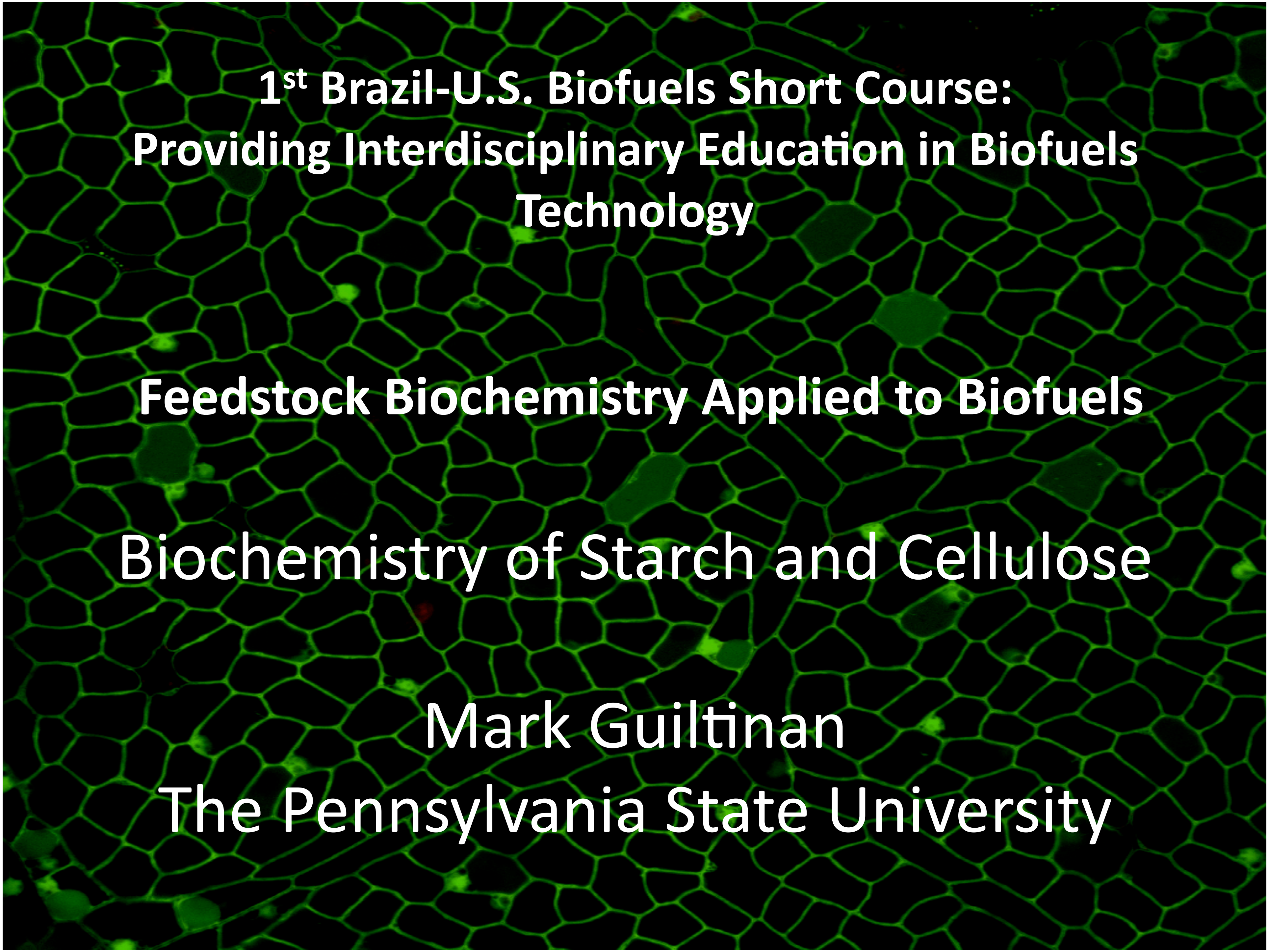
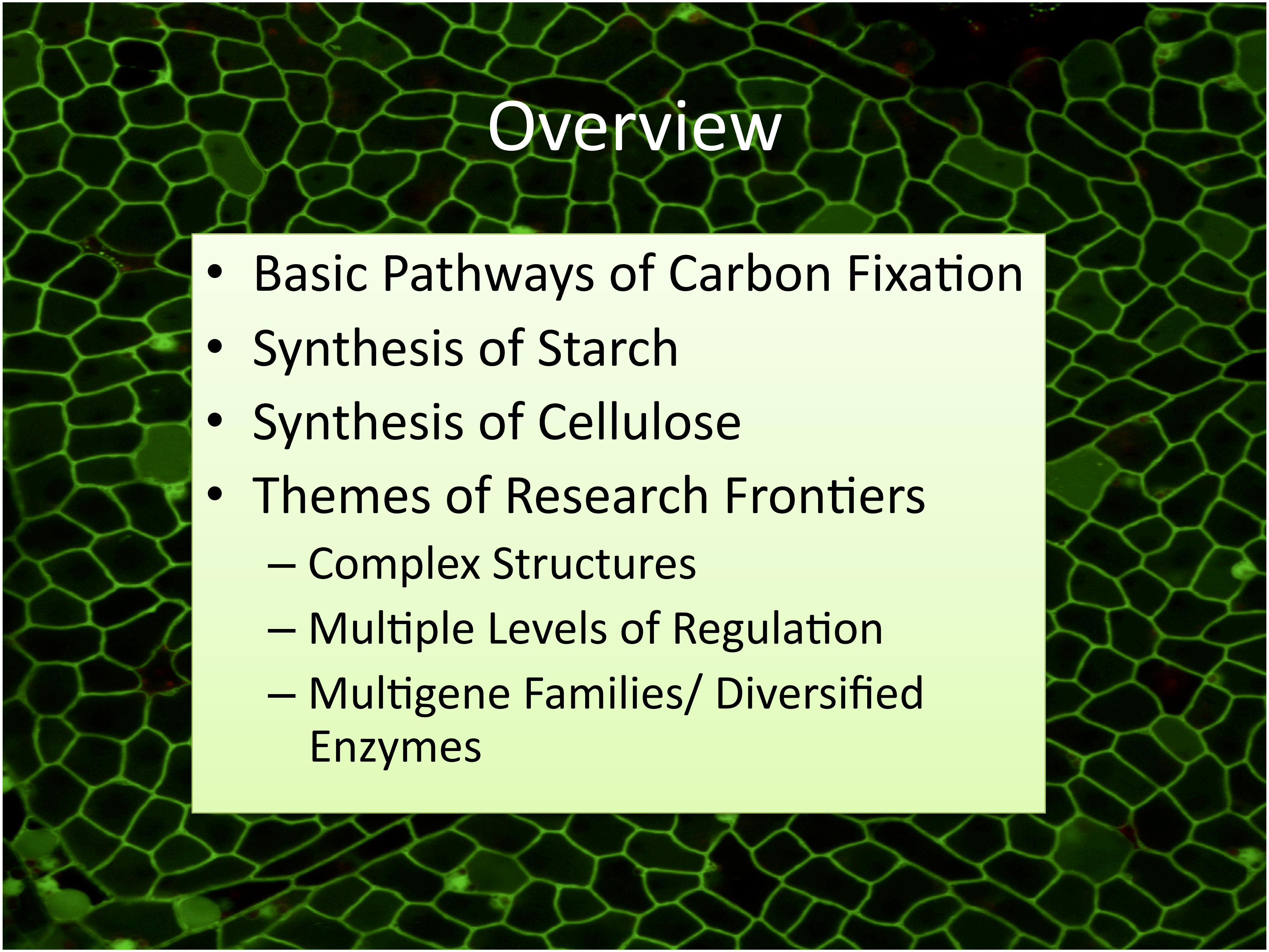


# 1st Brazil-U.S. Biofuels Short Course: Providing Interdisciplinary Education in Biofuels Technology

## Feedstock Biochemistry Applied to Biofuels

# Biochemistry of Starch and Cellulose

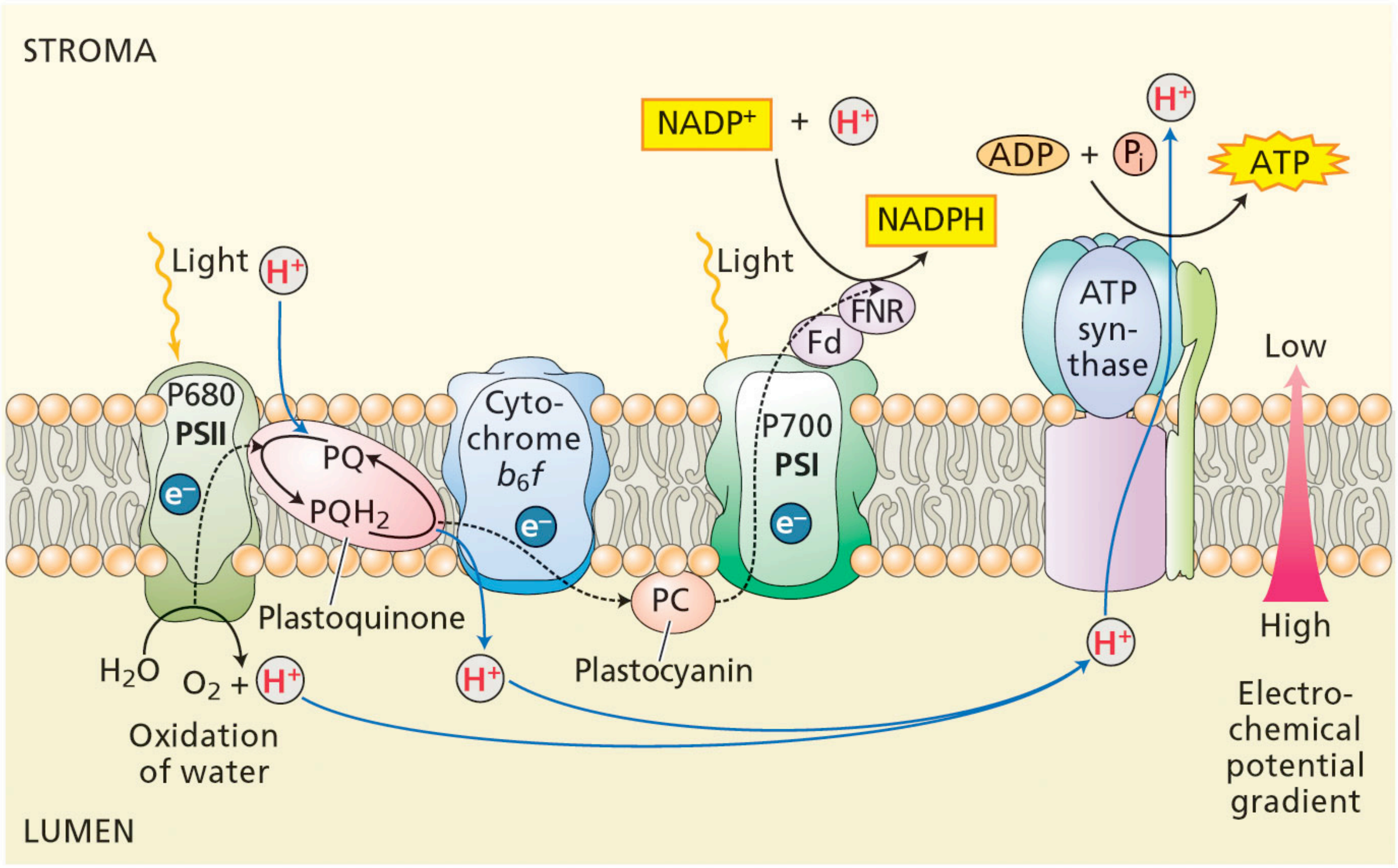
Mark Guiltinan
The Pennsylvania State University



# Overview

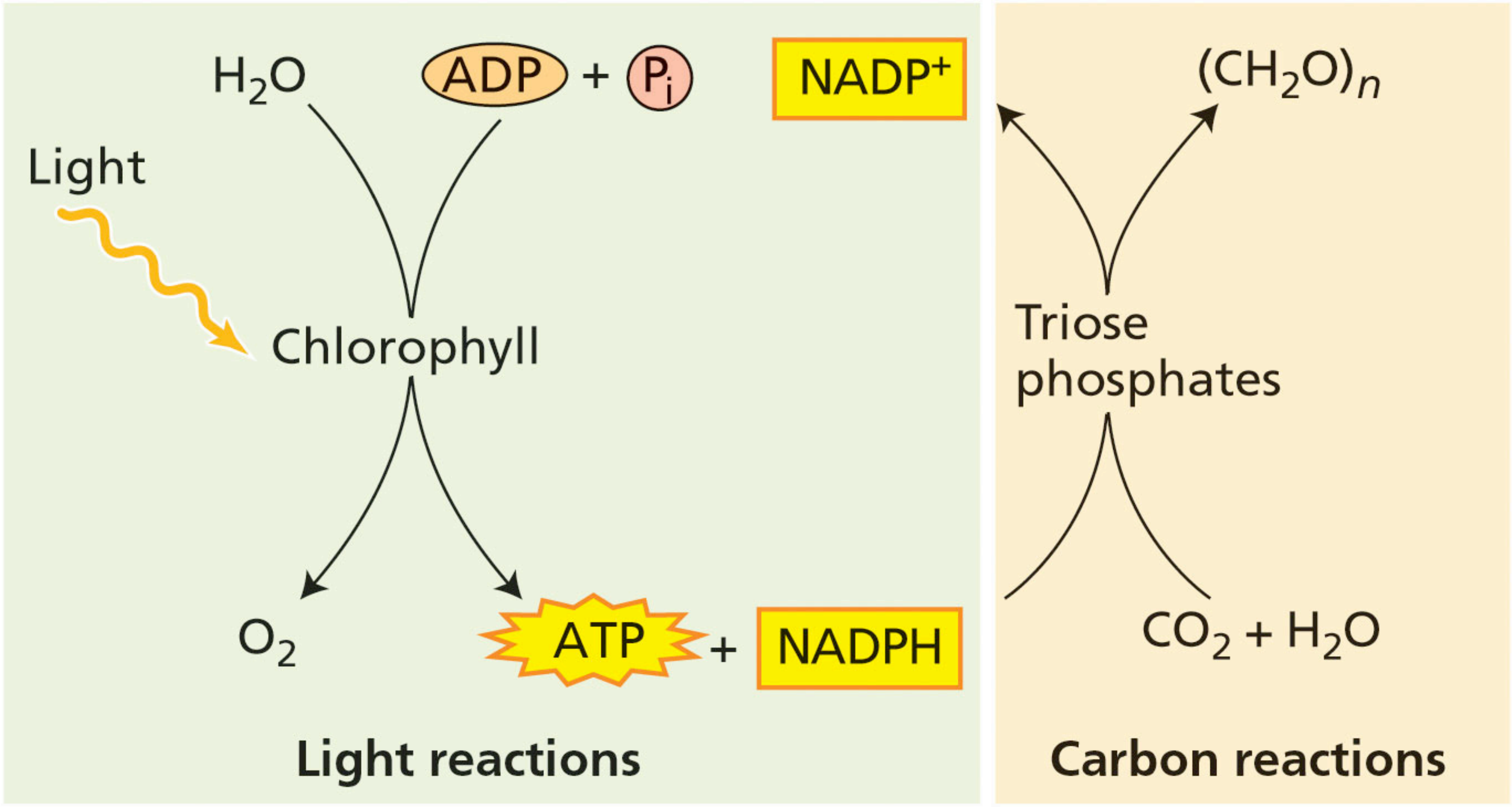
- Basic Pathways of Carbon Fixation
- Synthesis of Starch
- Synthesis of Cellulose
- Themes of Research Frontiers
  - Complex Structures
  - Multiple Levels of Regulation
  - Multigene Families/ Diversified Enzymes

# Photosynthesis: The Light Reactions
# Generation of ATP and NADPH



PLANT PHYSIOLOGY, Third Edition, Figure 7.22 © 2002 Sinauer Associates, Inc.

# The Dark Side of Photosynthesis
# The Dark Reactions (overview)



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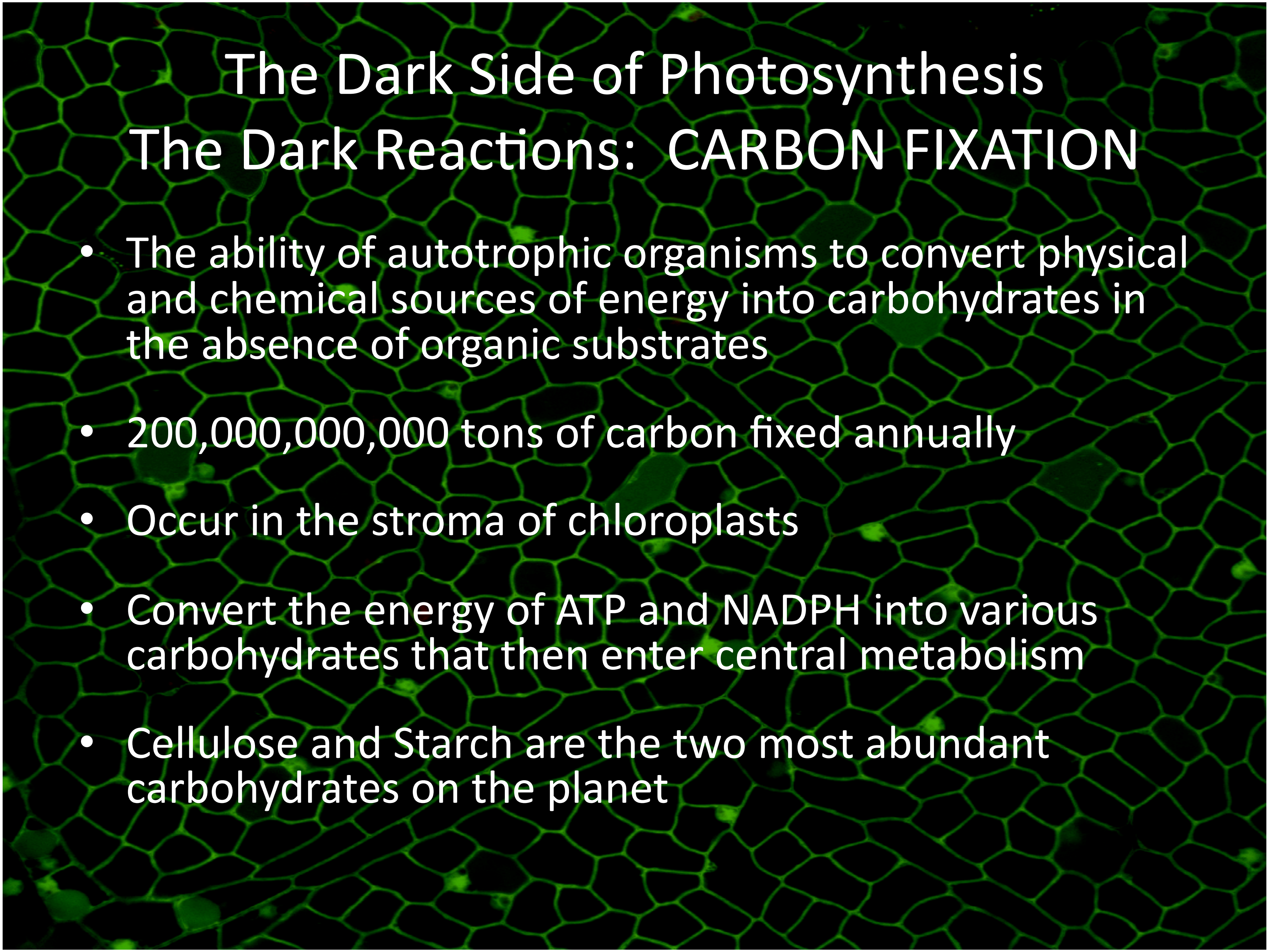


# The Dark Side of Photosynthesis
# The Dark Reactions: CARBON FIXATION

- The ability of autotrophic organisms to convert physical and chemical sources of energy into carbohydrates in the absence of organic substrates

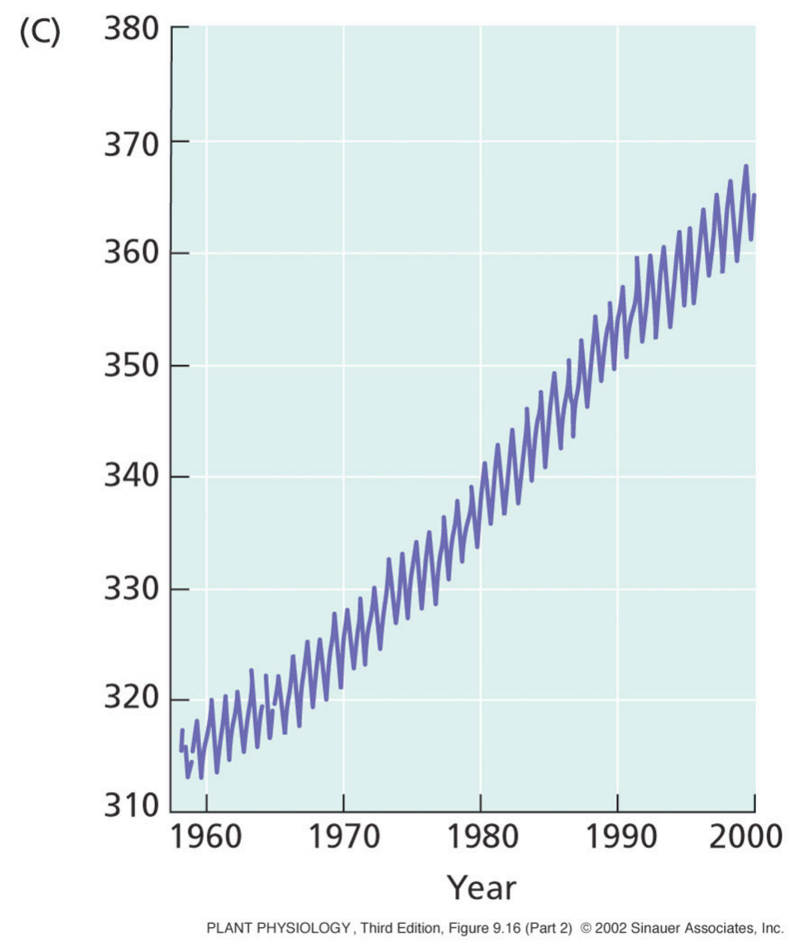
- 200,000,000,000 tons of carbon fixed annually

- Occur in the stroma of chloroplasts

- Convert the energy of ATP and NADPH into various carbohydrates that then enter central metabolism

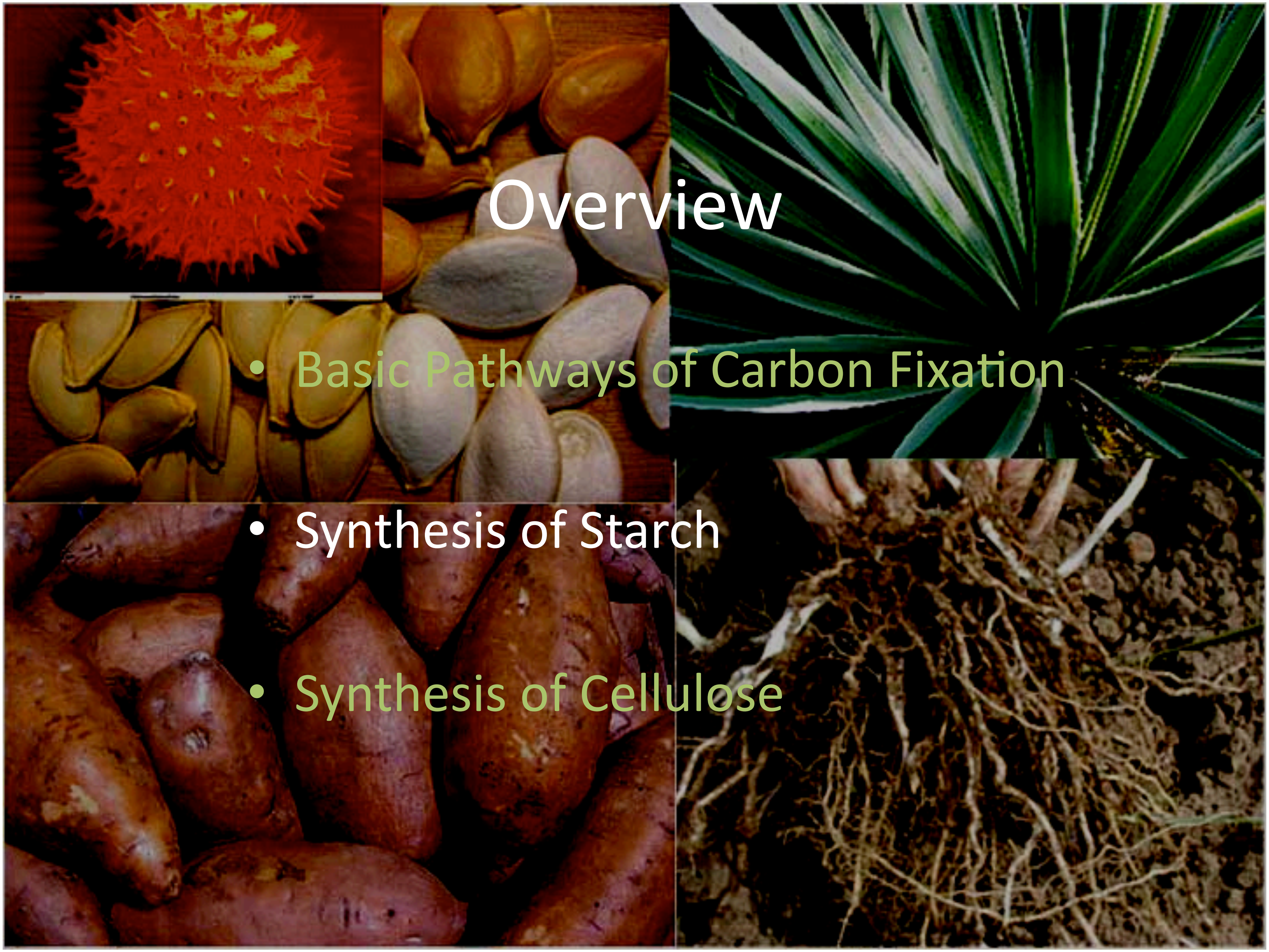
- Cellulose and Starch are the two most abundant carbohydrates on the planet

# The Dark Side of Photosynthesis
# The Dark Reactions: CARBON FIXATION



PLANT PHYSIOLOGY, Third Edition, Figure 9.16 (Part 2) © 2002 Sinauer Associates, Inc.

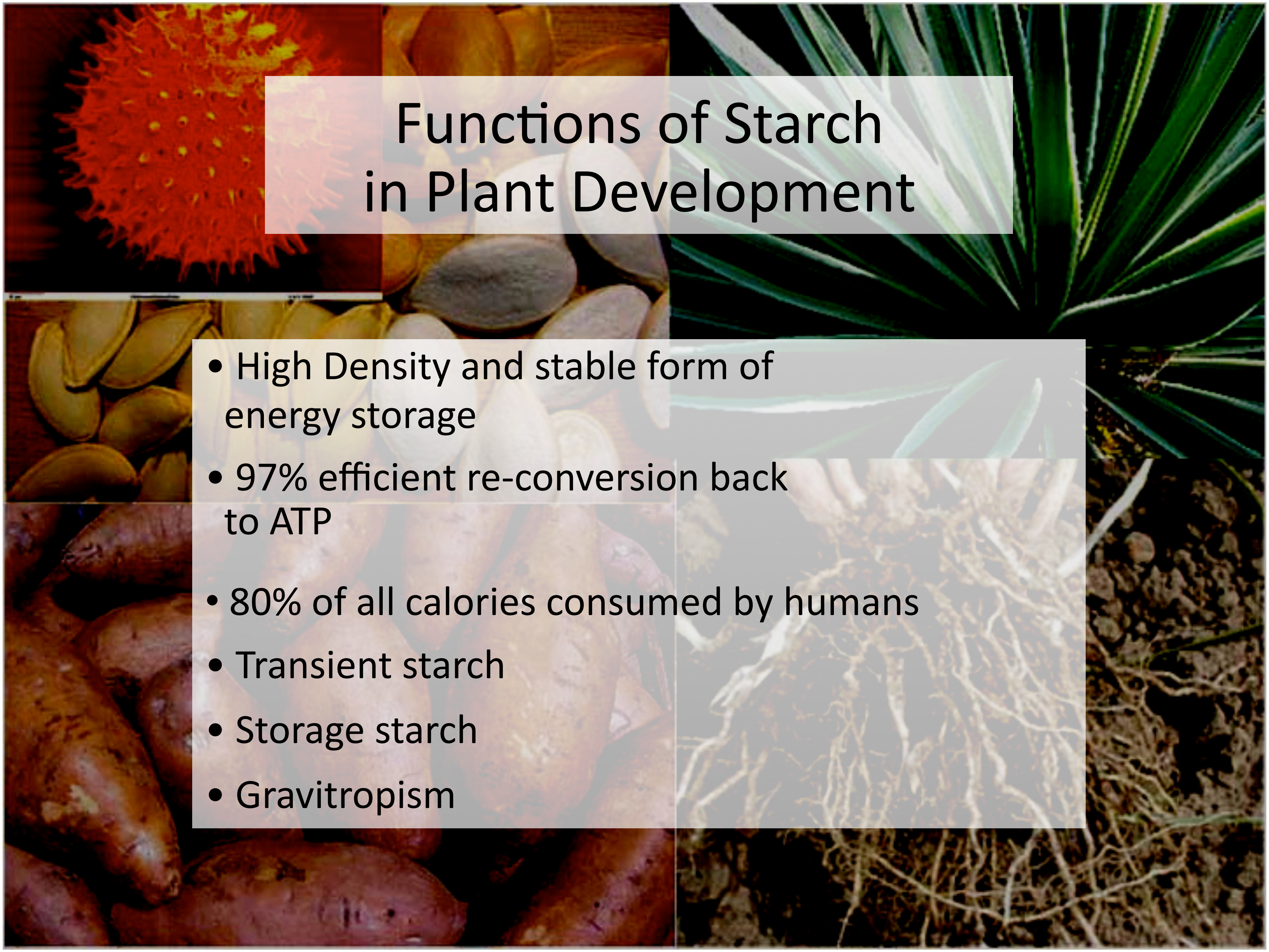
Seasonal concentration changes in atmospheric CO2 measured at Mauna Loa, Hawaii, low in October, high in May. (Taiz and Ziegler, Plant Physiology 4th ed.)



# Overview

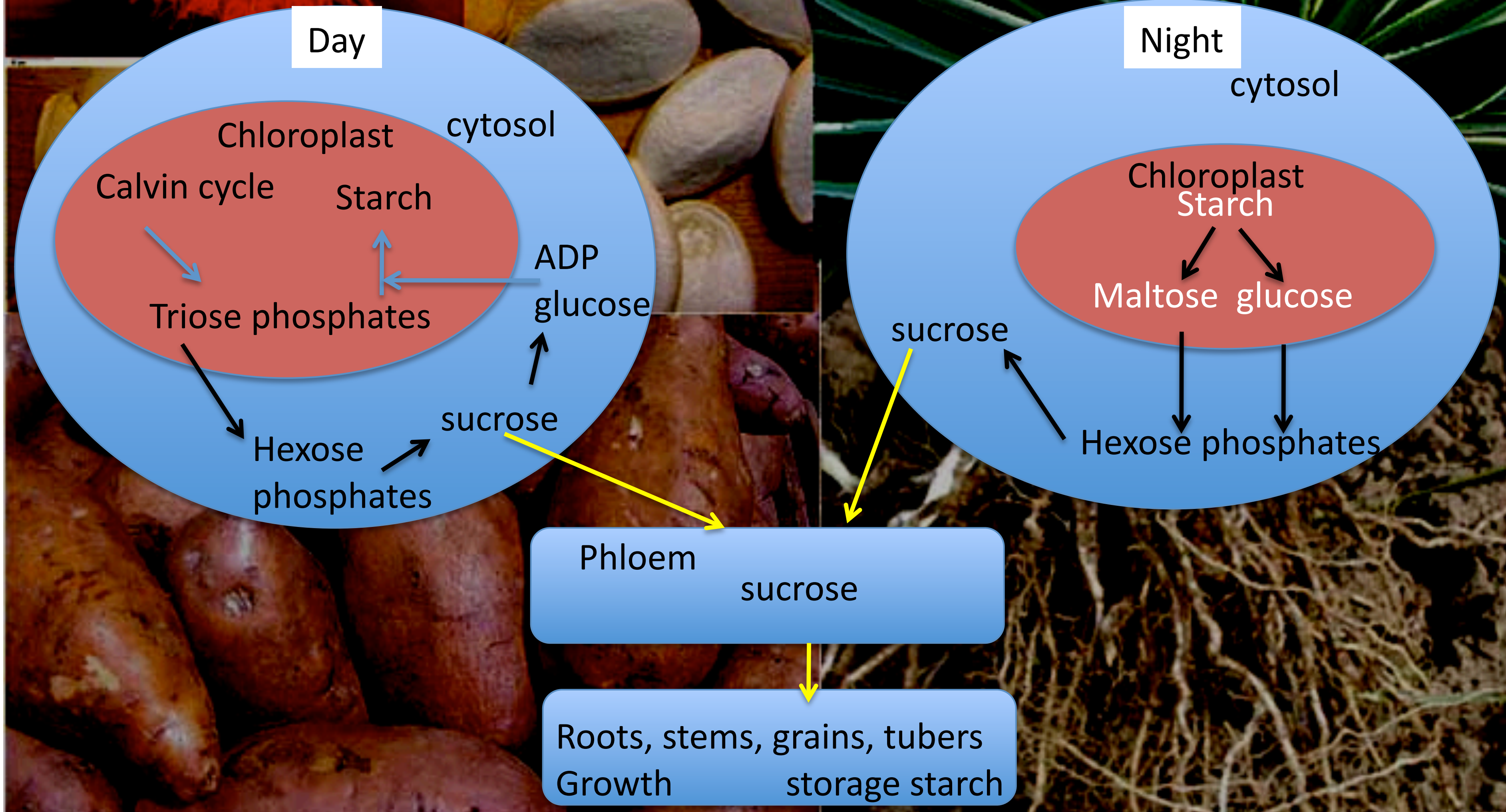
- Basic Pathways of Carbon Fixation
- Synthesis of Starch
- Synthesis of Cellulose

# Functions of Starch in Plant Development



- High Density and stable form of energy storage
- 97% efficient re-conversion back to ATP
- 80% of all calories consumed by humans
- Transient starch
- Storage starch
- Gravitropism

# Diurnal Cycling of Starch



Day

Chloroplast

cytosol

Calvin cycle

Starch

ADP glucose

Triose phosphates

Hexose phosphates

sucrose

Night

cytosol

Chloroplast

Starch

Maltose glucose

sucrose

Hexose phosphates

Phloem

sucrose

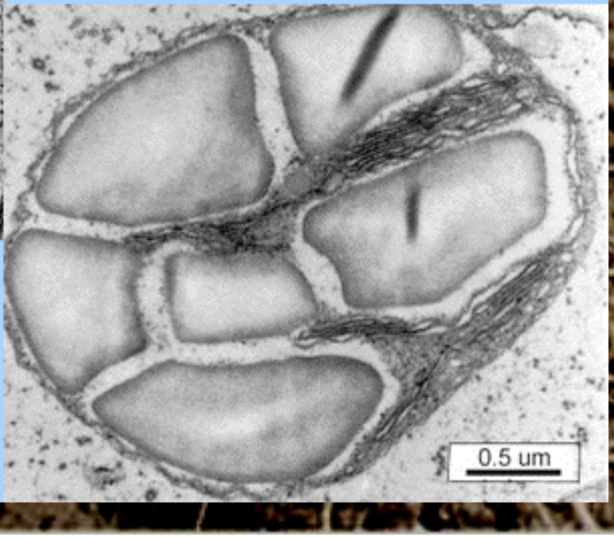
Roots, stems, grains, tubers

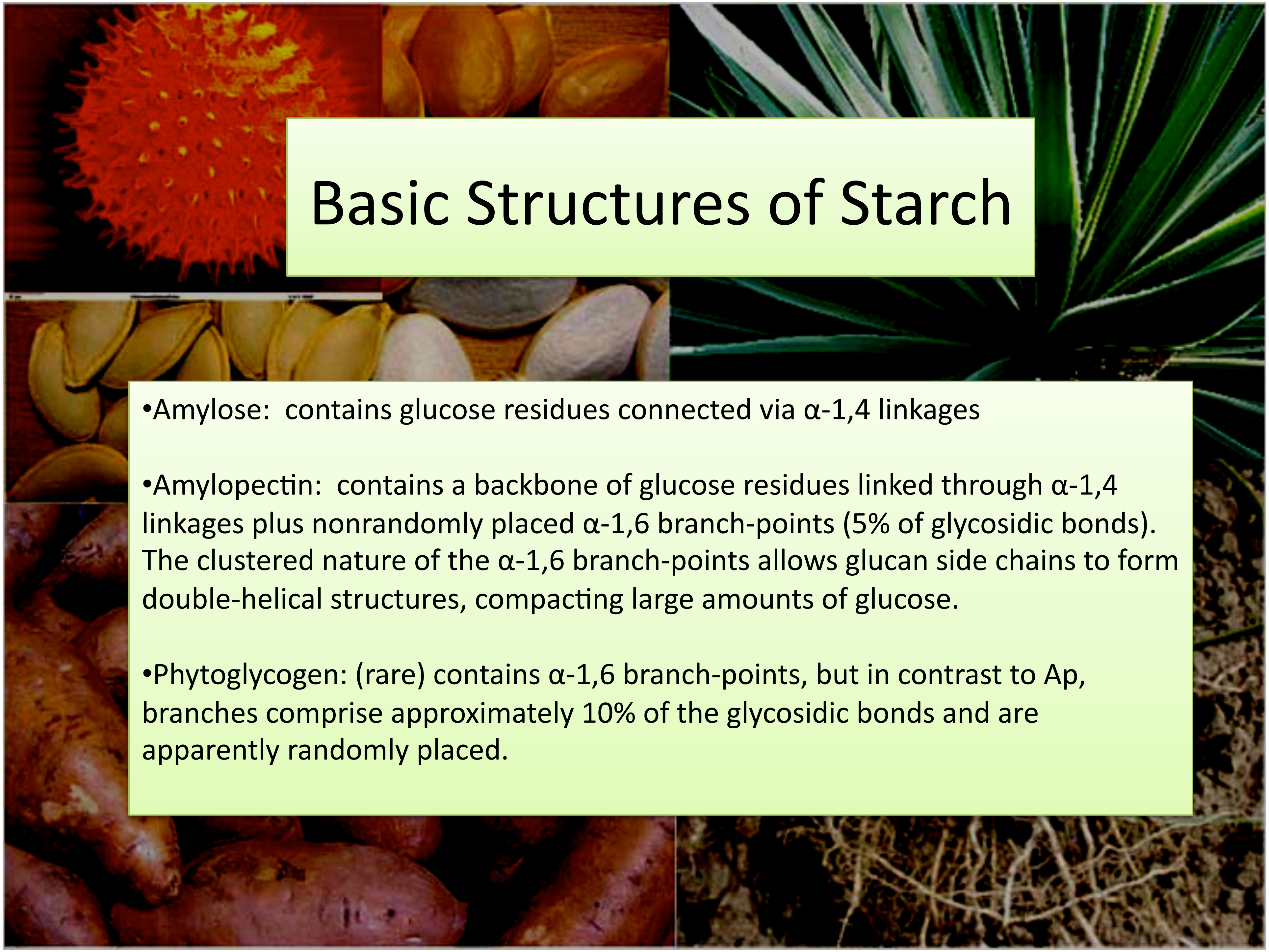
Growth storage starch

# Diurnal Cycling of Starch

http://faculty.uca.edu/~johnc/Chloroplast_and_microbodies.jpg

http://hcs.osu.edu/hcs300/jpeg/THYLAK.JPG





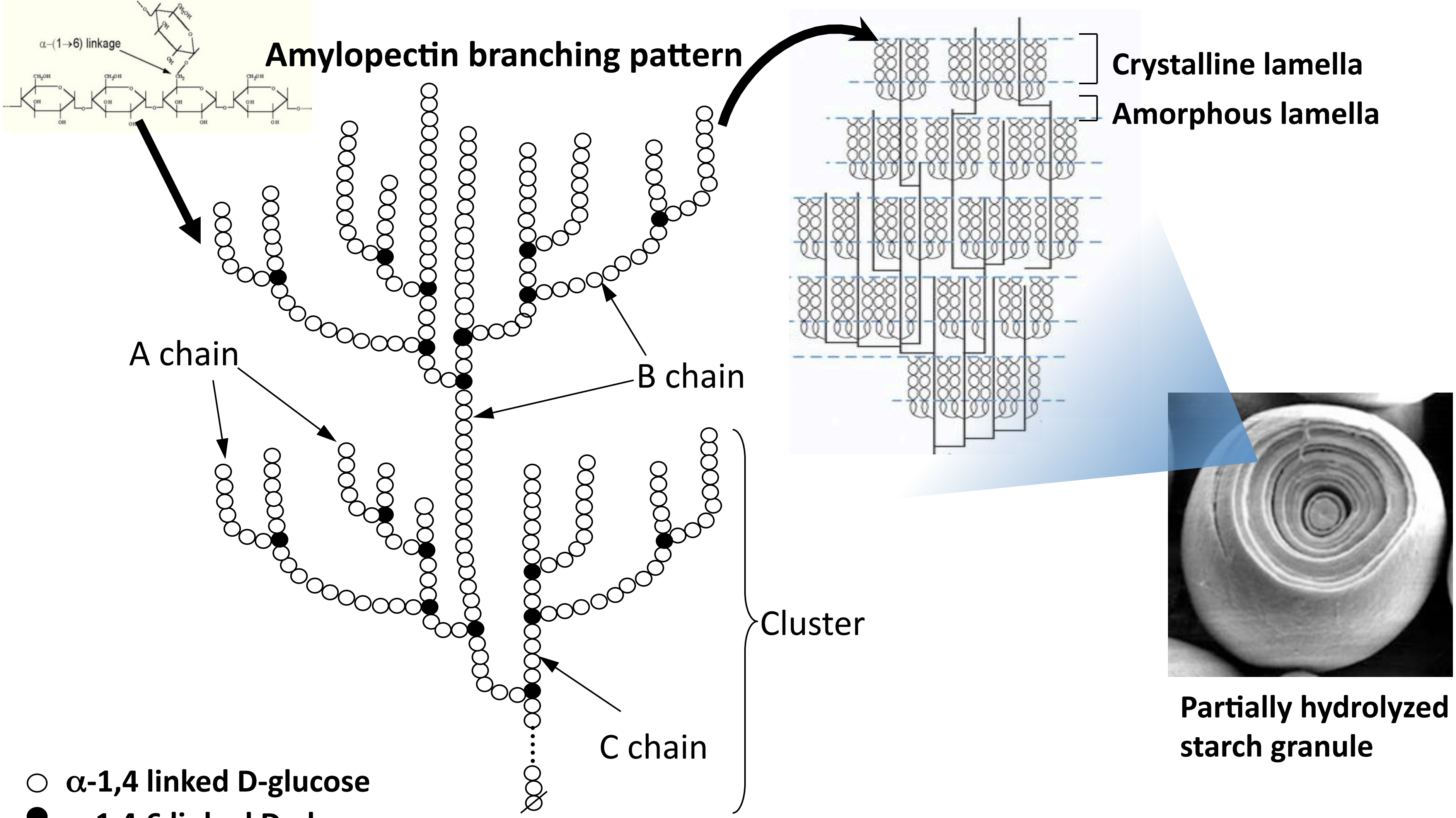
# Basic Structures of Starch

- Amylose: contains glucose residues connected via α-1,4 linkages

- Amylopectin: contains a backbone of glucose residues linked through α-1,4 linkages plus nonrandomly placed α-1,6 branch-points (5% of glycosidic bonds). The clustered nature of the α-1,6 branch-points allows glucan side chains to form double-helical structures, compacting large amounts of glucose.

- Phytoglycogen: (rare) contains α-1,6 branch-points, but in contrast to Ap, branches comprise approximately 10% of the glycosidic bonds and are apparently randomly placed.

# Hierarchical Order of Starch Structure



**Partially hydrolyzed starch granule**

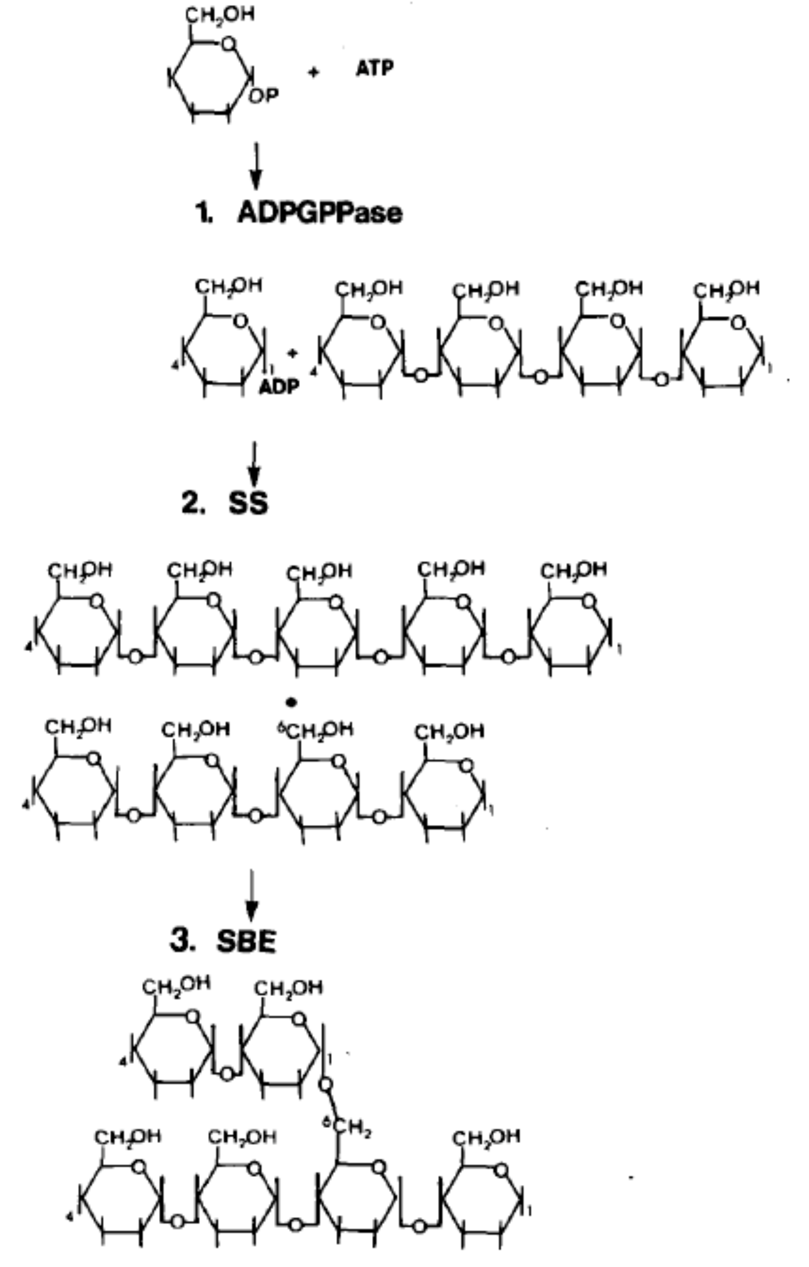
○ **$\alpha$-1,4 linked D-glucose**
● **$\alpha$-1,4,6 linked D-glucose branch points**
∅ **Reducing end**

Adapted from Ball & Morell 2003 & www.cheng.cam.ac.uk/research/

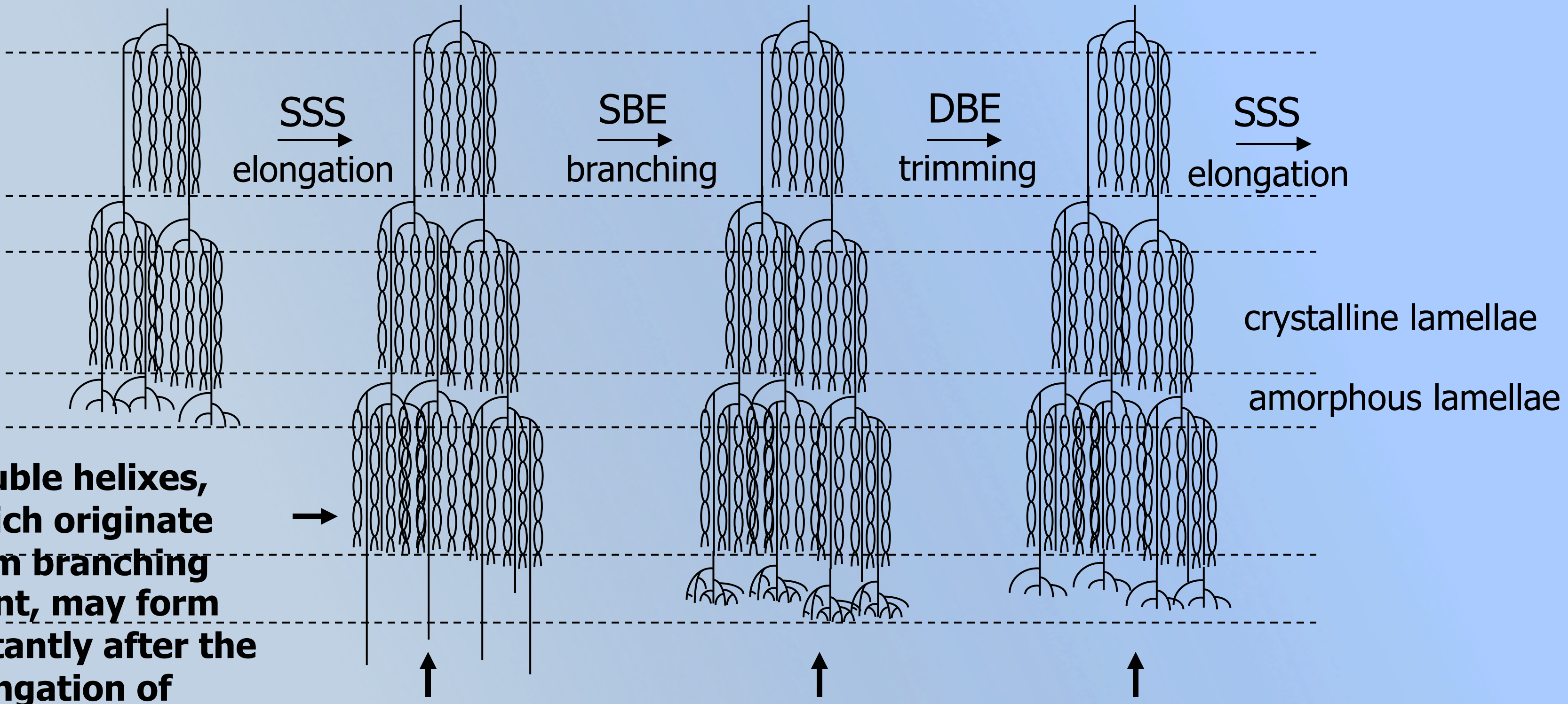
ADPGPPase catalyzes the formation of ADPglucose and inorganic pyrophosphate from glucose-I-phosphate and ATP (step 1).

Starch synthases (SS) add glucose units from ADPglucose to the nonreducing end of a growing $\alpha$(l-linked glucan chain by an $\alpha$ (l-4) linkage and release ADP (step 2).

Starch-branching enzymes (SBE) cut an $\alpha$ (l-4)-linked glucan chain and form an $\alpha$ (1-6) linkage between the reducing end of the cut chain and the C6 of another glucose residue, thus creating a branch (step 3).



# A Model of Amylopectin Biosynthesis



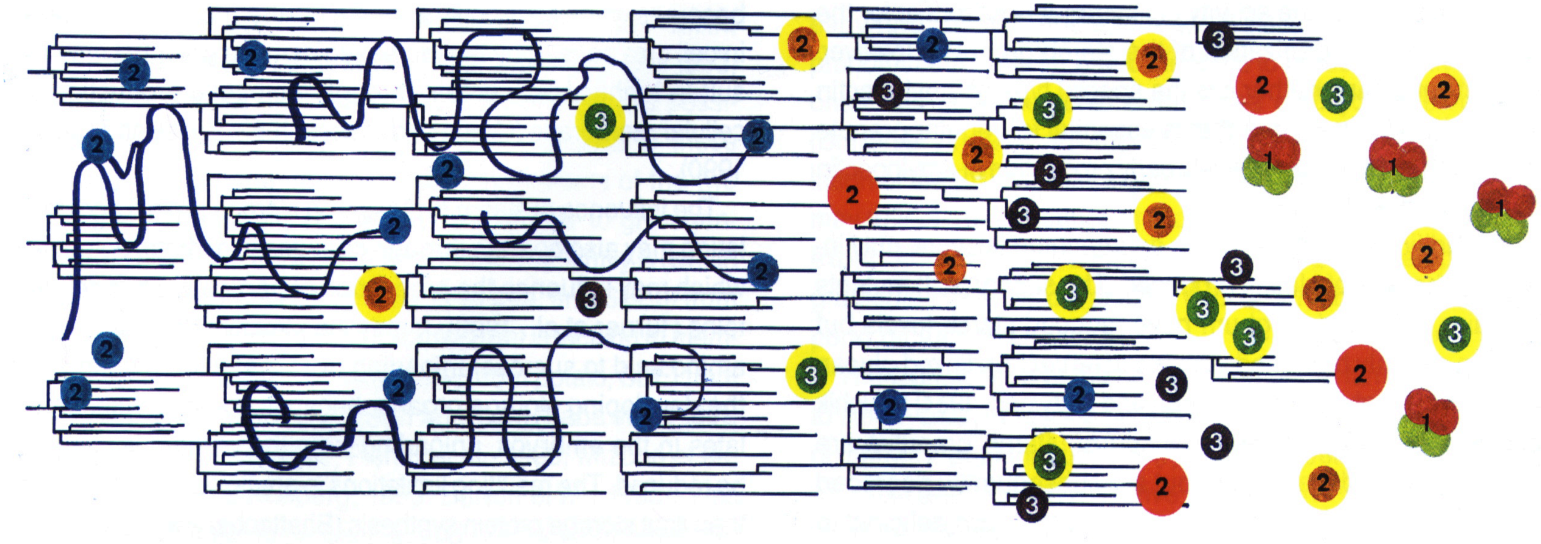
**Double helixes, which originate from branching point, may form instantly after the elongation of external chains**

**Single chains used by SBE for α-1,4 hydrolyzation and α-1,6 reattachment**

**Unique distribution pattern of branching points is determined by different SBE isoforms, and DBE trimming**

# Model for the Role of Isoforms in Starch Synthesis within the Starch Granule

(from Martin and Smith, The Plant Cell 7:971)



1) ADPGPPase
2) Starch Synthase
3) SBE
4) Debranching Enzymes

# Maize Starch Biosynthetic Enzyme Isoforms Encoded by Multigene Families

| | | |
|---|---|---|
| ADPG Pyrophosphorylase | Sh2 | |
| large subunit | Bt2 | |
| small subunit | | |
| Starch Synthase | SS1 | partitioned |
| | SSIIa | “ |
| | SSIIb | “ |
| | SSIII/DU1 | DU1 soluble |
| | GBSSI | Wx granule bound |
| Branching Enzyme | BEI | |
| | BEIIa | |
| | BEIIb | Ae |
| Debranching Enzyme | DBE | SU1 |
| | ZPU1 | |

# AGPase Represents a Rate-limiting Step in Starch Biosynthesis

AGPases are tetramers made up of 50,000 kd subunits

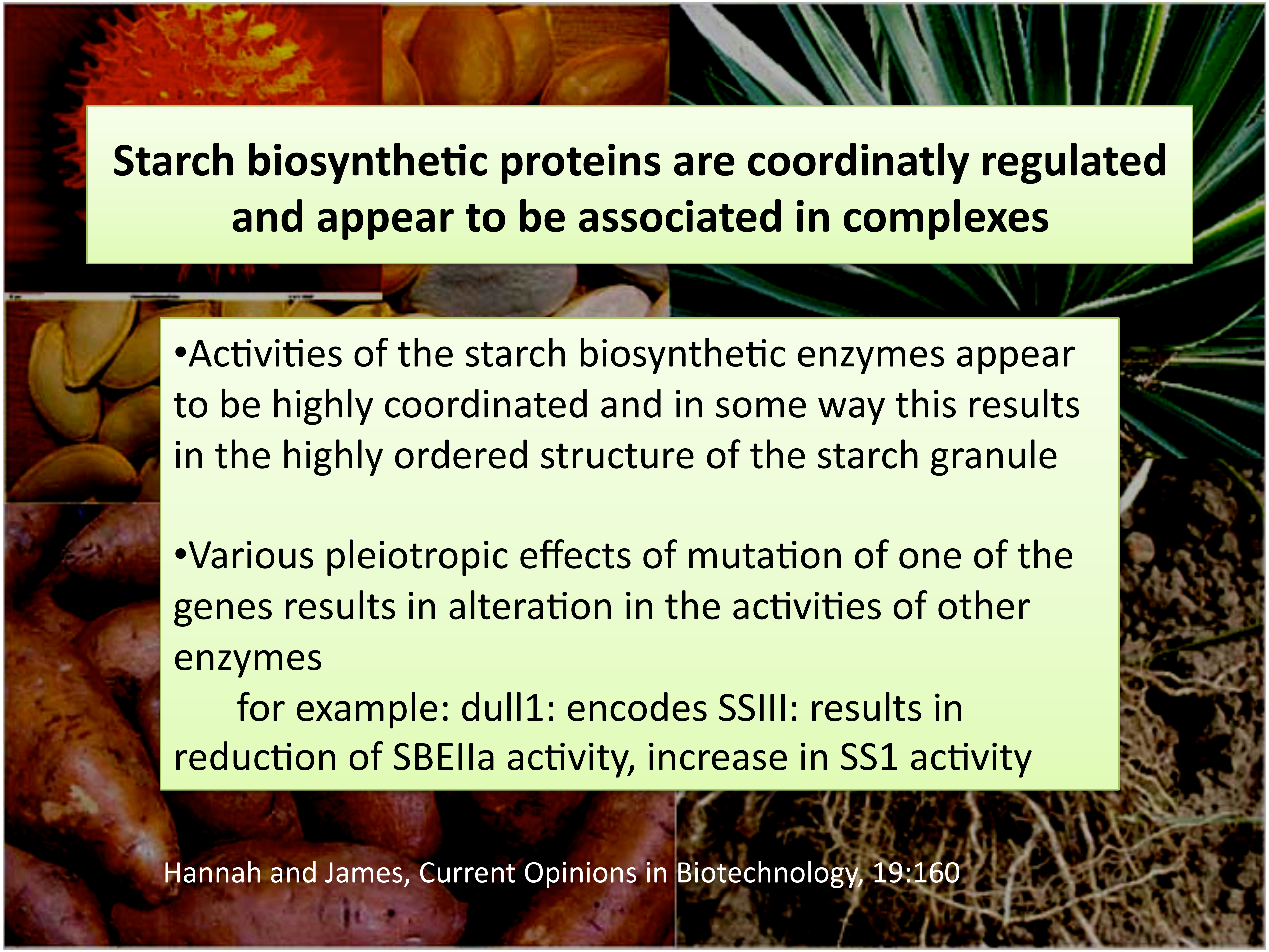
Two different subunits form the $\alpha_2\beta_2$ heterotetrameric plant enzymes

Although AGPase is in the plastids of spinach leaves and potato tubers it appears to be located in the cytoplasm in cereals

AGPase is allosterically regulated
  3-phosphoglyceric acid activated
  inorganic phosphate ($P_i$) inhibited
  mutant forms lacking inhibition increase starch production up to 35%

Heat sensitive
  Heat stable mutants increase starch content up to 38%

Hannah and James, Current Opinions in Biotechnology, 19:160



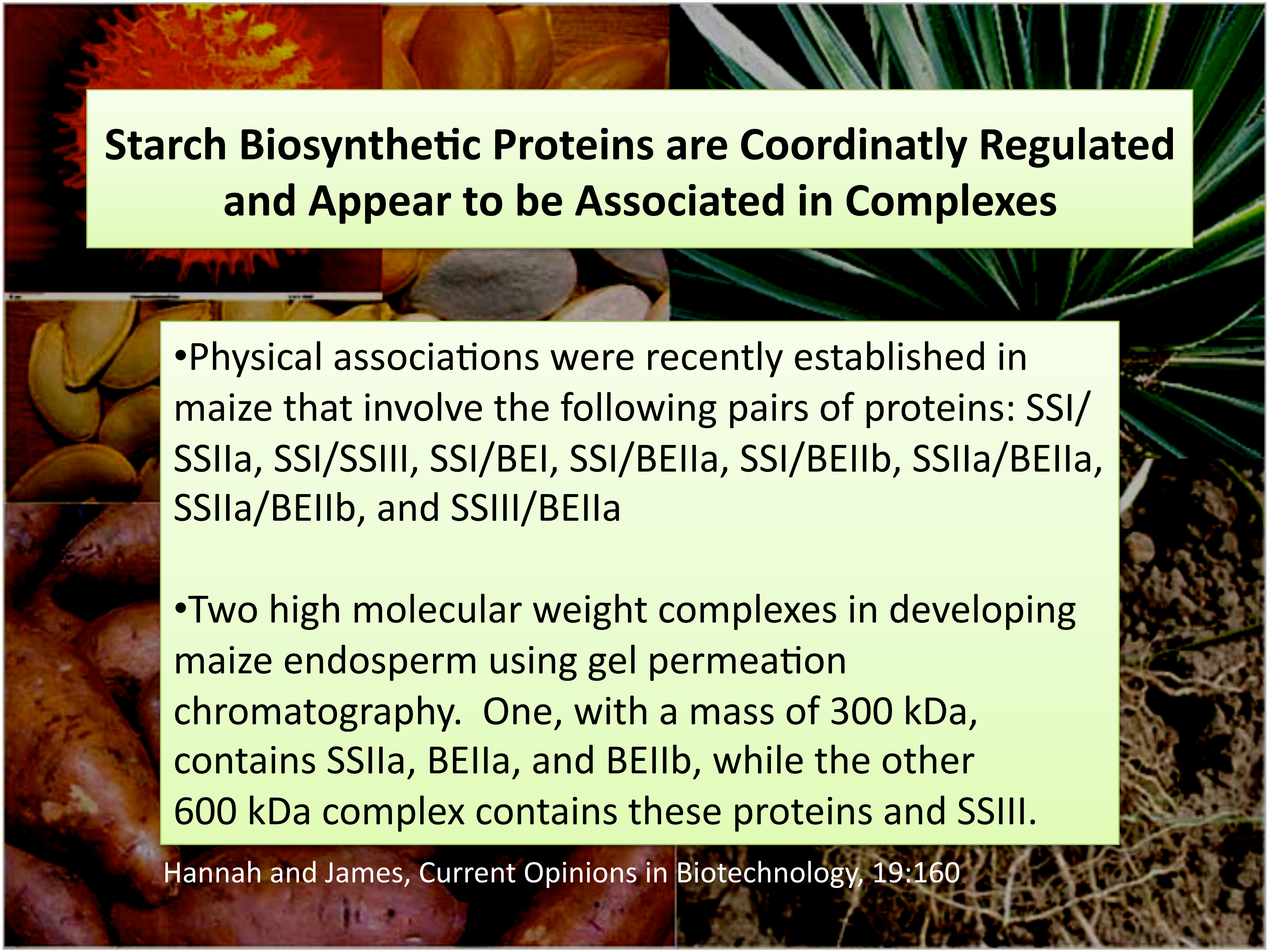
# Starch biosynthetic proteins are coordinatly regulated and appear to be associated in complexes

•Activities of the starch biosynthetic enzymes appear to be highly coordinated and in some way this results in the highly ordered structure of the starch granule

•Various pleiotropic effects of mutation of one of the genes results in alteration in the activities of other enzymes

for example: dull1: encodes SSIII: results in reduction of SBEIIa activity, increase in SS1 activity

Hannah and James, Current Opinions in Biotechnology, 19:160



# Starch Biosynthetic Proteins are Coordinatly Regulated and Appear to be Associated in Complexes

- Physical associations were recently established in maize that involve the following pairs of proteins: SSI/SSIIa, SSI/SSIII, SSI/BEI, SSI/BEIIa, SSI/BEIIb, SSIIa/BEIIa, SSIIa/BEIIb, and SSIII/BEIIa

- Two high molecular weight complexes in developing maize endosperm using gel permeation chromatography. One, with a mass of 300 kDa, contains SSIIa, BEIIa, and BEIIb, while the other 600 kDa complex contains these proteins and SSIII.

Hannah and James, Current Opinions in Biotechnology, 19:160

# Starch Branching Enzymes

- Transglycosylases
- Transfer alpha -1,4 linked chain to alpha-1,6 position
- No net synthesis of starch by their action alone
- Increase number of nonreducing ends in molecule
- Interact with starch synthases, DBEs and other enzymes in multi-protein complexes?
- Critical in definition of starch structure--> function

# Starch Branching Enzymes

- Three Isoforms: SBE I, SBE IIa and IIb in Maize. Multiforms in Rice, Pea, Arab.

- SBE I, 86 KD in Maize
  - Separated from SBE II by DEAE cellulose Chromatography
  - lower $K_m$ for amylose than SBE II
  - Preferentially branches long chains of alpha-1,4 glucan
  - different amino acid composition
  - distinct antigenic properties
  - present in both leaves and endosperm

# Starch Branching Enzymes

- **SBEIIa and SBEIIb, 89.6 and 84.9 kd**
  - similar in
    - amino acid composition
    - kinetic properties
    - antigenic properties
  - separated by 4-aminobutyl sepharose column
  - preferentially branch short chain of alpha-1,4 glucan

- **SBEIIa activity:**
  - present in both leaves and endosperm
- **SBEIIb activity :**
  - Not in vegetative tissues
  - absent in the *ae* mutant and leaves of wild type Maize

# Chain Transfer Specificity of SBE Isoforms

Takeda et al. (1993); Guan and Preiss (1993)

- ***In vitro*** branching of amylose
- SBE IIa and IIb transferred glucan chains of 7-9 residues
- SBE I transferred chains of 12-14 residues

- **How does this relate to amylopectin structure and isoform specific functions *in vivo* ?**

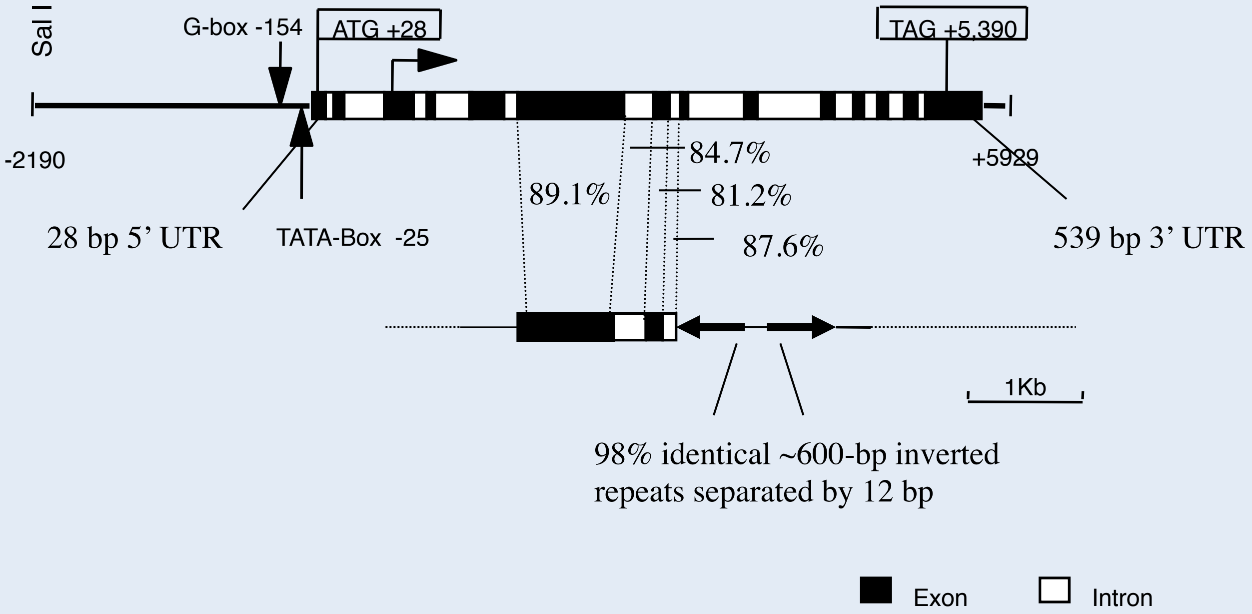
# SBEI Class

*Sbe1a*

14 exons, 13 introns
chromosome 5.03

*Sbe1b*

Pseudogene with limited
homology; Chr 6, bin 6.01

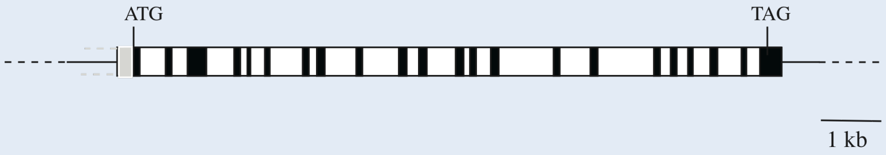


# SBEII Class

## *Sbe2a*

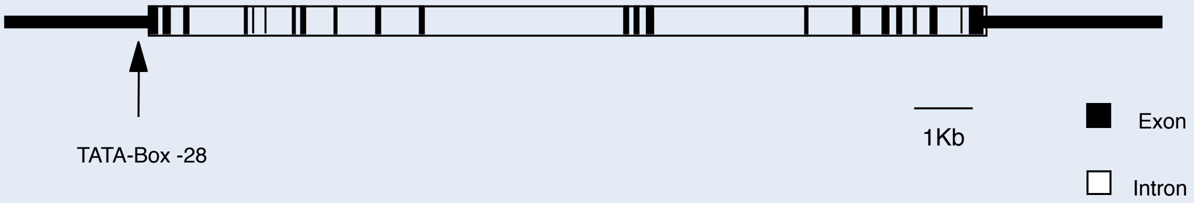
22 exons, 21 introns

Chromosome 2 (according to 1st phase sequence analysis)



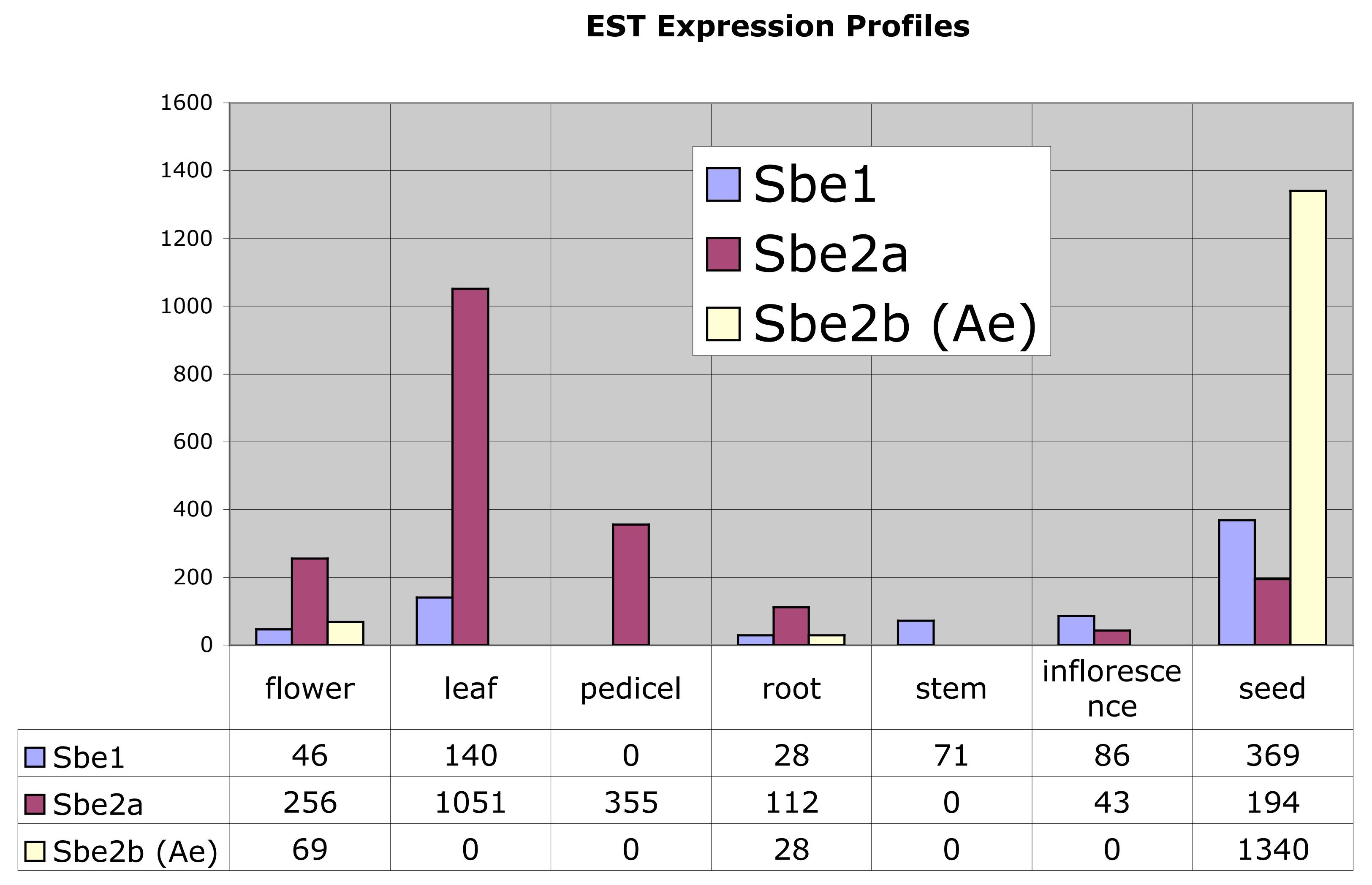
## *Ae* (*Sbe2b*)

22 exons, 21 introns, 76 bp to 4,020 bp
chromosome 5 bin 5.04-5.05



# SBE Gene Expression

## Data-Mining of Genbank EST Database

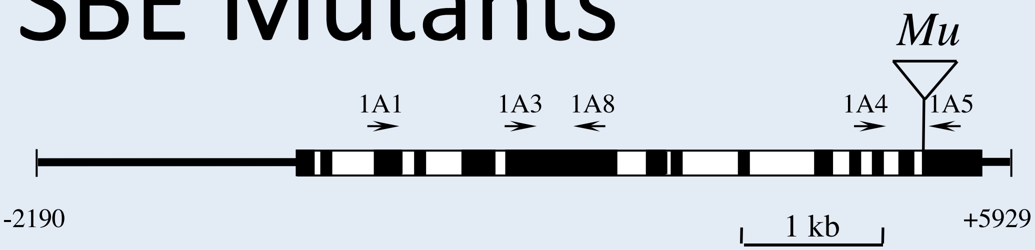


| | flower | leaf | pedicel | root | stem | inflorescence | seed |
|---|---|---|---|---|---|---|---|
| 🟦 Sbe1 | 46 | 140 | 0 | 28 | 71 | 86 | 369 |
| 🟥 Sbe2a | 256 | 1051 | 355 | 112 | 0 | 43 | 194 |
| 🟨 Sbe2b (Ae) | 69 | 0 | 0 | 28 | 0 | 0 | 1340 |

# SBE Mutants

*sbe1::Mu*

14th exon in the 761st codon

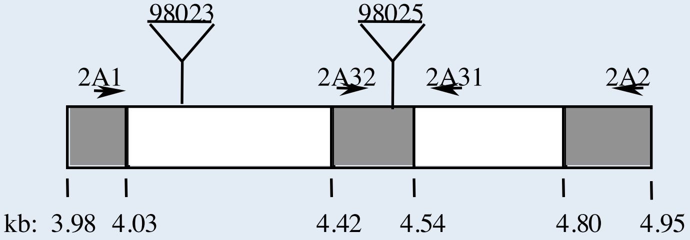


*sbe2a::Mu*

First intron line 98023

Second exon line 98025

Between codon 43 and 44

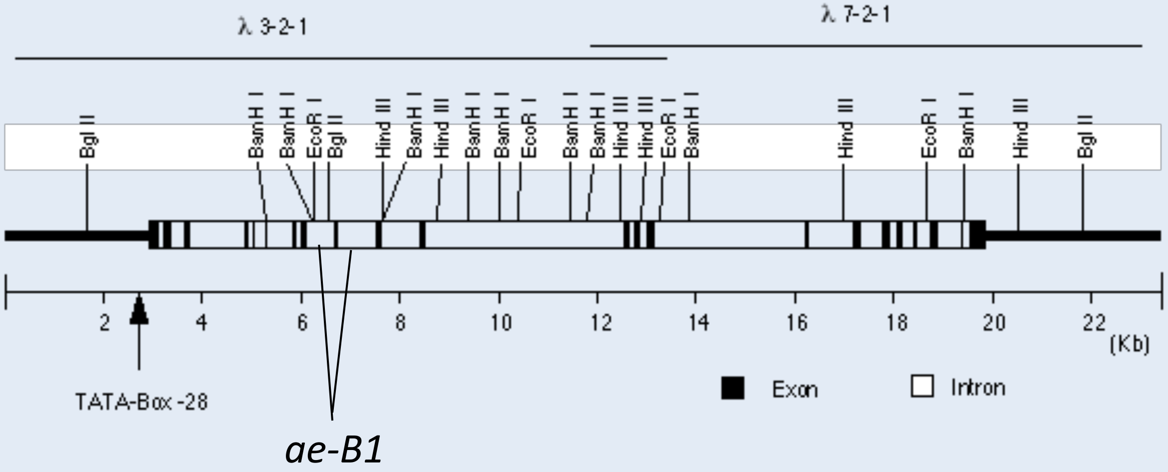


*ae-B1*

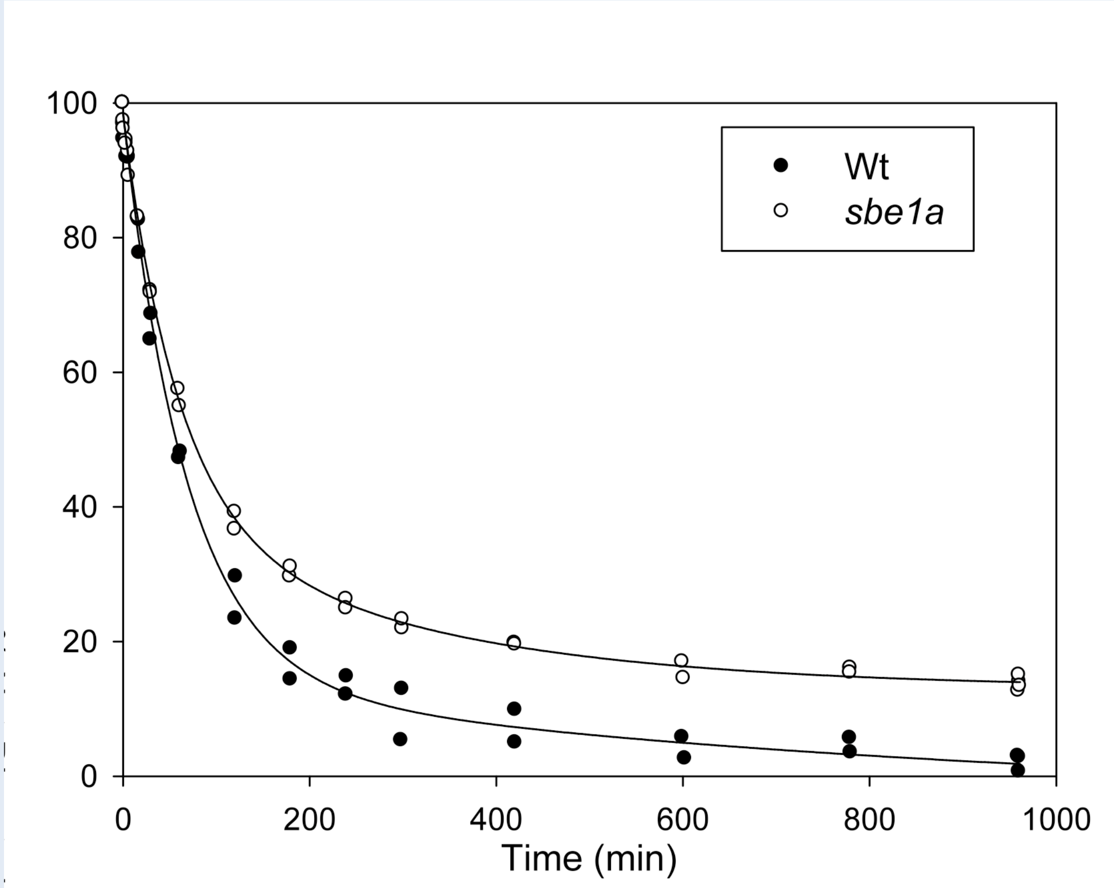
882 bp deletion spanning position 6613 in intron 8 to 7494 in intron 9

Exon 9 is entirely deleted.

2 bp "insertion" of the sequence "AA".



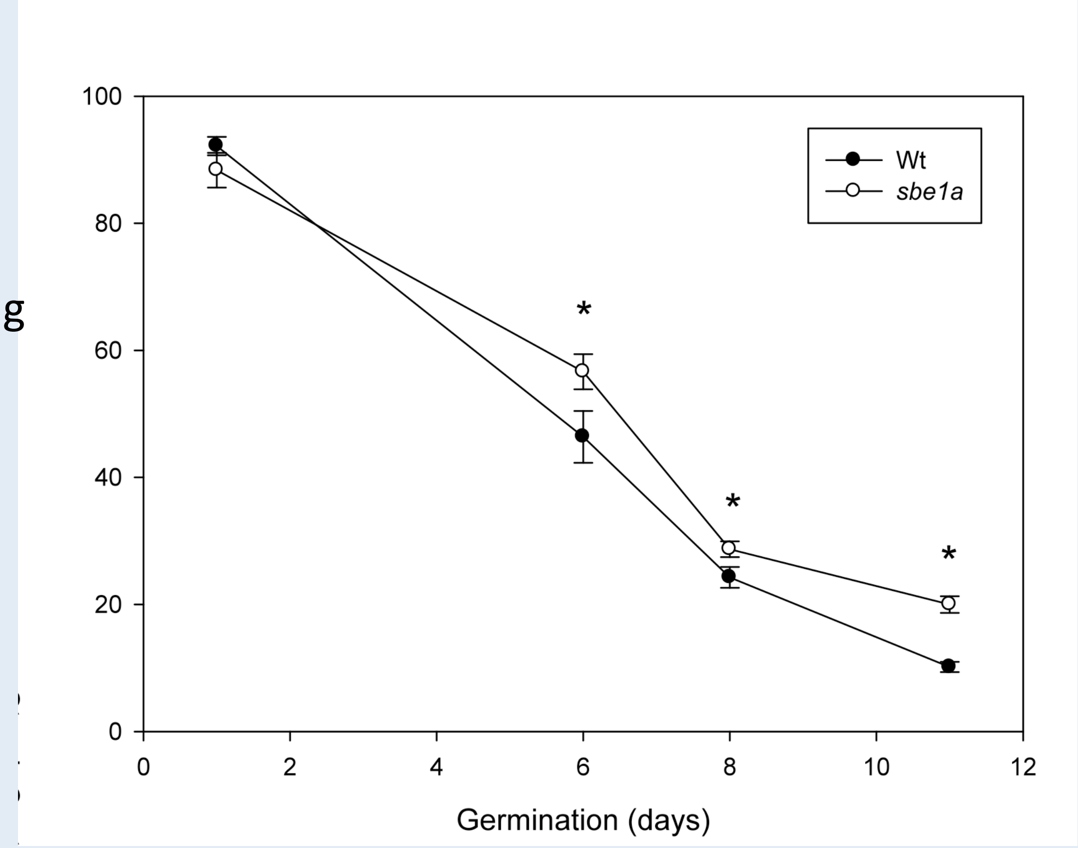
# Mutant Lacking SBEI Makes Starch That is More Resistant to Amylase Digestion



Time course of digestion of the resistant starch assay for Wt and *sbe1a* mutant starch from one biological replication. Curves shown are best fits of analysis of combined data from 2 independent digestions.

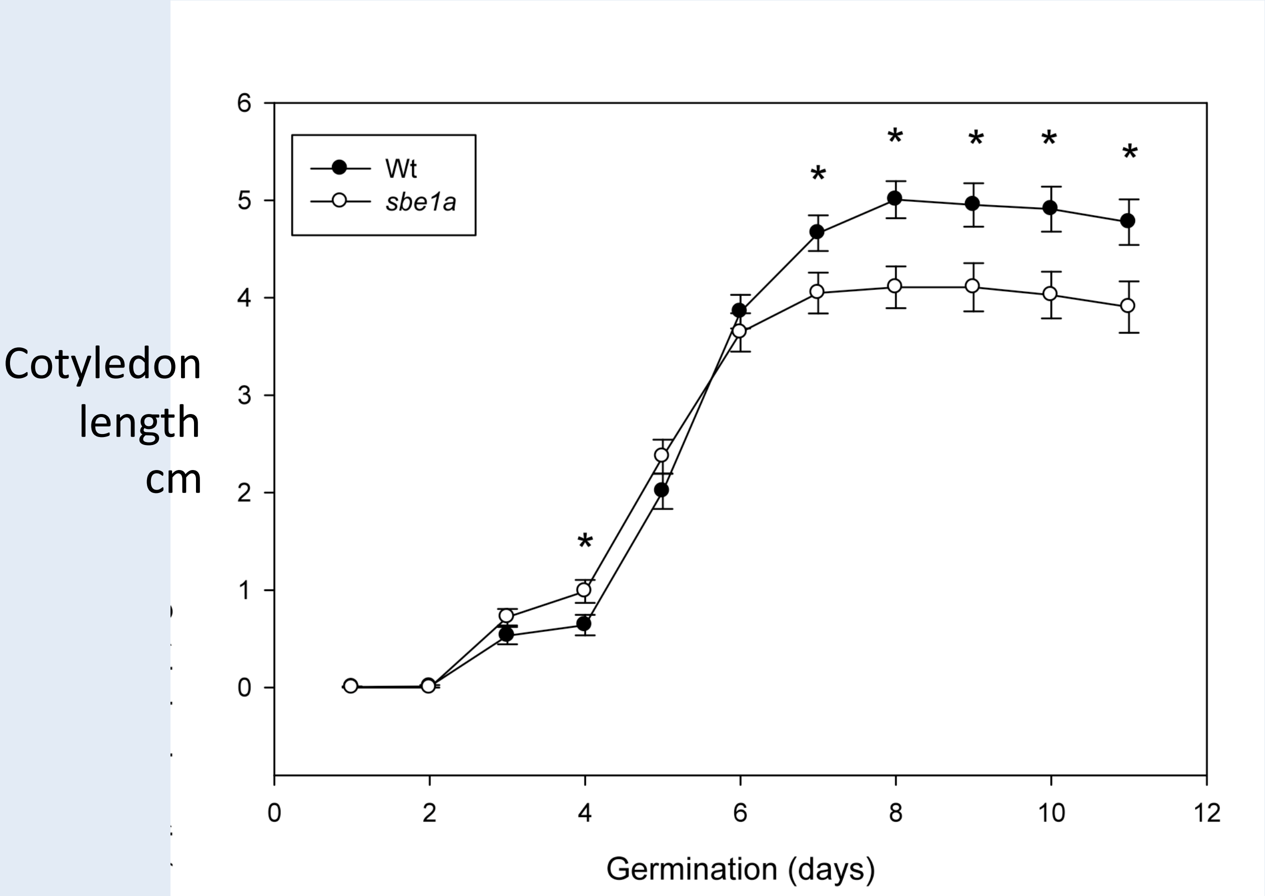
# Mutant Lacking SBEI Utilizes Starch Slower During Germination

% starch remaining



Germination of Wt and *sbe1a* mutant kernels: Starch content in the germinating endosperm was quantified at Day 1, 6, 8, 11, and percentage of starch content at each day against the dry weight of Day 1 kernels was plotted

# Mutant Lacking SBEI Reaches a Shorter Length During Germination

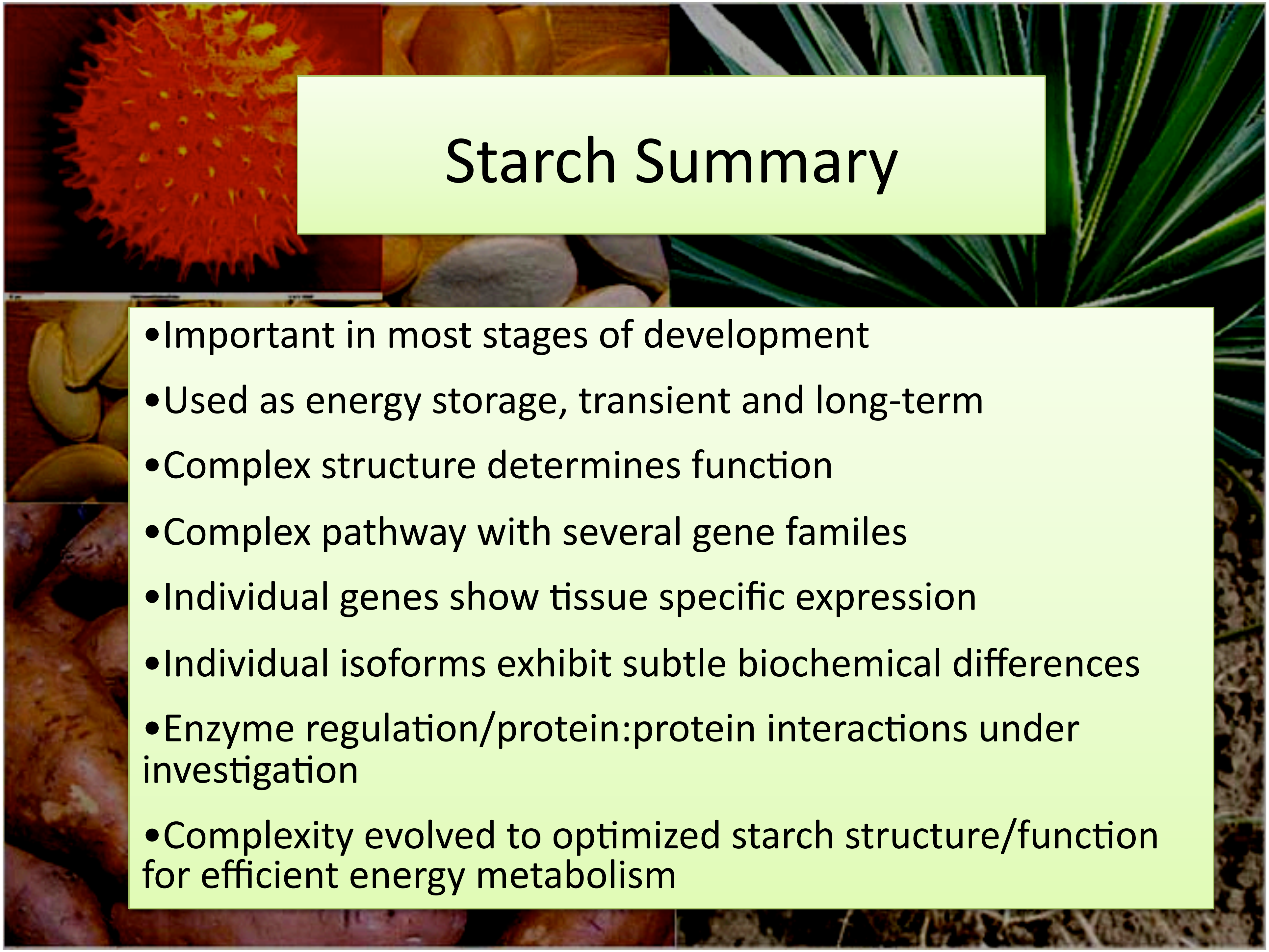


Germination of Wt and *sbe1a* mutant kernels: The lengths of the emerged cotyledons were measured on successive days during the incubation period

# Effect of sbe2a Null Mutant on Vegetative Development

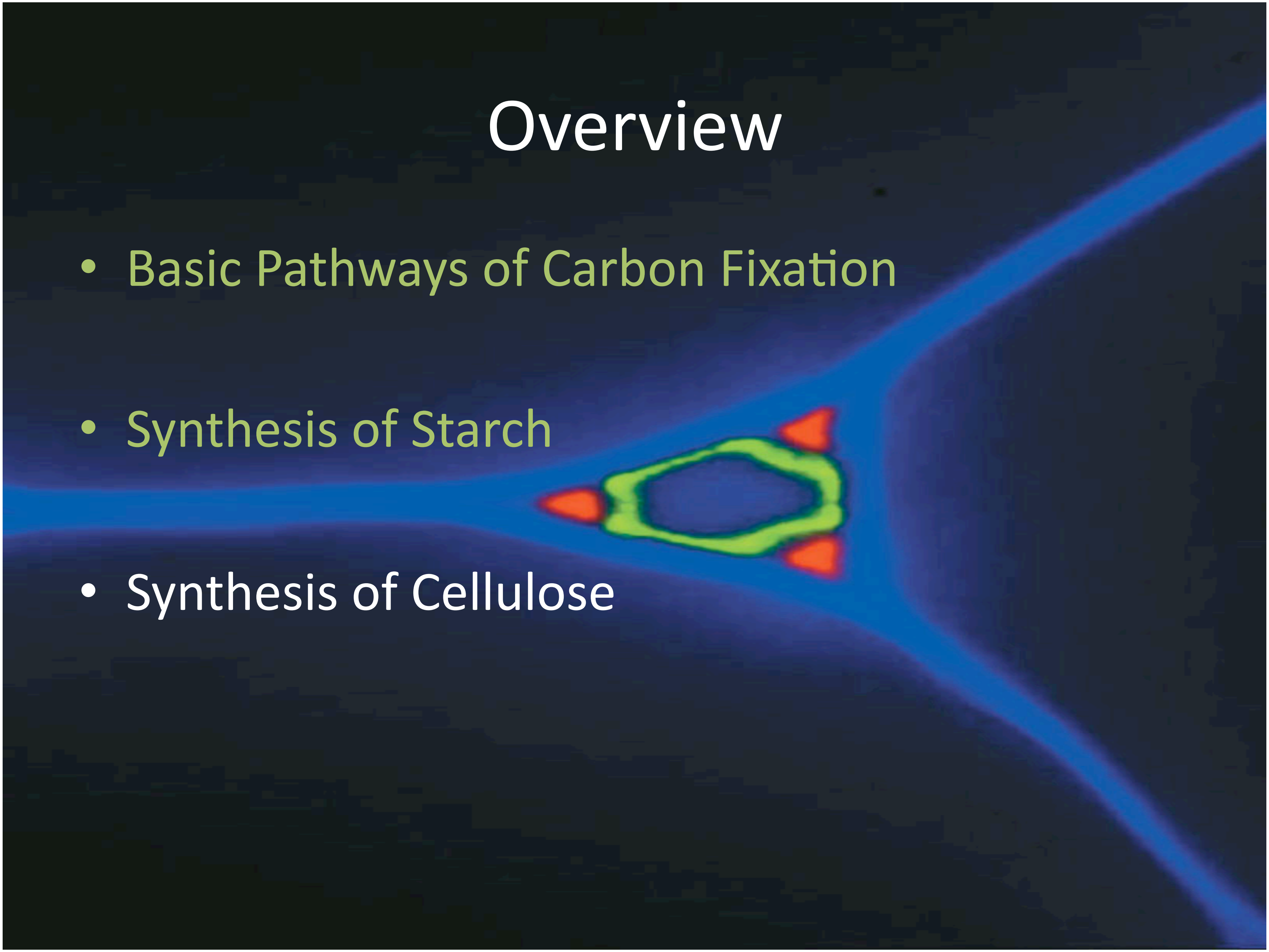
*Sbe1a/s; sbe2a-Mu/s*
(06-1094)

# Effect of sbe2b Null Mutant on Kernel Development



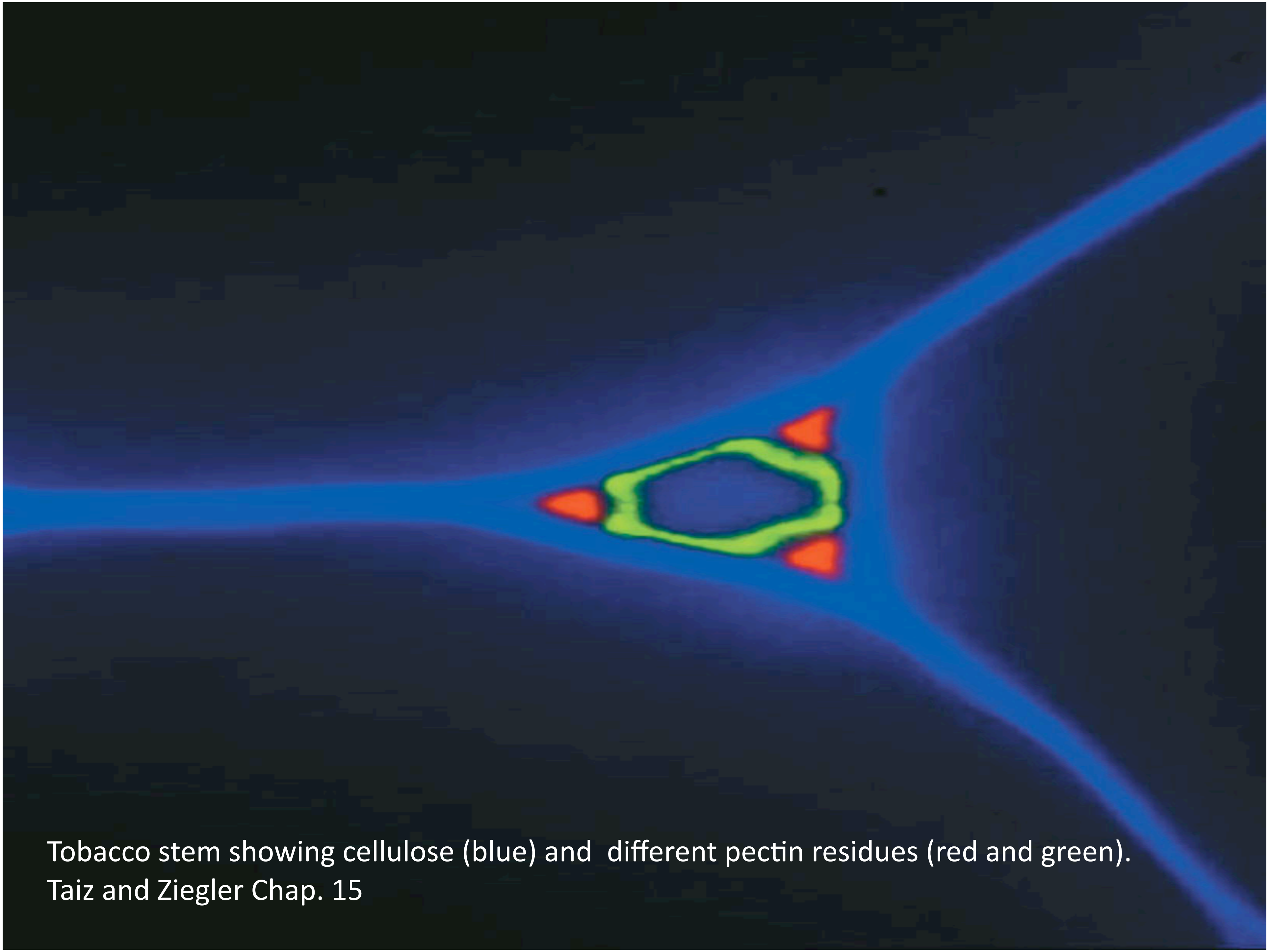
# Starch Summary

- Important in most stages of development
- Used as energy storage, transient and long-term
- Complex structure determines function
- Complex pathway with several gene familes
- Individual genes show tissue specific expression
- Individual isoforms exhibit subtle biochemical differences
- Enzyme regulation/protein:protein interactions under investigation
- Complexity evolved to optimized starch structure/function for efficient energy metabolism



# Overview

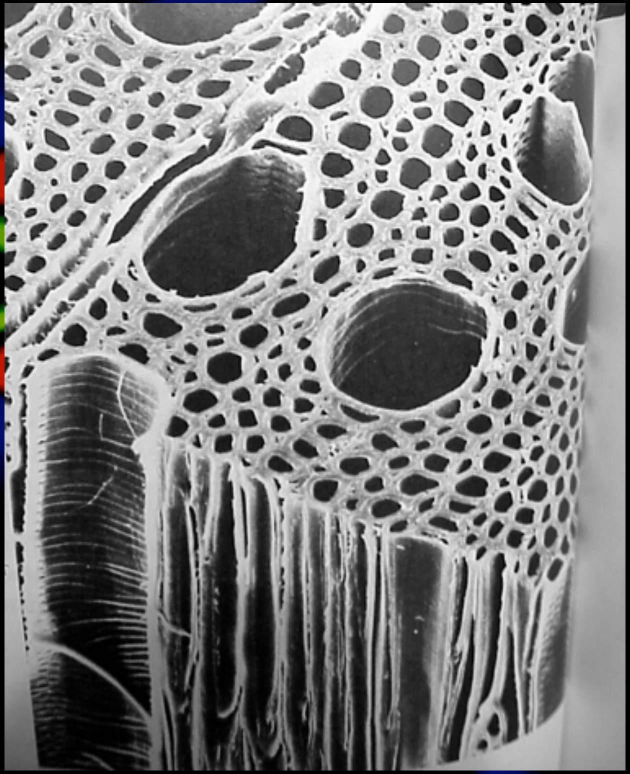
- Basic Pathways of Carbon Fixation
- Synthesis of Starch
- Synthesis of Cellulose



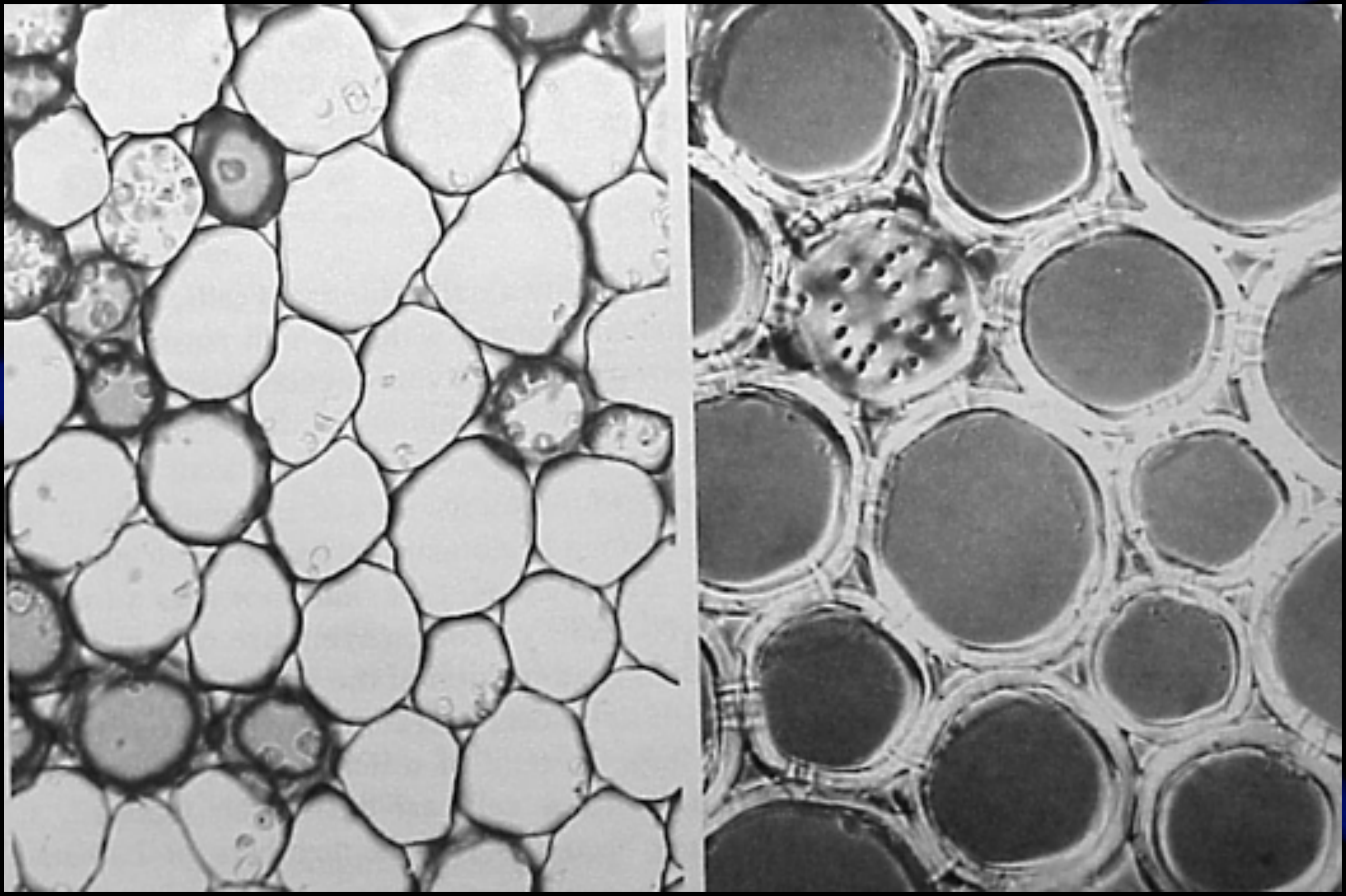
Tobacco stem showing cellulose (blue) and different pectin residues (red and green).
Taiz and Ziegler Chap. 15

# Biological Functions of the Cell Wall:

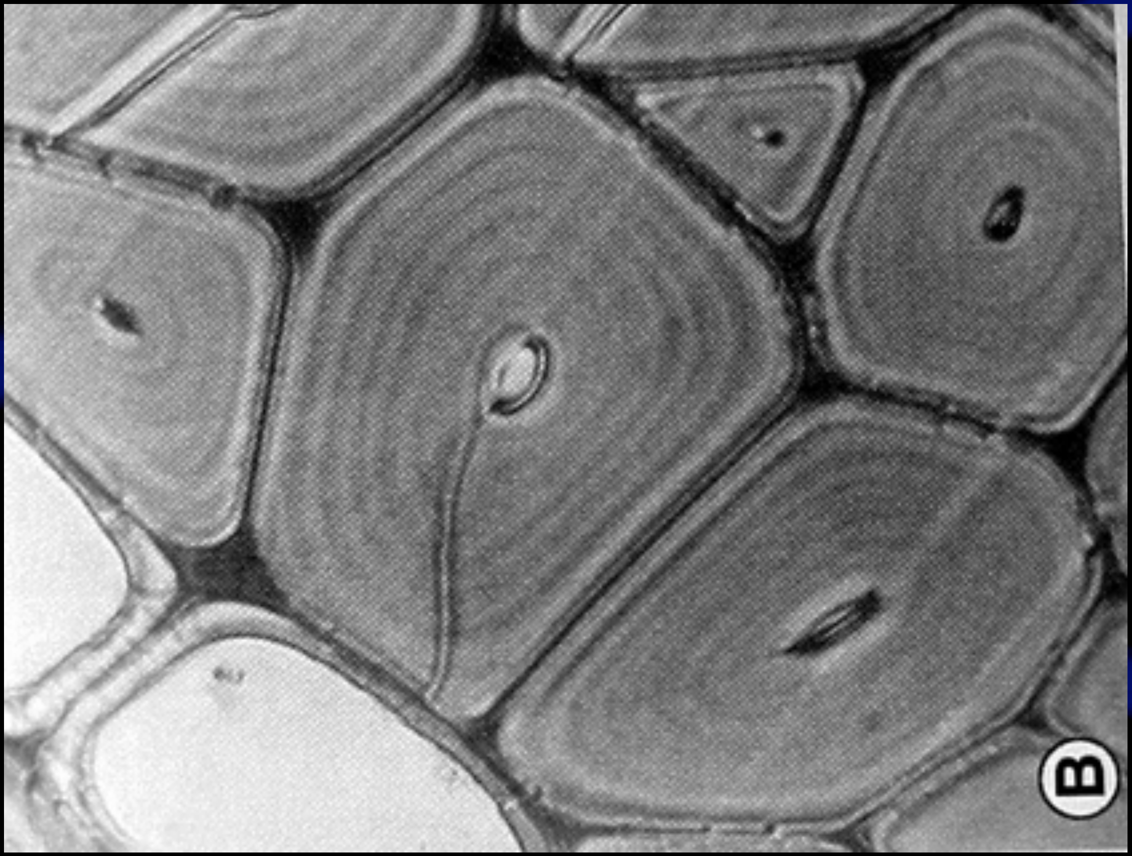
- Determines cell shape & size
- Increases structural strength
- Enables cells to have turgor
- Influences plant water relations
- Resists invasion by pathogens, insects



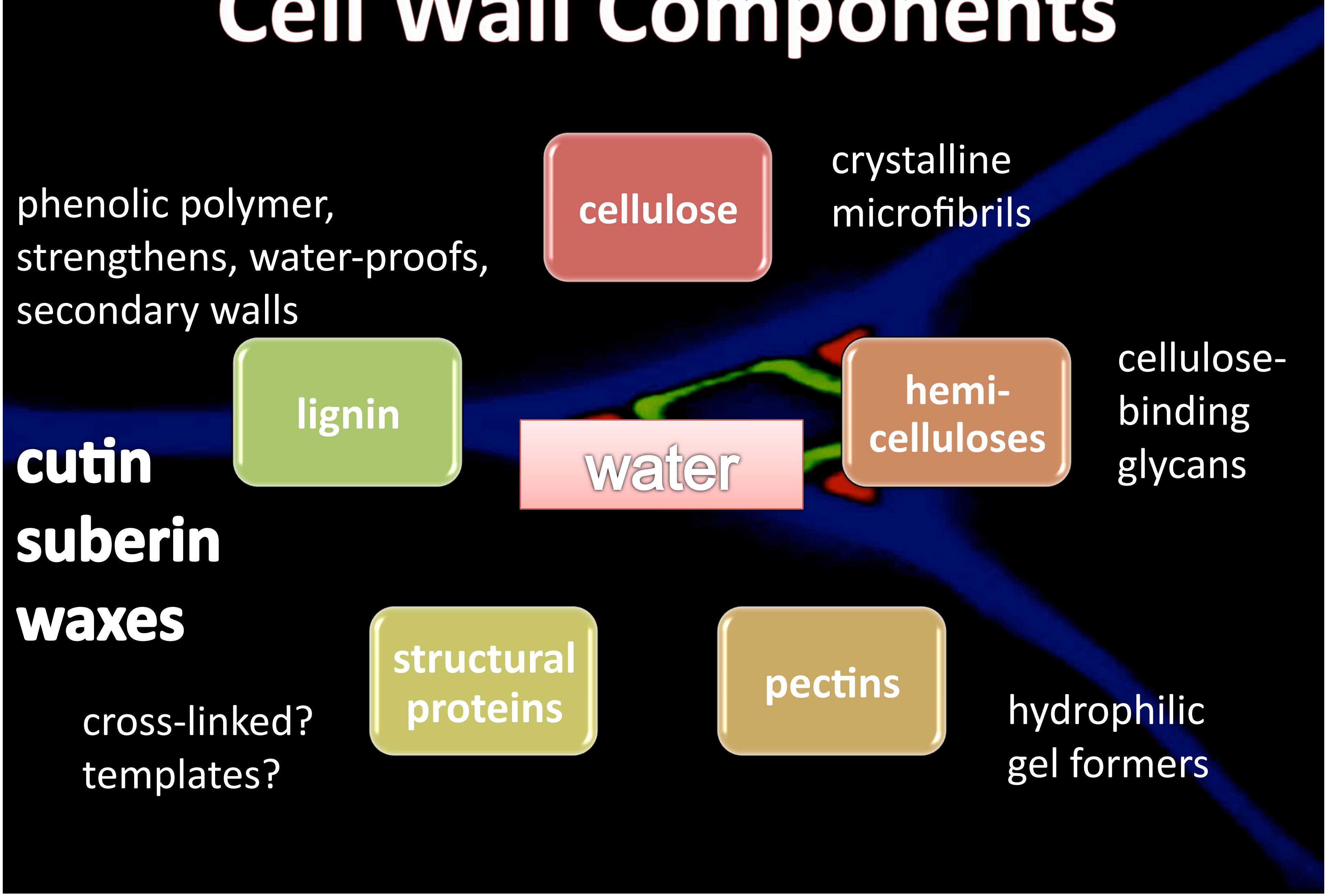
# Thin Walls / Thick Walls



# Thick-walled Fibers



# Cell Wall Components



**cellulose**

crystalline
microfibrils

phenolic polymer,
strengthens, water-proofs,
secondary walls

**lignin**

**water**

**hemi-celluloses**

cellulose-binding
glycans

**cutin**
**suberin**
**waxes**

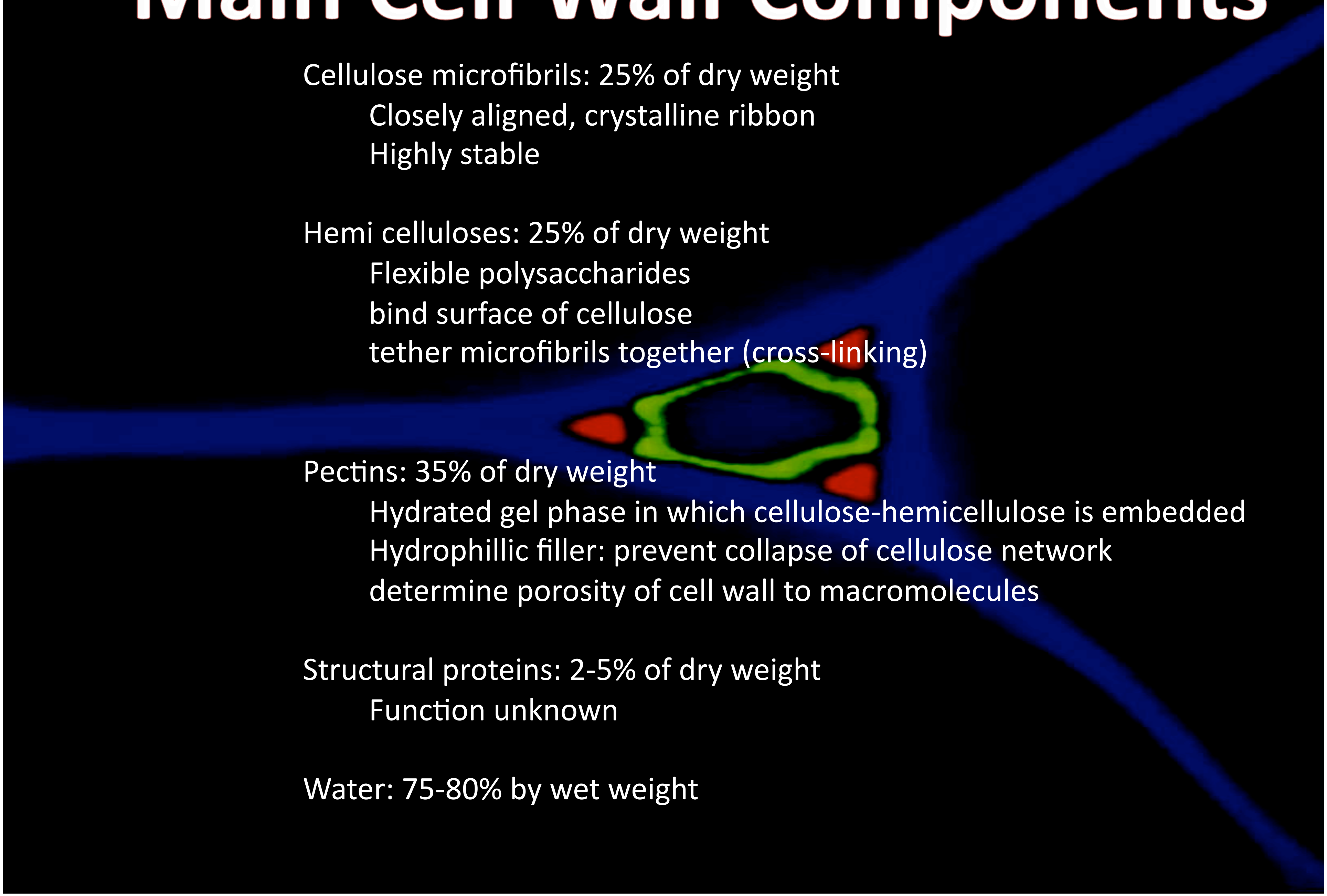
**structural proteins**

cross-linked?
templates?

**pectins**

hydrophilic
gel formers

# Main Cell Wall Components



- Cellulose microfibrils: 25% of dry weight
  - Closely aligned, crystalline ribbon
  - Highly stable

- Hemi celluloses: 25% of dry weight
  - Flexible polysaccharides
  - bind surface of cellulose
  - tether microfibrils together (cross-linking)

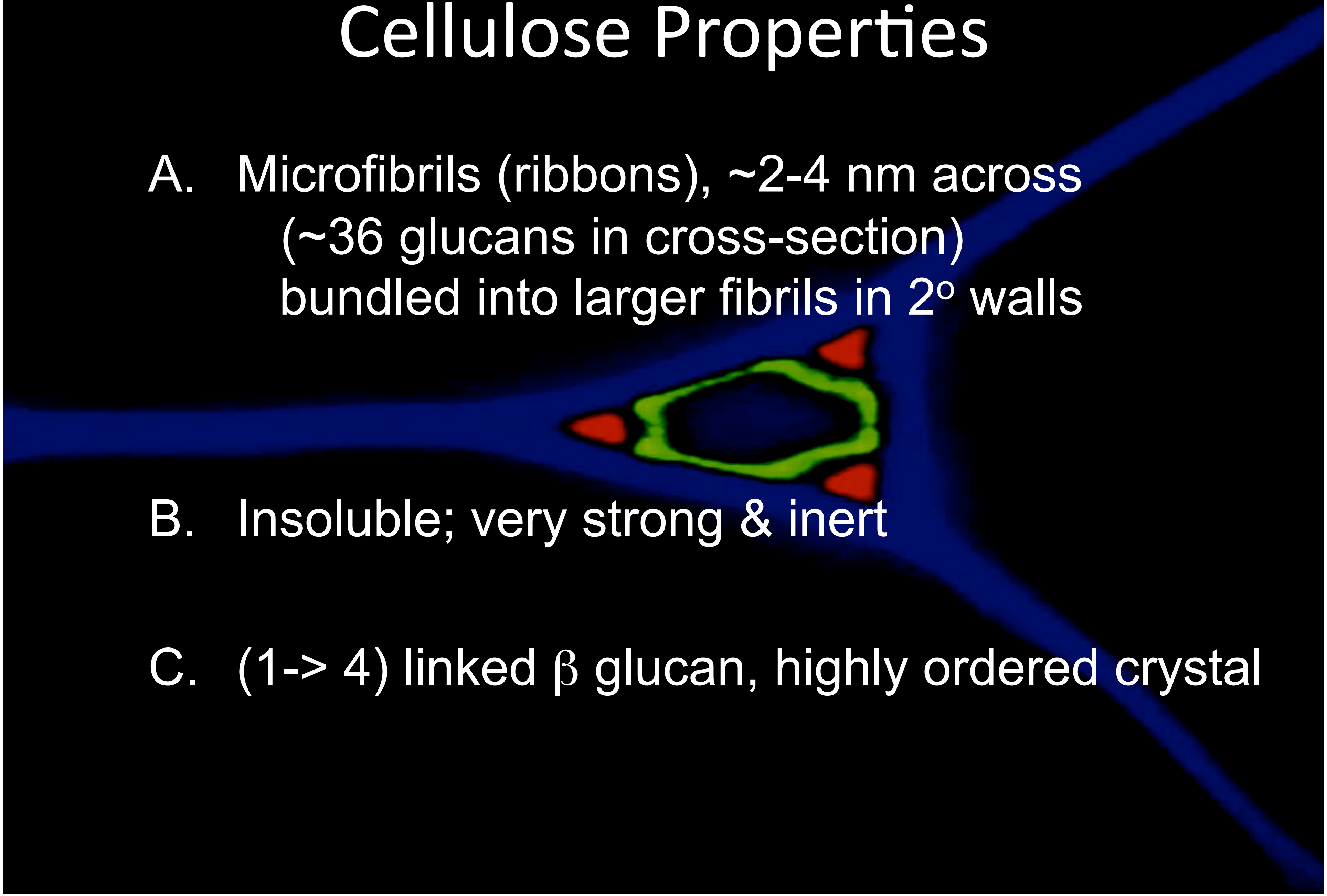
- Pectins: 35% of dry weight
  - Hydrated gel phase in which cellulose-hemicellulose is embedded
  - Hydrophillic filler: prevent collapse of cellulose network
  - determine porosity of cell wall to macromolecules

- Structural proteins: 2-5% of dry weight
  - Function unknown

- Water: 75-80% by wet weight

# Cellulose Properties

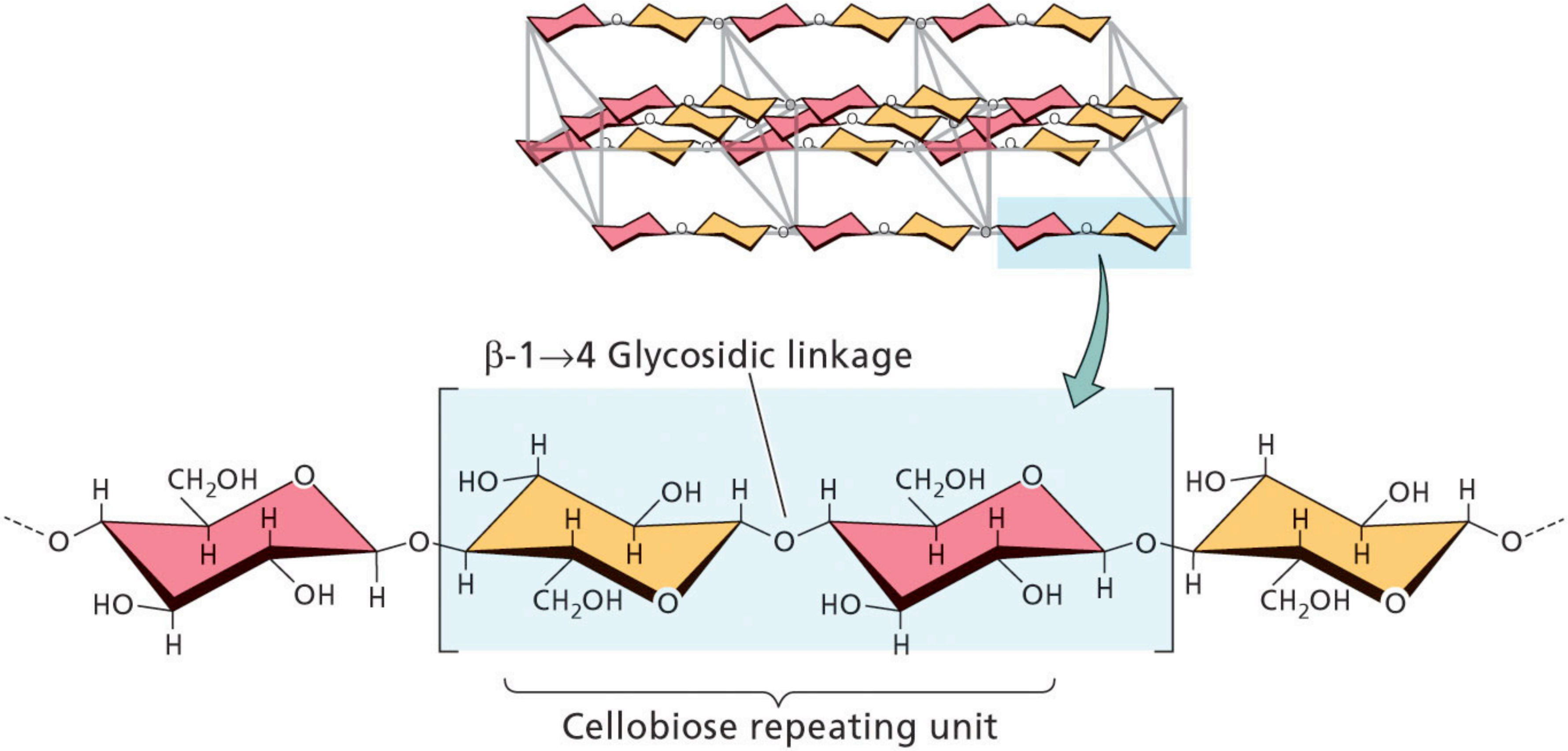
A. Microfibrils (ribbons), ~2-4 nm across
 (~36 glucans in cross-section)
 bundled into larger fibrils in $2^o$ walls



B. Insoluble; very strong & inert

C. (1-> 4) linked $\beta$ glucan, highly ordered crystal

# Cellulose Structure



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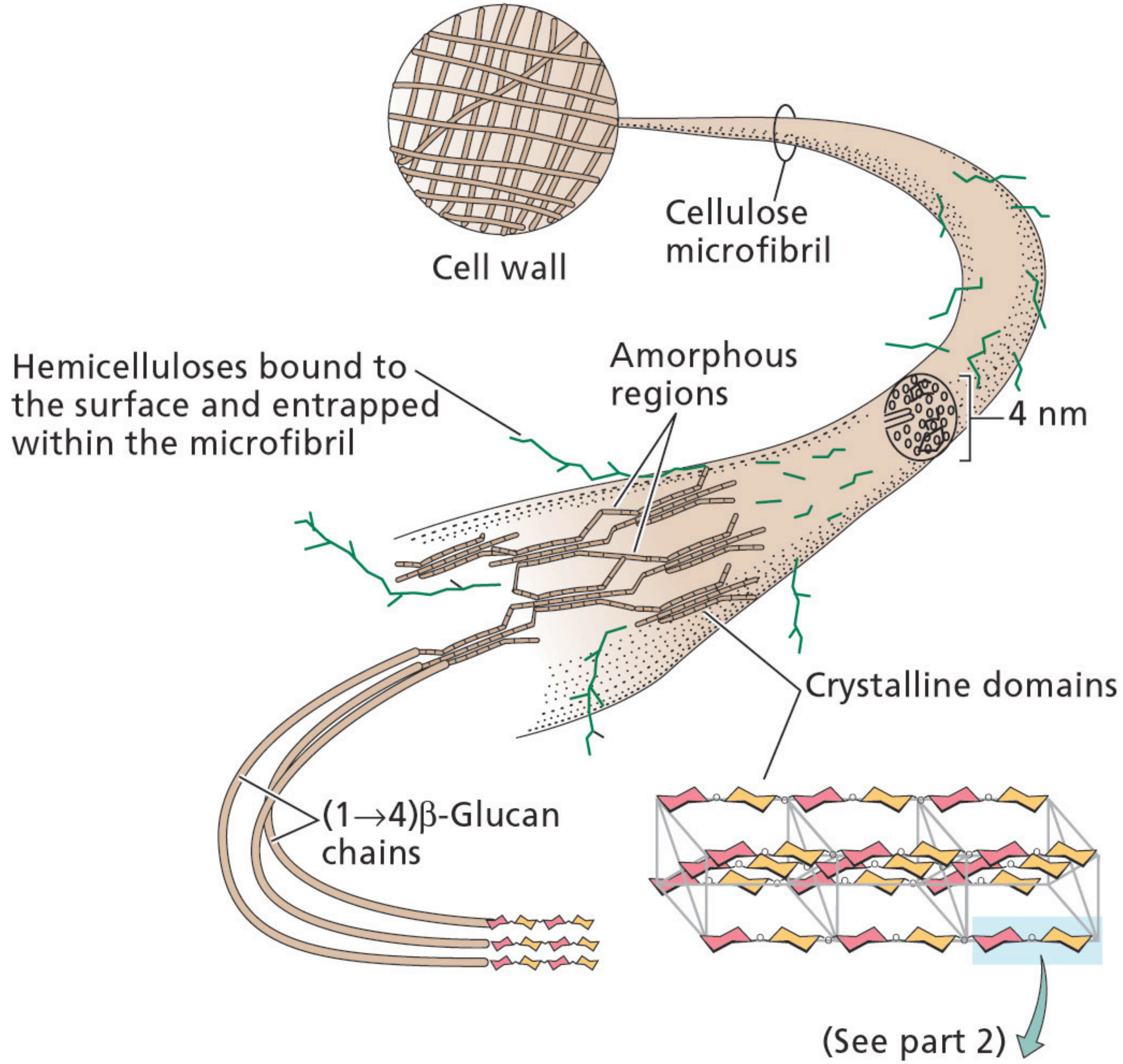

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# Model of Cellulose Synthesis

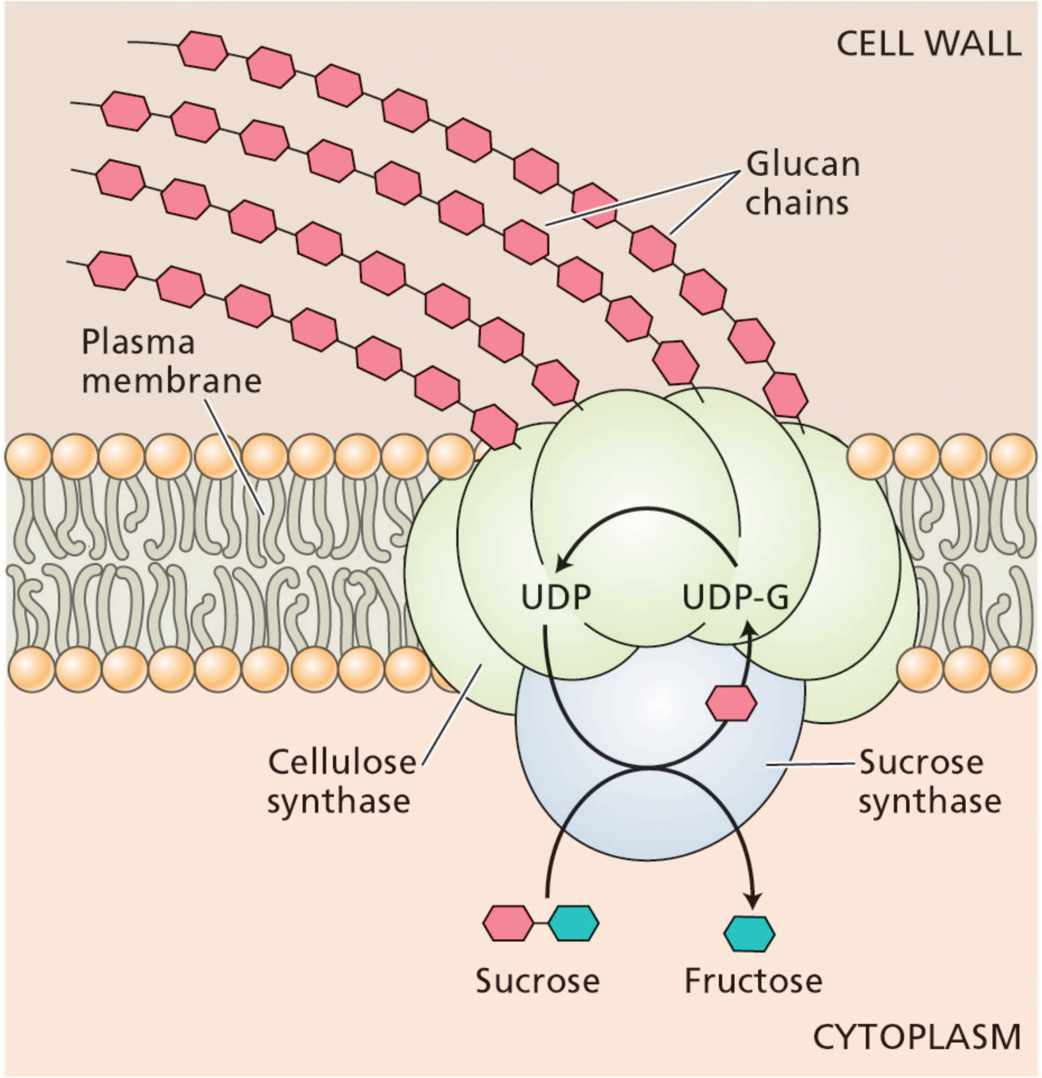
Sucrose synthase cleaves sucrose to form Uridine diphosphate-glucose

UDP-G is utilized by cellulose synthase to form 1-4 beta-D glycosidic bonds

Rosette of 6 CES proteins form CES particle

CES proteins encoded by 3 different genes

6 CES particles form rosette, which makes 36 glycan chains making up one cellulose microfibril



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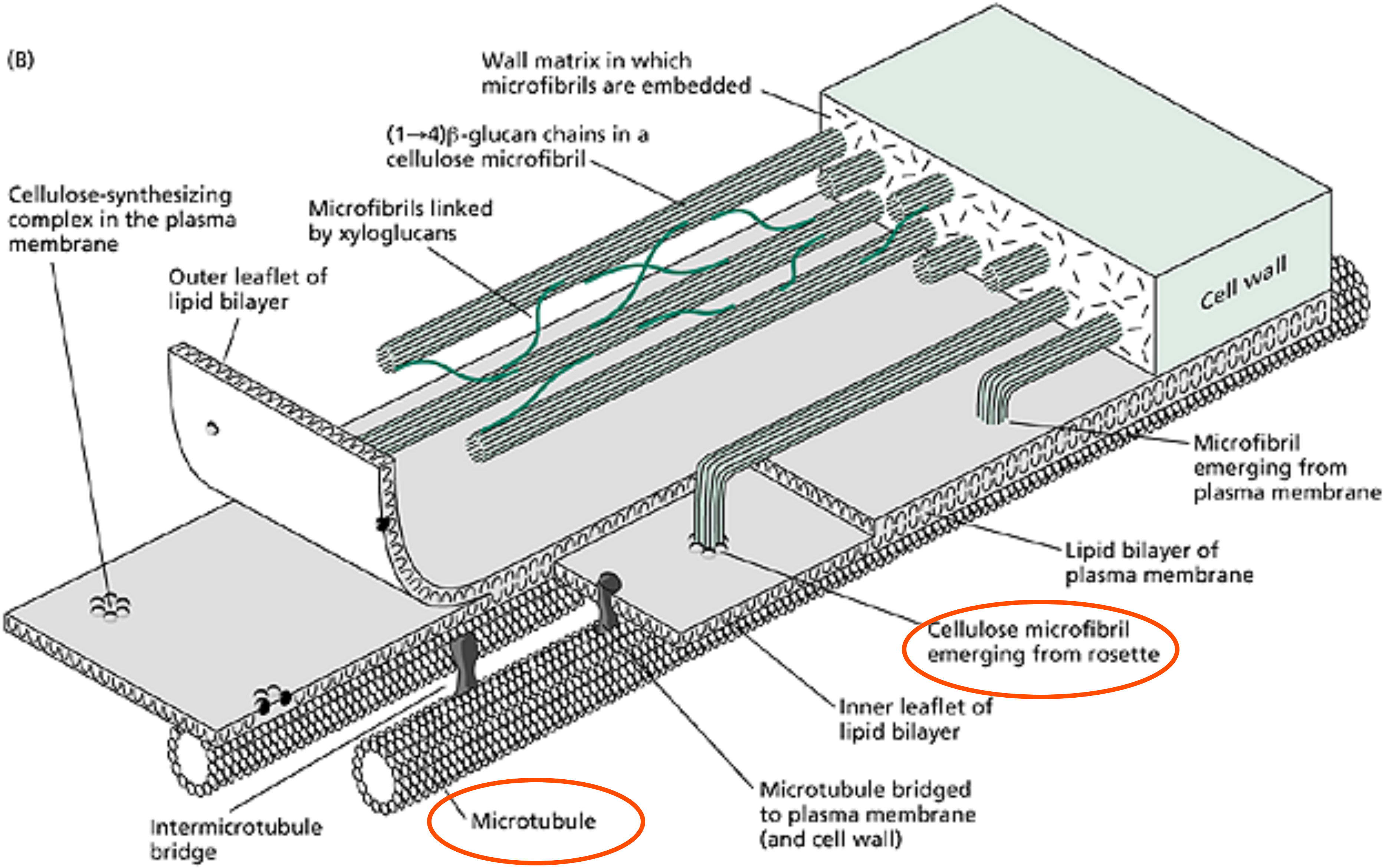
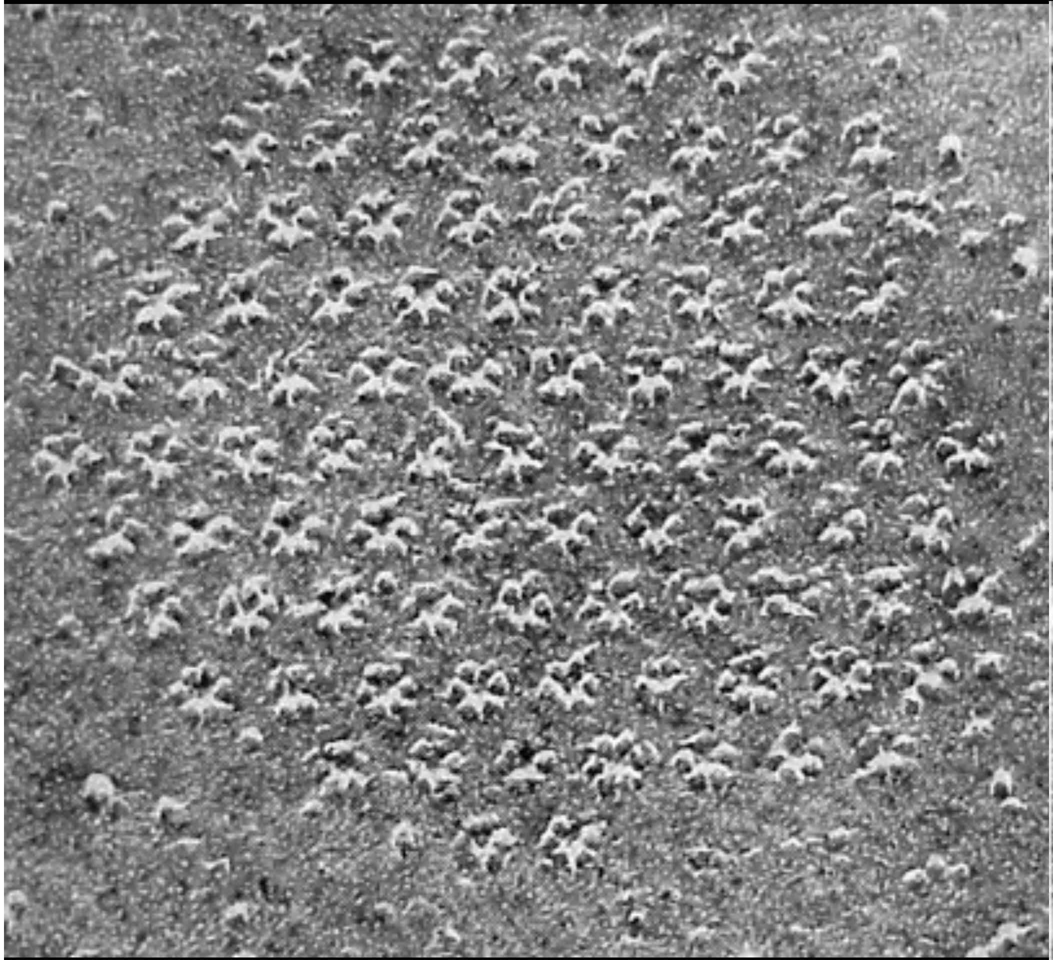
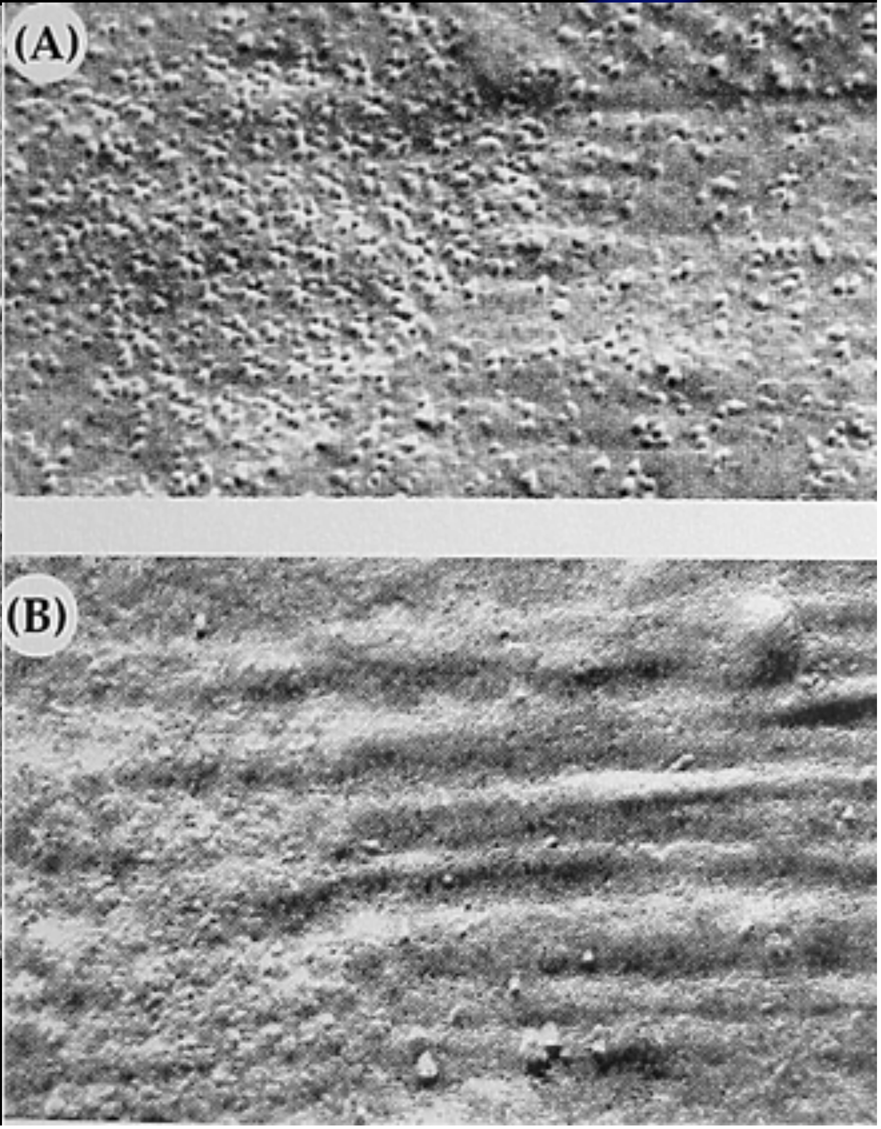
# Cellulose Direction Controlled by Microtubules



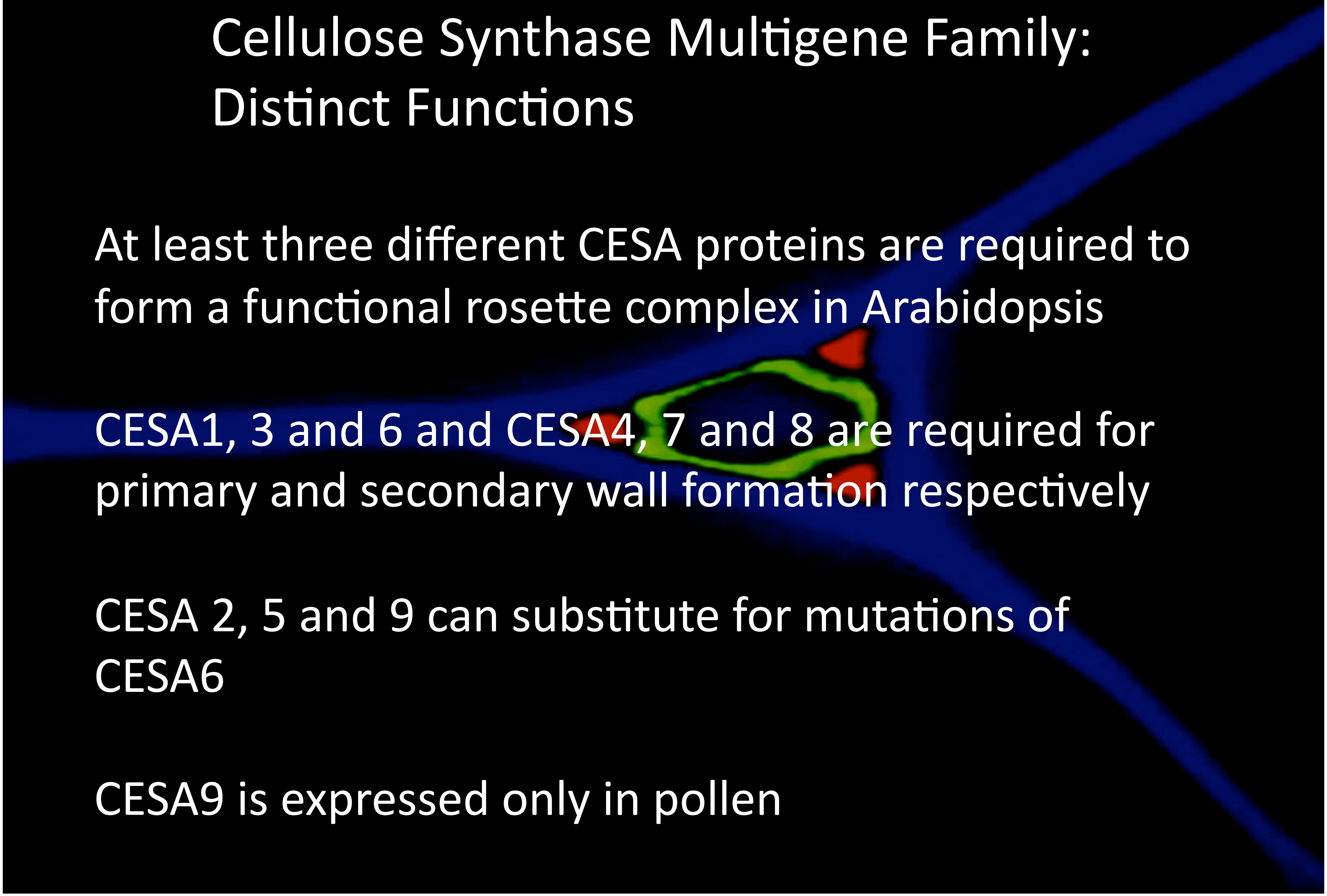
# EM Images of Cellulose Synthase Complex in Plasma Membrane



From Giddings et al. 1980



# Cellulose Synthase Multigene Family: Distinct Functions



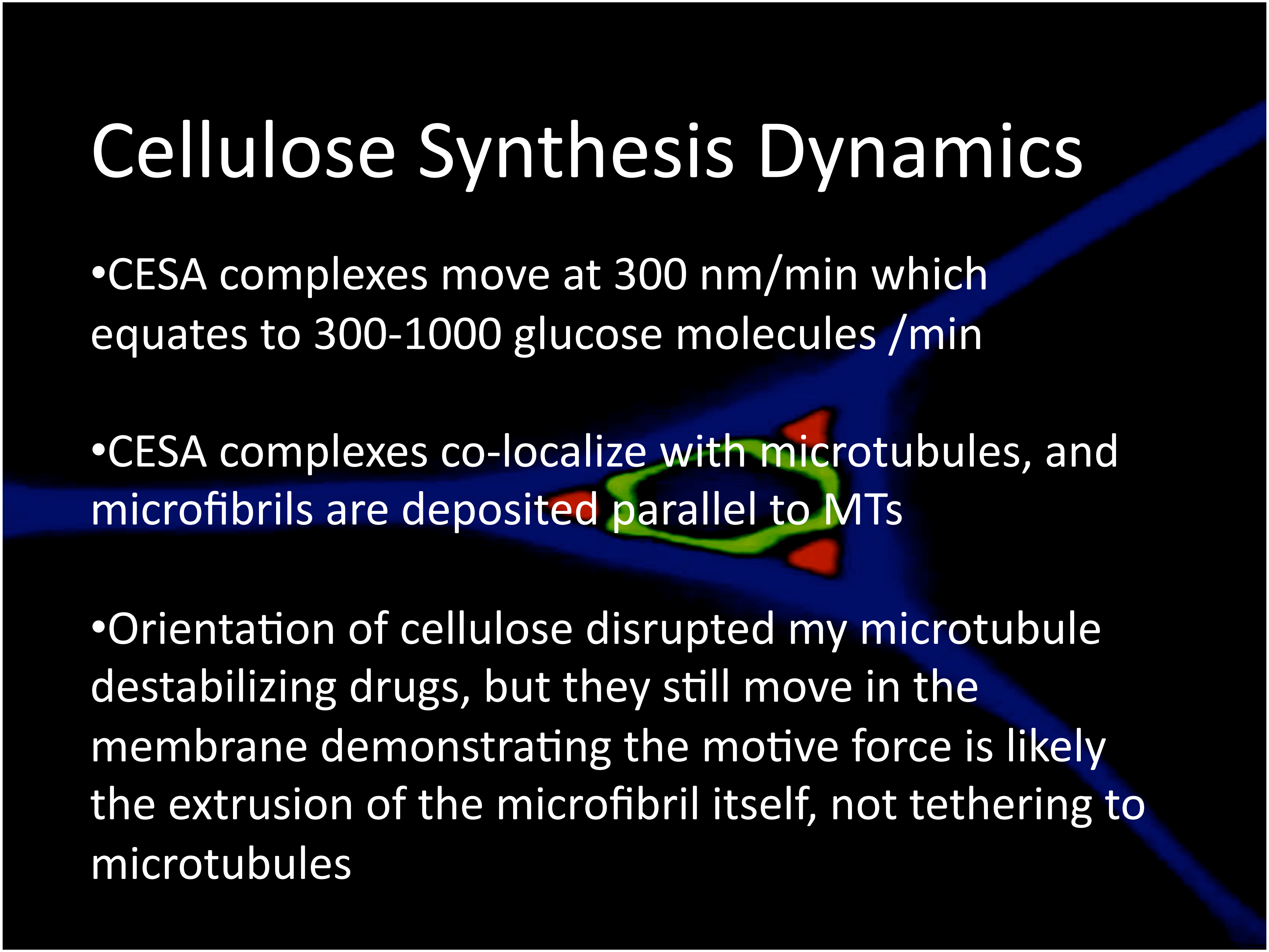
At least three different CESA proteins are required to form a functional rosette complex in Arabidopsis

CESA1, 3 and 6 and CESA4, 7 and 8 are required for primary and secondary wall formation respectively

CESA 2, 5 and 9 can substitute for mutations of CESA6

CESA9 is expressed only in pollen

# Cellulose Synthesis Dynamics



- CESA complexes move at 300 nm/min which equates to 300-1000 glucose molecules /min

- CESA complexes co-localize with microtubules, and microfibrils are deposited parallel to MTs

- Orientation of cellulose disrupted my microtubule destabilizing drugs, but they still move in the membrane demonstrating the motive force is likely the extrusion of the microfibril itself, not tethering to microtubules

# Matrix Polymers (Hemicellulose and Pectins) are Synthesized in The Golgi And Secreted Via Vesicles

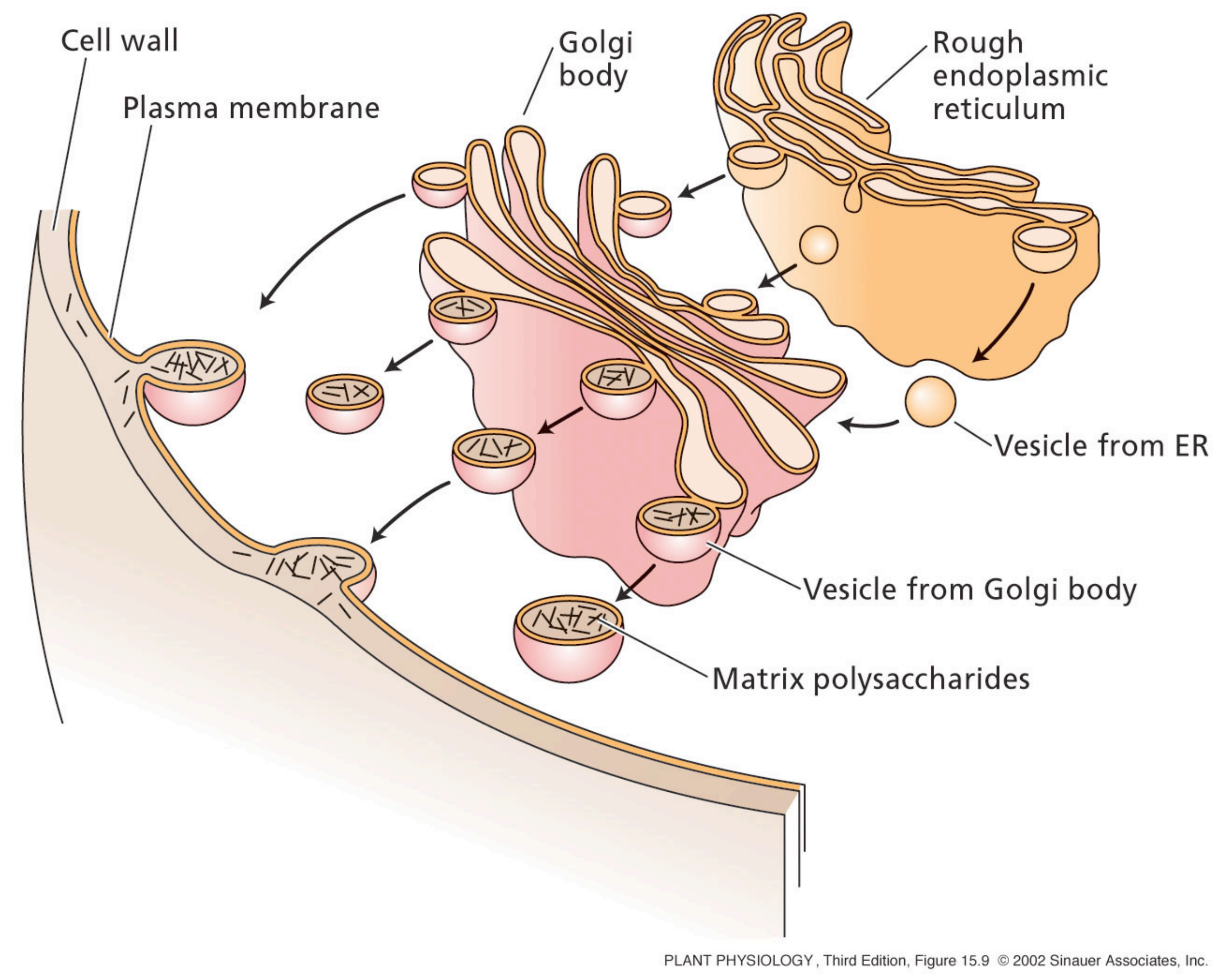
Glycosyltransferases
In golgi

Amorphous structure due to extensive branching and non-linearity

Bind cellulose tightly

Pectins contain sugars such as galacturonic acid, rhamnose etc

Very complex structures very with species and cell type



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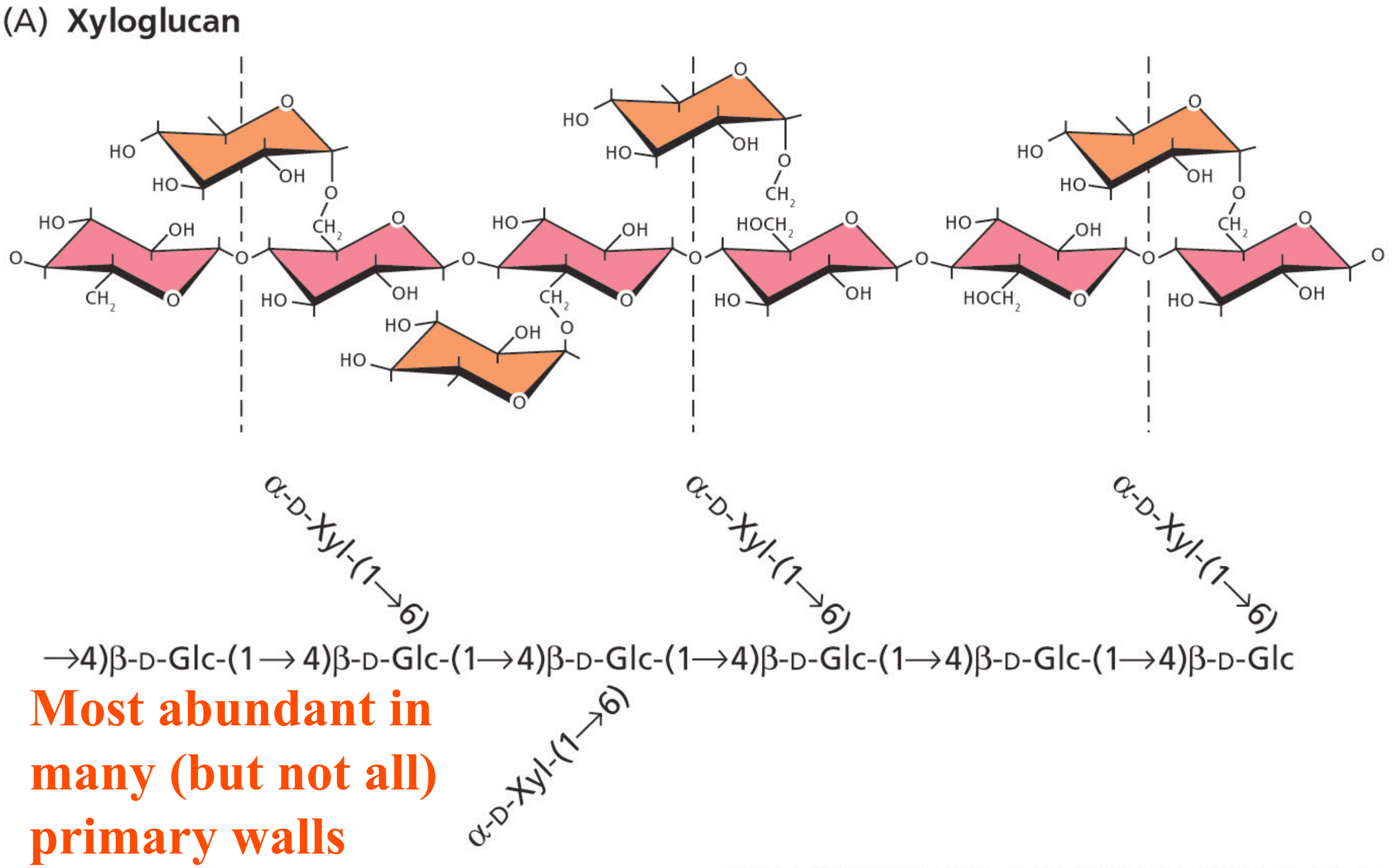
# Hemicellulose Structure



$$\begin{array}{c}\alpha\text{-D-Xyl-}(1\rightarrow 6)\ \ \ \alpha\text{-D-Xyl-}(1\rightarrow 6)\ \ \ \alpha\text{-D-Xyl-}(1\rightarrow 6)\\ \rightarrow 4)\beta\text{-D-Glc-}(1\rightarrow 4)\beta\text{-D-Glc-}(1\rightarrow 4)\beta\text{-D-Glc-}(1\rightarrow 4)\beta\text{-D-Glc-}(1\rightarrow 4)\beta\text{-D-Glc-}(1\rightarrow 4)\beta\text{-D-Glc}\\ \alpha\text{-D-Xyl-}(1\rightarrow 6)\end{array}$$

**Most abundant in many (but not all) primary walls**

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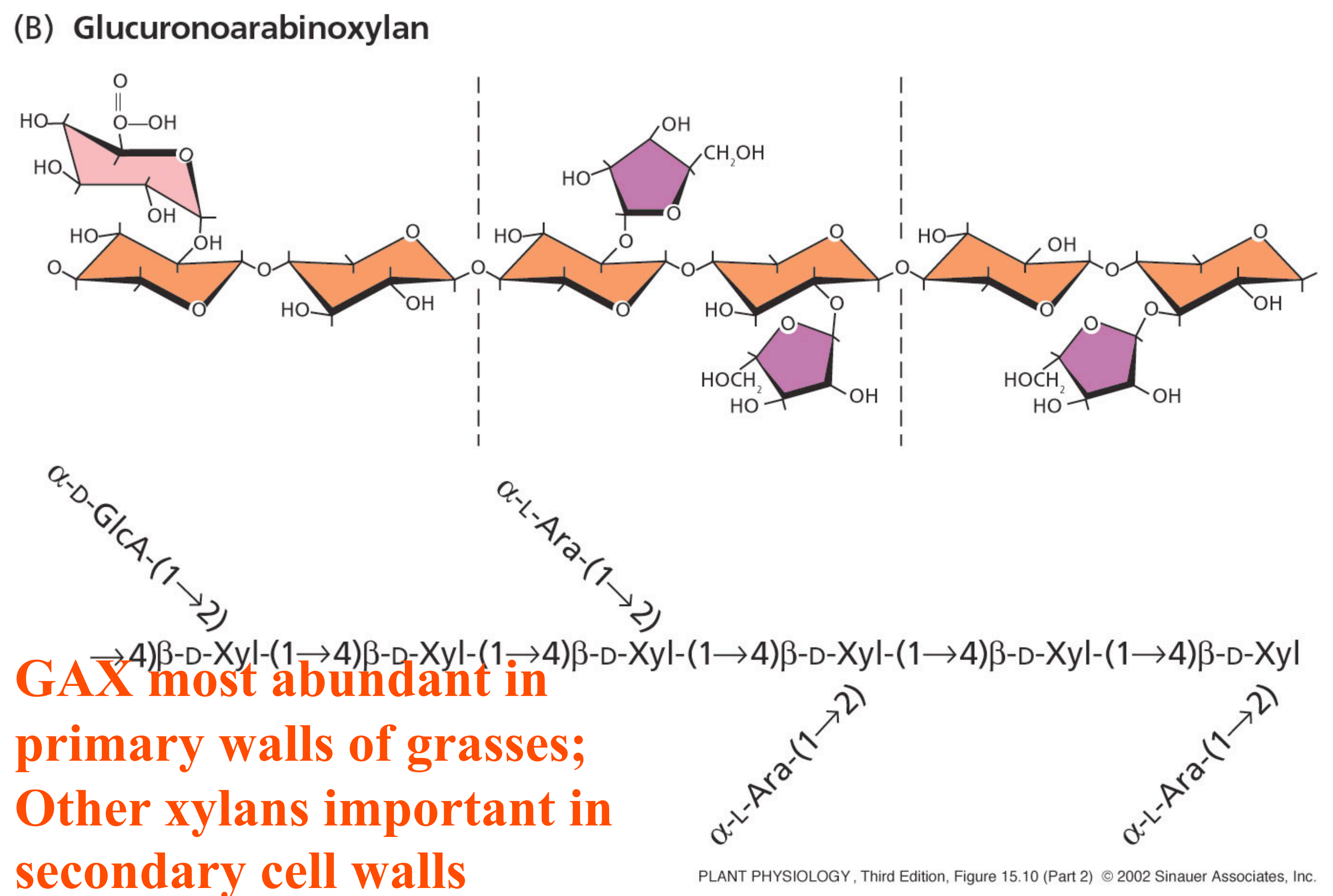

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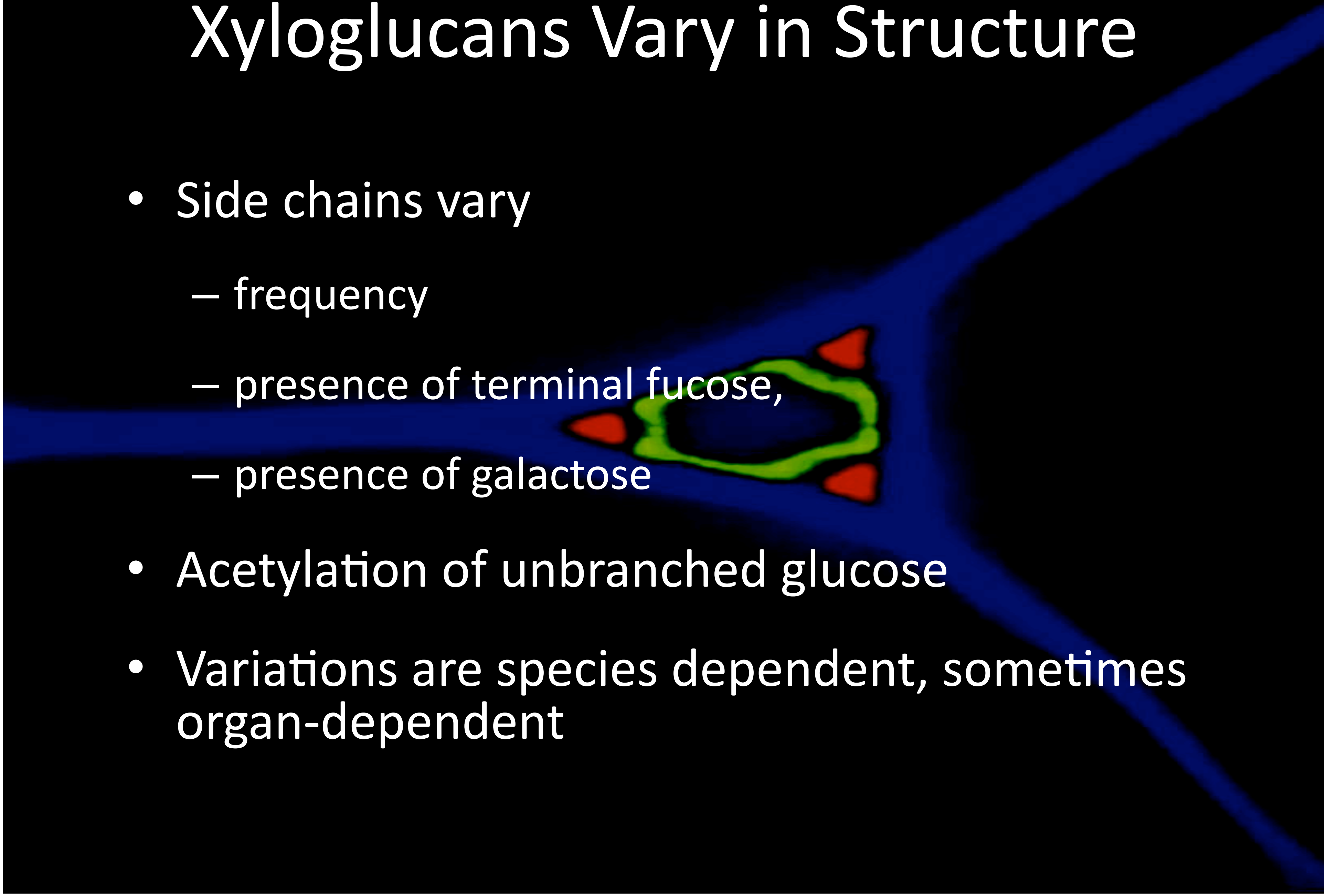
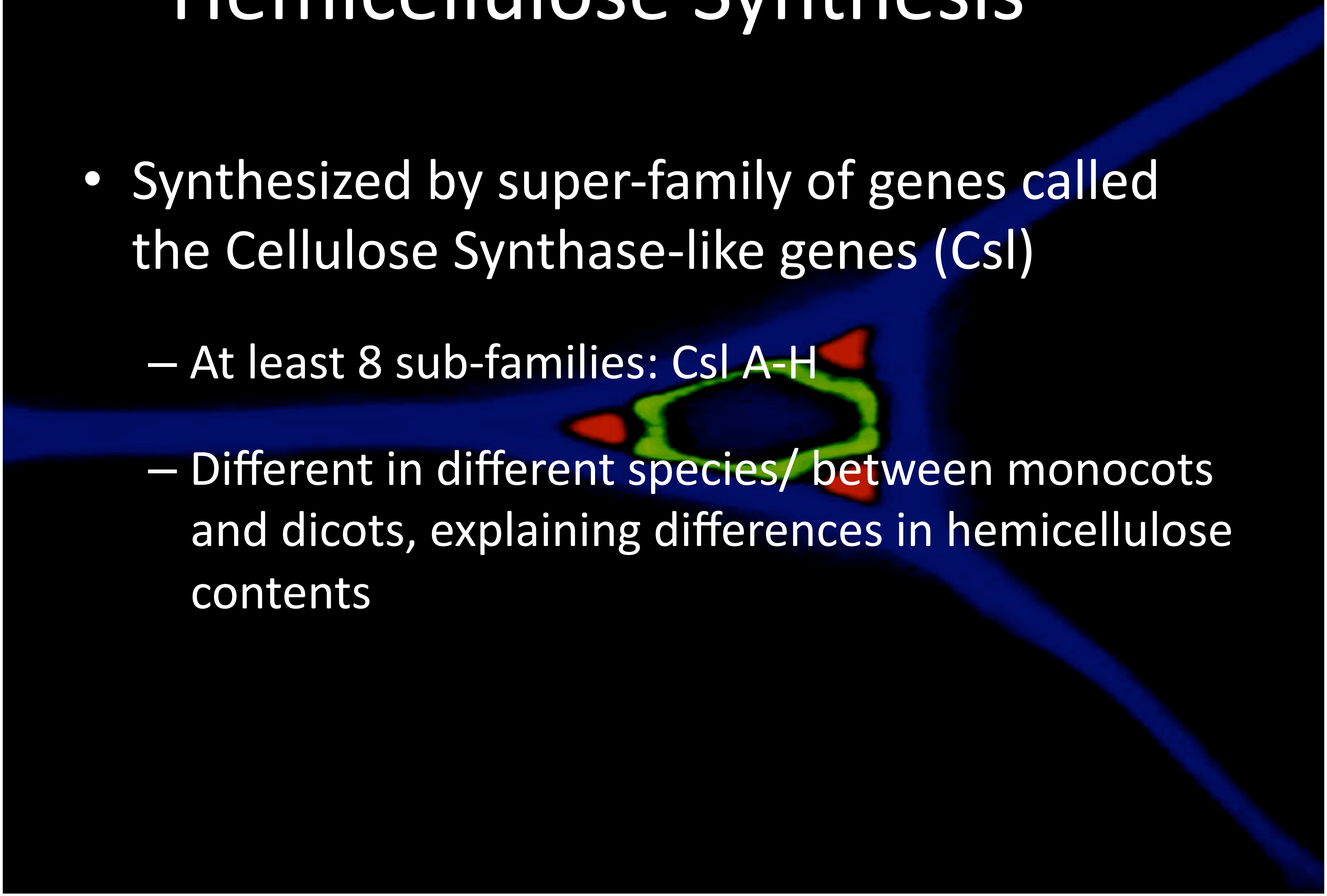
GAX most abundant in primary walls of grasses; Other xylans important in secondary cell walls

# Xyloglucans Vary in Structure



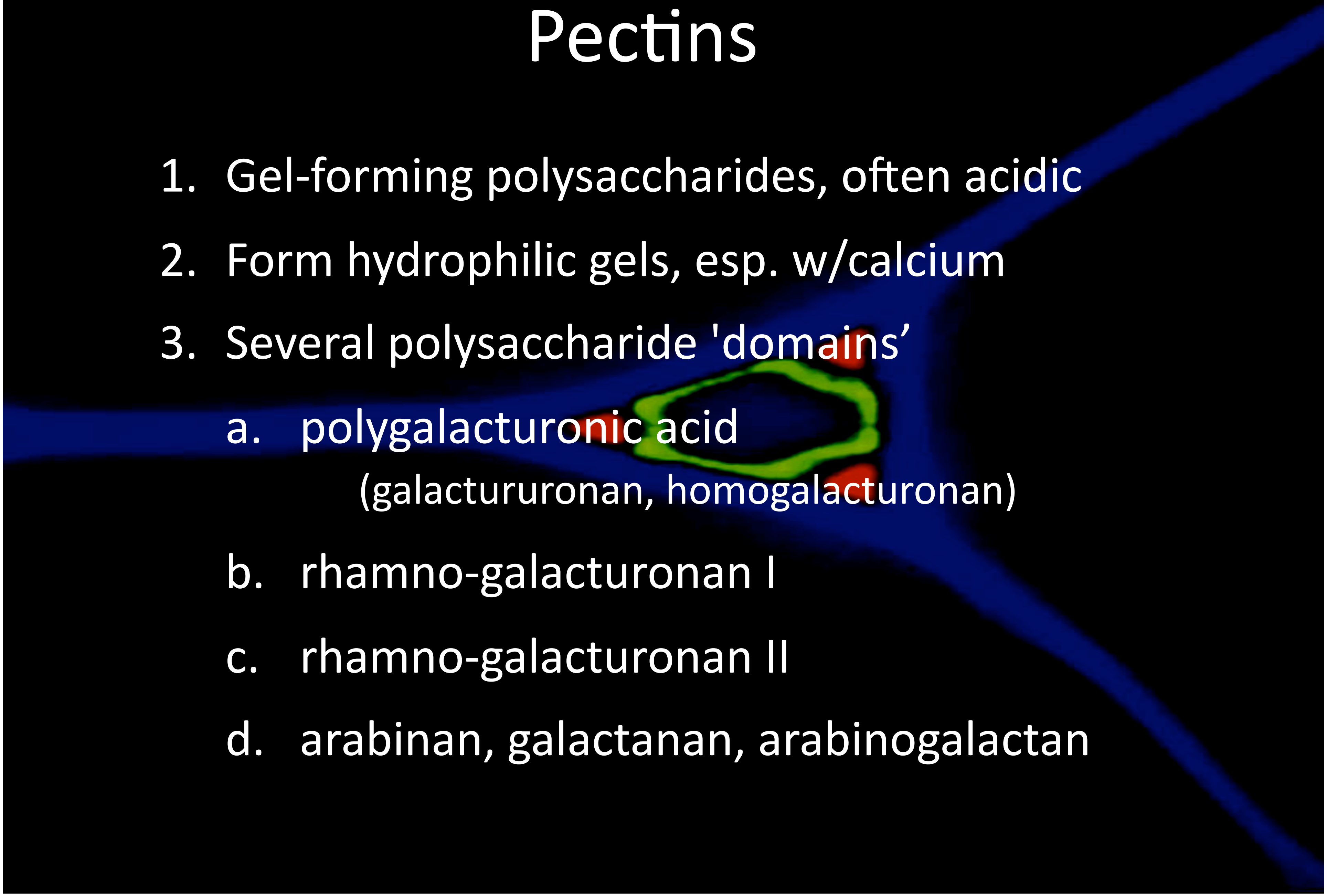
- Side chains vary
  - – frequency
  - – presence of terminal fucose,
  - – presence of galactose
- Acetylation of unbranched glucose
- Variations are species dependent, sometimes organ-dependent

# Hemicellulose Synthesis



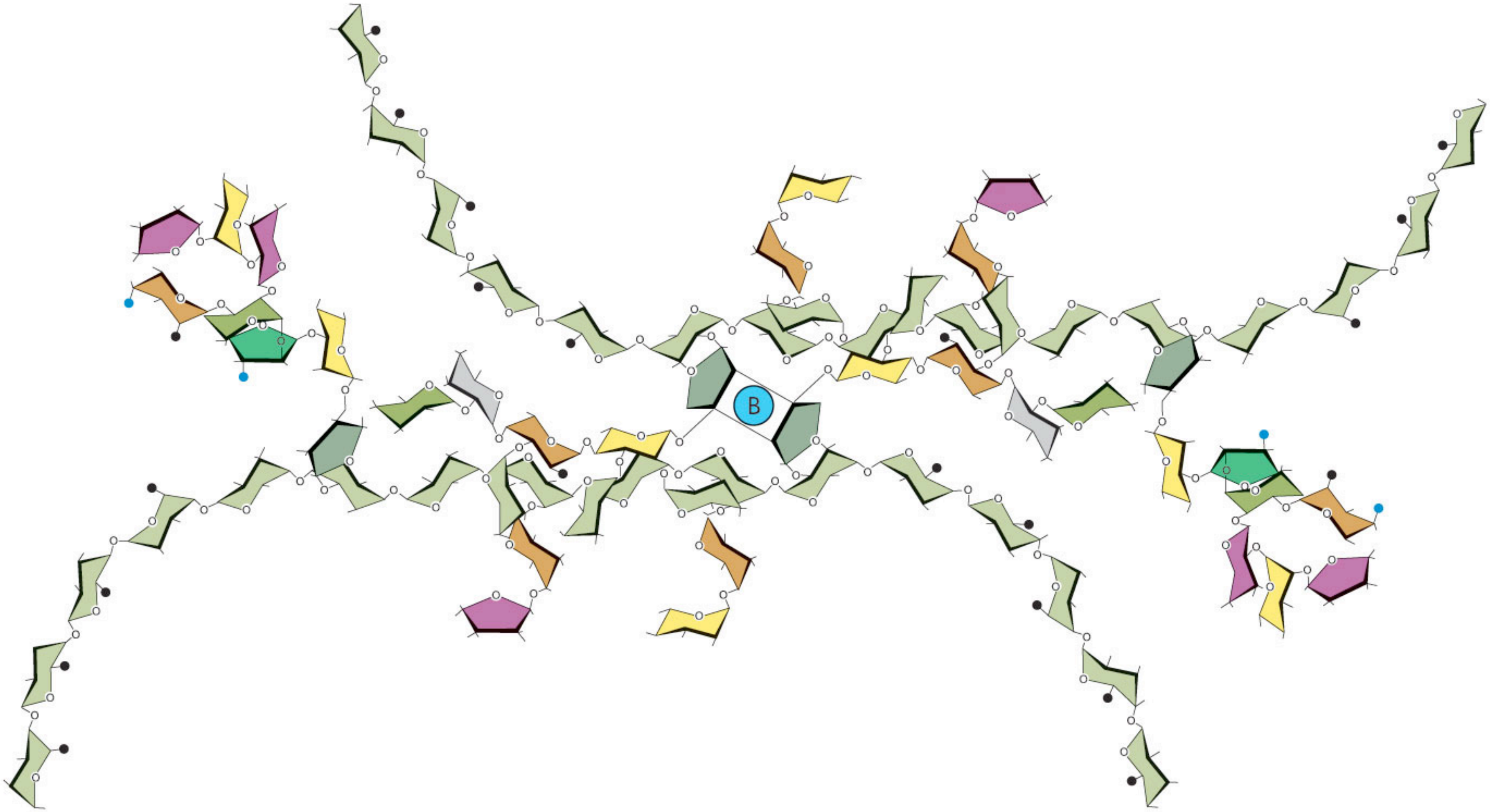
- Synthesized by super-family of genes called the Cellulose Synthase-like genes (Csl)
  - At least 8 sub-families: Csl A-H
  - Different in different species/ between monocots and dicots, explaining differences in hemicellulose contents

# Pectins



1. Gel-forming polysaccharides, often acidic
2. Form hydrophilic gels, esp. w/calcium
3. Several polysaccharide 'domains’
   a. polygalacturonic acid
      (galactururonan, homogalacturonan)
   b. rhamno-galacturonan I
   c. rhamno-galacturonan II
   d. arabinan, galactanan, arabinogalactan

# (C) Rhamnogalacturonan II (RG II) dimer cross-linked by borate diester bonds



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# Differences in Cell Walls Between Monocots and Dicots

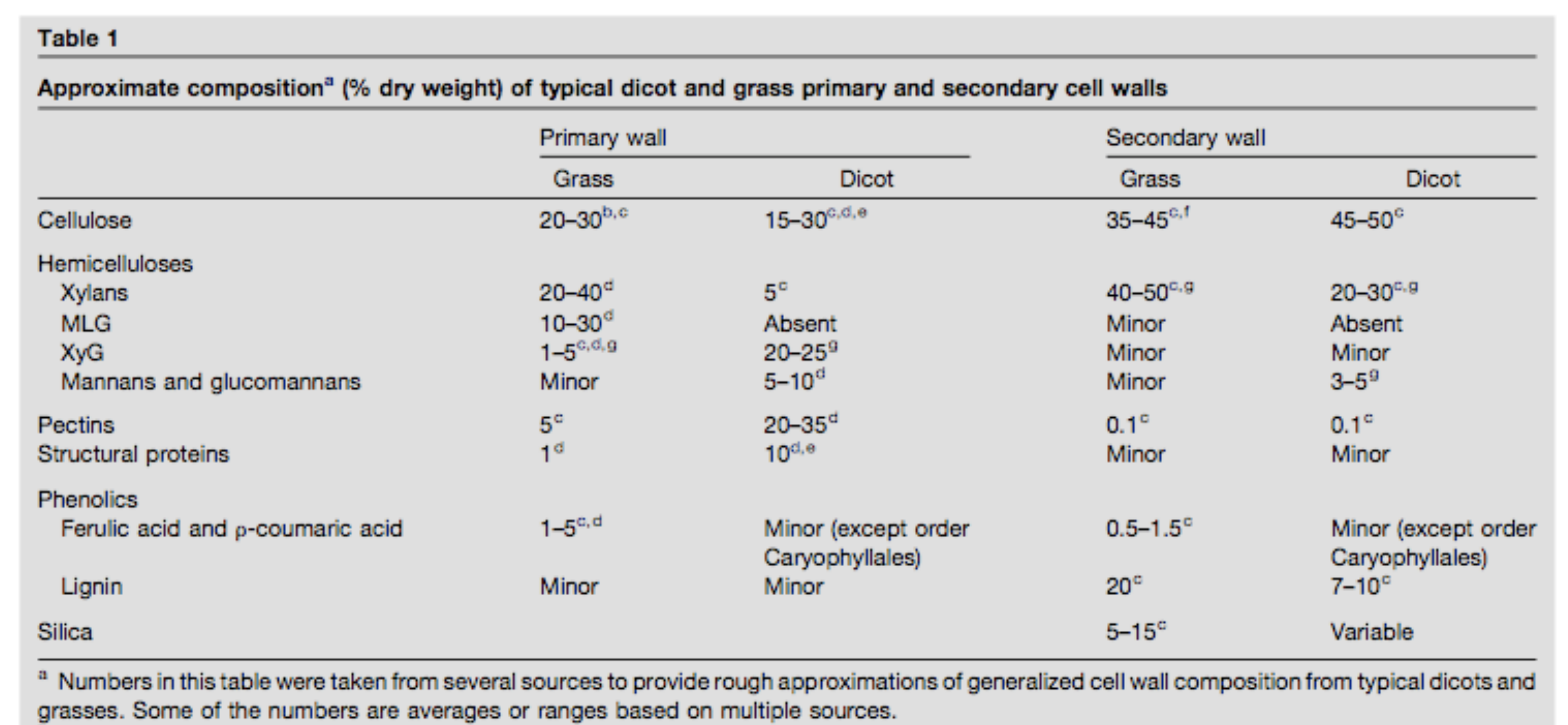


**Table 1**

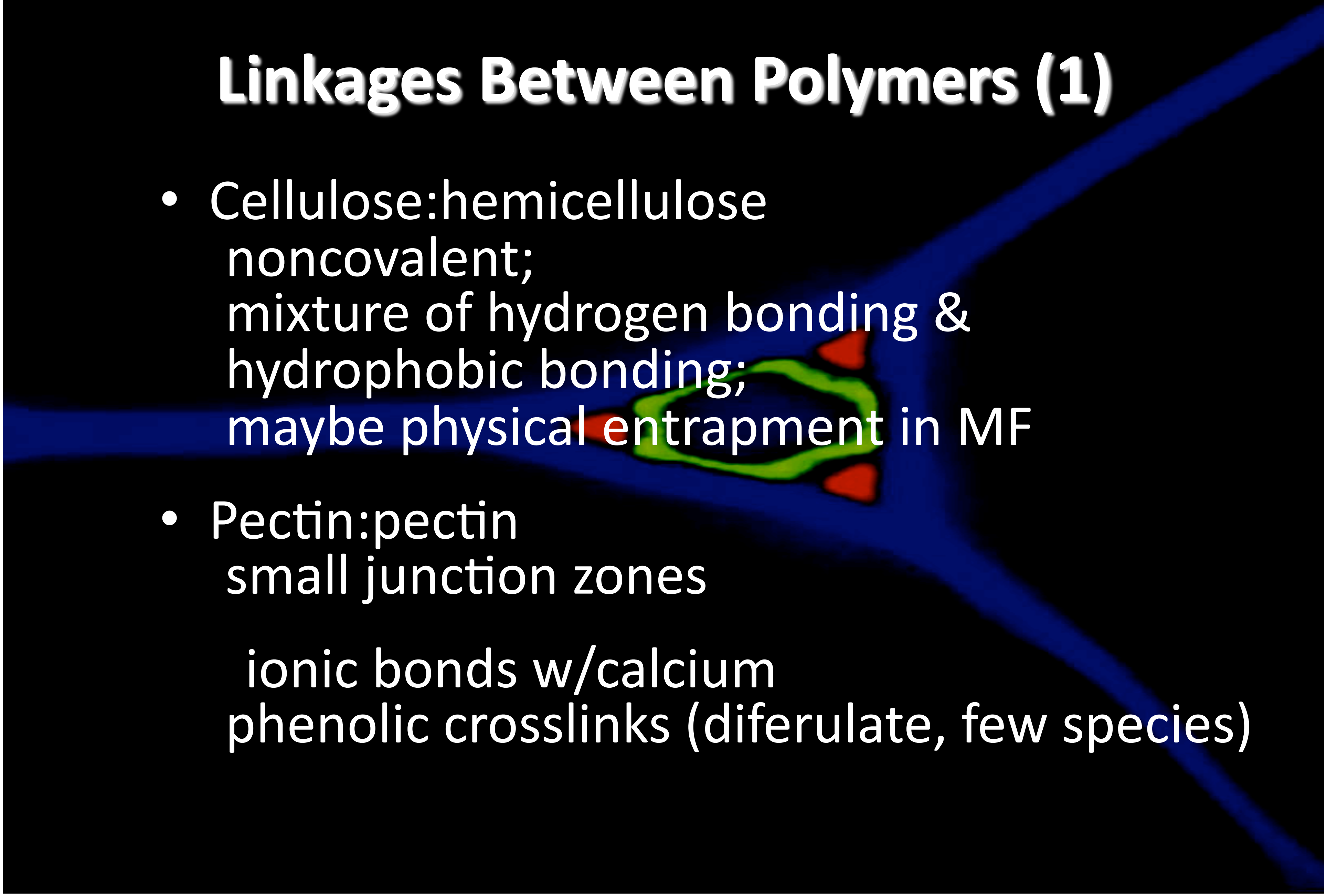
**Approximate composition[a] (% dry weight) of typical dicot and grass primary and secondary cell walls**

| | Primary wall | | Secondary wall | |
|---|---|---|---|---|
| | Grass | Dicot | Grass | Dicot |
| Cellulose | 20–30[b,c] | 15–30[c,d,e] | 35–45[c,f] | 45–50[c] |
| Hemicelluloses | | | | |
| Xylans | 20–40[d] | 5[c] | 40–50[c,g] | 20–30[c,g] |
| MLG | 10–30[d] | Absent | Minor | Absent |
| XyG | 1–5[c,d,g] | 20–25[g] | Minor | Minor |
| Mannans and glucomannans | Minor | 5–10[d] | Minor | 3–5[g] |
| Pectins | 5[c] | 20–35[d] | 0.1[c] | 0.1[c] |
| Structural proteins | 1[d] | 10[d,e] | Minor | Minor |
| Phenolics | | | | |
| Ferulic acid and *p*-coumaric acid | 1–5[c,d] | Minor (except order Caryophyllales) | 0.5–1.5[c] | Minor (except order Caryophyllales) |
| Lignin | Minor | Minor | 20[c] | 7–10[c] |
| Silica | | | 5–15[c] | Variable |

[a] Numbers in this table were taken from several sources to provide rough approximations of generalized cell wall composition from typical dicots and grasses. Some of the numbers are averages or ranges based on multiple sources.

Vogel, Current Trends in Plant Biology, **Volume 11, Issue 3**

# Linkages Between Polymers (1)

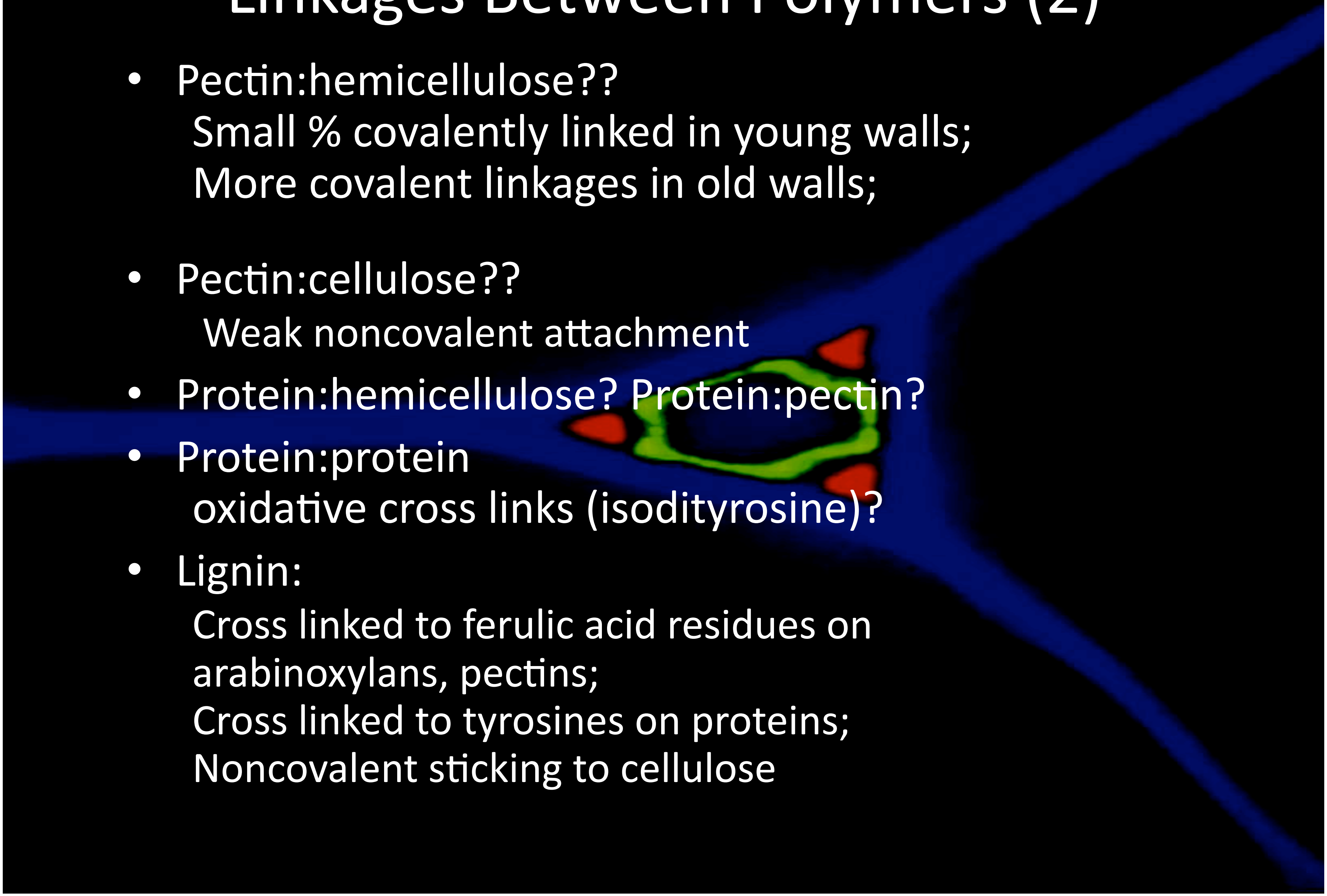


- Cellulose:hemicellulose
  noncovalent;
  mixture of hydrogen bonding &
  hydrophobic bonding;
  maybe physical entrapment in MF

- Pectin:pectin
  small junction zones

  ionic bonds w/calcium
  phenolic crosslinks (diferulate, few species)

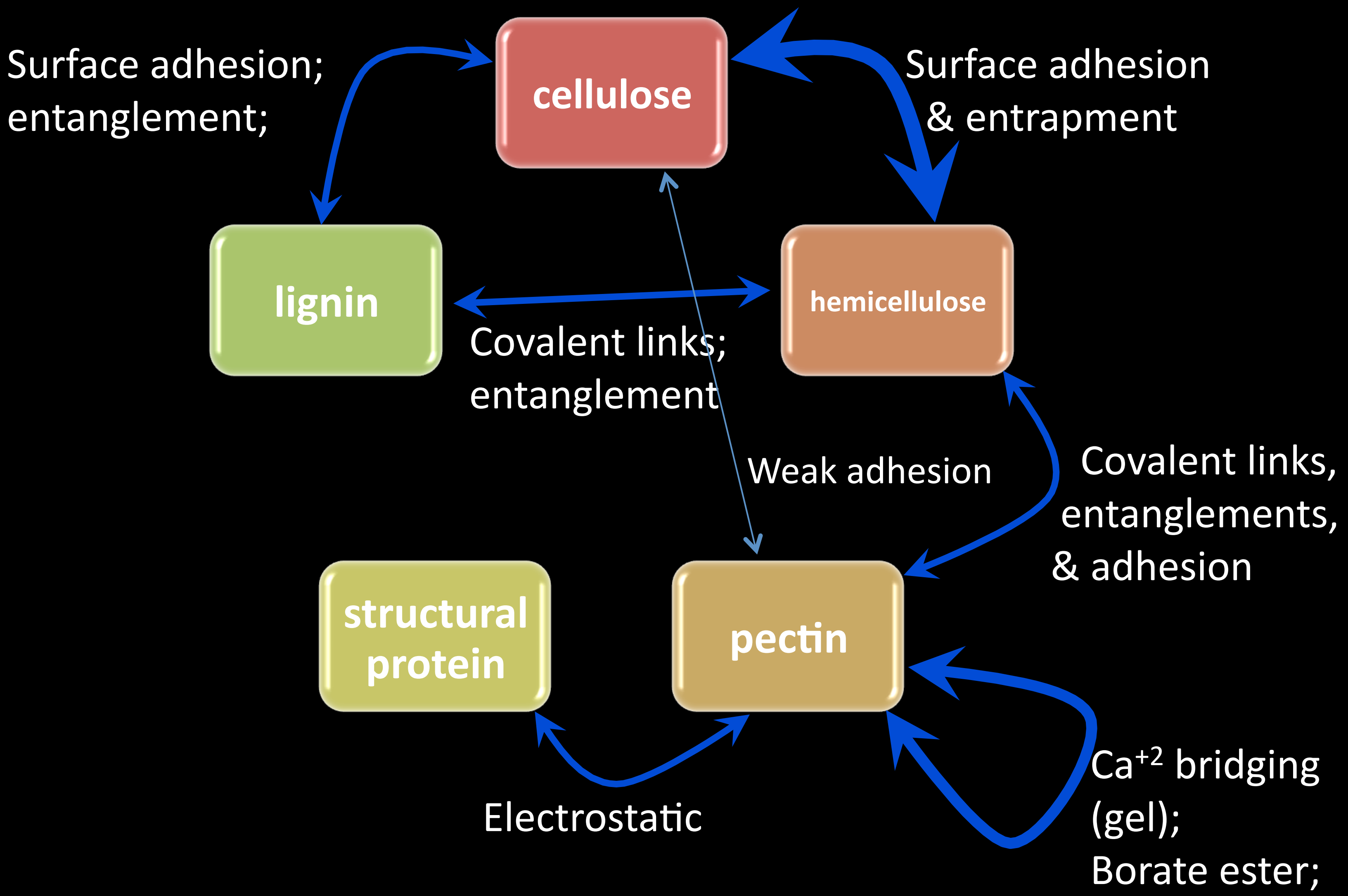
# Linkages Between Polymers (2)



- Pectin:hemicellulose??
  Small % covalently linked in young walls;
  More covalent linkages in old walls;

- Pectin:cellulose??
  Weak noncovalent attachment
- Protein:hemicellulose? Protein:pectin?
- Protein:protein
  oxidative cross links (isodityrosine)?
- Lignin:
  Cross linked to ferulic acid residues on arabinoxylans, pectins;
  Cross linked to tyrosines on proteins;
  Noncovalent sticking to cellulose

# Interactions Between Cell Wall Polymers



**cellulose**

**lignin**

**hemicellulose**

**structural protein**

**pectin**

Surface adhesion;
entanglement;

Surface adhesion
& entrapment

Covalent links;
entanglement

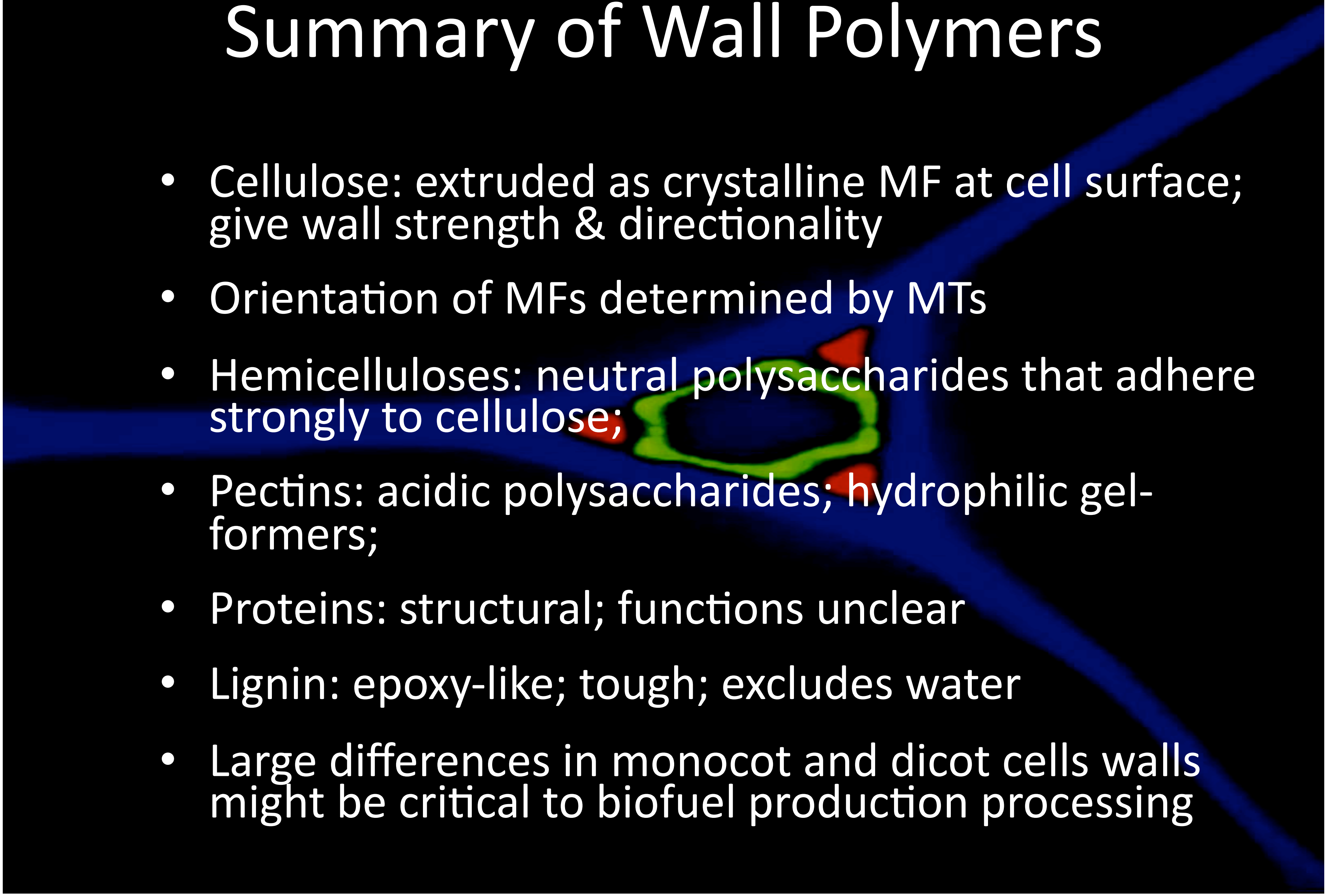
Weak adhesion

Covalent links,
entanglements,
& adhesion

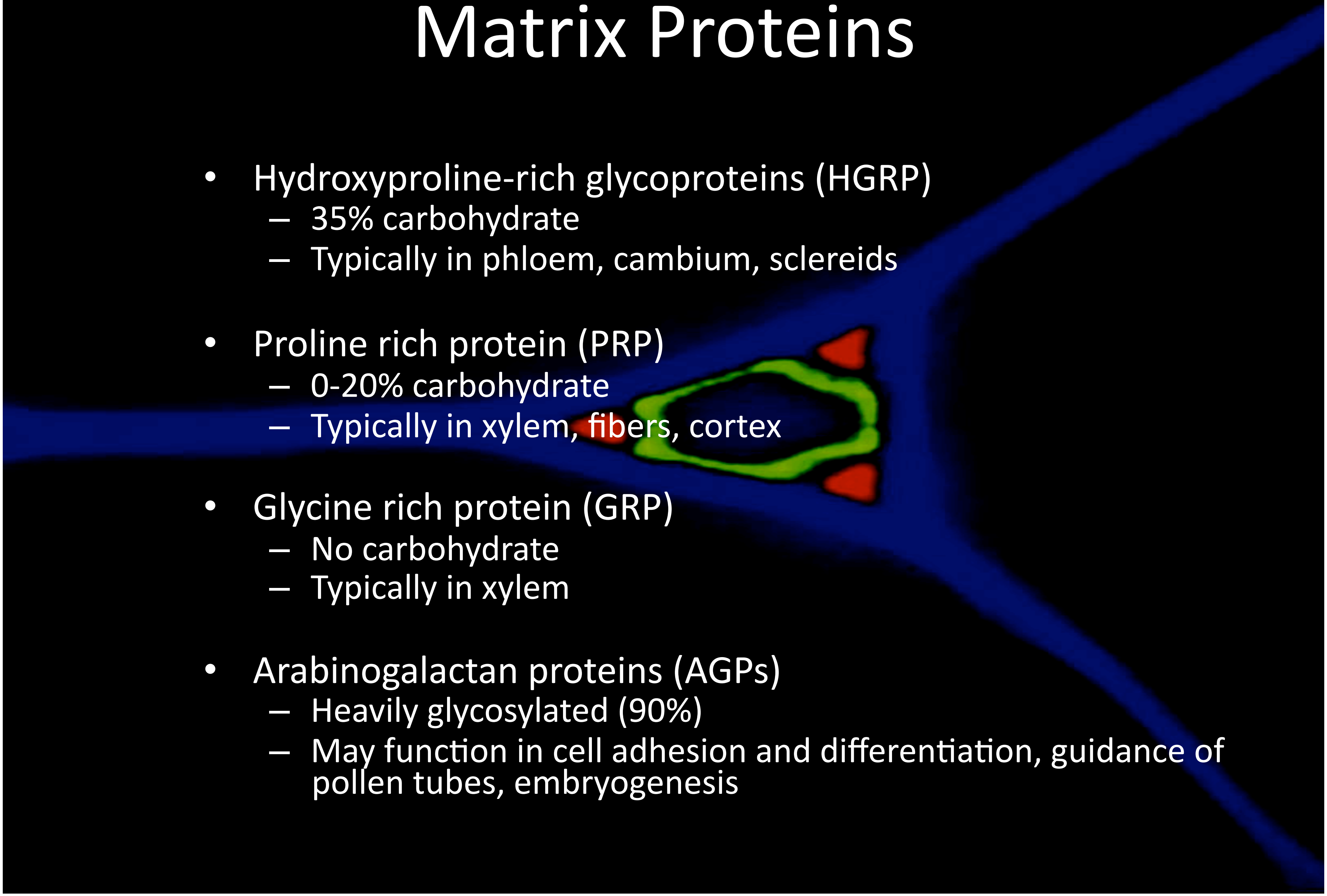
Electrostatic

Ca$^{+2}$ bridging
(gel);
Borate ester;

# Summary of Wall Polymers



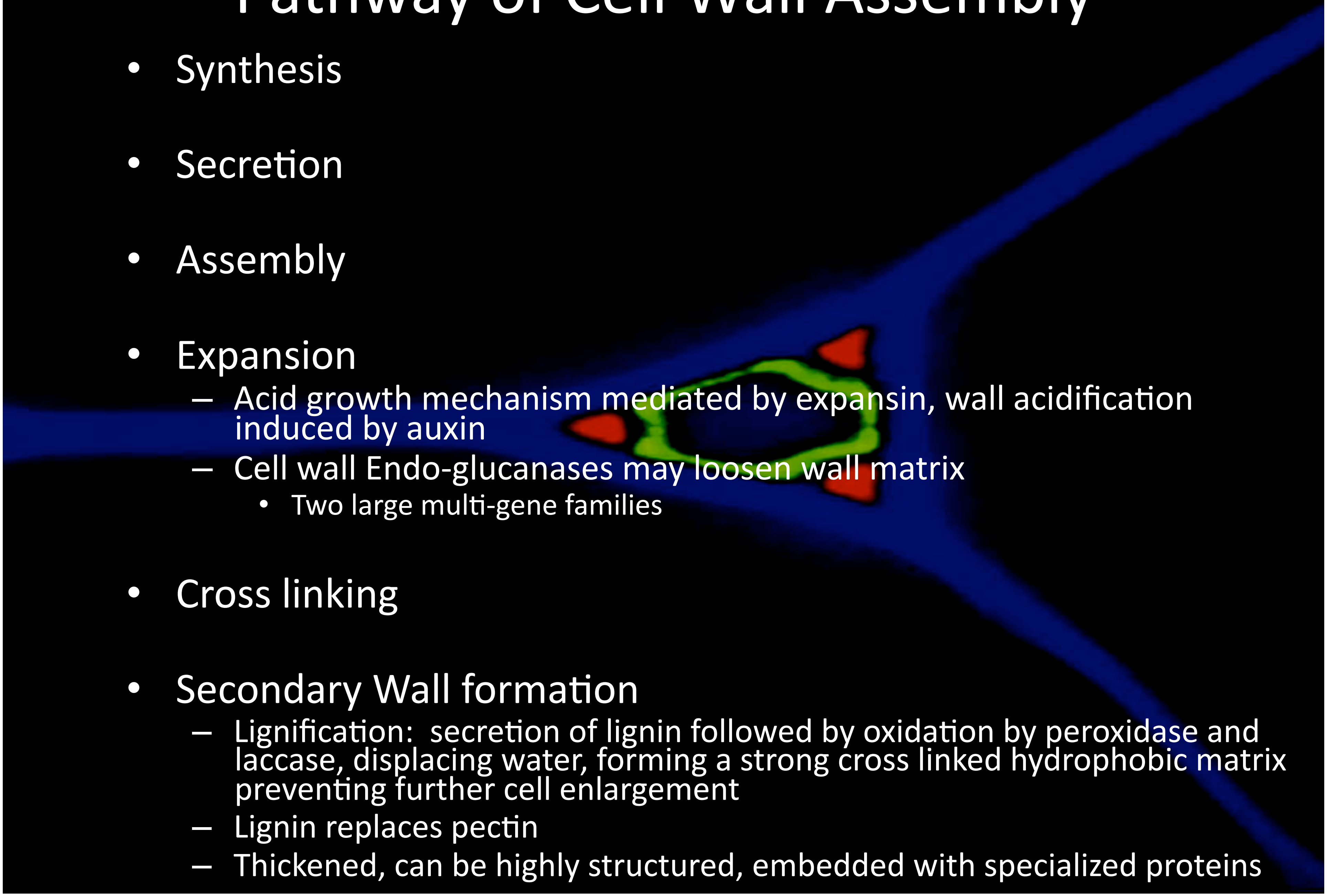
- Cellulose: extruded as crystalline MF at cell surface; give wall strength & directionality
- Orientation of MFs determined by MTs
- Hemicelluloses: neutral polysaccharides that adhere strongly to cellulose;
- Pectins: acidic polysaccharides; hydrophilic gel-formers;
- Proteins: structural; functions unclear
- Lignin: epoxy-like; tough; excludes water
- Large differences in monocot and dicot cells walls might be critical to biofuel production processing



# Matrix Proteins

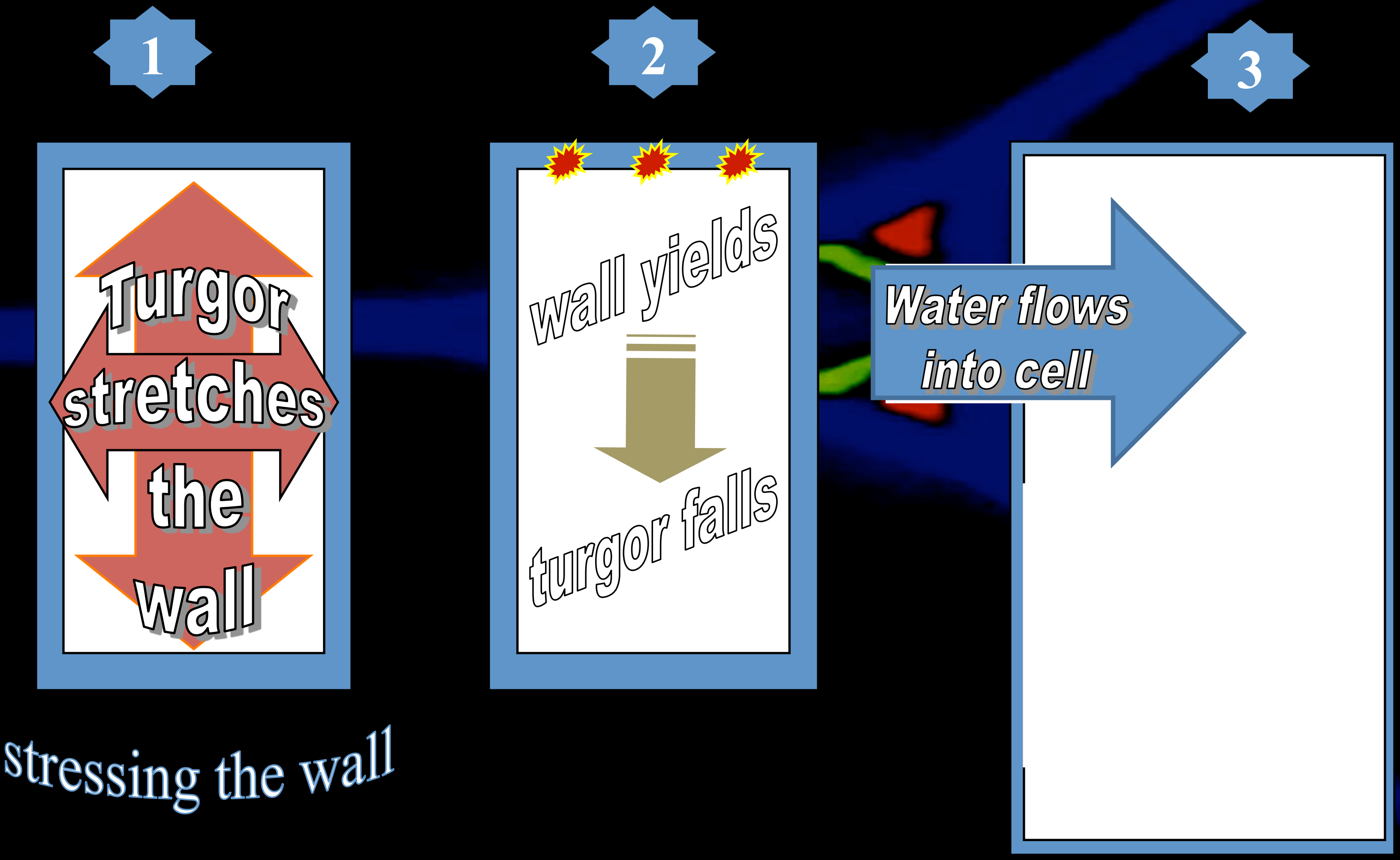
- Hydroxyproline-rich glycoproteins (HGRP)
  - 35% carbohydrate
  - Typically in phloem, cambium, sclereids
- Proline rich protein (PRP)
  - 0-20% carbohydrate
  - Typically in xylem, fibers, cortex
- Glycine rich protein (GRP)
  - No carbohydrate
  - Typically in xylem
- Arabinogalactan proteins (AGPs)
  - Heavily glycosylated (90%)
  - May function in cell adhesion and differentiation, guidance of pollen tubes, embryogenesis

# Pathway of Cell Wall Assembly



- Synthesis
- Secretion
- Assembly
- Expansion
  - Acid growth mechanism mediated by expansin, wall acidification induced by auxin
  - Cell wall Endo-glucanases may loosen wall matrix
    - Two large multi-gene families
- Cross linking
- Secondary Wall formation
  - Lignification: secretion of lignin followed by oxidation by peroxidase and laccase, displacing water, forming a strong cross linked hydrophobic matrix preventing further cell enlargement
  - Lignin replaces pectin
  - Thickened, can be highly structured, embedded with specialized proteins

# Elementary Biophysics of Cell Growth



1

Turgor stretches the wall

stressing the wall

2

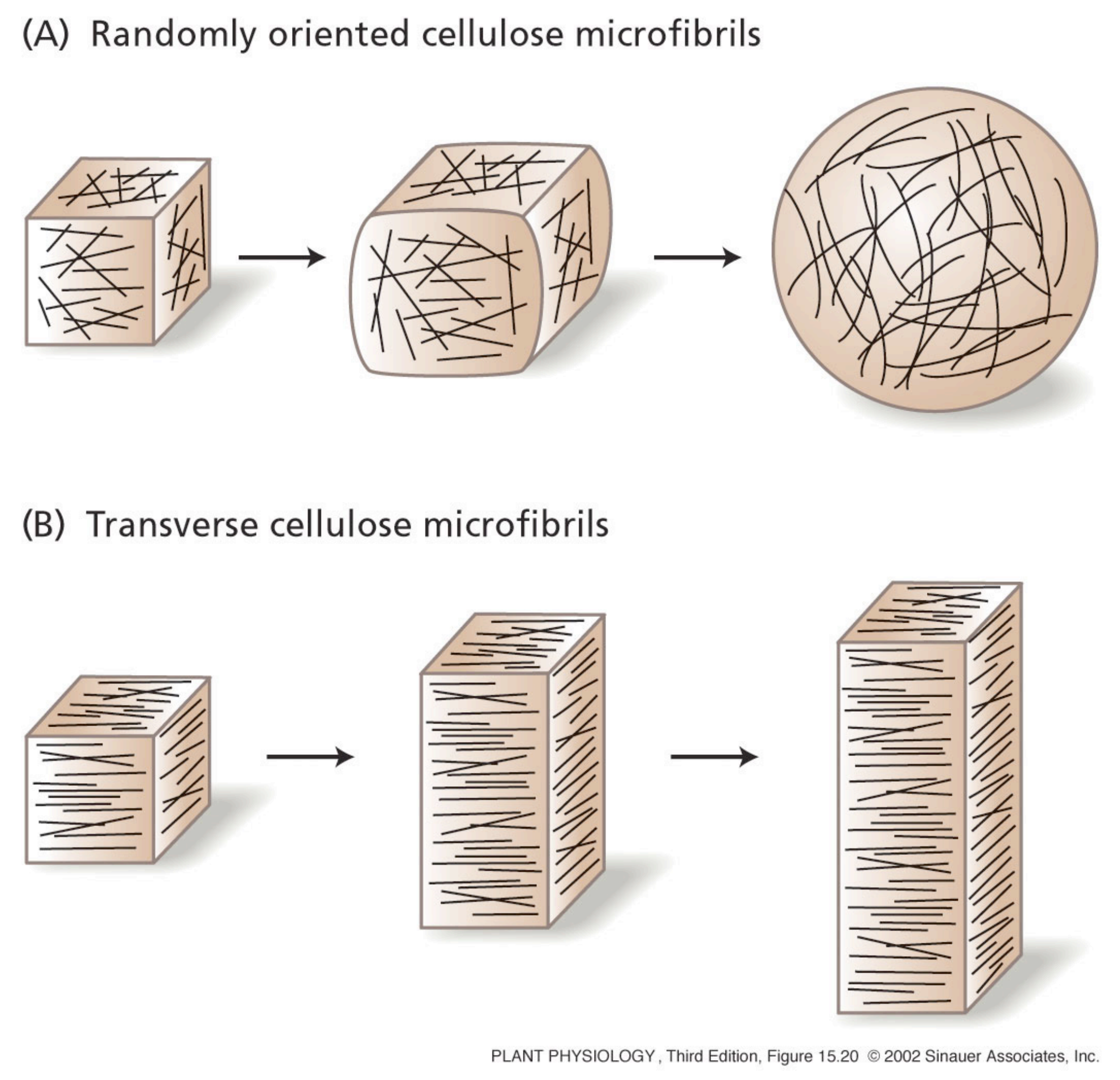
wall yields

turgor falls

3

Water flows into cell

# Elementary Biophysics of Cell Growth



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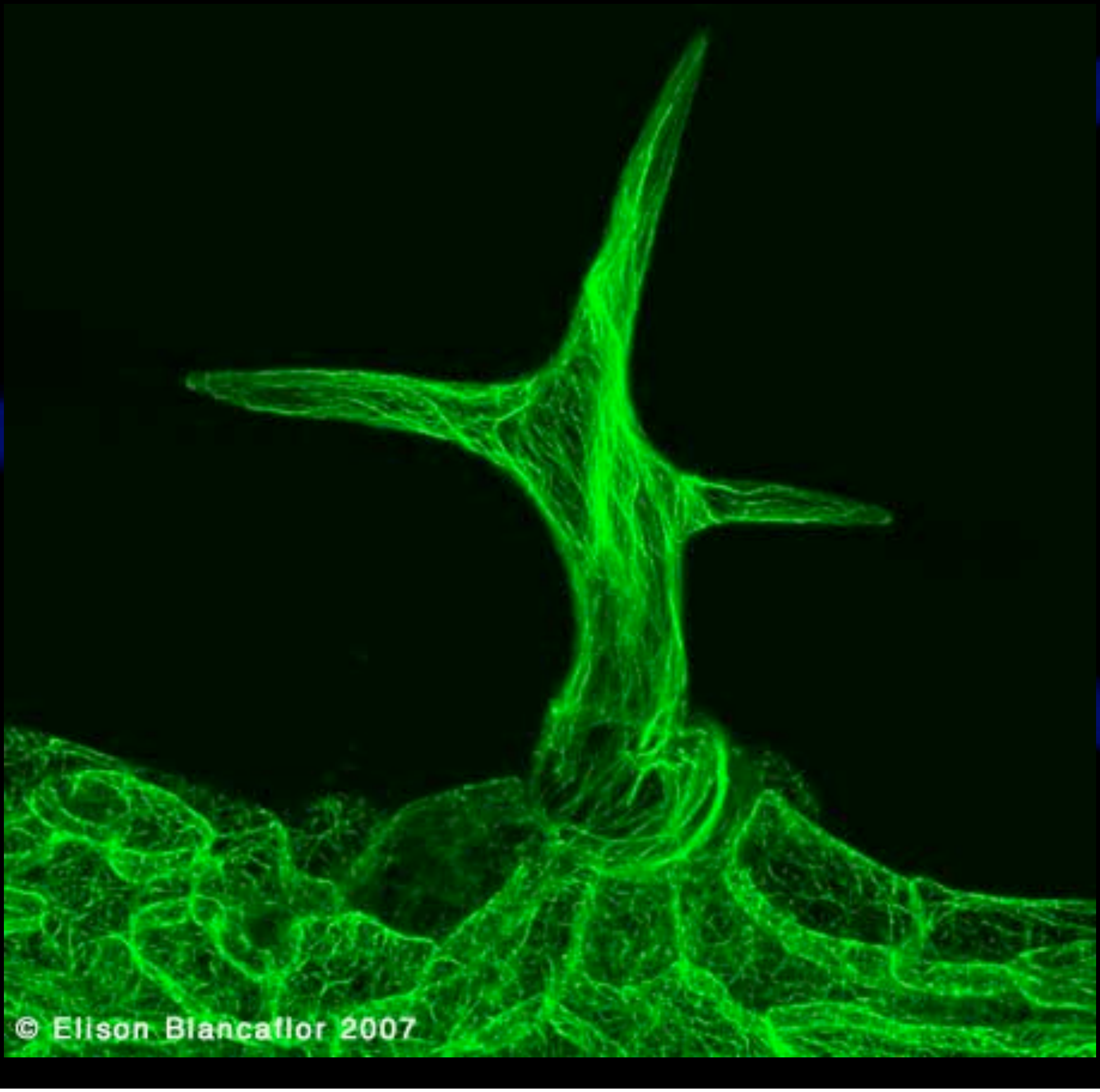
# Regulation of Cellulose Microfibril Orientation by Microtubules Controls Expansion to Produce Complex Cell Shapes



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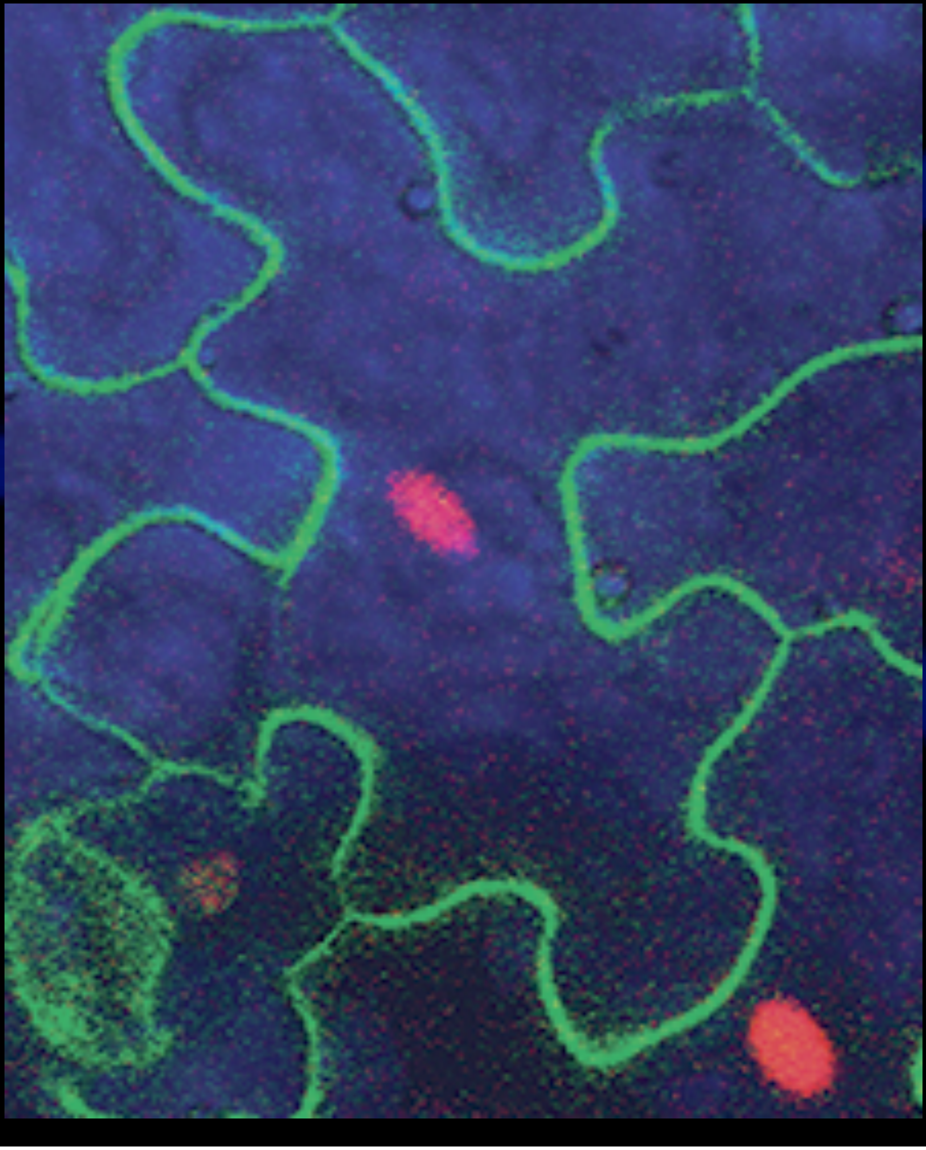
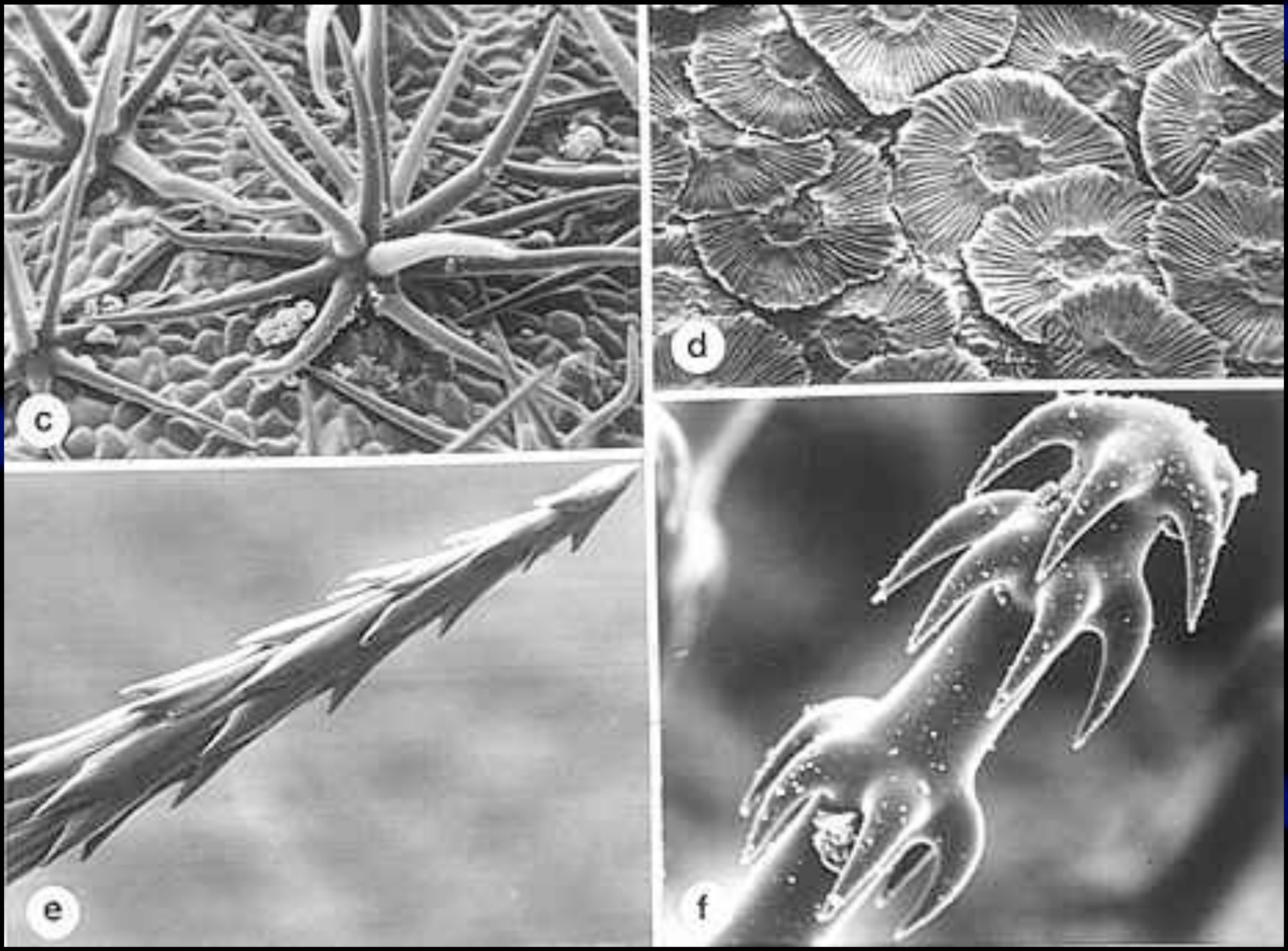
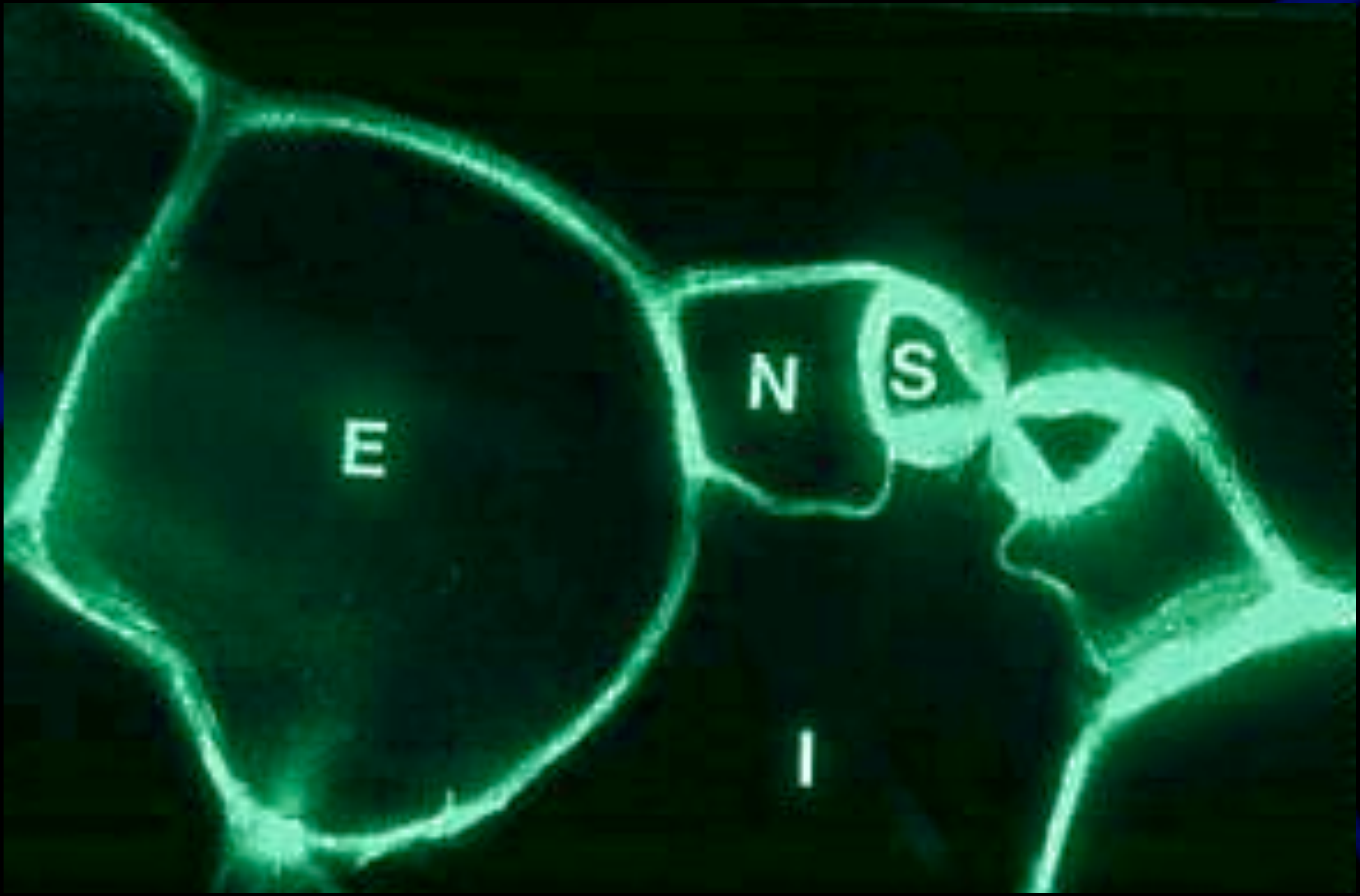
# Complex Cell Shapes



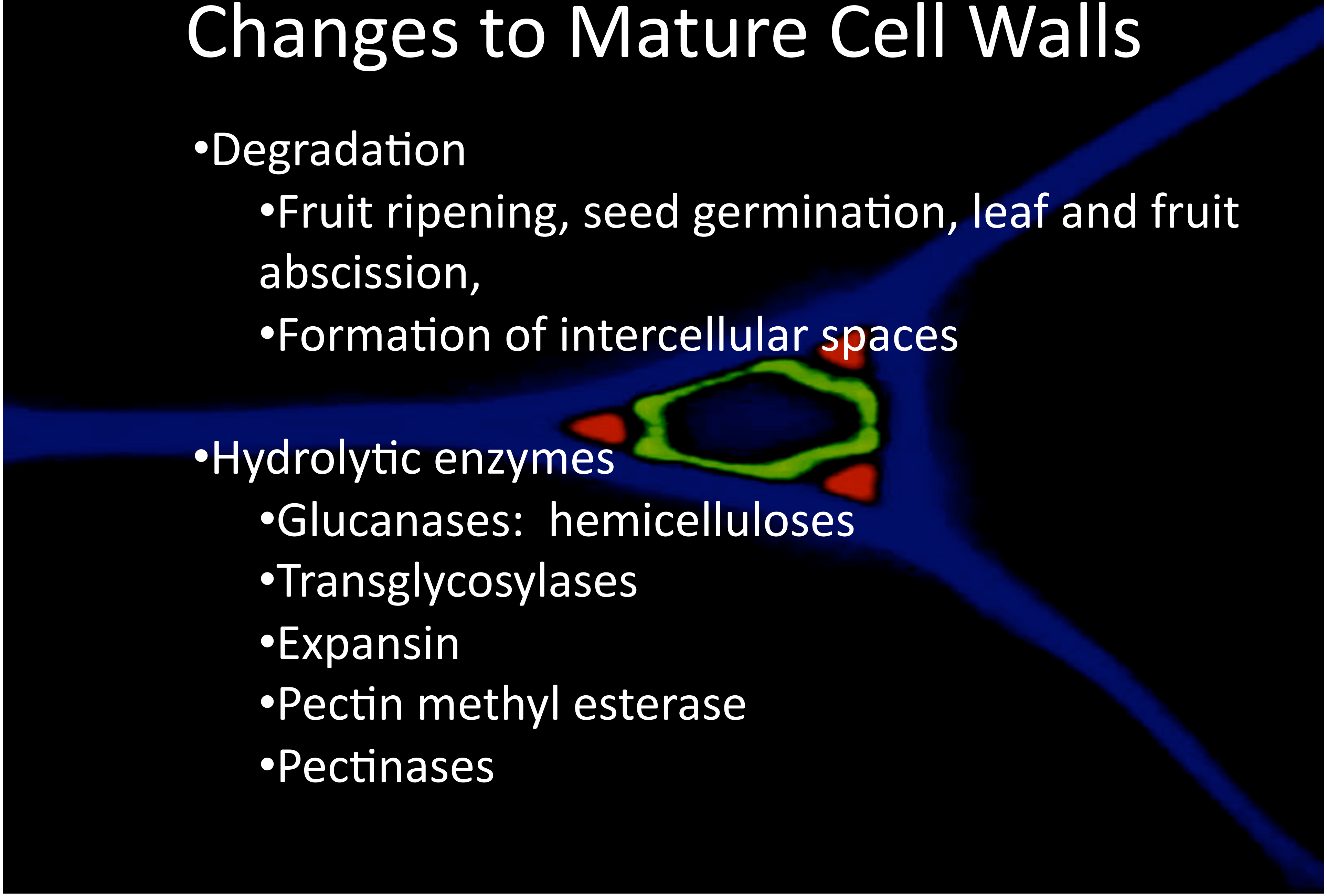
# Complex Cell Shapes



# Complex Cell Shapes



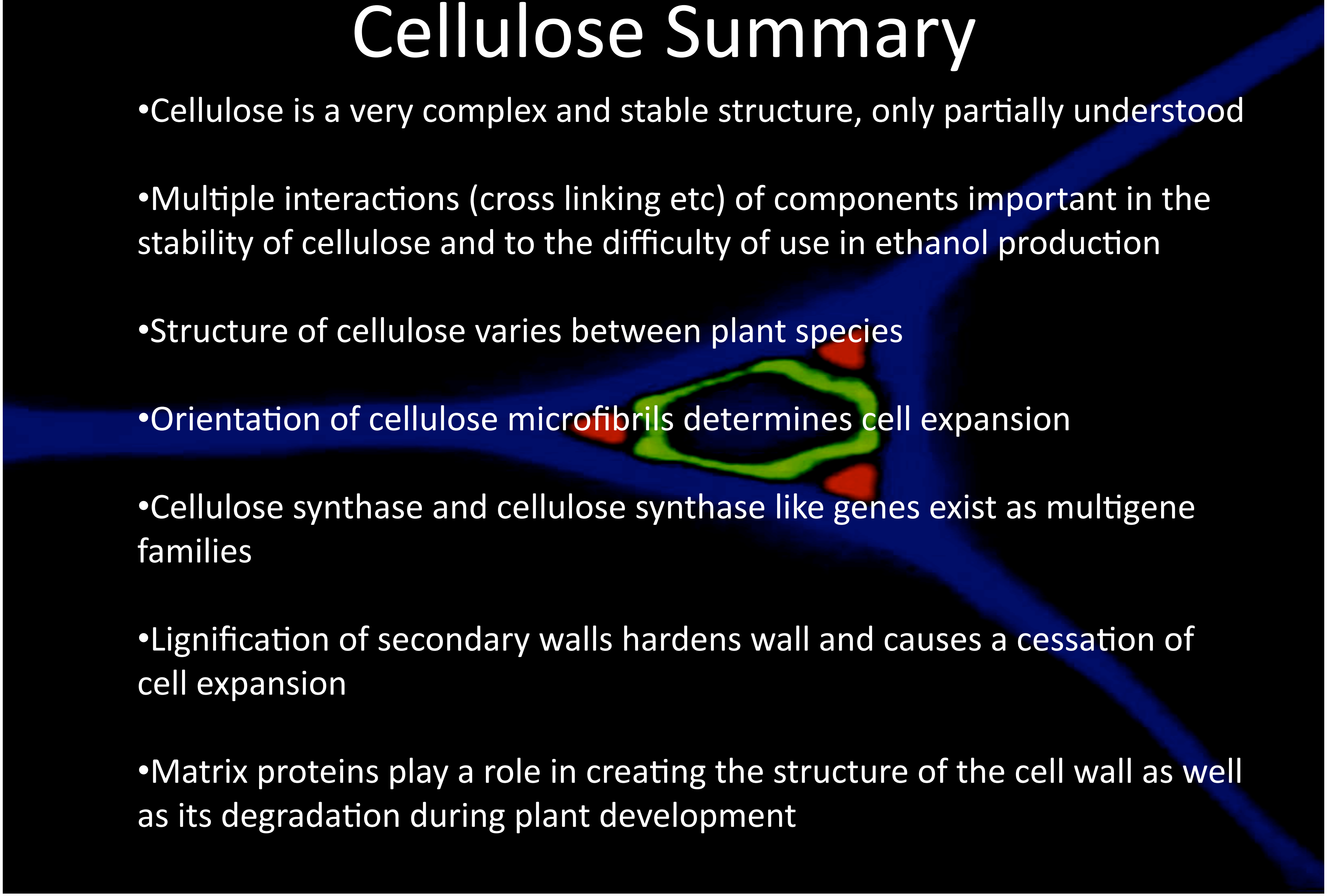
# Changes to Mature Cell Walls



- Degradation
  - Fruit ripening, seed germination, leaf and fruit abscission,
  - Formation of intercellular spaces

- Hydrolytic enzymes
  - Glucanases: hemicelluloses
  - Transglycosylases
  - Expansin
  - Pectin methyl esterase
  - Pectinases

# Cellulose Summary



- Cellulose is a very complex and stable structure, only partially understood

- Multiple interactions (cross linking etc) of components important in the stability of cellulose and to the difficulty of use in ethanol production

- Structure of cellulose varies between plant species

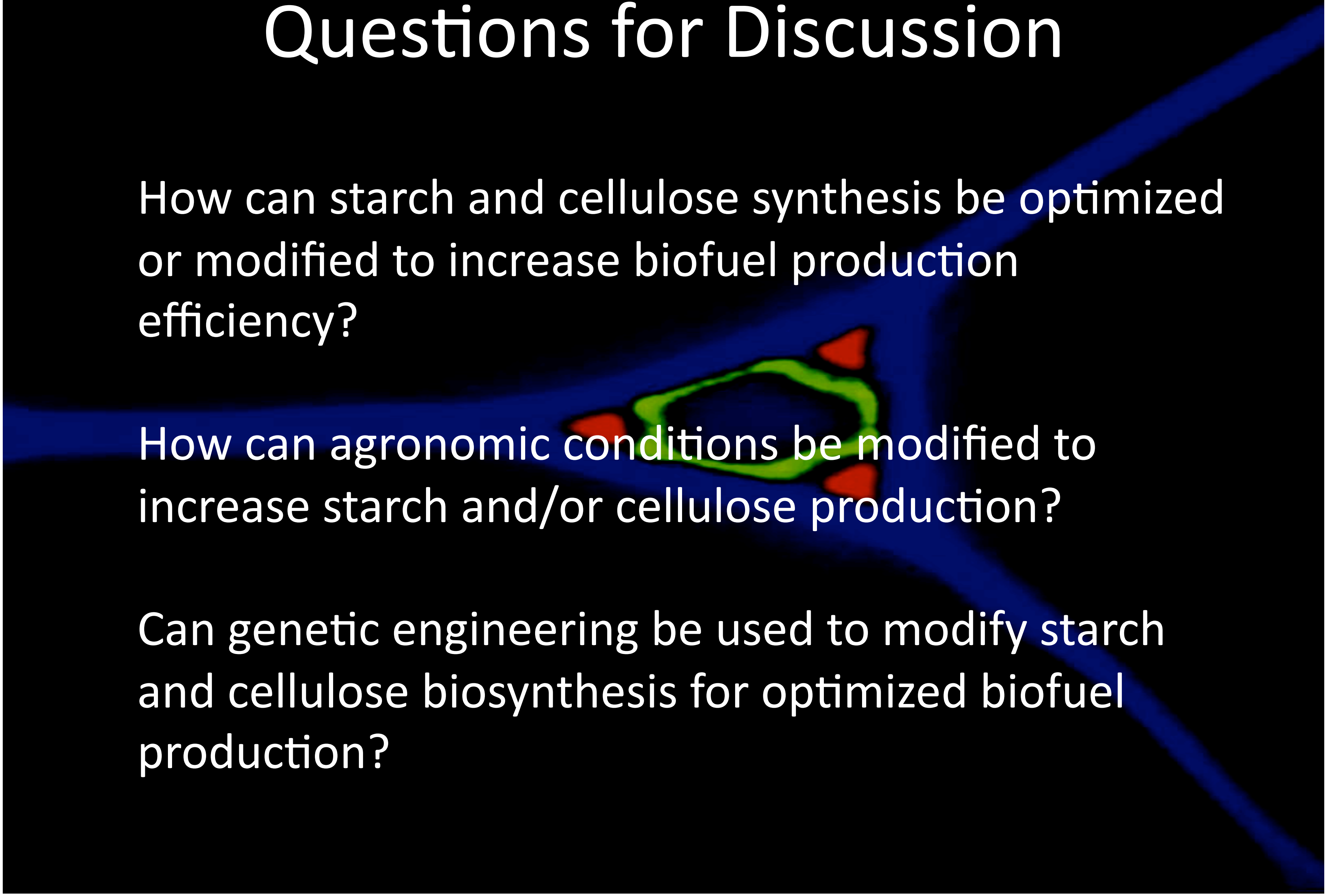
- Orientation of cellulose microfibrils determines cell expansion

- Cellulose synthase and cellulose synthase like genes exist as multigene families

- Lignification of secondary walls hardens wall and causes a cessation of cell expansion

- Matrix proteins play a role in creating the structure of the cell wall as well as its degradation during plant development

# Questions for Discussion



How can starch and cellulose synthesis be optimized or modified to increase biofuel production efficiency?

How can agronomic conditions be modified to increase starch and/or cellulose production?

Can genetic engineering be used to modify starch and cellulose biosynthesis for optimized biofuel production?

Thank You

Research on SBEs supported by the DOE Basic Biosciences Program