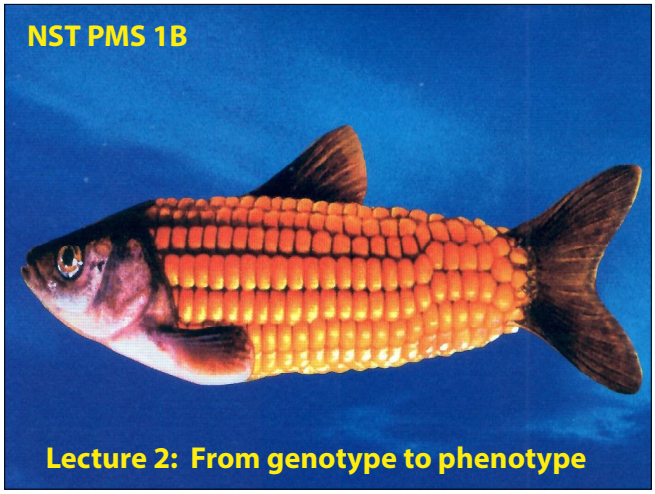

1

For revision of basic concepts and terminology in molecular biology - a free online Life Science textbook can be found at http://csls-text.c.u-tokyo.ac.jp/index.html

The Arabidopsis Book - another free online resource that covers more plant specific material can be found at http://arabidopsisbook.org

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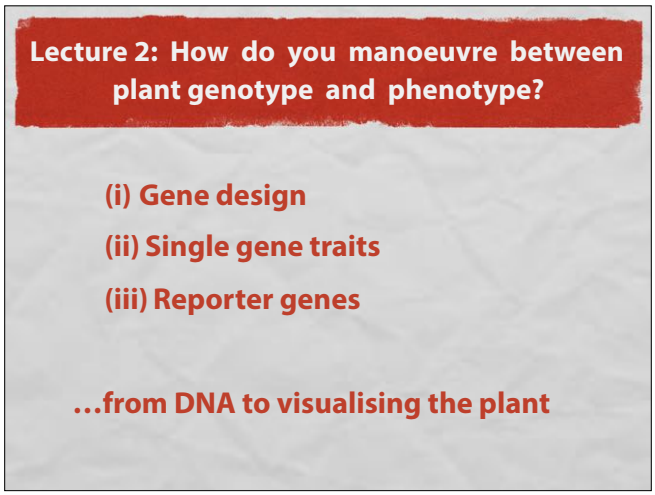


3

In the last lecture we discussed progress in agriculture to the point of the early 1980s, when the first plant transformation procedures were developed. This lecture will focus on the design of synthetic genes and the kinds of traits that can be engineered with single genes. Further, we will look at how reporter genes can be used to visualise gene activity and cellular and organismal properties - in other words, how they can be used to link studies of genotype modification and phenotype.

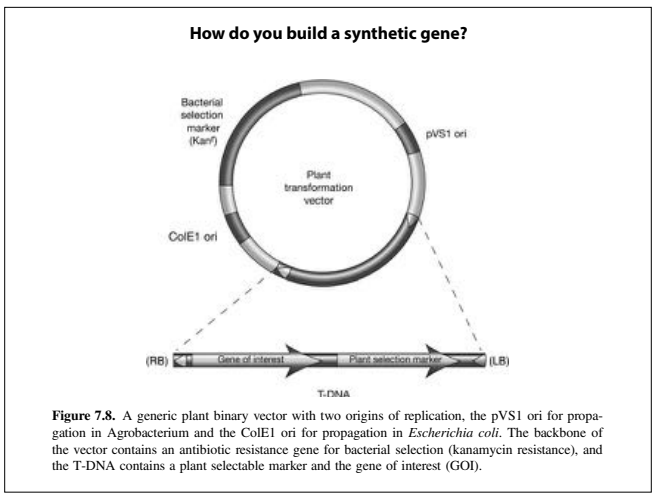
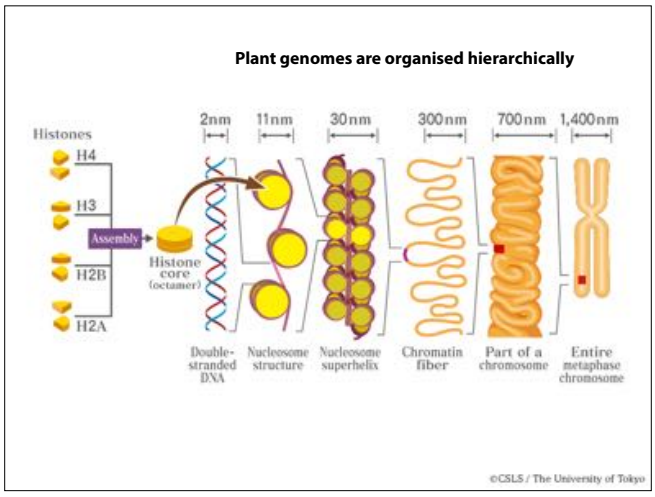


Figure 7.8. A generic plant binary vector with two origins of replication, the pVS1 ori for propagation in Agrobacterium and the ColE1 ori for propagation in *Escherichia coli*. The backbone of the vector contains an antibiotic resistance gene for bacterial selection (kanamycin resistance), and the T-DNA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

4

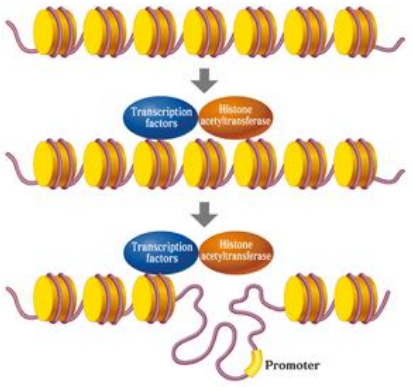
The previous lecture contains a description of how binary plasmid vectors were derived from tumourigenic Ti plasmids, and used for Agrobacterium-mediated plant transformation. These transformation vectors all contain a backbone with origins of replication and a bacterial selection marker. In addition, 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.



5

This diagram shows a simplified representation of different scales of organisation in eukaryote chromosomes. In vivo, double-stranded DNA is found wrapped around nucleosomes, composed of histone protein components in the form of octamer cores. In turn, nucleosomes form superhelical structures of 30 nm diameter, and these form loop structures packed onto chromosomal scaffolds. Chromosome structure is dynamic, with packing and unpacking of chromatin occurring as a part of gene regulation and the cell cycle.

When a foreign gene is inserted into a plant genome, it can inherit properties of the local chromatin



6

The Agrobacterium mediated transformation of a plant cell results in insertion of a foreign DNA segment into a random section of the plant gene. 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

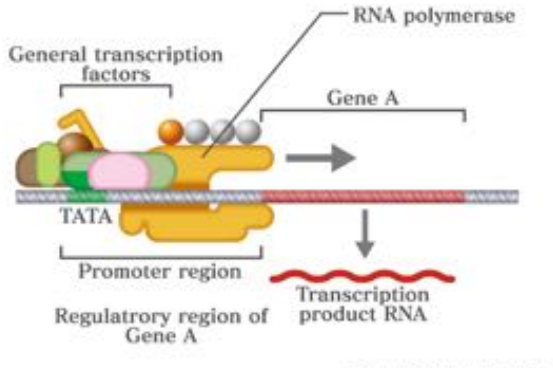
1. Specific sequences provide a key for interaction between DNA and host proteins, which ensure regulated conversion into RNA and protein.
These sequences are crucial for design of properly regulated synthetic genes.

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?

7

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.

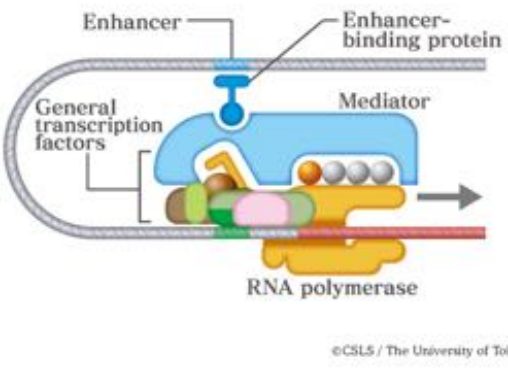
Core promoter elements for a plant gene



8

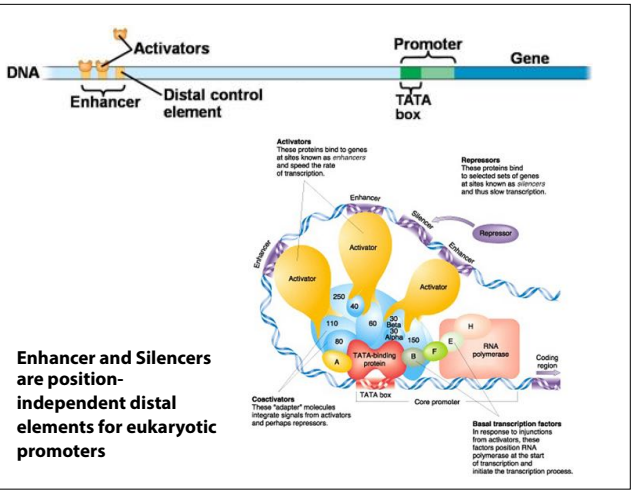
Eukaryotic protein encoding genes are transcribed by RNA polymerase II. The core protein components of RNA polymerase II bind to DNA immediately upstream of the transcribed sequence (red). DNA binding is associated with a conserved TATA-containing sequence (green). However, this complex is not sufficient to initiate transcription.

Transcription initiation requires interaction with distal promoter elements



9

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.

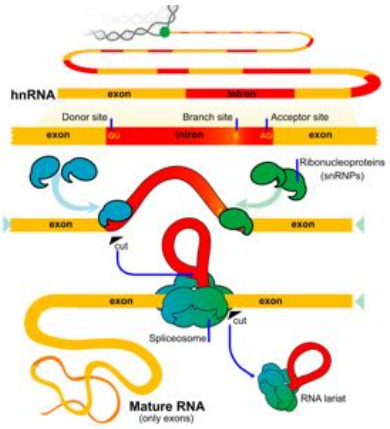


Enhancer and Silencers are position-independent distal elements for eukaryotic promoters

10

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.

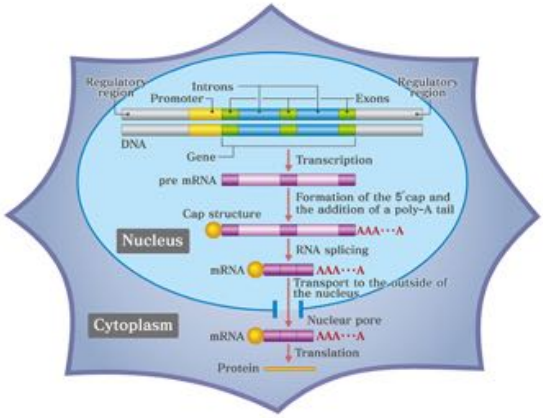
Plant genes generally contain introns (and DNA encoded signals to allow correct splicing and avoid cryptic splicing)



11

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.

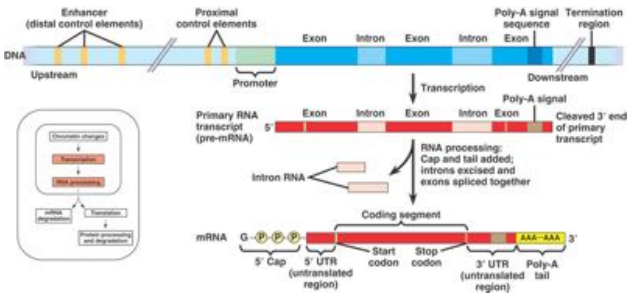
Processing, capping, polyadenylation and efficient translation of plant mRNAs requires appropriate DNA-encoded sequences



12

Synthetic gene design must incorporate sequences that mediate these molecular contacts during maturation, and avoid aberrant cryptic sites.

Plant gene structure

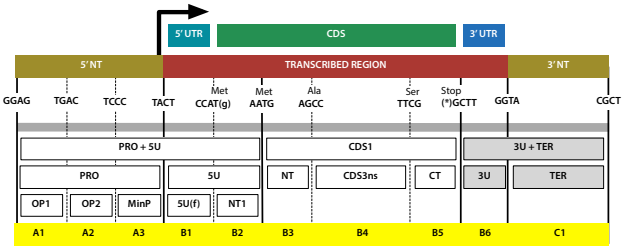


13

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.

A common syntax for assembly of plant DNA parts

Based on Golden Gate standard assembly and type IIs restriction enzyme splints.



Standards for Plant Synthetic Biology: A Common Syntax for Exchange of DNA Parts. New Phytologist 208:13–9. (2015)
by Patron, Nicola; Orzaez, Diego; Marillonnet, Sylvestre; Warzecha, Heribert; Matthewman, Colette; Youles, Mark; Raitskin, Oleg; Leveau, Aymeric; Farre-Martinez, Gemma; Rogers, Christian; Smith, Alison; Hibberd, Julian; Webb, Alex; Locke, James; Schornack, Sebastian; Ajioka, Jim; Baulcombe, David; Zipfel, Cyril; Kamoun, Sophien; Jones, Jonathan; Kuhn, Hannah; Robatzek, Silke; Van Esse, H Peter; Oldroyd, Giles; Sanders, Dale; Martin, Cathie; Field, Rob; O'Connor, Sarah; Fox, Samantha; Wulff, Brande; Miller, Ben; Breakspear, Andy; Radhakrishnan, Guru; Delaux, Pierre-Marc; Loque, Dominique ; Granell, Antonio; Tissier, Alain; Shih, Patrick; Brutnell, Thomas; Quick, Paul; Rischer, Heiko; Fraser, Paul; Aharoni, Asaph; Raines, Christine; South, Paul; Ané, Jean-Michel; Hamberger, Björn; Langdale, Jane; Stougaard, Jens; Bouwmeester, Harro; Udvardi, Michael; Murray, Jim; Ntoukakis, Vardis; Schafer, Patrick; Denby, Katherine; Edwards, Keith; Osbourn, Anne; Haseloff, Jim

14

These rules for modular description of gene architecture have been used as a basis for creating 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

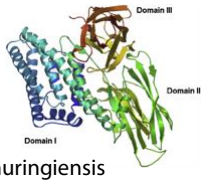
Over a dozen genetically modified (GM) plant species have been approved for commercial production in the US, and the single-gene traits that have been genetically engineered into them fall into five categories.

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

15

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.

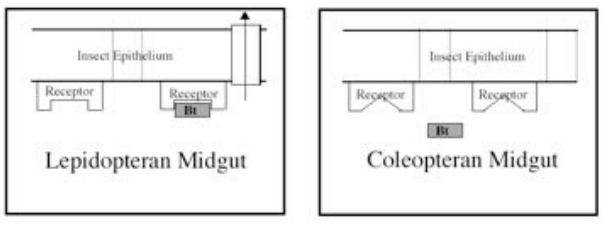
It is a highly selective toxin with no effect on mammalian cells. Bt based insecticides have been widely used in organic farming for over 50 years.

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

16

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.

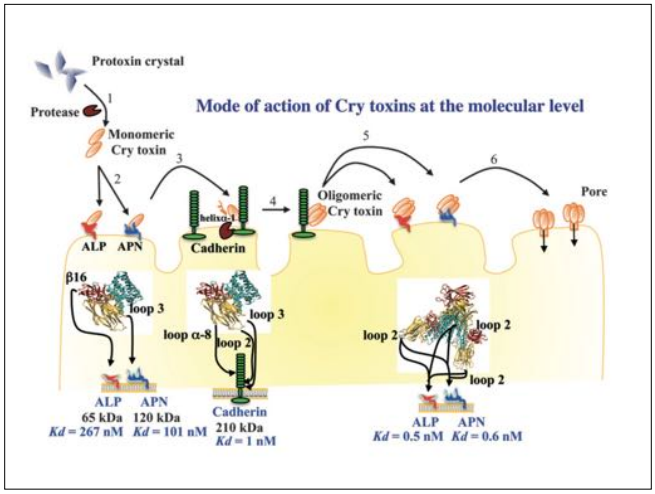
Details of Bt Action



Note: This illustration is for a "lepitodoperan-specific" Bt. Other Bt proteins, specific for coleopterans, exist as well.

17

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.



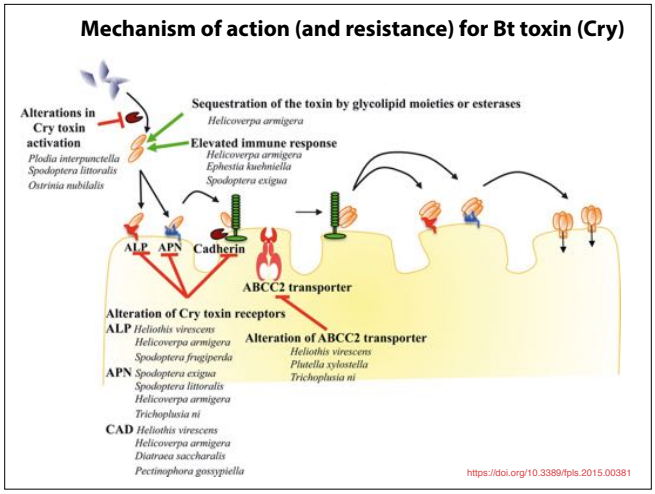
18

Mechanism of action of Bt toxins (3-domain Cry) in Lepidoptera. 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)

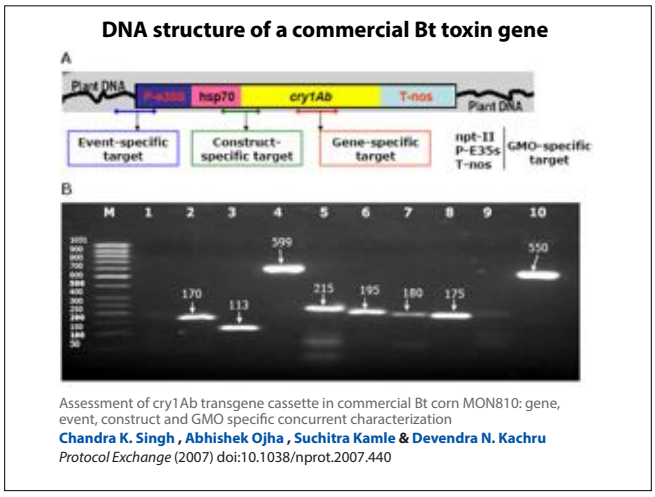
19

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



20

Schematic representation of the different mechanisms of resistance to Bt toxin (3d-Cry) described in lepidopteran insects.



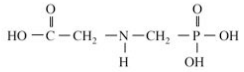
Assessment of cry1Ab transgene cassette in commercial Bt corn MON810: gene, event, construct and GMO specific concurrent characterization
Chandra K. Singh , Abhishek Ojha , Suchitra Kamle & Devendra N. Kachru
Protocol Exchange (2007) doi:10.1038/nprot.2007.440

21

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.

22

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

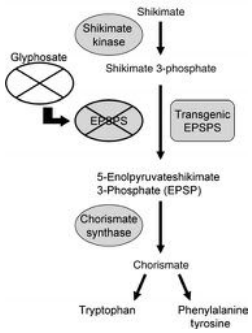


Figure 8.1. Resistance to glyphosate in RoundUp Ready™ plants is engineered by expressing a form of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS) enzyme that is resistant to the herbicide. In the absence of this transgenic enzyme, glyphosate inhibits the plant EPSPS and ultimately blocks 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.

23

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.

24

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.

Multiple herbicide resistance genes

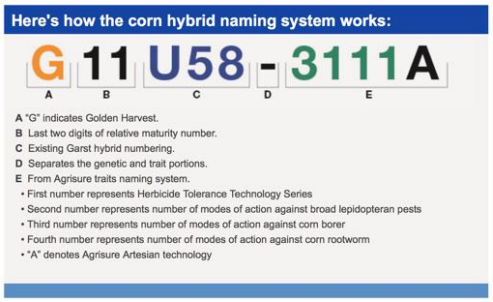
New varieties contain two herbicide-tolerant traits – one for glyphosate and one for dicamba herbicides. The addition of dicamba tolerance provides farmers with tools to manage glyphosate resistant and tough-to-control broadleaf weeds such as waterhemp, marestail, Palmer amaranth, giant ragweed, kochia and others.

https://www.pioneer.com

25

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

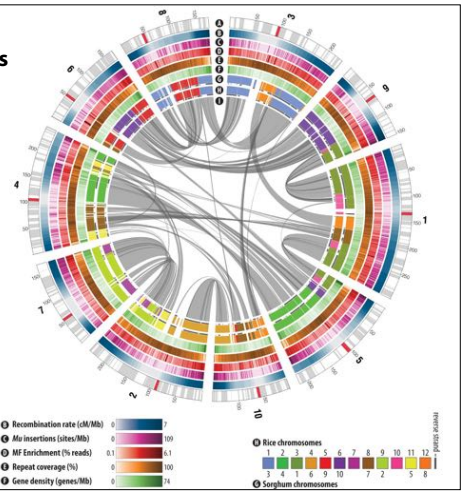


Syngenta

26

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.

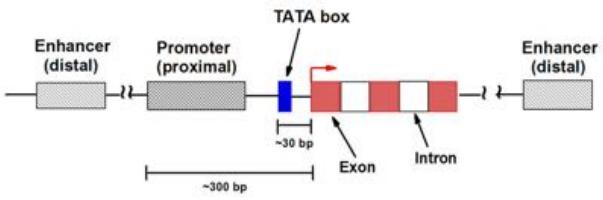
Maize genome
10 chromosomes
2.4 Gbp
32,000 genes



27

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.

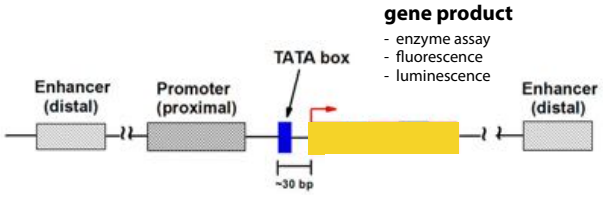
How can the activity of an individual gene be visualised?



28

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

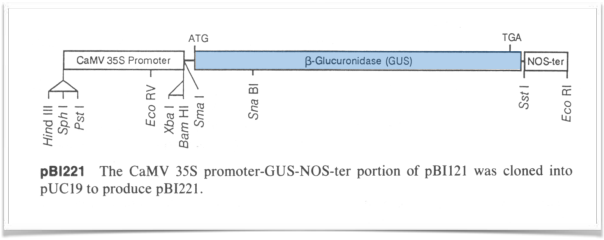


ß-glucuronidase
fluorescent protein
luciferase

29

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.

30

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.

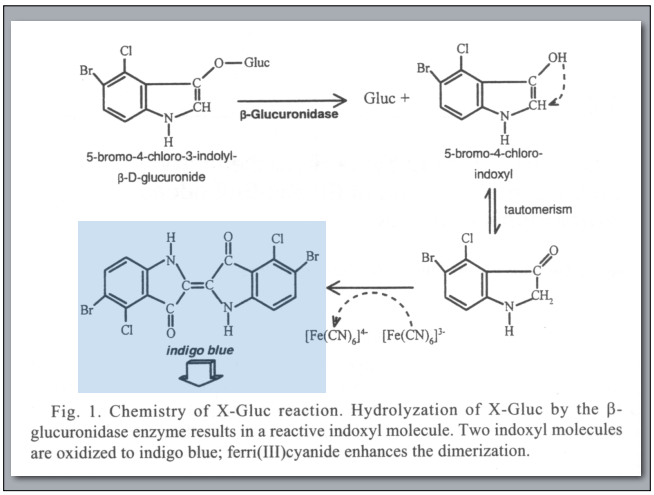
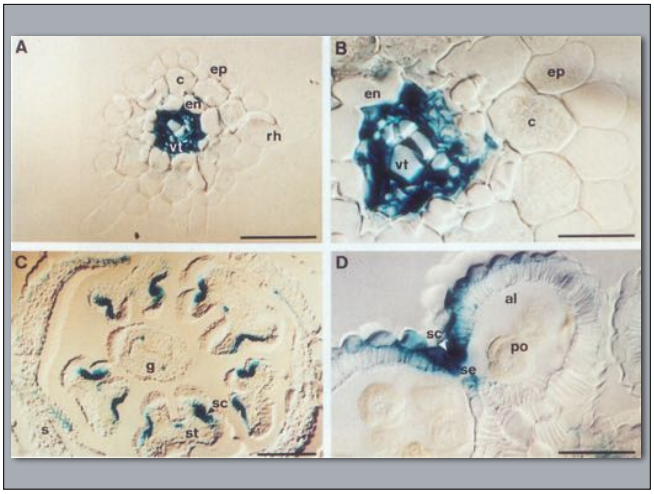


Fig. 1. Chemistry of X-Gluc reaction. Hydrolyzation of X-Gluc by the β-glucuronidase enzyme results in a reactive indoxyl molecule. Two indoxyl molecules are oxidized to indigo blue; ferri(III)cyanide enhances the dimerization.

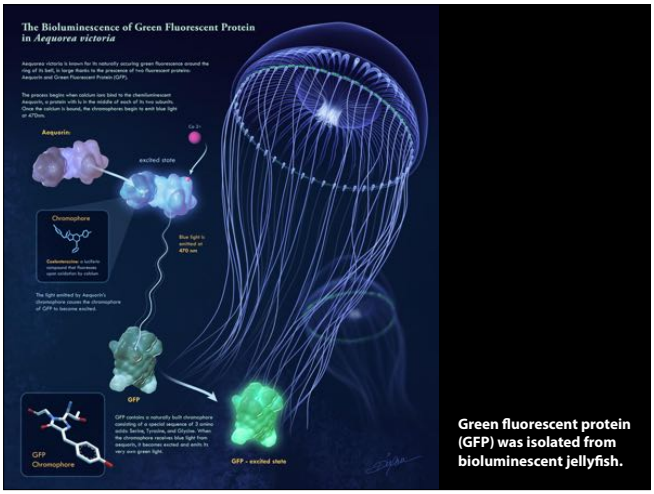
31

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.



32

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.

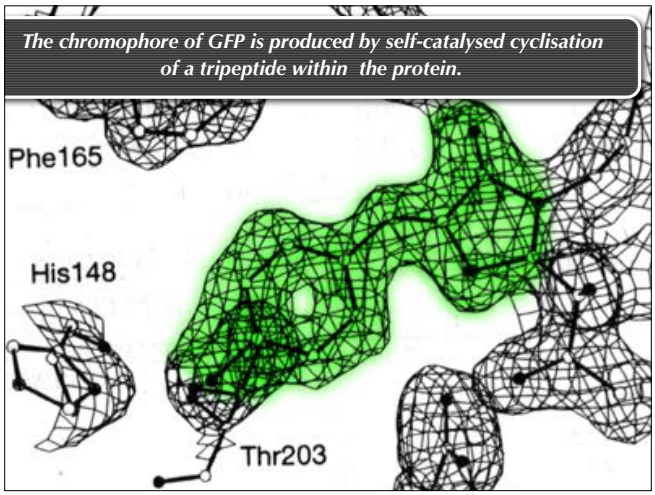
33

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).

3D structure of green fluorescent protein

34

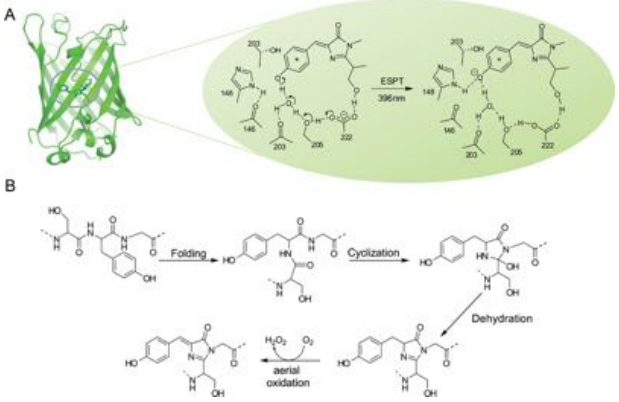
The green fluorescent protein consists of a barrel -like structure formed of beta sheets that surround a single alpha helix that descends through the centre of the protein. The barrel shape is capped by short alpha helical segments, and the outer part of the protein forms an effective solvent cage.



35

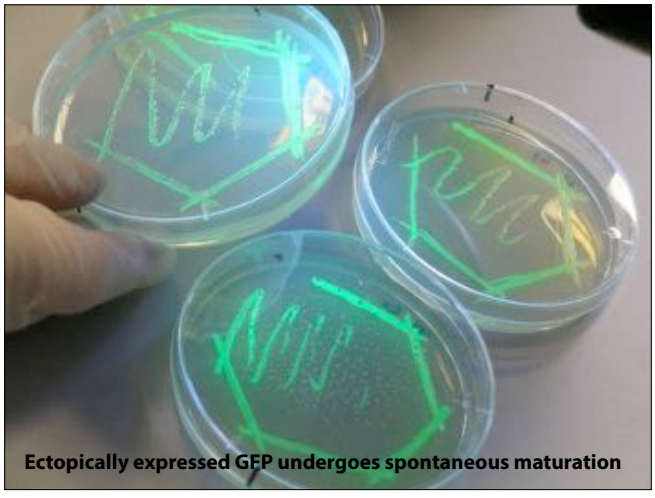
A highly unusual and characteristic chromophore is produced during folding and maturation of the protein. A tripeptide sequence, Ser-Tyr-Gly, undergoes cyclisation and oxidation to produce a multi-ring aromatic group on the alpha helix that runs through the centre of the protein.

Autocatalytic maturation of the peptide chromophore in GFP



36

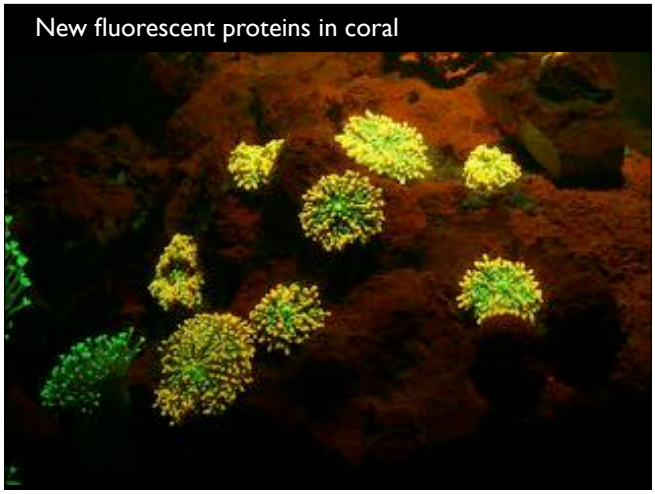
The maturation of the chromophore is autocatalytic, and occurs spontaneously in the protein is expressed in essentially any organism, if allowed to fold properly and have access to oxygen.



Ectopically expressed GFP undergoes spontaneous maturation

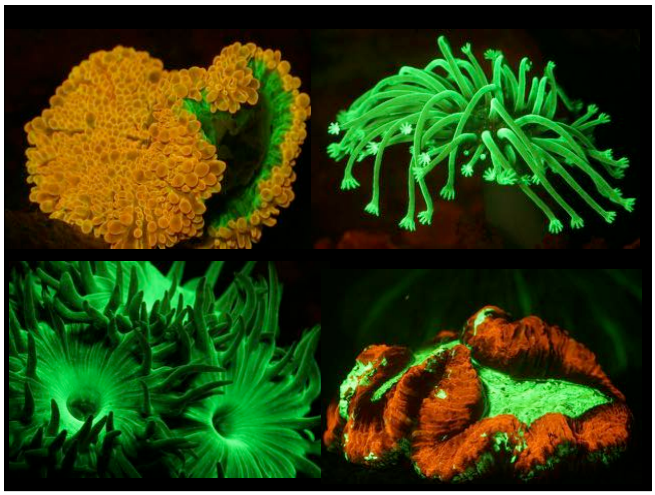
37

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.



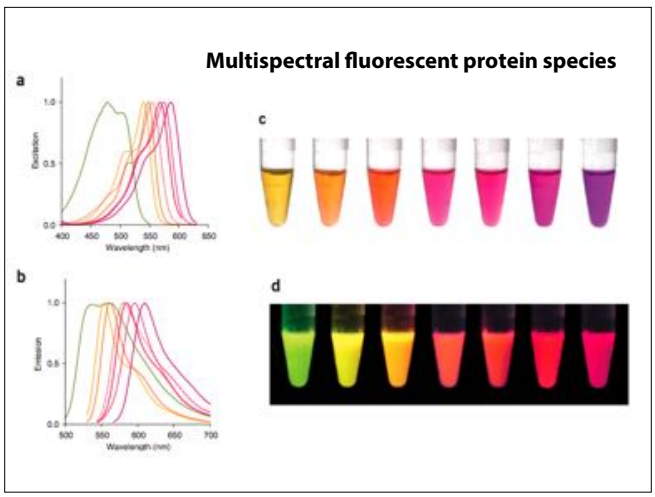
38

Many marine organisms use frozen proteins as part of by luminescent systems or to absorb light. For example, many coral express high levels of fluorescent proteins, which have a wide range of properties as fluorescent and pigmented molecules.



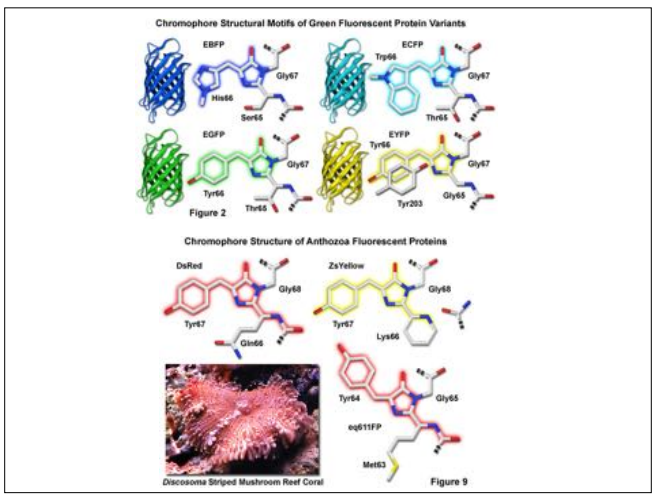
39

These are believed to play a role in natural colouring and light protection for the shallow water organisms.



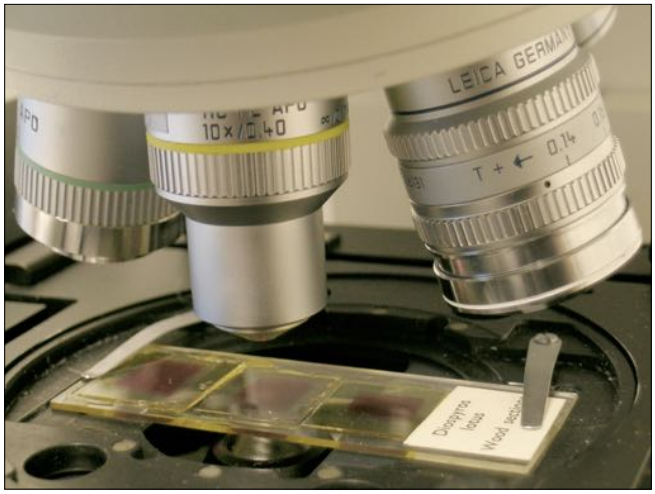
40

A wide range of fluorescent protein species have been domesticated for laboratory use, and provide a "paintbox" for reporter gene studies.



41

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.



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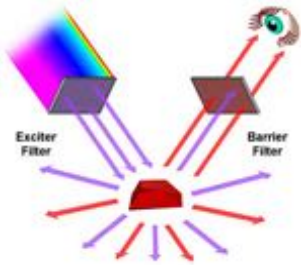
Fluorescent gene products can be detected by a wide range of microscopy and optical techniques.

Benefits of fluorescence microscopy with FP's

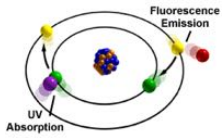
New optical and computing methods allow selective, non-invasive imaging of fluorescent labels within intact cells.

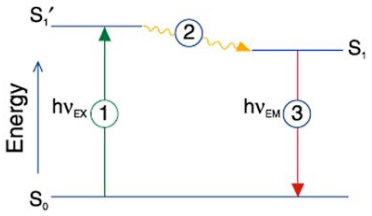
(i) Expression of fluorescent proteins allows live imaging
(ii) Fluorescent emission can be selectively filtered
(iii) Confocal imaging allows optical sectioning and 3D reconstruction



43

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.

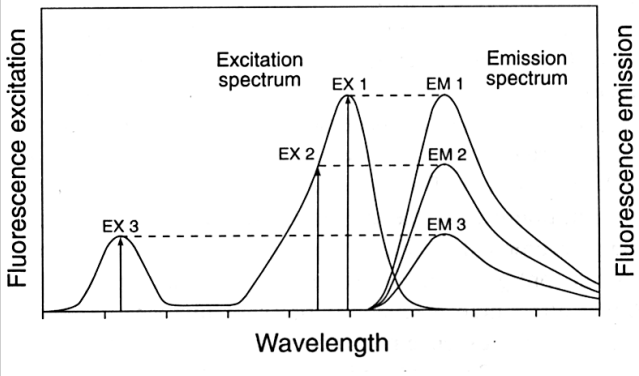




The light used for excitation of fluorescence is higher energy (shorter wavelength) than the emitted light

44

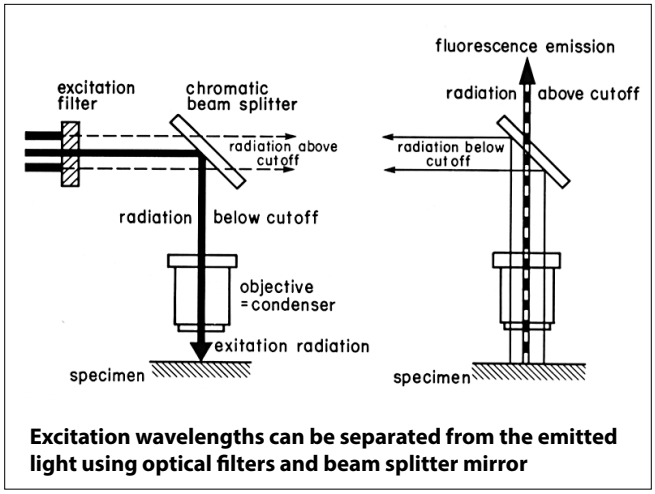
Excitation light excites electrons to an outer orbital. After relaxation the excited electron collapses back to the ground state and in doing so releases a photon to compensate for the loss of energy.



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

45

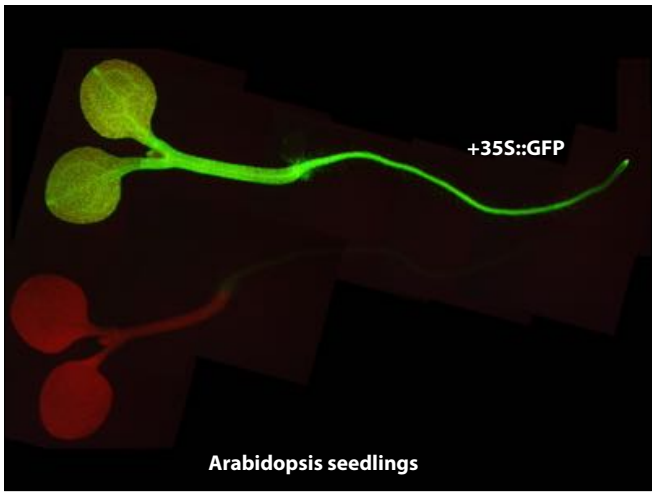
The difference between excitation and emission wavelengths allows the use of optical filtration to selectively block the excitation light and allow sensitive detection of the fluorescence emissions during observation.



Excitation wavelengths can be separated from the emitted light using optical filters and beam splitter mirror

46

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.



Arabidopsis seedlings

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

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Arabidopsis seedling apex

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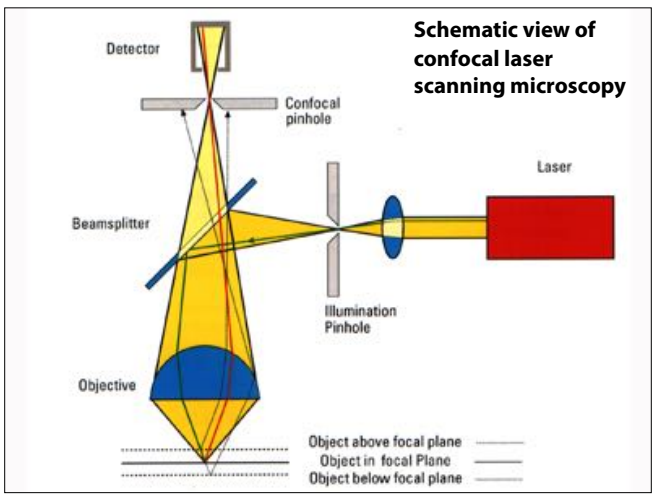
Higher magnification observation of a GFP transformed seedling, showing hypocotyl (stem), shoot apex and base of cotyledons (first leaves). Out-of-focus blur is evident.



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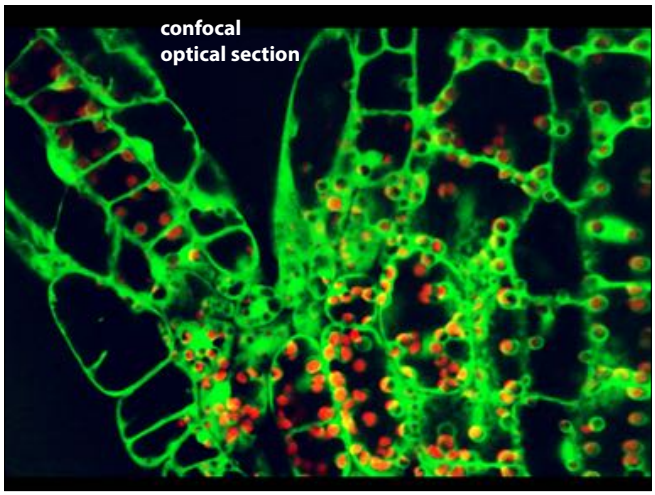
Confocal microscopy uses a laser beam for illumination. The laser illuminates the sample with a focussed spot, building an image as the beam is scanned across the sample. Fluorescent signals from the laser excitation are focused in the back plane of the microscope, passing through a small aperture (confocal 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.

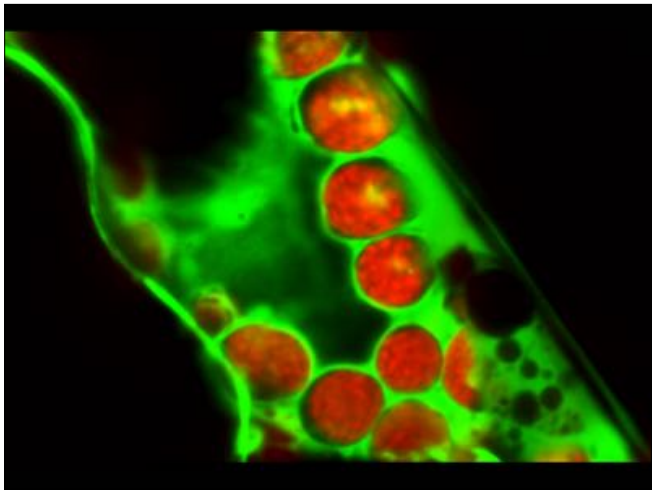
50

Modern confocal laser-scanning microscope



52

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).



53

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.

64

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.