PHA Accumulation in Sugarcane Plastids and Peroxisomes

BIOEN Workshop on Synthetic Biology
São Paulo Brazil
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SIIB Sugar Industry Innovation through Biotechnology

THE UNIVERSITY OF QUEENSLAND

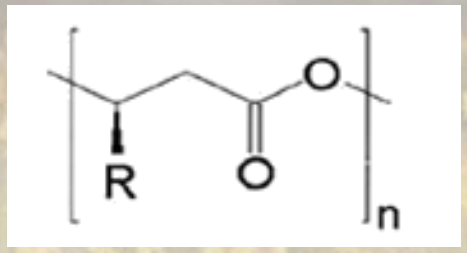
Metabolix Bio-Industrial Evolution™

BSES Sugarcane for the future

PHA in Sugarcane

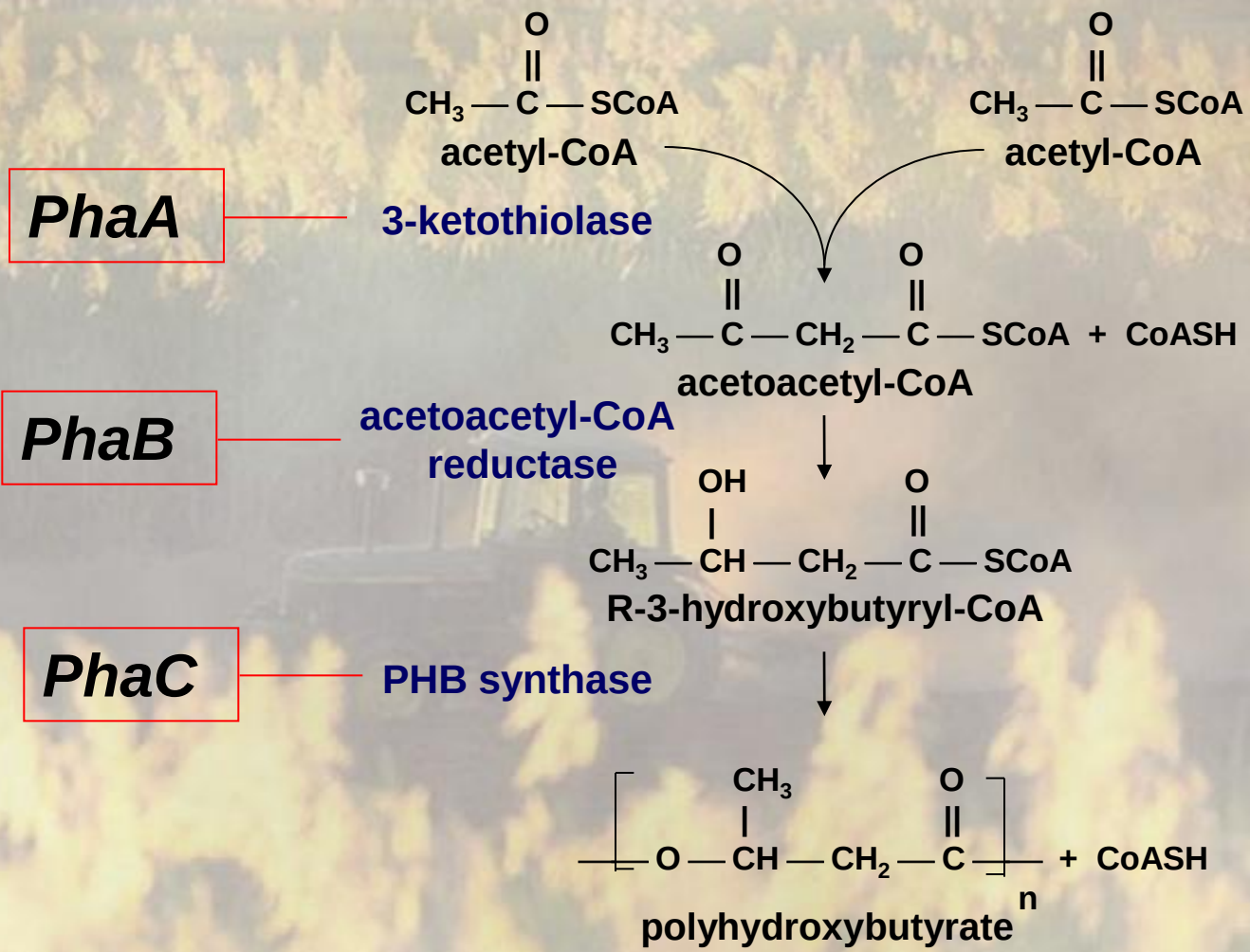
- Introduction
- High throughput screening
- PHB Pathway Enzymes
- Substrate Competition
- PHB Production in Peroxisomes
- Extraction and Characterization of PHB
- Conclusion

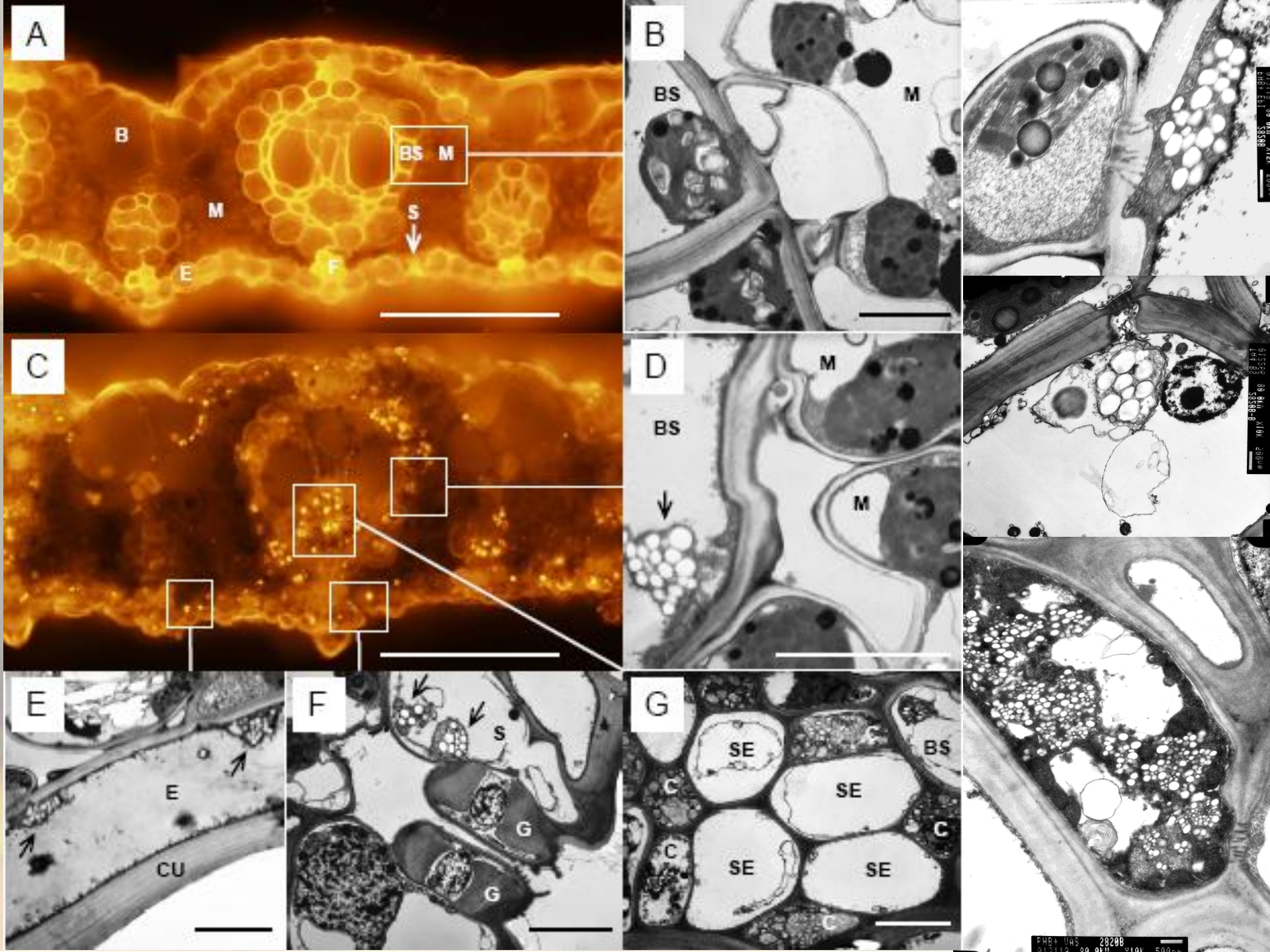
R groups in PHAs



- **scl-PHA**
  - **R = hydrogen — 3-hydroxypropionate (3HP)**
  - **R = methyl — 3-hydroxybutyrate (3HB) = PHB**
  - **R = ethyl — 3-hydroxyvalerate (3HV)**
- **mcl-PHA**
  - **R = propyl — 3-hydroxycaproate (3HC)**
  - **R = butyl — 3-hydroxyheptanoate (3HH)**
  - **R = pentyl — 3-hydroxyoctanoate (3HO)**
  - **R = hexyl — 3-hydroxynonanoate (3HN)**

PHB synthesis in *Ralstonia eutropha*




A
B
BS
M
S
E
F
B
BS
M
C
D
M
BS
M
E
E
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F
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G
SE
C
BS
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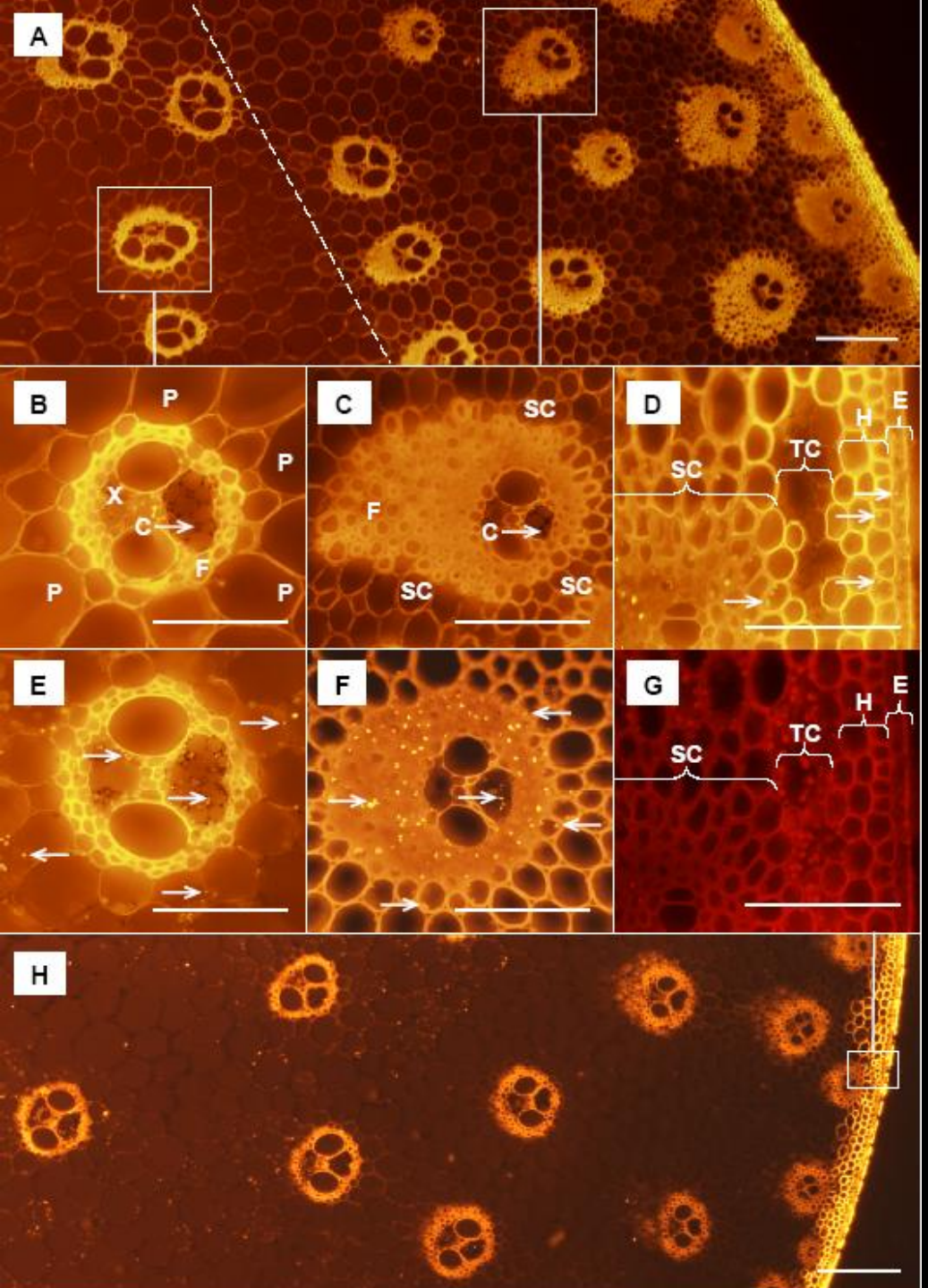
C
SE
SE
C



Sugarcane stalk internode

Sugarcane stalk node

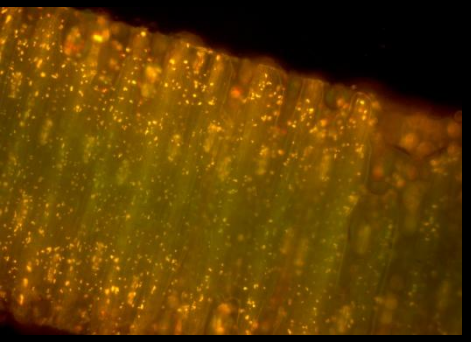
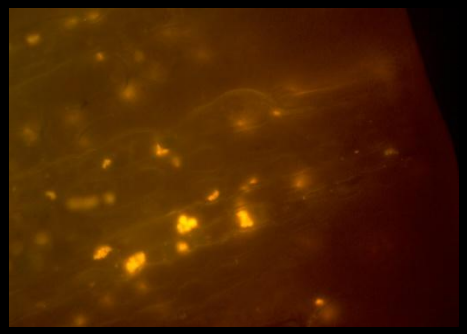
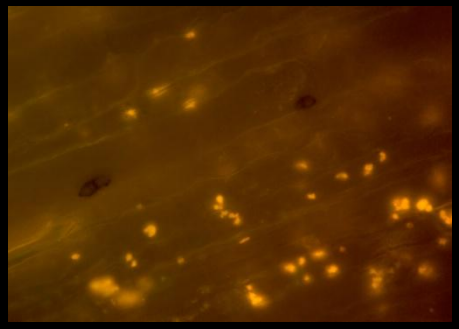
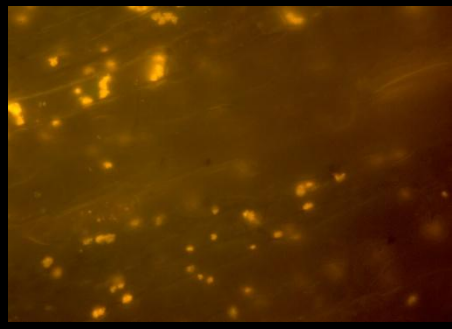
Petrasovits *et al.* (2007). Plant Biotechnol J 5:162-172

High Throughput Screening

1. **>10,000 independent transgenic sugarcane lines generated**
2. **4 Different promoters**
3. **Codon optimised**
4. **Megaconstruct**

High Throughput Screening

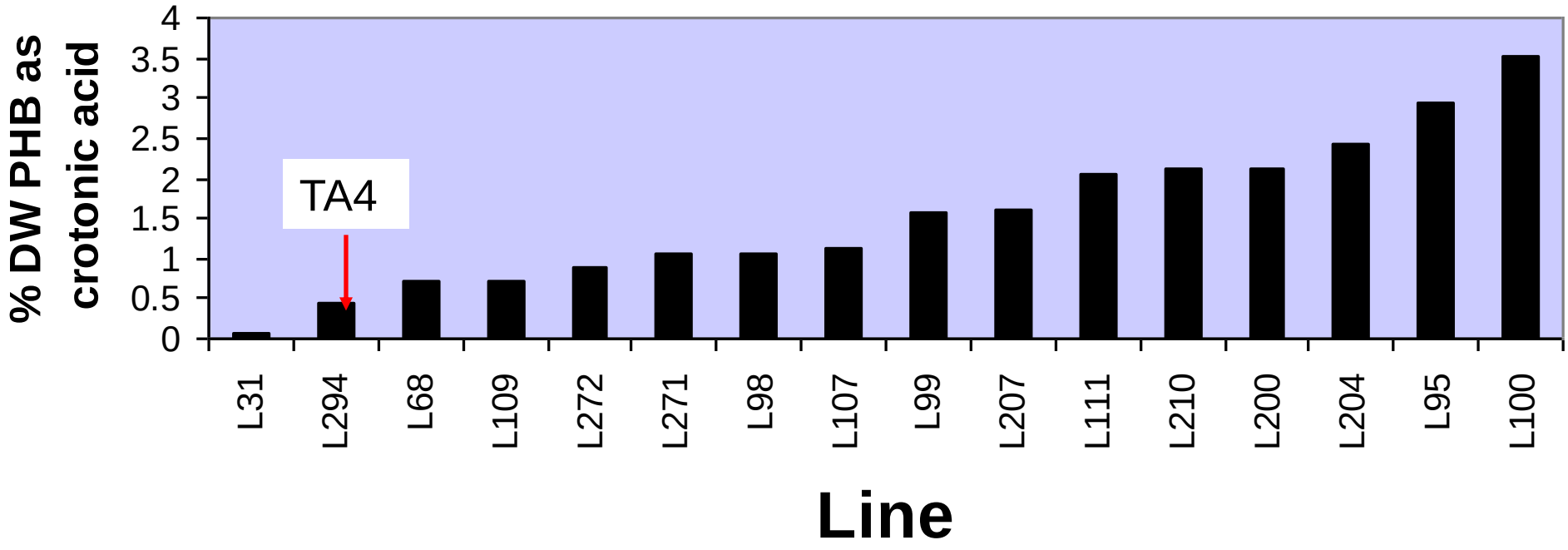
> 10,000 unique transgenic lines screened

Petrasovits et al. manuscript in prep

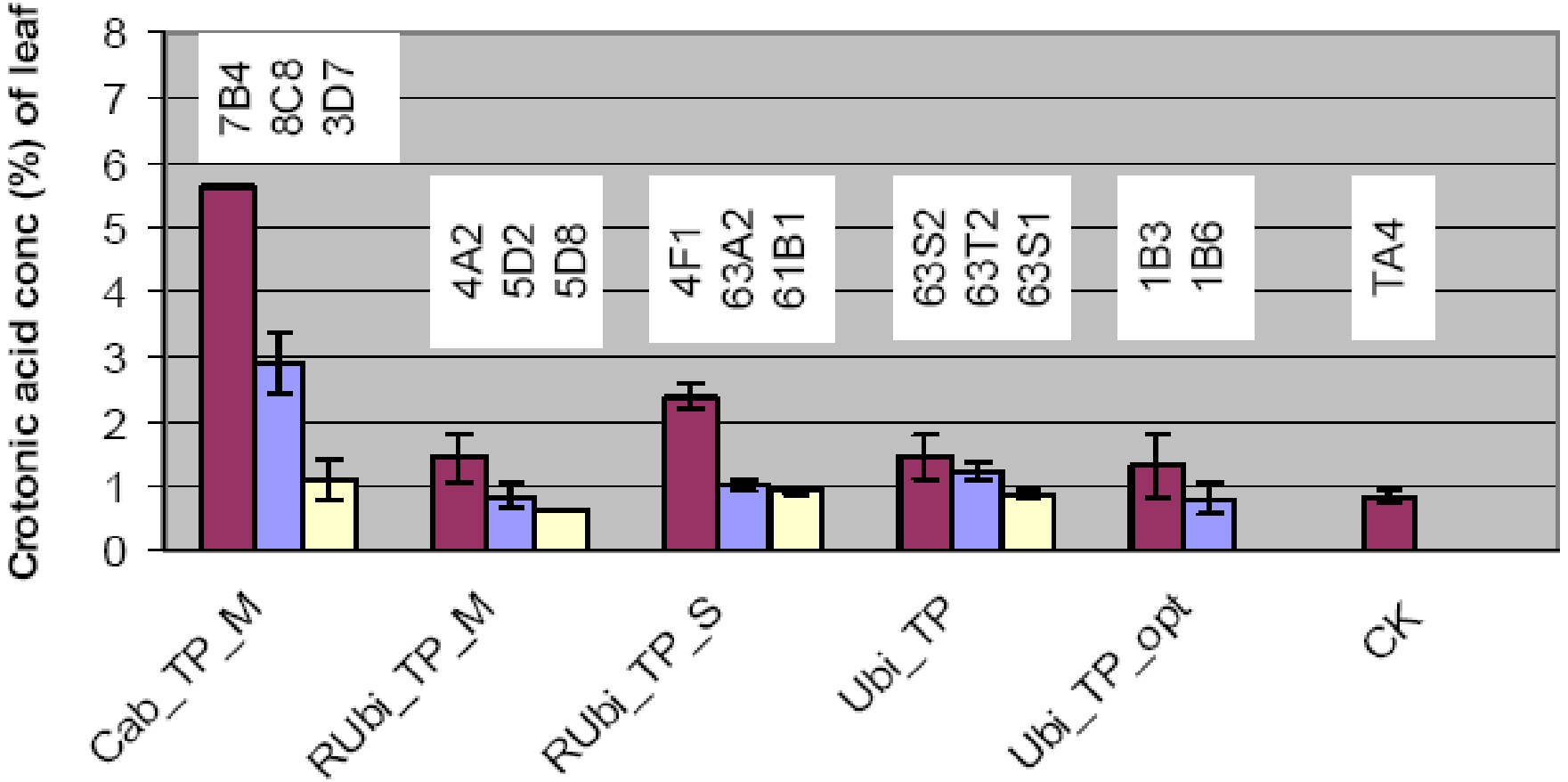
15 of the 300 lines were selected for further study

PHB content in GH lines at 3 months



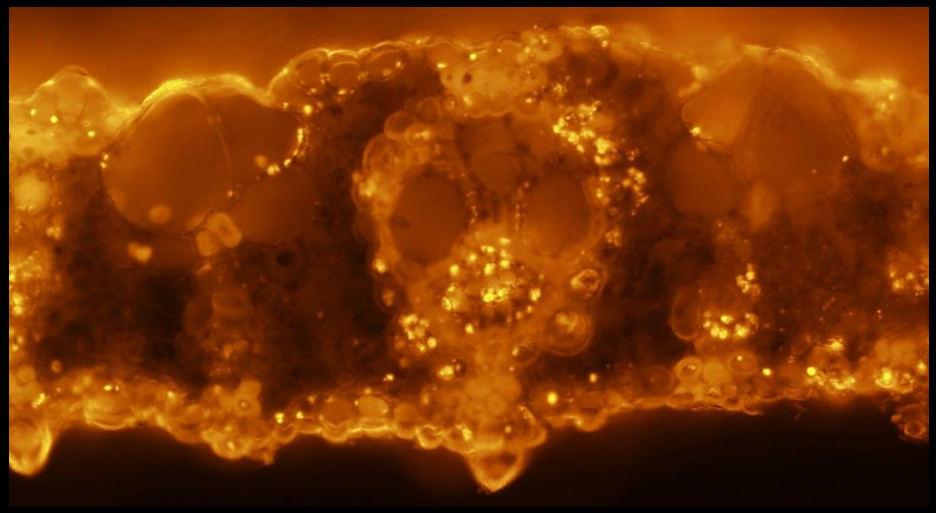
Petrasovits and Zhao et al. Manuscipt in prep

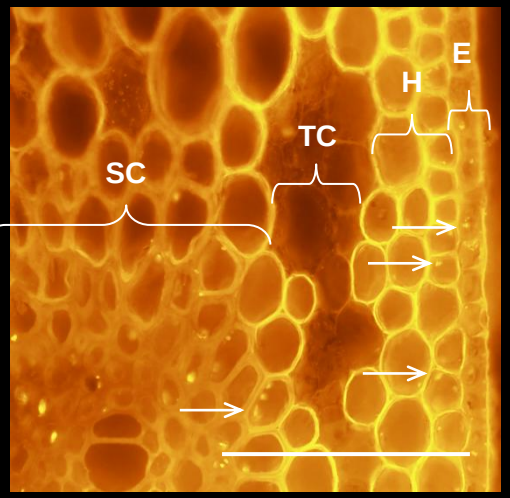
PHB as a (%) of leaf dry weight sampled from 3-month transgenic lines generated with various DNA constructs

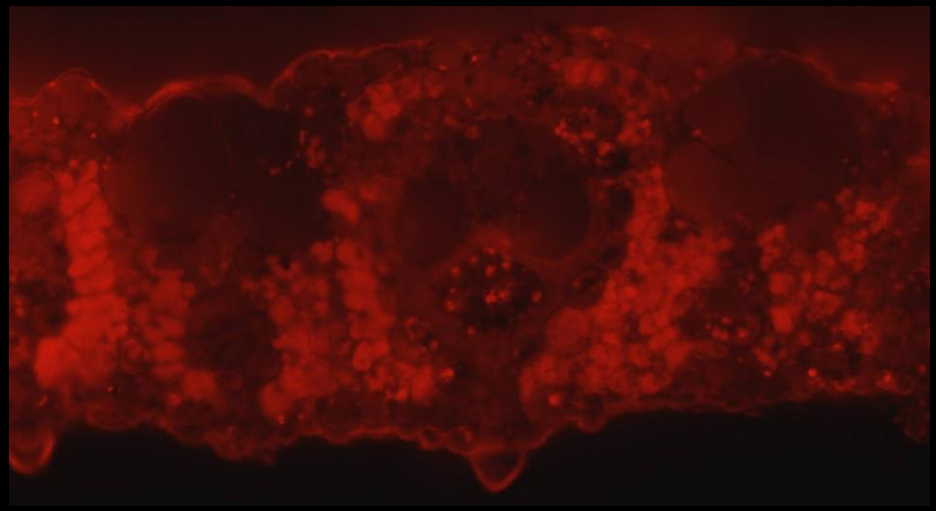


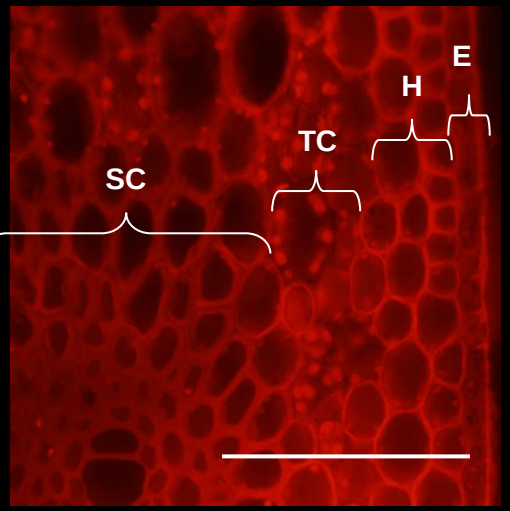
All plants are T1 and were generated by harvesting the original transgenic plant (T0) grown from embryogenic callus and by planting 4 replicate pots with 3 setts per pot.

Bundle sheath –vs- Mesophyll





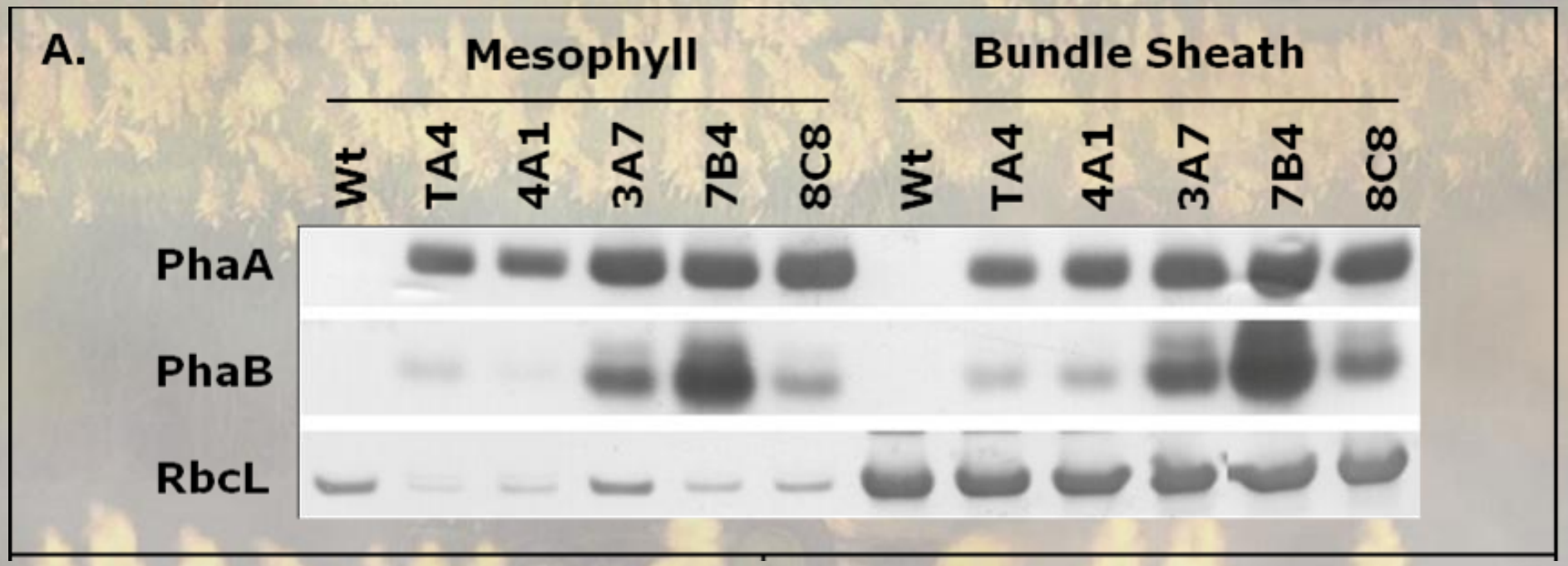




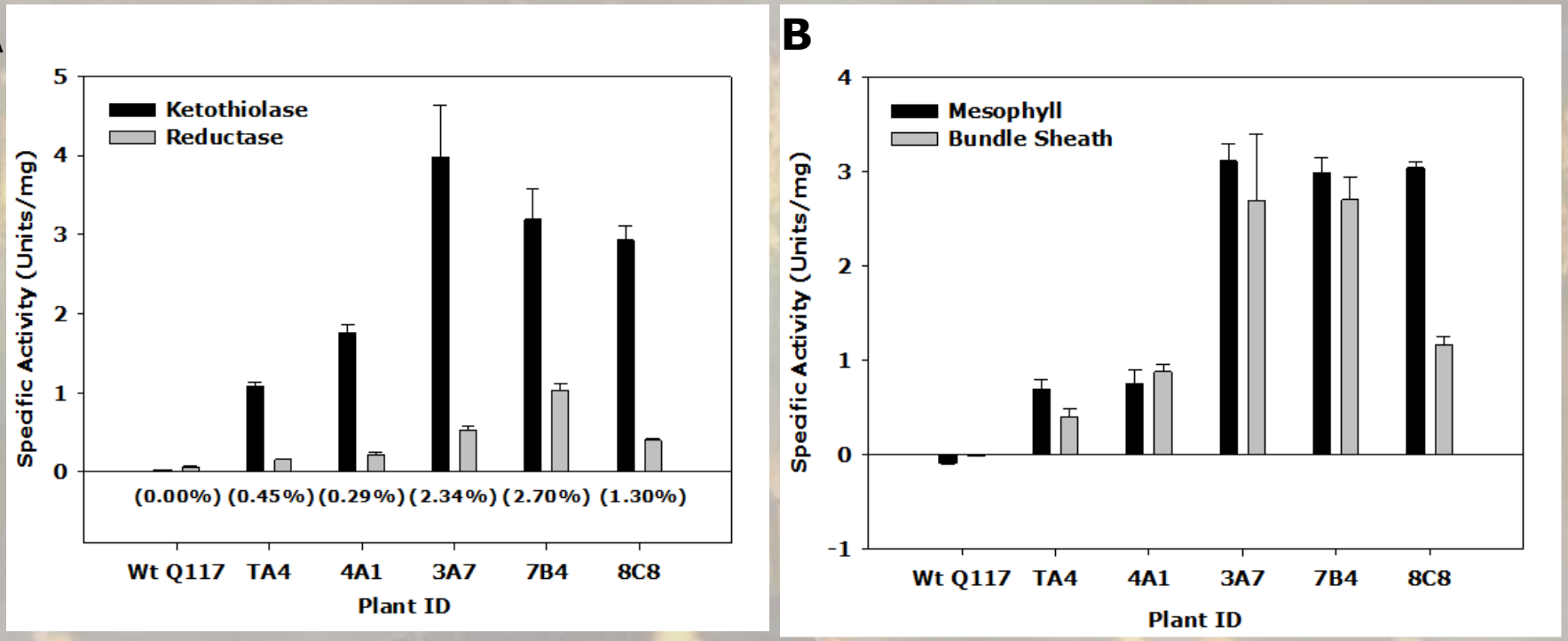
E, epidermal; H, hypodermal;
TC, thin-walled cortical cells;
SC, sclerenchymatous cortical cells/
Scale bars in all panels equivalent to 200 μm.

PHB Pathway Enzymes

- Enzyme activity levels
- In situ localization



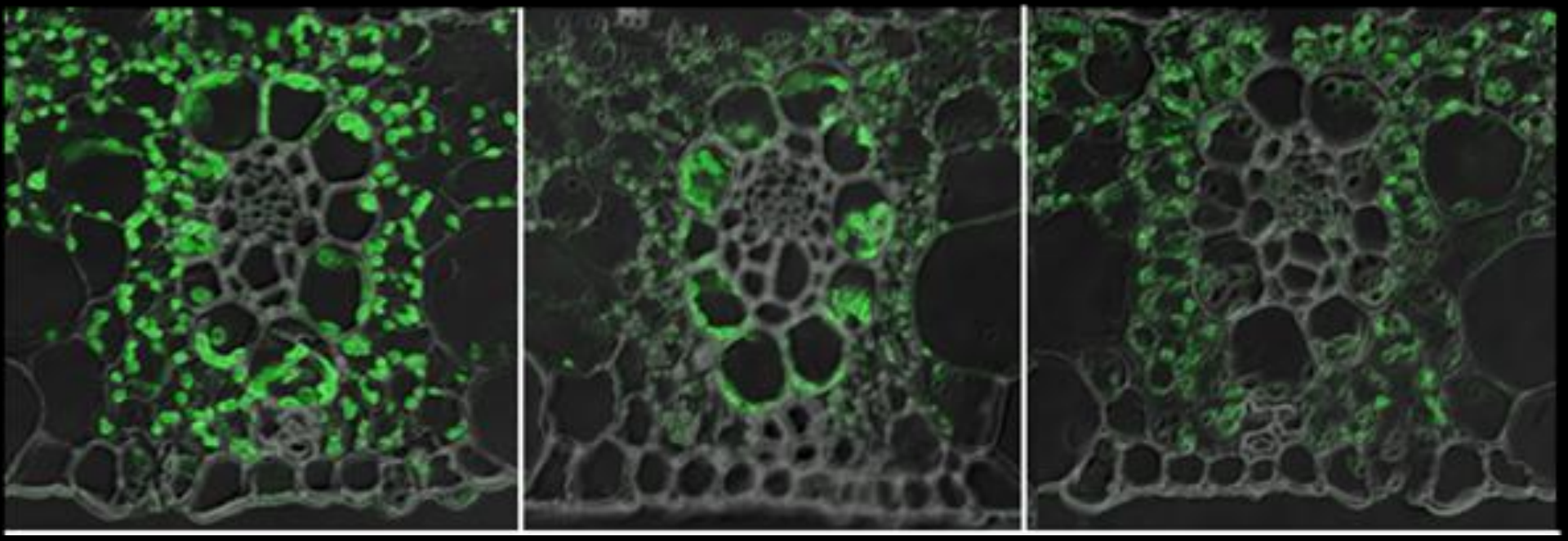
A. Expression of PhaA and PhaB in mesophyll and bundle sheath cells of PHB producing transgenic sugarcane plants. RbcL = Rubisco large subunit, shows minimal contamination of mesophyll fraction with bundle sheath proteins.
B. Expression of PhaC in TA4 mesophyl and BS cells
C. PhaA,B,C expresion in whole leaf extract. IC=unknown internal control protein.



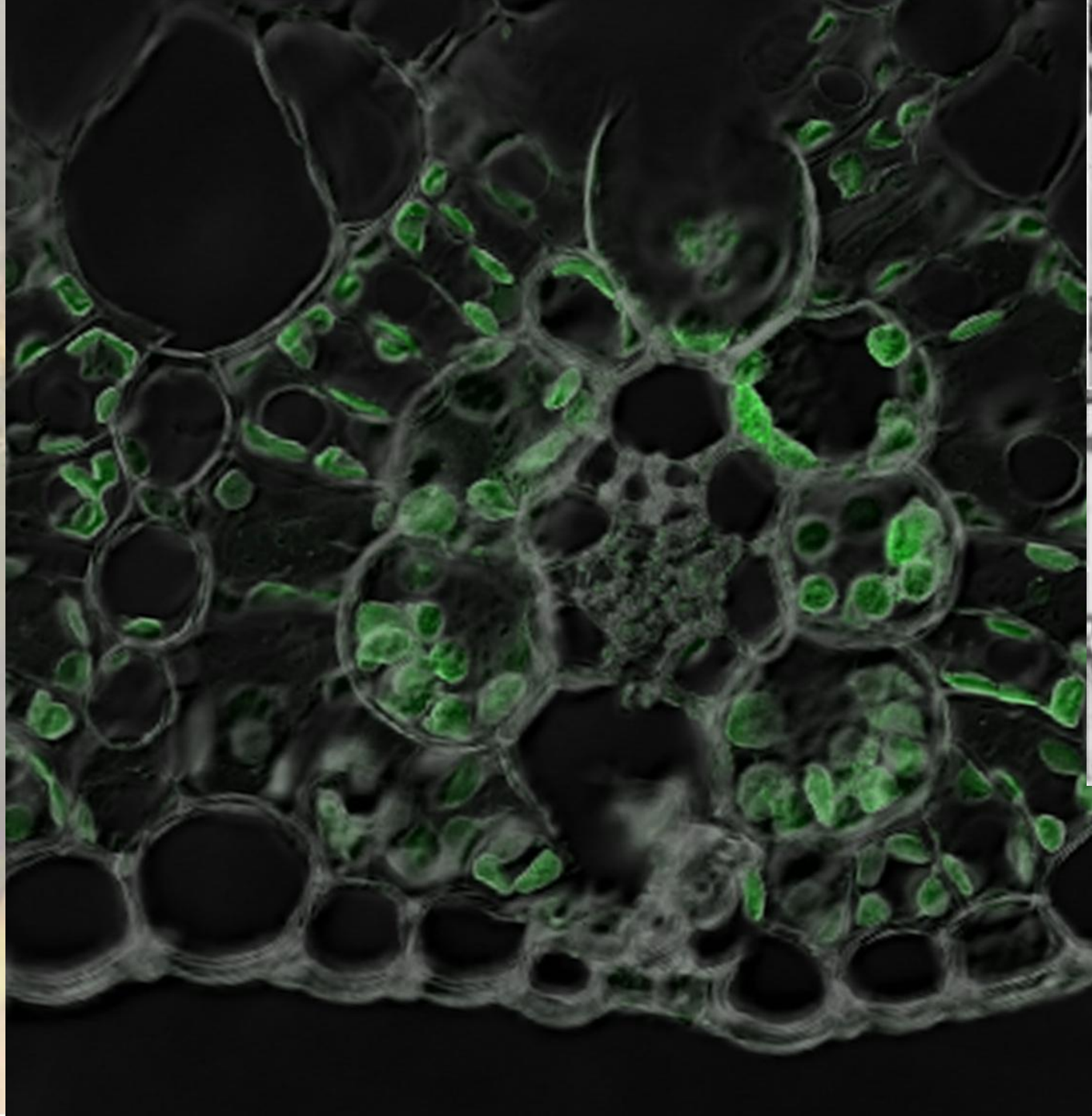
A. PhaA and PhaB enzyme activity in whole leaf protein extracts
B. PhaA enzyme activity in mesophyl and bundle sheath protein extracts

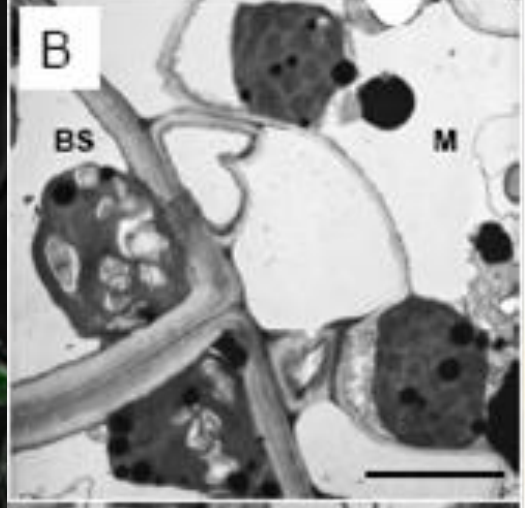
In situ Localization of PHB Pathway Enzymes in Sugarcane

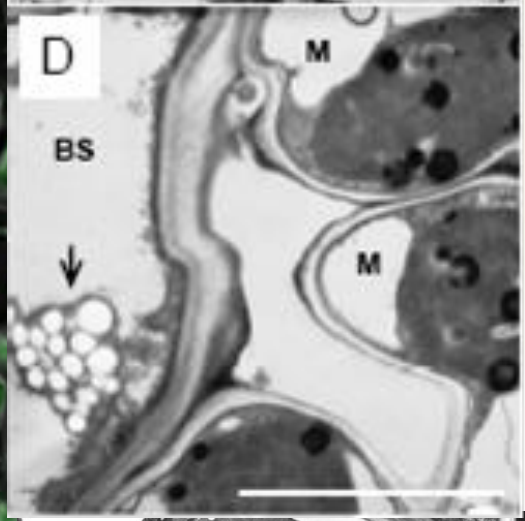
PhaA PhaB PhaC

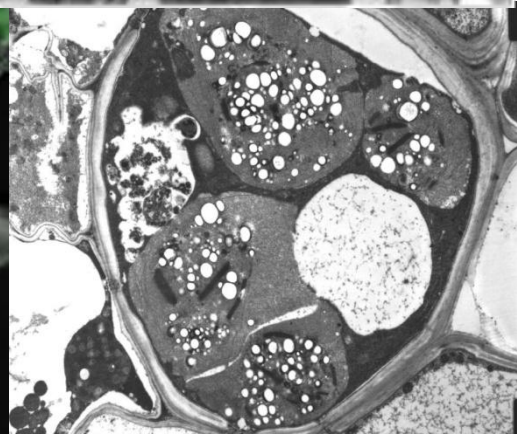


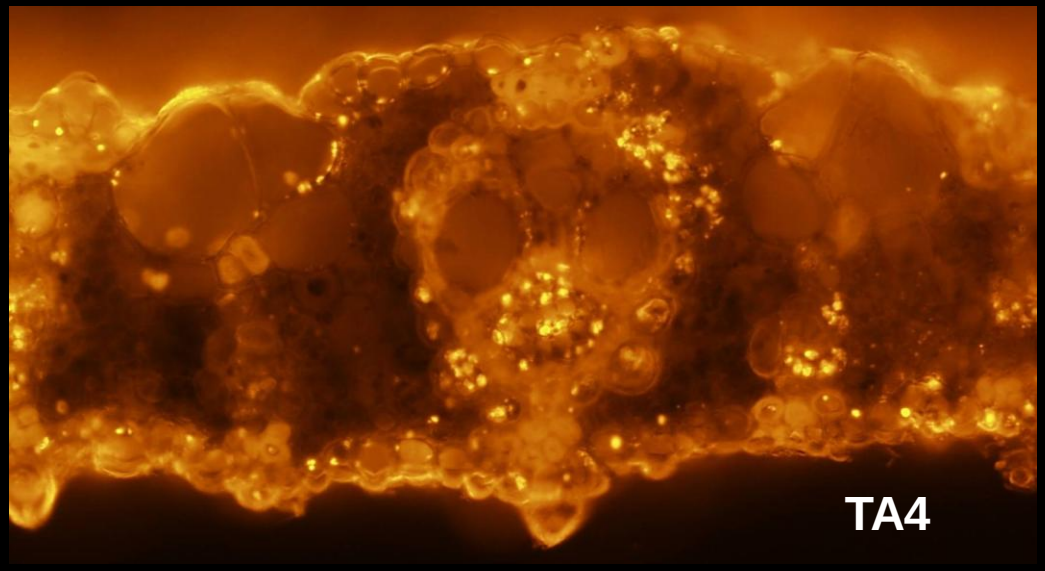
Localisation of PHB biosynthesis enzymes in transverse leaf section of transgenic sugarcane and switchgrass.




B
BS
M

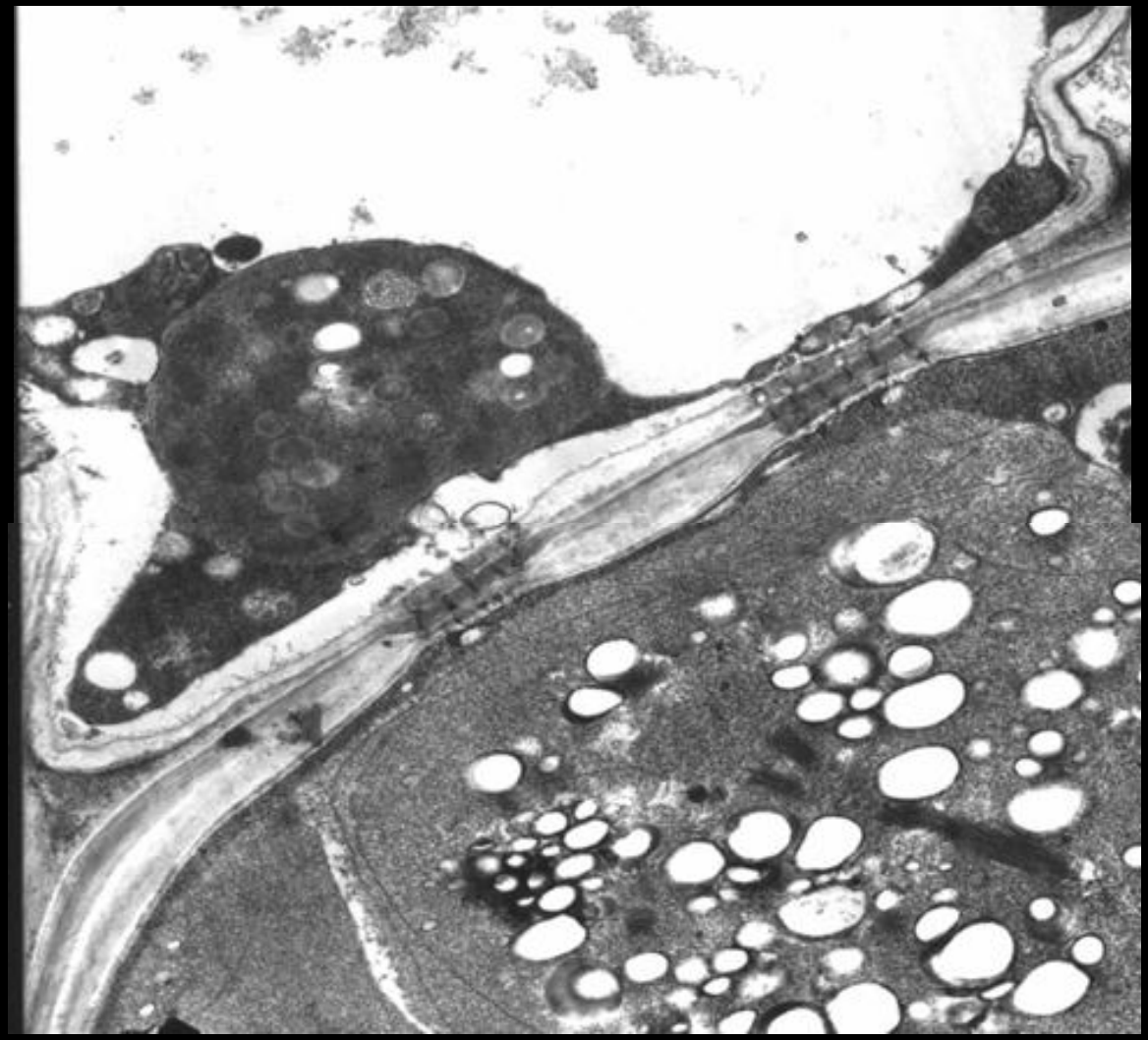

D
M
BS
M

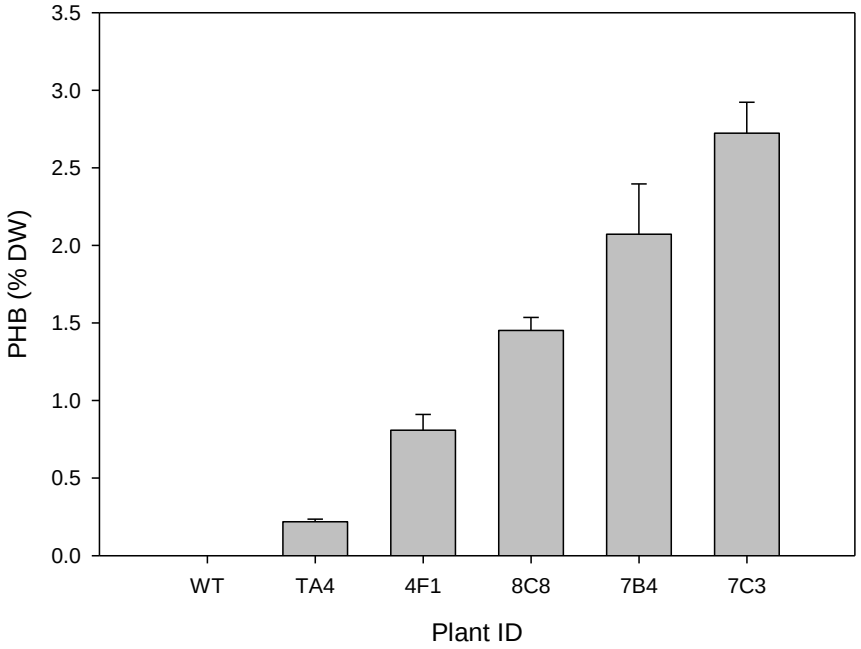


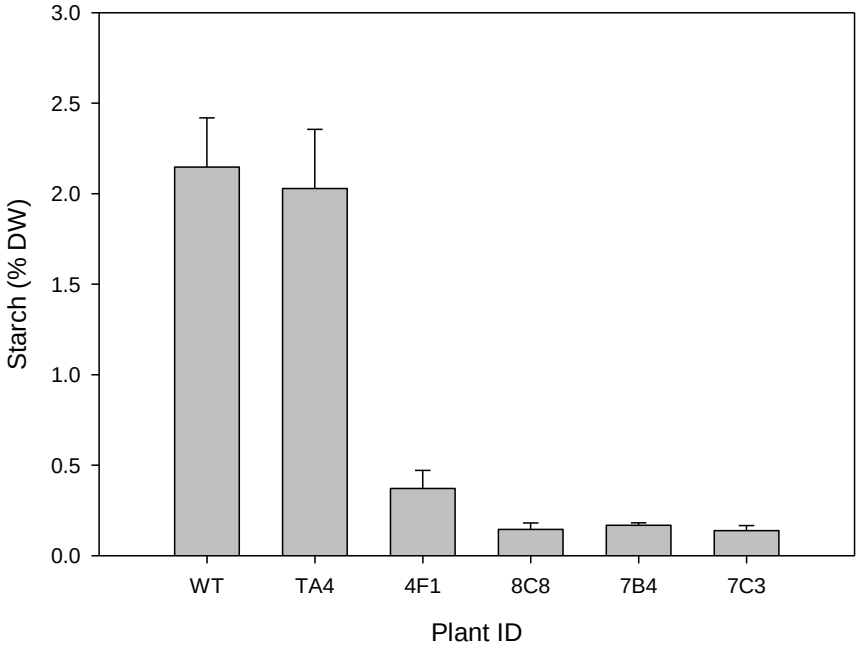


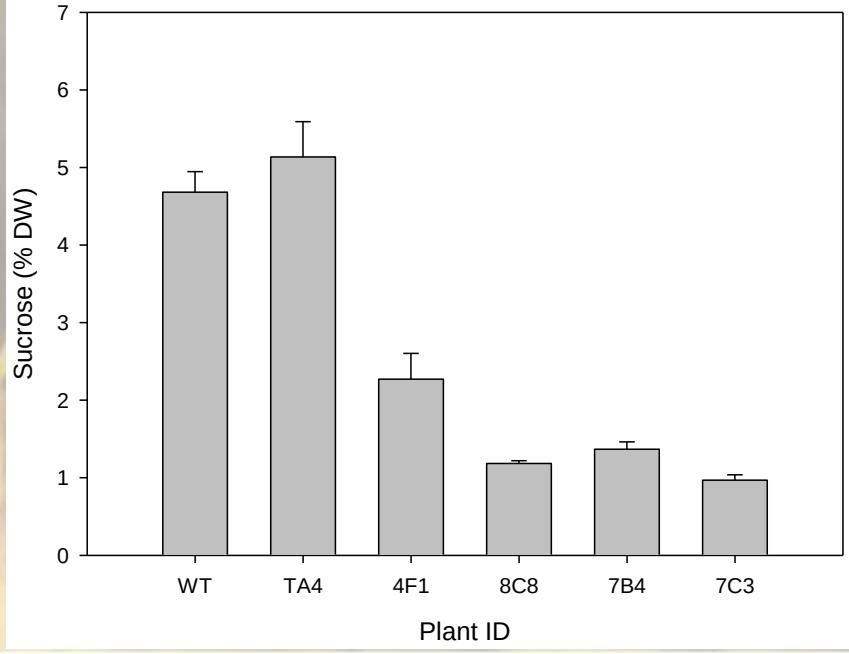
Sugarcane Leaf Producing ≥5% PHB

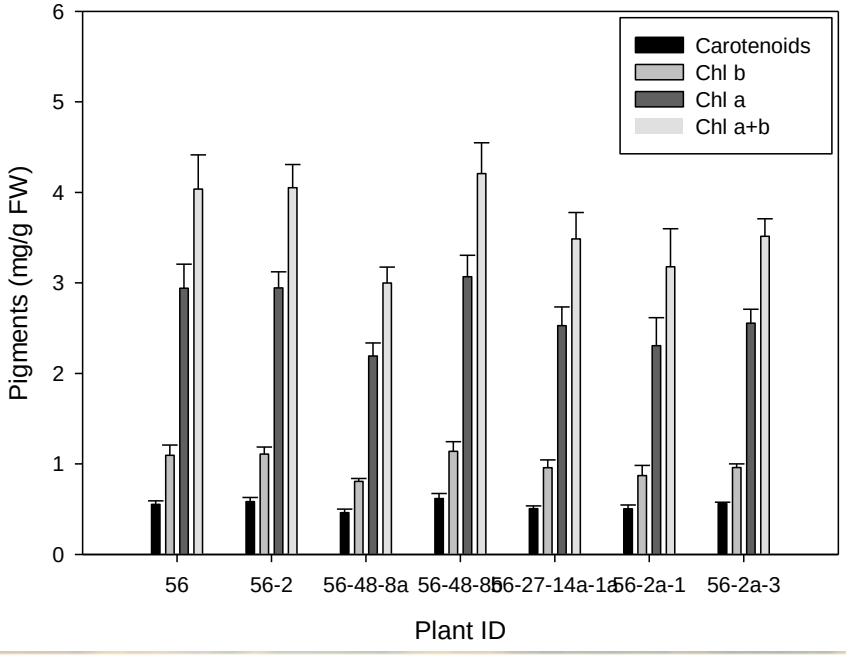
3A7 Leaf Section Showing PHB Granules in both Bundle Sheath and Mesophyll Cells




PHB (% DW)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WT
TA4
4F1
8C8
7B4
7C3
Plant ID


Starch (% DW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WT
TA4
4F1
8C8
7B4
7C3
Plant ID

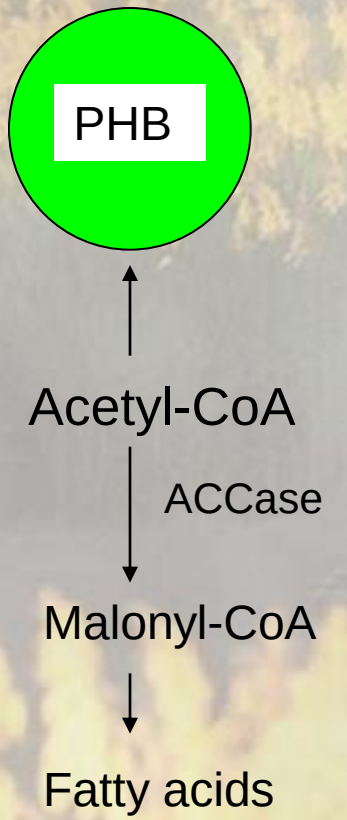

Sucrose (% DW)
7
6
5
4
3
2
1
0
WT
TA4
4F1
8C8
7B4
7C3
Plant ID


Pigments (mg/g FW)
6
5
4
3
2
1
0
Carotenoids
Chl b
Chl a
Chl a+b
56
56-2
56-48-8a
56-48-8b
56-27-14a-1a
56-2a-1
56-2a-3
Plant ID

Competition for Acetyl-CoA

- **ACCase Inhibitors**

Substrate limitation in mesophyll plastids?



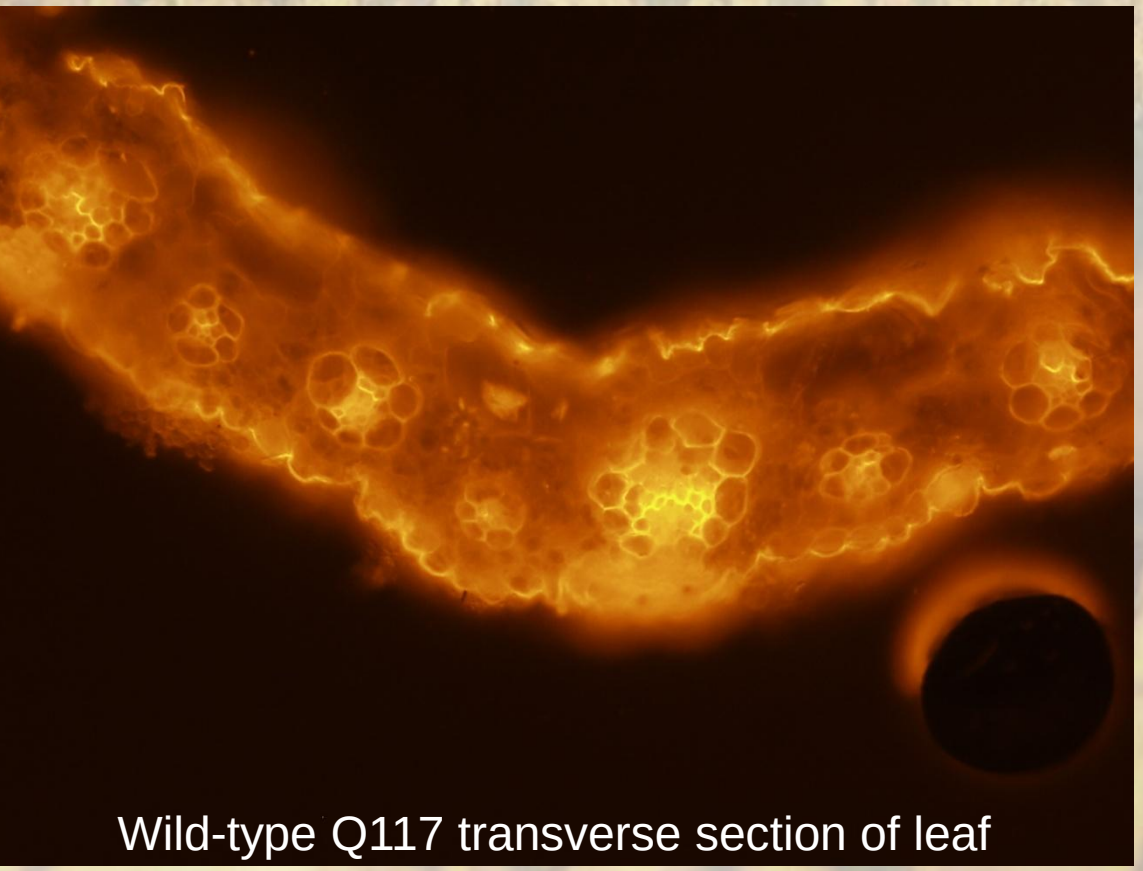
- Mesophyll plastids are the principal site of *de novo* fatty acid biosynthesis in plants
- The main competitor for PHB production in mesophyll plastids is fatty acid synthesis
- The first committed step of fatty acid synthesis is catalysed by acetyl-CoA carboxylase:

  $ATP + HCO_3^- + \text{acetyl-CoA} \longrightarrow \text{Malonyl-CoA} + ADP + Pi$

- ACCase is inhibited by Class A herbicides

Suzuki, Y., et al. (2002) *Bioscience, Biotechnology and Biochemistry* 66, pp. 2537-42

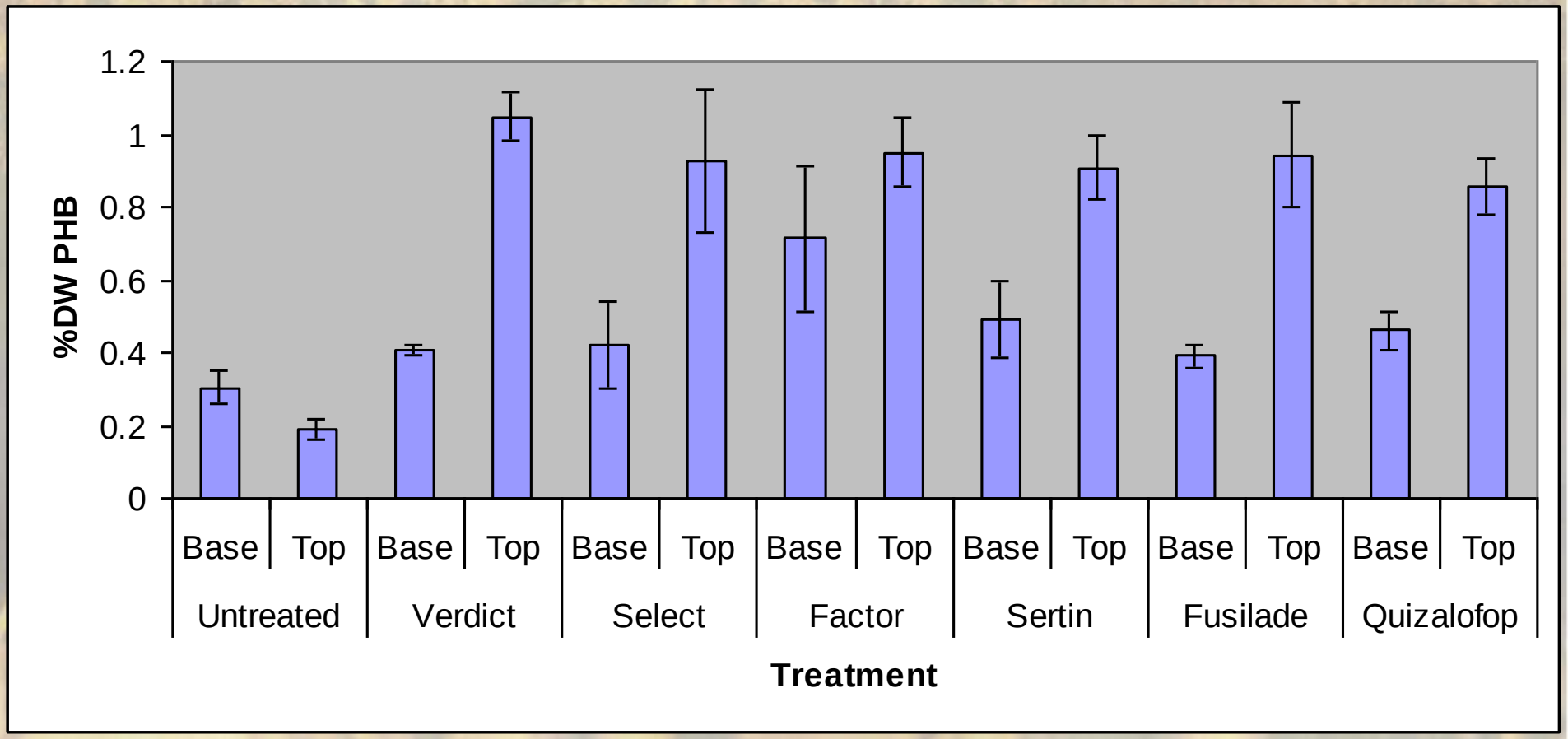
Do Acetyl-CoA Carboxylase (ACCase) Inhibitors Increase PHB Content in Mesophyll Cells?



Wild-type Q117 transverse section of leaf

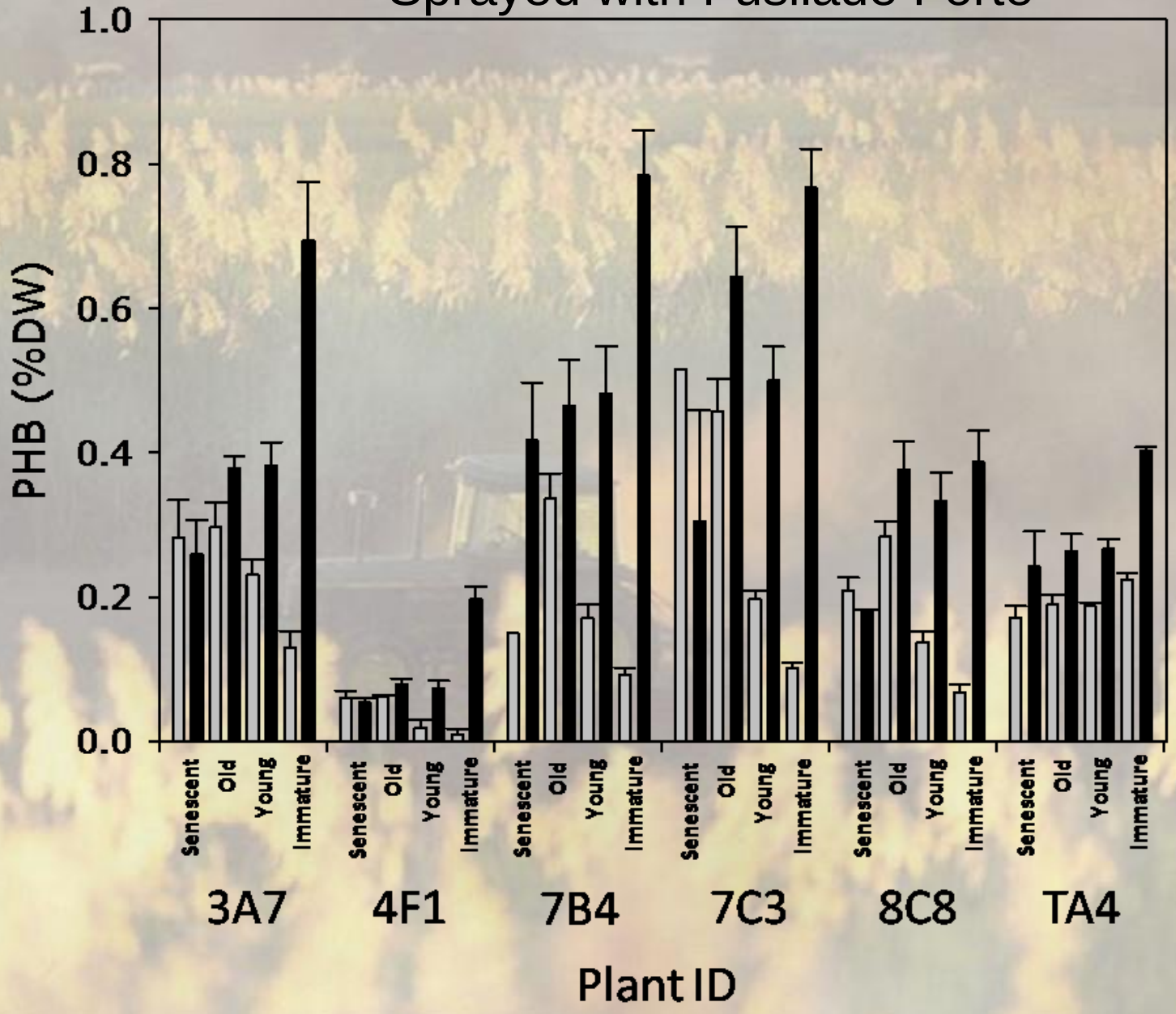
- An inhibitor of fatty acid biosynthesis
- Improve PHB biosynthesis in transgenic tobacco

Different herbicides applied to Line 8C8

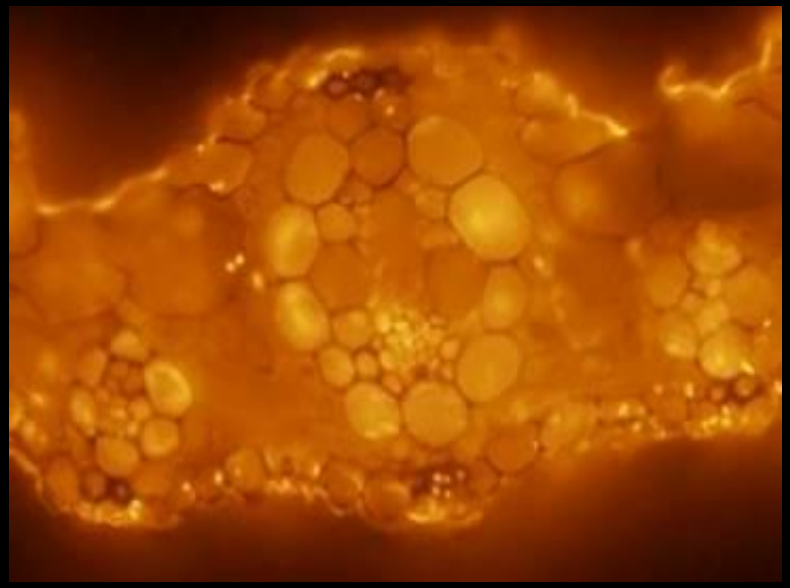


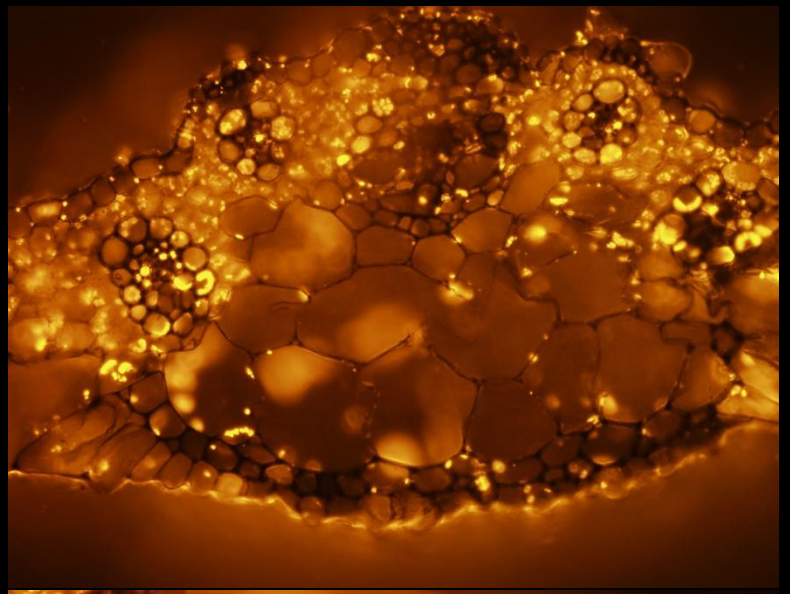
The error bars represent the standard error for three replicate plantlets.

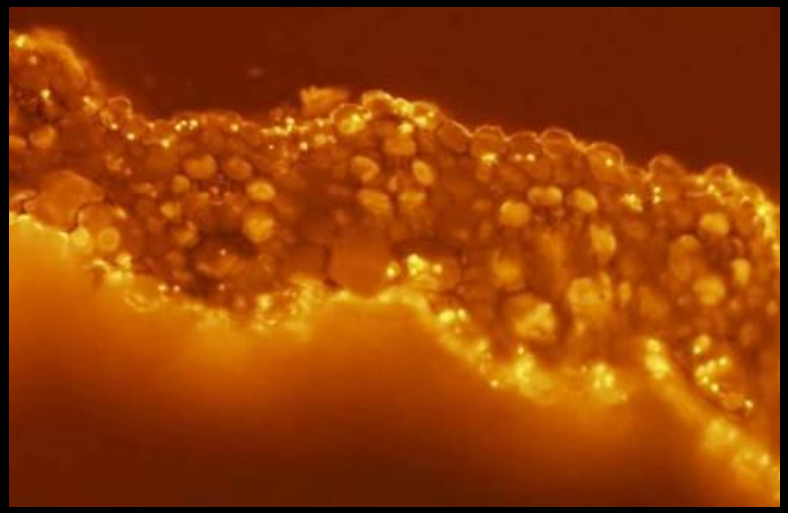
Four Month old Sett Germinated Sugarcane Lines Sprayed with Fusilade Forte

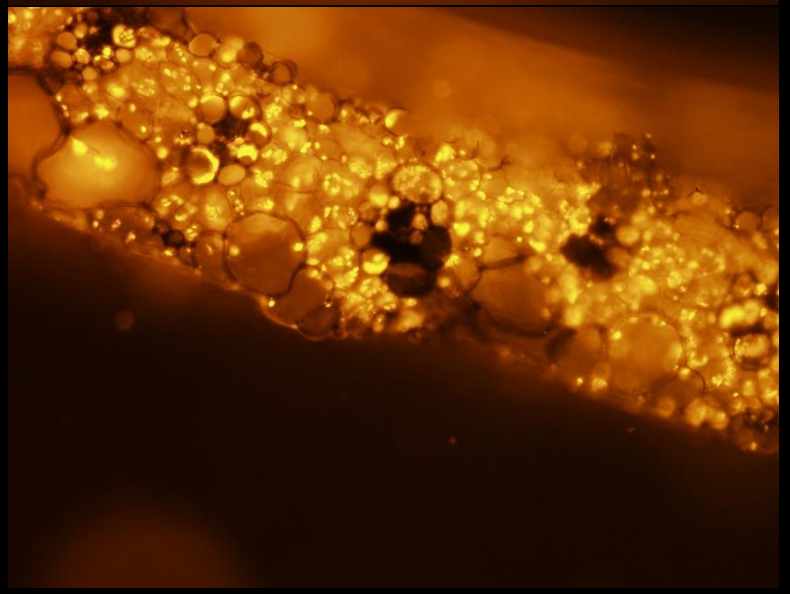


Transverse leaf sections of TA4, sprayed 16.9. and harvested 6.10.









Without ACCase Inhibitor

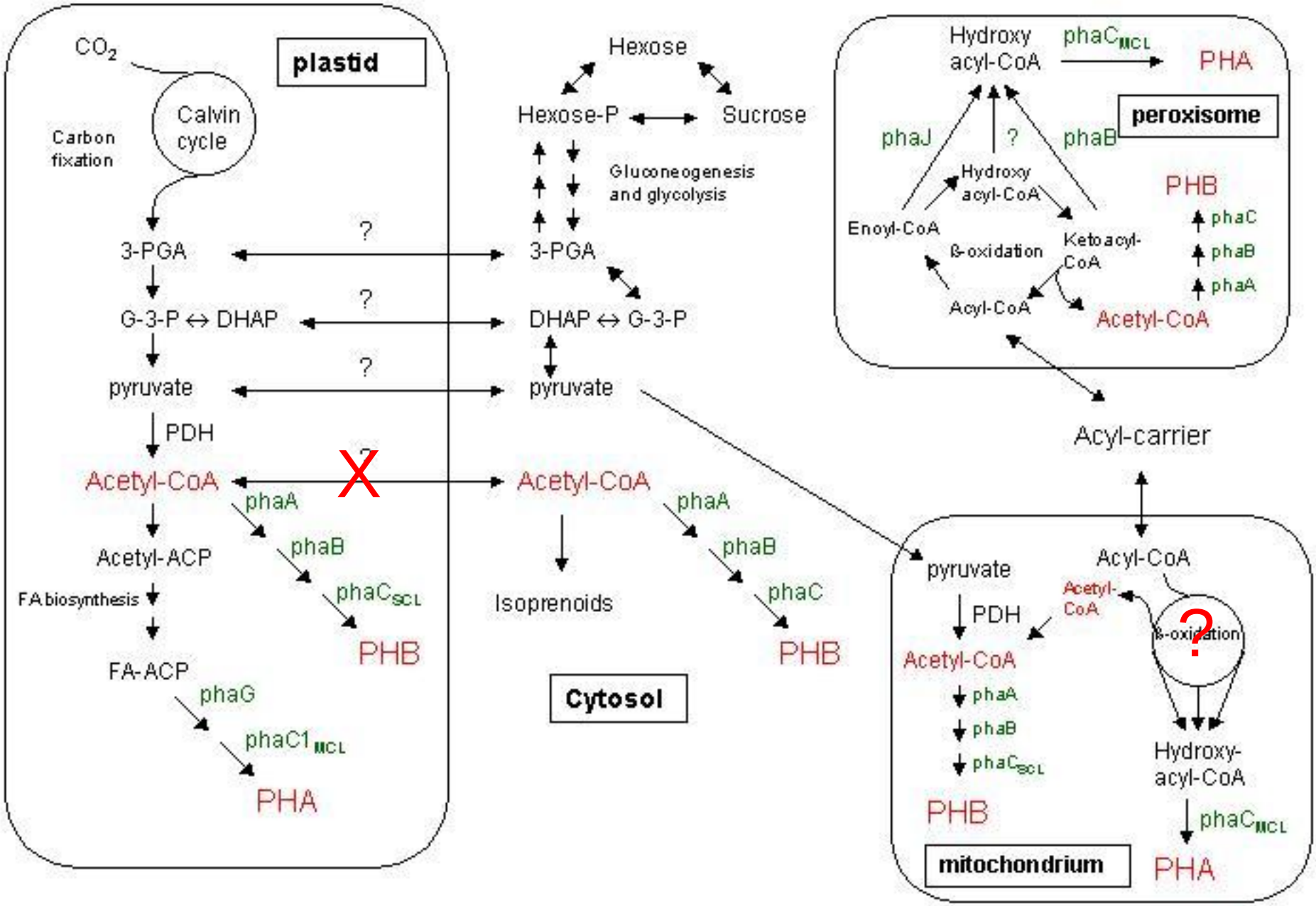
With ACCase Inhibitor

PHB Production in Peroxisomes

See Tilbrook et al PHB production in Plant Peroxisomes,
Session 9B p.71 Wednesday 11:30 -11:45

PHB Production in Peroxisomes

- scl/mcl PHAs
- PHB
  - ABC lines
  - FABC lines

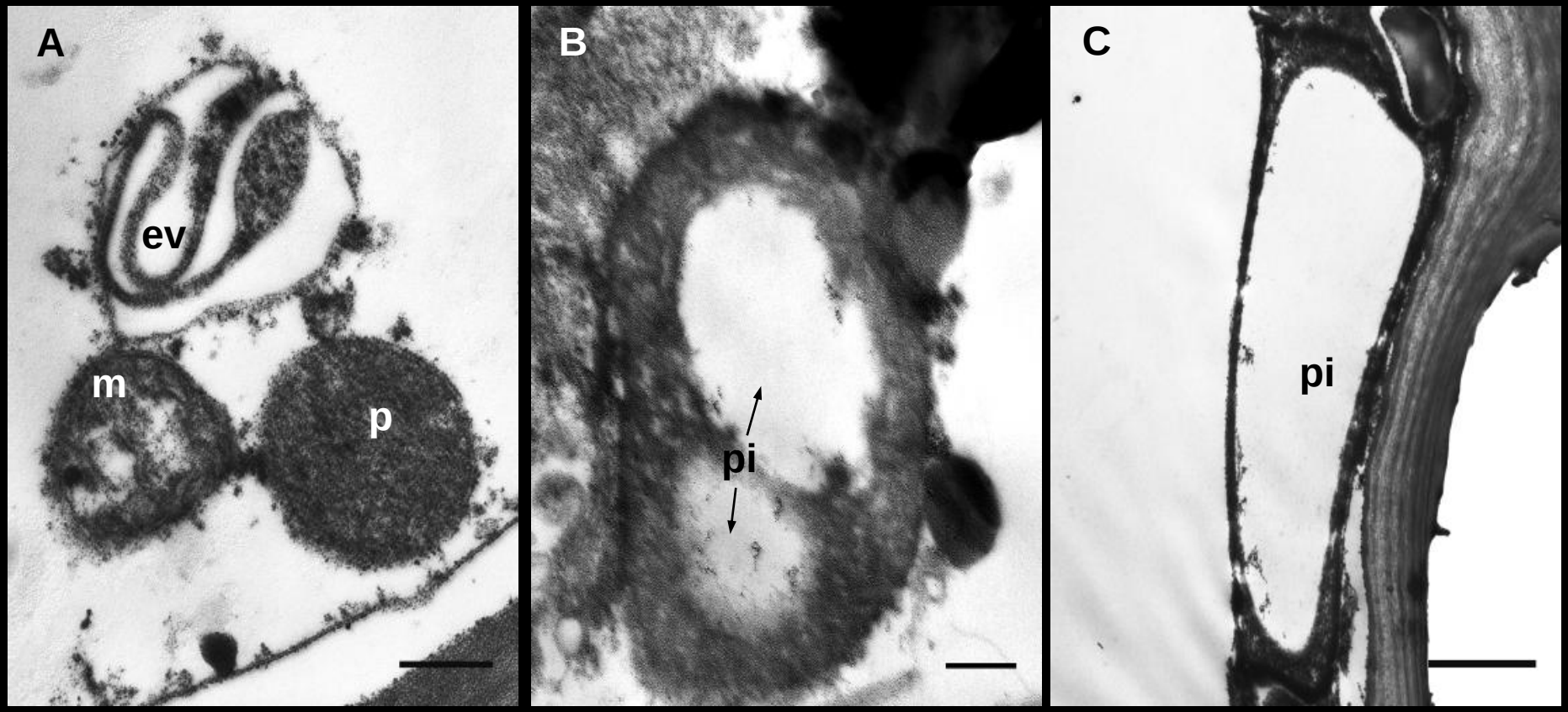


Simplified diagram of carbon flow in a sugarcane cell and PHA production possibilities

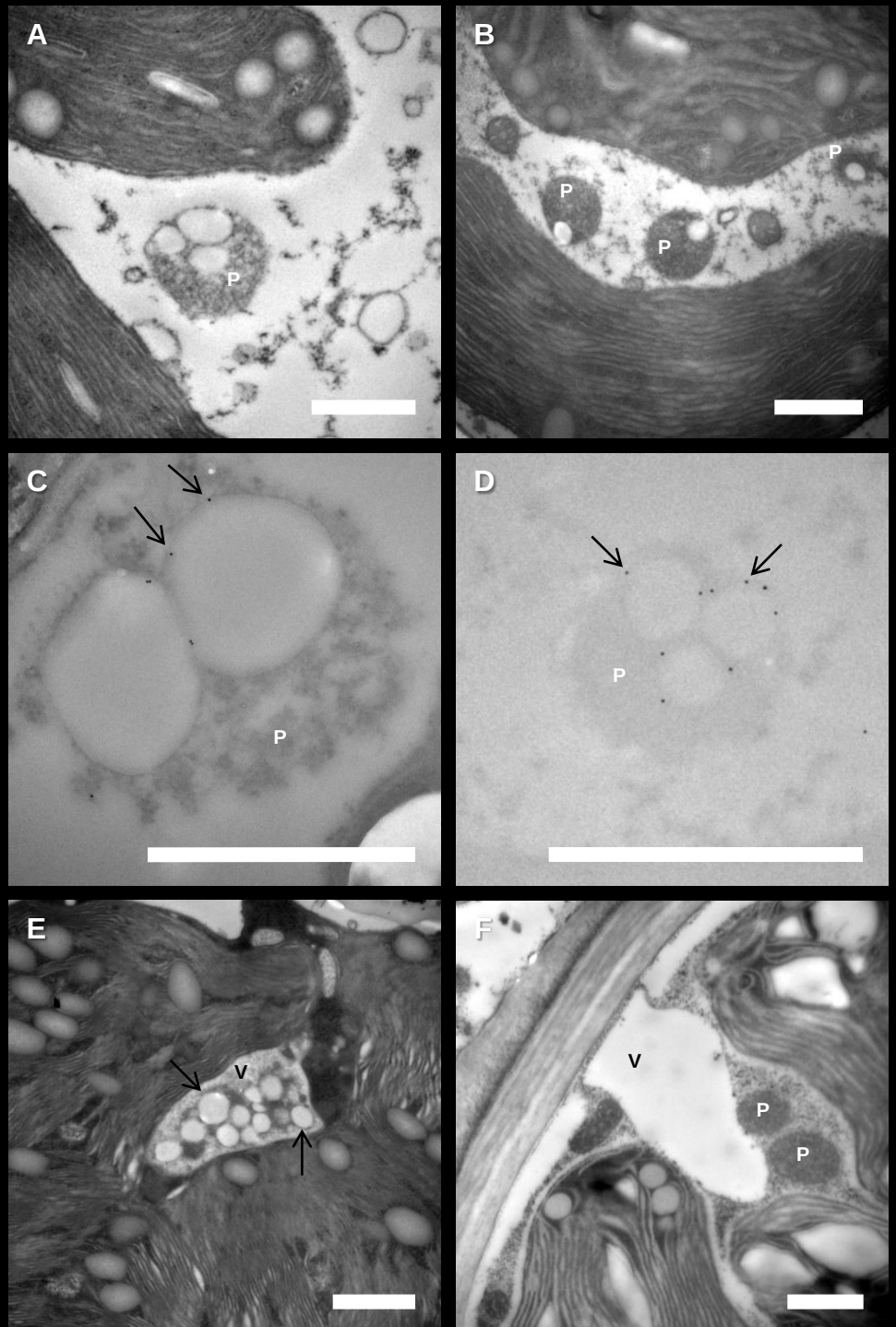
PHB/PHA Copolymer Production in Sugarcane Peroxisome

A - Wild type epidermal cell peroxisome

B & C - PHA inclusions in mesophyll peroxisome



p, peroxisome; ev, endoplasmic reticulum vesicle; m, mitochondrion; pi, peroxisomal inclusion
Scale bars: 200 nm
Anderson et al. Manuscript in prep.

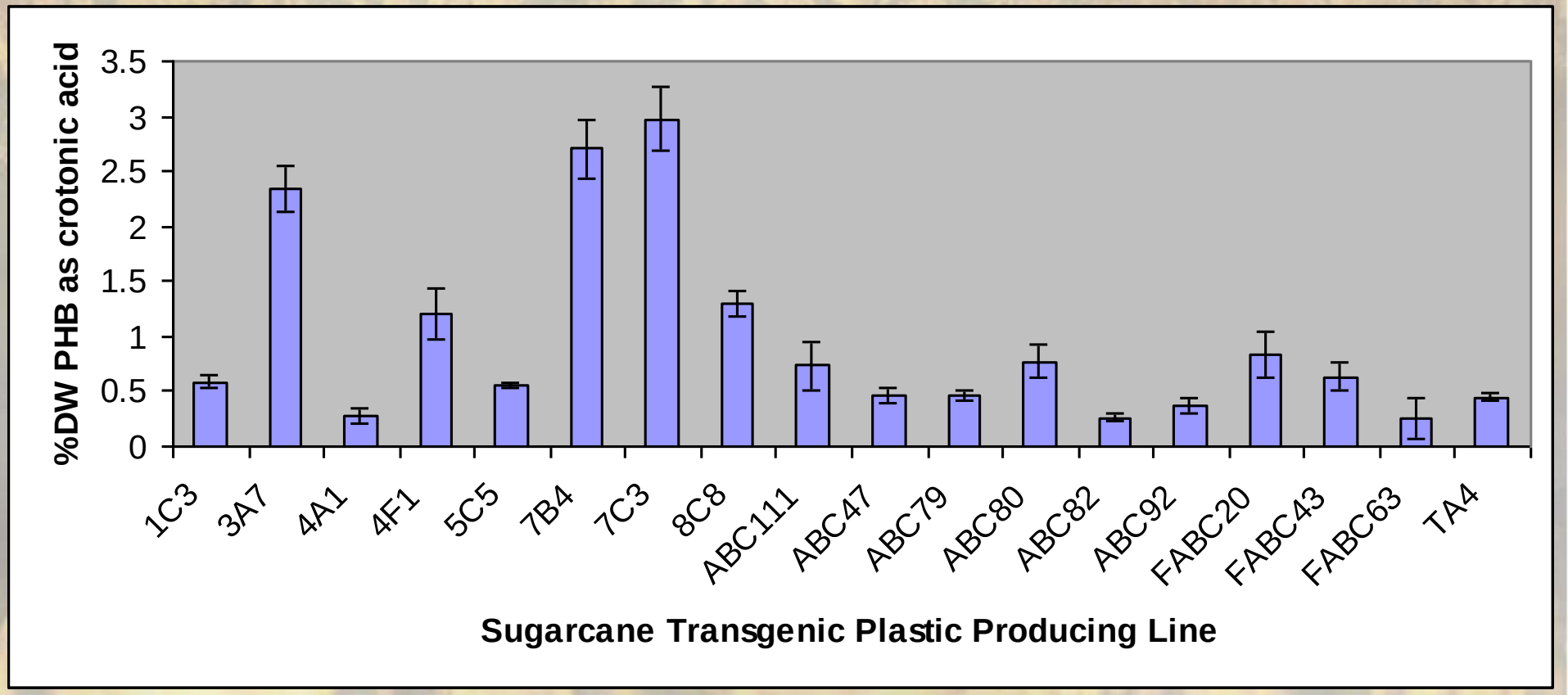


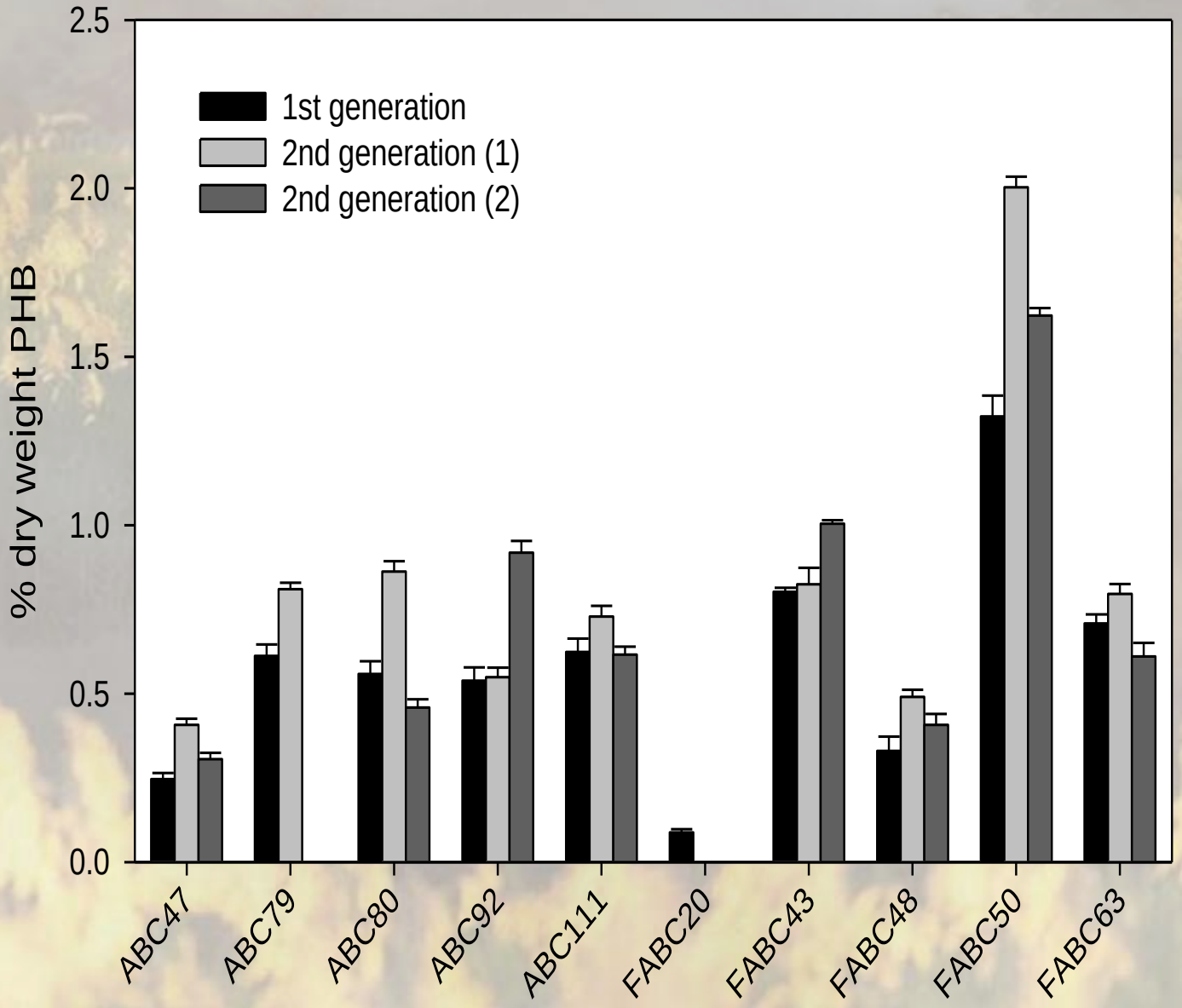
PHB in sugarcane peroxisomes

In situ localization of PhaC

PHB accumulation in sugarcane leaf cell vacuoles

2.5 Month Old Sugarcane Plants Sampled September 2009




% dry weight PHB
2.5
2.0
1.5
1.0
0.5
0.0
1st generation
2nd generation (1)
2nd generation (2)
ABC47
ABC79
ABC80
ABC92
ABC111
FABC20
FABC43
FABC48
FABC50
FABC63

Sugarcane PHB:
Extraction and Characterization

PHB Extraction from Sugarcane

Collect leaf sample

↓

Drying and grinding

↓

Digestion O/N, 50˚C, pH 5.0

↓

$CHCl_3$ extraction

↓

Precipitation, 8 vol EtOH, -80˚C

↓

GPC analysis

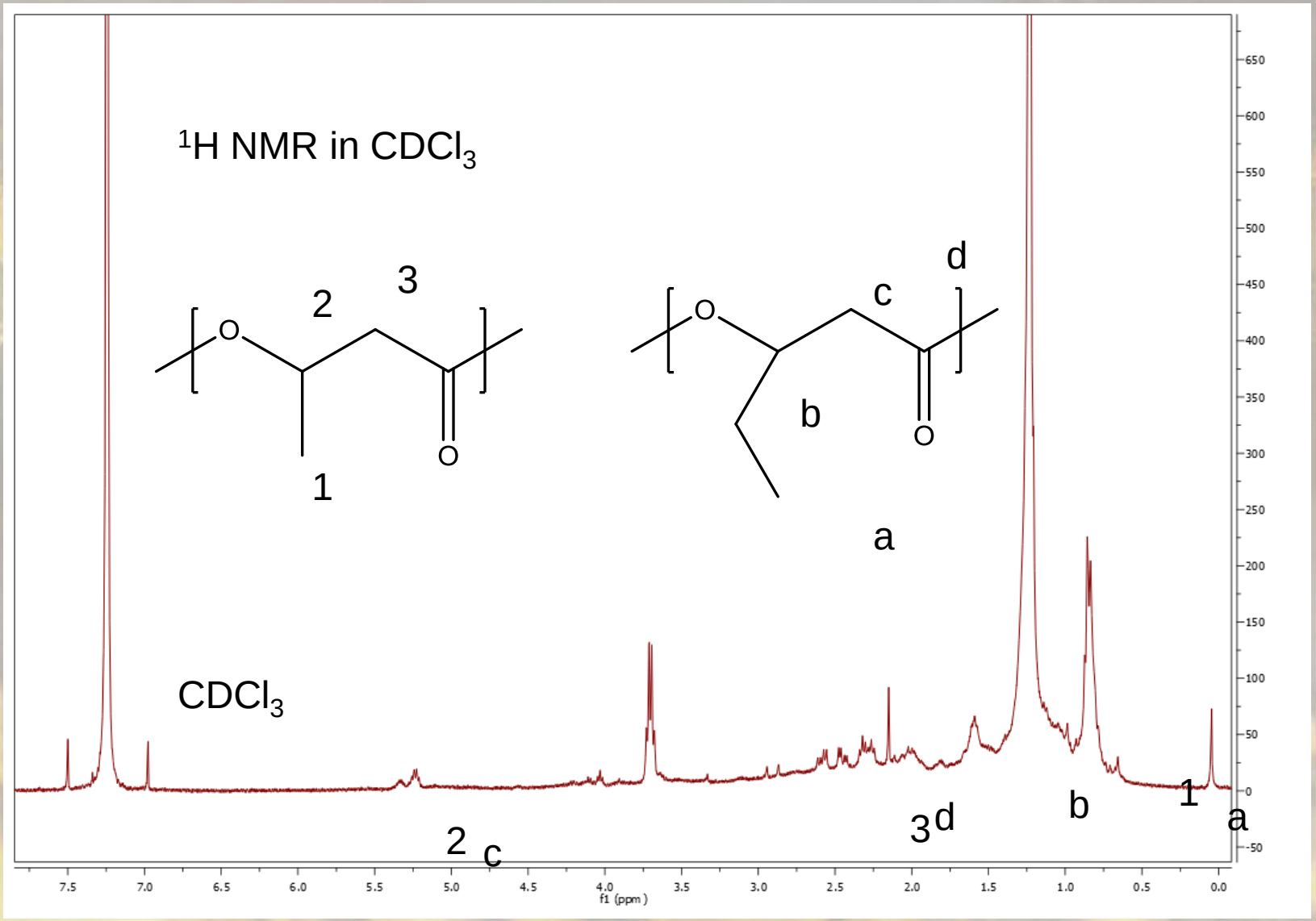
Sugarcane produces high-molecular weight PHB

Sample name	Location	Mw	Mn	Polydispersity
ABC 80	peroxisomal	2,174,126	1,804,292	1.205
ABC 111	peroxisomal	1,950,195	1,404,606	1.388
1C3	plastidic	(1,441,925)	(1,220,403)	(1.571)
3A7	plastidic	1,379,425 (1,322,753)	907,068 (882,701)	1.521 (1.499)
7B4	plastidic	1,265,280 (1,143,677)	857,532 (698,075)	1.475 (1.638)
7C3	plastidic	1,392,526 (967,618)	792,840 (564,383)	1.756 (1.714)
8C8	plastidic	1,309,025 (1,256,739)	837,491 (821,997)	1.563 (1.529)
TA4	plastidic	1,412,586 (823,186)	938,283 (525,961)	1.506 (1.565)
Q117	none	22,713	17,728	1.281

GPC results were supplied by: Dr Kristi Snell (Metabolix Inc.)

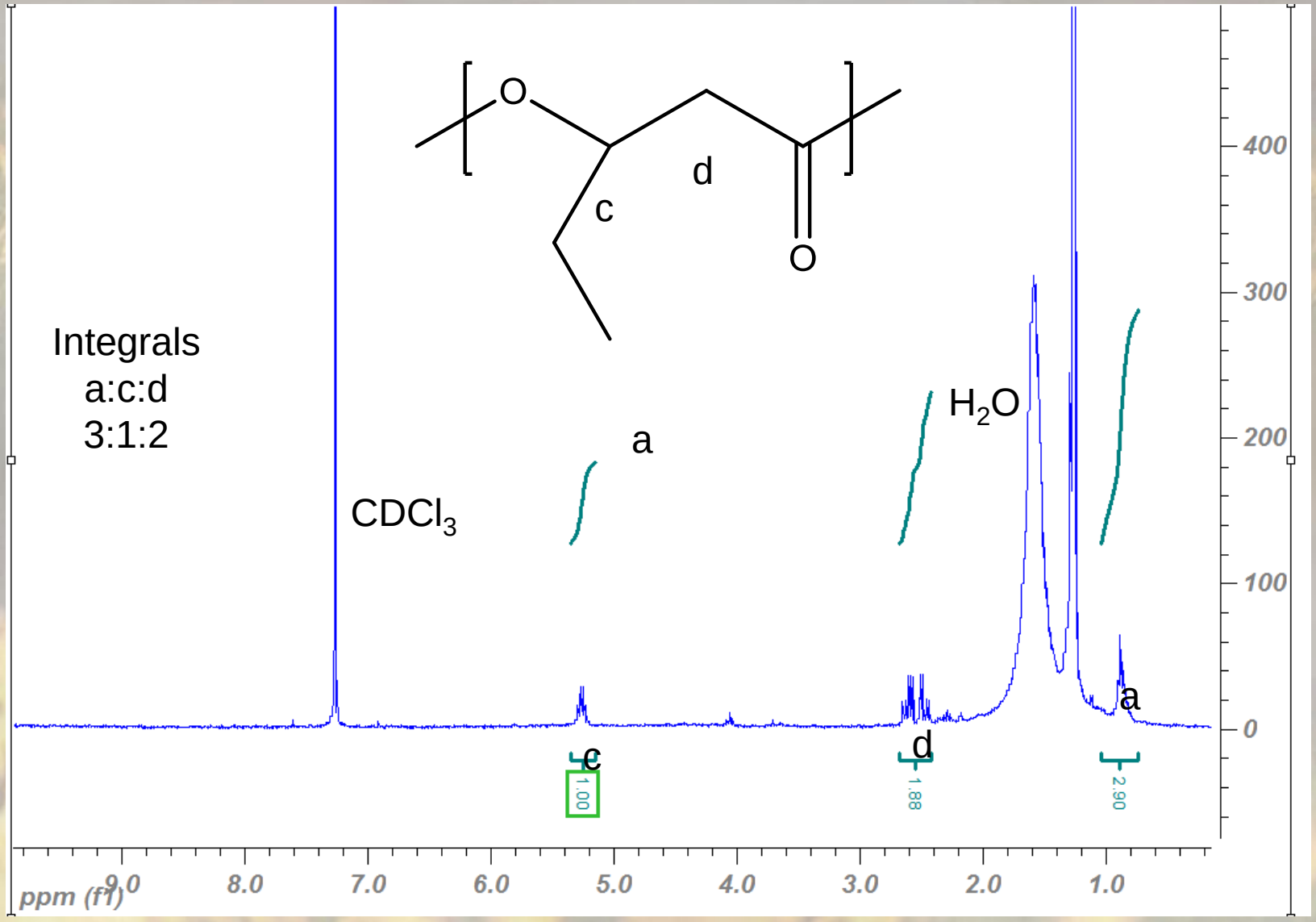
and Mr. Robert Russell (Australian Nuclear Science Technology Organisation (ANTSO)

Crude NMR of Extractables



Dr Kristofer Thurecht and Ms Tara Schiller – Australian Institute for Bioengineering and Nanotechnology

Precipitated in 1:9, $CHCl_3$:Acetone



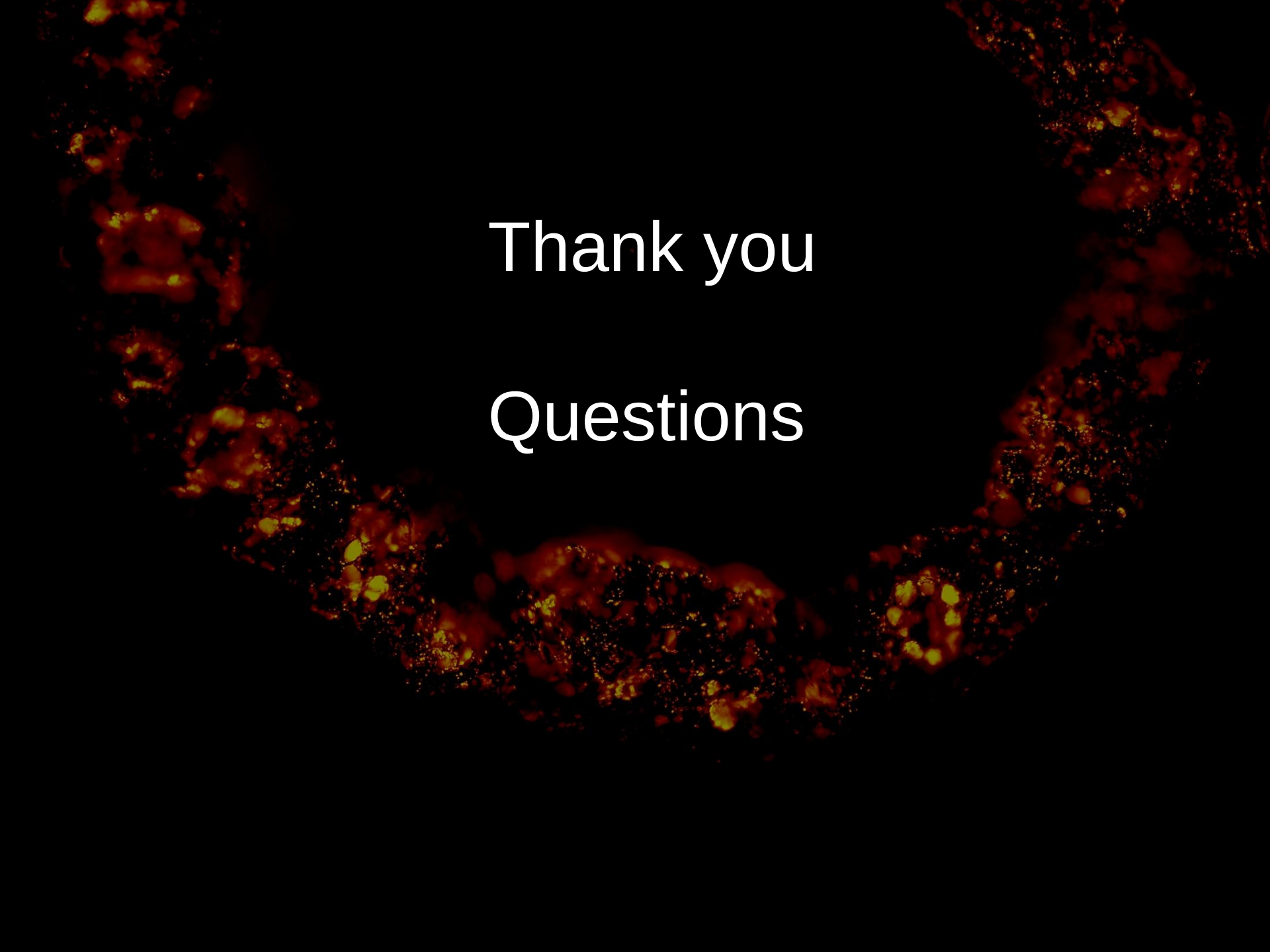
Dr Kristofer Thurecht and Ms Tara Schiller – Australian Institute for Bioengineering and Nanotechnology

Conclusions: PHB Cane

- Engineered sugarcane with a multigene pathway
- High throughput screening allowed detection of higher producing lines
- Enzymes properly targeted to both types of plastids
- Stronger promoter = more PHB
- ACCase inhibitor = more PHB
- Both plastidic and peroxisomal production results in PHB accumulation
- Getting very high MW polymer produced
- Possible PHB/PHV copolymer

Special Thanks to:

- **ARC Linkage**
- **UQ Chem Eng**
- **BSES Limited**
- **CRCSIIB**
- **SRDC**
- **Metabolix**


Thank you
Questions