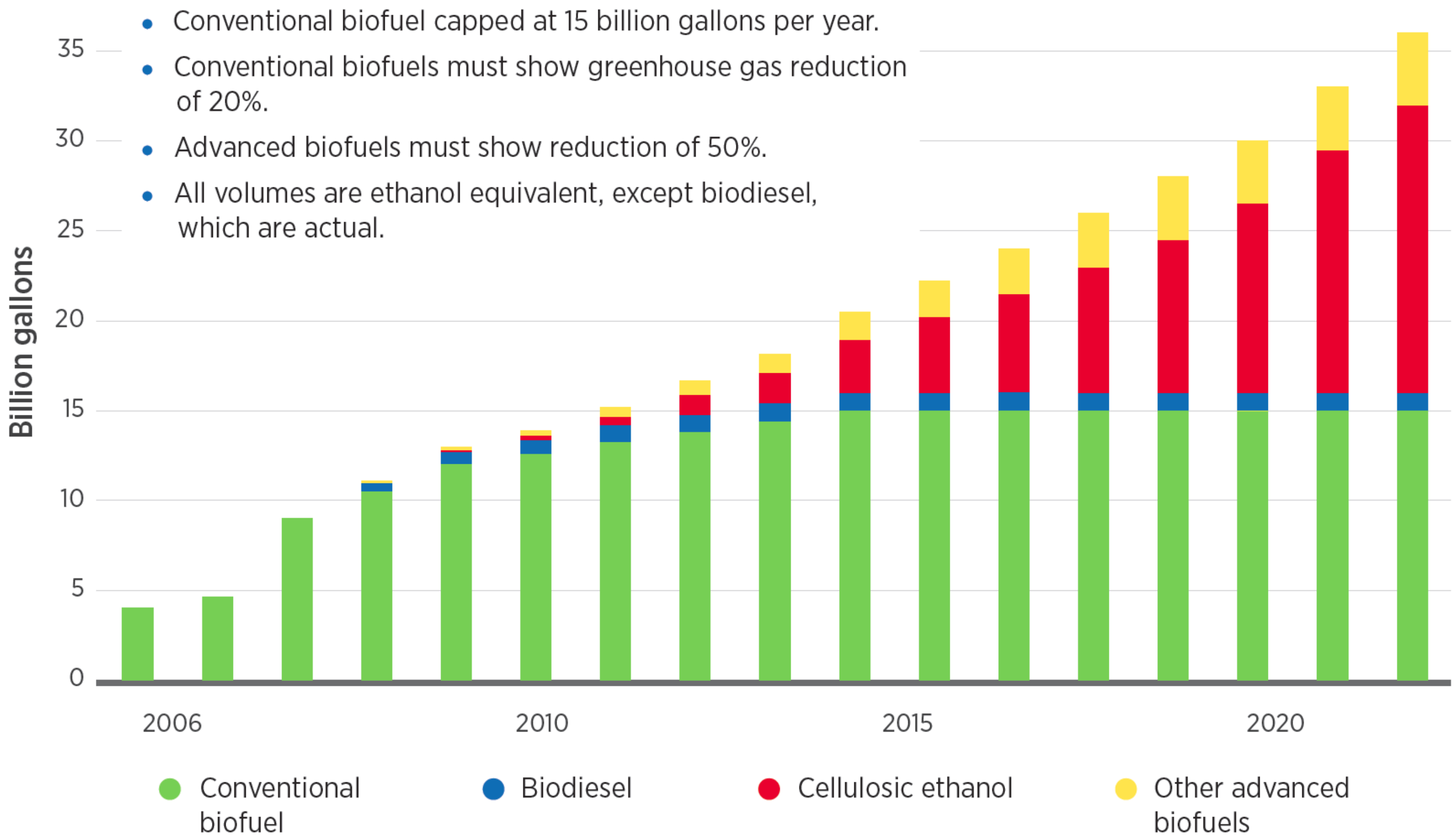
Justin Lenihan - Biofuels <https://sites.google.com/site/ee5352012/justin-lenihan>.

Carbon balance of biofuel life cycle



'Advanced Hardwood Biofuels Northwest' <http://hardwoodbiofuels.org/news-and-events/feature-stories/understanding-the-carbon-balance-of-biofuel-production/>

Increasing demand of biofuel from renewable biomass



2016 Billion-Ton Report

The opportunity space

The U.S. has ~billion tons of renewable biomass available annually that is a strategic national resource for the bioeconomy

U.S. bioeconomy is estimated at ~$250B/yr and expected to grow significantly over the next decade

Mobilizing and valorizing this resource through biomanufacturing could rapidly expand the U.S. bioeconomy

Biomanufacturing remains nascent in terms of robustness, scale and standardization

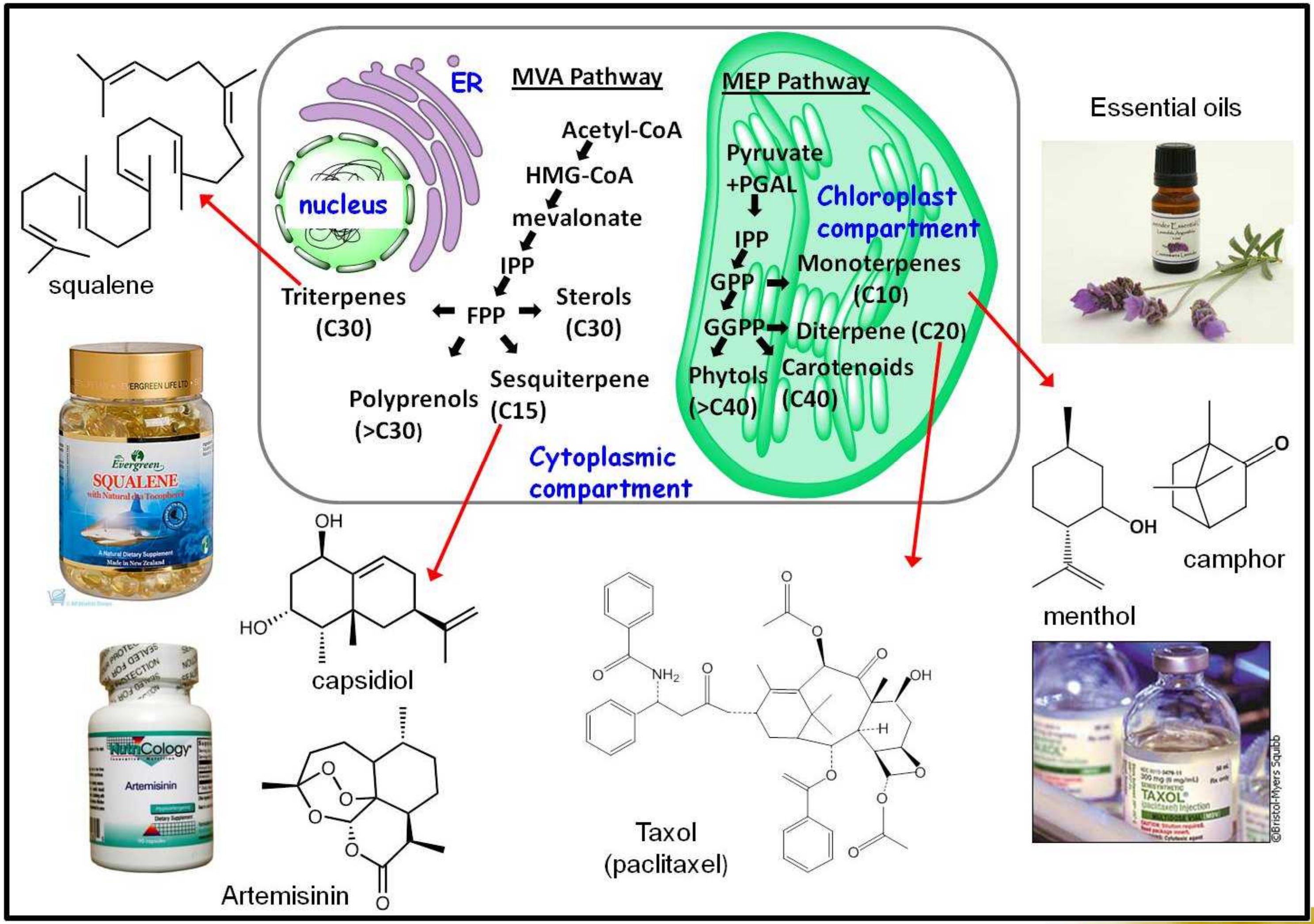
U.S. DOE, 2016 Billion-Ton Report

Rhodosporidium toluroides production platform for terpene and lipid bioproducts

Rhodosporidium toluroides (Pucciniomycotina)

- **Oleaginous and carotenogenic yeast**
- **Utilizes glucose, xylose, lignin-derived aromatics**
- **Produces up to 70% wt in TAGs, indicating the high level carbon flux through lipid pathway**
- **Produced high levels of carotenoids, indicating the high level carbon flux through MVA pathway**
- **20 MB genome published**
- **Genomic manipulation and transformation methods established**
- **Transgenic strains are stable**
- **Eukaryotic host suitable for additional modifications, such as oxidation and glycosylation**
- **Fast high density growth**

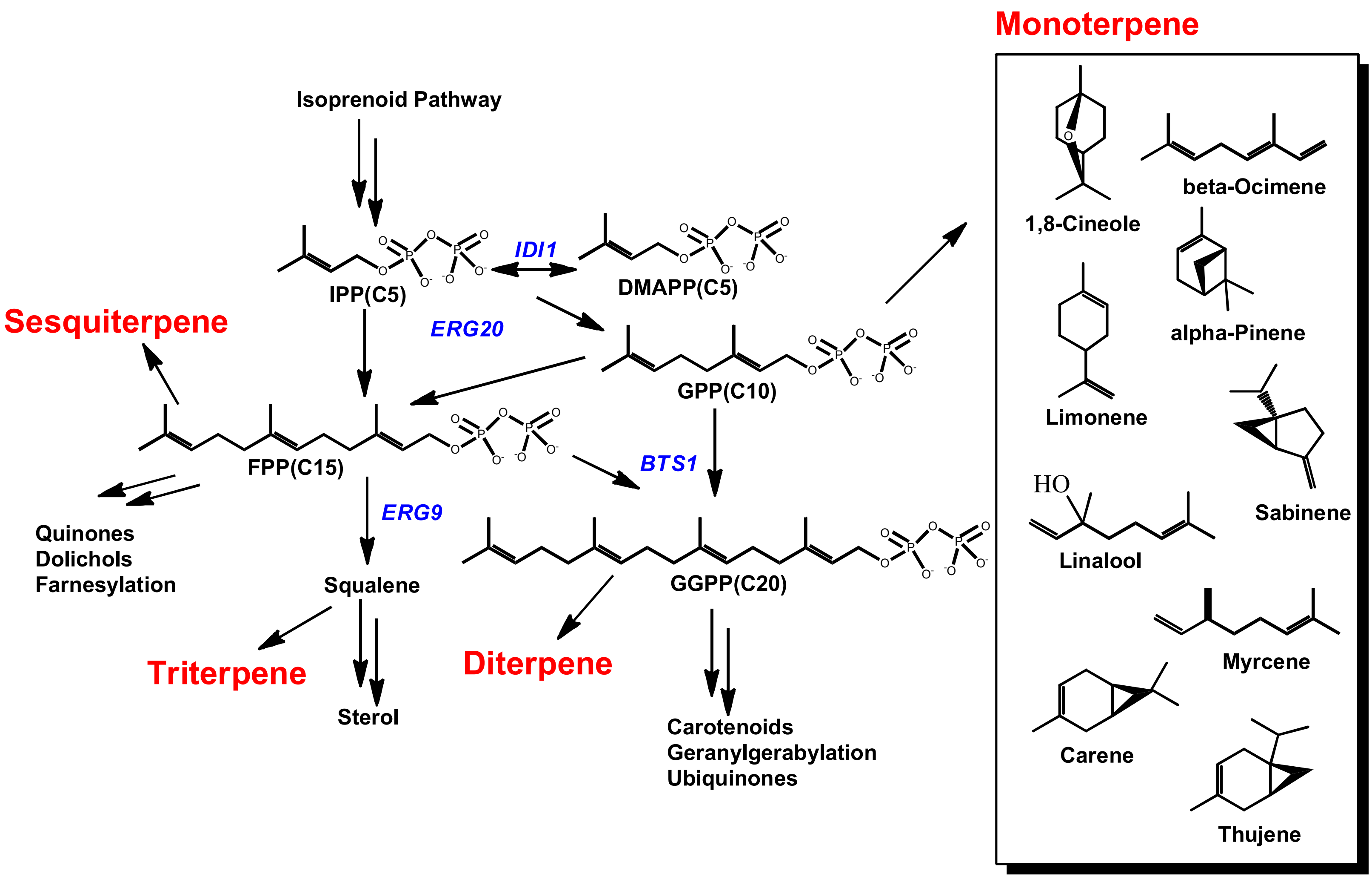
Chemical structure diversity and application of terpenes



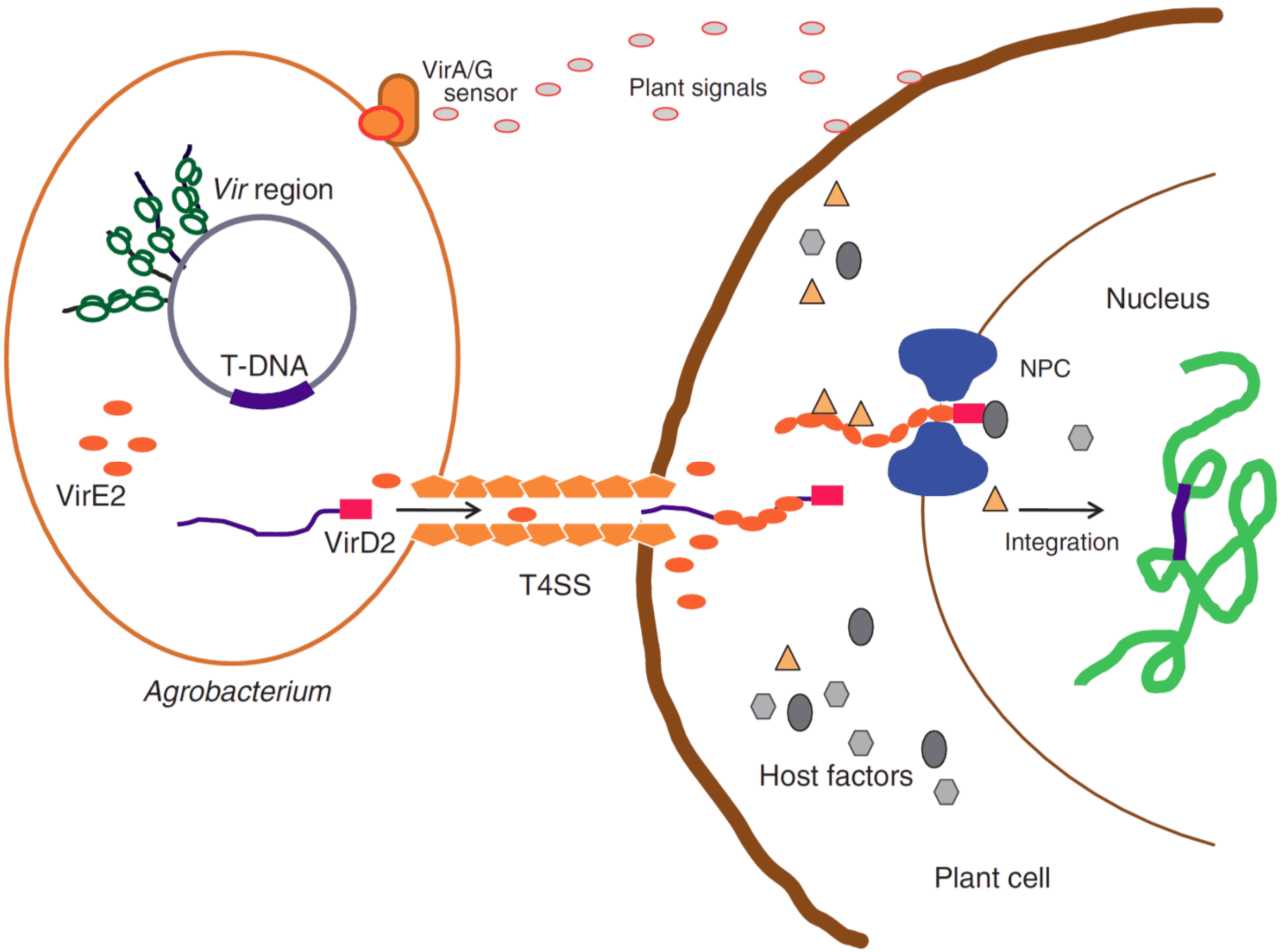
Terpenes as biofuel compounds

- Farnesene, Bisabolene as diesel fuel
- Pinene, Sabinene and Terpinene as jet fuel
- 1,8-Cineole
  - Fuel for Spark ignition engine as blend in or pure fuel
  - Could be utilized 100% as fuel in modified diesel engine
  - Octane boosting fuel additive
  - Blended in jet fuel

Schematic diagram of *R. toruloides* native mevalonate MVA pathway

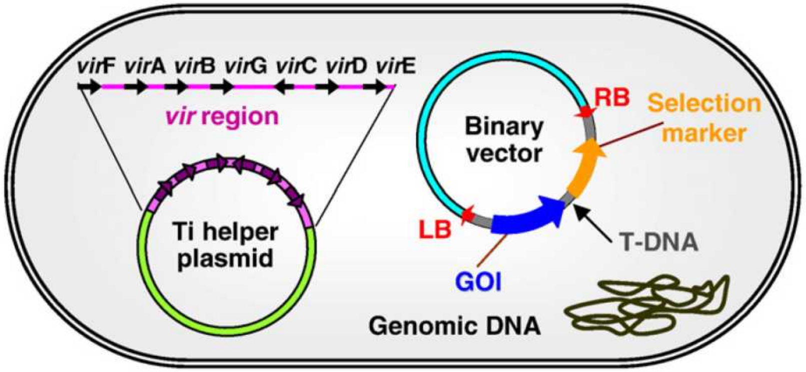


T-DNA transfer from *Agrobacterium tumefaciens* to a plant cell causes crown gall tumor



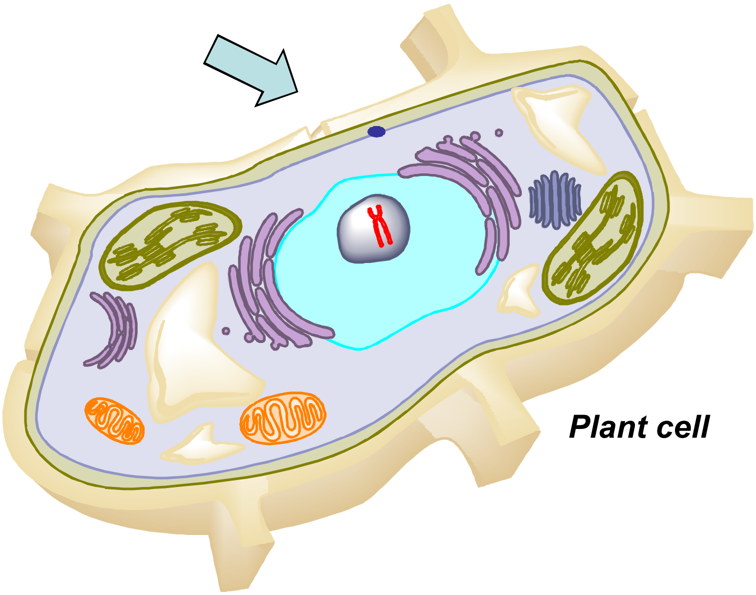
T Tzfira and Beer Sheva, *Brenner's Encyclopedia of Genetics, Second Edition* (Elsevier Inc., 2013)

Agrobacterium binary vector system for plant transformation



Agrobacterium

Transgenic plant



Plant cell

Benjamin Meyers et al., *Biotechnology Advances*, 28.6 (2010)

Agrobacterium Mediated Transformation (ATMT) of fungi

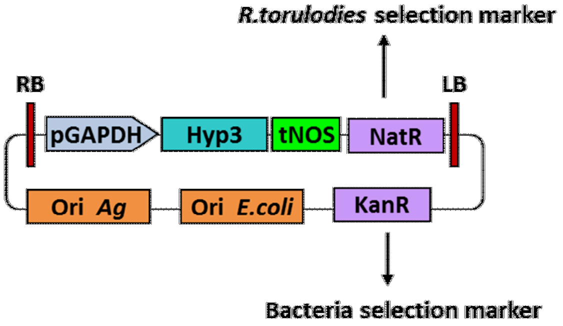
- *Rhodosporidium toruloides*
- *Saccharomyces cerevisiae*
- Filamentous Fungi
  - *Magnaporthe grisea*
  - *Fusarium oxysporum*
  - *Aspergillus niger*
  - *Trichoderma reesei*
  - *Colletotrichum gloeosporioides*
  - *Neurospora crassa*
  - *Agaricus bisporus*

Summary of Monoterpene synthases have been tested for monoterpene production in *R. toruloides*

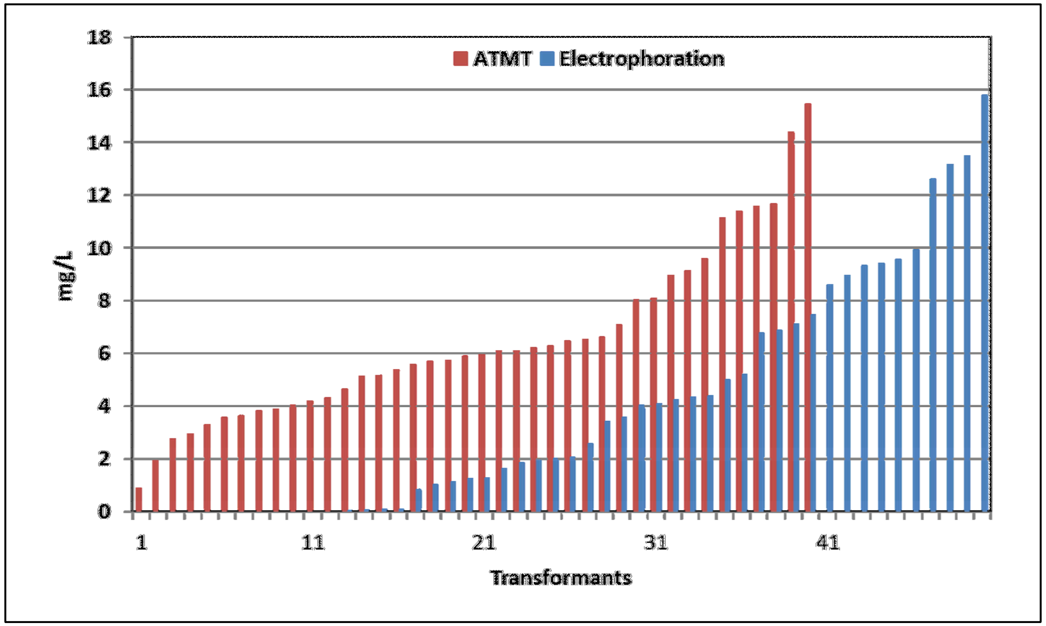
Product	Gene Name	Organism	Gene bank Access Number	Enzyme Kinetic for GPP	Product in *R. toruloides*	
					Dodecane Overlay	SPME
1,8-Cineole	*Hyp3*	*Hypoxylon sp. E74060B*	KJ433271.1	K_m=2.5±0.6 μM K_{cat}=0.295 S^{-1}	++	++
	SSCG_00536 CnsA	*Streptomyces clavuligerus*	DS570626.1	K_m=0.17 μM K_{cat}=0.079 S^{-1}	+	+
Ocimene	*ama0e23*	*Antirrhinum majus*	AY195607.1	NA	ND	+
	LcTPS1	*Licea cubeba*	HQ651178.1	NA	ND	ND
limonene	*ag10*	*Abies grandis*	AF006193.1	NA	ND	Detectable
Pinene	*PT30*	*Pinus taeda*	AF543530.1	K_m=47±9μM	ND	+
	AG3.18	*Abies grandis*	U87909.1	K_m=6μM	ND	Detectable
Myrcene	*amaOc15*	*Antirrhinum majus*	AY195608.1	NA	ND	ND
	ama1e20	*Antirrhinum majus*	AY195609.1	NA	ND	ND
	AG2.2	*Abies grandis*	U87908.1	NA	ND	ND
Linalool	*PaTPS-Lin*	*Picea Abies*	AY473623.1	NA	ND	ND
Sabinene	*RlemTPS2*	*Citrus jambhiri*	AB266585.1	NA	ND	ND
	SabS1	*Salvia pomifera*	DQ785794.1	K_m=7.4 μM	+	+
Carene	*PaJF67*	*Picea abies*	AF461460	NA	ND	ND
	TpsB	*Salvia stenophylla*	AF527416.1	NA	ND	+
Thujene	*LcTPS2*	*Licea cubeba*	HQ651179.1	NA	ND	ND

ND: No detectable <1 mg/L; +: <5 mg/L; ++:5-20 mg/L

Cineole productions in *R. toruloides* by two transformation methods ATMT Vs electroporation



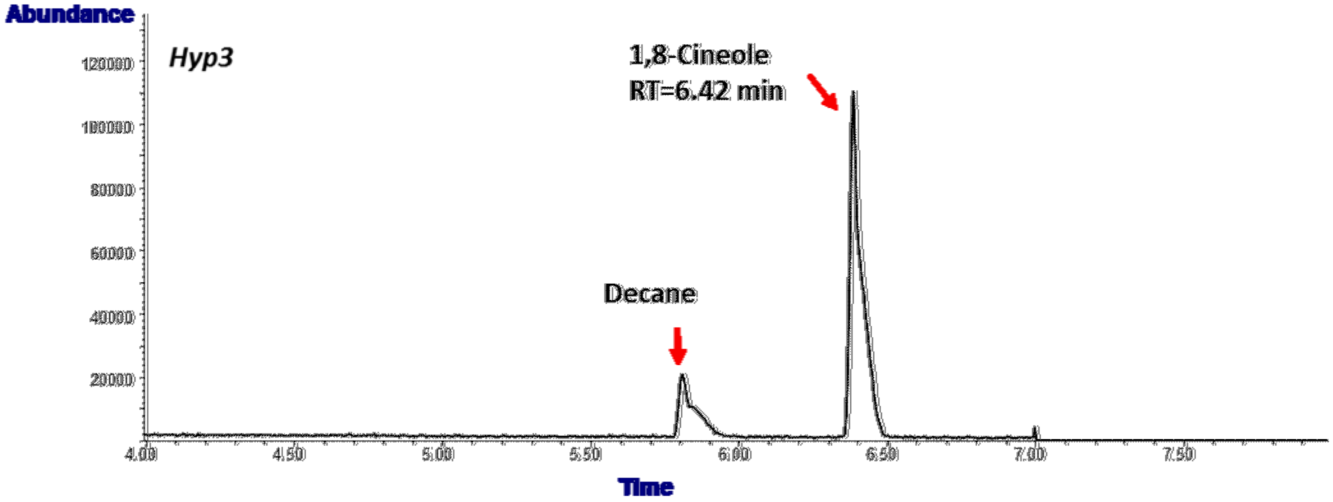
- Binary vector used to transform *R. toruloides* by ATMT method
- As template to PCR amplify T-DNA region to transform *R. toruloides* by electroporation method



Cineole production by fungal cineole synthase *Hyp3* in *R. toruloides*

Gene: *hyp3* from endophyte Hypoxylon

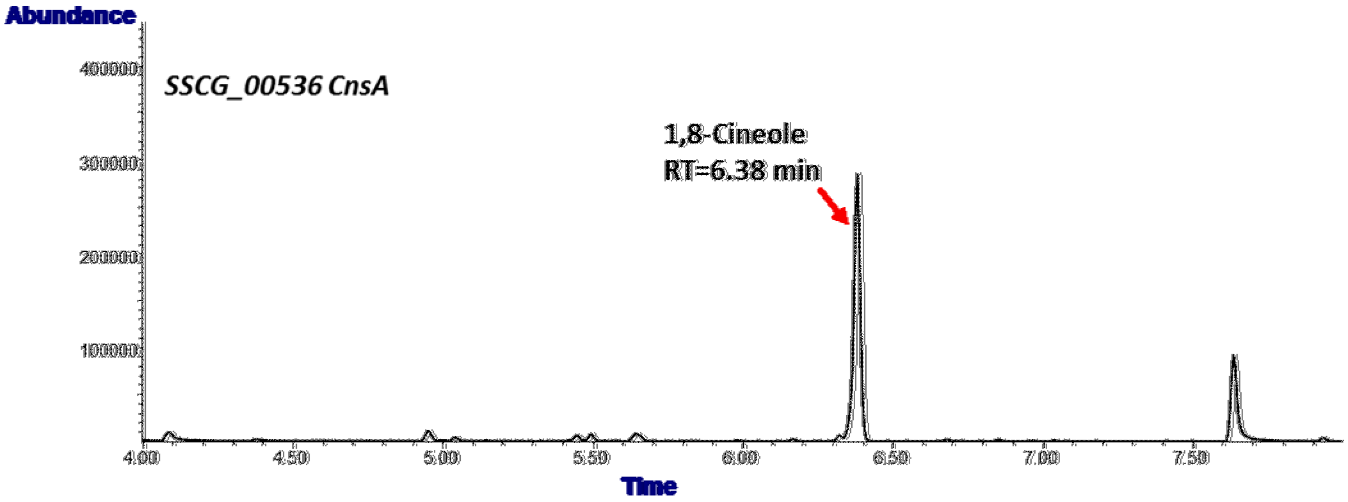
Product: 1,8-Cineole (Eucalyptol, RON 99.2)
Method: Dodecane overlay, direct injection



Cineole production by bacterial cineole synthase *SSCG_00536* in *R. toruloides*

Gene: *SSCG_00536 Cns from Streptomyces clavuligerus*

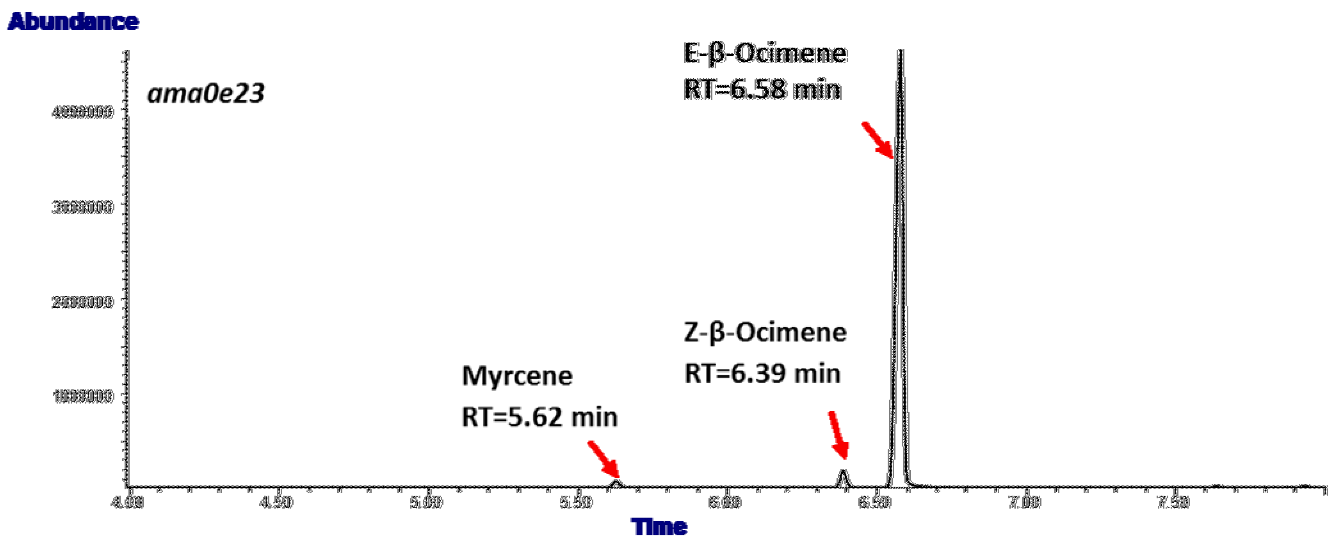
Product: 1,8-Cineole (Eucalyptol, RON 99.2)
Method: SPME



Ocimene production in *R. toruloides*

Gene: *ama0e23* from *Antirrhinum majus (snapdragon)*

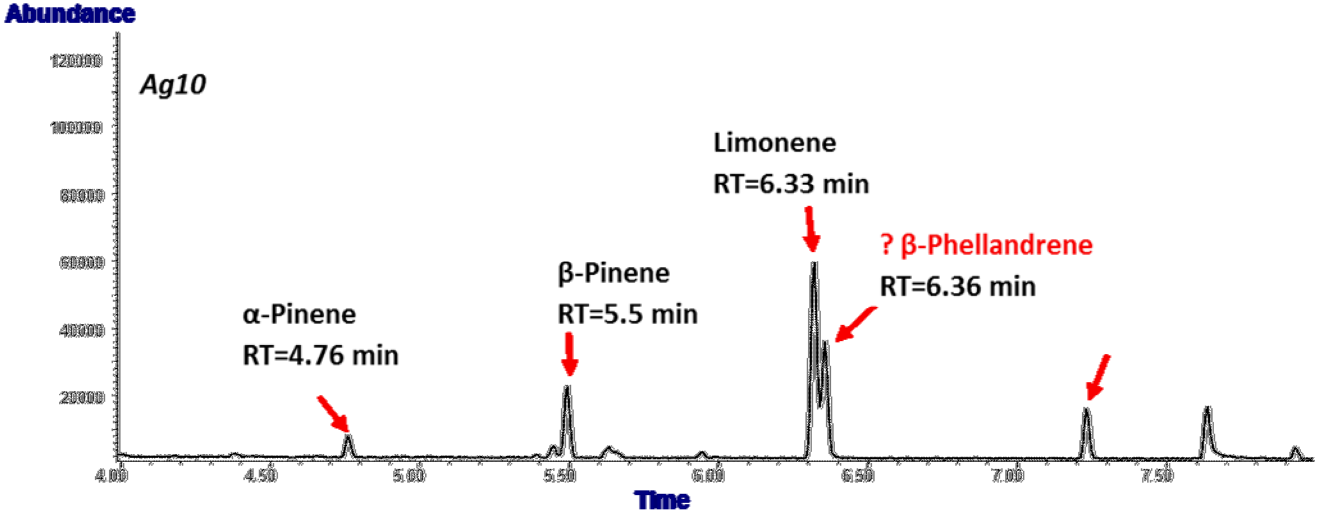
Product: Ocimene (RON 72.9)
Method: SPME



Limonene production in *R. toruloides*

Gene: *AG10 from Abies grandis (Grand fir)*

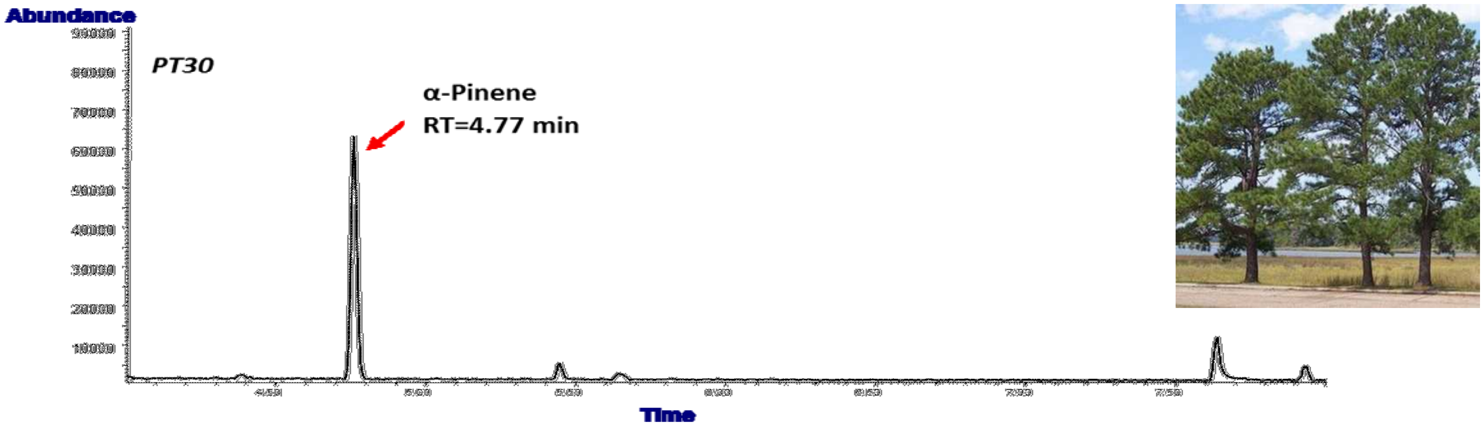
Product: Limonene (RON 87.1)
Method: SPME

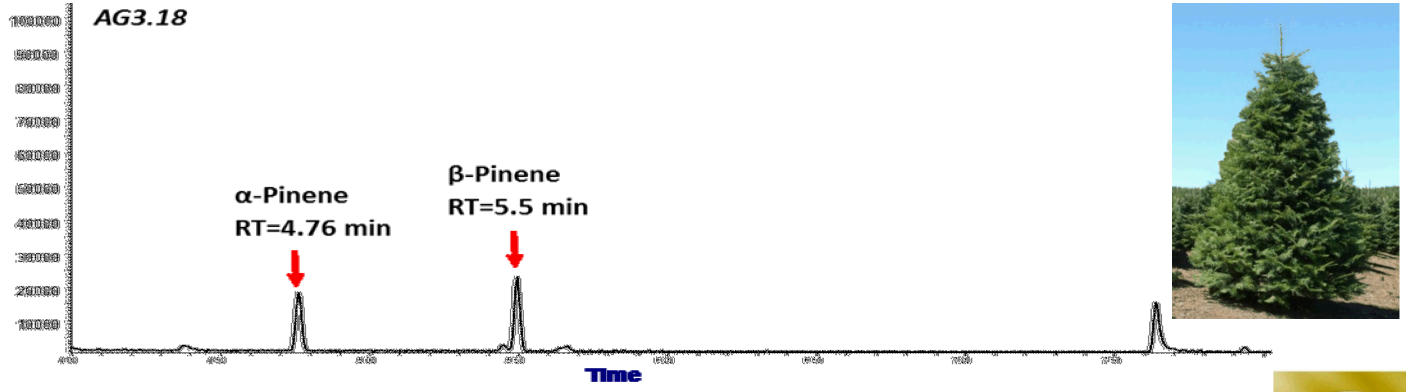


Pinene production in *R. toruloides*

Gene: *PT30 from Pinus taeda (loblolly pine) & AG3.18 from Abies grandis (Grand fir)*

Product: Pinene (RON 83.3)
Method: SPME

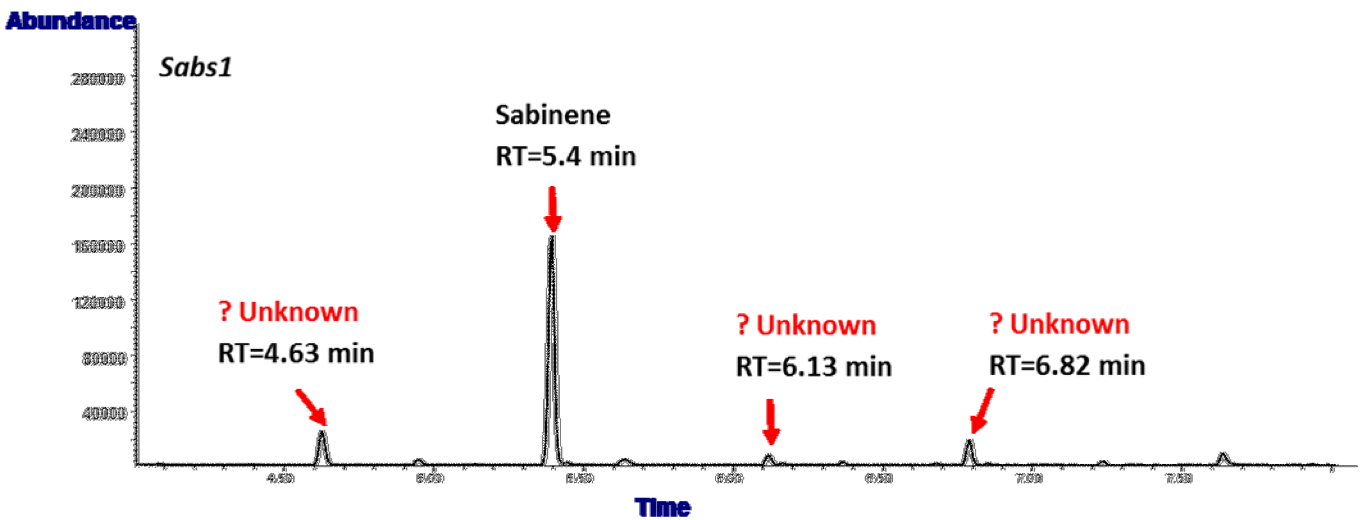




Sabinene production in *R. toruloides*

Gene: *SabS1* from *Salvia pomifera (Apple Sage)*

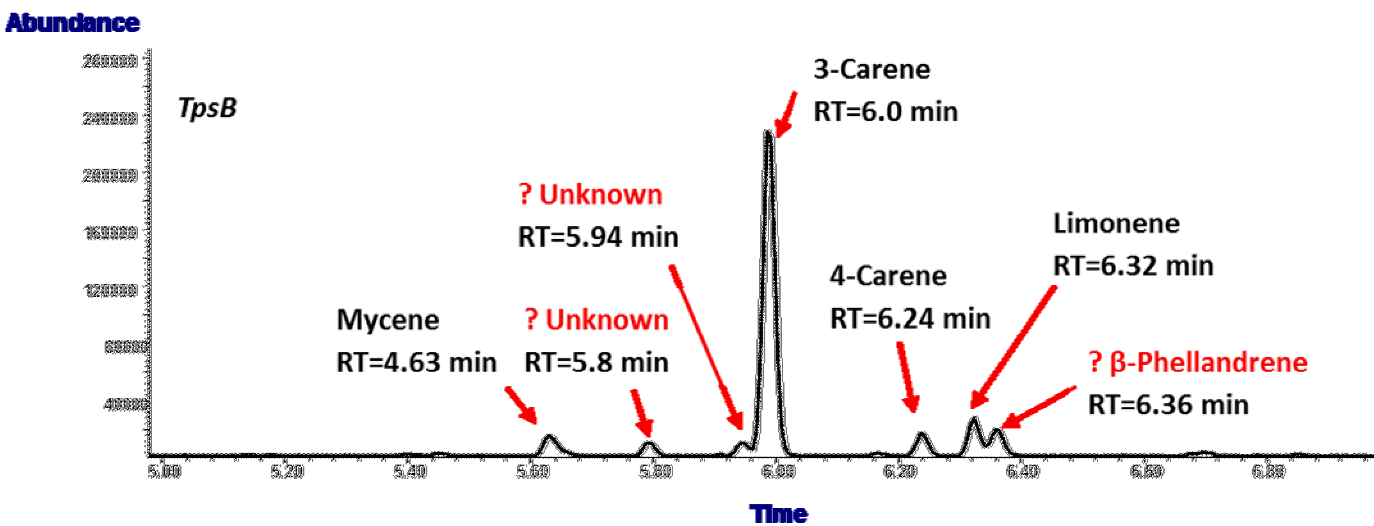
Product: Sabinene (RON 80.9)
Method: SPME



Carene production in *R. toruloides*

Gene: *TpsB from Salvia stenophylla (Blue Mountain sage)*

Product: Carene (RON 68.9)
Method: SPME



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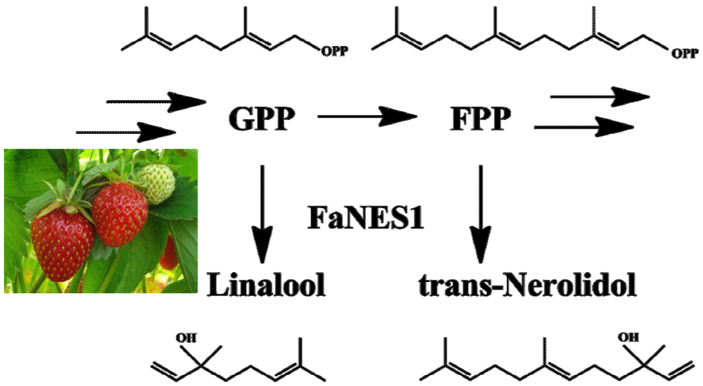
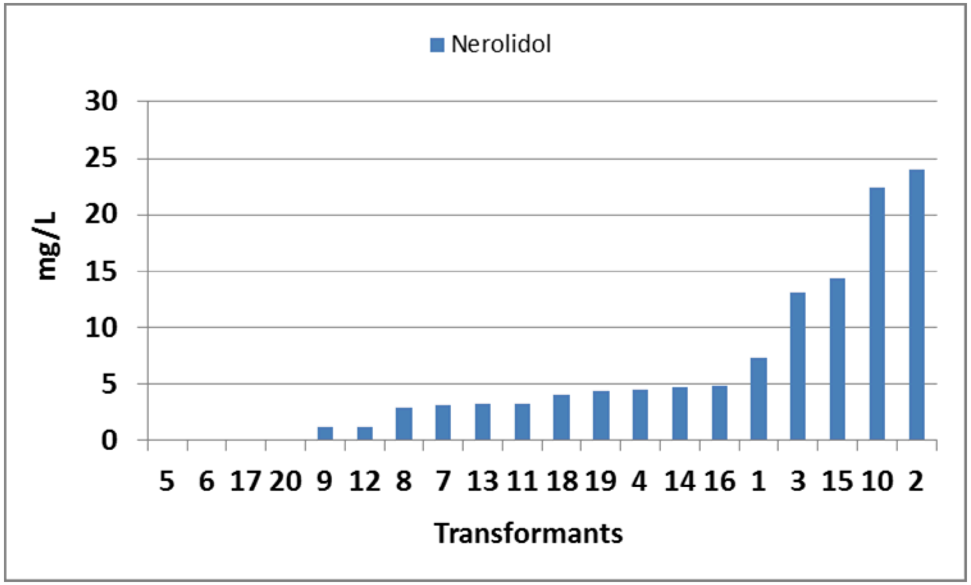
Measuring GPP substrate in *R. toruloides* by expression bifunctional strawberry terpene synthase

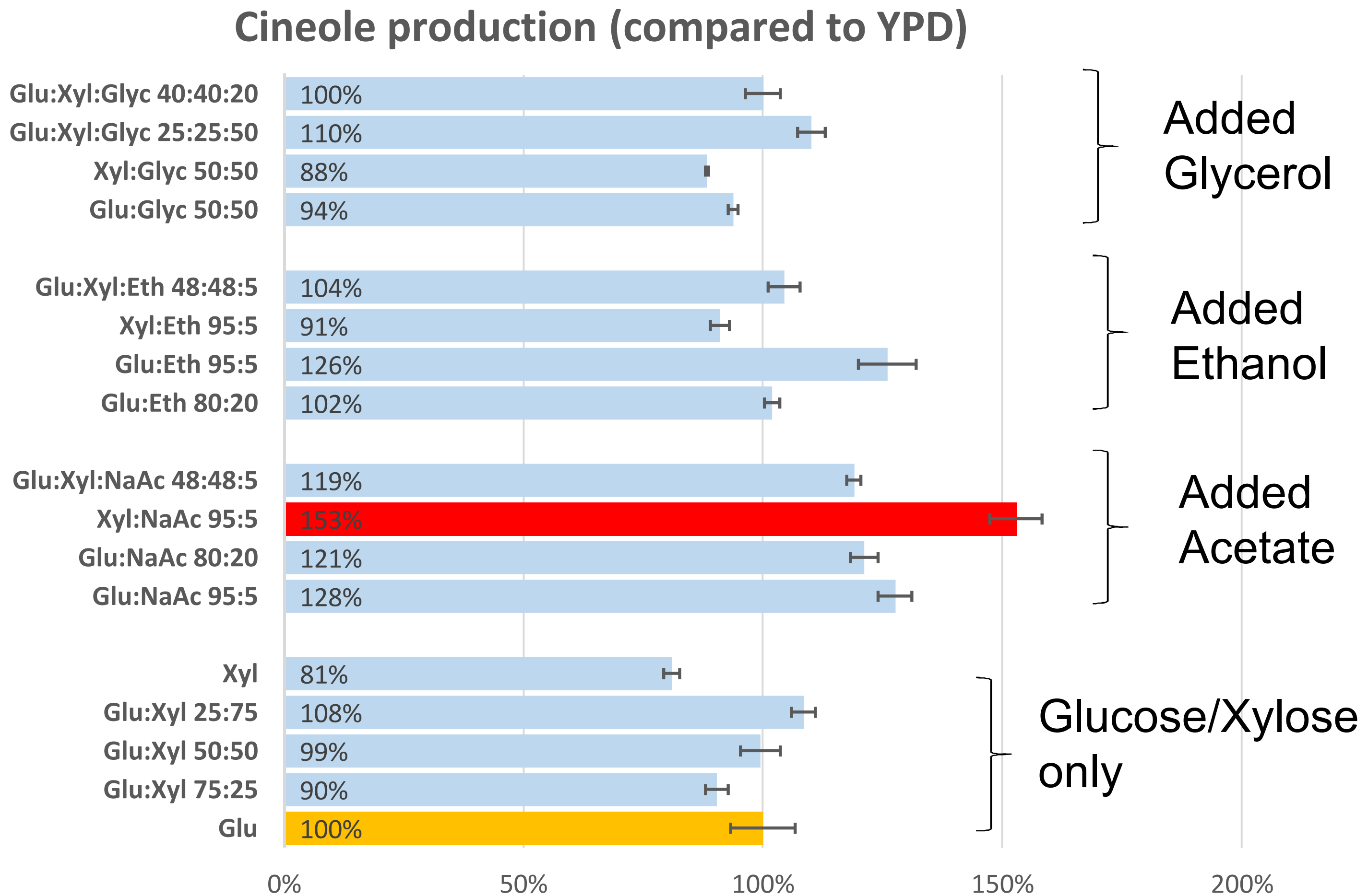


Bifunctional FaNES1 can utilize both GPP and FPP as substrate

Only C15 Nerolidol accumulated no detectable linalool produced. Limited GPP substrate in *R. toruloides*



Acetate Stimulates Cineole Production



Comparison fuel properties of the 1,8- cineole with a common biofuel ethanol

	1,8-Cineole	Ethanol
Research Octane Number (RON)	99.2[a]	109[a]
Motor Octane Number (MON)	91.0[a]	90[a]
Sensitivity (RON-MON)	8.2	19
Energy Density [MJ/L]	38.0[c]	20.2[b]
Heat of Vaporization [kJ/kg]	255[d]	919[d]
Vapor Pressure [kPa @25 C]	0.25[d]	7.833[d]
Water Solubility [g/L @21 C]	3.5[d]	Fully Miscible[d]
Oxygen Content [% of total mass]	10.4	34.7[d]
Anti-Knock Index AKI= ½(RON+MON)	95.1	99.5[d]
Boiling Point [°C]	176 [d]	78.5[d]
Freez Point/Melting Point [°C]	1 [d]	-114[d]
Flash Point [°C]	49 [d]	14[d]
OSHA hazards category	2 [d]	2 [d]

a: Octane Numbers for RON and MON determined by ASTM D2699 and D2700 respectively in collaboration with Intertek Group plc in Benicia CA.
b: Energy density based on the Lower Heating Values (LHV) first reported by Wallner et al and later in the NREL fuel properties database.
c: Energy density calculated based on reported energy densities for similar monoterpenes reported by Harvey et al [80] and later Meylemas et al.
d: Physical properties gathered from the NREL Co-optima fuel properties database.

Advantages of *Rhodosporidium* as a cell factory for terpene production

❑ Rhodosporidium natively has more terpene building blocks such as IPP, DMAPP, FPP than Saccharomyces

❑. Rhodosporidium can utilize both glucose and xylose as carbon source, so Lignocellulosic hydrolysates can be used as feedstock

❑Homologous recombination (HR) as well as random genes insertion with multiple copy numbers into genome with non-homologous end-joining (NHEJ) are both operated in *Rhodosporidium KU70* lines.

❑ Transgenic *Rhodosporidium* strain are stable than transformed *Saccharomyces* strain

❑Fermentable & scale-up able

Acknowledgements

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Co-Optimization of Fuels & Engines