Laimer

Teil 1

Plant tissue culture – Definition:
Plant tissue culture is a culture of isolated plant cells, tissues and organs under defined aseptic (keimfrei) conditions. Tissue culture produces clones, in which all product cells have the same genotype (clones) (unless affected by the mutation during culture).
Plant tissue cultures are used to produce recombinant proteins for therapeutic applications, e.g. plantibodies (monoclonal antibodies for a passive immune therapy), antigens (as edible vaccines) and blood clotting factor (Blutgerinnungsfaktor). But also for the production of plant materials, for plant breeding, for gene banks and for production of chemical compounds (secondary metabolites).
Important factors for a successful establishment and maintenance of plant tissue cultures:

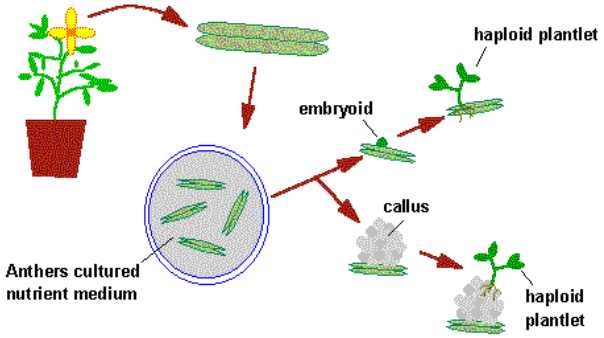
- sterile conditions
- temperature and light conditions
  = important exogenous factors which are influencing the growth and development; must selected according to the requirements of every species
- nutrient medium
  - solid – e.g. agar-agar (physiologically not completely inert, aeration insufficient, diffusion of toxic compounds too slow); activated charcoal (Kohle) adsorbs toxic compounds
  - liquid – requires artificial supports (filter bridges, perlite (Vulkanglas), Vermiculite (Silikate)
  - pH value – to do not affect cell membranes, to assure that salts remain in solution and available, to not influence negatively the uptake of substances, to affect the gelling ability of agar (4-7.0, usually set to 5.7 with 1n NaOH or HCl)
  - mineral salts, carbon source, vitamins, growth regulators (auxins, cytokines) – C, N and S are mainly present in the oxidised forms (CO_2, NO_3^-, SO_4^-), so they have to be reduced before they get integrated into the plant organic matter
  - macro- and micronutrients – according to their required relative amounts and importance

Callus cultures
are undifferentiated cell clusters with different texture, culture and consistency. There are two types – Compact (cells aggregated) and Friable (cells are loosely associated; provides the inoculums for suspension cultures).
A plant callus is a mass of unorganized parenchyma cells derived from plant tissue (explants = any part from a plant) for use in biological research and biotechnology (for testing new chemical compounds, for isolation of mutants, for gene expression studies). In plant biology, callus cells are those cells that cover a plant wound. Callus formation is induced from plant tissues after surface sterilization and plating onto in vitro tissue culture medium. Plant growth regulators, such as auxins, cytokines, and gibberellins, are supplemented into the medium to initiate callus formation or somatic embryogenesis. Plant callus is usually derived from somatic tissues. Plant hormones are used to initiate callus growth.
Plant calli can differentiate into a whole plant, a process called regeneration, through addition of plant hormones in culture medium. This ability is known as totipotency. Genes can be inserted into

callus cells using biolistic bombardment, also known as a gene gun, or Agrobacterium tumefaciens. Cells that receive the gene of interest can then be recovered into whole plants using a combination of plant hormones.
Callus cultures have different capacity for differentiation.

Suspension cultures
are usually initiated from callus cultures. Suspension cultures is a type of culture in which intact cells are maintained in suspension in the culture medium so that they are distributed evenly within it.
The inoculum density deteremines the inital lag phase (Latenzphase; Anlaufphase; in this phase the substances in the medium are analyzed and according to the special substances genes are switched on or off). There are different growth determination methods. Suspension cultures also have different capacity for differentiation.
Suspension cultures are more homogeny than callus cultures. They serve a large availability of material, have a high rate of cell growth and the conditions are easy to reproduce. Plant cell cultures in general are able to produce high-value secondary metabolites and other substances.

Anther cultures
use anthers (Pollensäcke) to culture haploid plantlets



+ technique is very simple
+ it is easy to induce cell division in the immature pollen cells in some species
+ haploids can be produced in large numbers very quickly
+ manipulating the medium components
+ culturing on a semi-solid medium
– within some species- the majority of plants produced are non-haploid
– in cereals very few green plants are obtained
– it is difficult to remove the anthers without causing damage
– sometimes a particular orientation is necessary to achieve a desired response

The anthers are used to induce androgenesis and embryogenesis – so it is an in vitro development/evolution process. The anther cultures allows the regeneration of haploid or dihaploid plants. NOT for production of secondary metabolites!

Protoplast cultures
Protoplast is a plant, bacterial or fungal cell that had its cell wall completely or partially removed using either mechanically or enzymatic means. But the plasma membrane is intact. → naked cells
These naked cells can be used for transient or permanent genetic transformation by introduction of trans-gene DNA, somatic hybridization by protoplast fusion of species or subspecies resistant to traditional cross-breeding, and isolation of sub-cellular organelles.

Protoplast cultures require regeneration of a cell wall to allow further cell devisions and thus growth. These cultures are used for experiments about cell wall regeneration and cell wall structures. Protoplast cultures are good for plant breeding – protoplast fusion (genome fusion).

Somatic embryogenesis

is a process where a plant or embryo is derived from a single somatic cell or group of somatic cells.
Somatic embryogenesis (SE) is a cloning technique based on tissue culture, where an unlimited number of identical embryos can be produced.
In general, plant embryogenesis is the process to develope plant embryos, being either a sexual or asexual reproductive processes that forms new plants. Natural embryogenesis is a result of sexual fertilization (embryo and endosperm together develop into a seed) and these embryos are called zygotic embryos (zygotic embryogenesis) and develop into seeds (and further seedlings). Plant cells can also be induced to form embryos in plant tissue culture – these embryos are then called somatic embryos. (somatic embryogenesis)
Somatic embryos are formed from plant cells that are not normally involved in the development of embryos (so from ordinary plant tissue). No endosperm or seed coat is formed around the somatic embryo. An example could be the generation of whole plants from single cells called protoplasts. First, the cells from tissue are cultured to form an undifferentiated mass of cells called callus. Plant growth regulators (auxins, cytokinins but also ammonium ions, high T and light induce somatic embryogensis) are used to induce the callus formation and further the building of somatic embryos. The indirect way is from callus with growth hormones to embryogenesis, the direct way is out of parenchymatic cells directly formed embryo.
Somatic embryogenesis is a model to understand the physiological and biochemical events that occur in the development process and is an alternative organogenesis for regeneration of a whole plant.

Stages of somatic embryogenesis:

1. Induction (with auxin)
2. Development and maturation
    a. Globular Stage: Embryo is small and round (multicellular)
    b. Heart Stage: shape changes to a heart shape with more cotyledon (Keimblatt) development (bilateral symmetry)
    c. Torpedo-shaped stage: consists of initial cells for the shoot/root meristem
    d. Mature Stage: embryo becomes cylindrical
3. Germination (conversion)

or

1. Initiation of embryogenic tissue (culturing of immature or mature zygotic embryos)
2. Proliferation or maintenance (establishment of embryogenic culture and continuous grow of tissue)
3. Maturation of somatic embryos
4. Germination of somatic embryos
5. Early growth ex vitrum (establishment of invitro-grown somatic seedlings)

Somatic embryo formation from cell and suspension culture:

1. culturing friable callus to a liquid medium
2. under agitation single cell break off and by division form cell chains and clamps which fracture again to give single cells and other small cell group
3. non-organized mass of vacuolated parenchyma cells arise

4. transformed into cytoplasma-rich cells which become embryogenic under the influence of auxin
5. embryogenic tissues then transferred into liquid medium – there the tissues proliferate and form the somatic embryo

Propagation of the selected cultivars – Stages (Skript laimer)

- Phase O (Debergh): Pre-conditioning of donor plants
- Phase 1: Initiation (initiation medium)
- Phase 2: Multiplication (propagation medium)
- Phase 3: Rooting (rooting medium)
- Phase 4: Acclimatization (to the greenhouse conditions; major challenges are the loss of humidity and infections)

Differences between zygotic and somatic embryo:

	Somatic embryo	**Zygotic embryo**
formed by	sporophytic cells	fertilized egg or zygote
covered by	no covering	seed coat
output	only form embryo	seed
nature of plantlets	weak	healthy
alike	mother plant	not like mother plant
propagation rate	high	comparatively low

Advantages /applications of somatic embryogenesis

- higher propagation rate (for clonal propagation, mass propagation)
- suitable for suspension culture
- plantlets from single genetically modified single cells
- somaclonal variation
- germplasm preservation (Bewahrung, Erhaltung)
- labour savings (Arbeitseinsparung) – generation of a whole plant from single cells
- organogenesis
- embryonic tissue can be maintained in liquid nitrogen (cryopreservation; -130 - -196°C) without loss of viability or change on genetic level
- synthetic seed technology possible (embryos are coated with synthetic chemicals → behave like a true seeds (clones))
- elimination of viruses possible
- genetic transformation

Disadvantages

- response is tissue specific
- confined to few species
- inability to generate large numbers of normal, free living plantlets
- maybe low frequency embryo production
- production of malformed embryos, incomplete embryo maturation
- may create unwanted genetic variation (due to somoclonal variation)

Development stages of somatic embryos. what is the advantage of somatic over zygotic embryos in secondary metabolite production?
Applications for somatic embryogenesis cultures?

Teil 2

Differences between plant cell suspensions and microbial cultures?

Features	**Microbial cultures**	**Plant cell suspensions**
size, individual cells, aggregates	1 – 10 µm, often	40-200 µm, frequently
growth rates	rapid, doubling times in hrs	slow, doubling times of days
inoculation density	low	high, 5-10 %
aeration	often high	low, 1 µmol, O_2 h^{-1}
shear sensitivity	insensitive	more sensitive
variability, stability	stable	unstable with considerable variation
extracellular production, extracellular polysaccharides, foaming	foaming	foaming and meringue (Schaumgebäck) formation
product formation	often extracellular	mostly intracellular into vacuole

Belüftung und Durchmischung: Pflanzenzellwand ist empfindlich gegenüber Schaumbildung durch Rührer, keine mechanischen Rührwerke, Produktakkumulation meist in den Zellen (vacuolen) schwerer hohe Expressionslevel zu erreichen Pflanzenzellen schwierig zu transformieren New Brunswick adoptierer Bioreaktor, weniger aggresiver Rührer Rollflaschen (keine scaleup, einfach, nicht sehr steril)

Plant cell suspension:
Lag phase, Exponential growth phase, linear log phase, stationary phase and necrotic phase. Metabolite production only occurs in the last 2 phases. Separation of growth and production phase requiring different culture conditions. In plant cell cultures the product formation occurs in the stationary and necrotic phase. Separation of growth and production phase requiringdifferent culture conditions. (Bild skript II seite 4)

Different culture systems of plant cell suspensions

- batch culture – single addition of nutrient medium
- open continuous culture - addition of nutrient medium and harvest of cell material
- closed continuous culture - addition of nutrient medium without harvest of cell material
- semi-continuous culture – frequent addition of nutrient medium and harvest of cell material

more detailed:

- batch culture – single addition of nutrient medium; discontinuous sigmoid growth
  example: two phase culture is being used for the production of Shikonin

- open continuous culture - addition of nutrient medium and harvest of cell material; cells are permanently in the log phase (no metabolite production)

- closed continuous culture - addition of nutrient medium without harvest of cell material; cells are immobilised in a perfusion culture system; eg. for biomass increase in the production of berberin
- semi-continuous culture – frequent addition of nutrient medium and harvest of cell material; eg. used for the recycling of already built up biomass for the biotransformation of β-methyl-digitoxine

Mass culture systems- Reactors

- Brunswick reactor: increasing stirrer blade→ decrease speed (plant cells are less robust)
- Roller bottle system: simple, no possibility for scale up
- Carboy system 30-134 l
- Glass fermenters, 130l and 600l pilot plant fermenter, STR
- 65-1600 l bubble type reactor (airlift): basal entrance of air→ agitation; reduced growth put down to reduced stirring

Natural products – are also called **secondary metabolites**, are composed of low molecular weight which are often restricted to special plant families or even genera. They are not important for the primary metabolism of plants, but in many cases of great importance for the survival of the plant in its environment. About one quarter of all prescribed medicines used worldwide is of plant origin. We can use these natural products as:

- pharmaceuticals
- cosmetics
- dye stuff
- food additives
- agrochemicals

Plants produce a high number of compounds used as pharmaceuticals or drugs: willow (aspirin), foxglove (digoxin), pacific yew (taxol), coffee and tea (coffein, theine).

10 most widely used plants derived pharmaceutical products

substance	**application**	**plant species**	**info**
steroids from diosgenin	inhibitors of ovaluation, cortisone preparations	*Dioscorea deltoidea*	production could be increased by addition of cholesterin (biotransformation); suspension cultures produce diosgenine in the stationary phase (up to 7.8 % of dry weight)
codein	analgetic	*Papaver somniferum*	in dried milk sap of poppy capsules; cures cough
antropin	anti-cholinergic	*Atropa belladonna*	=tropane alkaloids; in root cultures
scopolamine	anti-cholinergic	*Datura metel*	
Hyoscyamin	anti-cholinergic	*Hyoscyamus niger*	
digoxin	heart diseases	*Digitalis lanata*	callus and suspension cultures failed to produce the desired products
digitoxin		*D. purpurea*	
reserpin	against hypertony	*Rauwolfia serpentine*	

Pilocarpine	cholinergic	*Pilocarpus jaborandi*	
Quinine	anti-malaria	*Chinona ledgeriana*	

Malaria

Plasmodium falciparum is the causal agent of malaria. Plasmodium is transmitted by infected insects of humans. The bark of chincona contains chinine, a compound able to kill plasmodia. However, Plasmodium developed resistances against chinine, which makes the search for alternative antimalaria compounds necessary. Artemisia annua contains novel anti-malaria compounds. Artemisinin belongs to the endoperoxide class of anti-malarial drugs. Artemisa is grown in China, Vietnam and East-Africa countries. The extraction from dry leaves and flowers occurs with *n*-hexane. The active compound is mainly localized in glands of essential oils.

Secondary metabolites in tissue cultures?

Mass culture system for plant cells

Secondary metabolites are not directly needed by plants as they don't perform any physiological functions. They may include pharmaceuticals, flavours, fragrances, cosmetics, food additives, feed stocks and antimicrobial. Secondary metabolites are produced in high levels by plant cell cultures. SUSPENSION CULTURES! For example steroids from Diosgenin, shikonin, ubiquinone-10, berberine, nicotine and diosgenin.

Production of secondary metabolites seems to be linked to some degree of organization of plant cells, e.g. organs. Somatic embryogenesis represents a valid alternative if the substance is produced in organized tissues e.g. late developmental stages of somatic embryos of digitalis lanata synthesize cardenolides (a group of steroids), while early stages cannot.

Many secondary metabolites are toxic or repellant to herbivores and microbes and help defend plants producing them. Production increases when a plant is attacked by herbivores or pathogens. Production of s.m. depends on time and space (räumlich und zeitlich begrenzt) and can be initiated through the environment (medium).

There are 4 possible situations observed with regard to secondary metabolites

1. plant tissue cultures produced the expected products in sufficient amounts
2. plant tissue cultures did not produce the expected products in economically sufficient amounts
3. plant tissue cultures produced other than the expected products
4. plant tissue cultures modified added precursor molecules to the expected more valuable product (bio-transformation)

Not all cell types produce the desired metabolite. **Hairy root cultures** represent a valid alternative, if the substance is produced in the roots. Scientists have developed a form of root culture using *Agrobacterium rhizogenes*, the cause of hairy root disease. Cells transformed with some of the bacteria's DNA, causes the cells to be more sensitive to the hormones they produce. The cells form into roots. These roots grow very fast and produce the s.m. that ordinary roots produce. Roots often secrete the metabolites into the surrounding medium, making it easy for collection. Charcoal can be added to the medium, the metabolites are absorbed by the charcoal, and this stimulates even higher

production of the metabolite. Addition of precursors to the medium enhances product formation. Elicitors are the compounds of biological origin which stimulate the production of s.m., and the phenomenon is called ELICITATION.
The production of plant secondary metabolites by means of large-scale culture of plant cells in bioreactors is technically feasible. The culture of differentiated cells, such as (hairy) root or shoot cultures, is an alternative, but is hampered by problems in scaling up of such cultures. Metabolic engineering offers new perspectives for improving the production of compounds of interest.
Secondary metabolites are stored in the vacuole or in specialized cells (like öldrüsen); in cell culture 2-phase-system is done so that the products get enriched in the lipophilic phase (e.g. Shikonin). For s.m. stored in vacuoles of cells, two membranes have to be disrupted – with DMSO.

- Galantamine for Alzheimer's disease from Galanthus Woronowii – but now synthesized.
- Shikonin from Lithospermum erythrorhizon is antibiotic
- Protoberberin is also antibiotic and anti-inflammatory
- Taxol from Taxus sp.
- Paclitaxel is produced using the plant cell fermentation technology (with no chemical transformation)
- Gingseng (disease-healing) from field cultivation, but also plant cell and tissue culture methods (focusing on physiological and bioengineering factors affecting the productivity of ginseng biomass and useful metabolites)
- Berberine usually found in roots, rhizomes, stems and bark; anti-microbial, also dye wool leather, wood (yellow), also histology dye

Basic ways to **increase productivity**, thereby lowering costs are

- acquisition of highly productive cell lines
    - cell lines were selected by repeated cell aggregate selection and by protoplast selection from Coptis japonica for producing berberine and from Lithospermum erythrorhizon producing shikonin
- establishment of culture conditions and procedures that ensure maximum productivity of the cell line

Strategies for production of secondary metabolites

- selection of cell lines for high yield of secondary metabolites
- large scale cultivation of plant cells
- increase production of s.m. in plant cells
- biotransformation using plant cell culture is a possibility
- culture conditions must be optimized (sugar, hormones, vitamins, light, temperature)

Biotransformation

Conversion of one chemical into another by using biological system as biocatalyst is called biotransformation or bioconversion. Conversion of some less important substances to valuable medicinal or commercially important products. May involve many reactions. E.g. large scale production of cardiovascular drug digoxin from digitoxin by *Digitalis lanata*.

Teil 3

Plants as expression systems – foreign genes can be produced in plants by:

1. transient expression using virus-based vectors
2. permanent insertion into the plant genome
3. transient expression from Agroinfiltration

Transformationmethods

a) Infection with agrobacterium or plant virus – biological principal
b) gene gun - biolistic
c) transformation of protoblasts – physical principal

Characteristics of **plant viruses**:

- obligate parasites; multiply in living cells
- localized or systemic spread in plants
- nucleoproteins (nucleic acid plus protein)
- only one kind of NA (either RNA or DNA)
- many units of the same protein (protein subunits) make up the coat protein (capsid)
- few plant viruses have membrane envelop
- only NA (RNA or DNA) necessary for infection

Tobacco mosaic virus – TMV

Replication cycle of TMV:

1. TMV enters a wounded plant cell
2. TMV-Coatprotein stripped from RNA
3. host translates RNA Polymerase – RP generates anti-sense RNA
4. anti-sense RNA generates plus-sense TMV RNA
5. anti-sense RNA generates plus-sense sgRNAs (subgenomic) and expression of movementprotein (MP) and coatingprotein
6. plus-sense TMV RNA encapsidated form new particles reaching $10^6 – 10^7$ virions per cell
7. plus-sense TMV RNA wrapped with MP moves to an adjacent cell for another round of replication

Cell-to-cell movement of TMV:

1. MP binds to the viral RNA
2. host proteins and/or other virus-encoded proteins may be in the MP-complex
3. the MP-complex then moves from cell-to-cell through the plasmodesmata
4. when the complex enters a new cell, the MP (and other host proteins) are released from TMV RNA
5. translation of genomic RNA to express the replicase proteins and initiation of a new round of replication

Tranlocation of TMV

- Cell-to-cell movement (through plasmodesmata, multiply in parenchyma cells, 1mm/day, takes about 2-5 days
- Long distance movement (through phloem to growing regions, re-enters parenchyma cells)

Systemic vs. local infection of viruses

a) Systemic infection: pathogen is spread, mosaics not tissue restricted, 100.000-million particles/cell, some restricted to phloem, meristem tip free of virus!
b) Local infection: pathogen stays at the place of attack, plant restrict virus movement, hypersensitive reaction, cell death

Tobacco mosaic virus resistance gene, N

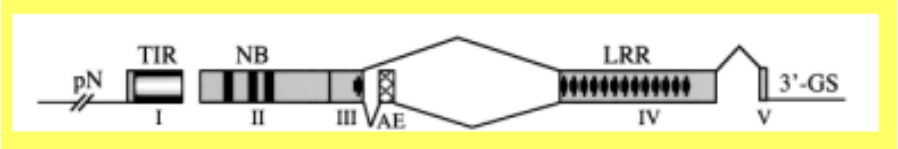
- N – for necrotic type response to TMV infection
- **N-gene** confers resistance to all but one Tobaccoovirus
- Infection with TMV induces hypersensitive response within 48 h at the site of infection
- **SAR** (systemic acquired (erworben) resistance): induction confindes (begrenzt) virus to a small region surrounding the necrotic spot
- SA (salicylic acid) inhibits TMV replication
- N-gene mediated hypersensitive response is temperature sensitive (HR occurs below 28°C)
- at temperatures higher than 28°C TMV spreads normally
- returning to permissive temperature allows restoration (wiederherstellung) of HR function
- N-gene was the first plant virus R-gene to be cloned
- N-gene resistance to TMV have **functions** in heterologous systems – transgenic expression induces necrotic lesions and restriction of virus spread and is reversibly inactivated by higher temperatures (e.g tomato!)
- therefore potential agronomic value, valuable tool for a fast forward genetic approach termed Virus induced gene silencing (VIGS – see later)
- TMV based vectors expressing GFP are tools for studies of virus movement and of N-gene mediated resistance to TMV.

N-Gene

- encodes two transcripts N_S and N_L by alternative splicing, both required to confer (verleihen) resistance
- N_S transcript encodes the full length N protein (131.4 kD)
- N_L transcript encodes a truncated (gekürzt) protein (75.3 kD) because of frame-shift in the reading frame
- the genome of TMV contains 6400 bp
- genomic RNA acts as mRNA for 2RdRp-proteins (RNA dependent RNA polymerases)
- movement protein and coat protein are encoded by subgenomic RNA

N-Protein contains 3 domains:

- N terminal TIR domain (toll-interleukin-1-recpetor homology)
- central NB domain (nucleotide binding)
- C-terminal LLR domain (Leucin rich repeat)



Virus induced gene silencing – VIGS

Early observations

- virus genes inserted into host will cross protect (reduce and slow infection)
- antisense RNA interferes with virus replication
- attempts to overexpress transgenes leads to silencing

RNA viruses carrying sequences homologous to a transgene or an endogenous gene can both be a triggers and target of PTGS (post transcriptional gene silencing); a vector engineered to carry host-endogenous sequences can be used to silence to function of those endogenous genes; PTGS (triggered by the recombinant viral vectors) will target both the viral vector and the host sequences corresponding to the insert. The phenotype of the plant silenced by VIGS for a particular gene mimics the **phenotype of loss of function mutant.**

Features of silencing

- dsRNA are essential but only an intermediate
- sequence specific - at least 20 nucleotides homology
- effective against exons
- si RNA are produced during silencing
- silencing spreads systemically via the larger class of siRNAs
- limitation: some viruses have a reduced ability to spread through the plant (to understand: GFP as a reporter gene)

Role of viruses – Potatoe Virus X (PVX) vector containing plant genes e.g. PVX-GFP will silence GFP in GFP tobacco
Is silencing important for plant virus replication? – plant viruses produce high amount of ds RNA, plants may recover from virus infection (silencing?), some plant viruses contain anti-silencing genes

VIGS – TRV
Tobacco Rattle Virus is a bipartite positive sense RNA virus. cDNA clones of RNA-1 and RNA-2 of TRV allow the cloning of target gene sequences for VIGS. RNA-1 and RNA-2 constructs are mixed with agrobacterium tumefaciens and infiltrated into the leaves; viral RNA gets synthesized and their transcripts serve as templates for further replication for viral RNA. Systemic infection by the virus then bring about VIGS of the targeted plant host sequences. TRV-based VIGS vector can be very effectively used in plants to identify gene functions e.g. tomato, petunia, potato, poppy...
TRV-based VIGS system can also be used to silence genes e.g. flower specific genes or fruit specific genes.

Plant viruses as vectors for TRANSIENT GENE EXPRESSION in plants

Synergistic interactions of some viruses can break resistance in double infections but also induce it. Some transgenes encode proteins involved in the suppression of gene silencing responses and probably other plant resistance responses.

Why use viruses as gene vectors?

- Production of commercially interesting products (high level of expression)
- Test effect of a gene on a plant by either expressing/inactivating

How to use viruses as gene vectors?

- mechanical inoculation of appropriate host plants
- harvest of biomass and purification of molecules of interest

Advantages of using transient expression system using viruses:

- Absence of position effect in the plant genome
- Infection with a virus based vector is far simpler and quicker than stable transformants (where gene in plant itself is changed)
- Higher flexibility with constructs
- Amplification by the viral vector gives higher level of expression

ds DNA virus

- e.g. CaMV Cauliflower Mosaic Virus (easy to manipulate, clonal viral DNA was infectious, high replication rate (efficient), but template switching and translation of ORFs complex and disruption sensitive, so not very stable)

ss DNA virus

- Bipartite Geminivirus (e.g. ACMV African Cassava Mosaic Virus or TGMV Tomato Golden Mosaic Virus) – choose vector according to the plant - infect dicotylenonous species

- Monopartite Geminivirus (e.g. MSV Maize Streak virus or WDF Wheat Dwarf Virus) - infect monocotyledonous species

ss RNA virus

- grow to extremely high titers in infected plants
- but: the development of RNA virus-based vectors with difficulties involved in genetically manipulating RNA at first; later: cDNA copies of RNA viruses were infectious on their own; plasmids containing full length cDNA clones of viral RNA are directly infectious to the plants; e.g. TMV, PVX (potato virus X), AIMV, CPMV Cowpea Mosaic Virus

Welche Möglichkeiten zur Genexpression in Pflanzenzellen kennen sie, vor und Nachteile? Vorteile von Pflanzen als Expressionssystem?

Transgene pflazen, akkumulation: gute ausbeute,permanente linie möglich, ökonomisches, scale up, lange produktionsdauer, keine wirkliche regulation des stoffwechsels möglich

Transgene pflazen, sekretion: Keine Reinigung nötig, containment gut(was auch immer das heißt), schlechte ausbeute+scale up, produktionskosten hoch

Transplantome Pflanzen Gute ausbeute, niedrige toxizität, containment,Keine glykosylierung, event. Horizontaler gentransfer
Virusinfizierte Pflanzen Gute ausbeute,kuze zeit, gemischte infektion Schlechte biosicherheit, limitation in konstruktgröße
Agroinfiltrtion Kurze zeit,
Zellkultur Kurze zeit, containment, secretion ins medium->keine aufwendige reinigung, regulation möglich Kosten hoch

RNA viruses as gene vectors
For proteins that need post-translational modified subgenomic expression vectors are used; the foreign gene is inserted after the subgenomic (sg) promoter followed by a second sg promoter for the CP; BUT homologous recombination of the 2 copies of the subgenomic promoter may lead to an exact deletion

Foreign peptides fused to viral coat protein geness
Synthetic oligopeptides might not be able to elicit an immune response;
Epitope presentation consists of a carrier molecule able to assemble into a macromolecular structure, e.g. capsid protein
TMV CP accumulates as much as 10% dry weight→ assuring high yield
Modified CPs display the foreign peptide on the surface→ immune response
Foreign genes may be expressed as readthrough fusions with a protein (sequence of interest is introduced after an amber stop codon downstream the CP gene) or from a subgenomic promoter

e.g. HIV-I, capsid epitope, CPMV
e.g. influenza virus, HA epitope, TMV

➔ Plant and animal disease control!

TMV as gene vector

- to produce therapeutical reagents
- e.g. malaria epitopes
- e.g. vaccine candidate against Hep C
- e.g. recombinant food allergens

CPMV as gene vector

- Cowpea mosaic virus - Bipartite positive sense RNA virus
- RNA 2: 60 copies of “large coat protein”, “small coat protein”
- Insertion of foreign DNA into the small coat protein of CPMV-RNA
- Infection of plants; extraction of modified virus
- Successfully applied for the presentation of antigenic sites of
    - Food and mouth disease virus (FMDV)
    - HIV-1
    - Human Rhinovirus 14 HRV-14

AIMV as gene vector

- Alfalfa mosaic virus
- Production of therapeutical virus
- e.g. HIV-1
- e.g. Experimental rabies vaccine (Tollwut)

PVX as gene vector

- for production of valuable reagents e.g. functional single chain antibodies

Virus like particles VLPs

are non-infectious, non-replicating viral protein shells produced using recombinant technology. To create VLPs, the viral genes that make up the viral structure (capsid proteins) are expressed in a production system. VLPs can trigger an immune response without any risk of infection!
When expressed in a suitable heterologous system, viral structural proteins involved in capsid formation often self-assemble to form three-dimensional, virus like particles composed of multiple viral antigens in the absence of other viral components usually required for virus assembly (such as structural or non-structural proteins and viral genomes).
Usage:

- as carriers for foreign epitopes (vaccines)
- as foreign antigen presentation systems

Pros and Cons

+ no risk of infection
+ no replication
− need of accurate heterologous system for production of VLPs
− knowledge of conditions about VLP assembly necessary

Agroinfiltration

First step of the protocol is to introduce a gene of interest to a strain of Agrobacterium. Subsequently the strain is grown in a liquid culture and the resulting bacteria are washed and suspended into a suitable buffer solution. This solution is then placed in a syringe (without a needle). The tip of the syringe is pressed against the underside of a leaf while simultaneously applying gentle counterpressure to the other side of the leaf. The Agrobacterium solution is then injected into the airspaces inside the leaf through stomata, or sometimes through a tiny incision made to the underside of the leaf.

Teil4

Different expressionsystems in plants for recombinant proteins:

Table 1 | **Comparison of production systems for recombinant human pharmaceutical proteins**

System	Overall cost	Production timescale	Scale-up capacity	Product quality	Glycosylation	Contamination risks	Storage cost
Bacteria	Low	Short	High	Low	None	Endotoxins	Moderate
Yeast	Medium	Medium	High	Medium	Incorrect	Low risk	Moderate
Mammalian cell culture	High	Long	Very low	Very high	Correct	Viruses, prions and oncogenic DNA	Expensive
Transgenic animals	High	Very long	Low	Very high	Correct	Viruses, prions and oncogenic DNA	Expensive
Plant cell cultures	Medium	Medium	Medium	High	Minor differences	Low risk	Moderate
Transgenic plants	Very low	Long	Very high	High	Minor differences	Low risk	Inexpensive

system	**Overall cost**	**timescale**	**Scale up**	**Product quality**	**glycosylation**	**Contamination risk**	**Storage cost**
Bacteria	Low	Short	High	Low	None	endotoxins	
Yeast	Medium	Medium	High	Medium	Incorrect	Low risk	
Animal cell culture	High	Long	Very low	Very high	Correct	Prions, virus, onco DNA	
Animals	High	Very long	Low	Very high	Correct	Prions, virus, onco DNA	
Plant cell culture	Medium	Medium	Medium	High	Minor diff	Low risk	
plants	Very low	long	Very high	high	Minor diff	Low risk	

Concerning glycosylation: there are certain residues in sugar moieties that are present in humans but not in plants; they need to be added; those present in plants but not humans need to be removed!

Plants as expression systems for recombinant proteins

- most economic producers of biomass
- Free of bacterial endotoxins
- Free of animal pathogens
- Eukaryotic protein mod allow proper folding, correct glycosylation and targeting
- cheaper than with animals

Therapeutical applications:

- Plantibodies: monoclonal antibodies for passive immune therapy
- Antigens as edible vaccines
- Blood clotting factors

Examples
Growth hormone expressed in tobacco: first human protein expressed in plants
IgG1 expressed in tobacco: by crossing plants that expressed heavy and light chain
Hep B virus envelope protein expressed in tobacco: first vaccine candidate
Interferon
Insulin

Gene transfer in plants:

- Plant protection

- Improvement of qualitative and quantitative traits (Merkmale)
- Improvement of processing parameter/properties
- Pharmaceutical application
- Biofuel plants
- Solutions for developing countries

Development of transformation
Input: improvement of agronomic traits, resistance against diseases or stress, release of environment in production, improved storage, cost reduction
Output: improved qualitative traits, change of fatty acid composition, vitamin content, taste, processing traits, undesirable compounds

In the first steps of biotechnology the agronomic traits were improved to enhance the yield (e.g. bt technology, herbicide resistance); now more and more there is work done on the quality features of plants themselves (fatty acid composition, aa,..) and their use as production factories (vit, enzymes, fatty acids, polymers..)

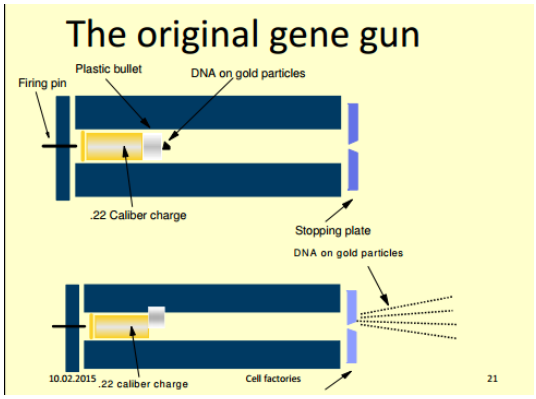
Requirements for the production of transgenic plants:

- first requirement: regeneration of plants from cell lines: organogenesis/somatic embryogenesis
- Second requirement: methods of gene transfer (agrobact., particle gun or protoplast; see later)
- Third requirement: A way to get plants back from only those cells that received DNA (use of antibiotic resistance genes)
- Fourth requirement: case by case confirmation (e.g. molecular analysis of transgenic grapevines to determine copy number)

Organogenesis= The development of adventitious organs or primordia from undifferentiated cell mass in tissue culture by the process of differentiation is called organogenesis.

Gene transfer in plants – methods:

- Integrating systems
  - Ti-plasmid vector (agrobacterium)
  - Transposable (vertauschbar) elements
- Direct gene-transfer
  - PEG-method
  - Electroporation
  - Microinjection
  - Microprojectiles
  - Laser beams
- Viral vectors
  - Caulimovirus
  - Geminivirus



The gold particle is slammed onto the stopping plats→ burst into particle and genetic information is blasted into the sample

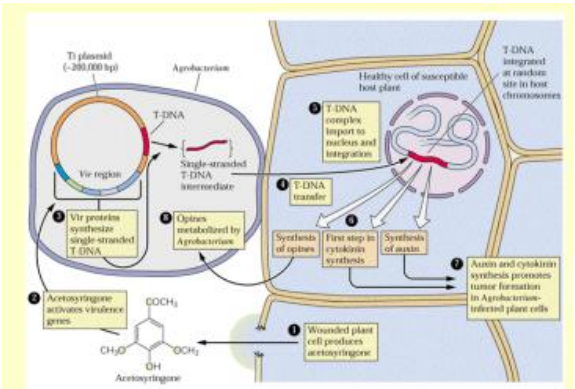
Agrobacterium tumefaciens

can transfer DNA into plant cells! The virulent ones have a Ti (tumor inducing) Plasmid with genes, the Ti plasmid integrates a segment of its DNA in plants after infection = T-DNA; A. tumefaciens has flagella (can swim); 25 vir genes are necessary for tumor induction; T-pilus to transfer T-DNA into the plant cell

Early discoveries: Tumorous plant cells were found to contain DNA from bacterial origin integrated in their genome. Furthermore the transferred DNA (named T-DNA) was originally part of a small molecule of DNA located outside the chromosome of the bacterium. This DNA molecule was called Ti plasmid

In vivo wounded, dicotelydonous plants (zweikeimblättrig) are infected! Recognition of the plant cell by the bacterium with help of pectines and glucanes on the surface. In lab → also monocotelydonous plants can be infected. A.t. induces extensive growth (wucherungen)

2 types: rhizogenes and tumefaciens
Gram-negative, soil-inhabiting bacteria, rod shaped, motile by flagella

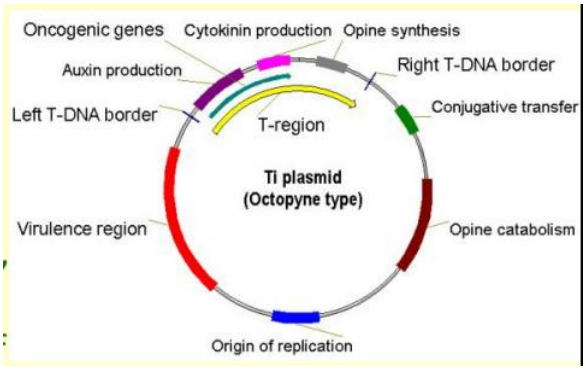


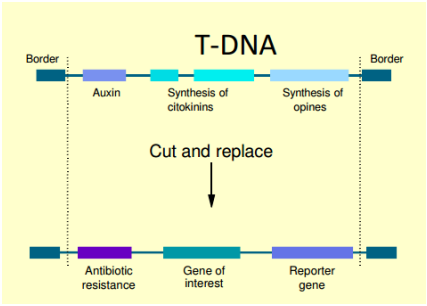
1. Wounded plant cell produces acetosyringone (and also vanillin, sinapin acid, katechol,...)

2. these secondary plant substances induce chemotaxy in the bacterium and activate virulence genes (vir genes) which encodes for proteins, that can enter the plant
3. attachment of bact. by cellulose fibrils
4. Vir proteins synthesize single stranded T-DNA with VirD2-protein at 5'end (for nucleustranslocation)
5. T-DNA excise (herausschneiden) and transfer
6. Into nucleus and integration (randomly) in host genome
7. Synthesis of auxin, opines and cytokinins (via activating of promoter which is recognized by the plant; the transferred sequences have a typical eukaryotic structure although they are from a bacterium). Auxin und cytokinin synthesis promotes tumor formation in plant cells
8. Opines metabolized by agrobacterium
9. Auxin and cytokinins in excess→ tumor formation (cell proliferation and gall formation)

Auxins and cytokinins are involved in <u>crown gall disease</u> caused by A. tumefaciens.

T-DNA= transferred DNA
Ti-plasmid =Tumor inducing plasmid





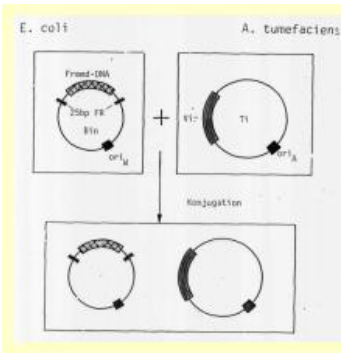
Plasmid size 200-300 kbp; plasmid destroyed at 37°C

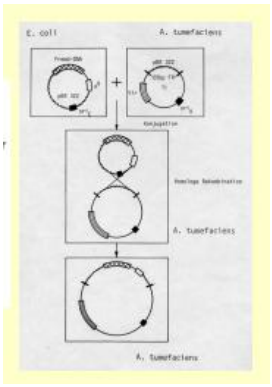
Basic elements from the Ti-plasmid were taken to design a vector:

- T-DNA border sequences; right border necessary for integration into the genome
- Vir genes required for transport of the T-DNA region to the plant
- Modified T-DNA region: tumor formation genes are removed and gene of interest GOI inserted;
- Transformed plants appear normal and are in most cases fertile

Chimeric genes = made from genetically different genes
Promoter-GOI-terminator: each part can derive from a different organism
Promoter: expression level, constitutive, tissue specific
Polyadenylation site: stabilizes the mRNA

The vectors will either integrate or stay as an episomal plasmid: co-integrative vectors vs. binary vectors:





A way to get plants back from only those cells that received DNA

Use of antibiotic resistance; the challenge is to get plants solely from those that were hit e.g. by a bullet→ if an antibiotic is added only those with the resistance gene will grow!

ARMG= antibiotic resistance marker genes; are of environmental concern→ risk assessment of GMOs (genetic modified organisms); e.g. how likely is horizontal gene transfer to microbes? ARMG need to be identified and phased out (ausmustern)

But the GMO panel considers the frequency of horizontal gene transfer from GM plants to other organisms as very low for all ARMGs considered.
The GMO panel has defined **3 risk classes** with different potentials to compromising human health and the environment;

1. group: include genes conferring resistance to kanamycin and hygromycin; no rationale for inhibiting or restricting the use of genes in this category Group: field trial only; e.g. ampicillin
2. group: includes resistance to chloramphenicol, ampicillin, streptomycin and spectinomycin; restricted to field trial purposes and not be present in GM plants
3. group: resistance to amikacin and tetracycline; only clinical usage, not present in GM plants (not on market or field trials)

Zinc finger nuclease

- Principle of zinc finger nuclease facilitated site-directed homologous recombination (gene targeting) and potential application of customized zinc finger protein technology
- Zinc fingers are protein domains formed by the coordination of 4 aa around a zinc atom – resulting in a unique finger-like structure
- Most common DNA binding protein motif
- Many transcription factors contain this motif

Application:

- Gene targeting

    - Specific gene knock out
    - Gene correction
    - Site specific insertion/integration
    - Site specific deletion
- Gene regulation
    - Upregulation
    - Downregulation

Transgenic fruit trees

Modifiable (veränderbar) traits:

- Resistance to abiotic stress
- Resistance to biotic stress
- Modified growth habit
- Altered nutritional qualities
- Altered processing and storage qualities

Transformation methods for fruit trees:

- Indirect
    - Ti/Ri plasmid of Agrobacteria
- Direct
    - Microprojectiles
- Combined methods

Regeneration systems for fruit trees:

- Adventitious shoot (austrieb) formation
    - From leaf discs
    - From immature zygotic embryos
    - From roots
- Somatic embryogenesis
    - From petioles (Blattstiel)
    - From immature embryos
    - From anthers (staubbeutel)

Examples:
e.g. Knap1 from Malus, kn1 homologue in maize and KNAT1 homologue in Arabidopsis transformed into M. domestica Jonagold via Agrobact.- mediated leaf disc transformation, resulted in a modified growth habit with reducec internode length
e.g. Virus resistant apricots: Sharka or Plum Pox Virus, ssRNA virus; flexible virusparticle
e.g. regeneration from somatic embryogenesis of fruit trees: Prunus subhirtella
e.g. Virus resistant grapevines (!)

- Insect transmitted virus: grapevine virus A (GVA), grapevine virus B (GVB)
- Nematode transmitted: grapevine fanleaf virus (GFLV), Arabis Mosaic Virus (ArMV)
    - GFLV: protein mediated (translation of an intact or modified protein) or RNA mediated (based on sense RNA (full or truncated) or antisense RNA)
    - GFLV remains a serious threat in vineyards → transformation remains an attractive alternative to develop GFLV-resistant rootstocks

Recent model for **genetic engineering** approaches for virus resistance:
post-transcriptional gene silencing in plants: the RNA virus is transcribed but recognized → dsRNA mediated DNA methylation and silencing

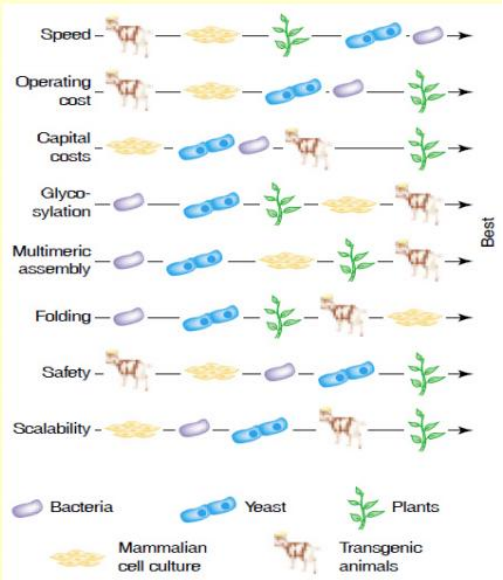
Example of successful GE: golden rice, insectresistant soja beans, herbizid-tolerant sugar beet, fungi-resistant potatoe, virus-resistant pumpkin

Teil 5

Plant made pharmaceuticals

Plant your pharmaceuticals and watch them grow!

Comparison of recombinant protein production systems



Comparison of different plant-based production systems

system	***advantages***	***disadvantages***
Transgenic plant, accumulation within plant	Yield, economy, scalability, establishment of permanent lines	production timescale, regulatory compliance
Transgenic plant, secretion from roots or leaves	Containment, purification	Scale, yield, cost of production facilities
Transplastomic plant	Yield, multiple gene expression, low toxicity, containment	Absence of glycosylation, some evidence of horizontal gene transfer
Virus based	Yield, timescale, mixed infections	Biosafety, construct-size limitations
Agroinfiltration	timescale	cost
Cell or tissue culture	Timescale, containment, secretion into medium, regulatory compliance	cost

Transplastomic plant= A transplastomic plant is a genetically modified plant in which the new genes have not been inserted in the nuclear DNA but in the DNA of the chloroplasts

plant	***advantages***	***Disadvantages***
Arabidposis thaliana	Accessible genetics, range of available mutants, easy transformation	Not used in commercial process (low biomass)
tobcacco	High yield, established transformation and expression technology, rapid scale up	Low protein stability, presence of alkaloids
potato	Edible, proteins stable in storage function	Has to be cooked
soybean	Economical, high biomass, expression in seed coat	Low expression levels, difficult to manipulate and to transform
wheat	protein stability during storage	low yields, difficult to transform and manipulate

There is several ways for pharmaceutical protein production: into the whole plant, cellular compartment (vacuole, ER), tissue specific (tuber, root, fruit) or exudate.

Therapeutical applications for animals or humans:

- Plantibodies
- Antigens as edible vaccines

Antibodies

Are the largest group of molecules in clinical trials, about 20% of all biopharmaceuticals are ab

Plantibodies = plant produced monoclonal antibodies, independent cloning of heavy and light chain in agrobacterium vectors; transformation of plant tissues (e.g. tobacco); regeneration of plants expressing independent chains; crossing for the production of progenies (früchte) producing both chains

Plant-derivied antibodies, the front runners
e.g. **CaroRx**: chimeric secretory IgA/G in transgenic tobacco plants through the expression of four separate transgenes; sequential crossing of plants that could express one of those; the AB recognizes the main adhesion protein of streptococcus mutans, which is a oral pathogen for tooth decay in humans; after application bacteria are removed and do no recolonize for several months

Animal vaccines

First animal vaccine: sterne vaccine against anthrax; sterne vaccine lacks the plasmid pX02 which encodes the protective capsule of the bacteria; problem: being a live bacteria; retains some of its virulence; still used; benefits outweigh the drawbacks

In veterinary medicine, vaccinology addresses a wider spectrum of challenges. These include the development of cost effective approaches to prevent and control infectious animal diseases, considering animal welfare and focusing on decreasing production costs of animal used as food.

Active vaccination using live virulent or attenuated vaccines is widely used, but has its **drawbacks**:

- low levels of immunogenicity
- high production cost
- antigenic variability between species
- possible transfer of genetic material to wild-type strains

Vaccination is also not feasible option for mammals post-weaning (nach abstillen) as wells as in animals such as broilers (Masthähnchen), which have a short life-span.

Vaccine composed of:

1. Attenuated or killed organism
    a. Low level of immunogenicity
    b. High production cost
    c. Antigenic variability between species
    d. Possible transfer of genetic material to wild-type strains
    e. Not feasible for animals post-weaning or broilers
2. Subunits of an antigenic protein from the disease causing organism

Plants can be used as the producers for edible vaccines: in the form of juice, powder or sauce, would be less complicated and easier than purification

Edible vaccines

- For immunisation of populations in developing countries (lack of cold storage)
- Alternative for diseases requiring life long repeats of vaccination

e.g. HepB, cholera, Norwalk virus, traveller's diarrhea (ETEC)

3 WHO goals:

1. Avoid the use of needles (oral vaccine)
2. Remove the need for cold storage (heat-stable)
3. Reduce cost per dose

Regrettably vaccines are not of big economic value and therefore drug companies focus their development efforts elsewhere; although they are of high social value they have lower economical value than other pharmaceuticals
Problematic: R&D costs are the same as for any other pharmaceutical, but activists demanded that companies would produce those vaccines at marginal (geringfügig) price→ pharma companies turned away from the production of vaccines→ "manufacturing gap"

hepatitis B virus infection is probably the single most cause of persistent viremia in humans; acute and chronic hepatitis, which can initiate hepatocellular carcinoma; Prevalence (Verbreitung) of this disease in developing countries→ need of efforts; 2 forms were available, both injectable and expensive (one from the serum of infected individuals, the other from yeast (recombinant antigen expressed))

Potential plant-derived parenteral or oral vaccines against HEP B virus
Alternative HBV vaccine:
Either: expression of the HBV antigene in a plant bioreactor e.g. tobacco→ purified VLPs for parenteral vaccines→ injection in human
Or: production of HBV antigen in edible plants e.g. lettuce→ powdered lyophilized tissue as a semi-finished product → oral formulation e.g. tablet as a booster vaccine

Edible vaccine: Plants were transformed with the gene encoding the hep B surface antigen (HbsAg), the same as used in the yeast derived vaccine; antigenic spherical particles recovered from these plants (are analogous to the recombinant hepatitis surface antigen derived from yeast); parenteral immunization of mice has shown that it retains (behalten) both T and B cell epitopes

Other **examples** of vaccines in edible plants:

- Spinach with rabies virus antigen
- Potatoes with e.coli vaccine
- Vaccine production in bananas
- Carrots and lettuce with Hep B antigen
- tomatoes with bronchitis-vaccine
- Hep B virus antigen (surface protein) in tobacco (early 90ies)

Oral immunization

Lymphoid tissues are localized in the gut; M cells recognized particulate antigens (virus, vaccines, bacteria); mucosal B cells migrate to the mucosal membranes (gut, lung...), mucosal plasma cells produce secretory IgA – oral immunisation results in antibody secretion

Plant based vaccines delivered orally
early work – tobacco was used as model plant system for expression of antigenic proteins; showing that the plant-derived protein had similar antigenicity as do human- and yeast- derived hep B surface antigen (HBsAg); following mice immunization study resulting in stimulation of T-cell proliferation; later it was demonstrated that B- and T-cell epitopes of HBsAg were preserved when the antigen was expressed in transgenic tobacco
e.g. Hep B virus in lettuce leaves (viral major surface antigen); no major safety concern, serum and mucosal immune responses were observed
e.g**. E.coli heat labile entereotoxin LT-B**: constructs carrying the gene encoding the binding subunit of E.coli heat labile enterotoxin (LT-B) were introduced into tobacco and potato plants. Heat labile enterotoxin is produced by enterotoxigenic E.coli (ETEC), the causal agent of an enteric disease, and also interacts with cholera toxin → LT-B is a candidate vaccine against both ETEC and cholera

Increase the expression in transgenic plants:

- Gene optimization
- Strong promoters
- Sub-cellular targeting signals
- Cross high expressing transgenic lines
- Etc.

Banana work

Plasmid containing cholera DNA and a.tumefaciens DNA→ agrobacterium infects the banana→ bacterial DNA is introduced into the plant cell →proteins like those found in the cholera bacterium are produced→ if eaten the body responds as if invaded by a cholera bacterium→ immune response

Why bananas? Readily available, common food staple, eaten raw, no cold storage, could be coloured differently for identification

Potato work

Cholera toxin B subunit (CTB) in potato tubers accumulated at high enough levels that induce both mucosal and serum immune response in mice. Humans given a plant-derived oral vaccine (fed raw transgenic potato tubers (knollen) carrying the recombinant LT-B antigen) produced both serum IgG- und mucosal IgA-specific antibodies

Plant engineering: choose as suitable plant, choose a plant that can be eaten raw
Regulatory: pre-clinical studies with mice; vaccine only a "food additive"

Other plant systems used in the production of vaccines: lupine, lettuce, soybean, corn, rice, wheat

Vaccine Stabilization:
GOAL: heat stable vaccines with a long storage life
APPROACH: Mimic nature's protein stabilization during plant development

Plant-based oral subunit vaccine for the **respiratory syncytial virus (RSV)**
RSV is a serious pathogen that causes bronchiolitis and pneumonia-type disesase in all human age groups. RSV disease occurs throughout the world; more severe in under-developed countries where it results in increased mortality

Transition to regulated biomanufacture
USDA regulated plant production→ GMP processing and FDA regulated→ adaption of food science to vaccine biomanufature: e.g. dehydration of plant tissues mimics nature to protect pharmaceutical proteins in the tissue

Developing GMP System:
Contained batch production and harvest → freeze dry→ grind, blend, sieve for uniformity→ package for delivery (e.g. pH sensitive coatings, aliginate)

Tomato juice can be encapsulated in alginate (Polysaccharid). Dried particles are stable. pH sensitive coatings are possible. Food process technology and ingredients are applicable (anwendbar).

Plant derived vaccines

Better products:

- Heat stable formulation for oral delivery
- Vaccines suitable for long term storage
- Suitability for multiple antigens

Attractive as manufacturing investments:

- Cost-effective food technology
- "scalable" biomanufacturing to limit initial capital cost; requirements as vaccine demand is established

Teil 6

Metabolic Engineering

is the modification of existing, or the introduction of entirely new metabolic pathways in organisms. M.E. in plants involves the modification of endogenous pathways to increase the flux towards particular desirable molecules. Introduction of new pathways enables to use nature's diversity to meet human needs in a sustainable way. (!)

Metabolic response (collect data)→ generate hypotheses→test hypothesis (new experiments)→metabolic behaviour (genetic, environmental,.. perturbations)→ metabolic response

Metabolic pathways are controlled at multiple level and any form of perturbation (störung) can have wide-ranging effects at the whole system level. To elucidate (erläutern) and characterize metabolic pathways rather in a holistic (ganzheitlich) manner than on a step-by-step basis. Direct studies at the level of the metabolome; modelling of a metabolic pathway on a genomic scale;

Single compound targeted approach→ compound class approach→the metabolome

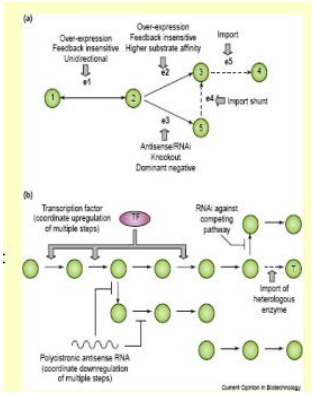
Three common strategies for introducing foreign genes into plant cells:

transformation	advantage	disadvantage
Agrobacterium	Effective, cheap, simple, can be used in germ line transformation; copy number of insertions low	Requires tissue culture regeneration; host range limited (by hypersensitive response)
Particle bombardment	Effective for transient expression; all plant hosts; to produce transplastomic plants	Requires tissue culture regeneration; copy number of insertions high→ gene silencing
electroporation	Effective for transient expression, all plant hosts; high DNA delivery	Requires tissue culture regeneration; copy number of insertions high→ gene silencing

Key focus areas:

- Nutritional value of foods; AA, vit, fatty acids content or iron levels increasing
- Polymer production: modified starch compostition (Amflora)
- Enhanced crop function: resistance to insects, disease, drought; enhanced fertility
- Mathematical modelling: lipid synthesis, primary metabolism, secondary metabolism
- Development of experimental tools: inducible promoters, proteomics, metabolic profiling
- Phytoremediation (Sanierung): heavy metal uptake, arsen, Se, Cd
- Pharmaceutical production: alkaloids, taxanes

Strategies for M.E.



top: single-step metabolic engineering

bottom: holistic approach,e.g. TF upregulation of multiple steps

Engineering primary metabolic pathways – Examples of successful M.E.?

- Carbohydrate metabolism: Centers on the reversible conversion of sugars into storage and structural carbohydrates (starch, cellulose)
- Starch: M.E. to increase the yield and modify the properties of starch (changing the proportion of components amylose and amylopectin)
- e.g. amflora: genetically modified starch-potato cultivar; renewable source for potato-starch industry; high amylopectin starch for improved paper quality
- high-amylose starch: improved trying or as a gelling agent and thickeners
- high-amylopectin starch: improved paper quality; improved freeze-thaw; adhesive manufacture
- low-amylose rice
- novel strategies: use of starch-binding domains to target recombinant enzymes to starch granules during starch biosynthesis
- Cellulose: valuable as pulp, fiber and as a starting point for the synthesis of commercially important polymers; “cellulose biosynthetic pathway”: not fully understood; several enzymes identified: sucrose synthase, CesA family of cellulose synthases and Korrigan cellulases
- e.g. inhibition of sucrose synthase in cotton→ inhibit fiber formation
- e.g. CesA genes → CesA proteins for cell-wall formation

- Fructans: storage carbohydrates; similar texture to fat but far fewer calories (→ interesting as substitute); at present the only commercially available fructan is inulin from chicory; bacterial and plant enzymes are introduced in plants to increase the fructan level; further fructan can be used in the protection of plants from abiotic stress
- Amino acid and polyamine metabolism: increase the abundance of essential amino acids (e.g. lysine, tryptophan...) for feed and food; other aa such as proline are associated with stress response; use of RNA interference (RNAi)

Lipid metabolism

Change the quantity and quality of fatty acids in plants for food industry; production of detergents, fuels, lubricants, paints and plastics; metabolic eng. Of seed oils is attractive because even extensive modification do not affect the growth and development of the vegetative part of the plant

Favourable: unsaturated fatty acids

e.g. transformation of oilseed rape by cDNA encoding enzymes for the desaturation and functional modification of long-chain fatty acids

Engineering secondary metabolic pathways

Challenges for plant metabolic eng.

- Compartmentalization: complicate metabolite and flux analysis, redundancy of pathways
- Current models lack predictive quality
- Dearth (Mangel) of inducible promoters
    - Constitutive promoters: e.g. 35S (viral), upiquitin (plant origin, high level expression, might vary), Actin, 2A11 (tissue specific, fruit); inducible: APase promoter
- Transformation of plants with multiple genes
- Lack of genetic characterization (only 2 sequenced plant genomes!)

Examples:

Terpenoids (Isoprenoids) are diverse polymers. E.g. Carotenoids are terpenoids – natural products like rubber.

Petunia was transformed with the (S)-limonene synthase gene from Clarkia breweri resulting in the production of linalool, which repels aphids (Blattläuse abweisen). Also in tomatoes.

Alkaloids

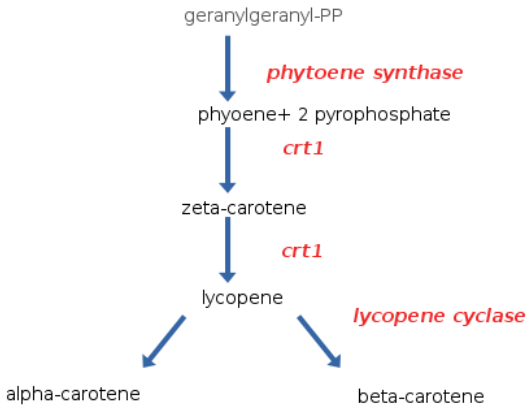
Perhaps the largest group of secondary products synthesized by plants. The pathways are extensively studied.

Isoquinoline, indole and tropane classes.

Golden rice

Golden rice is a variety of rice produced through genetic engineering to biosynthesize beta-carotene, a precursor of vit A, in the edible parts of rice (in endosperm). For areas with a shortage of dietary vit A. Golden rice differs from its parental strain by the addition of three beta-carotene biosynthesis genes. In the year 2000 golden rice was considered as a significant breakthrough in biotechnology! In 2005 – golden rice 2 was developed, which produces up to 23 times more beta-carotene than the original golden rice.

The rice plant can naturally produce beta-carotene in its leaves, where it is involved in photosynthesis. However, the plant does not normally produce the pigment in the endosperm, where photosynthesis does not occur. A key breakthrough was the discovery that a single phytoene desaturase gene (bacterial CrtI) can be used to produce lycopene from phytoene in GM tomato, rather than having to introduce the multiple carotene desaturases that are normally used by higher plants. Lycopene is then cyclized to beta-carotene by the endogenous cyclase in Golden Rice.



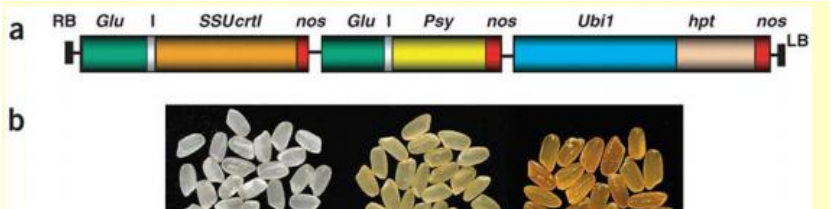
Synthesis of β-carotene from geranylgeranyl diphosphate requires 3 enzymes:

- Psy (Phytoene synthase) from Narcissus Pseudonarcissus
- Crt1 (Phytoene desaturase), synthesis of the red colour, from bacterium
- Lcy (lycopene beta-cyclase), synthesis of beta-carotene (yellow colour)

The transformation occurs with agrobacterium; the heterologous genes derive from daffodils (Narcissus P.) and a bacterium called erwinia; every gene is under the control of a promoter (endosperm-specific promoter, so they are only expressed in the endosperm); Gt1 = Endosperm specific glutenin

- Gt1 promoter-psy
- 35S promoter-crt1
- Gt1 promoter-lcy

Further a selection marker is introduced e.g. hygromycin resistance unter the control of the 35 S promoter; the enzymes can derive from different sources and will show a different beta-carotene yield (e.g. Np=narcissus p, Zm= zea mays) In the past: inserted Lycopene-beta-cyclase-gene from Narzisse; now: gene from maize



Nos=terminator

Future work:

- Introgression of transgenes into varieties (Introgression = movement of a gene from one species into the gene pool of another by a repeated backcrossing of an interspecific hybrid with one of its parent species)
- Evaluate metabolic trade-offs (= losing one quality or aspect of something in return of gaining another quality or aspect)
- Overcome public concerns
- Availability to 3rd world countries (careful: patents, should be available in every country)
- Change eating habits

Other examples of nutritional engineering:

- rice and potato with enhanced carotene level
- lysine-rich corn
- iron-rich lettuce (Kopfsalat)
- lycopene-enhanced tomatoes

Vitamin enhancement by simultaneously increasing the levels of carotene, ascorbate, and folate in corn endosperm, representing 3 entirely different metabolic pathways, was achieved in an elite breeding variety of corn by taking advantage of multigene engineering via direct DNA transfer.

Problematic with the eating habits in the developing world is that people rely on one staple food, which most often these foods are low in many nutrients; people have grown those foods for ages

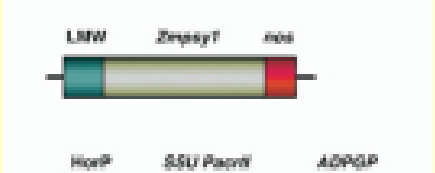
Multivitamin corn

The absence of key minerals in the grain reflects the fact that corresponding pathways are absent, truncated or inhibited in the endosperm

They are several approaches but all of those only target one single nutrient→ multigene engineering increasing the level of folate, ascorbate and carotene in corn

10-14 day old zygotic embryos of a corn breed were transformed bombarding them with metal particles; 5 constructs: the selectable marker bar and 4 cDNAs;

- Zmpsy1: zea may phytoene synthase 1
- Pacrtl: Pantoea ananatis phytoene desaturase



- Osdhar: Oryza sativa dehydroascorbate reductase
- EcfolE: E. coli GTP cyclohydrolase I.

Psy and crtl to increase the beta-carotene content
Dhar to increase ascorbate
Gch1 to increase folate levels

Compared to wt maize or golden rice the mulivit maize shows significantly increase in vitamins

M.E. for production of isoflavones in non-legume plants

Isoflavones = similar structure like oestrogen, so named phytooestrogens; secondary plant substances for defence against pathogens
legume = Hülsenfrüchte

Could distribute the health benefits of these phytoestrogens to more widely-consumed grains. Expression of a soybean isoflavone synthase gene (IFS) in Arabidopsis results in the synthesis and accumulation of the isoflavone genistein (=Östrogen) in leaf and stem tissue. The ability of the heterologous isoflavone synthase enzyme to interact with the endogenous phenylpropanoid pathway, which provides its substrate, was investigated in several plant tissue systems. In tissues that undergo naturally enhanced synthesis of anthocyanins, genistein production was enhanced. Induction of the flavonoid/anthocyanin branch of the phenylpropanoid pathway thorugh stress treatment also enhanced genistein production. Both previous effects were seen in dicot plant systems. Introduction of seed-specific promoter driven IFS (Isoflavone Synthase) and CRC (Catabolic repression control) genes resulted in corn plants where the isoflavone can be detected in the kernels (Kerne).

Novel pathways can even produce completely alien products! Two multifunctional cytochrome P450 enzymes and uridine diphosphate glucose (UDPG)-glucosyltransferase were transferred from sorghum into A. thaliana, resulting in the production of the cyanogenic glucoside dhurrin, which confers resistance to the flea beetle (Flo-Käfer).

PHA (polyhydroxyalkanoates)

Many MO in nature synthesize PHA as energy storage; PHA production in plants would not be possible without M.E., huge scale and favourable economics make plants very good targets; since they derive all their carbon from co2 in the air→ low cost biofactories

PHB can be produced in the plastids of A.th. but it is rather brittle (brüchig) with few potential uses

Phytoalexins PA

Induced biochemical defense

antimicrobial, sometimes antioxidative; after infection by MO plant is producing PA; only in the attacked region of the plant; PA are secondary plant substances; PA production is also induced by UV-light, heavy metals, high/cold temperature, injury of tissue!
>300 chemicals have phytoalexin properties

- Isoflavonoids in legumes
- Gossypol in cotton
- Resveratrol in grape
- capsidiol in pepper

Flavonoids

Polyphenolic secondary metabolites, derived from phenylalanine
Based on their structure they are divided in several classes: flavanones, flavonols, anthocyanins,..
Some flavonoids are specific for only a few plant species in the plant kingdom
Presumed health benefits→ growing interest
Biosynthesis:

1. conversion of precursor 4-coumaroyl-CoA into chalcone by chalcone synthase
2. chalcone derivatized to pigments, defence chemicals (PA), regulatory molecules

Act as pigments, defense chemicals and regulatory molecules
Have been used in GE since the 1990s since different modifications can be determined through a change in colour
Increasing the level of flavonoids in food plants can provide health benefits; antioxidant activity!, the consumption of many flavonoids species is more beneficial than the intake of only one species, in most cases every plant species only contains few classes of flavonoids→ Genetic engineering
e.g. in tomatoes by overexpressing the gene for chalcone isomerise, leading to an 80-fold increase in the flavonoid content in the peel and 20-fold increase in the paste.
tomato fruit contains chalcones and flavonols

Examples of engineering of the flavonoid pathway

- increased levels of flavonols in peel and flesh by ectopic expression of the CHI gene from Petunia
- Lc/C1 maize regulatory genes induced the expression of Flavonoids in tomato fruit flesh
- RNAi suppression of tomato regulatory gene DET 1 induced enhanced falvonoid content and high pigmented fruit
- Insertion of new branch of enzymes induced stilbene production on tomato
- insertion of new branches of flavonoid pathway further downstream via foreign structural genes

e.g. stilbenes (trans-resveratrol): cardiac helath; dietary source: red wine

Production of novel flavonoids in plants

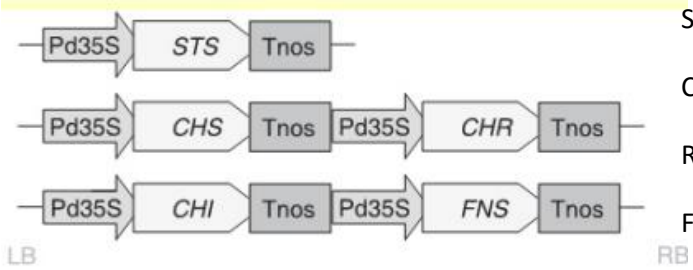
Wild type tomato contain 6-hydroxychalcones


Pathway engineering for healthy phytochemicals leading to the production of novel flavonoids in tomato fruit

Elio Schijlen[1,*], C.H. Ric de Vos[1], Harry Jonker[1], Hetty van den Broeck[1], Jos Molthoff[1], Arjen van Tunen[2,3], Stefan Martens[4] and Arnaud Bovy[1]

Stilbenes Flavones

Classes of flavonoids were targeted that are normally not present in tomatos; using structural genes from several plant species in combination transgenic tomatoes accumulating stilbenes, deoxychalcones or flavones were produced.



STS=stilbene synthase

CHS= chalcone synthase, CHR=chalcone Reductase

FNS= flavone synthase

CHI=Chalcone isomerase

Over-expression of flavonoid pathway genes (CHI=chalcone isomerase) led to a stimulated flux through the pathway and the introduction of new branches of this pathway. The dotted arrow represents the pathway leading to anthocyanins, which normally is not active in tomato fruit. These fruits displayed altered antioxidant profiles and an up to threefold increase in total antioxidant activity of the fruit peel.

To substantially increase the level of flavonoids TF can be introduced and expressed in tomato plants;

e.g. overexpression of Lc and C1 (encode TF) have shown to increase levels of flavonols but no anthocyanin accumulation was observed; TF show great potentials to enhance synthesis but their specificity may differ with different plant species (Lc and C1 control anthocyanin biosynthesis in maize)

e.g. delila (del) and rosea1 (ros) from the snapdragon (löwenmäulchen) where expressed in the fruits of transgenic tomatoes; binary vector containing del and ros1 each under the control of a fruit specific promoter E8; “Expression of Del and Ros1 causes the upregulation of genes required for anthocyanin biosynthesis and results in higher total antioxidant capacity”

→ functional foods!

Metabolic production of isoflavones in non-legume parts

Could distribute the health benefits of phytoestrogens to more widely consumes grains.

Expression of isoflavone synthase (IFS) gene in A.th. results in the synthesis and accumulation of the plant isoflavone genistein in leaf and stem tissue; was experienced in several plant tissues systems

Comparison of gene transfer systems:

1) Integrating System

Advantages and disadvantages of Agrobacterium (integrating system)

+ technically simple
+ relatively uncomplicated insertion events (low copy number, minimal rearrangements)
+ unlimited size of foreign DNA
+ efficient
+ adaptable to different cell types, culture procedures
+ transformants are mitotically and meiotically stable

− host range limited
− culture systems have to be adapted to agrobacterium

2) Direct DNA uptake

Electroporation

+ specialized vectors are not needed
+ high-efficiency transient expression
− only protoplasts can be used
− frequency of stable transformation is low
− in case of integrated DNA (stable transformants): high rearrangement
− copy number of insertions is high → gene silencing

Particle bombardment

+ effective for transient expression
+ all plant hosts
+ also for generation of transplastomic plants (DNA of chloroplasts is changed)
− requires tissue culture regeneration
− copy number of insertions is high → gene silencing

3) Virus-based vectors

+ Absence of position effect in the plant genome
+ Infection with a virus based vector is far simpler than stable transformants
+ Higher flexibility with constructs
+ Amplification by the viral vector gives higher level of expression
− no stable transformation
− biosafety
− construct size limitations