Al-Farabi Kazakh National University (KazNU)

Faculty of Biology and Biotechnology

Department of Molecular Biology and Genetics

Discipline: «Modern Problems of Plant Genetics»

Lecture 4

Plant Cell Culture Technology: Somatic Hybridization and Protoplast Fusion

Overcoming sexual incompatibility barriers via enzymatic protoplast isolation, PEG & electrofusion, symmetric/asymmetric hybrids, cybrids, and verification

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Aim and Structure of the Lecture

Aim: learn how wall-less plant protoplasts enable parasexual gene transfer across sexually incompatible species and cytoplasmic remodeling

KEY FOCUS

Lecture Plan & Modules

Key sections:
1. Rationale & Scope
2. Protoplast Isolation & Culture
3. Fusion Methods (PEG vs Electric)
4. Symmetric, Asymmetric & Cybrids
5. Selection & Hybrid Verification
6. Applications & Limitations

- **Rationale & Scope:** Overcoming pre- and post-zygotic sexual barriers between wild relatives and cultivated crops
- **Protoplast Isolation & Culture:** Enzymatic cell wall digestion (cellulase/macerozyme), osmotic stabilizers, and nursing systems
- **Fusion Methods (PEG vs Electric):** Chemical fusogens (PEG + high Ca^{2+}/pH) vs dielectrophoretic AC alignment and DC pulse fusion
- **Symmetric, Asymmetric & Cybrids:** Full nuclear fusion vs X-ray/UV-irradiated chromosome elimination vs organelle-only cybridization
- **Selection & Hybrid Verification:** Complementing auxotrophs/antibiotics, flow cytometry, and SSR/SNP/GISH cytogenetic confirmation
- **Applications & Limitations:** CMS transfer in Brassica, late blight resistance in potato, citrus hybrids, and somaclonal challenges

Overcoming Sexual Incompatibility Barriers via Parasexual Genetics

Conventional crossing often fails between crops and wild stress-resistant relatives — somatic fusion bypasses meiosis and fertilization

Part I: Core Mechanisms

Failure Before Fertilization

- Pollen fails to germinate on foreign stigma or pollen tubes arrest before reaching the ovule
- Differences in flowering phenology, flower morphology, or self-incompatibility loci

Embryo Abortion & Sterility

- Failure of hybrid endosperm development or genomic disharmony kills the early zygote
- Odd ploidy hybrids fail meiotic synapsis and are sterile or produce non-viable pollen

Part II: Applications & Balance

Seedless & Juvenile Perennials

- Triploid banana, seedless citrus, and recalcitrant potato lines cannot be crossed conventionally
- Somatic fusion combines traits without requiring sexual seed production

Maternal Organelle Inheritance

- In sexual crosses, chloroplasts and mitochondria come solely from the egg cell (~99% of angiosperms)
- Protoplast fusion mixes both parents' cytoplasms, enabling mitochondrial/plastid recombination and CMS transfer

Step 1: Enzymatic Isolation of Viable Plant Protoplasts

Removing the rigid cellulose–pectin cell wall without bursting the delicate plasma membrane (Cocking, 1960)

1 1. Tissue Source & Plasmolysis

Surface-sterilized leaf mesophyll or embryogenic callus pre-plasmolyzed in osmoticum

2 2. Enzyme Digestion

Cellulase R-10 (hydrolyzes cellulose) + Macerozyme R-10 / Pectolyase (middle lamella) in mannitol

3 3. Purification (Sucrose Cushion)

Filter debris (40–70 µm mesh) and float intact spherical protoplasts on 21% sucrose cushion

4 4. Viability Assessment

Fluorescein diacetate (FDA — bright green esterase fluorescence) and Calcofluor White (no wall)

5 5. Wall Resynthesis & Division

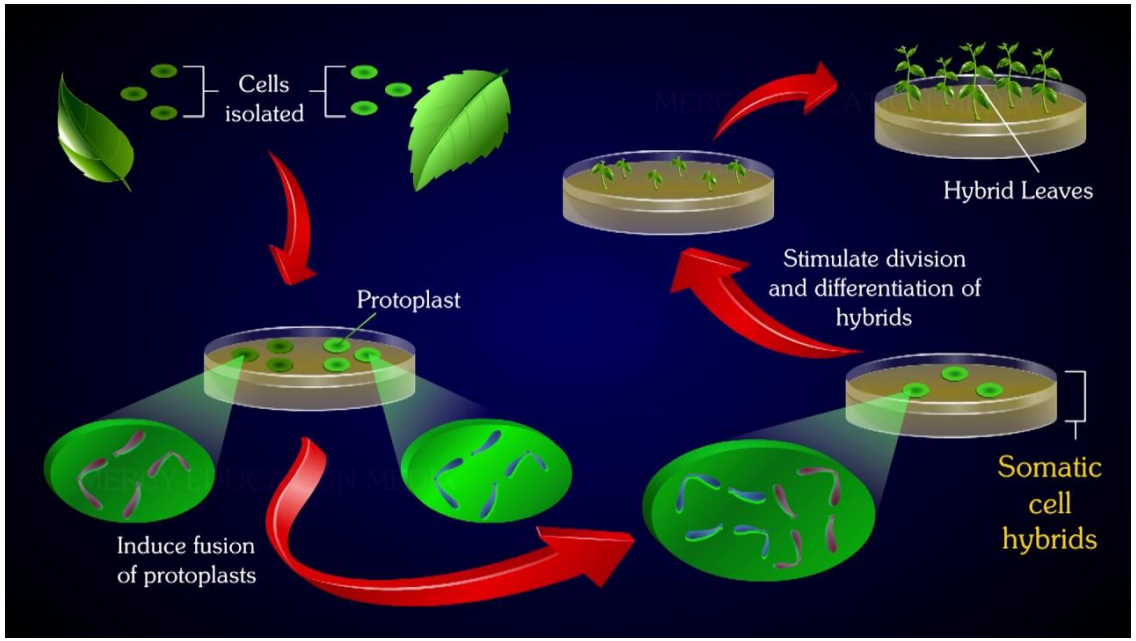
Within 24–48 h in agarose/bead culture, cell wall reforms and first mitotic divisions begin

Maintaining exact osmolarity (0.4–0.6 M mannitol, sorbitol, or glucose) is essential until cell wall cellulose microfibrils regenerate.

Two Main Mechanisms of Protoplast Membrane Fusion

Bringing negatively charged plasma membranes into tight physical contact to trigger reversible bilayer coalescence

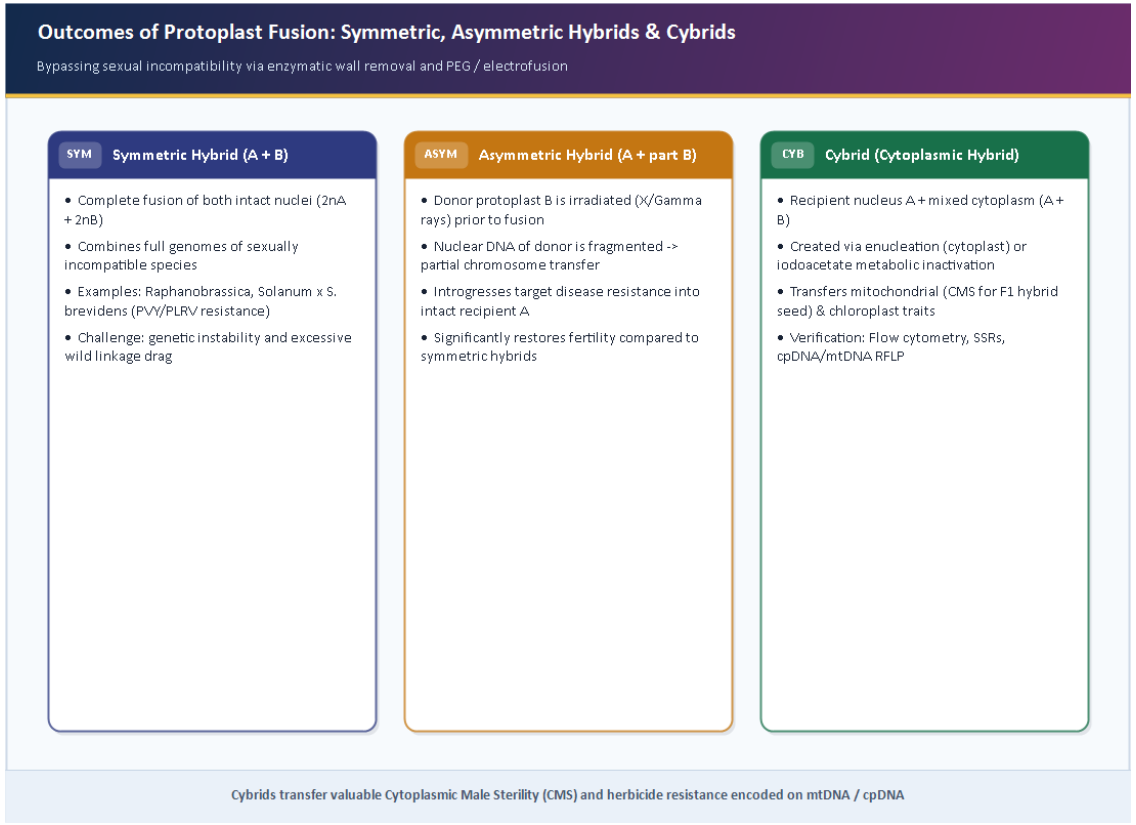
- **Chemical Fusion:** PEG + High Ca^{2+} / High pH: Polyethylene glycol (PEG 4000–6000, 25–40%) is a hydrophilic polymer that dehydrates membranes and forms molecular bridges
- Followed by high pH (9–10.5) and high Ca^{2+} (50–100 mM) elution buffer that neutralizes negative surface phosphate charges
- **Advantages:** inexpensive, requires no specialized pulse equipment, processes millions of protoplasts simultaneously
- **Disadvantages:** somewhat toxic to fragile protoplasts, higher yield of random multi-cell homokaryons
- **Physical Electrofusion (AC Alignment + DC Pulse):** Step 1 (AC field, ~1 MHz, low voltage): induces dielectrophoresis so protoplasts align neatly into «pearl chains»
- **Step 2 (Short high-voltage DC pulse, ~1–2 kV/cm for 10–50 µs):** creates transient reversible membrane nanopores at contact points



Two main mechanisms of protoplast membrane fusion (PEG vs Electro)

Genomic and Organellar Fate Following Heterokaryon Formation

Four possible cellular outcomes after two genetically distinct protoplasts (A and B) fuse their membranes



Genomic and organellar fate: symmetric, asymmetric hybrids & cybrids

- **Full Nuclear Combination (2nA + 2nB):** Both parental nuclei enter mitosis simultaneously (mitotic synchrony) and merge into a complete allotetraploid amphiploid genome; Advantage: combines all nuclear traits; limitation: often retains unwanted wild traits (e.g., glycoalkaloids, tubers yield penalty)
- **Partial Donor Genome Transfer (2nA + fragB):** Donor protoplasts (B) are fragmented via X-ray / γ-irradiation (50–300 Gy) prior to fusion with intact recipient (A); Randomly eliminates 70–95% of donor chromosomes while retaining tiny donor chromosome segments conferring disease resistance
- **Recipient Nucleus + Novel Cytoplasm (nA + mt/cp B):** Fusion of an enucleated cytoplast or heavily irradiated/iodoacetate-treated partner transfers chloroplasts/mitochondria only; Allows one-step transfer of Ogura CMS or atrazine-resistant chloroplasts without changing the elite nuclear cultivar!
- **Unique Biparental Cytoplasmic Sorting:** Unlike chloroplasts (which usually sort randomly to one type), plant mitochondria undergo active inter-parental DNA recombination; Yields novel mitochondrial configurations impossible in nature or sexual hybridization

Selection Strategies for Recovering True Somatic Hybrids

Because fused populations contain excess parental cells and homokaryons (A+A, B+B), stringent selection is mandatory

Selection Strategy	Genetic / Biochemical Basis	Example System
Biochemical Complementation	Parent A lacks gene X (albino/nitrate-reductase null); Parent B lacks gene Y; only hybrid grows on minimal medium	Chlorate-resistant NR^- tobacco × chlorophyll-deficient albino mutant → green colonies
Double Metabolic Inhibitors	Parent A treated with iodoacetate (glycolysis block); Parent B irradiated with X-rays (mitosis block)	Only A+B hybrids combine intact metabolism from B and intact nucleus from A to divide
Antibiotic / Herbicide Stacking	Parent A carries nptII (kanamycin resistance); Parent B carries hpt (hygromycin resistance)	Dual antibiotic medium eliminates both parental colonies and selects strictly A+B hybrids
Visual & Flow Sorting (FACS)	Green mesophyll protoplasts (chlorophyll autofluorescence) fused with colorless callus/root protoplasts (stained FITC)	Micromanipulator aspiration or dual-laser FACS physically isolates dual-fluorescent heterokaryons

From Selected Heterokaryon to Verified Fertile Somatic Hybrid

Rigorous molecular and cytogenetic verification confirms true nuclear/cytoplasmic constitution

1 1. Micro-Callus Formation

Nurse culture or agarose bead embedding yields green micro-calli within 4–8 weeks

2 2. Shoot & Root Regeneration

Transfer calli to shoot-induction (BAP/zeatin) and rooting media; verify hybrid morphology

3 3. Nuclear DNA Verification

Co-dominant SSR/CAPS/SNP markers & flow cytometry confirm both parental genomes (or fragments)

4 4. GISH & Organelle Assay

Genomic In Situ Hybridization (GISH) paints donor vs recipient chromosomes; cp/mt PCR verifies cybrids

5 5. Backcrossing & Introgression

Cross symmetric/asymmetric hybrids back to cultivated varieties to eliminate linkage drag

Genomic In Situ Hybridization (GISH) directly visualizes whole chromosome sets, monosomic additions, and intergenomic translocations in somatic hybrids.

Typical Frequencies and Yields Along the Protoplast Fusion Workflow

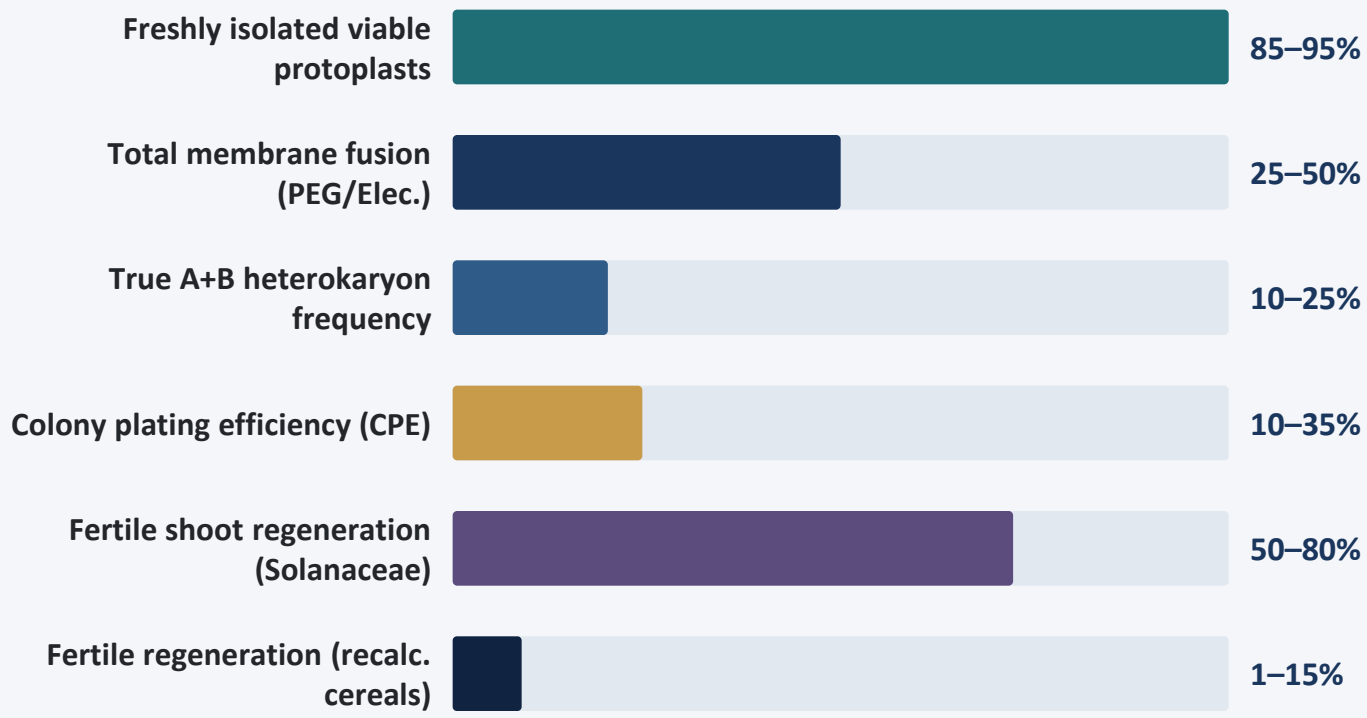
Why efficient heterokaryon selection and viability optimization remain essential in somatic hybridization

$1–5 \times 10^6$
Protoplasts per gram leaf

10–40%
Heterokaryon fusion rate

1960 / 1972
Cocking / Carlson (1st hybrid)

Representative Efficiency Metrics Across Protoplast Culture Stages (%)



Taxonomic Divide

- Solanaceae (potato, tobacco, petunia) and Brassicaceae regenerate shoots readily from leaf mesophyll protoplasts
- Cereals and many woody legumes rarely regenerate from leaf protoplasts, requiring embryogenic suspension cultures
- Modern applications increasingly use protoplasts for transient CRISPR RNP delivery rather than only somatic fusion

Major Agricultural Achievements of Somatic Hybridization and Cybrids

Real-world breakthroughs in brassicas, potato, citrus, and cereals

Fusion Partners	Hybrid Type	Transferred Agronomic Trait & Impact
Brassica napus × Raphanus sativus (radish)	Cybrid (Ogura CMS + normal cp)	Transfer of radish Ogura male-sterile mitochondria while replacing defective radish chloroplasts with B. napus chloroplasts (removes chlorosis)
Solanum tuberosum (potato) × Solanum bulbocastanum / S. brevidens	Symmetric / Asymmetric somatic hybrid	Introgression of broad-spectrum late blight (Phytophthora infestans), PVY, and frost resistance across 1EBN barriers
Brassica napus × Sinapis alba / Brassica oleracea	Asymmetric hybrid / Re-synthesized oilseed	Broadened genetic base for canola, Alternaria blackspot resistance, and clubroot resistance
Citrus sinensis (sweet orange) × Poncirus trifoliata	Allotetraploid somatic hybrid	Dwarfing rootstocks tolerant to Citrus Tristeza Virus (CTV), Phytophthora root rot, and salinity

★ Key Takeaway

- Real-world breakthroughs in brassicas, potato, citrus, and cereals
- **Comparison scope (4):** Brassica napus × Raphanus sativus (radish); Solanum tuberosum (potato) × Solanum bulbocastanum / S. brevidens; Brassica napus × Sinapis alba / Brassica oleracea; Citrus sinensis (sweet orange) × Poncirus trifoliata
- **Priority synthesis:** Citrus sinensis (sweet orange) × Poncirus trifoliata — Dwarfing rootstocks tolerant to Citrus Tristeza Virus (CTV), Phytophthora root rot, and salinity

Critical Evaluation: Advantages and Limitations of Somatic Hybridization

Balanced assessment relative to sexual wide crossing, transgenesis, and genome editing

Key Advantages & Unique Capabilities

- Transfers complex polygenic agronomic traits (drought, frost, durable blight resistance) whose underlying genes remain uncloned
- Bypasses sexual incompatibility, apomixis, juvenility, and male/female sterility barriers completely
- **Unique access to cytoplasmic genetics:** mixes and recombines mitochondrial & chloroplast genomes in one step
- In most jurisdictions, intraspecific/wild-relative somatic hybrids are regulated as non-transgenic conventional crops

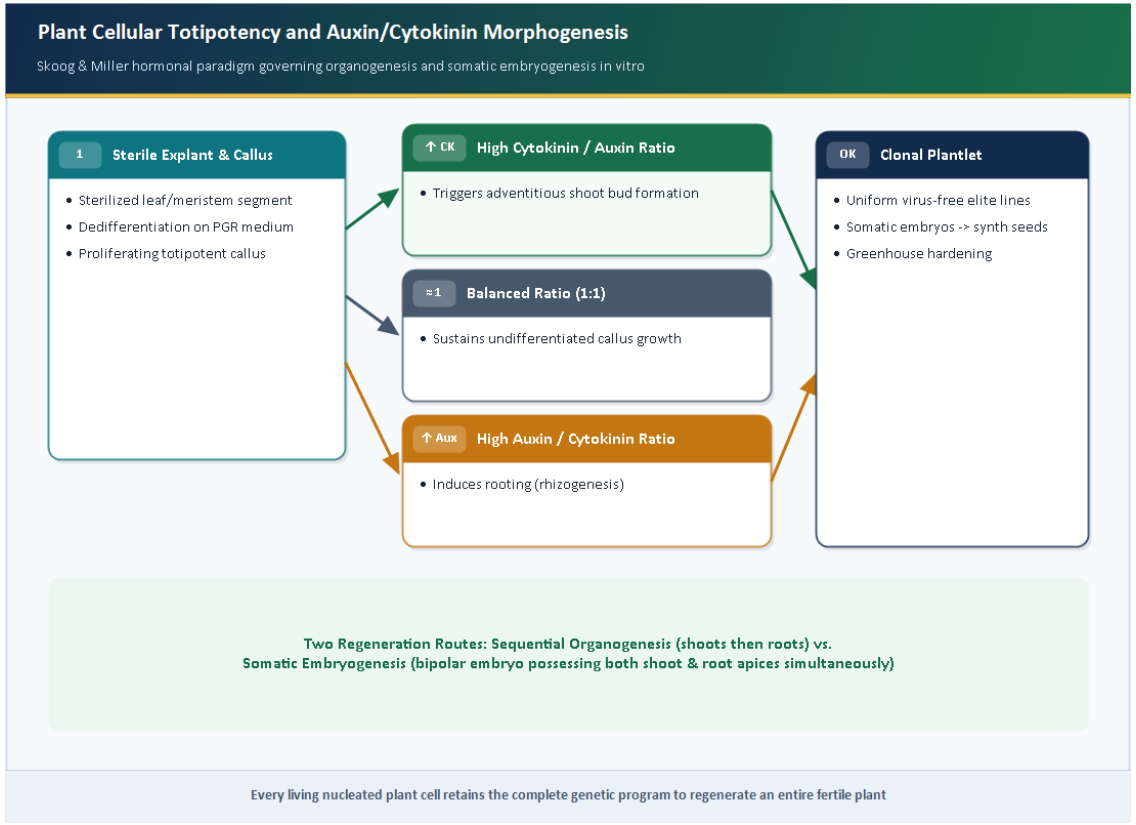
Biological & Technical Limitations

- Somaclonal variation during prolonged callus phase causes aneuploidy, chromosomal rearrangements, and epigenetic instability
- Genetic drag of undesirable wild alleles (poor tuber shape, high toxic glycoalkaloids/glucosinolates) requires extensive backcrossing
- Genomic disharmony and somatic incompatibility lead to developmental abnormalities or hybrid lethality in very distant crosses
- Low regeneration efficiency from mesophyll protoplasts in monocot cereals (wheat, maize) and forest trees

Modern Rebirth of Protoplasts in Precision Plant Genetics (2020–2026)

Protoplast platforms now serve a dual role beyond classical fusion: rapid CRISPR screening and transgene-free editing

- **TRANSGENE-FREE RNP — DNA-Free Genome Editing via PEG:** PEG delivers pre-assembled Cas9 protein + sgRNA ribonucleoproteins (RNPs) directly into potato, cabbage, and lettuce protoplasts; RNPs edit the target locus and degrade within 48 h — regenerated plants carry zero foreign plasmid DNA!
- **FAST sgRNA VALIDATION — 48-Hour Transient Editing Assay:** Instead of waiting 6–12 months for transgenic wheat or maize plants, protoplasts validate sgRNA cleavage efficiency in 2 days; High-throughput screening verifies Base Editors and Prime Editors before committing to whole-plant regeneration
- **ORGANELLE / MITO EDITING — Resolving CMS & Cytoplasmic Traits:** Protoplast microinjection and cybridization pair naturally with mitoTALENs and chloroplast base editors (DdCBE); Enables targeted repair or knockout of mitochondrial CMS genes without losing nuclear elite purity
- **SUBCELLULAR BIOLOGY — Single-Cell Localization & BiFC Assays:** Wall-free protoplasts allow rapid visualization of GFP fusion proteins, protein–protein BiFC, and hormone signaling dynamics; Supports scRNA-seq profiling without cell-wall enzymatic bias when studying cell-type identity



Precision protoplast regeneration via auxin/cytokinin morphogenesis

Distinguishing Somatic Hybrids, Cybrids, and Transgenic Crops

Structural composition, inheritance behavior, and regulatory treatment in modern biotechnology

Somatic Hybrids vs Transgenic Plants (GMOs)

- Somatic hybrids recombine intact or fragmented native genomes from two species (~tens of thousands of genes) via membrane fusion
- Transgenic plants insert 1 to a few specific cloned coding cassettes (often from bacteria or unrelated kingdoms) via Agrobacterium or biolistics
- **Asymmetric somatic hybrids act as an intermediate bridge:** transfer small native wild chromosome segments without recombinant DNA constructs
- Thus somatic hybrids generally avoid GMO biosafety dossier requirements in major agricultural jurisdictions

Precision Cytoplasmic Remodeling via Cybrids

- Sexual introgression of CMS requires 6–8 backcrosses to recover the recipient nuclear background (~7 years)
- Cybrid fusion eliminates the donor nucleus via X-rays or enucleation and replaces only mitochondria/chloroplasts in a single tissue culture cycle
- Moreover, cybridization corrects plastid–nucleus incompatibility (chlorosis) by recombining recipient chloroplasts with donor CMS mitochondria

Case Study: Introgressing Wild Late Blight Resistance into Potato (4x)

How somatic hybridization overcomes the 1EBN (Endosperm Balance Number) crossing barrier of wild diploid Solanum relatives

01 **1. Wild Donor Selection:** Diploid Solanum bulbocastanum (2x, 1EBN) carries broad Rpi-blb genes vs Phytophthora infestans

02 **2. Recipient Dihaploid (2x):** Generate dihaploid (2x) from tetraploid cultivar via anther culture or Solanum phureja inducer

03 **3. Protoplast Electrofusion:** Fuse mesophyll protoplasts (2x + 2x → 4x somatic hybrid) at cultivated ploidy level

04 **4. GISH & Phytophthora Test:** Verify chromosome stability, select blight-resistant hybrids with low solanine/glycoalkaloids

05 **5. Backcross to Elite 4x:** Cross fertile 4x hybrids to commercial tetraploid cultivars to release resistant varieties

Control Questions and Key Takeaways

Consolidating Lecture 4 concepts

? Control & Review Questions

- 1. Why does somatic hybridization succeed where conventional sexual crossing fails between cultivated crops and wild relatives?
- 2. Describe the reagents, osmotic control, and viability dyes used in enzymatic protoplast isolation.
- 3. Compare PEG-mediated chemical fusion and physical electrofusion (AC dielectrophoresis + DC pulse).
- 4. Differentiate symmetric hybrids, asymmetric hybrids, and cybrids, giving a real agricultural example of each.
- 5. What selection methods isolate true heterokaryons, and how does GISH/SSR verify regenerated plants?

✓ Key Takeaways

- Enzymatic digestion (cellulase + macerozyme) in mannitol osmoticum yields totipotent wall-free plant protoplasts.
- PEG + Ca^{2+} and electrofusion unite somatic cells parasexually across reproductive and EBN barriers.
- Cybridization transfers CMS mitochondria and replaces incompatible chloroplasts in a single step, bypassing multi-year backcrosses.
- Today, plant protoplasts are equally critical for DNA-free CRISPR RNP delivery and rapid functional screening.

Recommended Literature

Sources on plant protoplast technology, somatic hybridization, cybrids, and RNP delivery

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2 Additional Literature & Databases

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