NST PMS 1B: Origins of modern agriculture

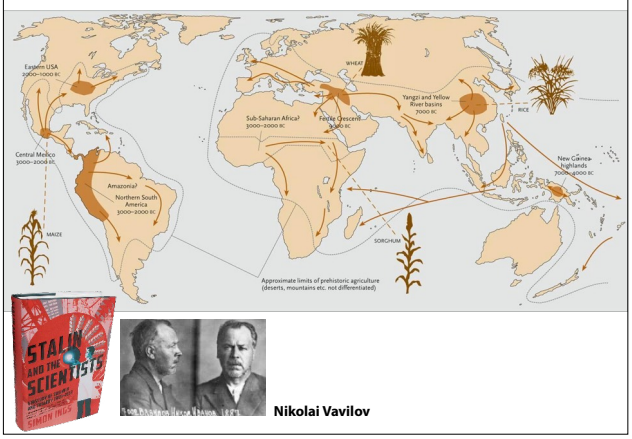
Prof. Jim Haseloff (jh295)

Lecture 1. **Plant breeding and transformation**
(i) Crop domestication, with maize as an example
(ii) Modern agriculture, hybrid maize and the rise of agribusiness
(iii) Green Revolution
(iv) Agrobacterium mediated plant transformation

Lecture 2. **From genotype to phenotype**
(i) Designing synthetic plant genes
(ii) Single gene traits: pest and herbicide resistance
(iii) Reporter genes
(iv) Microscopy

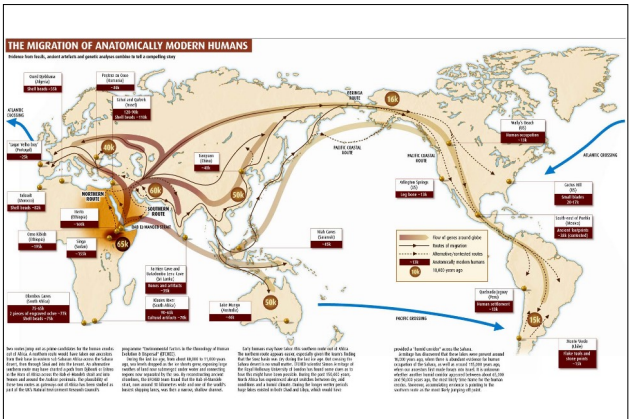
Lecture 3. **New tools for engineering future crop traits**
(i) Complex traits and breeding
(ii) Reprogramming regulatory networks
- Engineering new metabolic pathways
- Loss-of-function e.g. for reduced pod shatter
- Re-wiring networks e.g. modification of tomato plants
- Selective amplification of pathways e.g. expansion of structural tissues

Origins of world crops



Nikolai Vavilov

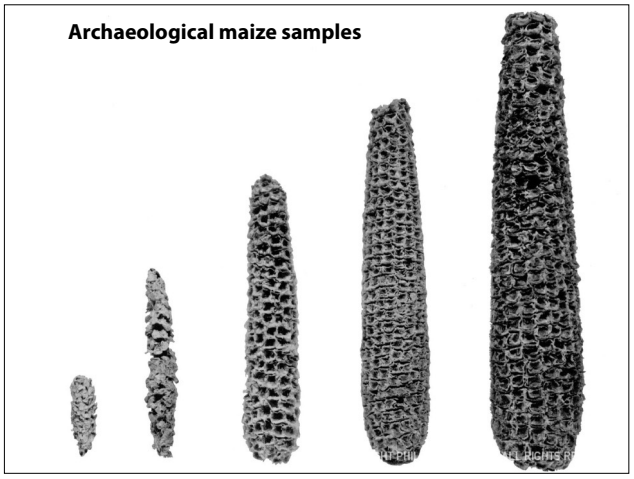
Nicolai Vavilov was a Russian biologist who first popularised the idea of geographical centres of diversity for the origin of modern crop species. These centres corresponded to areas of botanical diversity that coincided with the establishment of early human societies and plant domestication.



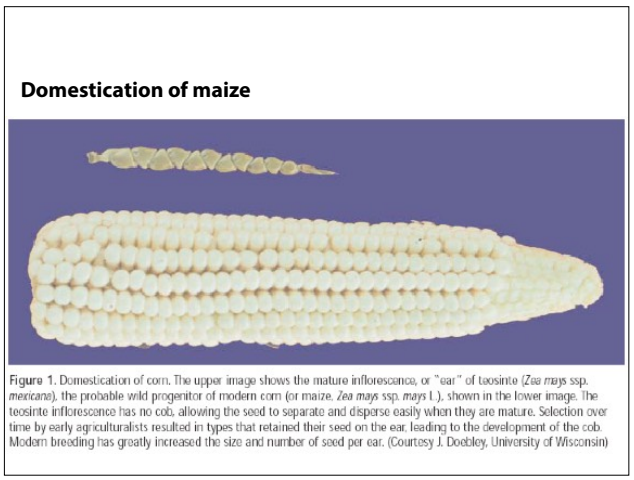
Human migration and establishment of population centres

Current theories for the evolution of anatomically modern humans, include origin in east Africa and successive waves of migration into Europe, Asia and the Americas - starting over 65,000 years ago. By 15,000 years ago modern humans had reached Mesoamerica. Over the following millennia, local people shifted from a nomadic lifestyle to an existence based on agriculture, and began the domestication of local plant species. In this lecture we follow the history of human use for one of these plant species, maize.

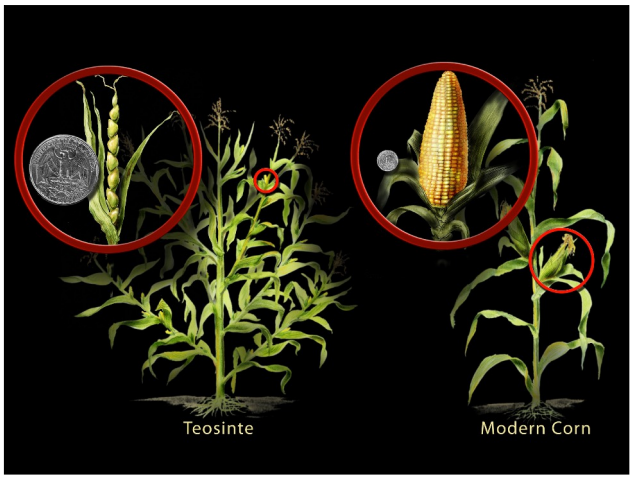
Diorama at the American Museum of Natural History showing an Aztec market in Tenochtitlán, the capital city of the Aztec empire in ancient Mexico - in the year 1519, immediately prior to the arrival of Europeans. By this stage maize had been grown and selected for around 7000 years, and could be found in recognisably modern form.



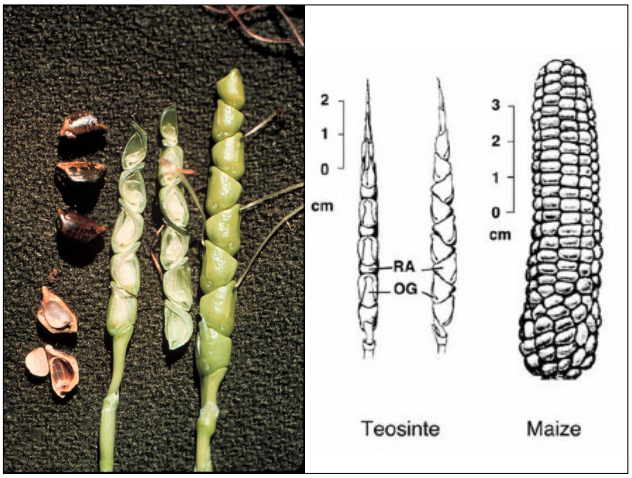
Zea mays cobs, plant remains found at the Tehuacan Valley, Puebla, Mexico; c. 5000 BCE to 1500 CE. Archaeological excavations have revealed a series of intermediate forms of maize, and these have been dated and can be arranged on a timeline - with cobs ranging from small vestigial forms through to large modern forms due to selective propagation of seed. Robert S. Peabody Museum of Archaeology, Phillips Academy, Andover, Massachusetts.



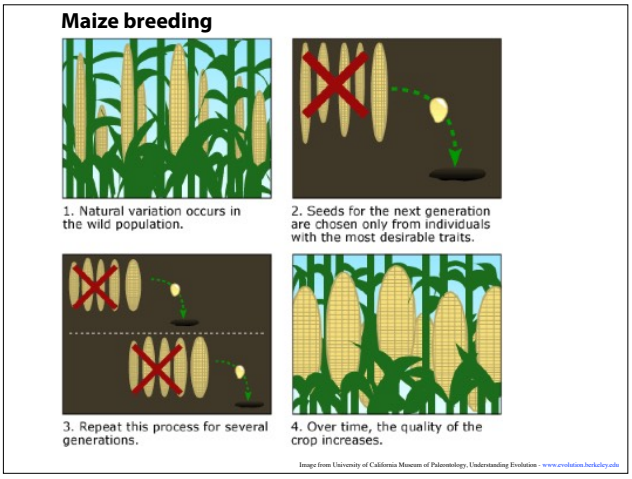
Early forms of maize strongly resemble teosinte, a plant endemic to Mesoamerica, and a subspecies of *Zea mays*. This likely progenitor has a strikingly distinct morphology, with smaller numbers of kernels arranged on a spike. It has been estimated that new varieties of maize been selected for over 9000 years. Modern varieties are characterised by a cob architecture with much larger numbers of kernels on each inflorescence.



The overall habits of teosinte and modern maize plants are strikingly different. Teosinte plants are more highly branched with multiple male and female inflorescences. Graphical representations are shown with a coin added for scale. Modern maize plants are taller with a higher degree of apical dominance, and are better adapted for modern agricultural practices.

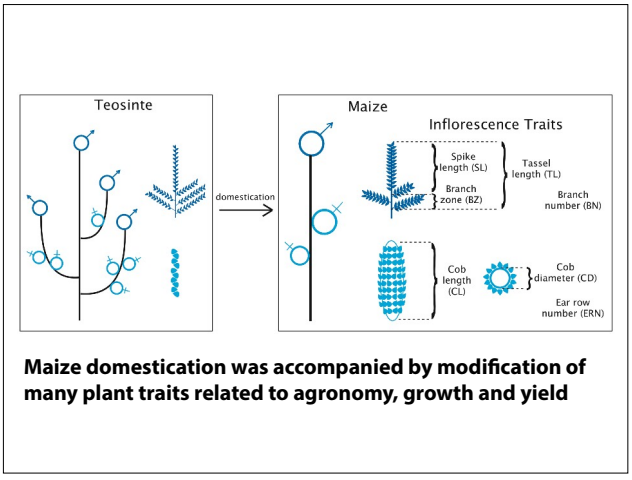


A close-up view of teosinte, showing the hardened integument that normally covers the seed. Harvested teosinte seed need to be broken open to release the nutritious kernel. The selection of improved varieties produced maize varieties that lacked this integument.

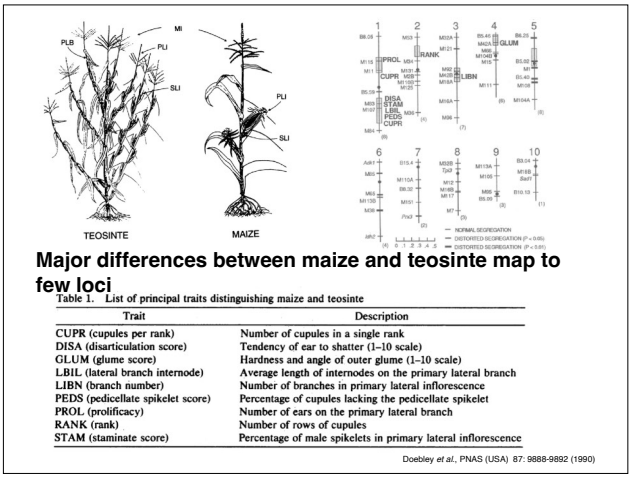


Wild teosinte and primitive maize varieties are self fertilising and true breeding plants. Genetic variation occurs naturally in these populations. Early agriculturalists simply chose plants with improved characters and selectively planted their seed. the cumulative effect of this simple selection procedure over many generations gave rise to plant lines with many improved characters.

Maize rapidly became a staple crop and took on greater, even religious significance. The application of crop selection gave rise to a myriad of new maize varieties.
The new maize varieties posesses highly individual characteristics were selected and maintained across Mesoamerica. Farmers developed local varieties with different characteristics. Some examples are shown here.
Traditional respect for the different properties of these local corn varieties has resulted in the historical maintenance of a large number of races or varieties. Today, the conservation of diversity in maize has been taken up by international seed banks such as CIMMYT (http://www.cimmyt.org)
Diverse true breeding populations of maize have been found widely across South America and North America, as a result of the historical spread of maize as a staple crop.



With the benefit of modern knowledge, one can take a more analytical approach to describe the changes that occurred during domestication of maize. Here, formal annotation is used to describe the inflorescences and branching of Teosinte and maize. Domestication saw the origin of a series of novel characters that corresponded to modification of the original plant morphology. These include creation of branching patterns and arrangement of female flowers and therefore kernels.



Major differences between maize and teosinte map to few loci

Table 1. List of principal traits distinguishing maize and teosinte

Trait	Description
CUPR (cupules per rank)	Number of cupules in a single rank
DISA (disarticulation score)	Tendency of ear to shatter (1–10 scale)
GLUM (glume score)	Hardness and angle of outer glume (1–10 scale)
LBIL (lateral branch internode)	Average length of internodes on the primary lateral branch
LIBN (branch number)	Number of branches in primary lateral inflorescence
PEDS (pedicellate spikelet score)	Percentage of cupules lacking the pedicellate spikelet
PROL (prolificacy)	Number of ears on the primary lateral branch
RANK (rank)	Number of rows of cupules
STAM (staminate score)	Percentage of male spikelets in primary lateral inflorescence

Doebley *et al.*, PNAS (USA) 87: 9888-9892 (1990)

Work from John Doebley's lab has mapped the genetic differences between teosinte and maize. Genetic mapping studies have identified genes known to be involved in vegetative branching, morphology and floral architecture. Strikingly it was estimated that around 90% of the difference in form between teosinte and maize could be accounted for by less than ten genetic loci.

Maize farming in the US Midwest circa 1900

Europeans adopted maize as a crop and the 1800s saw large plantings across the Midwest of the United States. Before 1900 farmers in the Midwest were highly self-sufficient. They looked to the outside world for things like salt and nails, but external inputs into crops were minimal. Fertiliser inputs were limited to manure, pesticides were unknown and crops were true breeding and seed corn was obtained from previous year's crop. County fairs included competitions for the highest yielding corn plants.

Genetic crossing to produce hybrid Maize

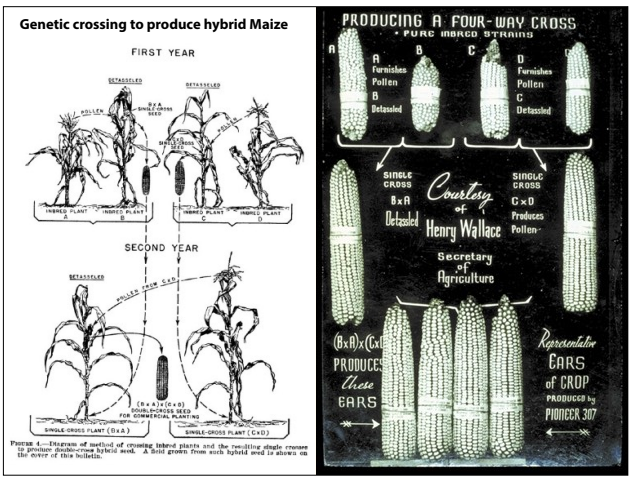


FIGURE 4.—Diagram of method of crossing inbred plants and the resulting single crosses to produce double-cross hybrid seed. A field grown from such hybrid seed is shown on the cover of this bulletin.

In the 1900s scientists like G.H. Shull observed that open pollinated inbred forms of maize became less productive over time. In contrast heterosis or out-crossing gave rise to highly productive progeny. (Maize plants have separate male and female flowers and detasseling of male flowers is a simple way of ensuring selective crossing). Through the 1920s, plant breeding stations were established to create parental inbred lines that could be used for different crosses and to create highly productive maize seed.

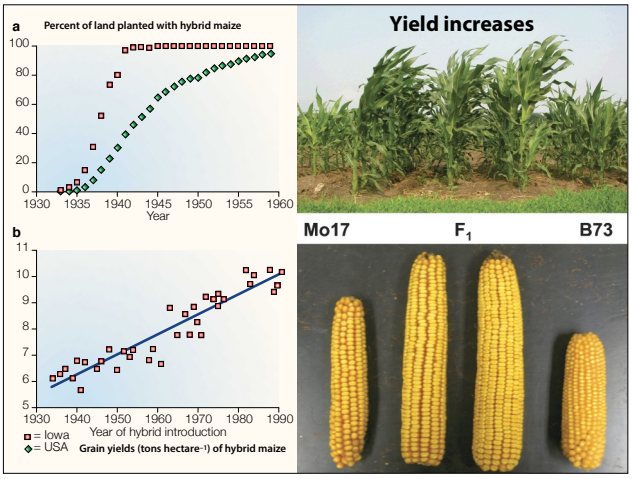
Roswell Garst: marketing and adoption of hybrid maize.

Growth of seed companies (like Garst Seed) and increasing use of fertilisers and pesticides.

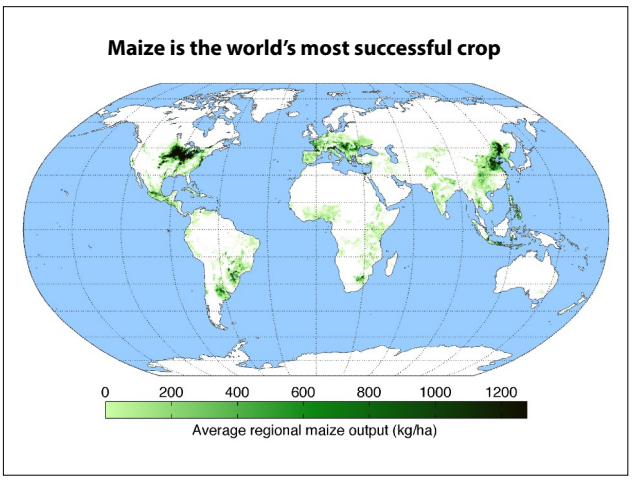
Beginning of modern agriculture and integration of industrialised approaches to food production.

Entrepreneurs like Roswell Garst helped transform US agriculture last century. He helped to establish sales of hybrid corn seed with the noted corn breeder Henry Wallace in 1930s in Iowa. Wallace established Pioneer Hi-Bred, and Garst established Garst seed. Farmers were previously highly self reliant - saving a portion of their crop for next year's seed, using manure for fertiliser, and using draft horses for ploughing and carting the hand-picked corn. Garst offered free bags of hybrid seed corn in return for half of the next seasons increased yield. When the new seed outperformed, he only accepted the cost of the seed corn - in return for a commitment for the following season. Farmers soon switched to purchasing seed corn for cash.
Eventually this led to the conversion of farming from an occupation, to an industry. There was a loss of diversity, from 786 varieties in 1903 to 52 in 1983 - and increased application of synthetic fertilisers, pesticides and herbicides. Machinery

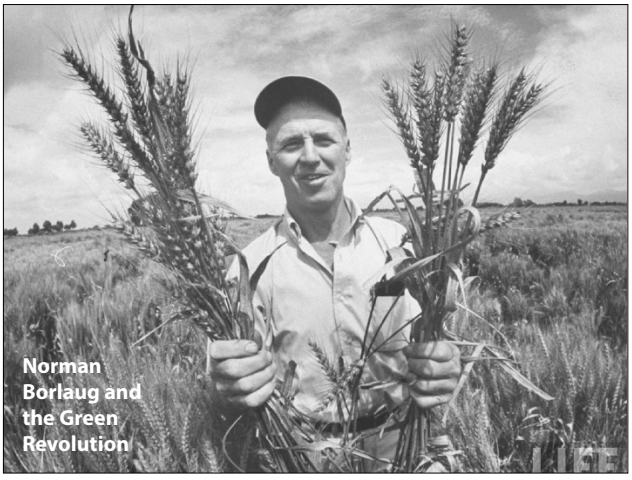
was invented for handling of the more uniform crops. Integration of these activities gave rise to agribusiness.



Hybrid maize seed saw rapid adoption in the US Midwest after its introduction in the 1930s. the overall percent plan planted with hybrid maize increased rapidly. In addition, new varieties of hybrid maize saw rapid increases in productivity over the coming decades. Photographs are shown of parental lines and hybrid progeny.



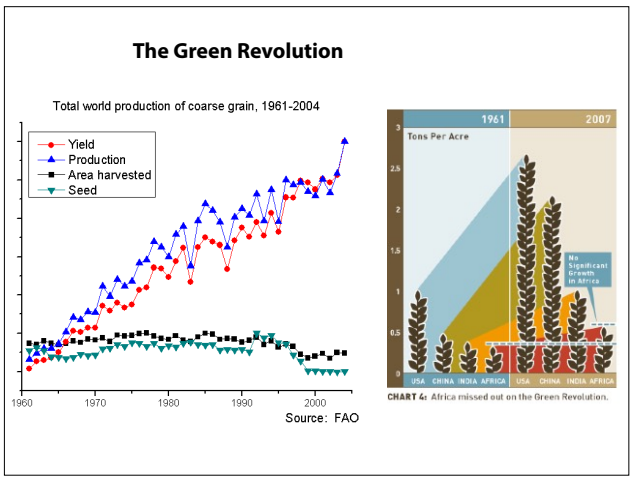
From its origin as a Mexican weed, worldwide production of maize is over 1 gigatonne per annum, more than wheat or rice. (http://www.fao.org/faostat/, and http://www.worldofcorn.com). The USA and China are the major producers of maize.



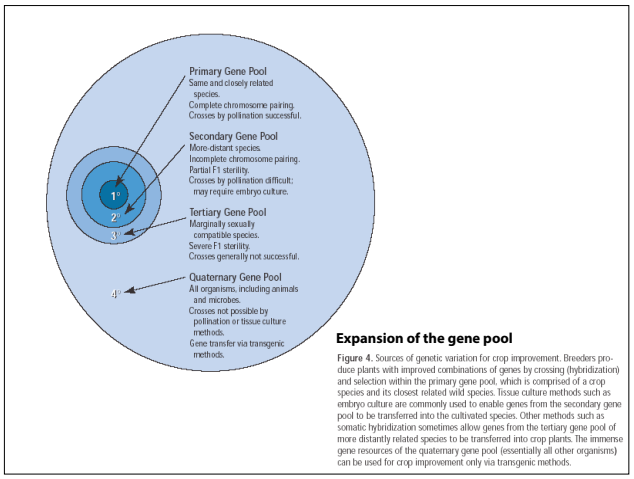
Selective breeding of other crops has dramatically improved their yields also. The decades following 1960's saw the breeding of highly productive new varieties of wheat. Many of these varieties were dwarf, which provided agronomic benefits and allowed commitment of more resources to seed production during growth. In addition, improved response to inorganic fertilisers and introduction of disease resistance through cycles of out-crossing and back-crossing contributed to new elite varieties.
Norman Borlaug was a pioneer of these efforts. He is shown here with Sonora-64, one of the semi-dwarf, high-yield, disease-resistant varieties that was key to the Green Revolution.

La Moisson (1874) - Léon Augustin Lhermitte) - with a graphic representation of a partly harvested wheat field in northern Europe. Note that the height of these wheat crops reached over waist height.

Modern wheat crops are much shorter, shown here with Norman Borlaug and colleagues at a trial field of Sonora-64, at what is now CIMMYT's CENEB station (Campo Experimental Norman E. Borlaug, or The Norman E. Borlaug Experiment Station), near Ciudad Obregón, Sonora, northern Mexico. The story of Borlaug career is inspiring, a short version can be found at https://en.wikipedia.org/wiki/Norman_Borlaug. He has been credited with saving a billion people from starvation, and his work has been extended to rice varieties.



From the 1960s, the worldwide production of grain has increased dramatically in yield and total production despite relatively constant area of cultivation and planted seed. The bulk of these increases have been seen in the developed world, China and India. The benefits of increased production have not been so widely seen in Africa.



Until the early 1980s, the genetic modification of crops required the introduction of new genes through sexual crossing and refinement of traits through breeding. Specialised breeding techniques can allow access to gene pools outside of the same species - but access is confined to closely related plants. The advent of techniques to create transgenic plants allows synthesis of effectively any engineered DNA construct and unconstrained modification of plant genomes. This breakthrough came in 1983 with the independent publication of the first Agrobacterium-mediated plant transformation papers from three groups.

Plant transformation

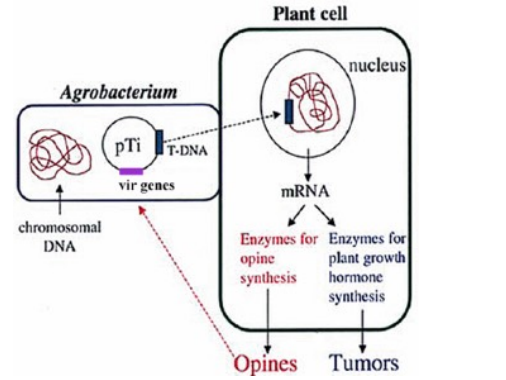
D.J. Pacumar et al. / Physiological and Molecular Plant Pathology 76 (2011) 76–81

Crown gall disease

Fig. 1. Crown gall tumor on an oak tree.

The current era of plant improvement is marked by the advent of new approaches to targeted genetic improvement of crops. This has its origins in the domestication of a bacterial plant pathogen, *Agrobacterium tumefaciens.* The natural oncogenic properties of Agrobacterium have been harnessed to engineer gene transfer.

Agrobacterium tumefaciens is capable of binding to plant cells, forming a conjugation complex and transferring a specific and delimited segment of DNA. Here, bacteria are shown attached to a potential recipient plant cell in an electron micrograph.
In a normal infection, conjugation of a bacterium with a susceptible plant is followed by replicative transfer of a specific segment of DNA called the T-DNA (shown in red) - from a region of a Ti plasmid into a recipient plant cell. The transformed cells are then programmed to proliferate.



Agrobacterium transfers genes for tumour growth and opine biosynthesis to plant cells

The transferred T-DNA includes genes that encode enzymes for synthesis of plant growth hormones. The ectopic production of the growth factors results in unregulated plant cell growth and formation of tumours. In addition, the T-DNA encodes enzymes for production of highly unusual metabolites, called opines. Growing tumours produce opines, which can diffuse into the surrounding soil.
Different races of *Agrobacterium* employ different chemical species of opine (e.g. nopaline, octopine, agropine, etc.) Bacteria of the same type encode genes for transport and metabolism of the corresponding opine on the Ti plasmid. (The Ti plasmid encodes both genes for bacterial expression and those destined for transfer to, and expression in the plant). In nature, Agrobacteria use plant transformation to create local sources of opine, as nutrient that they alone can consume.

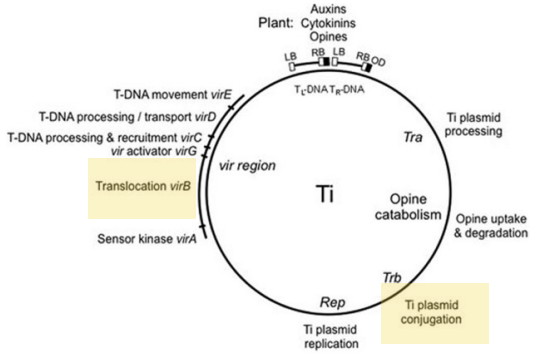
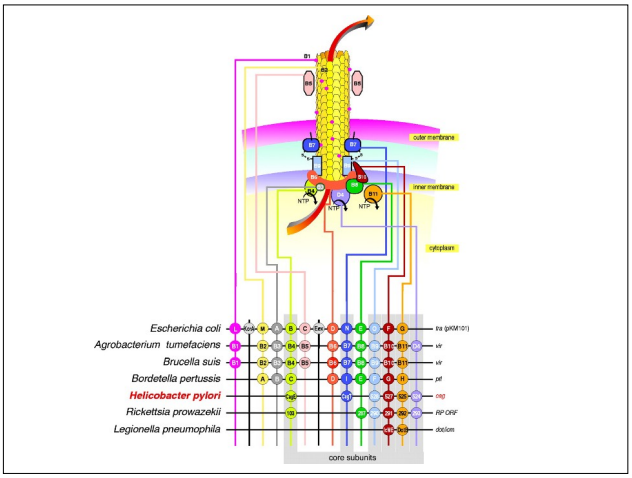
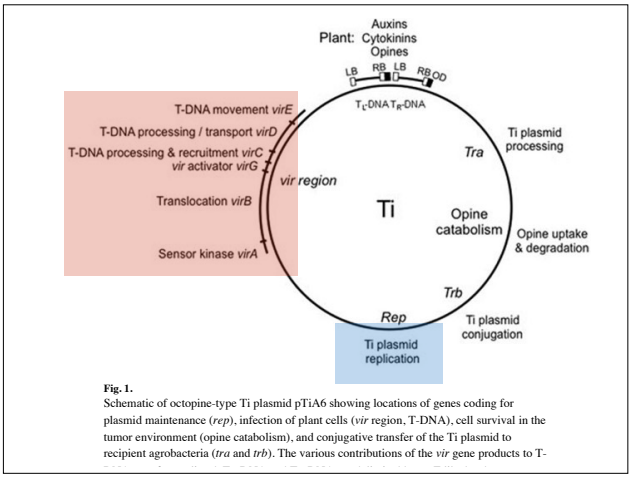


Fig. 1.
Schematic of octopine-type Ti plasmid pTiA6 showing locations of genes coding for plasmid maintenance (*rep*), infection of plant cells (*vir* region, T-DNA), cell survival in the tumor environment (opine catabolism), and conjugative transfer of the Ti plasmid to recipient agrobacteria (*tra* and *trb*). The various contributions of the *vir* gene products to T-

Agrobacterium tumefaciens exploits a plasmid-borne bacterial conjugation system (a multi-gene Type IV secretion system T4SS) in order to transform susceptible plant cells.
Agrobacterium strains contain large Ti (tumour-inducing) megaplasmids (~200kb), which have a modular structure, with grouped sets of genes that play distinct functional roles. First, they encode two different Type IV secretion systems (i) Trb for Ti plasmid transfer between bacteria, and (ii) virB for T-DNA transfer to plant cells (highlighted in yellow).



The Type IV secretion systems are encoded in multigene operons, which are highly homologous. Similar machinery is found for T4SS involved in conjugative transfer between bacteria, and between bacterium and plant.
Diagrammatic representation of the Type IV secretion system and conserved protein subunits found conserved among different bacterial species. The different elements of the secretion system come together to create a "hypodermic needle" like protein assembly on the surface of the bacterial cell that, in the case of Agrobacteria, facilitate efficient transfer of DNAs into recipient plant cells.



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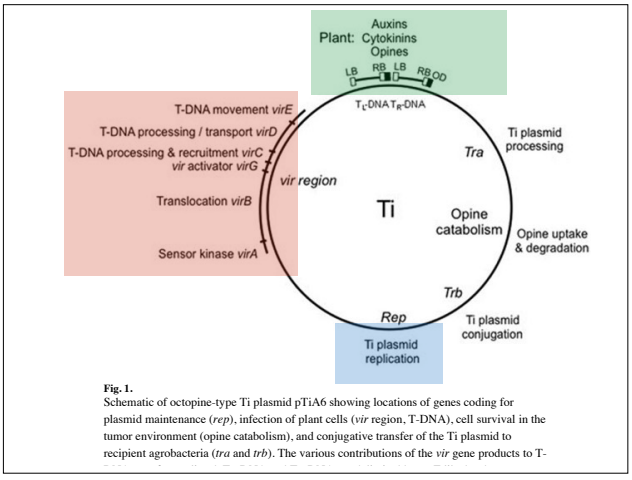
Second, the Ti plasmid encodes proteins and origin required for plasmid replication in the bacterial host (blue).
Third, the entire virulence region (vir) encodes proteins required for sensing wounded plant tissues, activating the vir operon, processing and transfer of the T-DNA in the recipient plant cell (including the Type IV secretion system). Shown in red.



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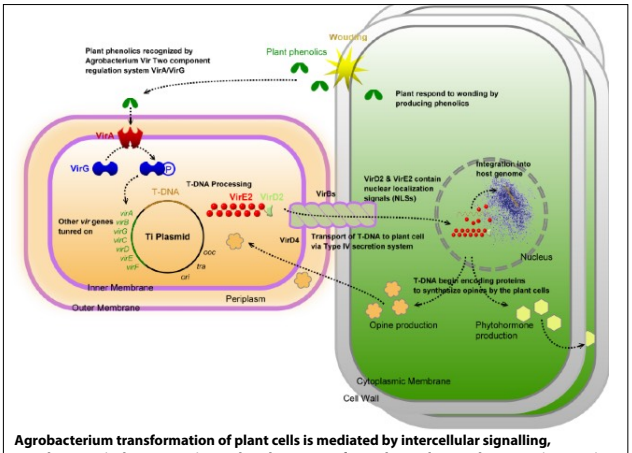
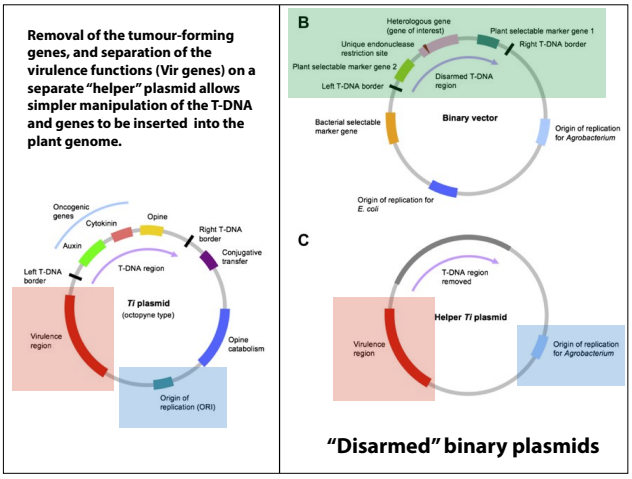
Fourth, the Ti plasmid contains one or more T-DNA (transfer DNA) regions (shown in green). Each is flanked by a specific 25 base-pair sequence, and these boundaries are termed left and right borders.



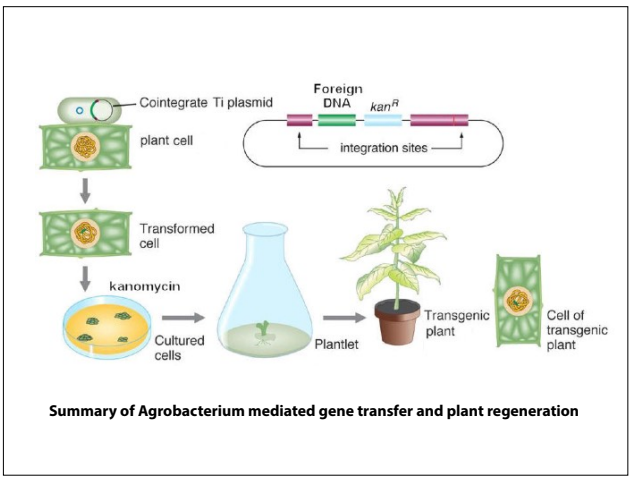
Agrobacterium transformation of plant cells is mediated by intercellular signalling, attachment, virulence protein catalysed DNA transfer to the nucleus and genome integration.

Diagrammatic representation of the induction process, mobilisation and transfer of the T-DNA segment into the recipient plant cell, and integration of the DNA into the host plant genome. Wounded plant cells liberate phenolic compounds, which are sensed by bacterial membrane receptors. These activate the signal transduction pathway and result in transcriptional activation of the virulence operon. Vir genes are responsible for recognition of the T-DNA segment at 25 base pair recognition sequences. Single-stranded DNA nicks trigger a specific replicative transfer of the T-DNA into the plant cell via the Type IV secretion system as a protein-coated single-stranded DNA complex. The defined T-DNA sequence is integrated randomly into the plant genome as a double-stranded segment. The T-DNA segment contains genes with plant control sequences. Once

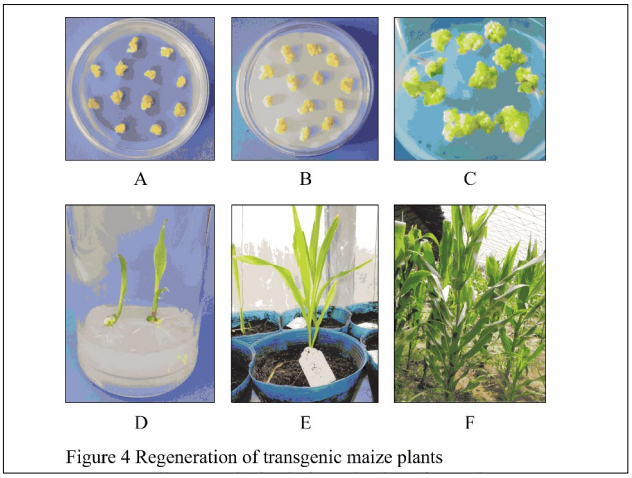
integrated, their expression gives rise to plant hormone and opine production.



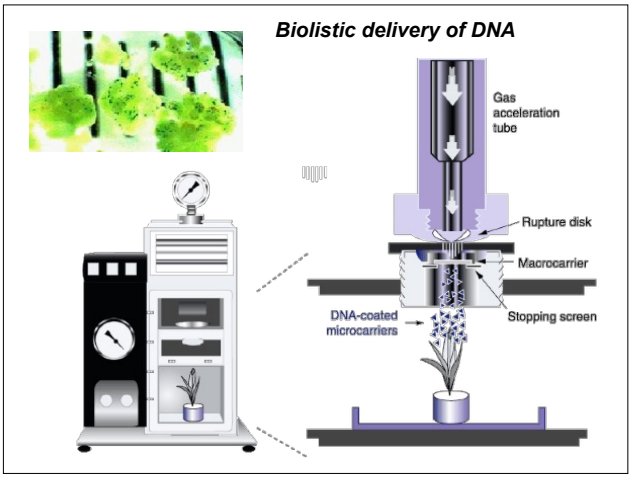
The native Ti plasmid can be disassembled according to its modular nature, and the functions required for tumourigenesis and opine production removed.
The functions required for DNA transfer to the plant can be maintained on a large disarmed plasmid. This is termed a helper plasmid. The gene functions required for DNA transfer can work *in trans* for a second smaller plasmid containing a customised T-DNA segment, along with compatible replication machinery and bacterial selection marker. This forms a binary plasmid system.
This allows simple engineering of new genes on a shuttle plasmid that allows Agrobacterium-meditated transformation of plants.



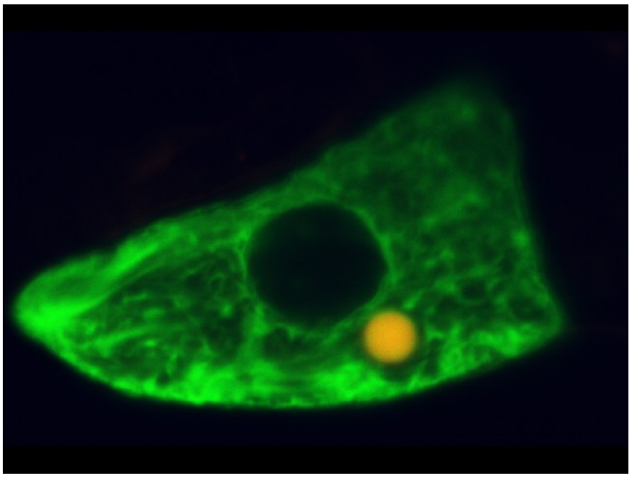
Plant transformation with a disarmed binary plasmid requires (i) co-cultivation of plant material with an engineered Agrobacterium strain, (ii) curing of the Agrobacterium by (microbial) antibiotic treatment, (iii) regeneration of plantlets from transformed cells under (plant specific) antibiotic selection. In this example, the engineered T-DNA contains kanamycin. (iv) Rescue of regenerated plants for grow and harvest transgenic seed. At this point transgenic plants can enter a breeding programme.



Returning to maize as an example, here are images of transformed and regenerating maize tissues, plantlets and fertile plants.



Agrobacterium-mediated transformation is not the only way to produce transgenic plants. For example, high velocity, biolistic delivery of DNA-coated microparticles (usually gold or tungsten) can also be used to produce transgenic plants and algae. This is the method of choice for transformation of organelles.



A confocal micrograph of a wheat embryogenic cell that has been bombarded with a colloidal gold particle coated with DNA containing an active gene for an ER-localised green fluorescent protein. In this example, DNA has been delivered to the cytoplasm of the cell, accumulated in the nucleus (unlabelled in the centre of the cell), and been transcribed. Messenger RNAs have been exported back to the cytoplasm, where they were translated and the green fluorescent protein product accumulated within the endoplasmic reticulum. Biolistic delivery is the transformation method of choice for chloroplast transformation.

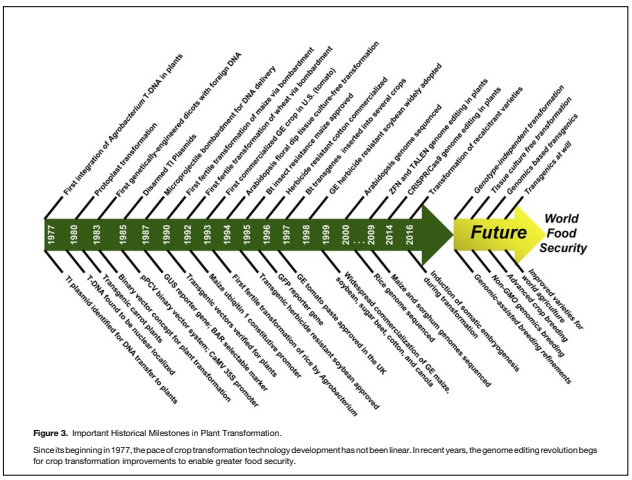
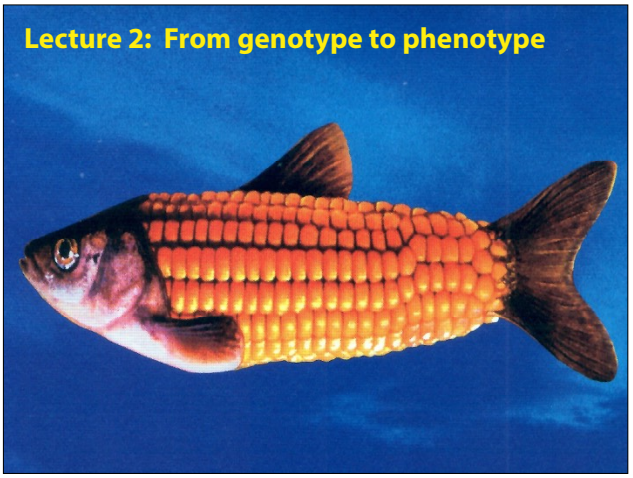


Figure 3. Important Historical Milestones in Plant Transformation.

Since its beginning in 1977, the pace of crop transformation technology development has not been linear. In recent years, the genome editing revolution begs for crop transformation improvements to enable greater food security.

Time line for recent milestones in transgenic plant work. The next lectures will explore some of these advances in more detail. We will explore gene structure in plants - i.e. how do you successfully build a new plant gene? - and look more closely at what types of genes are used in the commercial world, and how one employs reporter genes to explore the link between genotype and phenotype.



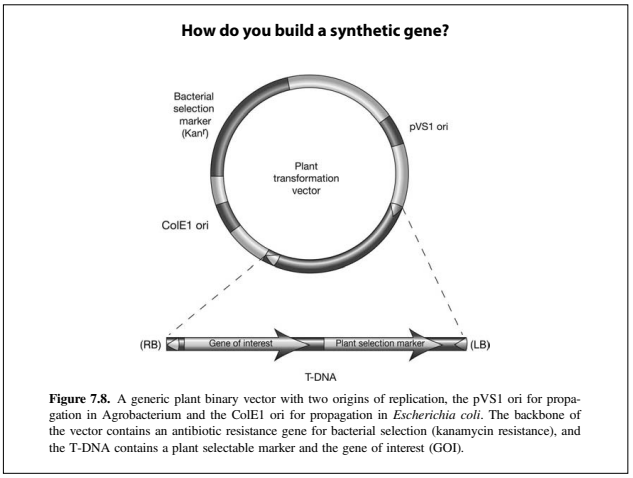


Figure 7.8. A generic plant binary vector with two origins of replication, the pVS1 ori for propagation in Agrobacterium and the ColE1 ori for propagation in *Escherichia coli*. The backbone of the vector contains an antibiotic resistance gene for bacterial selection (kanamycin resistance), and the T-DNA contains a plant selectable marker and the gene of interest (GOI).

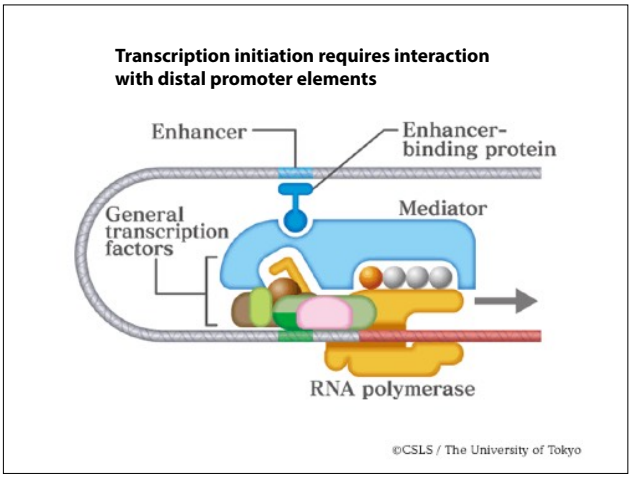
The previous lecture contains a description of how binary plasmid vectors were derived from tumourigenic Ti plasmids, and used for Agrobacterium-mediated plant transformation. These transformation vectors all contain a backbone with origins of replication and a bacterial selection marker. In addition, they contain a T-DNA marked for transfer to the plant by flanking 25 base pair repeat sequences, called the left border (LB) and right border (RB). The T-DNA can contain arbitrary DNA sequences, which would normally include a gene (or genes) of interest and a selection marker for rescue of transformed plants.
The Agrobacterium mediated transformation of a plant cell results in insertion of a foreign DNA segment into a random section of the plant genome. Any genes on the foreign DNA segment must contain control sequences that allow interaction with host transcription factors, RNA polymerase and other regulatory proteins for proper expression. In addition, flanking domains of plant chromatin can influence the activity of the foreign gene.

Rules for design of synthetic genes

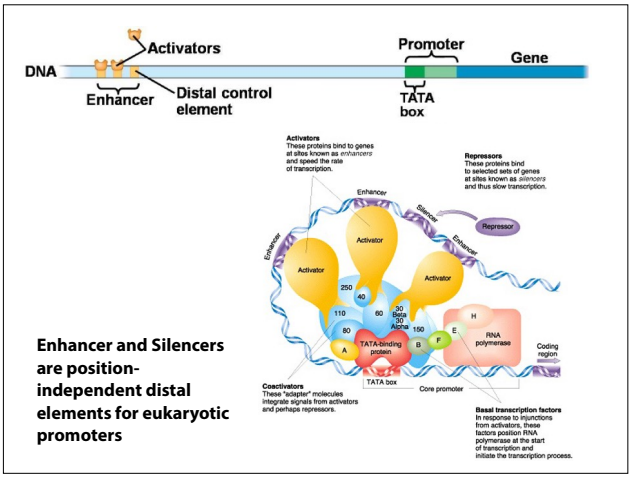
1. Specific sequences provide a key for interaction between DNA and host proteins, which ensure regulated conversion into RNA and protein.
These sequences are crucial for design of properly regulated synthetic genes.

2. How do you measure and validate the behaviour of a single transgene in a genome with 10,000's of other genes being expressed?

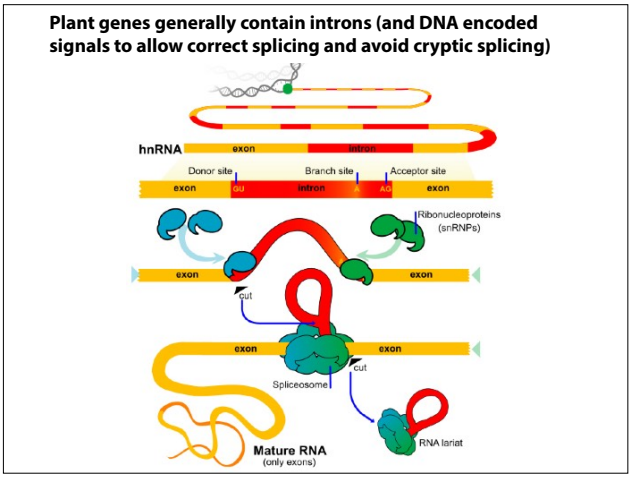
Control sequences for a synthetic gene must be sufficient to allow regulated transcription and efficient translation, and are the key to successful design of a synthetic gene construct. Once a synthetic gene is introduced into a plant there is the additional challenge of analysing its behaviour in situ.



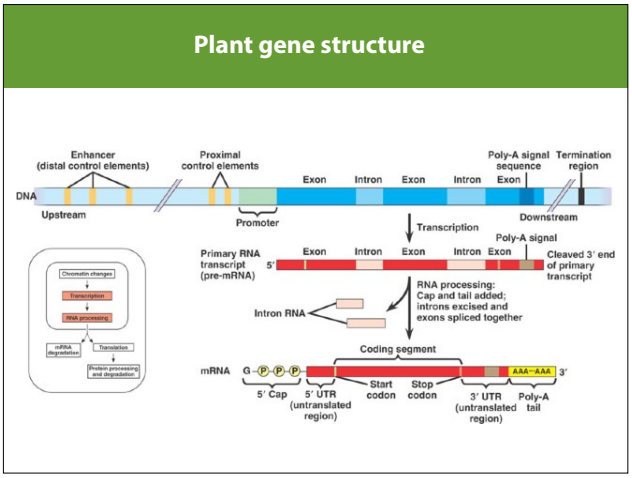
Eukaryotic protein encoding genes are transcribed by RNA polymerase II. The core protein components of RNA polymerase II bind to DNA immediately upstream of the transcribed sequence (red). DNA binding is associated with a conserved TATA-containing sequence (green). However, this complex is not sufficient to initiate transcription. Distal promoter elements, or enhancers (blue), contain binding sites for regulatory proteins that initiate molecular contacts with the core RNA Polymerase via mediator proteins. There may be many enhancers (or silencers) of transcription that can embody complicated genetic logic, and regulate the initiation of transcription.



Enhancer and silencer elements mediate DNA looping and formation of the active RNA polymerase complex. These elements can work at a distance, and be positioned upstream, downstream or even within genes. The proper transcription of a synthetic gene requires that appropriate DNA sequences are positioned adjacent to the coding sequence, in order to correctly mediate these precise molecular contacts with host transcription machinery.



Eukaryote genes undergo extensive post transcriptional processing. This includes the addition of a 7-methylguanylate cap at the 5′ end of the RNA transcript, and addition of a polyadenylate tail at the 3′ end of the transcript. Further, the majority of plant RNA primary transcripts contain introns that are removed by host spliceosome machinery. Spliceosomes are large ribonucleoprotein complexes that recognise RNA sequences at intron-exon junctions and branch sites, in order to precisely excise introns, and rejoin the mRNA via a series of transesterification reactions. These reactions are mediated by precise molecular contacts between host machinery and DNA/RNA sequences. Synthetic gene design must incorporate appropriate DNA-encoded sequences that mediate these molecular contacts during maturation and processing, to avoid aberrant cryptic sites and allow efficient polyadenylation and translation of mRNAs.



Conserved sequences features and their arrangement in plant genes can be used to define a map of functional domains. These are shown diagrammatically. Experiments have demonstrated these elements are functionally modular can generally be exchanged between different genes, if this sequence and position within the gene are respected. These rules for modular description of gene architecture have been used as a basis for creating common syntaxes for standardised plant DNA parts. The boundaries between modular domains were given arbitrary but constant definitions, compatible with schemes for modern, efficient assembly of genes via Type II restiction enzymes (Golden Gate, MoClo, Golden Braid, PhytoBricks). This has allowed stockpiling and exchange of common DNA parts for exchange and reuse in design of synthetic genes.

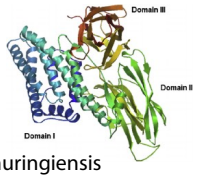
Single gene traits

Over a dozen genetically modified (GM) plant species have been approved for commercial production in the US, and the single-gene traits that have been genetically engineered into them fall into five categories.

Trait	Modified Plants	Gene Source
Insect resistance (Bt)	corn, cotton, potato, tomato	soil bacterium
Herbicide resistance	corn, soybeans, cotton, canola, sugarbeets, rice, flax	various bacteria, tobacco (modified)
Virus resistance	squash/zucchini, papaya, potato	plant viruses
Delayed fruit ripening	tomato	tomato, soil bacterium, or virus
Pollen control	corn, chicory, (radicchio)	soil bacterium

Genetically modified crops were first released for commercial use in the mid-1990s, a little more than 10 years after the first development in the laboratory. This first generation of crops were modified by the introduction of single gene traits, such as insect, herbicide and virus resistance.

Pest resistance



Bacillus thuringiensis (Bt) toxin

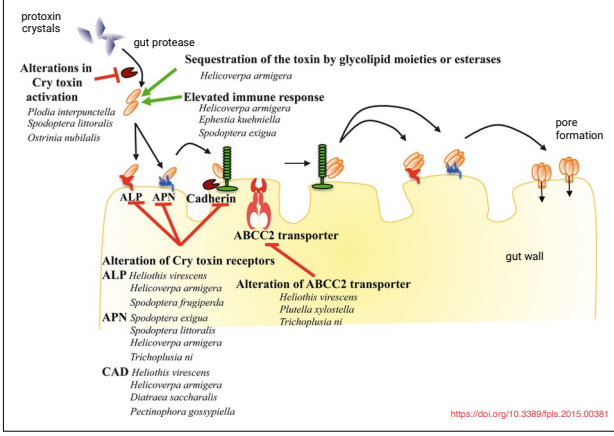
Bt toxin is a protein produced by Bacillus thuringiensis bacteria. On ingestion, and exposure to low pH and proteases in the insect gut, it binds to membrane receptors and causes water and ion leakage from epithelial cells lining the gut.

It is a highly selective toxin with no effect on mammalian cells. Bt based insecticides have been widely used in organic farming for over 50 years.

There are over 50 types of Bt toxin, each specific for different classes of insect.

Bacillus thuringiensis (Bt) strains produce a variety of protein toxins that are selective for different classes of insects. Bacterial extracts are widely used in organic farming for insect control. BT toxin can also be delivered by in vivo expression in transgenic plants.

Mechanism of action (and resistance) for Bt toxin (Cry)



Ingestion of BT toxin by insects results in processing and activation of the protein in the gut, followed by specific binding to transporters in the gut, and the formation of pores that cause uncontrolled water and ion leakage across membranes.
Mechanism of action of Bt toxins (3-domain Cry) in Lepidoptera - and different types of resistance to Bt toxin (3d-Cry) described in lepidopteran insects.
1. The larvae ingest the 3d-Cry protoxin, which is solubilized in the midgut lumen of the larvae due to high pH and reducing conditions and activated by gut proteases, generating the toxin fragment. 2. The monomeric 3d-Cry toxin binds ALP and APN receptors in a low-affinity interaction, the toxin is then located in close proximity to the membrane. 3. The monomeric 3d-Cry toxin binds the cadherin receptor in a high-affinity interaction and this interaction induces proteolytic cleavage of the N-terminal end of the toxin, including helix α-1 of domain I. 4. The cleaved 3d-Cry toxin is then able to oligomerise to form a toxin prepore oligomer. 5. The oligomeric 3d-Cry structure binds to ALP and APN receptors with high affinity. 6. The pre-pore inserts into the membrane causing pore formation.

Ears of Corn: The top is GMO (Bt transgenic), and the bottom is non-GMO. The Asian corn borer has caused damage to the ear, resulting in fungal growth (mold) and sprouting. These varieties were grown in the Philippines. (Source: Food for Thought Blog)

Bt toxin transgenic plants are highly unpalatable for target insect pests. However, pest resistance is highly specific, and pests can develop resistance to particular Bt toxins. Image: http://parrottlab.uga.edu/SIVB/HTML/102660-bt%20corn%20ears.html

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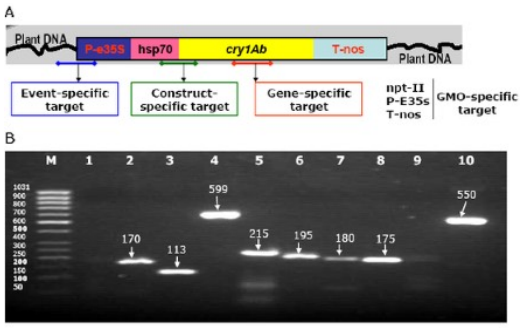
DNA structure of a commercial Bt toxin gene

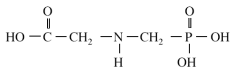


Assessment of cry1Ab transgene cassette in commercial Bt corn MON810: gene, event, construct and GMO specific concurrent characterization
Chandra K. Singh , Abhishek Ojha , Suchitra Kamle & Devendra N. Kachru
Protocol Exchange (2007) doi:10.1038/nprot.2007.440

Structure of a synthetic BT toxin gene used commercially by Monsanto in genetically modified lines of maize (e.g. MON810). The synthetic gene consists of the P-e35S promoter, hsp70 intron, cry1AB (Bt toxin) coding sequence and T-nos (nopaline synthase transcription terminator). Standard PCR assay for MON810. Lane M: 50bp marker, Lane 1: Env. Control, Lane 2: cry1Ab event specific (maize genome – P-e35S), Lane 3: cry1Ab construct specific (hsp-cry1Ab),Lane 4: gene specific (cry1Ab), Lane 5: npt-II, Lane 6: P-e35S, Lane 7: T-nos, Lane 8: hmgA, Lane 9: Neg. control, Lane 10: Pos. control (chloroplast tRNA)

Herbicide resistance

Glyphosate (Roundup)



Mode of Glyphosate Action

Glyphosate inhibits the shikimate pathway enzyme EPSPSase, an enzyme that acts late in that pathway. The pathway is responsible for, among other things, the biosynthesis of aromatic amino acids: phenylalanine, tyrosine and tryptophan. This pathway is also responsible for biosynthesis of such diverse plant compounds as phytoalexins, plastoquinone, alkaloids, cinnamate, coumarin and flavonoids

Mode of Glyphosate Lethality

Glyphosate rapidly moves to apical areas of the plant and inhibits protein synthesis. Cessation of growth happens almost immediately after the herbicide reaches the apical areas. Plants stop growing and many plant tissues and parts slowly degrade due to impaired protein synthesis. Symptomology on plants usually develops very slowly, with gradually increasing chlorosis, yellowing, and necrosis. Death ultimately results from dehydration and desiccation.

Tilling and cultivation of fields in agriculture is largely a mechanism for weed control. These can contribute to erosion and soil loss. There is much interest in no-till forms of agriculture, where application of herbicide can be used for weed control. In order to use this approach the crop species must be resistant to the herbicide.

Mechanism of herbicide resistance

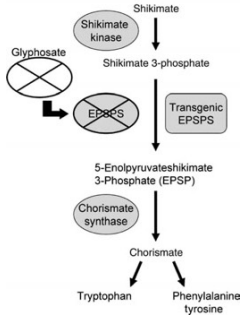


Figure 8.1. Resistance to glyphosate in RoundUp Ready™ plants is engineered by expressing a form of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS) enzyme that is resistant to the herbicide. In the absence of this transgenic enzyme, glyphosate inhibits the plant EPSPS and ultimately blocks the synthesis of chorismate, the branchpoint precursor to the essential aromatic amino acids: tryptophan, phenylalanine, and tyrosine. The transgenic EPSPS is unaffected by glyphosate, and can carry out the synthesis of EPSP leading to chorismate production.

Glyphosate inhibits a chloroplast enzyme required for aromatic amino acid synthesis. Resistance can be conferred by transgenic expression of an enzyme that is resistant to the herbicide. The new enzyme complements the glyphosate induced defect in amino acid synthesis.

An example of no till farming, where fields were not prepared by ploughing and seeds were directly drilled into the soil, and herbicide application was used for weed control. Stubble from the previous crop can be seen in the understory.
With the wide adoption of herbicide resistant crops, farmers have seen the emergence of herbicide resistant weeds. This has led to the use of multiple herbicide resistance genes for more robust weed control.

Stacking of transgenic traits in hybrid corn

Here's how the corn hybrid naming system works:

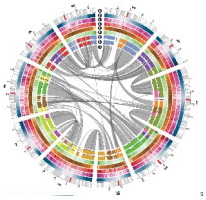
G 11 U58 - 3111A
A B C D E

A "G" indicates Golden Harvest.
B Last two digits of relative maturity number.
C Existing Garst hybrid numbering.
D Separates the genetic and trait portions.
E From Agrisure traits naming system.
- First number represents Herbicide Tolerance Technology Series
- Second number represents number of modes of action against broad lepidopteran pests
- Third number represents number of modes of action against corn borer
- Fourth number represents number of modes of action against corn rootworm
- "A" denotes Agrisure Artesian technology

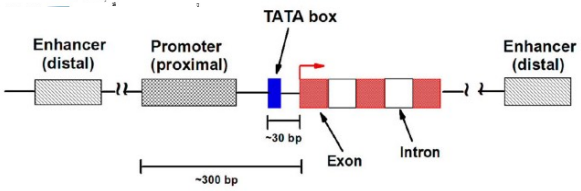
Syngenta

An increasing repertoire of single gene traits is being used in crops like maize soybean and cotton. Transgenic varieties contain stacked traits for herbicide resistance, and expression of BT toxins to confer resistance to a variety of pests. This has led to the development of systematic naming systems, like this example for a maize variety from Syngenta.

How can the activity of an individual gene be visualised?

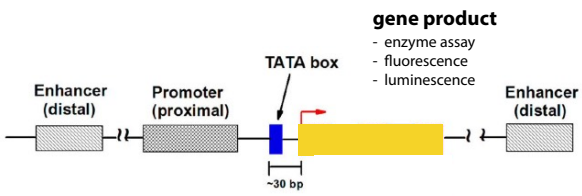


Maize genome
10 chromosomes
2.4 Gbp
32,000 genes



These examples of new commercial traits are due to the integration of synthetic genes into the maize genome. The maize genome consists of 10 chromosomes with 2.4 billion base pairs of raw DNA and 32,000 genes. The next part of the lecture deals with the challenge of following the behaviour of a single introduced gene in the context of the activity of the existing genome.
The conserved nature of gene structure in eukaryotes allows the replacement of modular functions. For example, the protein coding region (including introns and exons) of an existing plant gene can be replaced by an alternative coding region. this could include regulatory protein, enzyme or marker gene.

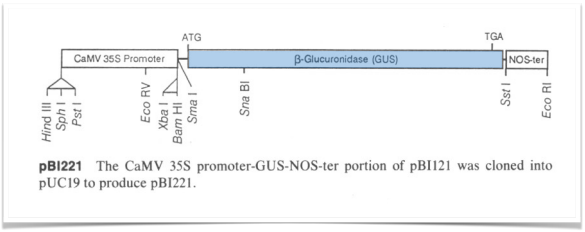
Reporter genes: markers for gene expression



ß-glucuronidase
fluorescent protein
luciferase

Marker or reporter genes are widely used as a means of visualising gene activity within intact plants. Reporter genes encode gene products that are not otherwise found in the genome, and can be easily measured or visualised. These include enzymes that can be histochemically localised and proteins that are intrinsically fluorescent or capable of emitting light.

Synthetic GUS gene for plant transformation



pBI221 The CaMV 35S promoter-GUS-NOS-ter portion of pBI121 was cloned into pUC19 to produce pBI221.

ß-glucuronidase (GUS) is a glycolytic enzyme from *E. coli* without a counterpart in most plant cells. Specific histochemical staining can be used to indicate the presence of the expressed gene product.

The coding sequence for the ß-gluronidase enzyme can be fused to chosen promoter and terminator sequences and expressed in planta. The bacterial enzyme is not normally found in plants, and is capable of cleaving ß-linked glucuronide groups from a variety of chemical substrates.

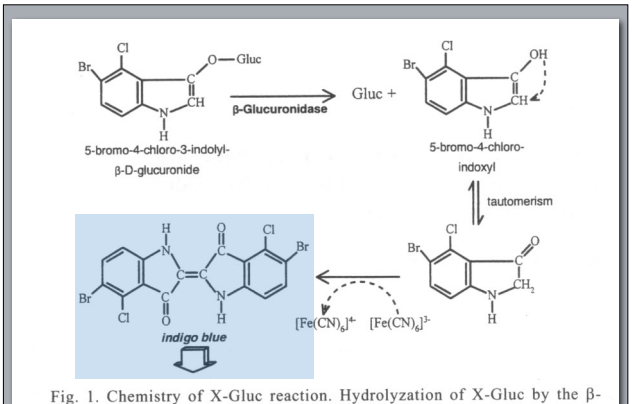
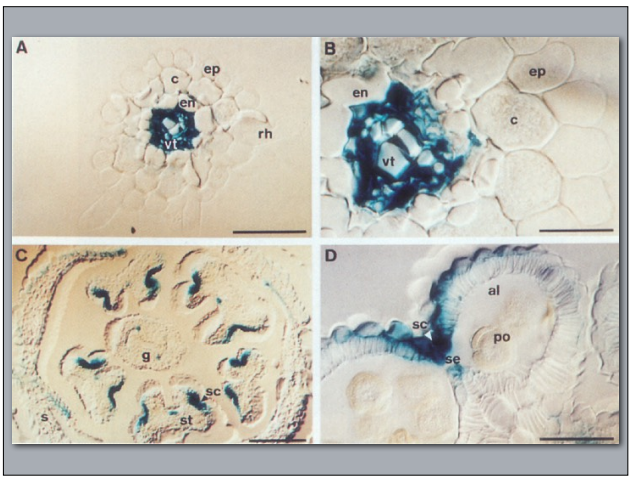
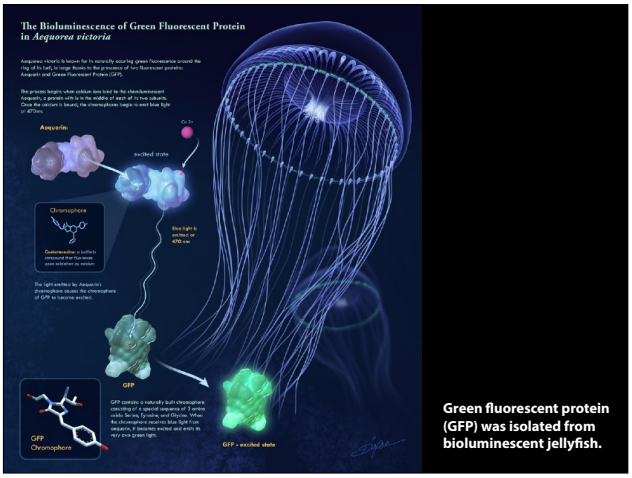


Fig. 1. Chemistry of X-Gluc reaction. Hydrolyzation of X-Gluc by the β-glucuronidase enzyme results in a reactive indoxyl molecule. Two indoxyl molecules are oxidized to indigo blue; ferri(III)cyanide enhances the dimerization.

X-gluc is the common name for a histochemical substrate for ß-glucuronidase - consisting of glucuronide linked to a potentially reactive moiety. The substrate is inactive in the absence of the enzyme. However action of the enzyme releases an activated indoxyl monomer, and spontaneous oxidation of two monomers produces an insoluble indigo blue product that is deposited at the site of the reaction.

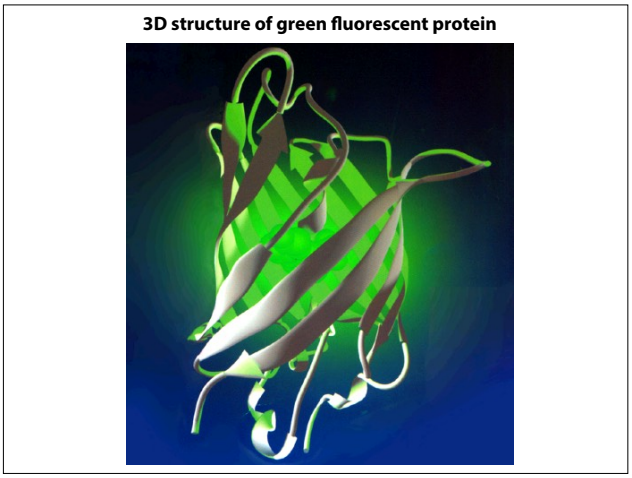


An example of histochemical localisation of ß-glucuronidase on an optically cleared cross-section of plant material. Histochemical localisation can allow simple and sensitive detection of gene expression in whole mounts due to clearing of pigments and light scattering elements from plant tissue. However the staining process is usually lethal, and it is difficult to localise the gene product at high resolution (e.g. resolve subcellular locations of the gene product).

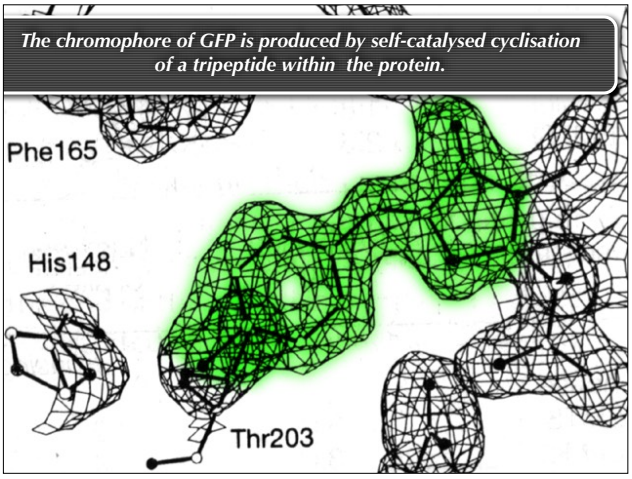


Green fluorescent protein (GFP) was isolated from bioluminescent jellyfish.

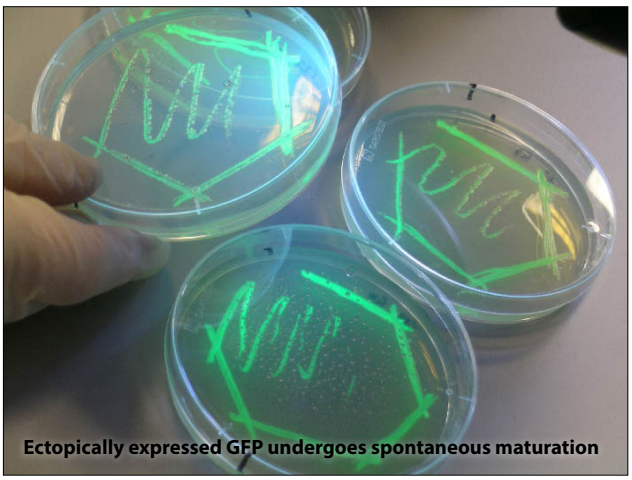
In contrast, certain gene products can be directly visualised. Green fluorescent protein was discovered in the bioluminescent jellyfish, Aequoria victoria. The jellyfish contain specialised light organs that contain calcium-activated photoprotein, aequorin. The photoprotein system emits blue light (470nm) under nervous system control. the green fluorescent protein which is maintained in close proximity to aequorin, absorbs the blue light and efficiently emits it as green (515nm).



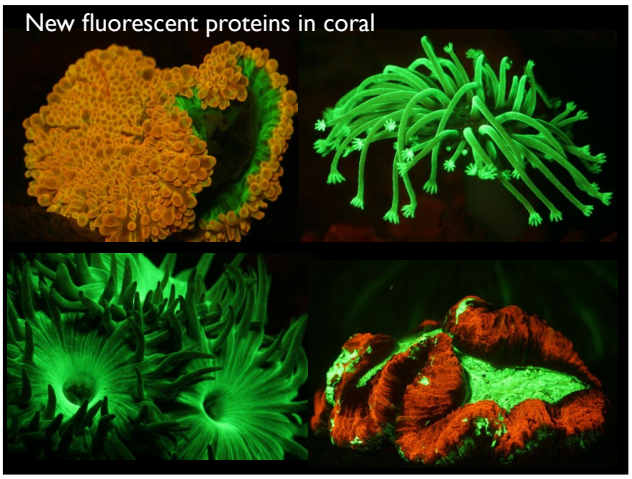
The green fluorescent protein consists of a barrel -like structure formed of beta sheets that surround a single alpha helix that descends through the centre of the protein. The barrel shape is capped by short alpha helical segments, and the outer part of the protein forms an effective solvent cage.



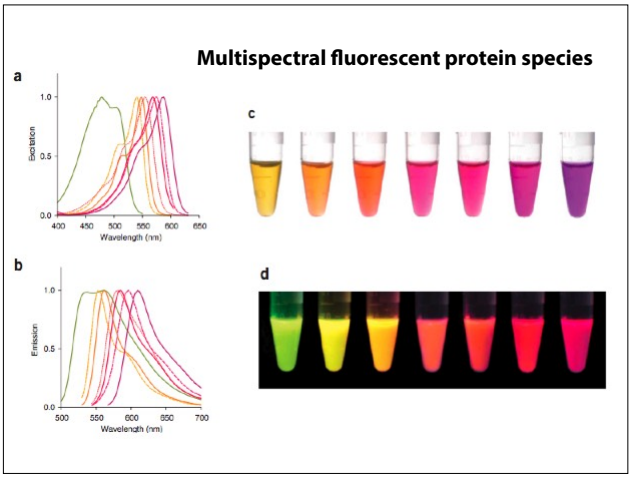
A highly unusual and characteristic chromophore is produced during folding and maturation of the protein. A tripeptide sequence, Ser-Tyr-Gly, undergoes cyclisation and oxidation to produce a multi-ring aromatic group on the alpha helix that runs through the centre of the protein. The maturation of the chromophore is autocatalytic, and occurs spontaneously in the protein is expressed in essentially any organism, if allowed to fold properly and have access to oxygen.



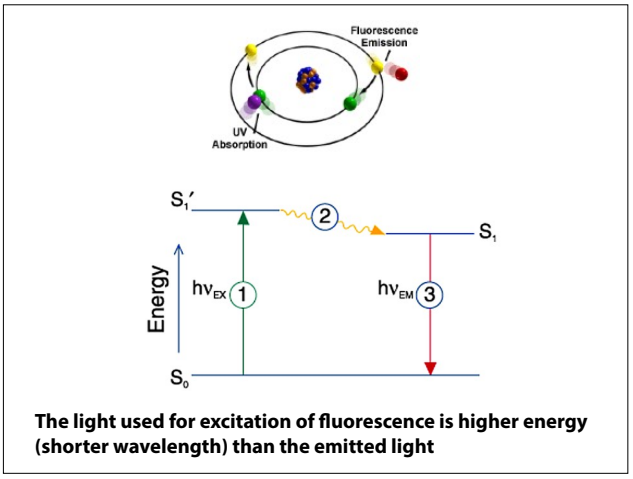
Therefore expression of green fluorescent protein results in production of a gene product that decorates or colours the cells. The protein generally does not have major toxic effects and living processes can be directly observed in labelled cells.



Many marine organisms use fluorescent proteins as part of luminescent systems or to absorb light. For example, many coral express high levels of GFP-like proteins, which have a wide range of roles as photoprotectants and pigments in these shallow water dwelling organisms.



A wide range of fluorescent protein species have been domesticated for laboratory use, and provide a "paintbox" for reporter gene studies.
The different optical properties of the fluorescent proteins are due to alterations in chromophore structure and in the arrangement surrounding amino acids in close contact with the chromophore.



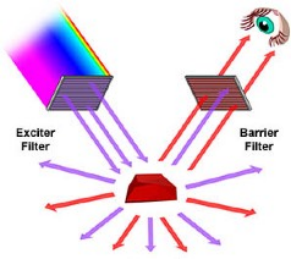
The light used for excitation of fluorescence is higher energy (shorter wavelength) than the emitted light

The physics of fluorescence: excitation light excites electrons to an outer orbital. After relaxation the excited electron collapses back to the ground state and in doing so releases a photon to compensate for the loss of energy. Fluorescent materials can be detected by a wide range of microscopy and optical techniques.

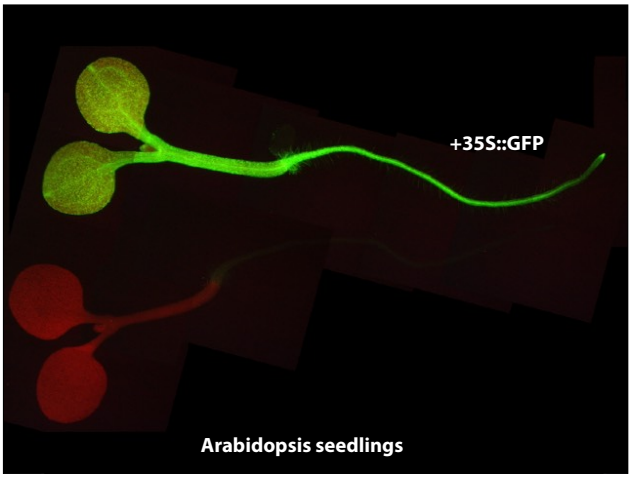
Benefits of fluorescence microscopy with FP's

New optical and computing methods allow selective, non-invasive imaging of fluorescent labels within intact cells.

(i) Expression of fluorescent proteins allows live imaging
(ii) Fluorescent emission can be selectively filtered
(iii) Confocal imaging allows optical sectioning and 3D reconstruction

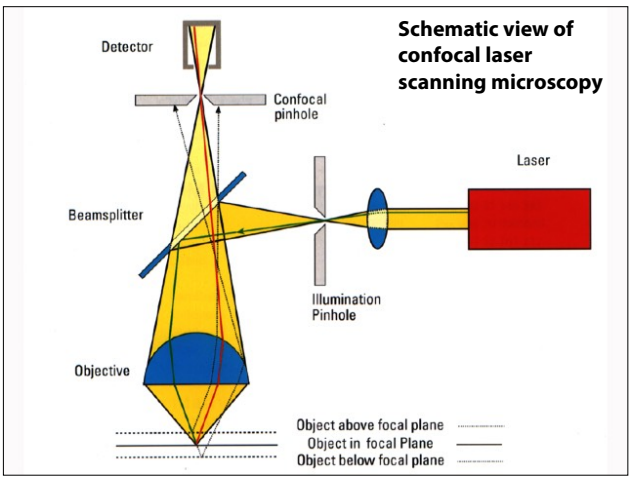


Fluorescence microscopy exploits the optical properties of a fluor to allow selective filtering of excitation and emission light. Fluorescence involves the absorption and re-emission of light energy. The energy of the excitation light is higher (shorter wavelength) than that emitted. The difference between excitation and emission wavelengths allows the use of optical filtration to selectively block the excitation light and allow sensitive detection of the fluorescence emissions during observation.
General principles for fluorescence microscopy. Excitation light is filtered to provide optimal excitation of a chosen fluor. The excitation light is directed at the sample by reflection from a chromatic beam splitter (dichroic filter). Light is focused on the sample through the microscope objective (acting as a condenser). any emitted light is collected by the objective and directed through the dichroic filter, and can pass through another optical filter before reaching the detector. in this way, low intensity emitted light can be detected sensitively - without being swamped by the excitation light.



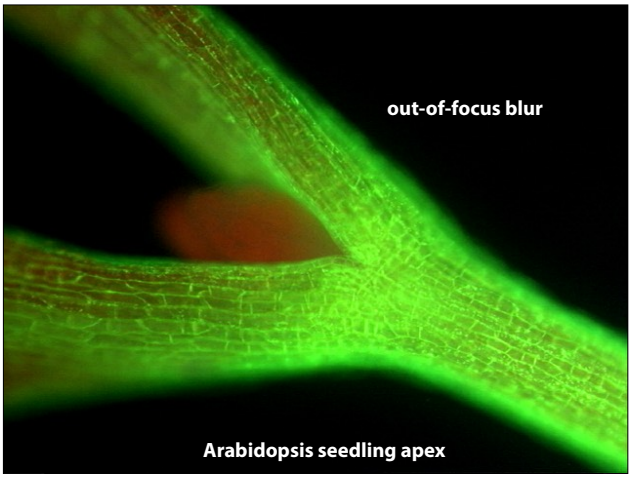
Wild type and green fluorescent protein (GFP) transformed Arabidopsis seedlings under a wide-field fluorescence microscope

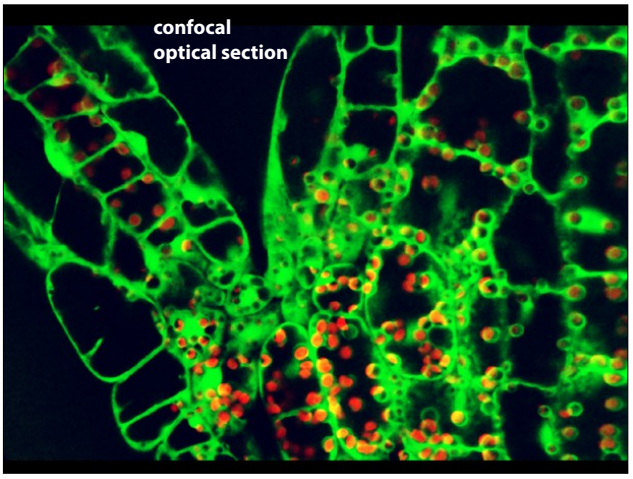
Higher magnification observation of a GFP transformed seedling, showing hypocotyl (stem), shoot apex and base of cotyledons (first leaves). Out-of-focus blur is evident.



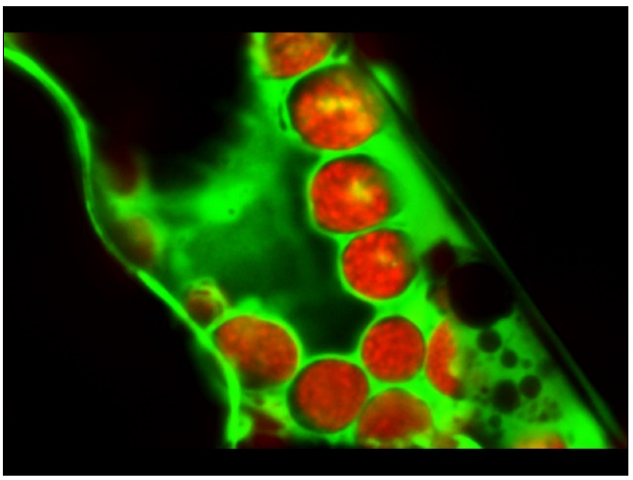
Confocal microscopy uses a laser beam for illumination. The laser illuminates the sample with a focussed spot, building an image as the beam is scanned across the sample. Fluorescent signals from the laser excitation are focused in the back plane of the microscope, passing through a small aperture (confocal pinhole). However, emission light from above or below the plane of focus in the sample is defocused and largely excluded from the detector, blocked by the confocal pinhole.

Modern confocal laser-scanning microscope



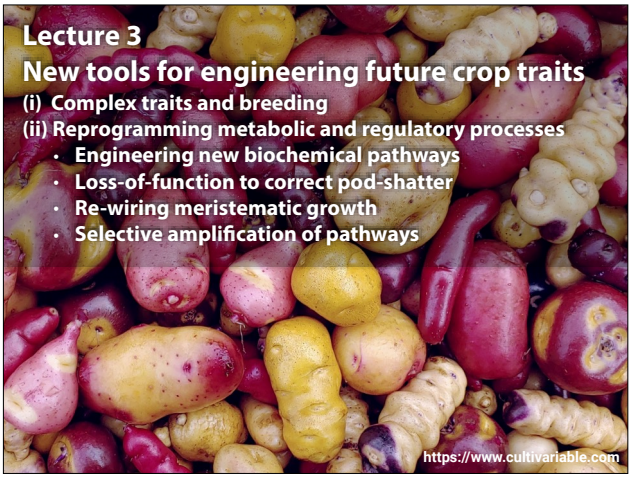


Out-of-focus blur is removed by confocal optics, effectively producing optical sections. The clarity of imaging allows the direct visualisation of subcellular features down to a fraction of a micron. Here showing the hypocotyl and the base of cotyledons of an Arabidopsis seedling expressing green fluorescent protein. The GFP is localised in the cytoplasm, and the optical section shows unlabelled vacuoles and autofluorescent chloroplasts (red).

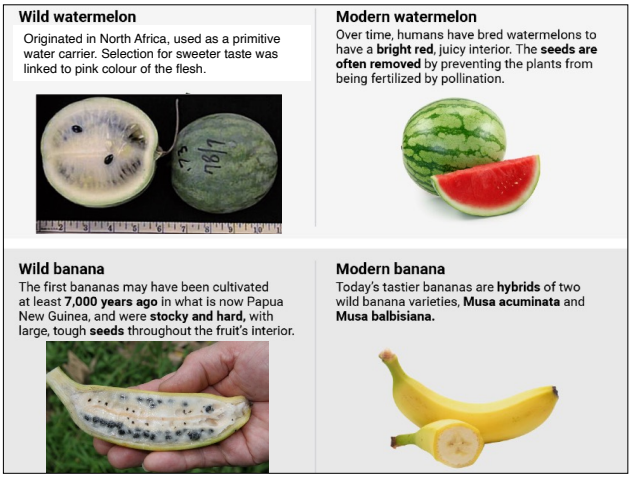


Confocal microscopy allows examination of cellular features at fine resolution, simply by changing objective or using digital zoom. Here showing individual chloroplasts in a hypocotyl cell in an Arabidopsis seedling.

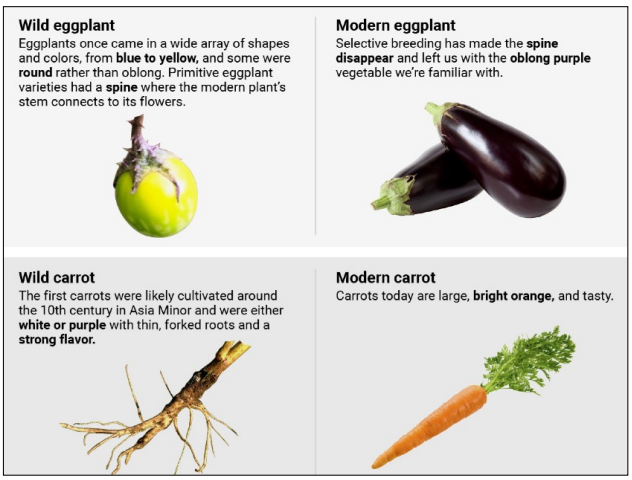
Green fluorescent protein can also be used to track whole plant gene expression. Here showing use of a labelled plant virus and tracking foreign movement across the plant.



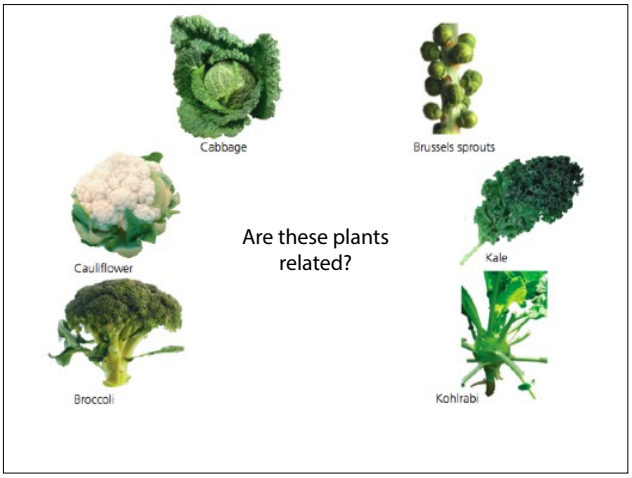
Modern approaches to gene editing and reprogramming of the expression of master regulators allow the prospect of rapid, targeted modification of plant architecture and other properties for crop improvement and possible domestication of new species.
Backdrop: Peru is home to over 4000 varieties of potato, with a wide range of different morphologies and characteristics. (Image from https://www.cultivariable.com)



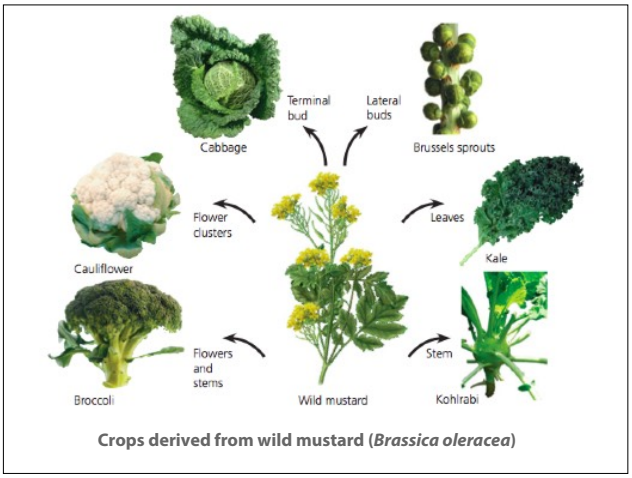
Ancient species are provided raw material for domestication of crop plants. Domestication has occurred over millennia, and often accompanied by substantial changes in phenotype. For example, melons were thought to have been originally used in prehistoric times as natural water carriers in northern Africa. The wild melons have a high water content but are bitter. The selection for sweeter tasting melons unintentionally produced pink flesh, as the genetic loci for colour and sweetness are closely positioned. In addition, bananas were first domesticated in Papua New Guinea. These were diploid and contained seeds. Modern bananas are triploid, sterile and seedless…and genetically homogeneous. (Images from https://jameskennedymonash.wordpress)



Eggplants, or aubergine, have been grown in southern and eastern Asia since prehistory. A relative of the nightshade family, domestication has led to changes in size, colour, alkaloid content and loss of spines.
Carrot was cultivated and used as a storage root similar to modern carrots in Central Asia beginning in the 10th century. The first domesticated carrot roots were purple and yellow, arriving in Western Europe and finally in England between the 11th and 15th centuries. Orange carrots were not well documented until the 15th and 16th centuries in Europe, indicating that orange carotenoid accumulation may have resulted from a secondary domestication event. (Images from https://jameskennedymonash.wordpress)
In each of these cases, centuries or even millennia of domestication was required to produce the productive and more palatable crop plants that we recognise today.

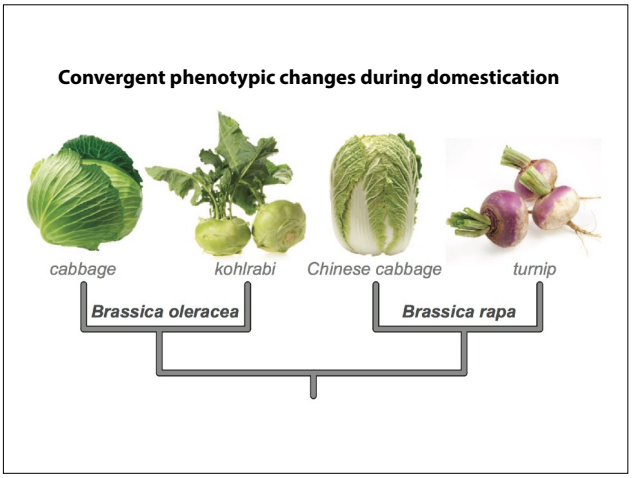


Plant genomes and morphology are highly plastic. For example, here are crops of derived from plants in the Brassica family. Many of these plants might be taken to be different species, however observed morphological differences are often due to selective breeding.



Crops derived from wild mustard (*Brassica oleracea*)

For example, all of these recognisably different vegetables are derived from the same ancestor species, *Brassica oleracea* or wild mustard. Breeding has led to the enhancement or exaggeration of particular features. For example the appearance of cauliflower is due to over-proliferation of shoot meristems, broccoli has a proliferation of floral buds, cabbage and Brussels sprouts have exaggerated vegetative meristems, and kohlrabi has a swollen stem.



Further, examples of convergent trait development can be seen in two *Brassica* species. For both *Brassica oleracea* and *Brassica rapa,* genetic variants have been selected independently for (i) indeterminate vegetative meristems and proliferation of leaves, and (ii) hyper proliferation of tissues at the base of the stem.

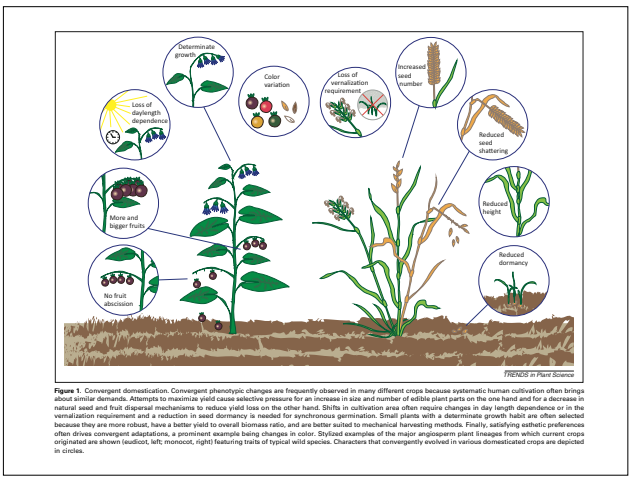


Figure 1. Convergent domestication. Convergent phenotypic changes are frequently observed in many different crops because systematic human cultivation often brings about similar demands. Attempts to maximize yield cause selective pressure for an increase in size and number of edible plant parts on the one hand and for a decrease in natural seed and fruit dispersal mechanisms to reduce yield loss on the other hand. Shifts in cultivation area often require changes in day length dependence or in the vernalization requirement and a reduction in seed dormancy is needed for synchronous germination. Small plants with a determinate growth habit are often selected because they are more robust, have a better yield to overall biomass ratio, and are better suited to mechanical harvesting methods. Finally, satisfying esthetic preferences often drives convergent adaptations, a prominent example being changes in color. Stylized examples of the major angiosperm plant lineages from which current crops originated are shown (eudicot, left; monocot, right) featuring traits of typical wild species. Characters that convergently evolved in various domesticated crops are depicted in circles.

Certain agronomic traits have common benefits in different crops. As a result, domestication has seen the parallel and convergent acquisition of traits in different species. For example, this diagram shows the benefits of similar traits in hypothetical dicot and monocot species - such as determinant growth, larger fruiting bodies and reduced fruit or seed loss.

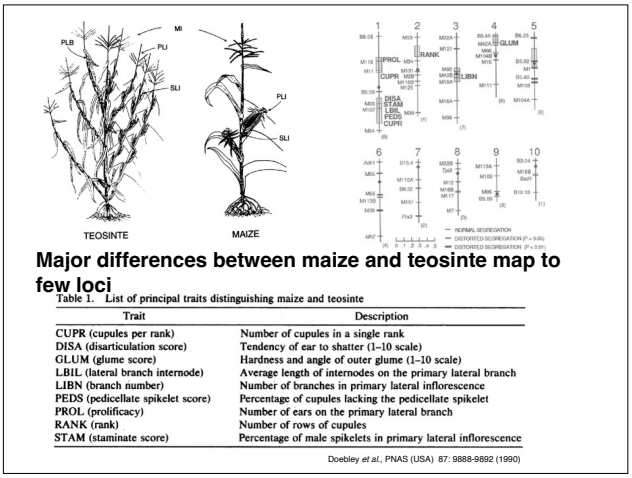
Crop traits

Traits that have been selected for by humans include:

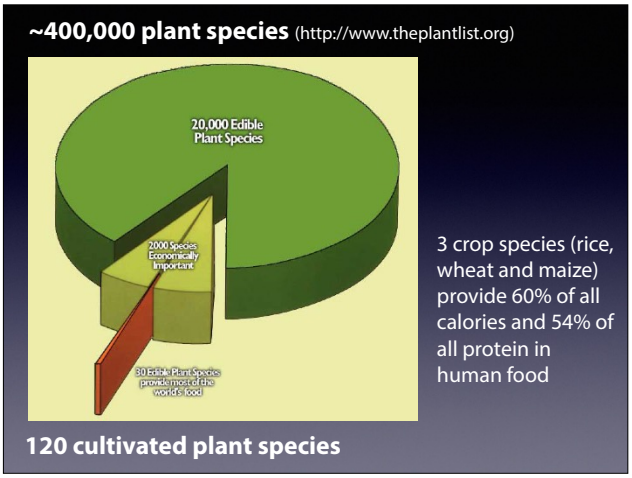
- Determinate growth habit (flowering occurs at the top of the plant, preventing further growth)
- Synchronous ripening, shorter maturity
- Lower content of bitter tasting and harmful compounds
- Reduced sprouting (higher seed dormancy)
- Improved harvest index (the proportion of the plant which is used); larger seed or fruit size
- Elimination of seeds, such as in banana
- Retention of mature seed on the plant (loss of grain shattering)

Many of these traits are multigenic and affect the shape and function of plant tissues and organs. If we want to engineer new crop traits in the future, we will need to understand the way DNA code is able to regulate plant growth and form.

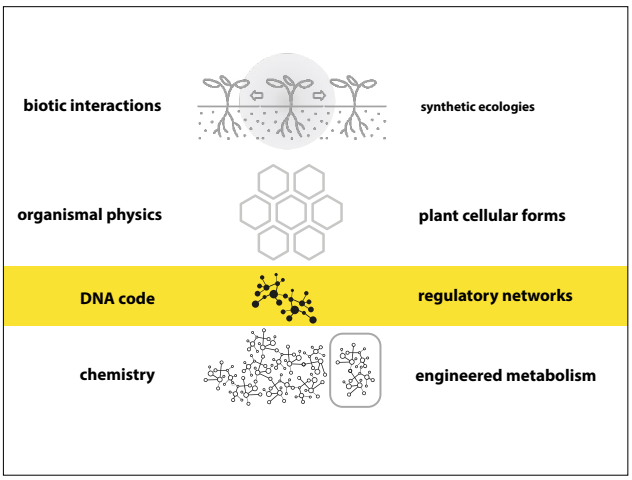
Many, if not most, of the important traits introduced during domestication are the result of coordinated changes in plant growth and form. While there may be simple genetic triggers for these changes, the modified traits are the result of programmed alterations in complex developmental and metabolic pathways.
What underpins programmed plant growth? Can these elements be easily reconfigured by human engineers?



As we saw in Lecture 1, work from John Doebley's lab has mapped the genetic differences between teosinte and maize. Genetic studies identified the relatively few gene loci account for around 90% of the difference in form between teosinte and maize. These cause differences in traits like vegetative branching, morphology and floral architecture.



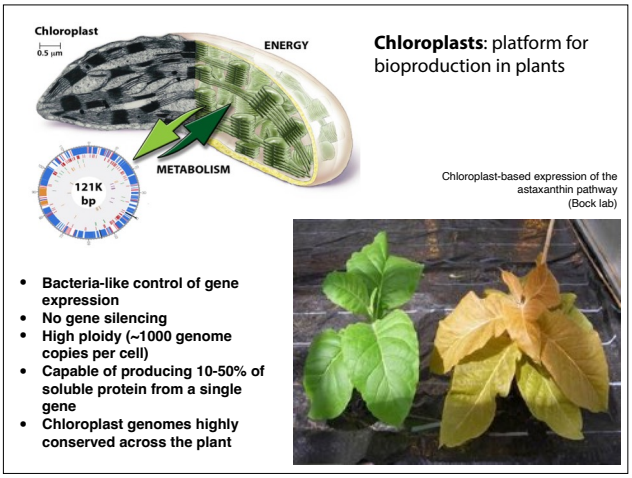
Crop plants sample a tiny fraction of total plant diversity. It estimated that there are around 400,000 plant species on Earth. Only around 20,000 of these have ever been used by humans as food, and only 2000 plant species have any economic importance as food crops. 30 species provide most of the world's food. Three species - rice, wheat and maize, provide 60% of calories and over half of the protein in human food. A vast potential reservoir of biological diversity remains untapped.



A major scientific challenge in the plant field is to better understand the dynamic interactions that give rise to precise developmental outcomes. In other words, to understand how one dimensional DNA code can be translated into four dimensional outputs. Success in this task will allow new approaches to the design and reprogramming of agronomically relevant traits in plants.

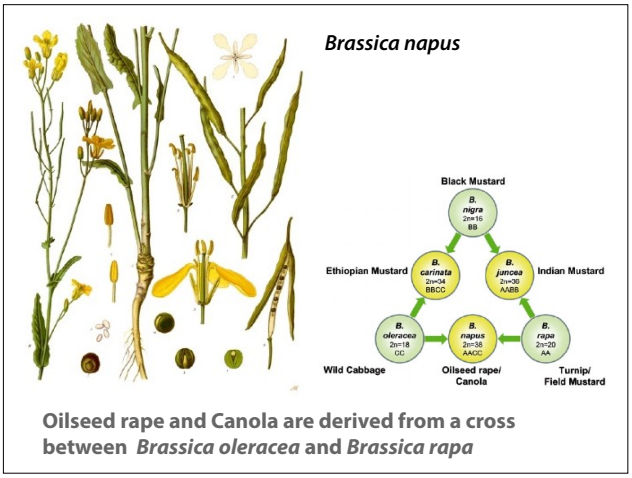
in addition, a wide variety of biochemical pathways are unique to plants, and produce secondary compounds, which often possess useful properties and are difficult or costly to produce by synthetic chemistry. There is much research activity aimed at both transferring plant-specific metabolic pathways to bacteria and yeast, and to re-jigging pathways in plants for higher yields and new products. Generally, plants can be grown for bioproduction - at much higher scale and cheaper than microbes.

The plant-based production of chemicals, materials and fuels figures prominently in plans for future models of the global bioeconomy. These plans invoke not just large scale bioproduction, but also systems for recycling of waste and circular economies.



Chloroplasts are responsible for the bulk of energy production and biosynthetic capacity in plant cells. They have simple, reduced genomes, yet are capable of prodigious levels of gene expression. The engineering of plastids figures prominently in current attempts to manipulate crop productivity and metabolic properties. An example is shown - transfer of the astaxanthin pathway into *Nicotiana tabacum* through chloroplast transformation. Astaxanthin is normally produced in algae, and contributes to the pink colour of some crustacea, flamingos and salmon flesh. In transplastomic plants, levels of the pigment accumulate enough to turn the entire plant pink. Lu, Y., Stegemann, S., Agrawal, S., Karcher, D., Ruf, S., Bock, R. (2017). Horizontal Transfer of a Synthetic Metabolic Pathway between Plant Species. DOI: 10.1016/j.cub.2017.08.044

Targeted loss-of-gene-function:
correction of pod-shatter through modified cell differentiation and tissue architecture



In a first example, we will look at *Brassica napus*, which has given rise to the oilseed rape crop, also known as canola. *Brassica napus* is derived from a cross between *Brassica oleracea* and *Brassica rapa*, and is thought to be a relatively new species, since the earliest reliable record appears only 500 years ago. Although feral populations are common, no truly wild populations have been recorded. Both *B. rapa* and *B. oleracea* have wide geographic ranges and geographically distinct centres of diversity. Molecular studies suggest that the maternal parent of *B. napus* was likely to be *B. oleracea*, due to similarities in the structure of their chloroplast genomes.

Canola is an oilseed crop. After planting and subsequent vegetative growth, the plants flower and set seed. The seed is harvested at the end of the growing season and pressed to extract oil.

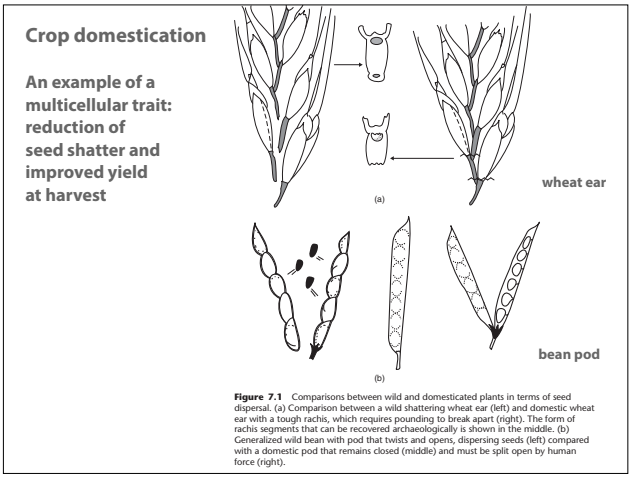


Figure 7.1 Comparisons between wild and domesticated plants in terms of seed dispersal. (a) Comparison between a wild shattering wheat ear (left) and domestic wheat ear with a tough rachis, which requires pounding to break apart (right). The form of rachis segments that can be recovered archaeologically is shown in the middle. (b) Generalized wild bean with pod that twists and opens, dispersing seeds (left) compared with a domestic pod that remains closed (middle) and must be split open by human force (right).

Wild plants rely on seed dispersal to maintain their population. In an agricultural context, this corresponds to seed shatter and losses in yield. A feature of the domestication of many seed crops is the selection for mutants that reduce seed shatter. Wheat seed are held in an ear with a central axis, or rachis. The rachis of wild type wheat plants contains abscission layers that result in breakage of the rachis and seed dispersal. Domesticated wheat have been selected for toughened rachis that allow retention of seed for harvesting. Similarly, domesticated crops with pod-borne seed are generally modified for reduced pod shatter and seed retention.

Oilseed rape is a relatively recently domesticated crop. Seed pods are often fragile in the weeks leading up to harvest. During this stage seed pods go through a process of dehiscence (splitting open), commonly known as pod shatter. This process can result in:

- substantial seed loss and decrease in yield;
- greater number of volunteers in next season's crop.

Pod shatter can result in substantial losses of yield (25-50%) for Canola and rapeseed oil crops - in adverse conditions (such as high winds) prior to harvest

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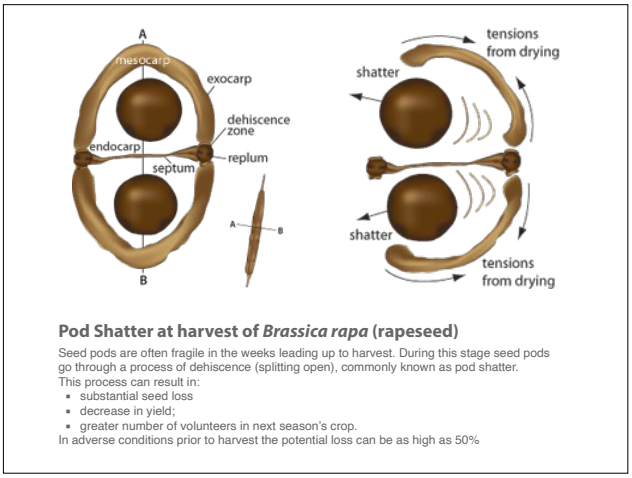
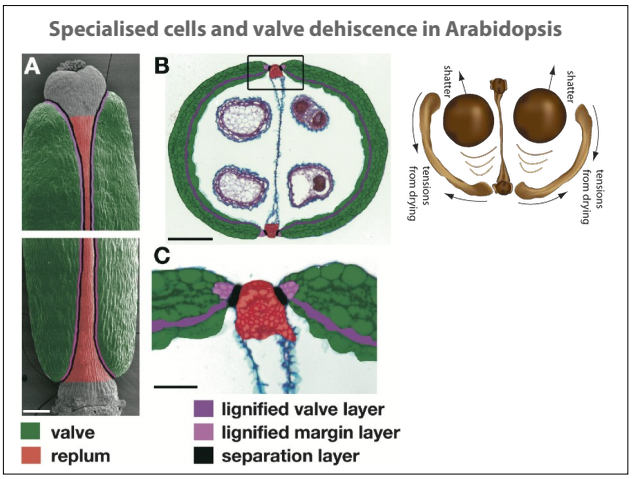
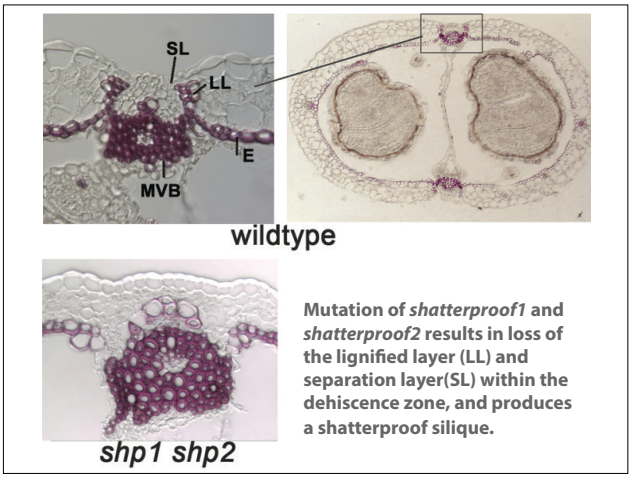


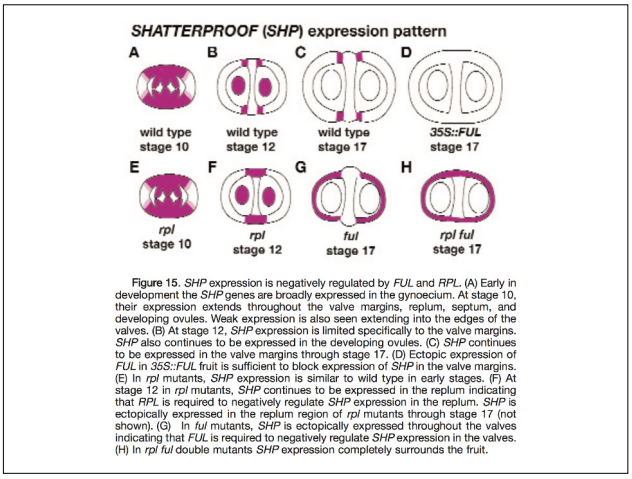
Plants within the Brassicaceae family share many common features. The chart shows overall leaf and fruit structure across the family. The seed pods of *Brassica oleracea* and *Brassica rapa* are similar to the model plant *Arabdopsis thaliana* - the world's best genetically characterised plant. Arabidopsis seed are carried in siliques (pods) that are formed late in flower development and expand after fertilisation and seed growth. They are formed by fusion of two carpels, to create joined chambers that contain multiple ovules - that after fertilisation will each form mature seed. S = stigma, the pollen receptive tissue at the apex of the female floral structure. Replum, support structure at the point of contact for the two valves. Analogous structures are found in *Arabidopsis*, *Capsella* and *Brassica spp*.



Coloured scanning electron micrograph of opening of an Arabidopsis silique (fruit). At maturity, the silique and seeds undergo desiccation. This causes a build up of physical tension within the walls of the fruit. The junction between the valves and replum is inherently weak (dehiscence zone), and eventually the valves tear apart from the replum at this junction at valve margins. The differentiation of specialised cells in the valve margins ensures that valve separation (dehiscence) occurs efficiently. In Arabidopsis, we see the presence of strong, lignified cells (i) as a layer within each valve, and connected to this, (ii) a strengthened layer at the valve margin. Desiccation causes tissue shrinkage and build up of tension in each valve. The lignified layers within the valves ensure that these forces are transmitted efficiently to the margins. Eventually, the cellular connections between valve and replum must give way, and the seed pod shatters, releasing the seed.



Genetic analysis of mutant plants, where seed shatter is defective, has allowed identification of key gene regulators. Notably, there are two MADS box transcription factors in *Arabidopsis* that play a redundant role in precisely specifying the lignified cells at the valve margins. If both genes are disrupted, these few cells at the junction of the valve and replum tissue are not specified properly. This precise and minor defect results in siliques that do not shatter normally, and the genes have been named Shatterproof 1 and 2.



1. There are regulatory genes expressed in the valve and replum that limit *Shatterproof* expression to the valve margin. These are the MADS box protein encoding gene Fruitfull (*Ful*) expressed in the valve, and the homeodomain protein encoding gene Replumless (*Rpl*), which is expressed in the replum.
Shatterproof gene expression is normally limited to the valve margin (C) in mature siliques. However, loss of Ful gene function results in expansion of SHP expression into the valve (G). Loss of Rpl gene function results in expansion of SHP expression into the replum (F).

2. There are genes downstream of Shatterproof 1 and 2 that are also required for formation of the lignified valve margin cells and separation layer. Examples of these are bHLH-class transcription factors, Indehiscent and Alcatraz. Strong mutant alleles of Indehiscent (e.g. *ind-2*) cause marked disruption of

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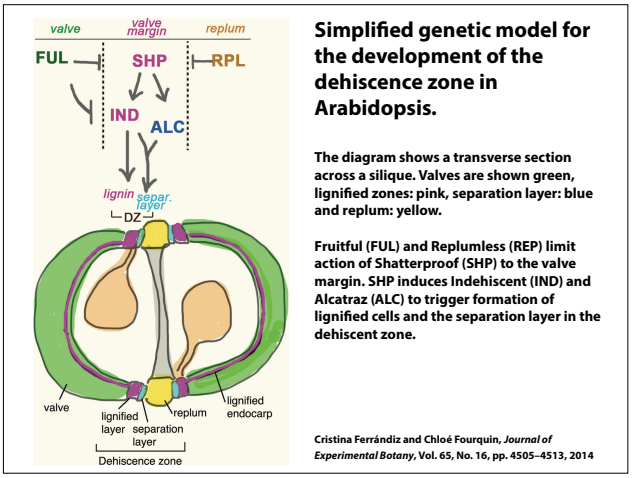
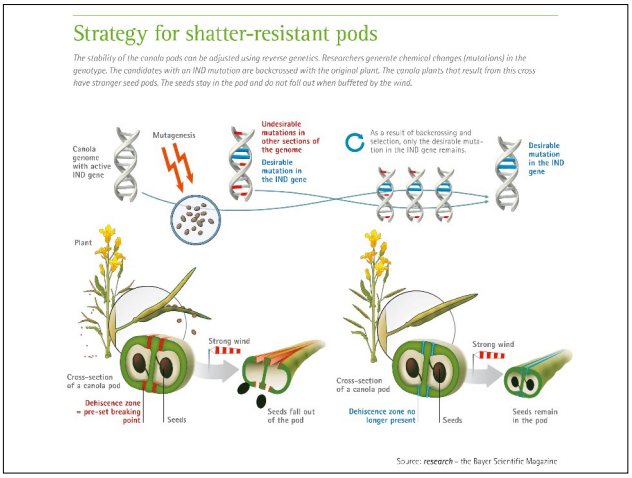


REPLUMLESS and FRUITFULL are expressed either side of the valve margin, and they act in concert to limit the domain of expression of the SHATTERPROOF proteins. In turn, SHATTERPROOF 1&2 regulate downstream functions required for specification of the lignified cell layer and separation zones in the valve margin. Mapping of the set of genetic interactions in Arabidopsis has provided a map for rational approaches to reduce seed shatter in Rape/Canola.



Understanding of the genetic and cellular processes involved in establishing dehiscence zone has led to the development of engineering strategies for reducing pod shatter in rapeseed varieties. In this example from Bayer, Canola lines have been selected with defects in the Indehiscent, IND gene. In addition, Canola lines with reduced pod shatter have been produced through expression of antisense genes and use of CRISPR/Cas9 induced gene knockouts.

Field trial of modified Canola with the "Pod Shatter Reduction" trait from Bayer. Trait engineering requires the careful balance of reduced pod shatter with the need for ease of seed separation during harvesting. Further, engineering of the *Brassica napus* genome can be complicated by its tetraploid (AACC) nature, and this is being aided by highly efficient CRISPR/Cas9 techniques for targeted mutagenesis.

Editing endogenous regulatory interactions: reprogramming mechanisms for control of meristem growth in tomato and related plants

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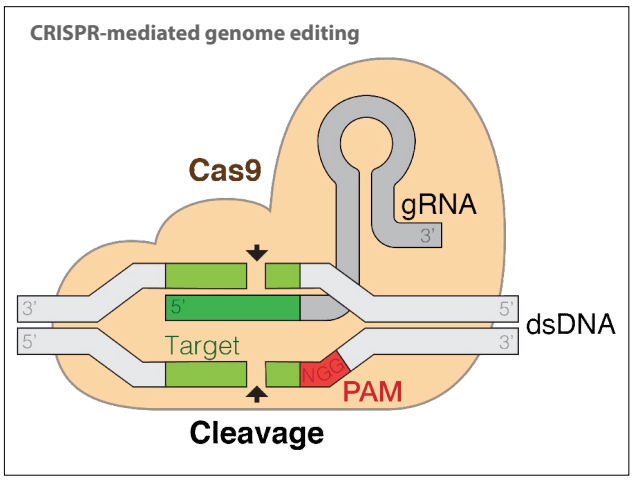
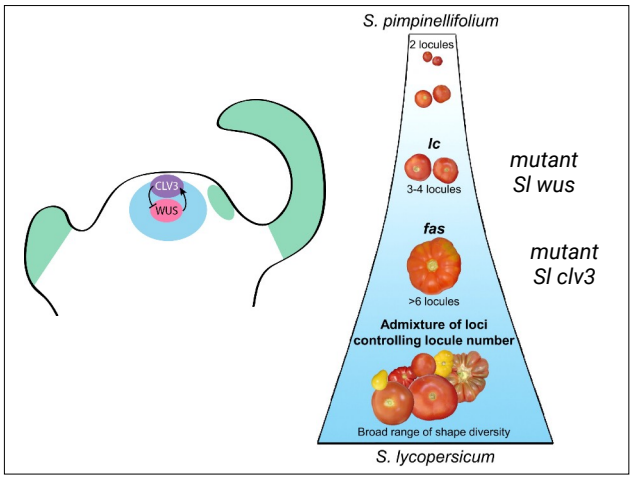
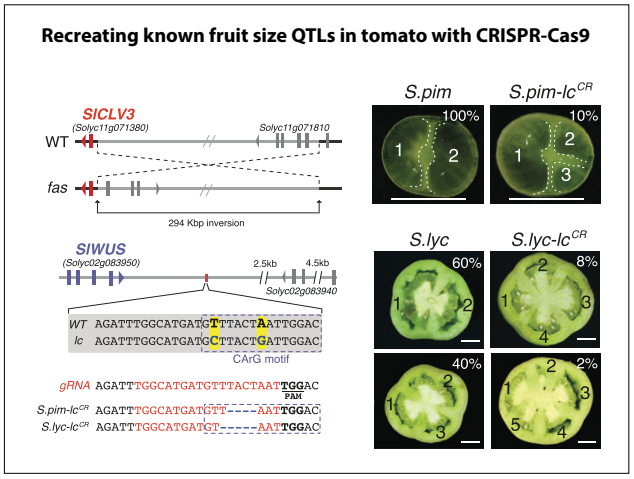


New genome editing tools like CRISPR have established a new class of genetically modified plants (and other organisms) which contain gene modifications, but no foreign genes. CRISPR technology allows the transient delivery of RNA-targeted endonucleases which are capable of catalysing specific dsDNA cuts in chosen sites within the host genome. The advent of these targeted mutagenesis tools has enabled new approaches, which do not depend solely on gene knock-out or loss-of-function.



The domestication of a crop plant like the tomato, has been accompanied by the selection and breeding of a wide variety of variants. These include plant varieties with profound differences in fruit size and shape, and plant architecture. For example, modern tomato varieties emerged from the wild Peruvian species (*Solanum pimpinellifolium*). Two mutant alleles which have played a major role in the breeding of large fruit sizes are *Locule number* (*lc*) and *Fasciated* (*fas*). The conserved Clavata3 (CLV3) - Wuschel (WUS) negative feedback circuit controls meristem and fruit size in plants. *Fasciated* encodes the *Solanum lycopersicum* CLV3 gene, and *locule number* (*Lc*) encodes the WUS gene.
Mutations in the fasciated (fas) and locule number (*lc*) fruit size QTL both contributed to increased tomato fruit size and locule number during domestication. Yellow arrowheads,

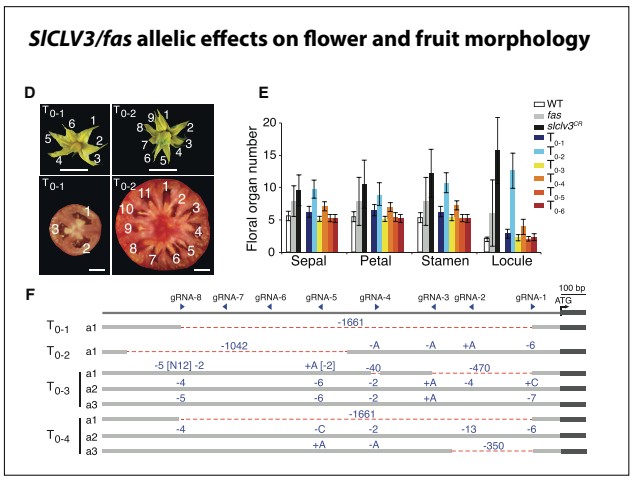
locules.



During domestication:

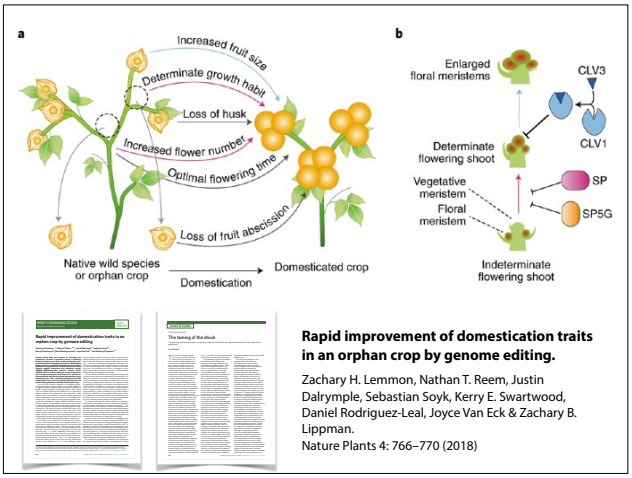
1. The fas mutation was caused by an inversion with a breakpoint 1 Kbp upstream of SlCLV3. (D)
2. The lc QTL (red rectangle) is associated with two SNPs (in bold) in a putative repressor motif (CArG, blue-dashed square) 1.7 Kbp downstream of SlWUS.

Mutations that affect the crop phenotype can be recreated and combined in different (or wild) varieties. CRISPR/Cas9-induced deletions in the CArG repressor motif are shown (blue-dashed square) in Solanum pimpinellifolium (S. pim) and Solanum lycopersicum (S. lyc). The gRNA target sequence is highlighted in red and the PAM site underlined. S.pim-lcCR plants produce fruits with more than two locules. S.pim-fasNIL S.pim-lcCR double mutants synergistically increase locule number.



Weak and strong effects on flower morphology and fruit size were observed among T0 lines. Number of floral organs and locules are indicated. (E) Quantification of floral organ number (mean ± SD; n>10) in T0, WT, fas, and slclv3CR plants. (F) Sequencing of SlCLV3 promoter alleles for all T0 plants. Deletions (–) and insertions (+) indicated by numbers or letters. T0-5 and T0-6 contained only WT alleles (data not shown). Blue arrowheads, gRNAs; a, allele.

With a judicious choice of regulatory gene targets, combinations of CRISPR-Cas9 agents could be used to modify shoot architecture and fruit properties. Here is an example of a tomato variety that is optimised for hydroponic growth in urban farming conditions, with a highly compact vegetative structure and "bunch-of-grapes" like fruiting structure with synchronised ripening. The technology is being further developed in the commercial sector, notably through the establishment of the new biotechnology company, Inari.



Rapid improvement of domestication traits in an orphan crop by genome editing.

Zachary H. Lemmon, Nathan T. Reem, Justin Dalrymple, Sebastian Soyk, Kerry E. Swartwood, Daniel Rodriguez-Leal, Joyce Van Eck & Zachary B. Lippman.
Nature Plants 4: 766–770 (2018)

Thus, knowledge of the genetic changes that underpin valuable traits can be used to recreate and extend selected phenotypes in related plant varieties - and even in related species. For example, Physalis (known as Cape Gooseberry or Ground Cherry) is another crop from the Solanaceae, related to tomato. The Lippman and Van Eck groups used similar approaches to modify fruit size, shoot architecture and other traits in this relatively unimproved crop. Targeted genome editing was used to create a rich set of variants that could be fed directly into breeding programmes.

Pathway engineering in plants:
introducing ectopic positive-feedback regulatory loops for hyper-expression of existing pathways

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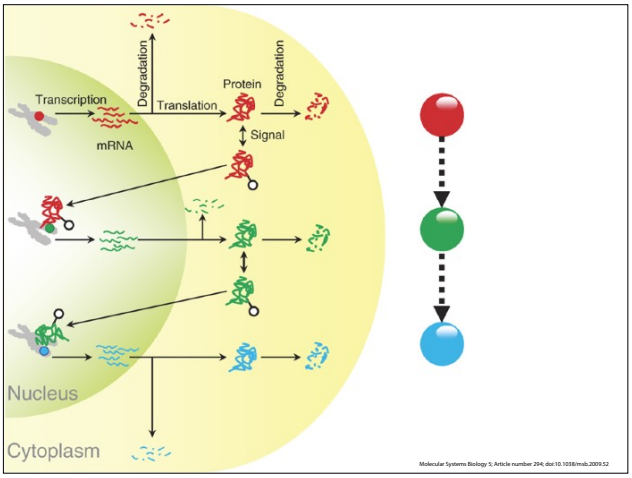
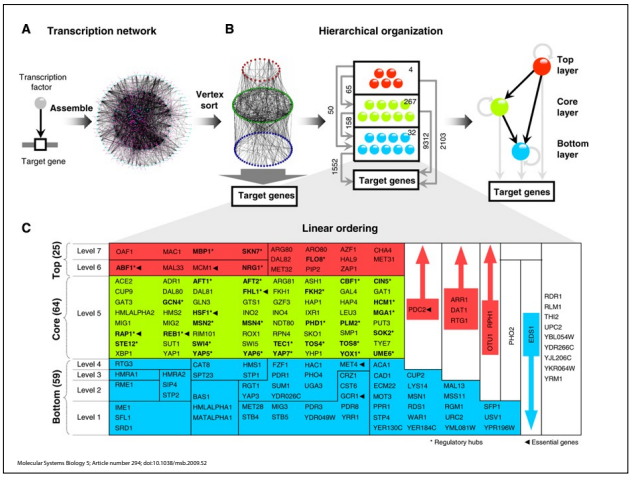


Molecular Systems Biology 5; Article number 294; doi:10.1038/msb.2009.52

Transcriptional cascades underpin gene regulatory networks. Transcription factors (TFs), denoted as nodes in a network (red and green circles), represent several entities (gene, mRNA, and protein) and events (transcription, translation, degradation, etc) that are compressed in both space and time. The series of regulatory events can be conveniently represented as a node in the network, although this does not capture the dynamics of these entities and the biological processes.



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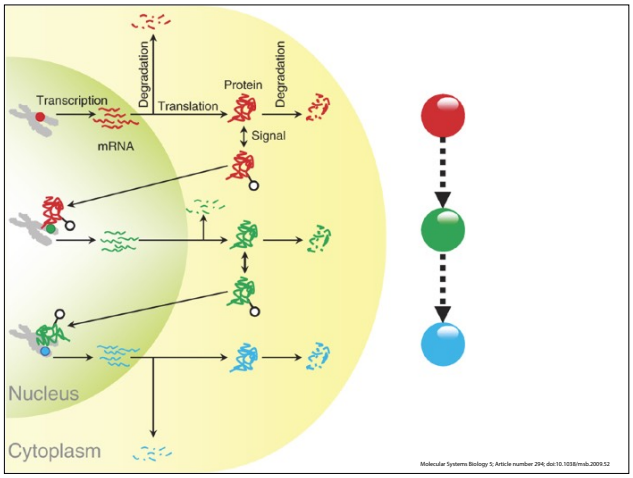
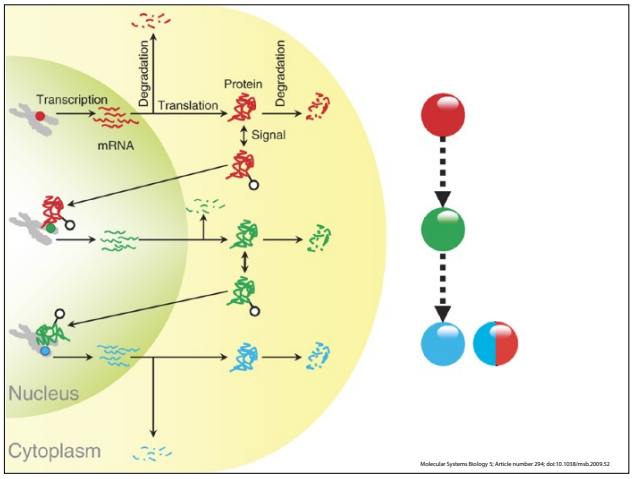
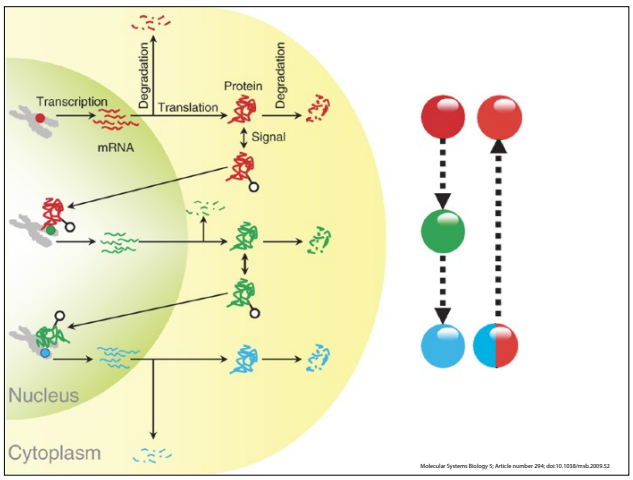
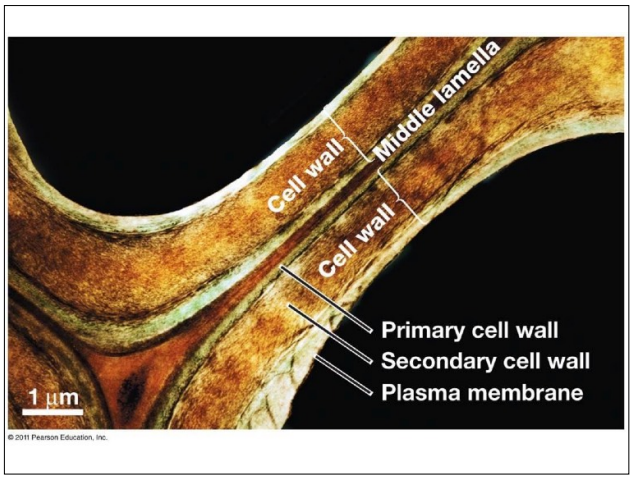
An example: hierarchical structure in the yeast transcription regulatory network. The organization of transcription factors in the network naturally clusters into three basic non-overlapping layers: the top (red), core (green), and bottom (blue). Thirty-two regulatory hubs are highlighted in bold and marked with a star (*), and nine essential TFs are marked with an arrow.



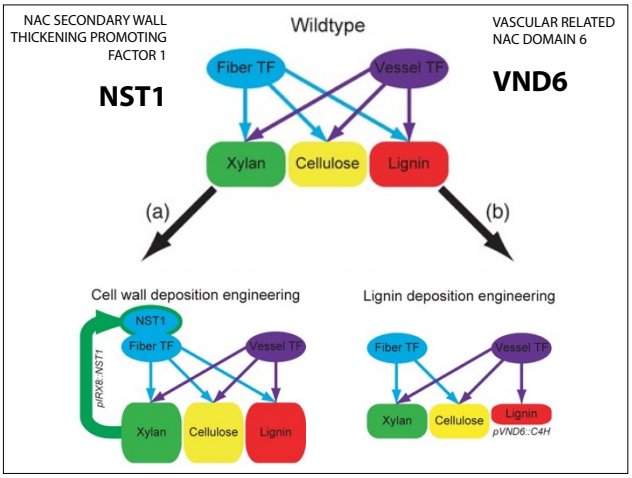




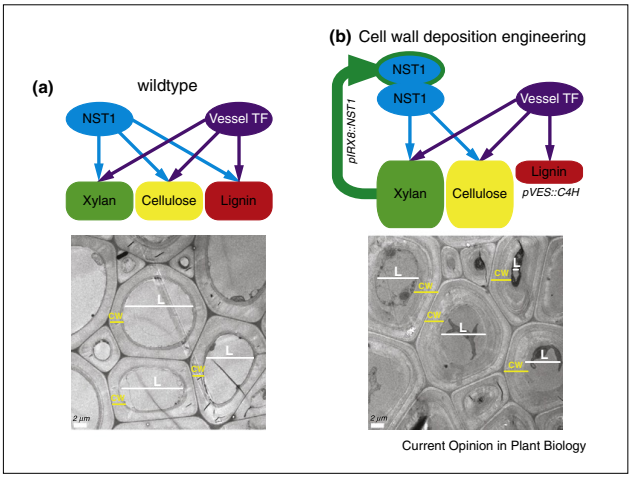
In this scheme, a gene from downstream in the regulatory hierarchy is identified. Its promoter (blue) is fused to a copy of the master regulator (red). Therefore when the master regulator is switched on during the normal course of development, there is a cascade of transcriptional control events that results in triggering of housekeeping genes, and in addition, triggering of a new copy of the master regulator. Results in expansion of the normal pathway.



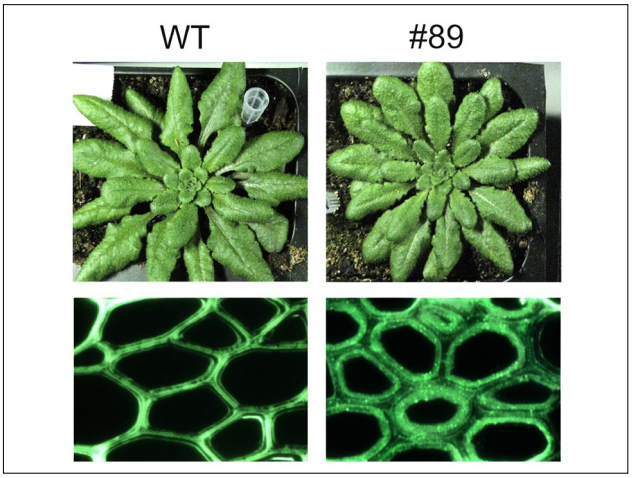
As an example, we will look at the engineering of transcription factors to modify cell wall composition in plants. The development of improved feedstocks for bioenergy production has focused on maximising primary cell wall and cellulosic material, and limiting amounts of lignin compounds during secondary wall growth.



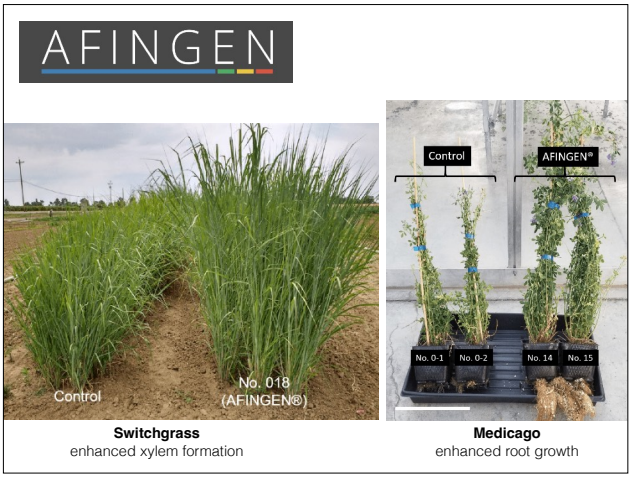
The two "arms" of vascular cell development are regulated by two transcription regulators, NAC Secondary Wall Thickening Promoting Factor 1 (NST1) and Vascular Related NAC Domain 6 (VND6). These genes show expression patterns that are limited to fibre and vessel cells, respectively.
In a two-way approach, Loque and colleagues interfered with the synthesis and deposition of lignin. (i) The promoter of a key lignin gene, *C4H*, was replaced by the vessel-specific promoter of transcription factor *VND6* in a *c4h* mutant. This rewired lignin biosynthesis specifically for vessel formation while disconnecting C4H expression from the fibre regulatory network. (ii) The promoter of the *IRX8* gene, a secondary cell wall glycosyltransferase, was used to express a new copy of the fibre transcription factor *NST1*, and as the *IRX8* promoter is induced by *NST1*, this creates an artificial positive feedback



Engineering of increased cell wall density and decreased lignin in Arabidopsis. Schematic of simplified regulatory network controlling secondary cell wall biosynthesis in vessel and fibre cells in plants and images from wildtype (a) and engineered (b) Arabidopsis plants depicting interfascicular tissues composed of fiber cells. Engineered plants were generated from a c4h defective mutant (mutant affected in the second lignin biosynthesis step) that was transformed with the wild-type version of the mutated C4H gene driven by a vessel-specific promoter which rescued the negative effect of low lignin content. Generated plants were further transformed with a construct (pIRX8::NST1) that led to higher expression of master transcription factor controlling secondary cell wall biosynthesis in fiber cells (e.g. NST1).



Production of transgenic plants with increased xylan content and decreased lignin content.



The biotechnology company AFINGEN® was set up to exploit the Positive Feedback Loop (APFL) platform - and they offer a robust path to improve commercially competitive crops with minimal cisgenic/intragenic manipulation and improved genetic stability compared to conventional bioengineering. By the selective amplification and/or reduction of target genes with unprecedented specificity and improved tolerance engineered crops (e.g. rice, corn, wheat, soybeans, canola, alfalfa, and sorghum) the modified plants can offer higher yields of biomass, more cellulose, and less lignin with healthy and robust plant growth. (Images from https://afingen.org/technology/)

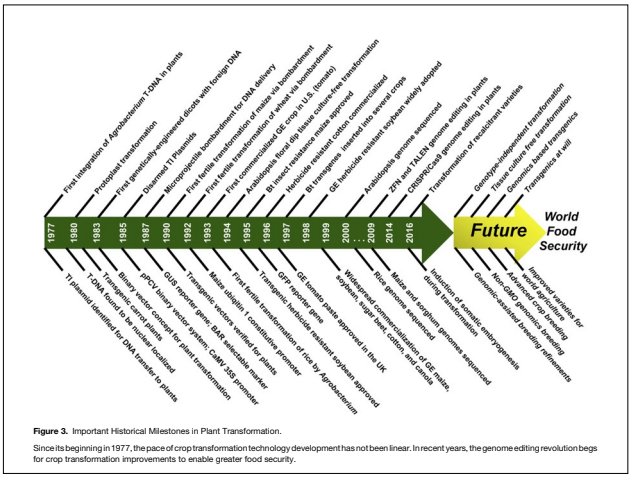


Figure 3. Important Historical Milestones in Plant Transformation.

Since its beginning in 1977, the pace of crop transformation technology development has not been linear. In recent years, the genome editing revolution begs for crop transformation improvements to enable greater food security.

The history of crop domestication has demonstrated the genetic plasticity of plants, and the benefits of manipulation of complex traits (e.g. microarchitecture of plant organs to reduce pod shatter). As our our ability to manipulate plant genomes improves, along with our understanding of plant development and growth - new possibilities for the rational design of plant improvements become feasible. This is very timely, as there is continued pressure to increase crop yields, due to constraints on the availability fertile land and water, and pressure from population growth and demand for improved food quality.