Unit- II

Genetic Engineering in plants: aim and scope for developing transgenic plant – Agrobacterium mediated gene transfer techniques to develop disease resistant and stress tolerant plants, Bt cotton, golden rice, and variegated banana. Direct transformation methods. Pros and cons of GM crops.

Steps in plant Genetic Engineering

Production of Transgenic plant

Isolate DNA → Genomic library.

↓

Gene Identification

↓

C DNA Synthesis (Splicing and resynthesis)

↓

Construction of VeeQG

↓

Transfer into plant (Recipient plant)

[Direct Transformation Method

asmi ectors

↓

Incorporation into Genome.

↓

Gene expression

↓

Selection of Transgenic plant cells.

← Plant regeneration

↑

[Molecular Verification of gene presence and expression]

↑

[Sexual pagation] [Asexual propagation]

↑

[Green House & Field Tests]

↑

[Ecological & Biosafety Analyses]

↑

[Regulatory Permits for large scale use]

↑

[Commercial deployment]

↑

[Continued monitoring of efficacy & safety]

Basic steps involved in g ng of Plants for Pest resistance / Bt cotton

Vector

Agrobacterium tumefaciens

Ti

Ti - Plasmid Vectors

Cointegrate Vector

Binary Vector

Foreign gene

Proteinase Inhibitor gene

Source: B. thuringiensis

Bacillus thuringiensis

PI gene

Source - Plant

Truncated Bt gene

PI gene.

CDNA

CDNA.

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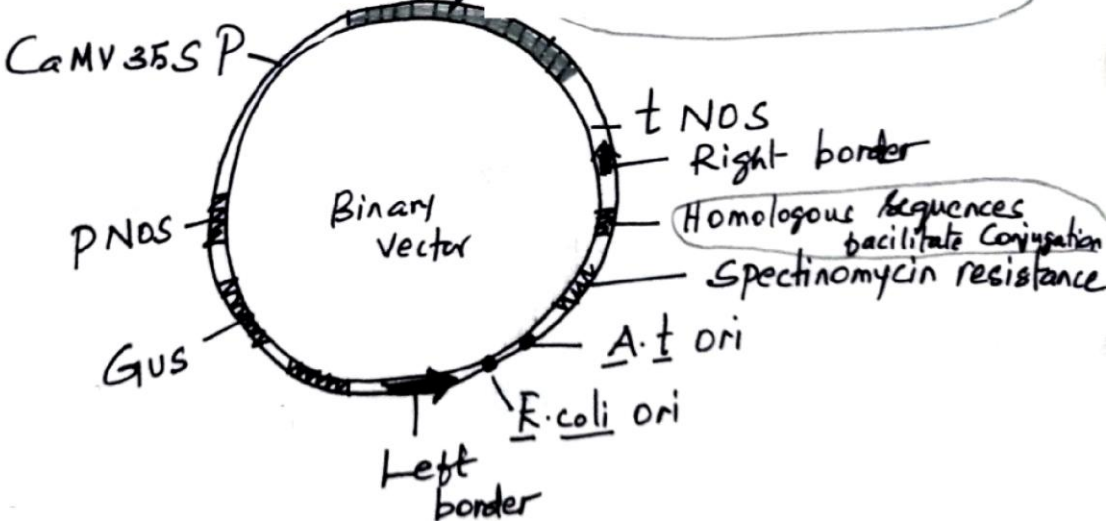
Gene of Interest

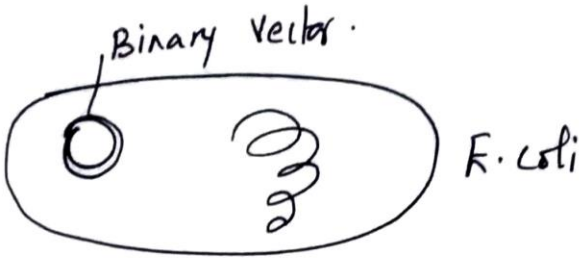
Bt toxin gene / PI gene



(Plant

Chemo Transformation (PEG, Ca^{2+}, g^{2+})

Electrotransformation.



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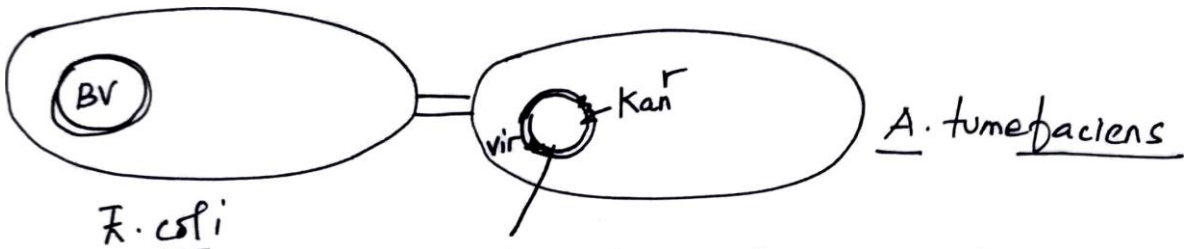
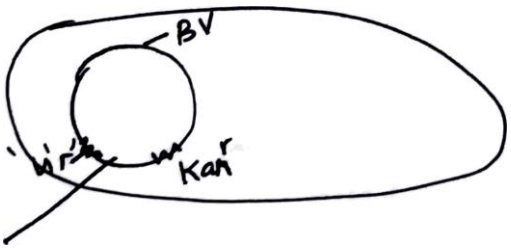
Selection of Transformed E. coli by growing them o‹ æ/,.1« «æ.›• r».Í«•›•ę
Spectinomycin (spc^r)

↓

Mobilisation of binary vector E. coli into



vir helper plasmid (dic-armed)



recombinant Ti-plasmid

↓

Selection Agrobacterium (Kan^r)

↓

Transformation of Foreign gene into plant genome

Co-cultivation

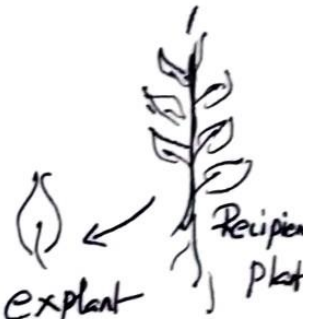
Recipient-Plant

ex-plant

Protoplasts

Transformed Agrobacterium & Plant Protoplasts

Leaf-disc Transformation



explant

Recipient Plant

Leaf discs

Leaf discs + Transformed Agrobacterium

growing in medium containing Ceftatoxin which inhibits Agrobacterium found on the surface of the plant.

A°e #ś»« ń•6•4 /**/ ^ A*k *•+*^*•

!*^^l*r $*a S^J are !!!'!' p

Selection of Transformed

↓

Transgenic Callus

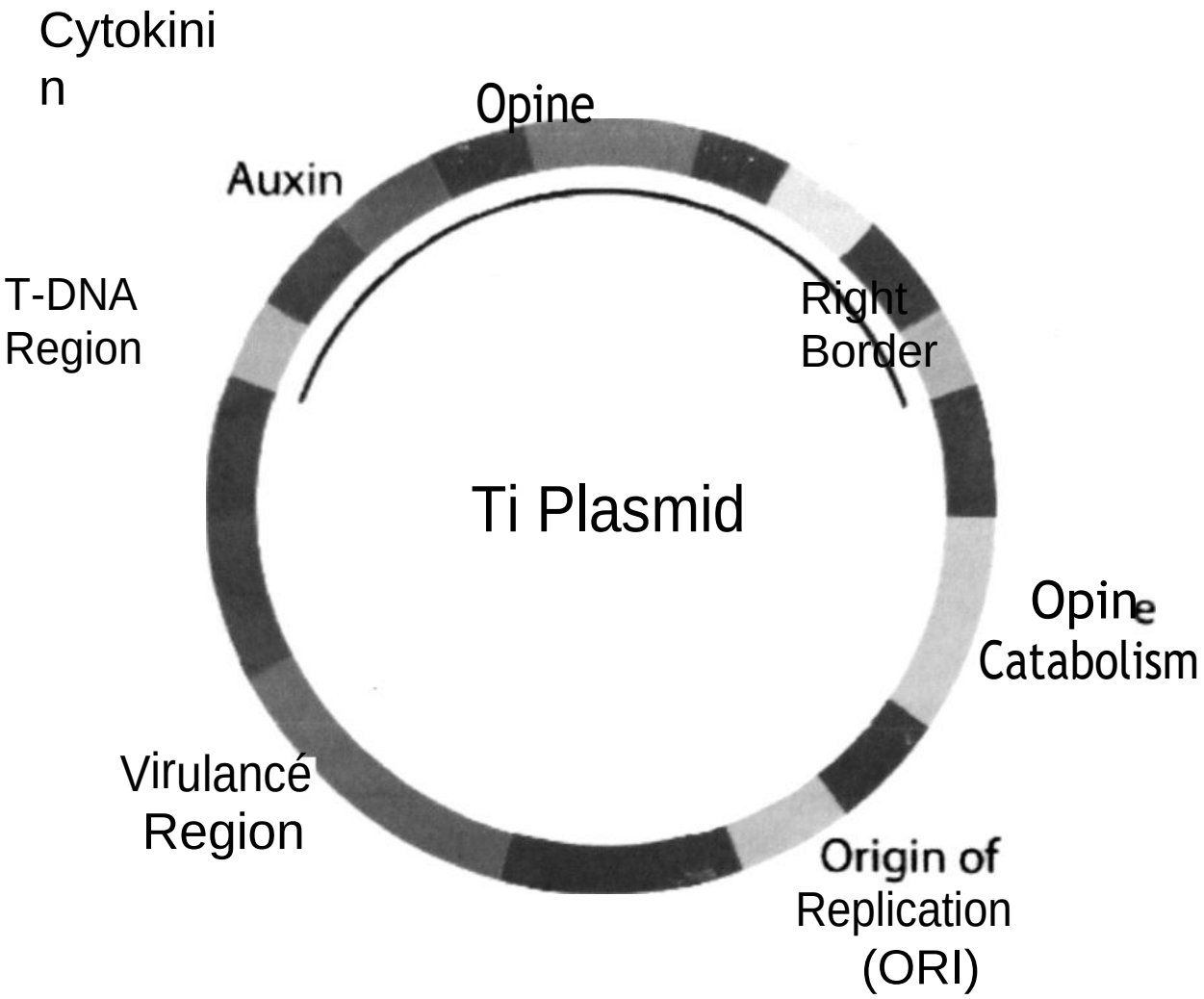
↓

Indirect Organogenesis

↓

Transgenic plant with Bt toxin gene.
(Pest resistant plant).

LeK Border


Cytokinin
Opine
Auxin
T-DNA Region
Right Border
Ti Plasmid
Opine Catabolism
Virulancé Region
Origin of Replication (ORI)

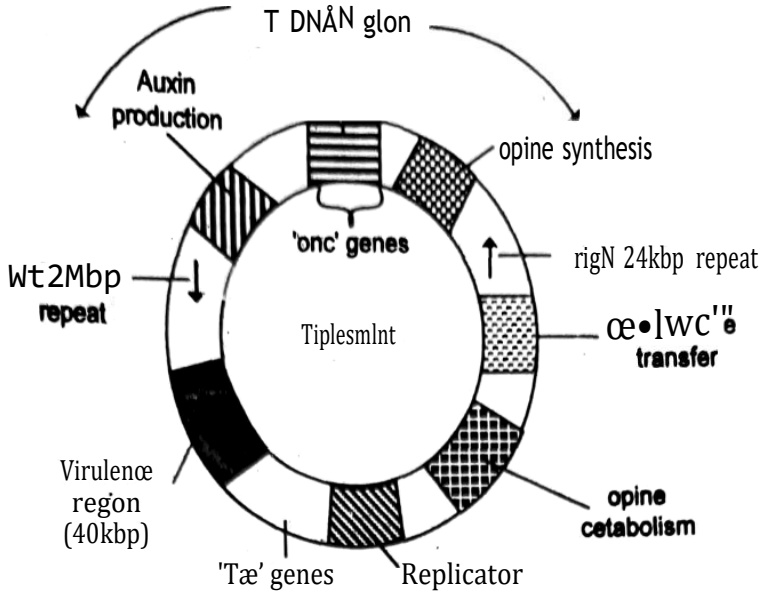


Fig. b.4 Genøtls Map of an octoplne TI plasmid

Røglonø of Tl•plmniłd Important IorTurnoñqenlclty

Qtr region, border repeats (RB, LB) and enhancer are involved in T-DNA transfer and the T-DNA with 'one' genes bring about symptoms on plants. The Vír' region contains 7 'vir' operons four of which (Vir A, Vir G, Vir B and Vir D) art abßohiteły eeøenäal for the transfer process, whereas the remnniøg three vir operons Qtr C, Qtr p•, Vir F} are necessary only in certain species. The integration of T-DNA within the active endogenous plant gene will inactive that gene which may intum cause phenotypic mutations. Two proteins encoded by the Vir A and Vir G mediate the activation of other Vir' genes in the presence of phenolic inducers (plant originJ. Qtr' genes are stent until they become induced by certain plant factors and these factors are phenolic compounds, acetosyringone, sinapic acid, e-hydroxyacetosyringone, flavonoids, syringaldehde and syringic acid. Chv A (codes for transport protein} and Chv B (235 kDa proteins of plànt origin are necessary for attachment of Agroß cterúnn to plant cell waíís.

Methods of Transformation of Plants with A containing Foreign Gene and Marker Gene)

1. **Co-cultivation**
2. £eaf-dice trandoræaäon methød
3. Direct methods (atiW-gun meŁod ţparticle gun bombardment; Electroporation)

TLRlasnÂ ÆaÆcÆilngvæöa Çlgc. 5.5a b 5.5b)

1. Bînaty eector
2. CoÓugrau xector

7t-ętaxndöartvÆVmär üyaamo

ÆŁouQ Łe 7i-plaßmid are esecúve as naÑral vectors; Łey have several

1. **The production of phytohormones by transformed cells growing in culture** @MV€f2t8 them from hertog *rr.*gencra*ted* into fn@*f* erefore, **the auxin** o ncgCnonmust berrmoved

2. **A gene coding for opine synthesis is not useful to a transg**znic**plant and may lower the final plant yield by diverting plant resources** /ore, the opine-synthesis gene shoU)d b *rE* **opine production.**

- Ti-plasmids are large (- 200kb). For r **DNA experiments, a much smaller version** $ preferred, so Jorge segments of D+ >t nre not se aentioJ *ä **cloning vector** otust be reznoved.

+. 7i plasmid does not replicate in *E.*‹x›ti. Therefore, in **developing Ti-plasmid-**beeed vec*tors, an origin* of replication cthat all be Uaed in ***E.coli* must be added.**

Thara azs two Yi-pfasmid Derived Clonlng **Vector Systems**

(I) The Binary Vector (Fig. 5.5a)

The binary vector contains **both *E.coli* and *A.tumefaciens* origins** of DNA replication but no 'rir' genes. it is ari A. ccii - A. fu. $J< C< * - * cloning steps are carried out in A. *aoli* before the vector is iritrodUced into fizzier. The recipient ñ. *tume**fa tens*** strain carries a modified (defectiPe; disarmed) Ti-plasmid contains a complete set of 'ver' genes but lacks portions of theT-DRN region. In this system, the defective Ti-plasmid synthesizes the 'vir' germ products that mobilize the T-DNA region of the binary cloning vector plasmid. By presiding the proteins encoded by the vir genes, the defective Ti-plasmid is acting as a helper plasmid, enabling the T-DNA from the binary cloning vector to be inserted OR› the plant ciiromOSOlMaI DNA. Right border is absolutely required for T-DRA ***integration into plant cell DNA.***

7arget gene

Plant selectable marker gen$_d$

'Vie' gènes

Fig. 5.6b Colntcgfate Vector Sytem

The components of co-integrate vector are: £. coli ori; right border; Bacterial selectable marker gene; plant selectable marker gene; target gene and homologous .DNA sequences.

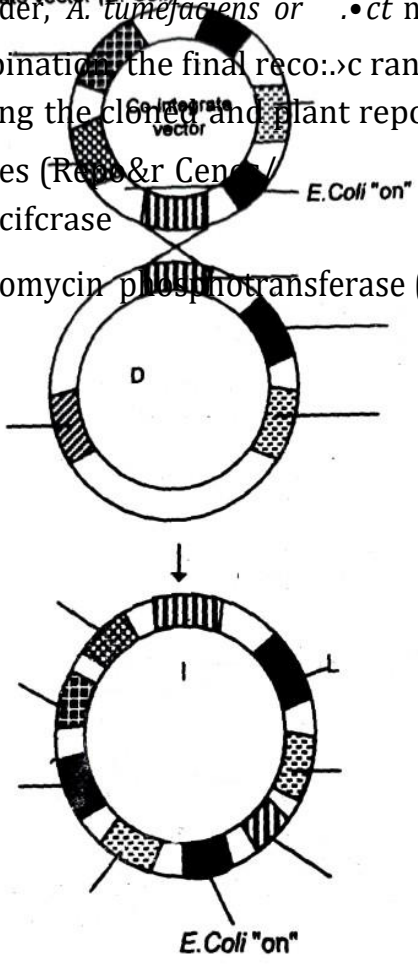
Tl la

Vir' gènes; left border, *A. tumefaciens or .•ct* nomologous DNA sequences.

Following recombination, the final reco:.›c rant plasmid has T-DNA left and right borders bracketing the cloned and plant reporter genes

List ofPlant Mâ6er Gènes (Reporter Genes)

Luc	Lucifcrase
npt'II	Neomycin phosphotransferase (Kanamycin resistance)



The Components of Disarmed -p smid

-

-

β-gal-β -

Gus-β -

o EPSPS (S . ,›y«i.yl shikimate 3-phosphate synthase)

dhJ/ Dihydrofolatc reductaee (glypho aate fi9jCt8f lC

FAR Phosphinothricin (herbicide marker)

COi2jmonly used proiriöters:Ca MV 195, 35S

grictz/ ••i yi»ne » n»• medv leAgrobacfe«• •'

Tobacc0, apium, asparagus, sugarbeet, turnip, oil SflCd, cucumbet, cO oL *‹'›ybean, cotton, sunflower, lettuce, tomato, alrarq, petunia, Phaaeolus, QOtBtO, ‹"Owpea, clover, etc,.

CO ?man.faits 'hlch eue ran»femed allô fée help o7TI Pleamld

Resistance to virus, fungal pathogens;
Herbicide tolerance,
Altered flower color,
Altered shelf life of tomato fruits,
Male sterility,
Cold tolerance,
Altered starch,
Oil composition,
Resistance to pathogenic bacteria,
Insect resistance,
Modified seed storage proteins sweeter taste,
Less fat etc,.

Conclusion

The *Agrobacterium* vector system is being used extensively for the transfer of *V&fiOtiS* traits to crop plants as well as for the study of gene function in plants.

OEVE1OPBENT OF INSECT RESISTANCE (PEST RESISTANCE) IN PLANTS

B.t Cotton

insecf (pests) are controlled by using chemical pesticides. Chemical pestici‹ic» are hi Qty inefficient because of these following reasons.

1. About 98% of the sprayed chemical is washed away from the plant surface and ends up in soil.
2. Chemical pesticides are not degraded efficienUy in the soil and their residues therefore build up and cause environmental pollution.
3. Have a broad spectrum of activity and are toxic to several non-target OfQ£tf2JSfTlS.
4. it is *very* difficult to deJiver chemical pesticides to highly vulnerable parts of plants such as roots (or) internal regions of stems and fruits.

Two Biotachnological Alternatives to Chemlcal Pesticides

!. /.nsecticir'aJ crystal proteins /rcm the bactcrïum

are toxic to specific groups of af

2. Proteinase inhibitor, which

high concentration.

(t8aclllUs Thuringlensl» (BI) Toxlns

Bacillus thuringiensis, a gram positive soil bacterium, produces upon SpOrtl latiOl3 a p£ti‘p s{ytj -o J t-t-yq} n}n wi]}j ivy srt-Î Îi-ÎfÎ£i1 activity. This protein is referred to as insecticidal crystal protein (lCP). When the larvae of susceptible insects ingest the lCP, a combination of alkaline pH and proteinases in the midgut solubilizes the protein and converts the harmless protoxin to the active toxin The effect of the toxin is very rapid; within few minutes of ingestion, the binding of toxin molecules to specific receptors in the midgut membranes disrupts ion-transport across the membrane and paralyses the midgu(fbl flCtÎOn. The larvae stop feeding almost instantlJ arid die. The *Bacillu il « g!'* harmless to higher animals, iricluding humans.

Biopesticides based on *Bacilluo* thurinpiensts art produced by growing the organisrri in liquid media in fermenters, allowing the organism to sporulate and form protoxin on depletion of nutrients, then drying the Qf oduct to a poWder. The pré can be Liusted ontc plants or sprayed onto them after the powder is emulsified ir- water.

Advanmyes		Sure and efficacy
Disadvantages	-	Does not persist in the environment. High cost of production; lois pers'stence; low stability

Geoefic *Engineering of OnciflHs Thuri!tgiensio Toxin* Genes *{ri9. s.e:j:*

Scientists *from* Plant a•tic lystc! s a Belgian Biotechnology company , reported the first successful gene -:›ti.on *•ansgenic plants with *BaSllus ttwriiigierusis* toxin. A full length protoxin gem: *›c!L11(* iOi 1155 amino acids, whereas a truncated version inclusive of *N-ter* ninnf *29 - 60T* ‹i iiiio acids was cloned under the control of the mannopine synthase promoter of Ti plasmid. Plants were transformed and selected using Kanamycin, since npt II confers Kan ycin resistance gene. It is fused to the C-terminal end of the truncated Bacillus *!M nets* gene. Since then a number of reports on genetic engineering of tomato, tobacGo, potato, afin tton have appeared.

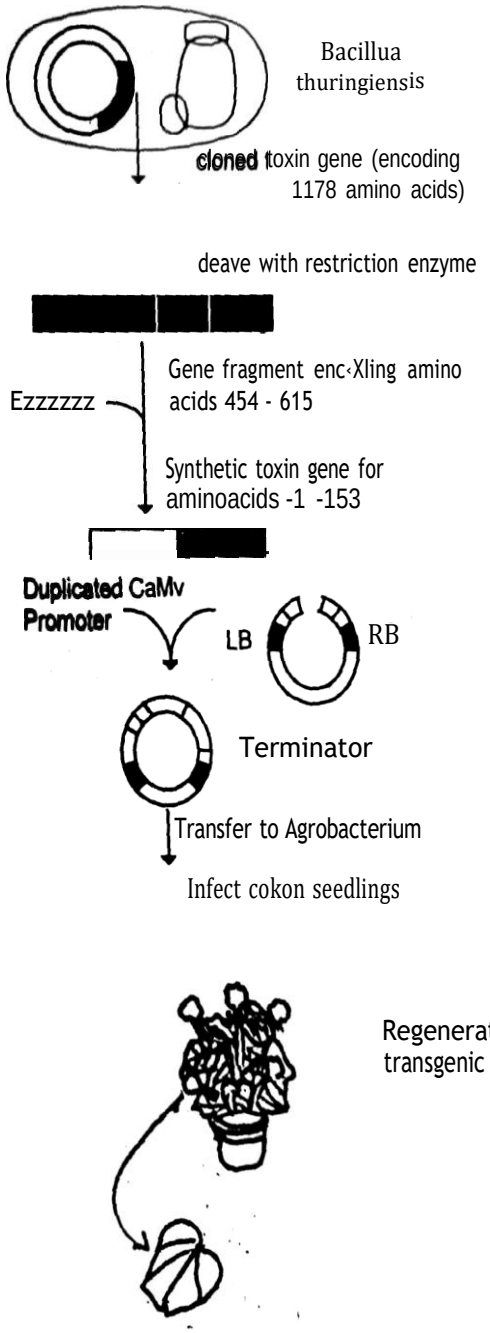
Drawback

The level of expression of *BaeilluB thuringieriBiB* gènes in transgenic plants is low, not sufficient for commercial exploitation. To overeome tliis, regions of A=1 richness, regions resembling plant introns, potential poly ‘A' signals and ATTr/ sequences were deleted from *Bacilluz* tñuringiertsis gene producing a truncated gene A 100-500 fold increase in the expression of the engineering (téuncated) the wild type gene was

2. Toxin proteins are produCed Withlfl the plants.
3. *Baeñllus ihwlngieti sis* toxins made in the plants would kill only target organisms, which feed on the transgenic planta.
4. *BO@llu8* tliuriitpiensis toxins act as loW c .‹centrations.

Ofsadvanfapçy

1. There is a feæ that *Badllus* J/›uriitpiensis resistant insects would evolve rapidly. To overcome these problem two different *Bacillus* thuringiensis toxin genes are introduced into a plant for the same target insect or introduce two types of insect resistance genes (Bacillus thiirinqiensis genes and proteinase inhibitor gene) into a plant.
2. *Proteinase* inhibitors: Many plants have evolved natural mechanism of defense against insects by accumulating proteinase inhibitors (proteins which inhibit the activity of proteinase enzymes) at concentration that will cause metabolic inhibition upon ingestion by insects. Compared to *Bacillus* tfuiringiensis toxins, proteinase inhibitors have a broader spectrum of metabolic inhibition and so can be used to control many different types of



Regenerated transgenic cotton

insects. Proteinase inhibitors (PI)

ht3ge ns, can not be us t0 c ontrol plant viruses, sinee viruses are araceMular obligate parasites.

Mechanism of T-DNA Transfer

The 'vir' region: The genes responsible for the transfer of the T-DNA region into the host-plant — is an 40 kb region found outside the T-DNA regions.

There are at least- 9 Vir-gene Operons.

Vir A - Phenolic sensor, phosphorylates & activates vir 'G'

Vir G - Transcription factor; responsible for induction of other vir genes

Vir B_1-B_{11} + Vir D4 → Components of Transfer apparatus

Vir C_1 - Overdrive, enhances efficiency of T-DNA transfer.

Vir D1 - T-DNA processing

Vir D2 - SS-Nicks (nicks) to T-DNA & directs T-DNA through Transfer apparatus.

Vir F_1 - Required for Vir F_2 Export from Agrobacterium spp to plant cells

Vir F_2 - - SS DNA-binding Protein
- prevents T-DNA degradation by Nucleases
- Involved in Nuclear targeting and passage through Nuclear pore Complex (NPC).

Vir F - Cell Cycle regulation

Vir J - T-DNA Export.

Steps:

1) Wounded plant cells release phenolic substances & sugars
2) The phenolic subs. & sugars are sensed by Vir 'A'
3) The Vir 'A', then activates Vir 'G' by phosphorylation.
4) Activated Vir 'G' induces the expression of all the other genes of 'Vir' Operon

Gene products of 'vir' genes are involved in a variety of processes.

a) Vir D_1 & D_2 are involved in T-DNA processing. The LB & RB are recognized by a Vir D_1 / vir D_2 Complex & Vir D_2 produces SS-nicks in the DNA. After nicking, Vir D_2 becomes Covalently attached to the 5' end of the displaced SS T-DNA strand. Vir C_1 assists this process. Vir D_1 & D_2 are also protects the T-DNA & helps export from Agrobacterium to plant cell. SS T-DNA is now Complexed with Vir D_2 & Vir F_2

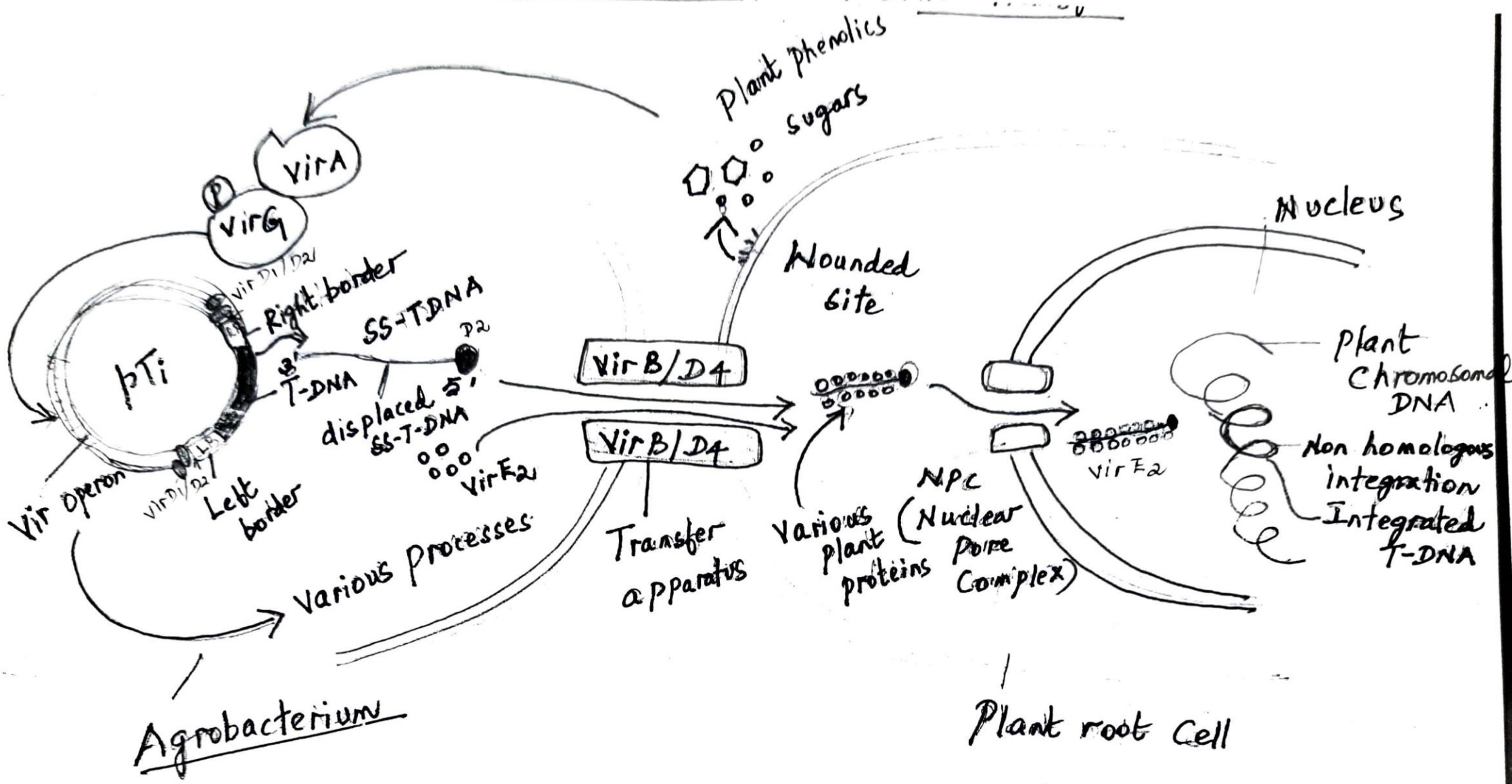
b) Vir 'B' products form the Transfer apparatus. The SS-T-DNA along with Vir D_2 & Vir F_2 are exported through the Transfer apparatus.

c) In the plant cell, the T-DNA becomes Coated with Vir F_2. Various plant Proteins interact either with Vir D_2 (Or) Vir F_2, which are attached to the

T-DNA & influence Transport and integration.

8) The T-DNA / VirD2 / VirE2 / plant protein Complex enters the nucleus through the NPC.

(9) Integration ~~of~~ into plant chromosome via illegitimate (non-homologous) recombination. | P.611



Mechanism of T-DNA Transfer ↑

Development of Insect resistance / Pest resistance

Insect Pests are controlled by using chemical Pesticides. Chemical Pesticides are highly inefficient (i) — about 98% of the Sprayed chemical is washed away from the plant surface and ends up in Soil (ii) Chemical pesticides are not degraded efficiently in the Soil & their residues therefore build up and cause environmental pollution; (iii) have a broad Spectrum of activity & are toxic to Several non-target organisms. — (iv) it is very difficult to deliver chemical pesticides to highly Vulnerable parts of plants Such as roots (or) internal regions of Stems & fruits.

Two biotechnological alternatives to chemical Pesticides: (i) Insecticidal crystal Proteins from

bacterium Bacillus thuringiensis (B.t) that are toxic to specific groups of insects at low conen. (ii) Proteinase inhibitors :- are harmful to insects when present in the diet at high concentrations.

(i) B.t toxins :- Bacillus thuringiensis, a Gram +ve soil bacterium, produces upon sporulation a parasporal crystals / insecticidal activity. This Protein is referred to as insecticidal crystal protein (Icp). When the larvae of susceptible insects ingest the ICP, a combination of alkaline pH & Proteinases in the midgut solubilises the protein & converts the harmless protoxin to the active toxin. The effect of the toxin is very rapid; within few minutes of ingestion, the binding of toxin molecules to specific receptors in the midgut membranes disrupts ion-transport across the membrane & paralyses the midgut function. The larvae stop feeding almost instantly & die. The B.t toxin is harmless to higher animals, including humans.

Biopesticides based on B.t are produced by growing the organism in liquid media in fermenters, allowing the organism to sporulate & form protoxin on depletion of nutrients, then drying the product to a powder. The powder can be dusted onto plants (or) sprayed onto them after the powder is emulsified in water.

Advantages – safe & efficacy.

Disadvantages - does not persist in the environment high cost of production; low persistence; low stability.

Genetic engineering of B.t toxin genes:

Scientists from Plant Genetic Systems, a Belgian Biotechnology Company, reported the first successful generation of transgenic plants / B.t toxin. A full-length Protoxin gene coding for 1155 amino acids, whereas a

truncated version inclusive of N-termind 29-607 amino acids was cloned under the control of the mannopine Synthase Promoter of Ti plasmid T-DNA. Plants were transformed & selected using kanamycin; since npt II confers kanamycin resistance is fused to the C-termind end of the truncated B.t gene. Since then a no. of reports on genetic engineering of tomato, tobacco, potato, Cotton have appeared. Drawback: the level of expression of B.t genes in transgenic plts is low not sufficient for commercial exploitation. To overcome this, regions of A=T richness, regions resembling plant introns, potential poly 'A' signals & ATTTA sequences were deleted from B.t gene producing a truncated gene. — A 100-500 fold increase in the expression of the engineering (truncated) gene over the wild type gene was observed.

(38)

There are 2 advantages in using transgenic plants c B.t genes for pest resistance:

Advan: B.t toxins act at low conc.

(i) B.t genes in a transgenic plant could be expressed in all parts of the plant including roots & internal regions of stems & fruits.

(ii) toxin proteins are produced within the plants.

(iii) B.t toxins made in the plants would kill only target organisms c feed on the transgenic plant.

Disadvantages: (i) there is a fear that B.t resistant insects would evolve rapidly. To overcome this problem 2 different B.t toxin genes are introduced into a plant for the same target insect / introduce 2 types of insect-resistance genes (B.t gene & Proteinase inhibitor gene) into a plant.

(ii) Proteinase inhibitors: Many plants have evolved a natural mechanism of defence against insects by accumulating Proteinase inhibitors (Proteins c inhibit the activity of Proteinase engymes) at concn. that will cause metabolic inhibition upon ingestion by insects. Compared to B.t toxins, Proteinase inhibitors have a broader spectrum of metabolic inhibition & so can be used to control

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wide variety of
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including
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NPT - Plant Selectable marker gene

Homologous Sequence : Recombination (integration of 2 plasmids) is possible. - Conjugation.

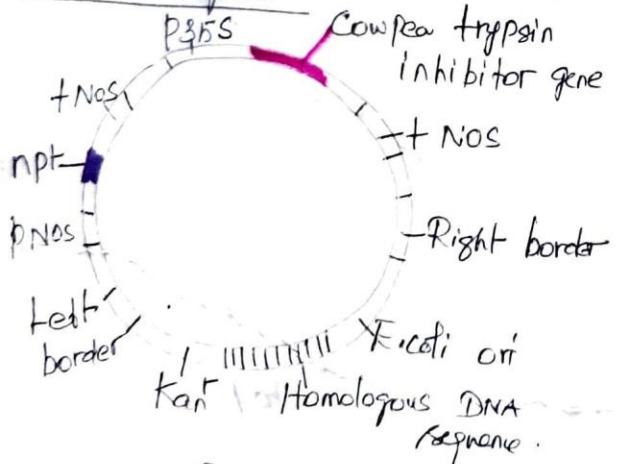
40 P Nos - Promoter from nopaline Synthase gene.

↗ Combination of low dose of chemical insecticide & ↘ Protection of B.t toxins by the plants gave good Protection in some cases.

To ↑ the level of expression of B.t toxin gene, an isolated insecticidal toxin gene was modified by site-directed mutagenesis to change any DNA Sequences that might inhibit efficient transcription (or) translation in a plant host (Partially modified gene) — 10-fold ↑ in the level of expression was observed, i

In the Second attempt, a fully modified version of the insecticidal toxin gene was designed & chemically Synthesized. This fully modified gene Contained codons more commonly used by plants. It was also modified to eliminate any potential mRNA Secondary Structure (or) chance plant Poly adenylation Sequences, which might decrease gene expression. — 100 fold ↑ in the level of expression of B.t toxin gene was observed.

Binary cloning vector carrying a Cowpea trypsin inhibitor gene



kan^r - Kanamycin resistance gene.

By combining B.t toxin gene & P.I gene → 20-fold ↑ in the insecticidal activity was observed than that of the B.t toxin gene alone.

(fusion Proteins)

Development of virus resistance in Transgenic plants:

Plant viruses cause considerable crop damage & significantly reduce yields; and lowered Product quality. Chemical methods that are available to control other types of plant pathogens, cannot be used to control plant viruses since viruses are intracellular, Obligate parasites.

Several safe agricultural practices currently used to limit virus diseases are (i) using virus-free seeds; (ii) Controlling insect vectors that spread plant viruses; (iii) Controlling weed species that serve as alternate hosts for viruses (iv) Using plant cultivars resistance to viruses.

Engineering virus resistance into crop plants is a prime area in crop biotechnology. Several approaches have been taken to introduce virus resistance into desired crop plants: < Host-encoded resistance / Resistance using virus-encoded genes.

Host encoded resistance: Resistant genes are isolated from resistant plants & introducing them into susceptible crop plants. Major limitation is: All the resistance genes have so far been characterised genetically and not at the molecular level.

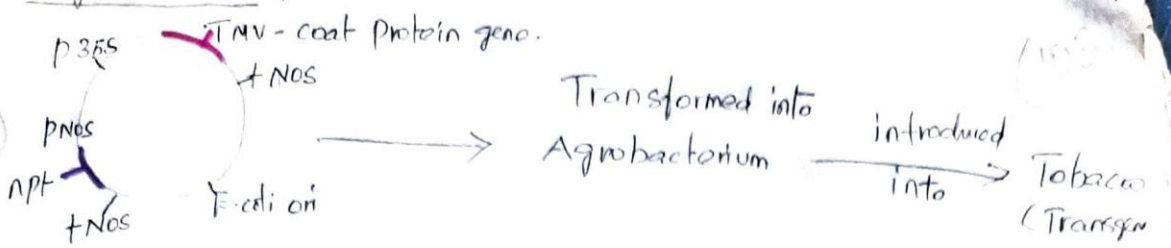
Resistance using virus-encoded genes: Pre-infecting the plants ~~c~~ mild virus strains (Cross-protection) - has been successfully used in the protection of tomatoes against Tobacco mosaic virus (TMV) and citrus trees against Tristeza virus.

Transgenic plants are generated ~~c~~ Produce components of the virus that confer cross protection without causing viral disease.

(i) Introduction of Virus coat protein gene:

TMV coat protein severely inhibited the translation of TMV RNA in vitro. Taking a clue from this, a chimaeric gene was constructed & the vector containing TMV coat protein gene was introduced into transgenic plants through

Agrobacterium.



P35S - is a constitutive promoter; i.e. expressed all the time & is a promoter from the genome of Cauliflower mosaic virus. Transgnic plants are become resistant to TMV infection. Following this remarkable success, coat-protein mediated protection has been successfully engineered for over 20 different viruses. for eg; Alfalfa mosaic virus (AlMV); Potato virus X; Potato virus Y; tobacco streak virus; tobacco rattle virus. This approach is very successful for viruses c single-stranded RNA genomes & is not successful in the case of double stranded & single stranded DNA viruses. Functional coat protein is not essential for conferring protection (Coat protein genes with deletions (or) mutations that affect coat protein function also confer resistance).

Vaccination with viral Coat-protein genes

Using this approach, researchers have developed virus-resistant transgenic tobacco, alfalfa, tomato & Potato plants. (+) → Page No. 104

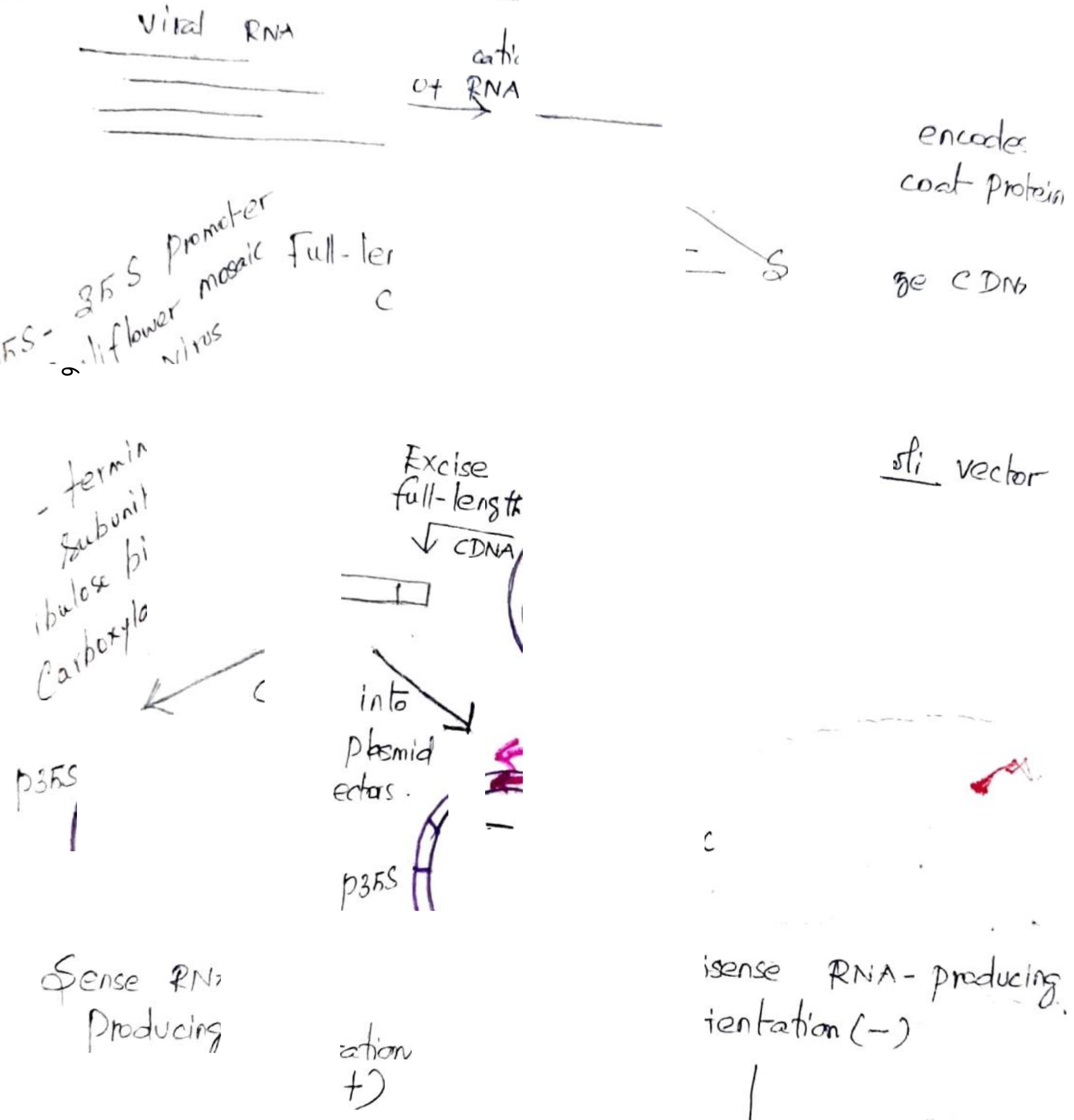
(ii) Antisense RNA: Using genetic engineering the complementary DNA strand of a gene sequence can be inserted in reverse orientation (3' → 5' as opposed to 5' → 3') into a vector under control of a suitable promoter. Such a complementary sequence in reverse orientation is termed as an Antisense gene. If such an antisense gene is expressed in a cell, the mRNA transcribed from it will also be antisense and will be complementary to the mRNA transcribed from the normal gene. The normal mRNA & antisense mRNA may hybridise (bind together) & the translation of the normal mRNA may be blocked (Ribosomes Cannot translate mRNA in hybridised form). Thus Engineering an antisense gene into a cell may result

seems hold promise for DNA viruses ier's replication & transcription takes place the nucleus g- refer xt page

in formation of antisense mRNA and blocking of gene expression (10,)

(ii) Viral antisense genes can be introduced into plants. Such an se mRNAs may block replication of the viruses. Antisense RNA approach was initially attempted for single-stranded RNA viruses.

Procedure for in roduct ... us coat Protein gene into plan



introdu d into tobacco plants ... d into ... o Plants

Result: Protect from vira particle accumu ion & did not show Symptoms of ral infection regard of heth r the inoculum f the cha enge Virus high or low. ... cted only ... Concentration ... challenge ... n the Inoculum

Steps: 1. Isolation of RNA 4.
2. In vitro enzymatic conversion of RNA 4 into double (44) stranded cDNA.
3. Insertion of full-length cDNA sequences into cloning vectors in both orientations ⊆ are under the control of p35S & t RBC.
4. Formation of separate transgenic plants carrying the cDNA sequence in one of the two possible orientations.

iii - Replicase — 54 kDa protein (a part of the replicase enzyme of TNV) introduced into the transgenic tobacco plants ⊆ conferred a very high level of resistance ~~in~~ tobacco against TNV.

iv - Movement Protein — Transgenic tobacco plants expressing a mutated 30 kDa movement protein showed a reduction in the final yield of infective TNV particles in infected plants. [A wild type 30 kDa movement protein of TNV binds to TNV-RNA & some host proteins & enables the ribonucleoprotein complex to pass through the plasmodesmata, thus mediating cell to cell spread of virus.]. It was proposed that the mutated (defective) 30 kDa protein of the transgenic plants competes ∈ the wild-type TNV-encoded 30 kDa protein and thus lowered the spread of the virus. The movement protein strategy seems to work for the single-stranded DNA viruses (Gemini viruses). A recombinant movement protein having parts from tomato golden mosaic virus & African cassava mosaic virus has been shown to severely interfere with the spread of both viruses.

v - Transmission Protein — Insect vectors spread plant viruses from one plant to another. In the case of aphid-transmitted CaMV, aphid transmission factor (protein) (helper component) bind ∈ the insect spread protein of virus & spread diseases from one plant to another. A mutant helper component was introduced into transgenic plants. In transgenic plants mutant helper component competed with the normal aphid protein for binding & thereby prevent the spread of insect-transmitted viruses.

vi - Disease attenuation with satellite RNA:

Satellite RNAs infect plants only with the help of

(DI) helper viruses. Satellite RNAs are encapsulated together with the respective helper viruses. Satellite RNAs either ↑ the severity of the symptoms caused by the helper virus (or) attenuate the symptoms caused by the helper virus. The latter property (attenuation) of Satellite RNAs has been used in biological control of spread of certain viruses.

For eg, when cucumber mosaic virus (CMV) infects pepper plants, severe symptom appear. However, when CMV is coinoculated with a Satellite RNA, the disease symptoms are attenuated & the yield of the plants is higher. Drawback of this approach - first infection of the Satellite RNA with a mild virus causes a penalty in yield. Additionally a mutation in the host helper virus may convert a mild strain into a virulent strain & may lead to the spread of the virulent strain. One way to avoid the need to use a helper virus is to introduce the DNA sequence corresponding to Satellite RNA into plants such that the symptom-attenuating Satellite RNA is expressed in transgenic plants. Transgenic plants expressed the Satellite RNA & this conferred protection against CMV and tomato asperny virus (TAV). Satellite RNA expressed in transgenic plants competed with the helper virus (CMV) for the limiting quantities of replicase & thereby reduced the replication of the infective virus.

Disadvan: Satellite RNAs that attenuate symptoms in one crop may cause severe disease in another crop plant; Satellite RNAs mutate very rapidly; recombination between Satellite RNAs have been observed.

Defective interfering DNA (DI) DI DNAs / RNAs are very rare in plants. Like Satellite RNAs, DI nucleic acids can intensify (or) ameliorate the symptoms of their respective parental viruses.

African Cassava Mosaic viruses, a gemini viruses have two ss- DNA genomes (A & B). (ACMV)

A Subgenomic 'B' (DI) component of ACNV was engineered into tobacco plants & are susceptible to ~~Ca~~ ACNV. The DI of ACNV interfered with the replication of both A- and B- DNAs in the transgenic tobacco plants & ameliorated the symptoms of virus infection.

(46)

Ribozymes] are small RNA molecules, derived from the satellite RNA of tobacco ringspot virus (TRSV) (or) certain viroids & viroid-like satellite RNAs, which promote catalytic cleavage of RNA. Such ribozyme sequences can be incorporated into the genomes of mild viruses such that ribozyme-containing sub-genomic RNA active against a severe virus [Cross-protection].

Resistance using animal genes] Genes for mouse monoclonal antibodies engineered into plants - functional antibodies were assembled in transgenic plants. (Plantibodies). Attempts are made to introduce genes for plantibodies against viral proteins such as coat proteins & replicase, so that multiplication of viruses within plant cell can be limited. (Using ~~anti~~ animal immune mechanism to confer immunity to plants).

(X) Antisense RNA approach: Tomato Golden mosaic virus (TGMV) replicase-coding sequence was cloned in its antisense orientation under the control of CaMV 35S Promoter and introduced into tobacco plants using Agrobacterium. Transgenic plants that expressed the antisense RNA of TGMV replicase showed resistance to TGMV.

Drawbacks: (i) RNA viruses are replicated in cytoplasm. high level of genome-sense mRNA thus necessitating ch higher concentrations of antisense mRNA;
ssociation of sense mRNA with proteins at all stages.

Transgenic plant:

Engineering plants for Disease resistance:

Plant diseases are due to either viruses (or) Fungal pathogens (or) Bacterial pathogens.

Virus-resistant Transgenic plants:

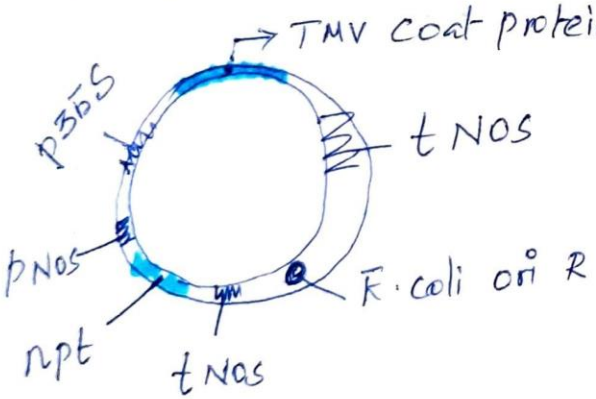
Plant viruses cause massive crop damage and reduce the quality and yield of crop plants. Viruses cannot be control by chemical methods since they are intracellular obligate parasites.

Engineering virus resistance into crop plants is a prime area in crop biotechnology.

Resistance using Viral genes:

Transgenic plants are regenerated by introducing viral genes: Cross protection.

Introduction of viral Coat-protein gene:



p35s Promoter: Constitutive Promoter; expressed all the time.

npt: plant selectable marker gene.

↓

Transformed into Agrobacterium & then introduced into the Tobacco plant.

↓

Transgenic plant with TMV Coat protein gene

↓

Become resistant to 20 different types of plant-viruses.

e is introduced into to
hydrolysis of Chiti

Bacterial pathogen resistant Crops:

The genes introduced so far to confer resistance against bacterial pathogens are

* Antibacterial magainins
* Cecropins
* Bacteriophage T_4 Lysozyme
* Thionins (α- thionin gene).
* Toxin - inactivating enzyme
* H_2O_2 - generating gene.
* Tabtoxin - specific acetyltransferase
* Phaseolotoxin
* OCTase gene.
* Tomato Cf-9 gene.
* Tobacco N-gene
* Arabidopsis RPS 2 gene.
* Tomato Pto gene.

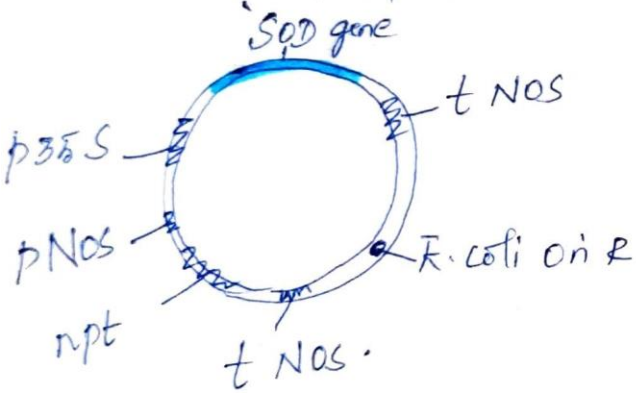
Development of stress-resistant plants:

Plants have to evolve physiological strategies to cope with stresses. During stress, at the molecular level, Oxygen radicals are produced inside the cells, which are highly toxic to cells. Hence, there is a need to create Transgenic plants, that are able to tolerate increased levels of Oxygen radicals; and thereby the plants can withstand various forms of environmental stress.

The mechanism

Superoxide Anion / Oxygen radicals $\xrightarrow{\text{'SOD'}}$ Hydrogen peroxide $\xrightarrow{\text{Peroxidase / Catalase}}$ $O_2 + H_2O$

Transgenic plant with 'SOD' gene



Introduced through Agrobacterium → Transgenic plants → Synthesize SOD & also acquire tolerance to Oxygen-radical damage; Caused by Light stress, heat, drought, UV-stress.

SOD - Superoxide Dismutase

ible

le Vaccine.

an System.

F & Used Vi

infectious

in

stable

lly an

vaccin

inactivated form of the

antigenic material present vaccine bacterial

infection.

stimulates the System

Specific vaccines are produced in plants as a result of transient (or) stable expression of foreign genes.

Genes encoding antigens of bacterial and viral pathogens can be expressed in plants. Transgenic potato tubers expressing a bacterial antigen stimulated humoral & mucosal immune responses when they were provided as food.

Plants could be a useful system for producing vaccines, because large amounts of antigen could be produced at a low cost, instead of sophisticated, expensive, cell-culture based expression systems.

* Plants are used as vehicles to produce vaccines.

Strategies for Production of Vaccines in plant tissues

Antigen from Pathogenic organism (virus, bacteria, Parasite)

Stable

Str. gene

Structural gene.

Direct

Particle gun bombardment

Indirect method

Plant Transformation vector for foreign gene expression

Agrobacterium mediated genetic transformation and regeneration of Transgenic plants

Stable integration of gene into nuclear genome.

Extraction of foreign antigens

feeding of edible plant tissue

Immunogenicity studies

Transient (plant viral vectors)

DNA fragment encoding B-cell & T-cell antigenic epitope

Fusion Protein

Epitope + virus Coat protein

Recombinant virus.

Infecting plant with recombinant viruses.

Transient expression of foreign Antigen.

Feeding of edible part

Isolation of Chimeric Viral parties from leaf tissues

Cowpea Mosaic virus

TMV

TMV/CPMV Capsid protein

Disease	Causative Organism	Antigenic epitope	Strategy	Method	Vector
Food poisoning	Streptococcus :S. mutans	SpaA	Stable	Indirect	disarmed Agrobacterium Ti-plasmid
Hepatitis	Virus	HBs Ag	"	"	"
~~Che~~ Diarrhea	Vibrio cholerae	Cholera toxin B Subunit	"	"	"
~~E. coli~~ Diarrhea	E. coli	Heat labile enterotoxin B Subunit	"	"	"
Malaria	Fusion protein strategy Sporozoites (parasite)	epitopes derived from malarial sporozoites	Transient	"	TMV Capsid Protein
Immune Contraception	Mammalian Oocytes	Zona pellucida ZP3 protein	"	"	TMV Capsid protein
Foot & Mouth disease	"	Virus epitope	"	"	~~Cauli~~ Cowpea Mosaic Virus Capsid protein
Sexually transmitted	Animals	epitopes derived from human	"	"	

Stable

Transient

Drawback /challenges

To ↑ the oral
imm
anti
↑
of
Stab
Prote
Larv
tiss

Allergy
tolerance
antigens must be
addressed

Difficult/less Easy to initiate Genetic Transformation Studies

Greater yield

Recovery of virus-li
Particles from leaf
extracts is easy,
simple, possible
Purification of virus - simpler

Fusion-protein strate
may be a better strat
because is foreign
proteins which are h
immunogenic

* Thermostability

Golden Rice.

riginated in Switzerland
tenoid Biosynthesi / Provitam A
duction in rice grains

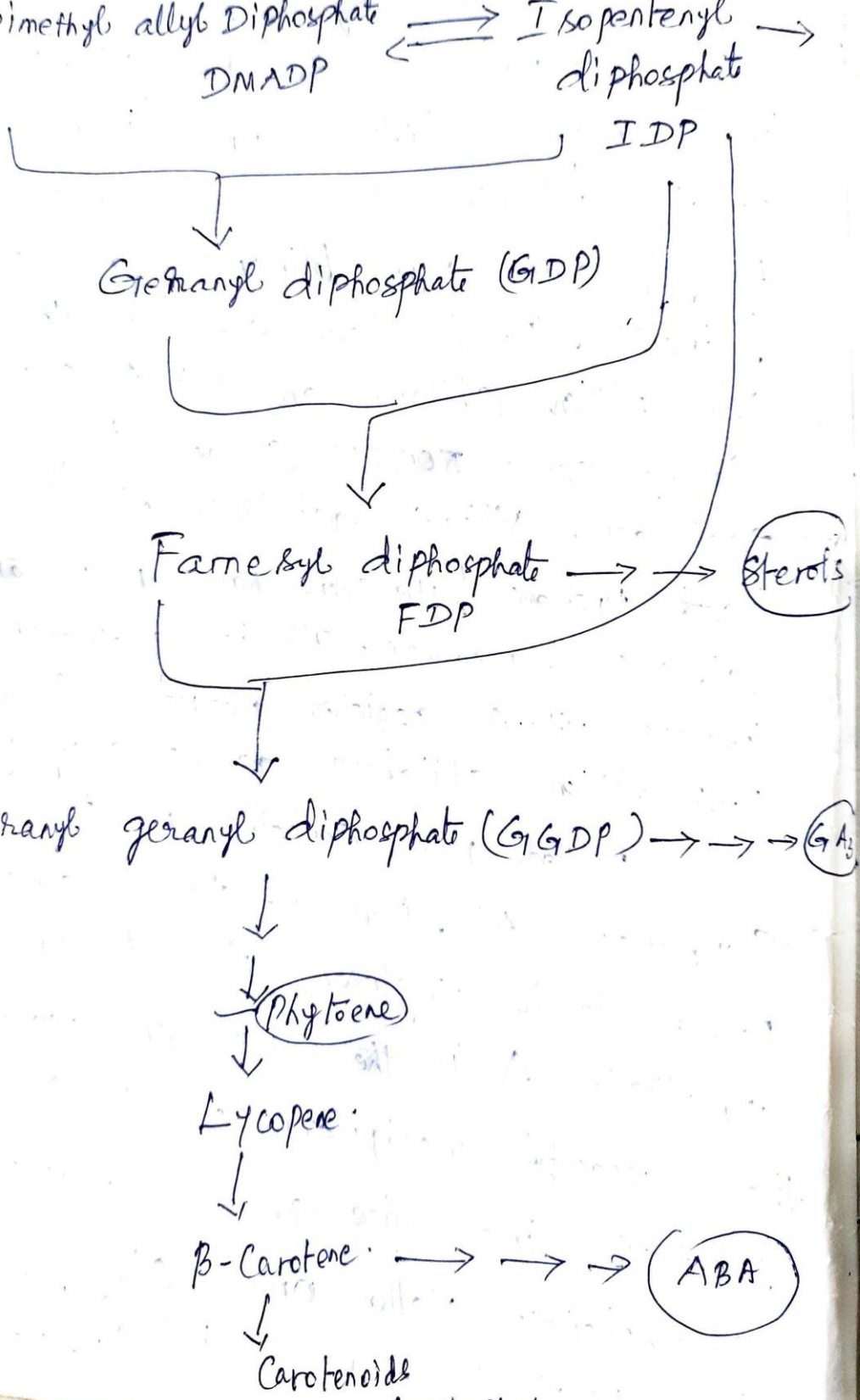
Rice is the most important food crop in the world & is eaten by 3.8 billion People in most of the regions, rice forms a staple component of the diet, Vit. A defic is a major nutritional problem. Deficiency of this vitamine can cause symptoms ranging from night blindness to total blindness. It has been estimated around 4 million children are Vit-A deficient, using about 500,000 Children to go blind each year. Vit. A deficiency lead to other health problems like diarrhoea, respiratory diseases & measles. It is estimated that improved vit-A ~~deficient~~ nutrition could prevent 2 million Childhood deaths every year.

Milled rice contains no β-Carotene Provitamin A, . One of the Solutions for this problem is to engineer rice to produce provitamin A in the rice endosperm. Since Successful manipulation of β-Carotene pathes in the rice grains gives the characteristic yellow/orange Colour, rice which is genetically enriched in Provitamin A'

has been described as Golden rice

Biosynthetic Pathway of Provitamin A

The synthesis of Lycopene & β-Carotene originates from the isoprenoid pathway.



Biosynthetic pathway of Provitamin A is a continuation of the Lycopene pathway. Immature rice endosperm is capable of synthesizing GGDP, but subsequent stages of the pathway are not expressed in this tissue.

Creation -

Phytoene synthase gene (psy gene) from daffodil + rice-endosperm specific promotor

↓

Transgenic plants (Phytoene could be synthesized from GGDP in the rice grain).

However, 2 subsequent steps are required to convert Phytoene to β-carotene.

Phytoene desaturase & Carotene desaturase } → to introduce the double bonds to form Lycopene

Lycopene β-cyclase → to form rings in β-carotene

A Bacterial Carotene desaturase gene was found to be capable of introducing all

four double bonds.

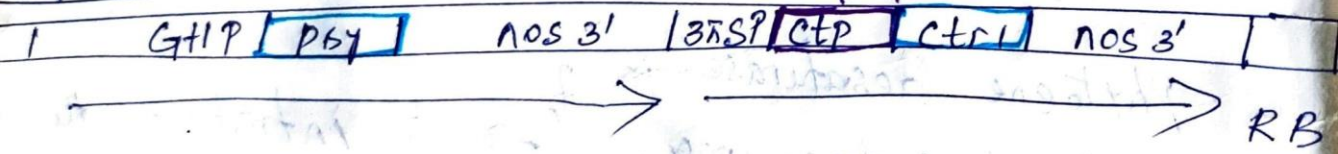
Manipulation of Golden rice requires the introduction of three genes

(i) Phytoene Synthase (psy) (synthesis of Phytoene)
(ii) Carotene desaturase (crtI) (introduce double bonds to lycopene)
(iii) Lycopene β-cyclase (lcy) — form rings in β-carotene

Gene Constructs for the production of Golden rice:

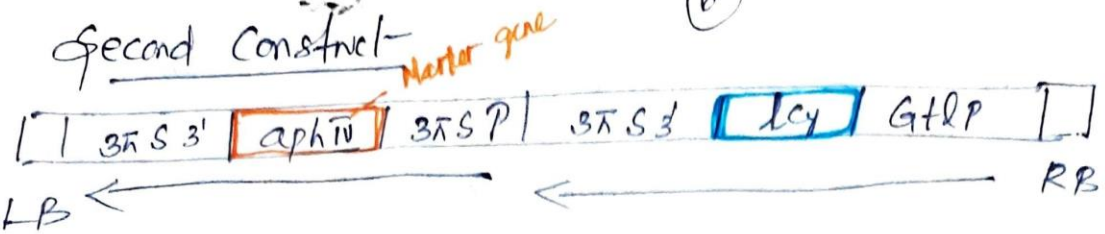
Two independent Constructs were made for the most successful prodn. of Golden rice. The first one contains a 'daffodil' Phytoene Synthase (Psy) gene fused to a rice glutelin promoter with a bacterial Carotene desaturase gene (crtI) driven by the 35S promoter. ↳ Bacteria Erwinia (source).

First Construct:



The second construct contains the kanamycin-resistance aph IV selectable marker with the lycopene β-cyclase gene (lcy) daffodil also fused to an endosperm-specific rice glutelin promoter.

Second Construct-



The first construct was inserted into the vector pZPsC. Both the enzymes were targetted to the plastid (the site of GGDP Synthesis), the psy gene by its own transit peptide & the Ctr I gene by fusion to a pea rbc S transit peptide sequence.

The Lycopene β-cyclase gene from daffodil with a functional transit peptide was inserted into a vector pZLcyH under the control of the rice endosperm-specific glutelin promoter, along with selectable marker gene (aph IV).

Rice immature embryos were inoculated with a mixture of Agrobacterium LBA4404 containing each of the two plasmids. A total of 60 hydromycin-resistant regenerated lines were selected at random, all of which were shown to contain the pZLcyH (2nd Construct). Of these, 12 were found to contain the pZPsC cassette (first Construct).

Most of the seeds from these transgnic lines containing both constructs were found to be yellow, indicating Carotenoid synthesis. A highest-producing line was found to contain 1.6 µg β-Carotene / g endosperm.

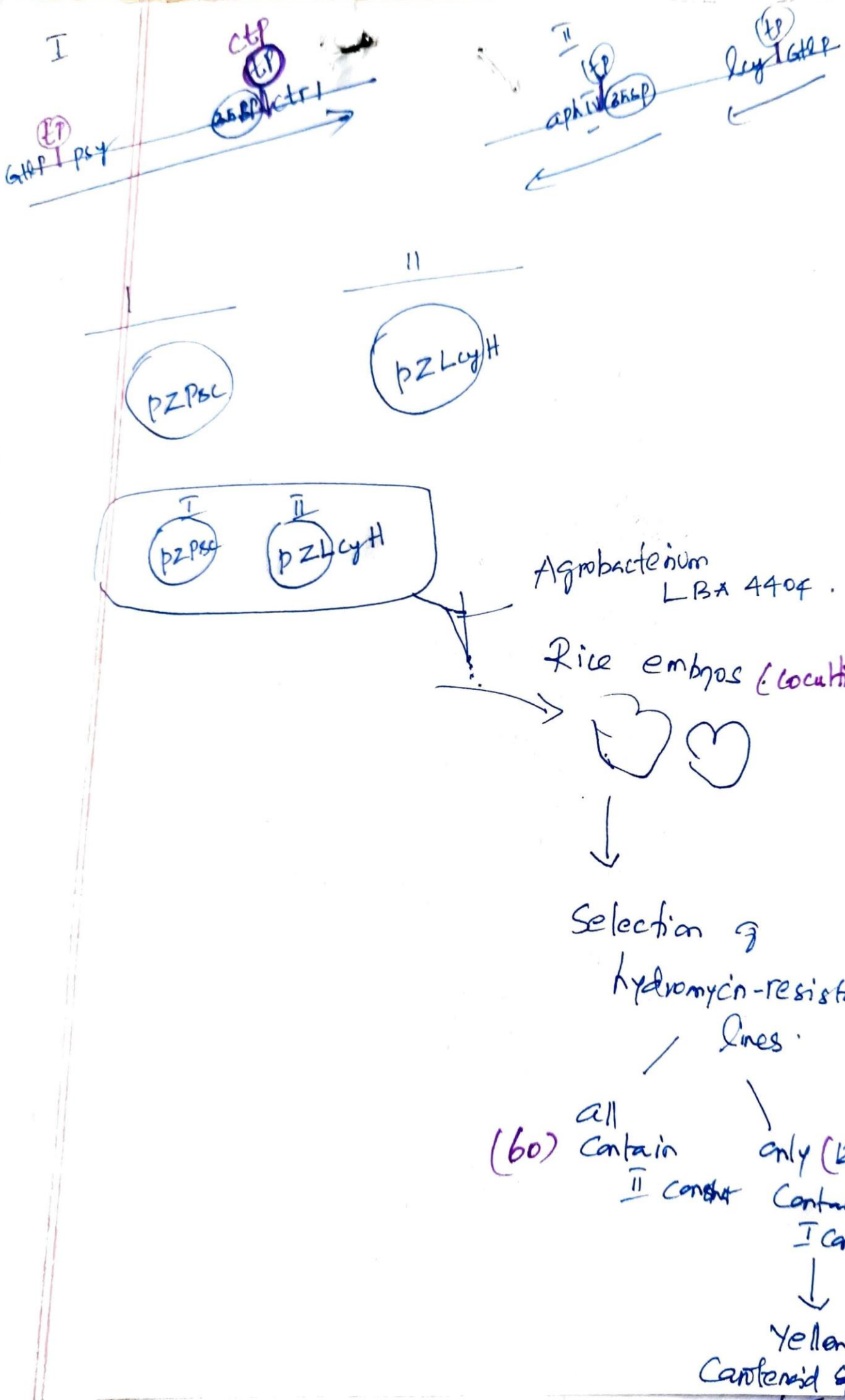
Drawback:

1) The genetic manipulation of the multistep pathway, via the insertion of 8 genes into rice, requires several years of intensive work.

2) The homozygous line should produce atleast 8 µg/g provitamin A, which corresponds to 100 µg retinol equivalents daily intake, assuming 300 g rice consumed/day. But the prodn. is less (insufficient) in Golden rice to provide the complete dietary requirement.

3) Poor Public acceptance.

4). The next challenges faced by the developers of Golden rice are (i) IPR, Patenting & Technology Transfer


I
II
pZPSC
pZLcyH
Agrobacteium
LBA 4404.
Rice embryos
Co cultivation
Selection of
hydromycin-resistant
lines.
(60) all
Contain
II Constr
only (12)
Contain
I Constract
Yellow
Carotenoid syn.

: position to Golden rice.

1. Benefits only rich.
2. It will be given free of charge to farmers (not possible).
3. It cannot be re-sown. (due to Terminator Tech.) Farmers have to depend everytime to Companies for seeds
4. Only Biotech. Industry gets benefits.
5. negative side-effects may be there.
6. It cannot be grown without any additional input.
7. It may reduce agricultural biodiversity.
8. " " " natural "
9. It may affect the ecosystem.
10. Always have a risk to Consumer health.
11. Golden rice is not meeting the Complete dietary requirement for Vit. A.

Direct transformation methods:

1. $CaCl_2$ & PEG mediated transformation
2. liposome mediated transformation
3. Electroporation
4. Transformation using microprojectiles.

The first 2 methods are applicable only to protoplast, the microprojectile bombardment method can be used on intact tissues. Electroporation & microprojectile bombardment methods have been found to be most effective direct transformation methods.

Electroporation: Protoplasts of large number of monocots & dicots have been successfully transformed by ~~ect~~ electroporation. Electroporation is a process whereby electrical pulses of high field strength are used to reversibly permeabilise cell membranes to facilitate uptake of large molecules, including DNA. Direct transformation vectors are different from the Ti plasmid vectors in that they do not have border repeat sequences. Direct transformation vectors generally have a plant transformation marker & a reporter

gene such as luciferase, chloramphenicol acetyl transferase (CAT) or β-glucuronidase. Advantages: Convenient; low toxicity & equal efficiency in dicots & Monocots. Disadvantage - is that it is effective only in protoplasts, from ≤ intact plantlets need to be regenerated; technically difficult in many crop plant species; risk of somaclonal variations because plts are regenerated through calli.

High voltage produce large pores on the membrane, through ≤ the DNA (direct transformation vector) in the medium diffuses spontaneously.

Particle-Gun bombardment: A major problem of introducing foreign DNA into the protoplast is the risk of somaclonal variations, since the plts are regenerated from microcalli. It is advantageous if intact plant tissues can be transformed & plants regenerated from transformed tissues. Bombardment of DNA-coated microprojectiles using particle gun allows the transformation of intact cells of embryos, shoot apices and other tissues.

The technology involves coating gold/tungsten spheres (microprojectiles) with DNA (foreign genes) and spreading the particles on the surface of a mobile plate (or) plastic/nylon bullet (macroprojectile). Under partial vacuum, the macroprojectile is fired against a retaining plate ~~or~~ either by an explosive discharge (ballistic device) (or) by using shock waves initiated by a high voltage electric discharge (electrostatic device). The macroprojectiles are retained by the retaining plate, while the microprojectiles (1-3 μm in diameter) pass through due to their small size and higher density & penetrate the target plant tissue. By this way calli, embryo, pollen, epidermis, shoot apices are getting transformed. The major advantage of this technique is its ability to introduce genes into intact tissues thereby obviating the need for protoplast isolation and plantlet regeneration from protoplasts. Limitations: high cost of equipment; high level of variations in transformation efficiencies; low frequency of achieving stable transformation.

Transferring genes into cells

Watson - p. 214 - 220

Organelle transformation turned out to be very difficult ; either by Agrobacterium (or) by PEG-mediated transformation / electroporation. Successful Chloroplast transformation was first achieved in the green alga Chlamydomonas. Chloroplast transformation by this technique occurs at a very low frequency when compared with the transformation of nuclear genomes by this same method. Transformation of mitochondrial genome is also being attempted using this technique.

Chemical treatment (of protoplasts): with PEG at high pH, with CaCl2 / MgCl2 leads to uptake of foreign DNA.

Microinjection of DNA into cells & protoplasts: by using capillary micropipettes - the DNA is directly delivered into specific cell compartments. Most direct & most precise method of DNA delivery.

Macroinjection of DNA into plants: Plasmid DNA containing a selectable antibiotic resistance gene is suspended in buffered medium & injected with a syringe needle directly into the lumen of the developing inflorescence. Microspores at a specific time are capable of taking up DNA.

Electrical and laser modification of cell membrane permeability: Like electroporation. Lasers are used to puncture holes in plant cell walls and membranes, that facilitate the uptake of DNA.

DNA transfer via the growing pollen tube: plasmid DNA carrying a selectable antibiotic resistance gene is simply applied to the cut surface of the stigma sometime after pollination.

DNA uptake into imbibing zygotic embryos: Uptake of DNA by imbibing cereal & legume embryos. Application of solution containing plasmids to the exposed surface of an embryo, whose testa has been mechanically removed by grinding.

Fibre mediated DNA delivery to plant cells: Silicon carbide fibres coated with DNA have been used to deliver DNA to cells of

maize and tobacco in suspension culture.

Electrofusion: electric-field induced cell to cell fusion.

Liposome-mediated transformation: A major problem in introducing DNA directly into the protoplasts is the presence of enzymes that degrade DNA. So it needs a good protection for exogenous DNA, so that DNA makes a safe journey from external medium to the recipient nucleus & such protection will facilitate its stabilization in recipient cytoplasm. The use of liposome is a new innovation to facilitate the uptake of nucleic acid without any degradation. Liposomes are the liquid crystalline structure obtained when amphipatic lipids such as phospholipids are dispersed in water (or) aqueous salt solution. Each liposome is a bilayered completely enclosed sac-like vesicles. It is possible to enclose the nuclek acid within liposome & can readily transport through biological membrane of the protoplast. Liposomes can protect the enclosed nucleic acid from the degradation by the enzymes of the recipient protoplast.

Fusion between the bacterial spheroplasts & plant Protoplast: This technique has also been used as a method of introducing DNA into plant cell.

Pros con Genetic Engineered crops

ros positiv impa cons (N ati pects)

positive of Crops:

commercialization of transgenic plants

chin about million hectares and

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about tal ba co cul ation

the of china of

Trans ons an

Transgenic cco; already ed in chin the

propert

Res tan vi

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application

tan MV potato

chin ap d ales rege ion refine

wo kin gulations

— — anigens delaye fruit

ripening) transgen ato approv

commercial expl ation USA

→ World wide, there have been 100s of field trials with transgenic crops. over 38 different crop plants have been tested in field in 31 countries. over the past 10 years, regulations governing the release of transgenic plants have been developed in different countries. There will be a harmonized, equitable and responsible regulations, so that transgenic plants and their products can be transported between countries for research & commerce.

Negative aspects

→ Environmentalists have been concerned about the risk of new crop escaping from cultivation and displacing natural vegetation

→ Markers (Kanamycin^r, Herbicide markers) contained in the Genetically Engineered food stuff passed to bacteria in the human gult making bacteria resistant to Kanamycin & related antibiotics.

→ kan^r gene passed on to other living organisms resulted in ecosystem damage. It is possible to remove kan^r gene? by co-clone, 'cry' gene. 'cry' gene (bacteriophage P_1) which catalyzes recombination event that excise DNA sequences carrying kan^r gene.

cry gene itself hazard u

New gene combination ht harm the en ronment

Plants exchi fed on variatio + ce

plants regener | from le rot genetically

homogenou

ansformation on with vector sp sm

difficult.

Harm to non target organisms

plants disturb the existing ecological balan

pt vectors are derivati of pathogeni microorgani

or the sources for disease transmission

the field

GEP could resul the displacemen res an

plants

There was wide concern about the competitiveness

plants wi|

common concern of pub to create ex pathogeni

new pests new weeds E

ccidental transfer genes c weeds &

Species are ely related

transgenes for herbi stance, sect disease

istance may transferred to other crop plan by ros pollination

The use of GE *pseudomonas* *syringae* against fros damage

→ during developing plts for virus resistance, recombinant viruses were made. They may ↑ the severity of diseases.

→ GE plants disrupt the ecological balance.

→ Not all the plants regenerated from protoplast / single cells.

→ cost of prodn. of transgenic plts is high.

→ Differences in release & commercialization criteria could inhibit International Trade.

→ There should be labelling plant products containing genes from humans & animals. i.e: ethically sensitive genes

→ There is no equility of fees being charged by regulation agencies. Denmark - 28,000 us

USA - 0

Such restrictions discourage scientists from developing countries; inhibit innovative research in some countries.

→ There is still uncertainly in some parts of the world about the acceptability of antibiotic resistant genes in food products.