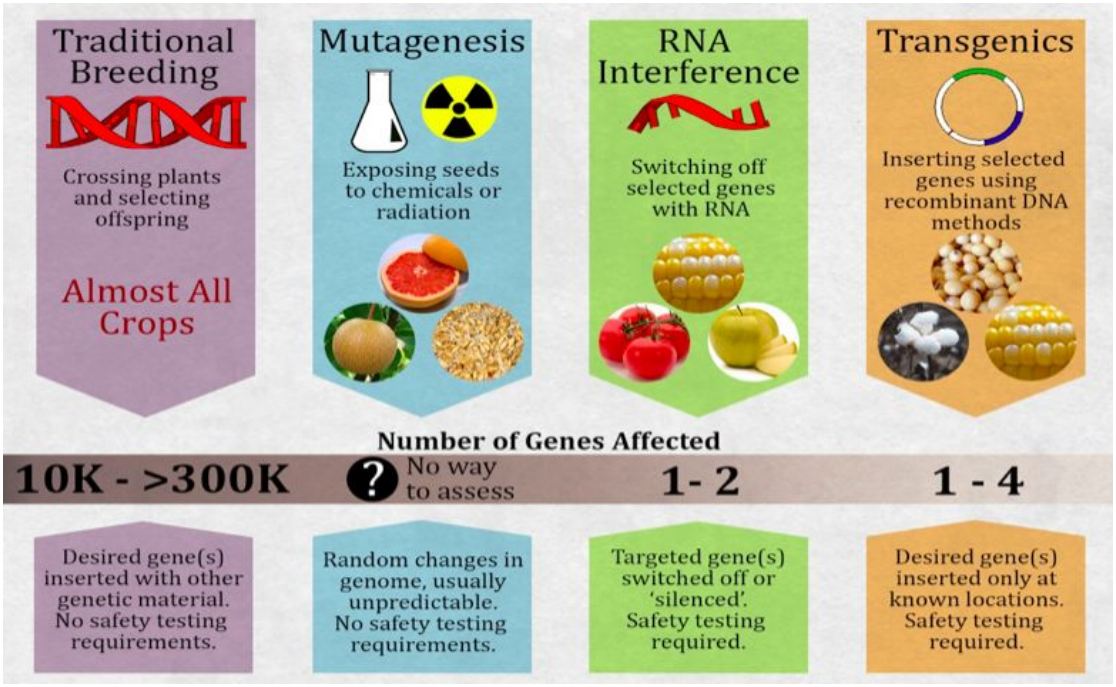
lecture 1: introduction

- plant biotechnology is any application of science/technology to plants
- without using it, a lot of people will be hungry
    - improve crop yield/agricultural productivity, use plants as a biofactory to make immunizations and antibodies (biofactories), phytoremediation, biofuels, protecting biodiversity, medicinal plants
- agricultural productivity
    - look at resistance to pests, herbicides, pathogens
    - control nutrition content (ie. golden rice), ripening & appearance
- plant biofactories
    - take gene of interest from any organism and put it in a plant to get a product
    - includes output of biopharmaceuticals (diagnostic kits, edible vaccines, biogenerics -- biogeneric sources of drugs), nutraceuticals (when something in food that may have a medicinal property), cosmeceuticals, agriculture
- phytoremediation
    - plants can extract heavy metals from the soil; they can be absorbed and degradation or volatilization
- biofuel
    - potential for us to not rely on oil; not necessarily realistic because of falling crop yields could cause some parts of the world to be hungry in exchange for biofuel production -- increased need for combination of both food and fuel
    - miscanthus is the best crop for biofuel (most cost efficient)
- protect biodiversity
    - DNA banks as an alternative to seed banks
    - molecular markers to measure or assay biodiversity w/ DNA fingerprinting; you can check for genetic drift and
    - introducing genes to prevent population bottlenecks (?); important in agriculture and horticulture ie. to increase biodiversity in ornamental plants (blue rose)
        - genes from pansy and iris were introduced and rose genes preventing blue pigment were turned off using RNA silencing
        - replacing a couple genes allowed for blue rose production
- genetic modification of crops
    - traditional breeding
        - crossing plants
    - mutagenesis

        - expose seeds to mutagenic chemicals or radiation and check out what the results are; very random/stochastic
    - RNA interference
        - turn off specific genes
    - transgenics
        - inserting selected genes using recombinant DNA tech



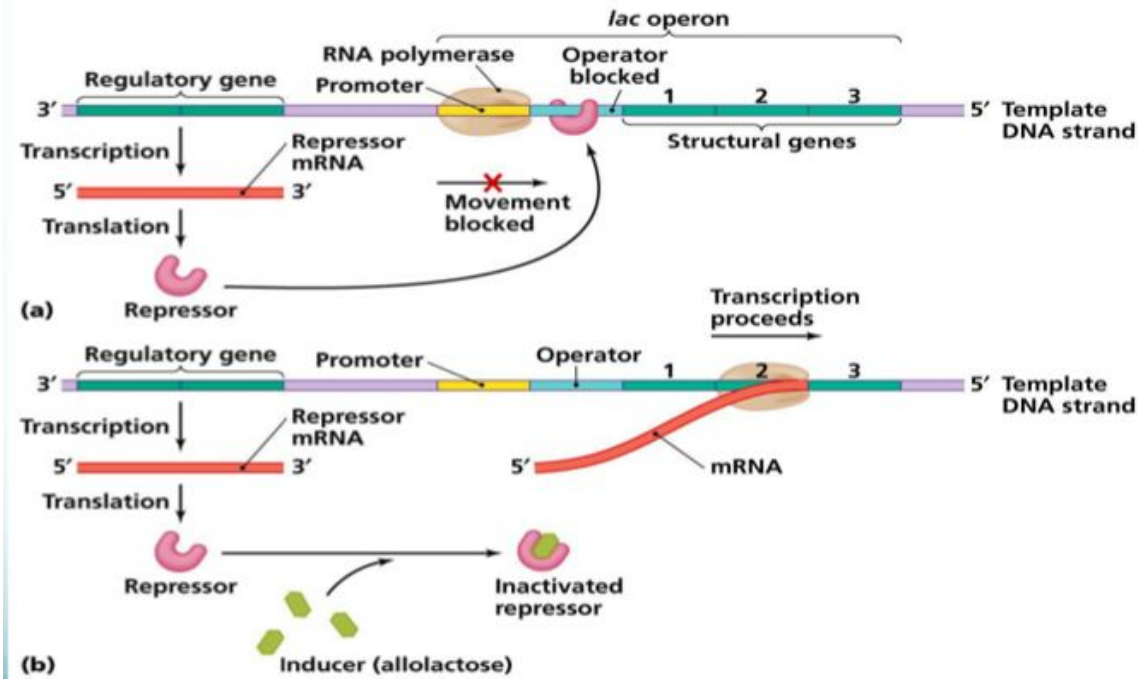
- cannabis biotech
    - focusing on epilepsy and neuropathic pain
    - THC is one of 80 to 100 different cannabinoids in marijuana, each with unique effects because humans have unique cannabinoid receptors

lecture 2: plant genomes

- plants contain 3 genomes: nuclear, chloroplast, mitochondrial
    - we usually modify the nucleus
    - plant has 20-100 chloroplast and thousands of mitochondria :. they are better for overexpression
- regulation of gene expression happens on multiple levels
    - chromatin conformation; gene transcription; nuclear RNA modification, splicing turnover and transport; cytoplasmic RNA turnover; translation (upstream nucleotides); post-translational modification; protein localization; protein turnover
    - chromatic conformation

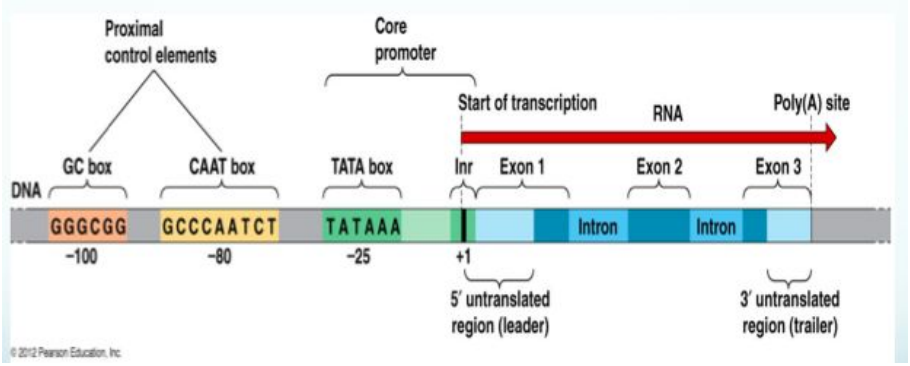
- keeping chromatin in folded conformation prevents RNA pol from binding
- growth during certain times of the year involves the unwinding of DNA
- acetylation of lysine residues on histone proteins alter the ability of the DNA to bind (changes +ve charge to -ve so DNA can't bind to it anymore)
    - methylation and acetylation of the DNA via enzymes also works
- if you're going to express your gene, the chromatin can't be folded up

- gene transcription of DNA in prokaryotes
    - emphasis on the promoter: the section of the DNA where RNA polymerase binds
        - based on DNA sequence of promoter, absence of inhibitors, and presence of transcription factors
    - ie. lac operon in E. coli
        - important bc we may be using some of the promoters
        - operons are collections of genes involved in a metabolic process
            - lactose metabolism



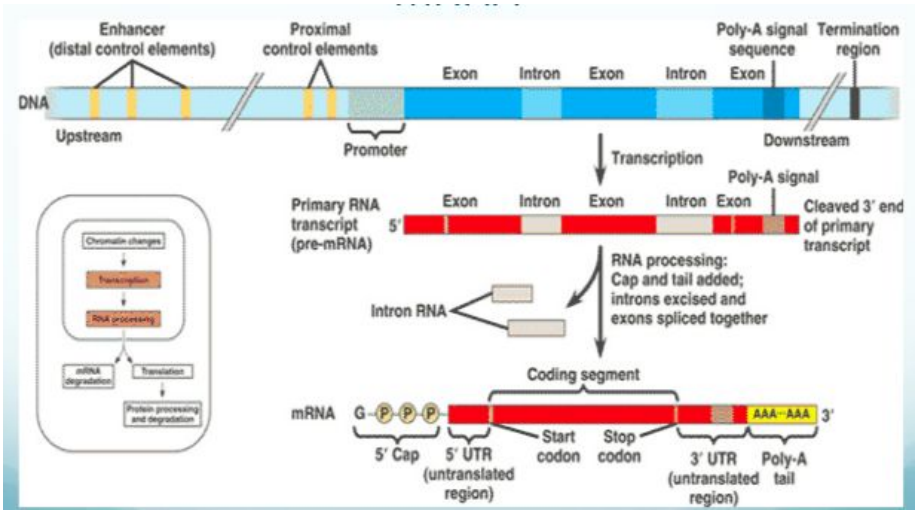
    - LacI regulatory gene that makes a protein (monomer) that binds the operator region of DNA

        - operator is right in front of the promoter
        - promoter is where RNA pol binds
    - LacI (inhibitor) gene always expressed that binds the operator and blocks the binding of RNA pol and transcription in the absence of lactose
        - despite the inhibitor, there's always a tiny smidge of leaky expression regardless
    - RNA pol binds the TATA box of the promoter, a consensus sequence
        - consensus sequence: the most common sequence you'll find in all promoters of E. coli
        - each promoter has a different strength of RNA pol binding which affects the amount of gene expression
        - TATA sequence not always TATA
    - the LacI gene forms a tetramer repressor which binds the operator, RNA pol binds the TATA sequence which is blocked by the LacI repressor
        - in the presence of lactose the repressor is inactivated because allolactose (lactose byproduct) binds the tetramer which changes the structure
            - prevents operator binding
        - allolactose is the inducer
- eukaryotic gene expression
    - at the TATA box the RNA pol binds
    - upstream activator binding sites located; transcription factors
        - CAAT box, GC box
    - 5' UTR (leader) followed by expressed exons and removed introns



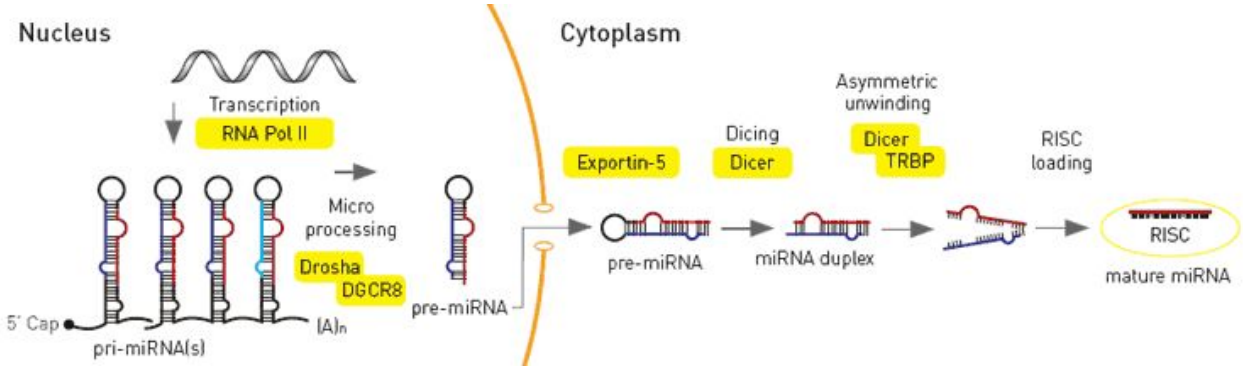
- eukaryotic expression and processing of mRNA

    - you can have multiple exons which can be combined in different combos (alternative splicing) which allows for different functions by removing whole sections of catalytic power from the protein



- nuclear RNA modification, splicing turnover and transport
    - multiple versions of one gene can be produced by selective splicing to make different combinations of exons from the same gene/mRNA
    - all sorts of different combinations to create a different protein
- cytoplasmic RNA turnover
    - turnover is altered by Cap and PolyA tail which increase stability
    - specific sequences in the mRNA decrease stability
        - AU rich regions
    - hormones and environmental factors alter longevity
- translation via upstream nucleotides/ORFs
- post-translational mods
    - plants are easy to produce but if you want to turn them into a biofactory they have to have the correct post-translational modifications
    - phosphorylation by kinases is a process that goes back and forth all the time
        - equilibrium
        - a reversible process; dephosphorylation by phosphatase
    - addition of modified carbohydrates (not simple carbs) are very important to protein function
    - phosphorylation, glycosylation, SUMOylation, ubiquitination
- protein localization

        - you may want to put it in a vacuole or secreted extracellularly
        - use the right localization/transport sequences to direct it
    - protein turnover
        - tryptophan/tyrosine/proline protein sequences increase/decrease stability
- post-transcriptional gene silencing
    - using RNA itself to stop the expression of RNA
    - best when you already have RNA and you want to prevent the expression; short nucleotide sequences that do not code for proteins but produce RNAs that silence genes; accomplished two ways:



- microRNA (miRNA)
    - self-regulatory machinery the plant itself uses to remove its internal RNA
    - miRNA genes are transcribed by RNA pol II as primary transcripts (pre-miRNA)
        - this base-pairs with itself to form a hairpin structure with a loop
    - the pre-miRNA is processed by a protein complex containing an RNase III enzyme, which is transported to the cytoplasm where it is processed by a second RNase III enzyme (DICER)
        - forms a mature miRNA which is incorporated into the ribonuclear protein to form the RNA-induced silencing complex (RISC)
    - this pre-miRNA structure is chopped at the hairpin loop by a DICER-like enzyme which produced a miRNA duplex that is methylated & exported from the nucleus
    - once being unwound and cleaved by DICER, a single strand of RNA forms a RNA induced silencing complex (RISC)
    - RISC binds a near-perfect/perfect complementary sequence and signals it for cleavage or translational inhibition of mRNA targets
- small interfering guide RNAs (siRNA)
    - a dsRNA is formed through RNA pol transcription, which forms a loop (shRNA)

- the shRNA is recognized by RNA binding domains in the dimetric DICER complex (which also contains RNAse endonuclease domains) which cleaves the hsRNA into 21-28 nt siRNA duplexes containing overhangs that are bound by the DICER PAZ domain
- now the siRNA-DICER complex is exported out of the nucleus where DICER recruits argonaute (which has helicase activity) to form the RISC
    - due to the helicase activity there are now two ssRNA strands and only one is retained (the guide strand vs passenger strand)
- the guide strand recruits RISC to the surface of mRNAs that are homologous to the siRNA sequence and the catalytic RNase degrades the mRNA
    - mRNA is targeted for deletion bc they no longer have 5' cap or PolyA tail on the ends
- control of gene expression by methyl jasmonate; signal transduction
    - involved in the wounding of a plant & its similar to mammalian process with arachidonic acid
    - signal transduced from receptor by MAPKs; co-silencing of two MAPKs (MPK1 and MPK2) compromises the defence response against wounding/larvae & also decreases production of jasmonic acid
        - :. applying methyl jasmonate to co-silenced plants rescued them and indicated that jasmonates are the signal responsible for changes in gene expression
    - when systemin is detected, the cytosolic calcium concentration increases and linolenic acid is released from the cell after a phospholipase has been activated
        - systemin binds a receptor which phosphorylates and cleaves off the fatty acid which eventually forms methyl jasmonate
    - linoleic acid is converted to jasmonic acid and activates defensive genes; production of jasmonic acid is induced by systemin and also upregulates systemin precursors
        - creating a feedback loop that amplifies the defence mechanisms
    - because methyl jasmonate is volatile, it can also activate systemic acquired resistance in neighbouring plants
    - the methyl jasmonate goes into the nucleus and binds and alters gene expression
- choice of promoter to regulate the transgene expression
    - 35S promoter from cauliflower mosaic virus is constitutive

        - some promoters have light-responsive elements/promoters
        - hormone-sensitive promoters exist too (ie. abscisic acid promoter - ABA)
    - the sequencing of plant genomes allows for many plant promoters to be discovered
- arabidopsis thaliana is the model organism in plant sciences, analogous to E. coli
    - dicotyledon, member of the mustard family, short life cycle, small model plant, produced lots of progeny, easy to transform, genome sequenced

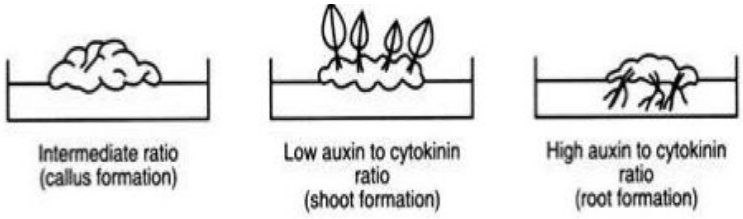
lecture 3: plant tissue culture

- plasticity evolved because plants are sessile and have long lifespans :. they have to be adaptable and be able to change their metabolism, growth, and development
    - one type of organ or tissue can change to another type
    - in tissue culture, one type of organ can be used to generate another type
    - a single genotype used to generate multiple phenotypes
- totipotency is maintenance of the ability to initiate cell division from almost any tissue in order to regenerate lost organs or undergo different developmental pathways
    - like a stem cell that has not irreversibly differentiated
    - a totipotent cell isolated from cells or tissues can generate an entire plant
- plant tissue culture techniques
    - use a plant tissue culture and don't use a seed because you want to get identical clones but w/ seeds you get different combinations
    - done through the formation of a callus resulting from wounding a plant
        - the cells are very totipotent and not organized in any particular way :. the have no meristem
        - abnormal tissue with the potential to produce roots and shoot growth & embryos that develop into plantlets
        - organogenesis may occur
            - the development of adventitious organs or primordia (embroid) from undifferentiated cell mass (callus) in tissue culture
    - this can also be performed with pieces of a plant
- plant tissue culture labs typically contain
    - media preparation room
    - culture media, washing powder, disinfectant
    - aseptic transfer chamber area
    - environmentally controlled culture room
    - analytical room

- acclimatization room
- ph meter, balances, electronic hot air oven, microscopes, centrifuge, filter sterilizing equipment, and a laminar airflow cabinet

- nutrition/media components
  - culture media is composed of three basic elements:
    - essential elements or mineral ions, supplied via a complex salt mixture + iron
      - macroelements/nutrients
        - calcium - plant cell wall component, pectin
          - promotes root development
        - iron - chlorophyll synthesis :. photosynthesis component
        - magnesium - central to chlorophyll and enzyme activator
        - nitrogen - plant growth rate, component of nucleic acids, chlorophyll, amino acids, proteins, alkaloids, hormones
        - phosphorus - meristem & fast-growing tissue, DNA/ATP comp
        - potassium - normal cell division
          - promotes meristematic growth
        - sulfur - deep green foliage
          - promotes root development
      - microelements/nutrients
        - boron - movement of sugar/water
          - promotes fruiting and cell division
        - chlorine - promotes photosynthesis
        - copper - energy conversion & chlorophyll synthesis
        - iodine - added as potassium iodide
        - manganese - promotes growth
        - molybdenum - converts ammonia and helps nitrogen fixation
        - zinc - enzyme activator, IAA production
          - promotes root growth
    - organic supplements
      - carbohydrates providing a source of fixed carbon
      - if you're growing a callus in the dark, you need to provide with sugar
        - usually sucrose

        - also D-mannitol, D-sorbitol, and hexitols
    - growth regulators
        - auxin - naturally indole acetic acid (IAA) but IAA is heat sensitive so use 2,4-D instead
            - induces cell division and root formation
            - used in callus induction
        - cytokinins - typically zeatin but also KN, BAP, 2iP or adenine derivatives
            - promotes cell division and shoot proliferation



        - gibberellins - inhibit callus growth but can be used for shoot formation
        - abscisic acid - heat stable but light sensitive; used in embryo culture and somatic embryogenesis
        - ethylene gas - naturally occurring, controls ripening and inhibits growth
- gelling agents - purified agar or difco bacto agar
- amino acids - all L-amino acids
- antibiotics - fungicides, bactericides, made fresh and added after autoclaving media
    - for selection when engineering
- natural complexes - coconut milk, yeast extract, malt extract, tomato juice, potato extract, casein hydrolysate, fish emulsion
- antioxidants - citric acids, ascorbic acids
    - reduces excessive browning of explants
    - adsorbents like PVP and activated charcoal are also used checking excessive browning
- aseptic techniques like sterilization (destruction of living matter), disinfectant (chemical agent used to kill pathogens without sterilizing matter to which the chemical is applied), & sanitization (substantially reducing and then maintaining the microbe population in the air and on objects in lab to acceptable levels)

lecture 4: culture types

- types of culture
    - callus culture

        - an unorganized, growing and dividing mass of cells generated from explants, using media and providing a carbon source (ie sucrose) all to make sure it doesn't dedifferentiate
            - must have carbon source and be grown in the dark bc photosynthesis stops with dedifferentiation (light causes differentiation)
                - this means the metabolic profile will not match that of the donor
            - composed of unspecialized parenchyma cells
        - compact callus vs friable callus, where molecules that hold it together haven't been generated; increase friable callus by using lower gelling agent concentration
- cell-suspension culture
    - friable callus where the cells are only loosely associated w/ each other has been used as an inoculum to form cell-suspension cultures
        - grown in equivalent liquid media in conical flasks
    - subculture dilution is empirically determined for each culture type otherwise the cells may die bc they're very temperamental
    - biomass will continue to increase until stationary phase is reached
- protoplast culture
    - similar to cell suspensions but the cell wall has been enzymatically or mechanically removed, typically originating from leaf mesophyll cells or cell suspensions
        - protoplast can take up macromolecules, viruses, & cell components by phagocytosis
    - very fragile and easily damaged but easily transformed
    - protoplasts can regenerate into complete plants and protoplasts with the same or different origins can fuse together & the fusion product may even form a plant
        - somatic hybridization via PEG
- root culture
    - established in vivo from root tip explants that have been cultured on simple media
        - indeterminate organs with potentially unlimited growth
    - you can genetically modify it to produce novel products (ie. pharmaceuticals) in the root
- shoot & tip meristem
    - used for clonal propagation (identical plants)

        - isolate the meristem and cryopreserve it and then warmed it up and after four weeks to see shoot tip elongation; after 8 weeks rooting occurs
            - change the ratio of auxin and cytokinins to change the meristem from stoop tip production to rooting
- embryo culture
    - use embryos as explants to form a callus cultures or somatic embryos
    - most common method for monocotyledonous plant regeneration
- microspore culture
    - using pollen or anthers as an explant to produce a callus and immature/mature embryos from haploid tissue
    - using just pollen can cause variable ploidy level occurring from cell fusion, using anthers lowers this risk
- whole plant regeneration
    - somatic embryogenesis - formation of a whole plant from somatic tissues
        - indirect somatic embryogenesis - callus is produced from explant tissue and then embryos are produced from the callus (or from a cell-suspension produced from the callus)
            - formation of callus is done on high 2,4-D concentrated media which initiates the embryo formation
            - embryo production is turned into a cell suspension when the callus is repeatedly subcultured in liquid media with low 2,4-D concentrated media that also contains abscisic which
        - direct somatic embryogenesis - young trifoliate leaves are used as an explant hen they are removed from the plant and chopped into small pieces that are washed in a plant growth regulator-free media
            - pieces are places in liquid medium and supplemented with 2,4-D and maintained in this medium for 10-15 days
            - washing the explants and replacing the old medium with a medium containing PEG
                - results in development of somatic embryos
            - mature the embryos on medium containing abscisic acid
    - organogenesis

- production of an organ from explants or callus
    - relies on the inherent plasticity of plant tissues and is regulated by changing the components of the medium
- begin by inducing shoots with a high auxin to cytokinin ratio and the root the shoots by changing the ratios

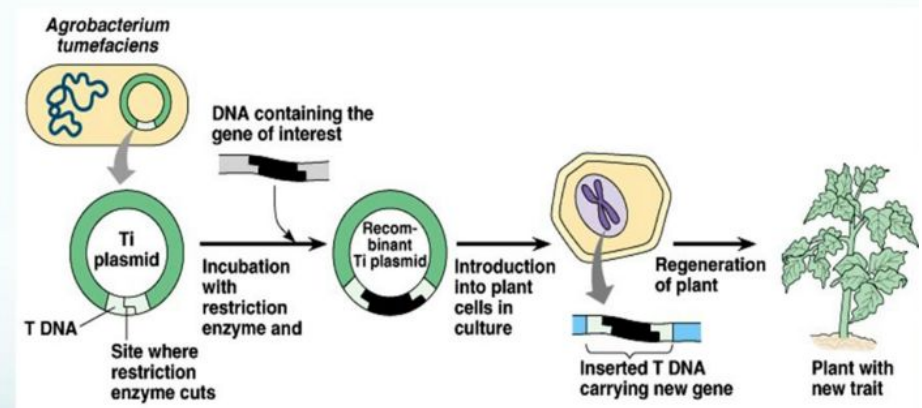
LECTURE 5: techniques for plant genetic modification and transformation

- transformation - the stable integration of transgenes into the genome of a plant
- palindrome - a structure that occurs in dsDNA where the sequence of two strands exhibits two-fold rotational symmetry about the axis
    - recognized by restriction endonucleases
- restriction endonucleases - naturally occurring restriction enzymes that cleave the phosphodiester backbone of the DNA at specific palindromes, so that the DNA is cut into more manageable sized pieces
    - enzyme name comes from the species it was isolated from
    - some yield sticky ends and some yield blunt ends
- recombinant DNA - DNA molecules that contain covalently linked segments derived from two or more sources of DNA
- sticky ends - a single stranded overhang at the end of a piece of DNA resulting from cleavage from a restriction enzyme
- DNA ligase - reforms the phosphodiester backbone left after annealing two complementary sticky ends
- plasmid - small circular dsDNA that is not part of the main circular DNA chromosome of the bacteria and :. can replicate independently
    - typically carry genes that may benefit the survival of the organism (ie. antibiotic resistance) and can be transmitted between bacterial species
    - they are often used to create recombinant DNA and transform cells; must contain antibiotic res and an origin of replication to do this
        - sometimes also contain a multiple cloning site (MCS) which is a cluster of restriction enzyme sites that allow for the ease of DNA insertion which is located in the beta-gal gene that allows for blue/white colony selection
- cloning - the process of inserting DNA into a plasmid and making identical copies of DNA that can be used to turn bacteria into protein factories
    - bacterial cloning involves inserting a desired piece of DNA into a plasmid
    - plasmid and gene are cut with the same restriction enzyme and run on an agarose gel; the bands are cut out and purified from the gel and then allowed to join together in a ligation reaction catalyzed by DNA ligase

- a plasmid will contain a gene for antibiotic resistance and a gene for beta-gal production
    - these are used to identify if the plasmid is present in the potential transformants
        - grow on medium containing an antibiotic and only bacteria w the resistance gene will thrive
    - also identifies if the plasmid contains the insertion
        - blue colonies will be formed if the plasmid doesn't contain the insertion bc the beta galactosidase gene, whose product appears blue, has not been interrupted
        - white colonies will be formed if the plasmid does contain the insertion bc the beta galactosidase gene is no longer functional because it has been interrupted
- polymerase chain reaction (PCR)
    - increase the amount of any given DNA without cloning the DNA
    - any chosen DNA can be amplified and it does not have to be separated from the rest of the DNA sample beforehand
        - just add necessary primers to amplify a specific section
        - primers allow access to the denatured DNA
        - each PCR cycle consists of three temperature changes
            - 1. denaturation @ 90 deg C
                - the DNA unwinds to allow the primers access to bind
            - 2. annealing @ 54 deg C
                - the forward and reverse primers anneal to the DNA
            - 3. extension @ 72 deg C
                - the polymerase synthesizes the complementary strand
                - starting from the 3'-OH
        - these three cycles are repeated 30-40 times
        - can be loaded and ran on agarose gel and visualized with ethidium bromide/UV
    - PCR has many uses
        - identification of disease w PCR RFLP
            - restriction fragment length polymorphism analysis is a technique that demonstrates the difference between samples of homologous DNA molecules from differing locations of restriction enzyme cut sites

    - the DNA sample is digested by a restriction enzyme and the resulting restriction fragments are run on agarose gel and separated according to their lengths
- amplification of unique genes in transgenic plants to ID them as transgenics
- amplification of gene for cloning; add restriction sites to the primers
    - amplify your gene of interest with PCR primers containing restriction enzyme site ends, mix the PCR product with supplied restriction enzyme digested vector and transform into competent E. coli

        - amplification of a region of genetic testing
        - detect GMOs using PCR primers that are your promoter and terminator sequences
- know RFLP and other mechanisms/procedures in detail
- Agrobacterium tumefaciens
    - gram -ve soil bacterium found in the rhizosphere of plant roots consuming nutrients
    - causes crown gall disease in wounded plants via chemotaxis of sugars and phenolics
    - one of the few examples of inter-kingdom gene transfer
- Ti plasmid
    - ability of crown gall in the presence of crown gall; Ti stands for tumor inducing
    - transfer DNA (T-DNA) region encodes proteins for the synthesis of auxin and cytokinin, which cause cell proliferation and also encodes genes for the biosynthesis of opines and agropines, which are used as a fuel source by the Agrobact
        - these are sugar derivatives that only the agrobacterium can metabolize
    - other genes are not transferred to the plant genome but allow for transfer of sugars into the bacteria
    - there are additional genes outside of the T-DNA region that are very important for transfer of the T-DNA into the plant

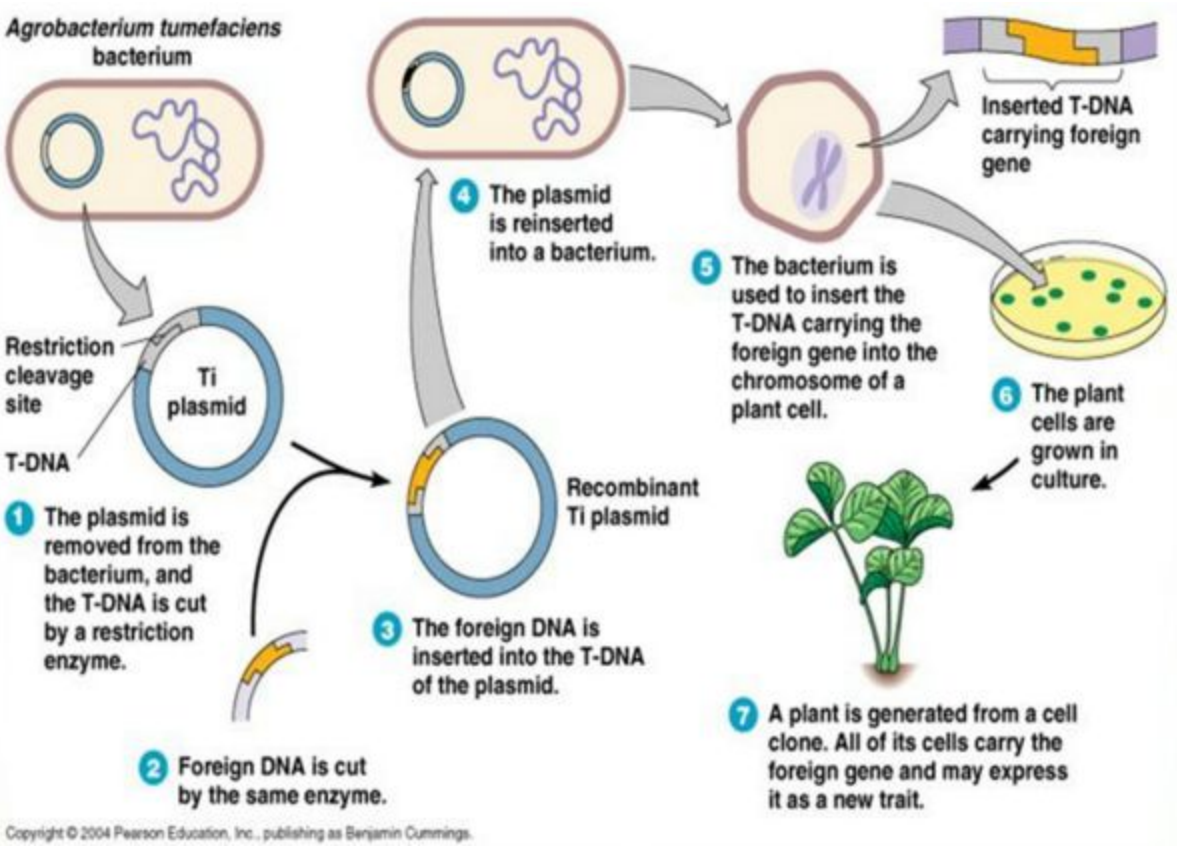


- ti plasmid features:
    - contains one or more T-DNA regions
        - defined by the presence of left and right border sequences
            - any DNA between the borders will be transferred into the genome of the plant
        - contains oncogenes
            - auxA and auxB are involved in the production of auxin IAA and cyt encodes an isopentyl transferase that catalyzes the most important step in cytokine synthesis
        - also contains genes for opine production, tumor size determining genes,
    - contains virulence (vir) genes
        - genes responsible for the transfer of the T-DNA into the host plant
        - nine vir gene operons:
        - VirA is a kinase that auto-phosphorylates itself and VirG in the presence of phenolic compounds which :. activates VirG to initiate transcription of all other virulence genes
            - :. you must always constitutively produce VirA and VirG
            - VirG is a transcription factor
        - VirB1 to B11 are proteins that form the membrane structure to allow the DNA to be transferred out of the agrobacterium and into the wounded plant
            - creates a pore that goes into the plant
        - VirC1 encodes an overdrive binding protein that increases T-DNA transfer efficiency
        - VirD1 modulates VirD2 activity

        - VirD2 nicks the T-DNA and directs the T-DNA through the VirB/VirD4 transfer apparatus
            - also applies nuclear targeting to the T-DNA apparatus and protects the 5' end of T-DNA from nucleases
        - VirE1-2 acts as single stranded binding proteins to prevent degradation of the ssDNA
            - VirE1 is the chaperone that guides and exports VirE2
            - now the T-DNA is leaving as a ssDNA :. it will have lots of random phosphates and base pairs bound to it, making it very sticky
            - to avoid this, VirE2 binds as a ssBP
        - VirF destroys the single stranded binding proteins (VirE2)
            - also regulates cell cycle and elongates S-phase
        - VirJ exports the T-DNA
    - contains an origin of replication (ori)
    - contains a region enabling conjugative transfer
    - contains genes for the catabolism of opines
- wounded plant cells release phenolic compounds and sugars that are detected by VirA, which activates VirG by phosphorylation; VirG induces the expression of all the genes in the vir region; VirD1-D2 are involved in ssT-DNA production, protection, and export; VirB products form the transfer apparatus; the ssT-DNA associated with VirD2 and VirE2 are exported together through the transfer apparatus; in the plant cell, the T-DNA is coated with VirE2; plant protein interactions with VirD2 and VirE2 induce transport & integration so that the T-DNA/VirD2/VirE2/plant protein complex enters the nuclear pore; illegitimate recombination integrates the T-DNA into the plant chromosome

- the process of Ti
    - signal of wounded plant recognized by bacteria
        - the signal is phenolic compounds (involved in healing) are released
    - attachment of bacteria to plant cell to polysaccharide
        - some plants have mutations in this so the agrobact bind ineffectively
    - induction of vir genes that initiates signal transduction
        - initiated by transmembrane protein VirA in the bacteria
    - initiated by virA
        - a membrane linked kinase that phosphorylates VirG, the transcription factor for the other vir genes.

        - once VirG is phosphorylated it will go bind the DNA on the Ti plasmid and transcribe virulence genes as a transcription factor
    - production of T-strand
        - left and right hand borders are recognized and clipped by VirD1-D2 (not always)
    - transfer of T-DNA-VirD2 complex out of cell through VirB channel with VirE2 (ssDNA binding protein) and VirF (helps to destroy VirE2 proteins later)
        - VirD2 is an endonuclease that goes through the VirB channel, including VirB2 (ssBP)
    - transfer of T-DNA
        - VirD2,E2 into plant cell nucleus and integration into the chromosomal DNA via illegitimate recombination
            - plant DNA repair process
        - uses nonhomologous recombination so it doesn't go in at the same site
- turn the Ti plasmid into a tool:
    - remove as many natural genes as possible to make room for gene constructs
        - if it gets too big it won't be stable!
        - so remove the auxin & cytokinin synthesis genes
    - keep left and right borders
    - add a selectable marker
        - such as antibiotic resistance or herbicide resistance or GFP
    - keep virulence genes
        - they're involved and cutting out and transferring T-DNA; essential
        - not always on the same plasmid; sometimes you have multiple plasmids
    - now you have a <u>disarmed genetically engineered Ti plasmid</u>
        - has the L and R borders, an AmpR gene, polylinker regions (for the gene of interest insertion), virulence genes
        - advantageous over direct gene transfer bc there is less rearrangement and a lower transgene copy number



- steps to transform tobacco (indirect method of transfer)
    - select plant tissue to transform; leaves for tobacco
    - genetically engineer gene into derivative of Ti plasmid.
        - lacZ polylinker pPTV to identify which E. coli has the gene construct
    - select strain of Agrobacterium for transformation of genetically engineered plasmid
        - not all strains will work for all plants! requires consideration
    - incubate leaf tissue in culture containing Agrobacterium for 30 minutes.
        - bacteria bind
    - wash off and place explant on solid MS media for 2 days
        - DNA transfer occurs; the media must contain a marker (ie. kanamycin)
    - wash explant in antibiotic solution and plate on agar with selective marker (kanamycin)
    - auxin and cytokinin are added for organogenesis
        - not coming from the Ti plasmid, coming from the media
- <u>direct</u> <u>methods</u> <u>of</u> <u>transfer</u> - disadvantageous because they have such a high rate of transfer and your insert too much of your DNA
    - particle bombardment (gene guns)

    - also known as biolistics method
    - gene coated gold or tungsten particles and then propelled at high speeds with a gene gun and into plant tissue, where it can integrate into the genome
        - used on grain crops (ie. cereal) such as Bt maize
    - too much DNA insertion leads to high copy number and gene rearrangement
    - DNA integration occurs infrequently and is totally random
    - transient expression of proteins
    - transformation of rice:
        - gene gun used for transformation of rice; callus induction; dehulled rice sterilized in ethanol and bleach placed on solid media-25C 2 weeks & bombard with two plasmids
        - one with gene of interest (GOI), the other with selectable marker
            - using two plasmids is helpful when you want to eliminate your selectable marker
        - coated on gold particles maintain callus on selective media
        - transfer to light for 3 weeks then transfer to rooting media.
- electroporation
    - low efficiency & requires careful optimization; does not damage cells
    - can be used on plant cells, protoplasts, callus cultures, and immature embryos
    - relatively low copy number
- PEG-mediated DNA uptake into protoplasts
    - cell wall has been removed so they can be transformed w naked DNA by exposure to PEG and calcium ions
    - makes the membrane permeable
    - problem: protoplasts are really fragile and it can be tricky to revive
        - all these methods can read to multiple copies
    - requires optimization with a regenerable cell suspension that may not be available
- plastid transformation
    - chloroplasts are maternally inherited and :. easier to maintain
    - can select for chloroplasts are transformed with 100% transformation efficiency
    - up to 10,000 copies of chloroplasts per cell
        - increases gene expression a lot!

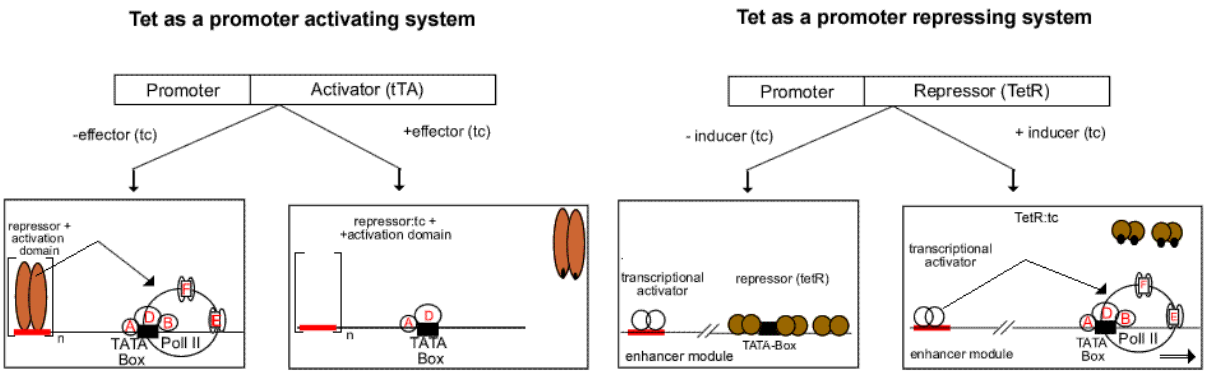
        - no gene silencing in chloroplasts (a eukaryotic mechanism)
            - can have a polycistronic message; multiple genes under one promoter
        - you'll get correct protein folding
        - plasmids can be designed for site-spec homologous recombination
    - silicone carbide fibers
        - very simple technique involving no special equipment
        - take fibers and DNA in a buffered solution and vortex them with a cell suspension
            - requires regenerable cell suspension

LECTURE 6: TRANSFORMATION

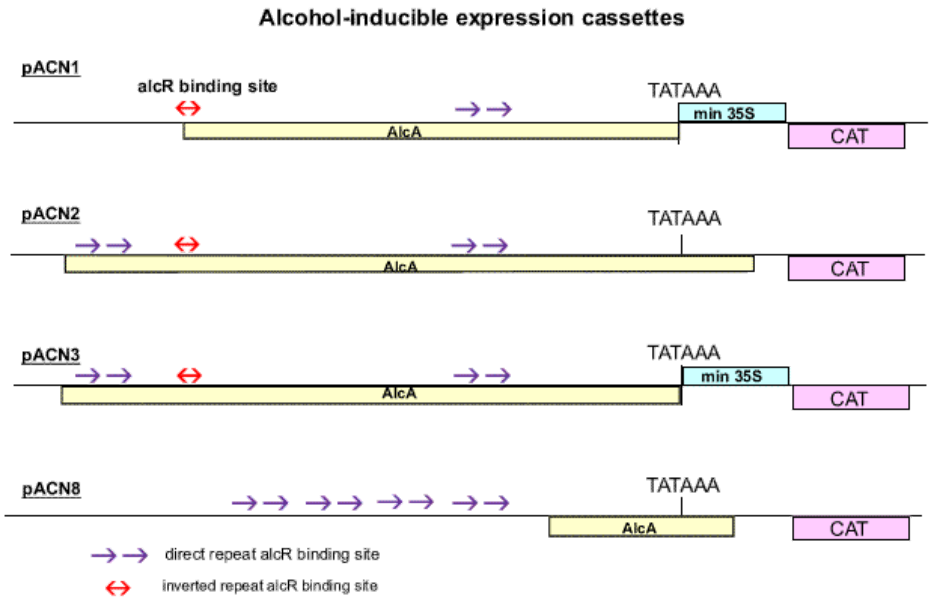
- plasmid vector for E. coli
    - small, high copy number, contains unique restriction sites
    - insertion restriction sites in the middle of a B-gal gene
        - blue/white colour selection (white will be what you want)
    - antibiotic res selectable marker
- agrobacterium plasmid Ti
    - only need regions flanking the T-DNA in the Ti plasmid to integrate into the host
    - can remove oncogenes but keep virulence genes to allow the GOI into the cell
- what we want in a plant vector:
    - replication in E. coli and agrobacterium, either with one or two oris
    - selectable markers
    - border seq of the Ti plasmid
    - gene of interest
- promoters and terminators
    - must have promoters and terminators that function in plants
    - a foreign gene can't use it's original promoter :. must have a plant promoter
    - the promoter will vary in
        - strength (how tightly RNA Pol binds)
        - tissue specificity
        - inducibility mechanism
    - there must be a 3' terminator seq to stop the process
- Ti plasmid promoter
    - Nos gene involved in nopaline synthesis
    - promoter and terminator used

    - from a bacterial source but able to function in plants too!
    - constitutive promoter
        - cauliflower mosaic virus also full
    - you can follow it with another gene (ie. one from an animal)
- 35S promoter
    - most common promoter
    - CAAT box upstream of the TATA box
    - from cauliflower mosaic virus
    - most common promoter for plant vectors
    - higher expression in dicots than in monocots
        - for monocots use the ubiquitin
    - constitutive promoter that can't be turned on/off unless it's altered
- NOS (nopaline synthetase) promoter
    - NOS promoter (and terminator)
    - used a lot with glucocorticoid receptor or anytime you want constitutive expression
    - always going to be made but produced even stronger when the plant is wounded
        - because it's from agrobacterium plasmid
    - an example of its use is to make a protein that is going to be a regulator of your GOI
    - first artificially used regulator in plants
- inducibility of promoters
    - we don't always want constitutive promoters
    - three classifications:
        - non-plant derived control so that it is independent of plant processes but requires the addition of an inducer
            - completely independent of w/e happens in the plant
        - plant derived control that responds to environmental signals
        - plant derived promoter based on developmental controls
- steroid receptors as a tool to identify the direct target genes of trans factors in plants
    - glucocorticoid receptor in plants
    - no dexamethasone (dex)
        - transcription factors fuse to the hormone binding domain of the glucocorticoid receptor (TF-GR) in inactive state due to an interaction with the heat shock protein 90 (HSP90) complex
    - hormone binding leads to disruption of interaction btwn GR and HSP90 and allows TF-GR fusion to enter the nucleus

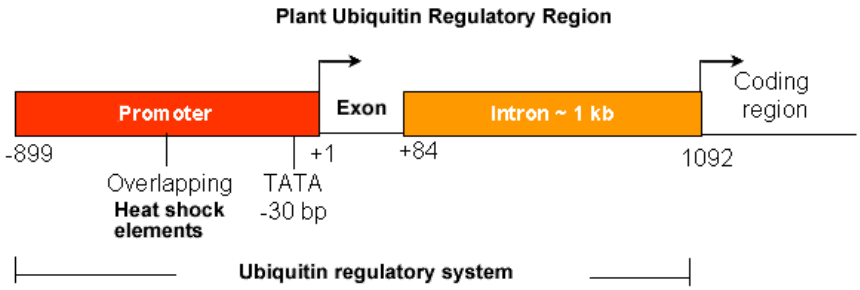
- once inside the nucleus they bind specific DNA sequences and activate or repress gene transcription



- tetracycline promoters
  - TetR is under constitutive control w 35S promoter
  - the TetR repressor protein binds to and blocks transcription
  - when tetracycline is added the repressor comes off and allows RNA Pol to bind
  - used in tomatoes, potatoes, and tobacco
- converted TetR promoter to a gene activator (modification of TetR system):
  - has a lack of versatility as a promoter repressing system :. it was modified to form an activator system
    - turns things on instead of turning them off
  - TetR fusion to herpes simplex virus protein 16 (V16)
    - forms a tetracycline transactivator fusion protein (tTA)
  - in the absence of tetracycline, tTA binds to tet operator seqs placed upstream of the TATA box in the target promoter :. activating transcription
  - in the presence of tetracycline, tet forms a complex with tTA which releases the operator :. turns off gene transcription
- ethanol inducible promoter
  - fungus Aspergillus nidulans has a well-characterized positive operator system
  - alcohol dehydrogenase I promoter (alcA gene) uses transcriptional activator protein AlcR
  - binds target seq with alcA gene promoter in the presence of ethanol, ethyl methyl ketone or other alcohols/ketones (ethanol derivatives)
    - inducers of gene expression
  - alcohol-inducible cassettes
    - alter the number of repeats to alter the strength of the promoter



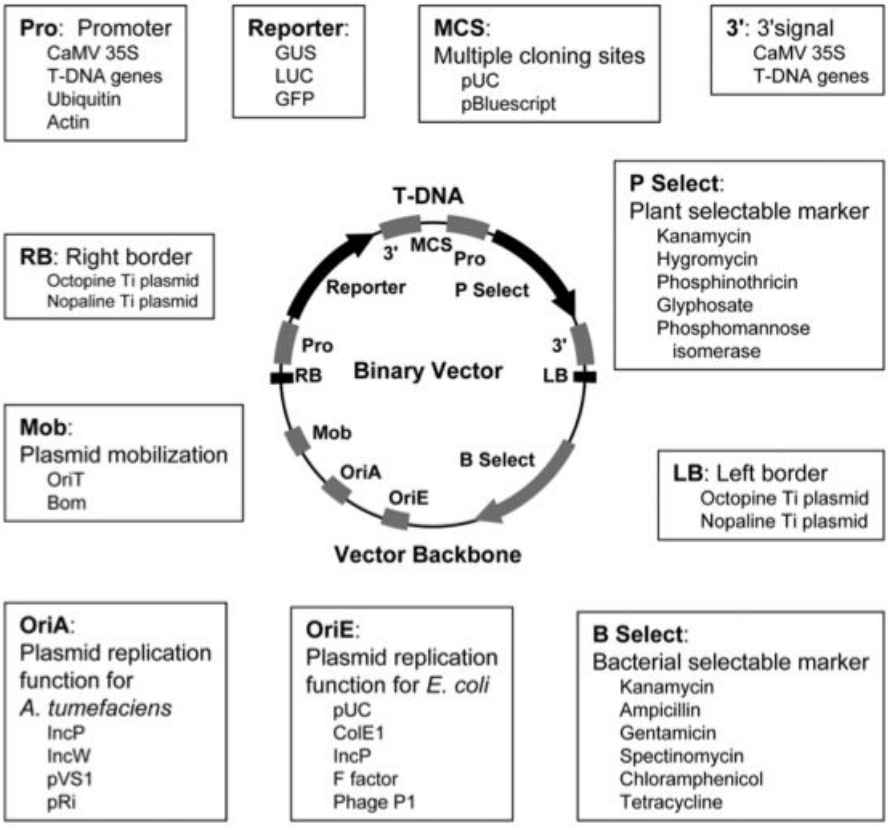
- ubiquitin promoter
    - identified in maize regulatory region that controls the expression of Ubi-1 gene
    - this seq contains a TATA box located @ -30m two overlapping sequences similar to consensus heat shock element located at the -140 and -104 position
    - heat shock elements of the regulatory region enhances the expression of ubiquitin protein in response to temperature stress
    - good option for having something constitutive in a monocot



- plant-derived environment inducible promoters
    - fusion of core S35 element and sequences that respond to various enviro factors
        - wound inducible methyl jasmonate
        - pathogen induced salicylic acid
        - heat schol temperature inducible
- developmentally induced plant derived promoters

        - SAG12 or SAG13 are senescence activate promoters when the plant is dying or under severe stress
            - cytokinin production
        - chimeric 35S-abscisic acid inducible promoter
        - chimeric 35S-auxin inducible promoter
        - synthetic promoters
            - promoters are always cis-acting elements that always need to be right in front of the DNA
                - you can have multiple cis-acting elements
- selectable marker - to verify the transformation/insertion of a GOI
- negative selectable markers
    - selectable marker confers decreased toxicity
        - nptII confers resistance to kanamycin
        - resistance to bleomycin etc. can be used at a lower level
        - herbicides sprayed on a plant (if it grows it was transformed)
            - chlorsulfuron and bialaphos
    - these are disadvantageous because you don't want to spray with herbicides unless you have to (or use antibiotics unless you must b/c antibiotic resistance)
- positive selectable markers
    - GFP (green fluorescence) for visual screening
    - Ipt gene from agrobacterium allows growth in the absence of cytokinin
- reporter genes
    - easy to assay and nondestructive :. widely used in plants
    - beta glucuronidase (uidA or gus)
        - very sensitive & qualitative
        - X-gluc blue precipitate for immunohistochemical localization
        - 4-MUG quantitative fluorescence
    - GFP
        - very sensitive & qualitative
        - GFP used in grapevines to show the removal of antibiotic resistance marker protein
            - exchanged antibiotic resistance for GFP
    - luciferase (lux or luc)
        - firefly enzyme that catalyzes oxidation of luciferin which results in the emission of light
        - bacterial luciferase genes luxA and luxB from Vibrio harveyi are used
    - chloramphenicol acetyltransferase gene (CAT)

    - used rarely now
- ori in plasmids
    - need replication of origin that works for both E. coli and agrobacterium
    - can use one ori that works for both or use two separate oris to ensure high copy number
- binary vector system
    - two plasmids for transfer
    - plasmid with vir genes (maintained in plant strain to be transformed) separate from plasmid with T borders surrounding your GOI
    - beneficial because it allows you to have a smaller plasmid with your GOI :. stability

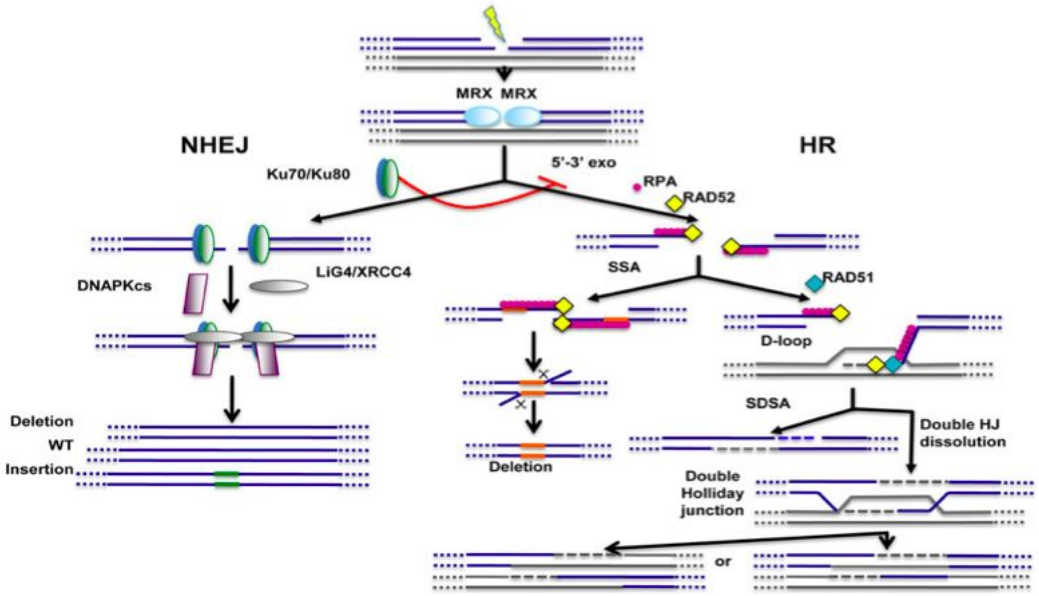


- gateway cloning
    - recombination into vector using Att borders from phage lambda
    - don't need to use restriction enzymes to get DNA to destination
    - instead use recombination system from phage lambda
    - use a common border that will allow the lambda to become lysogenic and recombine into their chromosome
- factors affecting efficacy
    - arrangement of genes in multiple copies should not be inverted

        - each gene has it's own promoter
        - copy number is empirically determined to lead to higher or lower expression
        - transgene position in the genome is random with T borders; not all positions lead to good expression
        - transgene features may need altered codons in plants (preferential codon usage)
- gene silencing
    - occurs inadvertently in transgenic plants
    - reduce the copy of transgenes
    - avoid same promoter and terminator (repeating sequences)
        - different promoter/terminator for each thing you're expressing
    - avoid transgenes or promoters with similar sequences to endogenous plant sequences
        - otherwise your gene may be shot down instead of expressed
    - post-transcriptional and caused by dsRNAs leading to RNAi
    - RNAi vectors
        - can intentionally silence gene expression by designing plasmids to cause dsRNA formation
            - insertion of the message in the antisense direction
            - antisense RNA is made and binds to sense RNA strand to produce dsRNA
        - you can also design the vector to produce an inverted repeat
- "clean gene technology"

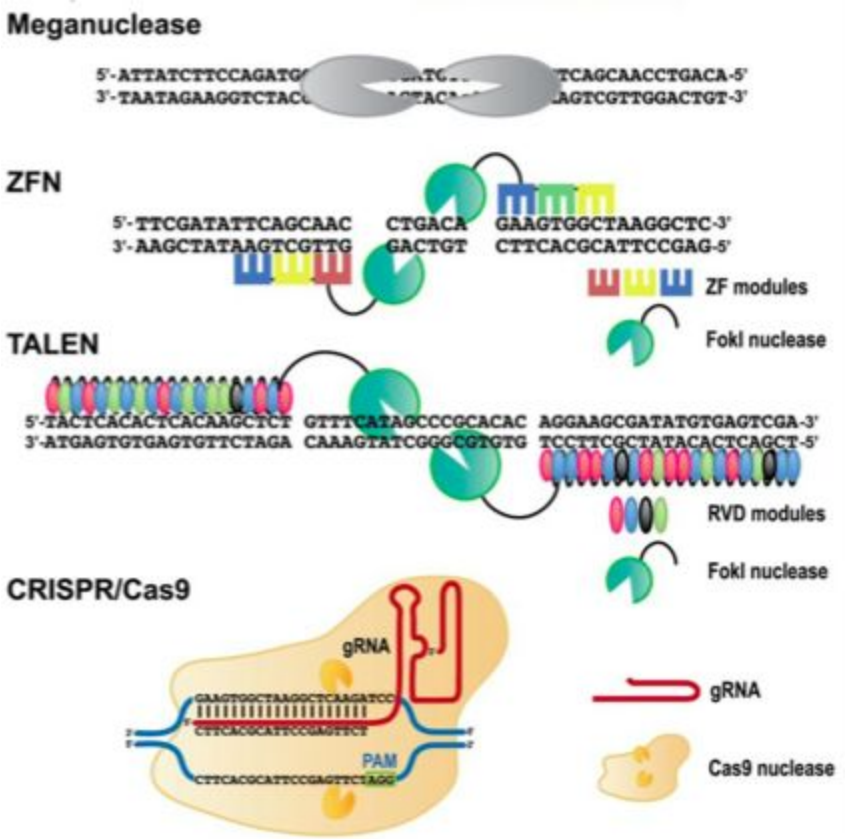
lecture 7: transformation II

- agrobacterium allows gene editing but it has non-spec integration & has cost/time issues
- GEEN
    - engineered nucleases
    - allows for very specific knock-outs
    - mutations and modifications can be put in by recombination after the DNA has been damages/cleaved; create a ds break with an engineered nuclease
    - you can put in a whole new gene in the middle of the gene with cleavage in it
    - non-homologous end-joining (if DNA is cut once) & homology-directed repair (if cut twice)
        - you get a large deletion with HDR because the repair mechanisms chew the ends



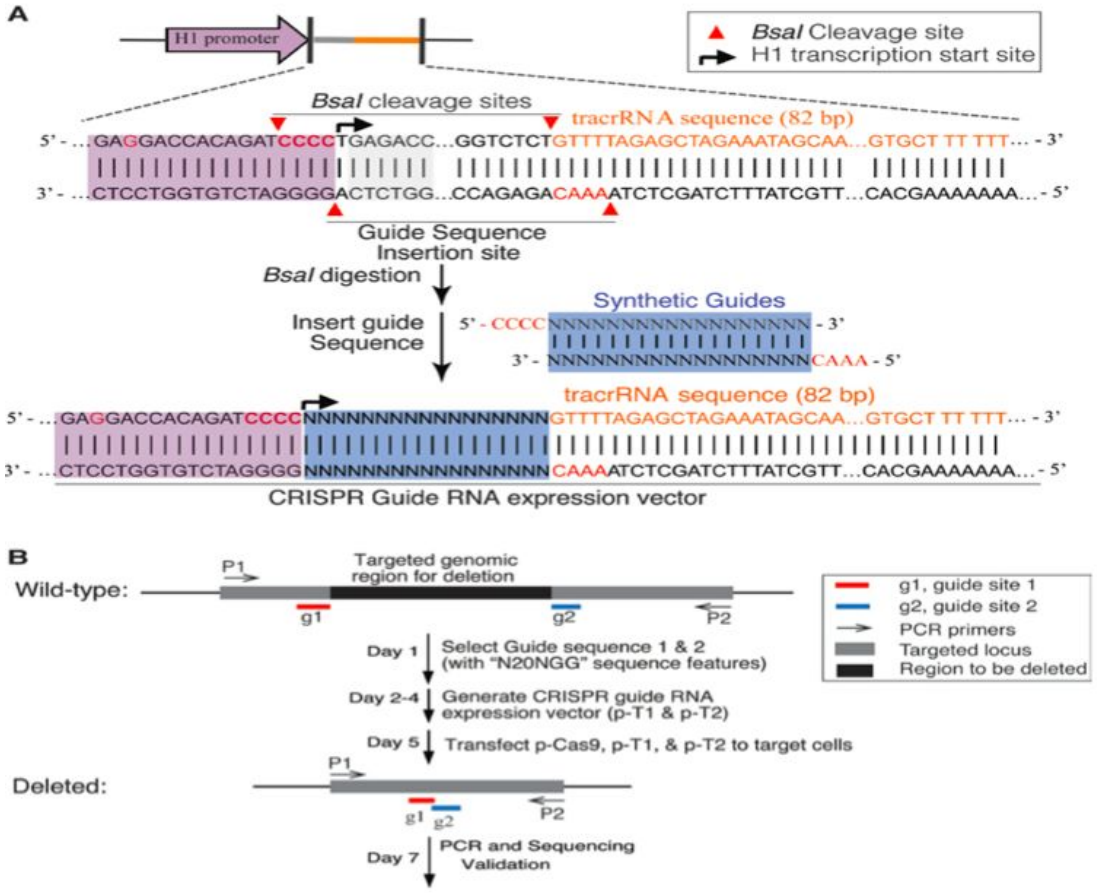
- (above) DNA repair mechanisms
- engineered nucleases to alter specific chromosomal genes (GEEN)
- really expensive because you make your own nuclease
    - meganucleases
        - cuts a much larger specific site
    - ZFN zinc-finger nucleases
        - designed to go to a specific site; genetically engineered protein
        - only good for one site (that you designed it for)
        - has zinc finger motif with recognition site for specific bases
            - each of the zinc fingers recognized different base sequences
        - can use non-homologous end-joining or homologous repair to repair a double strand break that was made using the fokl domain
        - NHEJ is more prevalent in plants
        - after the break, exonucleases remove some DNA and leaves overhangs; add in a larger stranded piece of dsDNA by taking advantage of HR
    - TALENs transcription activator like effector nucleases
        - designed to go to a specific site; genetically engineered protein
        - only good for one site (that you designed it for)
        - discovered in bacteria found in plants
        - uses tale to recognize a dipeptide sequence so link many together and use it to recognize a specific site
            - FCDs recognize specific sites and the rest progresses in the same way as zinc finger nucleases
        - when you covalently link the FokI to the TALE nuclease

        - the TALE identifies the sequence and the FokI makes the break
    - RGENS RNA guided engineered nucleases
        - CRISPR is better because all you have to make is RNA :. much more affordable
        - clustered regularly interspersed short palindromic repeats



- CRISPR
    - clustered regularly interspaced short palindromic repeats
    - being openly shared as opposed to the high regulatory control of TALENs
        - less expensive
    - gRNA (guide RNA) is in the clustered regularly interspersed repeats that has homology to viral DNA so that if there is viral DNA within the cell, it will be recognized by the sequence and bound :. being digested
    - cas9 is a nuclease that cuts DNA that is bound by the ss small gRNA
    - the cas9 unzips the viral DNA and replaces it with the gRNA if the gRNA is a match the viral DNA will be cleaved at two sites
        - the cell will try to repair the cut DNA but it's error-prone
        - tracrRNA (trans activating RNA) and crRNA (CRISPR RNA) are linked together to form gRNA

- PAM (protospacer adjacent motif) is a tri nucleic acid seq that is complementary to your target DNA's protospacer
    - the gRNA always begins with an RNA version of the protospacer seq
- CRISPR in nature
    - Cas9 nuclease naturally makes a ds break with its two subunits (HNH & RuvC) which each cut on different strands
        - must have the protospacer
        - by making a mutation in the CRISPR subunits you can make it so that only one strand of DNA is cleaved
            - creates Cas9 nickase
        - beneficial because it's much easier to get specificity with two gRNA targeting two distant sites :. success rate goes to dramatically
        - how does the success rate go up?
    - you can also inactivate the Cas9 completely so that it is guided to your DNA but will not cut it; attach another domain to it



- a cassette was designed where you can drop in your gRNA really easily

- you just need to synthesize your two single strands; they will hydrogen bind for you
- two targets; begin by selecting the sequence in the gene where your PAM sequence will occur; generate CRISPR gRNA expression vector where your synthetic gRNA is covalently attached to the transacting RNA seq (you'll have two plasmids -- one for each target); transfect the cell with a plasmid that contains your Cas9 (probably a nickase) and also your plasmids containing the gRNA for target one and target two; if you're not inserting any DNA then the genome segment between the two ss nicks will be targeted for deletion
- introduction of a mutation into rice using HDR and CRISPR
    - identify mutations with RFLIP because introduced gene contains two REN sites
- DNA-free CRISPR system
    - because people are wary of genetic engineering
    - to avoid agrobacterium and plasmids, which can leave behind DNA in the plant genome
    - Cas9 protein and gRNA are directly placed into plant protoplasts of Tobacco, rice, arabidopsis & lettuce using PEG
    - very unstable because there's no DNA involved; very unstable but this is beneficial because it doesn't linger for long but stable mutations were still generated and maintained through generations
    - you can't put in genes, just knock them out
- maize

LECTURE 8: HERBICIDE TOLERANCE

- used in combination with other genetically engineered resistances
    - insect resistance (Bt)
- in order to feed the population that we have now, the amount of genetically modified food being produced will increase, i order to feel the world
- GM use increasing within my lifetime
    - most prominent is herbicide resistant soybeans
- herbicide resistance was first because we already knew a lot of herbicide pathway info :. it was easiest to study because of all the genes available for testing
    - it only takes one gene to add resistance
- improved ability to use broad spectrum herbicides that would normally destroy crops
- much more toxic to plants that it is to humans

- plants are autotrophs there are many pathways to disrupt; many found in chloroplasts
    - photoreduction of CO_2
    - photooxidation of water
    - photophosphorylation
    - production of amino acids, lipids, NA, starch
        - we will focus on amino acids and how to help plants still produce amino acids in the present of a herbicide
        - alter amino acd production in plants so we can kill the weeds and still grow crop
    - assimilation of sulphur and nitrogen
- ALS (acetolactate synthase) inhibitors most broad-spectrum herbicide (ie. resolve Q)
    - first enzyme branched chain AA pathway, making leucine, isoleucine, and valine
        - ie. sulphonylureas
    - RoundUp
        - inhibits enzyme that makes aromatic amino acids, auxin, lignin, alkaloids, flavonoids
        - kills most weeds because it inhibits an enzyme that is necessary to the production of so many different compounds
        - mode of action
            - phosphoenolpyruvate (PEP) and shikimate-3-phosphate combined with EPSP synthase enzyme leads to the synthesis of 5-enolpyruvylshikimate-3-phosphate (EPS)
        - we need to make our plant tolerant to the EPSP enzyme
            - 1. oversaturate the plant with target protein
                - overproduction of target protein of EPSP synthase so that we're making so much of it in our plant so that the plant isn't impacted in the same way as the weed is
                    - more of our target than the inhibitor
                - gene duplication is more likely than single site mutation in euks
                - overexpression of ESPS enzyme in petunias is naturally selected
                - GM cDNA isolated then fused to the 35S promoter and nos terminator & then transformed in petunias using agrobacterium
            - 2. mutate the target protein

- introduce a target protein that is resistant to the herbicide
- change the active site so that when the enzyme encounters the herbicide, it will not act on it
- genetically resistant strain of agrobacterium found in waste at a glyphosate manufacturing CP4
    - bacteria had developed resistance
- the gene was cloned from this bacteria and transformed into plant chloroplasts (need signal peptide seqs on the protein)
    - initial experiments didn't target very well bc agrobacterium didn't have all the right signalling seqs
- to make roundup ready maize
    - mutagenized corn was selected in tissue culture for resistance; the gene had the correct chloroplast transport sequences (so it would be transported where it needed to go) in the resistant strain
    - maize fused to it

- 3. detoxification of herbicide once absorbed into plant
    - chemical alteration of the herbicide so that it becomes harmless; does not require a lot of knowledge
    - find naturally occurring glyphosate oxidase (GOX) genes in soil bacteria and added the correct peptide sequences for transport

- monsanto employs multiple strategies at once

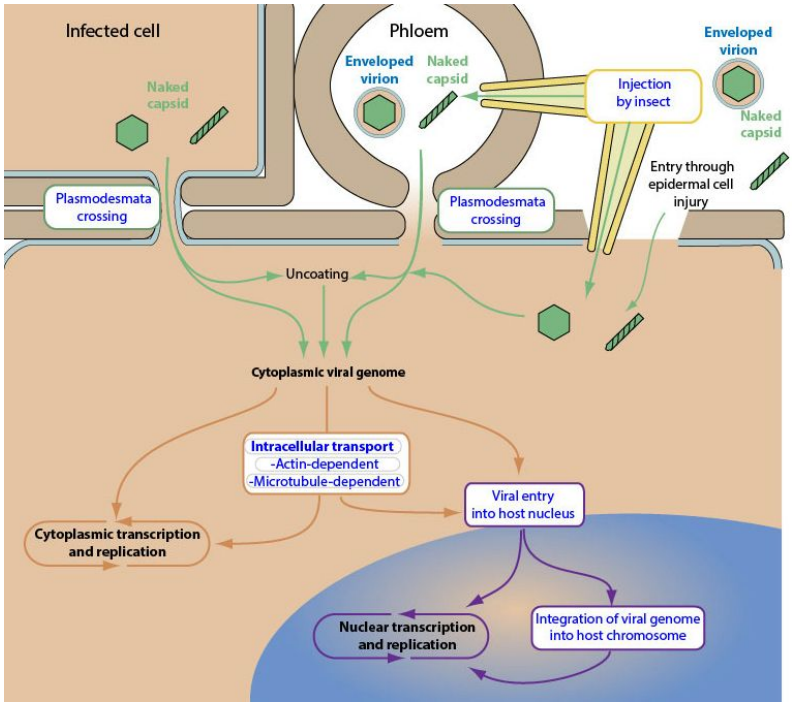
PLANT DISEASE RESISTANCE

- Plant-pathogen interactions
    - Symbiotic relationships between microorganisms and hosts (ie. rhizobia)
    - Microbe causes disease in the host plant
    - No infection develops because the plant is resistant to the pathogen
    - The host plant shows some resistance to the infection, so the pathogen is able to grow and replicate but infection symptoms are minimal
- Types of pathogens
    - Necrotroph
        - Organisms that kill the host and feed on the contents
    - Biotroph
        - Organisms that require a living host to complete life cycle
    - Hemibiotroph
        - Parasitic in living tissue that continues to live in dead tissue too

- Non-host resistance
    - Entire species is resistant due to a mechanisms conserved across species
    - Very complex, multiple genes involved; robust
- Host resistance
    - Interaction between single plant protein and single pathogen protein
    - Causes hypersensitive response (HR) that stops disease spread
- Prokaryotes
    - Phytoplasma - lack cell walls
        - Saprophytic - decomposers of dead organism matter
        - Intracellular pathogens
        - ie. Asters yellows
    - Agrobacterium
        - Crown galls and hairy roots
        - Transfers T-DNA into cells through wound sites
    - Blights, soft rot (enzymes), wilt (blocked vasculature)
- Fungi and mold
    - Most common pathogens
    - Many saprophytes
    - Oomycetes (water molds) classified differently from fungi
    - Ie. Phytophthora infestans responsible for potato blight
    - Ie. Aspergillus flavus - produced deadly Aflatoxin
        - Aflasafe outcompeted Aflatoxin producing fungal network
        - Defects on the aflatoxin biosynthetic pathway produce safe microbe that can displace toxin producing strains
- Natural disease resistance
    - Anatomical defenses
        - Wax, bark, & cuticle to prevent breach of cells
        - Many pathogens must invade plants through wounds
    - Protein and chemical protection
        - Antimicrobial proteins
        - Defensin and defensin-like proteins
            - Cause increased branching or slow growth
            - Found in seeds during germination to create microenvironment around plants
    - Inducible systems
        - Synthesis of proteins in response to attack detected when pathogen breaches cells
        - Elicitor response - local response induced by pathogen molecules (elicitors)
            - Wall fragments released induce genes to repair the cell and produce antimicrobial proteins
            - Pathogenesis related (PR) proteins, including anti-microbials
            - SN1 is an antimicrobial peptide that is active against bacterial and fungal pathogens in the potato

        - Chitinases cause destruction of pathogen cell wall, which releases chitin and other sugars that bind to other receptors and causes the synthesis of phenolic compounds (terpenes) toxic to pathogen and plant cells
    - Recognition-dependent disease resistance - specific proteins produced by the pathogen (avirulence gene product - avir) interact with resistance gene products produced by the plants
        - Hypersensitive response (HR)
        - Resistance gene product binds avirulence gene product
        - Gene-for-gene race-specific response that switches on defence system
        - Pathogen associated molecular pattern (PAMP) is when a molecule associated with the pathogen (ie. flagellin) interacts with its protein kinase PAMP which starts the MAPK (mitogen activated protein kinase) cascade to alter gene expression to close stroma
        - A series of kinases (MAPKK and MAPKKK) cascade and a transcription factor is activated that regulates defence genes against fungi or bacteria
    - Elicitor response and recognition dependent disease resistance lead to a cascade of reactions that invoke the hypersensitive response (HR) that induces systemic resistance and passes signal onto other plants
- Systemic responses
    - Induction of local defence pathways leads to induction of intercellular signals that produce systemic response or systemic acquired resistance (SAR)
    - Cells at infection site release salicylic acid (SA) into the phloem where it travels to other cells and induces proteins
    - Jasmonic acid and ethylene travel between plants and induce various pathways
        - Similar to when the plant experiences abiotic stress
    - Initiation phase and maintenance phase
- Biotechnological approaches
    - Pathogenesis related (PR) proteins that act against fungal infection (ie chitinases and glucanase to destroy cells wall of just fungi) expressed under 35S constitutive promoter or prp1-1, an inducible potato promoter that doesn't impact the metabolic load of plants so much
    - Antimicrobial proteins such as defensins that have a lytic activity against bacterial membranes have been expressed in transgenic plants

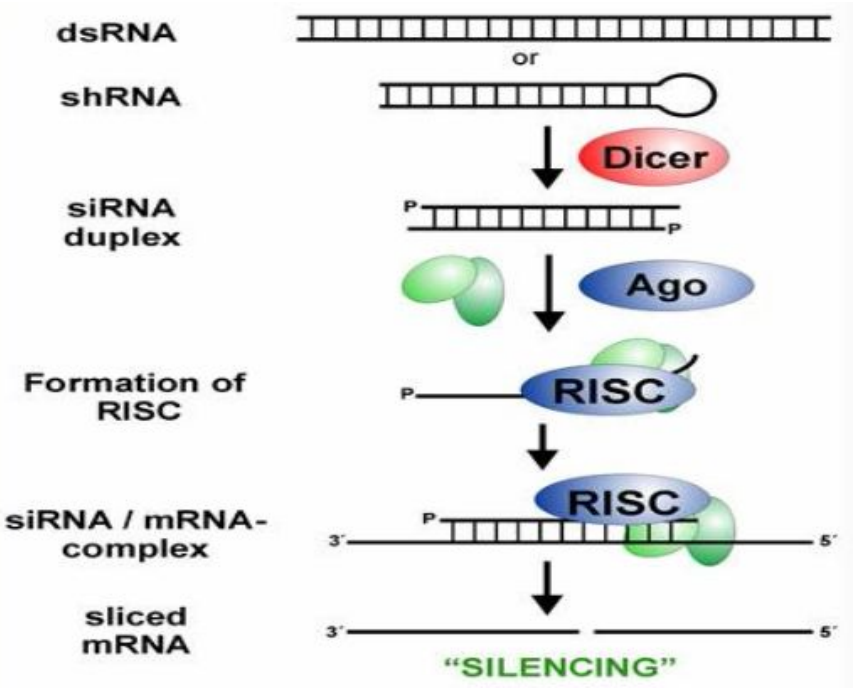
VIRAL DISEASE IN PLANTS



- Targets of viral life cycle
    - Entry, replication, packaging, transfer through plasmodesmata
        - Apoplastic pathway (through cell wall)
        - Symplastic pathway (through cytoplasm)
- ssRNA +ve sense
    - Bromoviridae
    - Comovirus
        - Cowpea mosaic virus (CPMV)
            - 2 ssRNA
            - Used for protein production and delivery vesicles
    - Potyviridae
    - Tobacco mosaic virus
        - One ssRNA genome with tRNA structure at 3’ end
        - Subgenomic (sg) RNA
        - Replicase complex contains:
            - Polymerase
            - Helicase & methyltransferase protein
        - Replication of the -ve sense RNA becomes template
- ssRNA -ve sense
    - Bunyaviridae
    - Rhabdoviridae
- dsRNA
    - Reoviridae
- dsDNA
    - Caulimoviridae
    - Geminiviridae

- Mechanisms to stop transmission
    - Infect plant with mold yet similar virus
        - Closterovirus citrus tristeza virus
        - Zucchini yellow mosaic virus (ZYMV)
        - Cucumber mosaic virus (CMV)
    - Develop resistant strains by breeding elite lines of two-gene resistance
        - Intron-hairpin RNA (ihpRNA) constructs made on sequences of BNYV 5' UTR or RNA-2 or the flanking sequence of P21 CP
    - Pathogen derived resistance - PDR (transgenic approach)
        - Engineer pathogen sequence into host genome
        - Theory is that it will be expressed at the wrong time in the cycle of the virus and disrupt the process (incorrect)
    - Interactions involving viral proteins
        - Use CP protein of TMV for cross-viral protection
        - Level of resistance sometimes related to amount of CP produced
        - Sometimes transcriptional silencing happens, but several different resistance mechanisms are at work
            - Interaction between CP and TMV-like virus
            - CP inhibiting uncoating
            - RNA silencing effect
- Arabis mosaic virus nepovirus (ArMV)
    - Transferred by nematodes, +ve sense mRNA virus
        - Shoots have shortened internodes, zig-zag growth, multiple shoots/node
        - Leaves are malformed, abnormal veins, fan-like, yellowing
        - Berry production decreases, some tendrils produce 1-2 leaves
    - Produce high levels of CP using strong promoter, transcription, translation enhancers to correlate amount of CP expression with enhanced resistance
    - Primers used to PCR the CP sequence
    - Ligated into E. coli plasmid with a CaMV promoter and a Nos terminator & transformed using Agrobact + kanamycin selection
    - Plants were mechanically infected with ArMV and necrotic lesions were counted, ELISA demonstrated lower levels of antigen
- Effects of RNA in pathogen derived resistance (PDR)
    - Satellite RNA
        - Plant viral sat RNAs require a helper virus for multiplication
        - May reduce the effect of virus on plants
            - Not required for viral replication but may affect disease symptoms
        - More satellites lead to protection, so produce transgenic plants containing sat sequences
        - Reduces CMV symptoms but not very effective
        - Worked better when combines with CP mediated resistance
            - Sometimes sat. sequences worsened virus

- Antisense RNA and ribozymes
    - Negative sense RNA and ribozymes bind to cleave target sense RNA sequences
    - Constructs express -ve sense RNA to hybridize viral sequence to interfere with viral replication
        - Based on sequence homology
    - May be due to PTGS



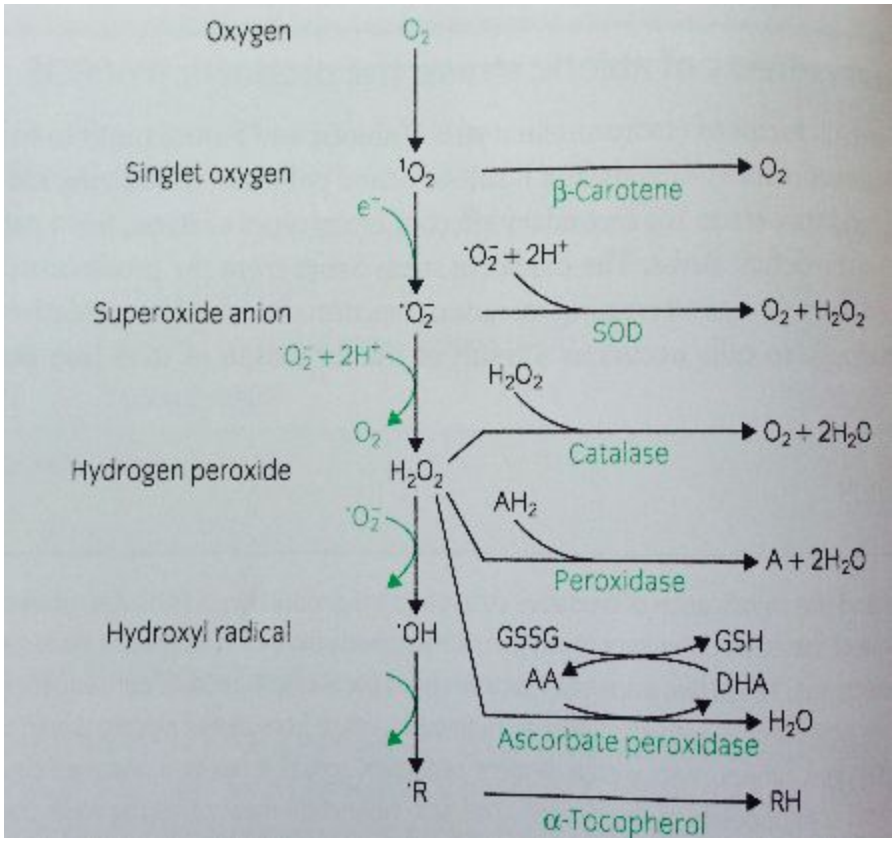
    - Post translational gene silencing (PTGS)
        - The explanation for RNA effects
        - Transgenic petunias and co-suppression of the endogenous and introduced gene
        - Viruses get past this system because they contain proteins that suppress gene silencing mechanisms
            - Potyvirus has HcPro that stops established gene silencing
            - CMV has 2b protein that stops process initiation
        - Effects of CP constructs are due to gene silencing
        - Used in CMV virus of tobacco
            - Inserting 4 viral inverted sequences into a single construct and introducing it into tobacco to make it resistant to all four viruses using PTGS
- non-PDR approaches
    - Target protein translation of the virus
        - Introduce a gene for ds strand dependent protein kinase that inhibits viral replication by inactivating viral translation of initiation factor eIF

        - Linked to be under control of wound-inducible promoter which made a transgenic strain resistant to CMV and several other viruses
    - Naturally occurring mutants of eIF (another translation initiation protein) confer resistance to YMV
- Geminiviruses
    - Begomovirus - transmitted by insects
    - DNA-dependent replication

STRESS TOLERANCE
- Abiotic stress
    - Intense light, herbicides, ozone
    - Temperature stress (heat, chilling, freezing)
    - Other water deficit stresses include drought and salinity
- Biotic stress
    - Pathogens, pest damage, wounding
    - Plants can adapt to enviro based on genotypic plasticity
    - Damage happens with extreme variations from the regular enviro
- Water-deficit stress
    - Drought, salinity, heat, cold
    - Water potential must match transpiration rates or the plants suffer
    - Water potential
        - A measure of potential where water flows from high potential to low potential compartments
        - Water is driven through the plant from the soil (high water potential) to the atmosphere (low water potential)
        - If soil potential starts to reduce, a deficit between the supply by the root to the leaf occurs and wilting :. Dehydration
        - Reduce the water potential in the leaf to maintain a necessary gradient to maintain the flow of water from soil
        - Control water potential
            - Concentrations of solutes that create solute or osmotic potential
            - Physical pressure of cell or tissue boundaries that form the turgor potential
            - Osmotic potential is lower than water potential and turgor pressure is the difference between them
            - As leaf turgor pressure falls, the stomata close and the leaf wilts as the cell collapses
        - Solutes can accumulate to adjust to the movement of water
            - Osmotic adjustment
- Causes of water deficit
    - Drought decreases water pressure
    - Hot and windy enviro increases transpiration
    - Salinity causes soil to hold in water

    - Freezing reduces water potential
- Shell of hydration
    - Water deficit increases the concentration of toxic ions and the loss of the protective shell around vulnerable molecules
    - Salt disrupts the shell and leads to protein denaturation, but compatible solutes (osmoprotectants) do not disrupt protein structure
- Osmolytes/osmoprotectants
    - Reduce osmotic potential without disrupting shell of hydration
    - Includes
        - Sugars and sugar alcohols - mannitol, sorbitol, pinitol, oligosaccharides such as trehalose and fructans
        - Zwitterionic compounds - amino acids like proline, quaternary ammonium compounds like glycine betanine
            - Carry positive and negative charges
    - Basic strategies for engineering stress resistance focus on the production of osmoprotectants in transgenic plants
        - Synthesis of glycine betaine in tobacco
            - Glycine formed by a two stage oxidation of choline, catalyzed by choline monooxygenase (CMO) followed by glycine betaine synthesis by betaine aldehyde dehydrogenase (BADH)
                - Both enzymes induced by osmotic stress
                - Cloned into transgenic plants
                - Transgenic plant more salt-tolerant than control, had higher levels of glycine betaine
    - Osmoprotectant transformants may produce higher levels of osmoprotectants or scavenge ROS or inhibit plant growth at higher levels (trehalose and sorbitol)
- Salt stress tolerance approaches
    - Salt increases with poor land and water
    - Saline prevents water efflux from cells
    - Na and Cl are toxic which causes a limit on osmoprotectant usage
    - Halophytes (salt tolerant) vs glycophytes (salt sensitive) plants
        - Glycophytes increase osmoprotectants and halophytes increase Na+ transporters
    - NA+/H+ antiporters
        - Uses energy to pump Na+ against gradient
        - Transform tobacco with gene for antiport protein and grew plants in high salt where salt accumulated in leaves but not fruit
        - Increase in ROS
- Cold stress tolerance
    - Induced in cold resistant plants by slowing decreasing temperature
        - Acclimation
    - Involved chloroplast targeted COR protein but many versions of this protein need to be used to protect the plant :. Gene pyramiding

- Cold tolerance genes contain similar areas of regulation in promoters
    - C-repeat CRT element
    - Dehydration response element (DSE)
    - Low temp responsive element (LTRE)
        - Transcription factor CBF1 binds all these elements to activate transcription
        - All called COR for cold-responsive genes; COR regulon
    - Transgenic arabidopsis with CBF1 constitutively expressed under 35S promoter do not require cold acclimation
        - Expression of CBF1 with DRE (dehydration responsive element) binding domain improved resistance to drought, salt, freezing
        - :. Pathways interconnected
    - COR and heat shock regulons
        - COR genes have regulatory elements called CRT (C-repeat) or LTRE (low temp responsive element)
            - CCGAC
                - Repeated sequence linked to rought resistance element DRE (dehydration responsive element)
        - CRY/LTRE/DRE all have transcription CBF1
            - CBF1 part of a family of transcription factors induced by low temperatures
            - Leads to cold acclimation changes such as osmolyte increases including prolines and soluble sugars
- Heat stress tolerance
    - Modulated mostly by molecular chaperones involved in prot folding
    - Highly conserved throughout most species
        - HSP - heat shock proteins
- Oxidative stress
    - A secondary effect
    - Reactive oxygen species (ROS) are generated by most stresses
        - Damage proteins and membranes
        - Caused by the production of free radicals that cause a cascade

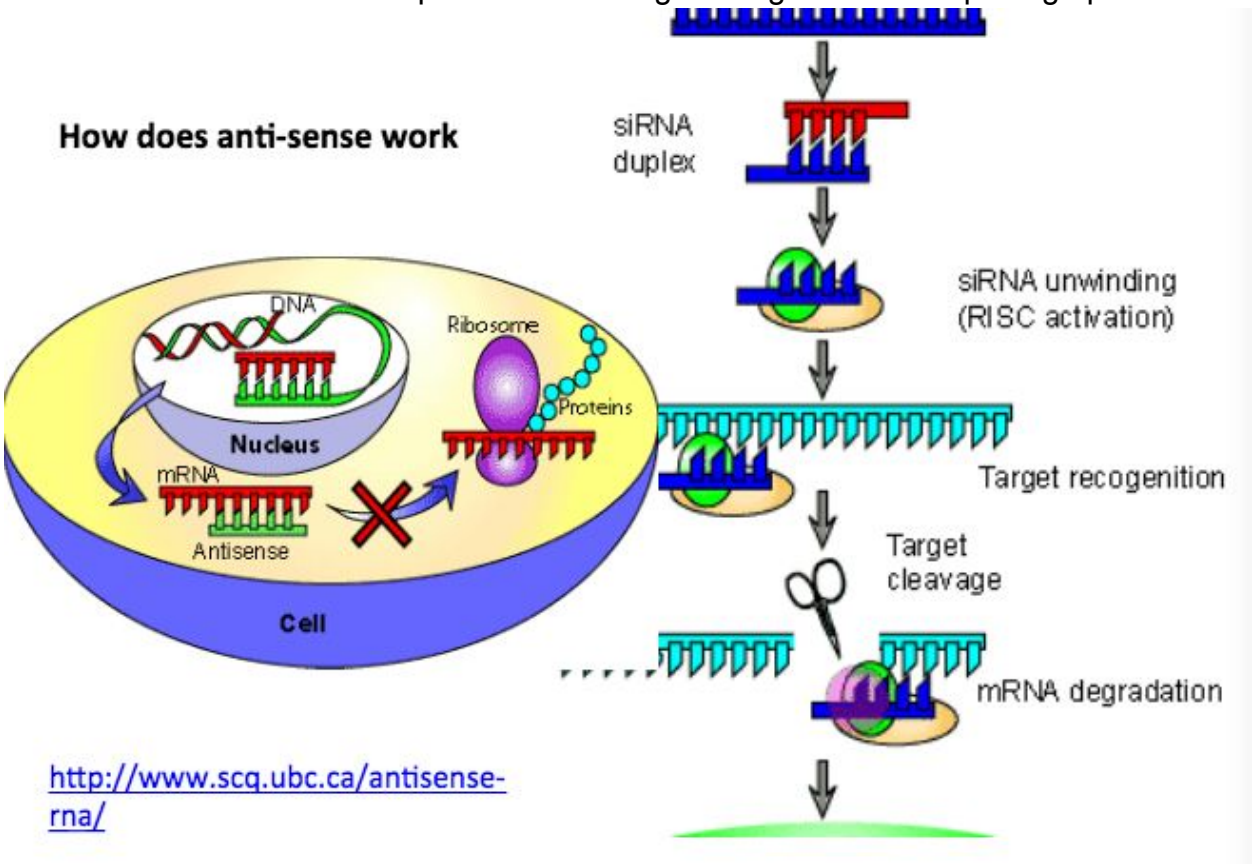


- Reduce free radicals with enzymes like superoxide dehydrogenase (SOD) catalases, peroxidases
    - Transgenically express enzymes that scavenge ROS
- Reduce free radicals by antioxidants like beta-carotene, vitamin C, vitamin E
    - Transgenically produce antioxidants

CROP YIELD AND QUALITY

- Manipulation of fruit ripening
    - Fruit ripening is an active process that begins with a burst of respiration, ethylene production (triggers ripening), softening (due to polygalacturonase PG and pectin methyltransferase PME), changes to colour (loss of chlorophyll, increase in lycopene), and flavour improvement due to starch broken down into sugar
        - Process discovered by comparing cDNA library from ripe tomato to cDNA library from green tomato
        - Clones that hybridized with ripe but not green investigated
            - Phytoene synthase, polygalacturonase, ACC oxidase
    - FlavrSavr tomato
        - Delayed softening of fruit due to PG that causes cell wall degradation, encoded for by Tom6
        - Antisense PG under control of CaMV35S (constitutive) and nos terminator, to knock down PG

- Expression of antisense gene will be constitutive and expression of endogenous gene will be ripening-specific



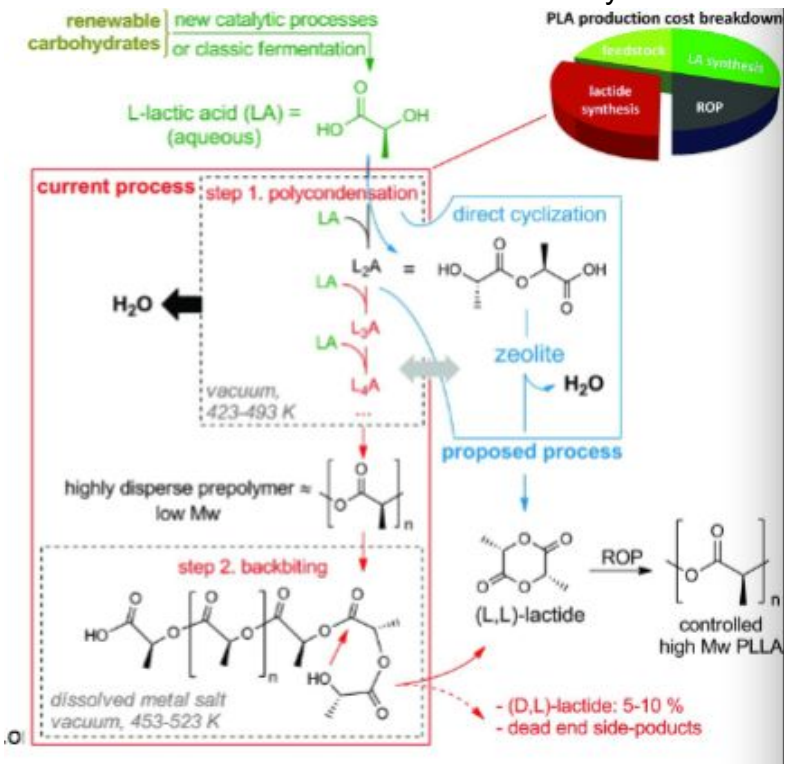
- Targeting ethylene biosynthesis (pTOM13)
  - pTOM13 encodes ACC synthase, several genes modulated by transient burst of ethylene synthesis
  - Reduction in the levels of ethylene produced led to delays in the ripening process
  - pTOM13 antisense transgenic tomatoes decrease spoilage
- Modification of colour (pTOM5)
  - pTOM5 encodes phytoene synthase, an enzyme necessary for lycopene formation (red pigment)
  - Antisense pTOM5 expression lead to yellow and dwarfed plant; a desirable crop trait
  - Constitutive expression of pTOM5 produced lycopene and had a shorter height
    - Gibberellin synthesis reduced by phytoene synthesis pathway channeling carbon into lycopene pathway
      - Gibberellins responsible for stem elongation

- Golden rice - engineered nutritional content
  - Vitamin A deficiency
  - Genetically engineer rice with provitamin A

        - Rice lacks enzymes to synthesize beta carotene from IPP so add beta carotene pathway genes, a vitamin A precursor
            - Daffodil gene phytoene synthase
            - Bacterial gene phytoene desaturase & carotene desaturase
            - Daffodil gene lycopene beta cyclase
        - Fused genes to rice endosperm promoter so enzymes produced in rice grain
- Enhanced photosynthesis
    - Target rubisco because it has a low efficiency
        - Replace with Rubisco from cyanobacteria b/c it's more efficient
        - Result not better & could transfer to weeds

MOLECULAR FARMING

- Candidate gene fused into expression vector, introduced into plant to make transgenic plants, planted, harvested, extracted and purified, PMPs
- Starch
    - Normally consists of 20-30% amylose (unbranched chains of glucose) and 70-80% amylopectin (branched molecules)
        - Branch in synthetic pathway
    - Triose phosphate generated from the calvin cycle is converted to hexose phosphate and then ADP-glucose (a substrate for starch synthesis)
    - Gene silencing to make high-amylose starch in barley
        - RNA hairpin produced with three members of the starch branching enzyme family (SBE) to silence all amylopectin production
        - A small amount of enzyme was still produced



- Bioplastics
    - From lactic acid a lactide is produced, and a metal catalyst makes PLA
        - Polylactic acids
        - Polylactic acid is an alternative to petrochemical based plastics
            - Polylactic acid has lactide as a key building block
                - Cyclic dimer of lactic acid
        - Zeolite based catalytic process converts lactic acid into lactide
        - Selectively control condensation
- Cyclodextrins from starch
    - Produced from starch fermentation
    - Ring with a hydrophobic pocket that can solubilise hydrophobic pharmaceuticals in high concentrations, which will be released when injected into patients at lower concentrations
- Polyfructose (inulin)
    - Soluble fructose polymers that can be used as a carbohydrate reserve
    - A soluble fiber or prebiotic that contains few calories
    - Exceptional amounts produced in hairy root cultures
    - Begin by transferring donor fructose from a donor sucrose to an acceptor sucrose molecule, to form ketose by sucrose-sucrose fructosyltransferase
- Improving plant oils
    - Long chain fatty acids but short to medium chain fatty acids desirable
    - Transgenic rape seeds produce eruic acid
        - Longer chain fatty acids
        - Oleochemical not suitable for consumption; industrial preparation
    - Coconut and palm kernel oils and lauric acid used in cosmetics and soaps
        - Shorter chain fatty acids
        - Fatty acid length terminated by hydrolysis lauroyl-ACP
            - Introduction of this gene into rapeseed caused fatty acid termination to come sooner and lauric acid to accumulate in the seed oil
        - Expression delta 6 desaturase gene
- Protein production systems
    - Nuclear expression - constitutive
        - Extract foreign protein
    - Tissue specific
        - Feed edible protein to patients
    - Targeted to seeds
        - Seed specific promoters
        - Extract foreign protein from specific tissues
    - Targeted to chloroplasts
        - Increase production
- Virus systems
    - TMV for production of antibodies
        - scFv antibody sequence located adjacent to translation initiation sequence

        - Infectious RNA used to transfect plants to produce subgenomic sgRNA in plants that acts as a template for expression of scFv
- Overcoat system
    - Construct a recombinant plant virus with a protein epitope
    - Introduce epitope sequence into coat protein seq within a vector
    - Infectious RNA was made and transfected into plants
    - Virus capsids containing the expressed proteins were produced
- Oleosin system
    - Protein produced as a C-terminus fusion to oleosin (an oil body associated protein)
    - Separate protein of interest and oleosin by cleaving with a protease and crushing seeds into an aqueous buffer
    - Centrifuge to separate into three fractions
        - Oleosin and pharmaceutical in top layer, which can be siphoned off and re centrifuged to separate out the pharmaceutical
    - Insulin and epidermal growth factor produced this way