Genetic engineering for biotic stress tolerance in plants

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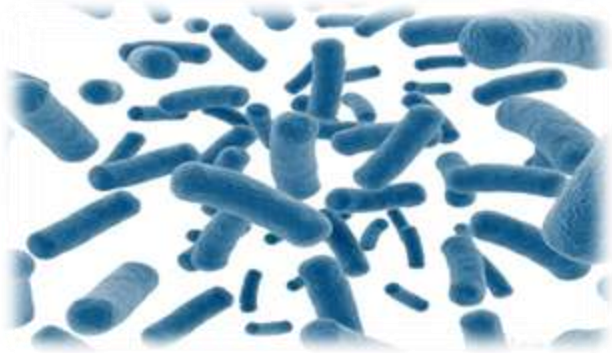
Biotic stresses in plants

- Weeds
- Insects
- Fungi
- Bacteria
- Virus



Genetic engineering for herbicide resistance

Genetic engineering for herbicide resistance

- First major achievement from genetic engineering in plants
- Glyphosate, glufosinate, sulfonylureas etc. have been successfully developed
- Commercially cultivated in USA and other countries

Contd.

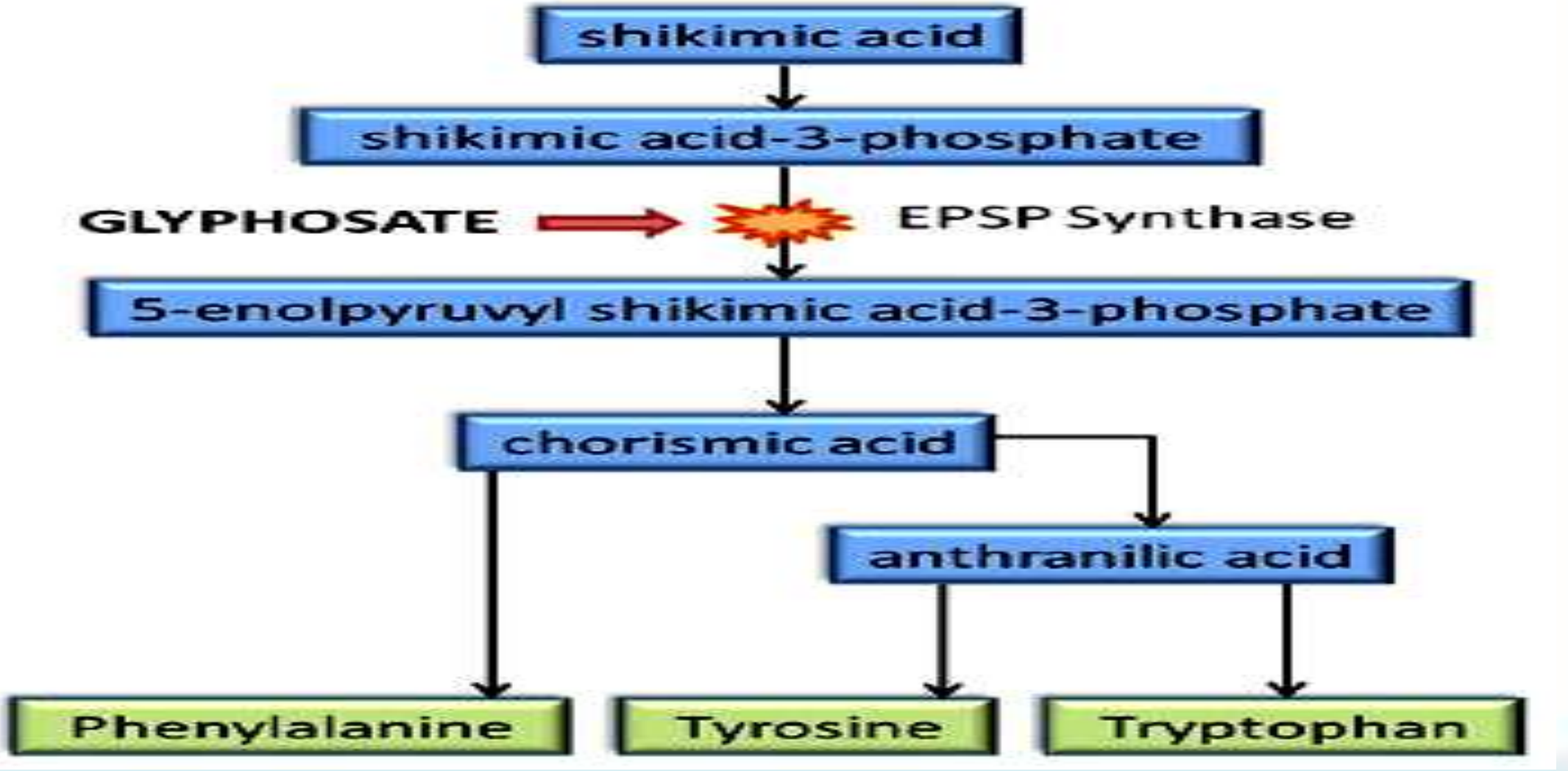
- **Three approaches:**

➢ Incorporating Insensitive EPSPS

➢ Over expression of target molecules

➢ Incorporating molecules which can degrade the herbicides

Glyphosate action in plants



Strategies for Glyphosate resistance

- ✓ Overproduction of EPSPS enzyme
- ✓ Encode an EPSPS enzyme that is tolerant to glyphosate
- ✓ Produce an enzyme that inactivates glyphosate
- ✓ Use a combination of 2 and 3

Contd.

Organism	Source of glyphosate tolerant strain/EPSPS enzyme	Mechanism of glyphosate tolerance
Tobacco	Cell lines selected for resistance to glyphosate	Overproduction of EPSPS due to gene amplification
Carrot	Cell lines selected for glyphosate resistance	Overproduction of EPSPS due to gene amplification
Salmonella typhimurium	Mutant strain	Tolerance of EPSPS to glyphosate (pro at position 101 replaced by Ser)
E. coli	Mutant strain	Tolerance of EPSPS to glyphosate (Ala at position 96 replaced by Gly)
Agrobacterium sp.	Natural strain CP4 (tolerant to glyphosate)	High tolerance to EPSPS to glyphosate and tight binding to PEP

Contd.

Organism	Source of glyphosate tolerant strain/EPSPS enzyme	Mechanism of glyphosate tolerance
E. coli	Site-directed mutagenesis	Tolerance to EPSPS to glyphosate due to amino acid replacement
Arabidopsis thaliana	Site-directed mutagenesis	Tolerance to EPSPS to glyphosate due to amino acid replacement
Petunia	Site-directed mutagenesis	Tolerance to EPSPS to glyphosate due to amino acid replacement
Achromobacter sp. Strain LBAA	Natural isolate	Inactivation of glyphosate by glyphosate oxidoreductase enzyme

Contd.

- *P. hybrida* EPSPS has been overexpressed in crops
- But increased level of glyphosate tolerance was not enough to protect these transgenics from herbicide under field conditions

Contd.

- Mutants of *S.typhimurium* and *E.coli* showed tolerance to glyphosate
- Several mutant forms of *E. Coli* and petunia EPSPS were expressed in Transgenic tobacco, soybean, canola, tomato
- Level of tolerance was inadequate for commercial use
- Because of mutant form of EPSPS has less affinity for PEP

Contd.

- Agrobacterium strain CP4 has glyphosate tolerant EPSPS enzyme
- Transferred into soybean
- Showed no visible injury up to 1.68 kg acid equivalent per ha glyphosate
- Expressed in cotton and other crops

Contd.

- Glyphosate is extensively and rapidly metabolised by GOX
- The gene encoding GOX isolated from *Achromobacter* sp. LBAA and expressed in transgenic plants
- Provided excellent tolerance to glyphosate in vegetative and reproductive stage
- Gene GOX has expressed in combination with *Agrobacterium* sp. CP4 EPSPS gene

Contd.

Before

After

Eg. RoundUp Ready Canola

Contd.

Gene for herbicide resistance	Source organism	Confers resistance to the herbicide	Mechanism of action
aroA	*Agrobacterium* sp. Strain CP4	Glyphosate	Encodes EPSPS enzyme that is sensitive to glyphosate
bar	*Streptomyces hygroscopicus*	Bialophos/Basta/Glufos inate	Acetylation of phosphinothricin
bxn	*Klebsiella ozaene*	bromoxynil	Converts herbicide into 3,5-dibromo-4-hydroxy benzoic acid
gox	Achromobacter sp. Strain LBAA	Glyphosate	Detoxifies glyphosate by oxidising it into amino athlyphosphonic acid and glyoxylate
Hra, csr1-1, C3 and ahas3r genes	Arabidopsis thaliana, N. tabaccum, yeast, E. coli	Sulfonylureas and imidazolinones	Encode acetoacetate synthase (ALS) that is insensitive to herbicides

Contd.

Classes of herbicide	Compound/ Herbicide	Transgene	Crops	Company
Glycine	Glyphosate (round up)	Maize resistance gene	Maize	Monsanto
Phosphonic acid	Phosphinothricin (basta) (Liberty)	Bar gene;pat detoxification	Maize, Rice, Wheat cotton, Potato	Bayer crop sci.
Sulphonyl urea	Chlorosulphurea	Mutant plant acetolactate synthase	Rice , Tomato	Dupont-pioneer hi-bred
S-triazine	Atrazine (lasso)	Mutant plant chl Psba gene	Soybean	Dupont
Phenoxy carboxylic acids	2,4-d	Monooxygenase detoxificaion	Maize , Cotton	Bayer crop sci.

Limitations

- Transgenic food product may allergy to human
- Affect the soil flora
- Developing super weeds
- Affect natural germplasm

Genetic engineering for insect resistance

Contd.

- **Approaches:**

➢ Incorporating delta endotoxin sequence from *Bacillus thuringiensis*

➢ Incorporating plant derived genes like lectins, proteinase inhibitors etc.

Bacillus thuringiensis

- *Bacillus thuringiensis* (Bt) is a Gram-positive, aerobic, sporulating bacterium
- Synthesizes crystalline proteins during sporulation
- Crystalline proteins are highly insecticidal at very low concentrations
- Crystalline structure of the inclusion is made up of protoxin subunits, called delta-endotoxins
- Proteins are non-toxic to mammals and other organisms

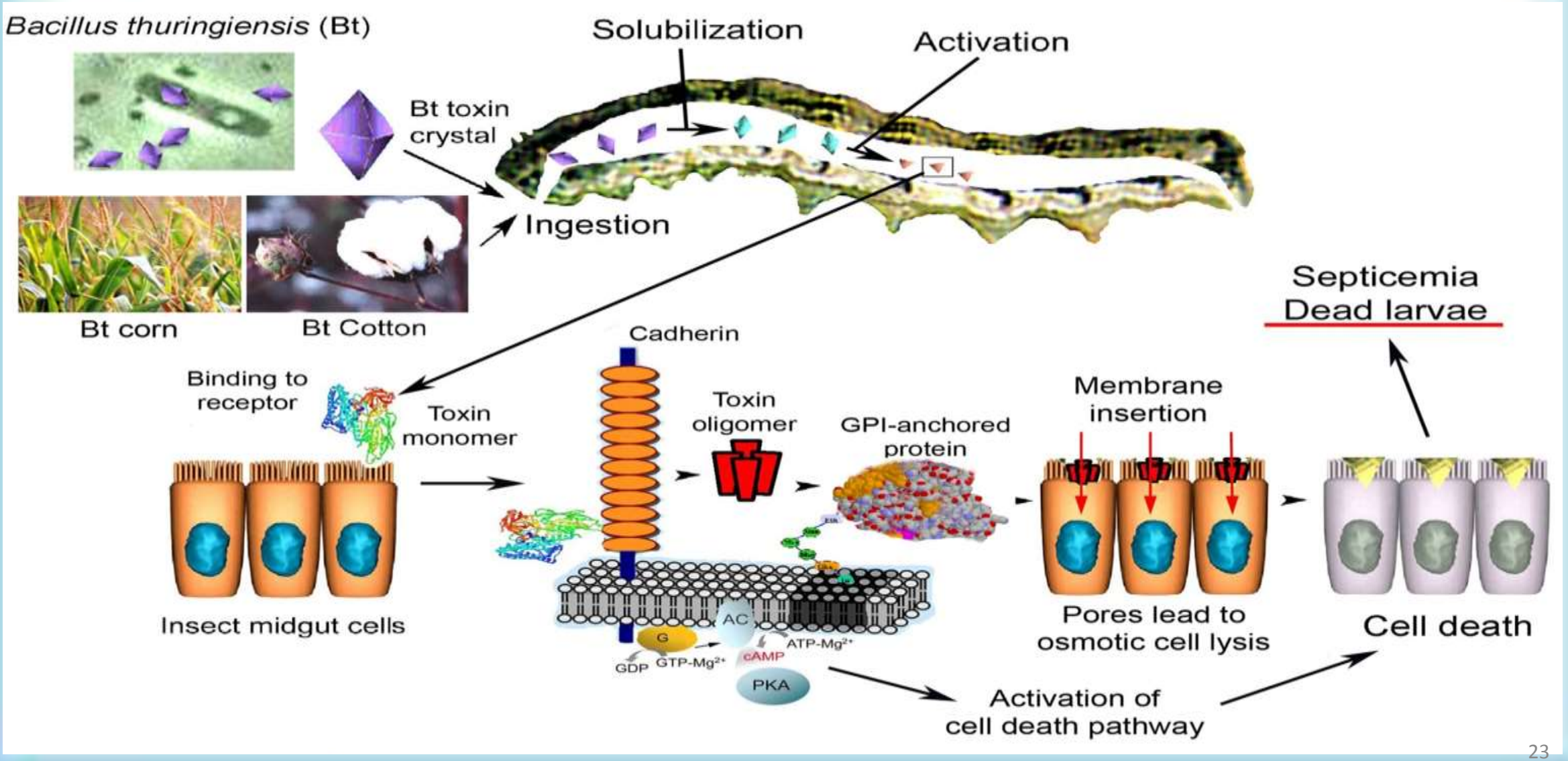
Contd.

- *cry* gene of *B. thuringiensis* produces protein, which forms crystalline inclusions in bacterial spores
- Responsible for insecticidal activities of bacterial strains
- Cry gene I to VI: based on insecticidal activity

Contd.

- Delta-endotoxins are solubilized in the insect midgut and are activated by gut proteases by a combination of alkaline pH (7.5 to 8.5)
- Cleave the protein into a smaller polypeptide, the toxin
- Toxin binds to the surface of epithelial cells in the midgut, inducing lesions that destroy the cells
- Lead to the death of the insect

Toxic action of cry proteins



Expression of cry genes in plants

- *crylA* genes transferred to tobacco, potato and tomato
- First report on response of transgenic tobacco plants to insect *Manduca sexta* and *Heliothis virescens*
- Field trials with transgenic tobacco has revealed plants are resistant to *M. sexta* and *H. virescens*
- Plants with moderate resistance in greenhouse proved highly resistant to insect attacks in field

Published field trials of Bt-expressing transgenic crops

Crop	Bt.	Target pest
Tobacco	Cry1A	Heliothines, *H. zea*
Potato	Cry1A Cry3A	Tuber moth Colorado potato beetle
Cotton	Cry1A	Pink bollworm
Maize	Cry1A	European cornborer *H. Zea* 23 Lepidoptera
Poplar	Cry1A	Gypsy moth

Limitations

- *cry* gene may fail to provide adequate pest control in field
- *H. zea* populations destroyed transgenic cotton expressing *cryIA(c)* gene
- Cotton crop was destroyed by *H. armigera* and *H. punctigera*

Contd

- **Possible causes:**

➢ Inadequate *cry* gene expression

➢ Reduced *cry* gene expression due to environment factors

➢ Presence of local *cry* protein resistant insect populations

➢ Development of resistance in insect pest due to inadequate management

Protease inhibitors

- Interfere with the digestive enzymes of the insect
- Results in the nutrient deprivation causing death of the insects
- According to their specificity, proteinase inhibitors (PIs) can be divided in four classes

➢ Serine protease inhibitors
➢ Cysteine protease inhibitors
➢ Aspartic and metallo-protease inhibitors
➢ Bifunctional alpha-Amylase1 protease inhibitors

Contd.

- Serine and cysteine-proteinase inhibitors inhibits mainly lepidopteran and coleopteran
- Most active inhibitor identified is cowpea trypsin inhibitor (CpTI)
- Transferred into at least ten other plant species
- Protein is an effective antimetabolite against a range of field and storage pests

Contd.

- CpTI-transformed tobacco, field-tested and caused significant larval mortality of cotton bollworm

- Serine proteinase inhibitors resulted in up to 100% mortality of first-instar cotton leaf worms when expressed in tobacco

alpha-Amylase inhibitors

- Insect larvae secrete a gut enzyme alpha- amylase to digest starch
- Blocking the activity of this enzyme by α-amylase inhibitor, the larvae can be starved and killed
- α-Amylase inhibitor gene isolated from bean has been successfully transferred and expressed in tobacco
- Provides resistance against Coleoptera

Lectins

- Lectins are carbohydrate-binding proteins found in many plant tissues
- Abundant in the seeds and storage tissues of some plant species
- Involves specific binding of lectin to glycoconjugates located in the midgut of the insect
- Tobacco plants expressing a pea lectin were shown to be toxic to the Lepidoptera *Heliothis virescens*

Insect chitinases

- Chitin is an insoluble structural polysaccharide that occurs in the exoskeletal and gut lining of insects
- It is believed to protect the insect against water loss and abrasive agents
- Larvae feeding on the transgenic plants exhibited several growth aberrations and died prematurely

Other insect-resistance genes from plants

- Tryptophan decarboxylase from periwinkle was expressed in tobacco
- Induced synthesis of tryptamine and tryptamine-based alkaloids
- Pupal emergence of whitefly decreased as a result of feeding on such plants

Genetic engineering for fugal and bacterial resistance

Contd.

- Hosts and pathogens show gene-for-gene relationship

- **Two types:**

➢ Incompatible reaction

➢ Compatible reaction

Incompatible reaction

- Found in biotrophic pathogens (obligate parasites)
- Smuts, rusts
- Alleles for resistance in host and those for avirulence in pathogen produce specific compounds, which recognise each other
- When these specific compounds interact, produce resistant response in host

Contd.

- Associated with hypersensitive reaction
- Triggered by certain unique molecules, called elicitors, of pathogen origin
- Elicitors are recognised by receptor-like molecules present in resistant plants
- Initiates several biochemical reactions

Contd.

- Produce active oxygen species
- Phytoalexin biosynthesis
- Cell wall reinforcement
- Release of hydrolytic enzymes/inhibitory poteins
- Results in rapid cell death

Compatible reaction

- Alleles for susceptibility in host and those for virulence in pathogen produce specific compounds, which interact with each other to produce susceptible response
- Lack of interaction between products of genes specifies resistance response in host
- Found in heterotrophic pathogens

Strategies for resistance

- Genes encoding insensitive target enzymes
- Genes specifying toxin inactivation
- Expression of antibacterial peptides
- Expression of bacterial lyzozymes
- Genes specifying programmed cell death
- RNAi targeting pathogen/parasitic genes

Contd.

- Expression of heterologous phytoalexins
- Genes encoding ribosome inactivating proteins
- Expression of heterologous thionins
- Ectopic expression of pathogenesis related proteins
- Ectopic expression of chitinase
- Modification of host proteins required for infection by pathogen

Genetic engineering for virus resistance

Contd.

- Coat protein gene
- cDNA of satellite RNA
- Defective viral genome
- Antisense RNA approach
- Ribozyme-mediated approach

Contd.

- Based on strategies on genes derived from pathogenic viruses themselves

- Pathogen-derived resistance: disease resistance generated by employing pathogen genes

Coat protein gene

- Transgenic plants having virus coat protein gene linked to strong promoter
- Tobacco, tomato, alfalfa, sugarbeet, potato etc.
- First plant: 1986: tobacco, coat protein from TMV strain U1
- Expression of virus coat protein gene: confers resistance to concerned virus and gives a measure of resistance to other related virus

Contd.

- Effectiveness of coat protein: affected by amount of coat protein produced in transgenic plants and by concentration of virus inoculum

- Resistance is due to blocking of process of uncoating of virus particles, which is necessary for viral genome replication as well as expression

GM released for commercial cultivation using CPMR

- Tomato resistant to TMV
- Tomato mosaic virus (ToMV) and cucumber mosaic virus (CMV)
- Cucumber resistant to CMV
- Squash resistant to zucchini yellow mosaic virus (ZYMV) watermelon mosaic virus (WMV2)

Contd.

- Cantaloupe resistant to ZYMV, WMV2 and CMV
- Potato resistant to PVX, potato virus Y (PVY) and potato leaf roll virus (PLRV)
- Papaya resistant to papaya ringspot virus, PRSV
- Pea early browning virus, potato virus Y and cucumber mosaic virus

Other viral genes

- Other than coat protein
- Done for potyviruses
- Genes used: potyvirus genome-linked proteins (*VPg*)
- VPg-protinases (*Nla*)
- RNA-dependent RNA polymerases (*Nlb*)
- Cylindrical inclusion proteins (*Cl*)
- Do not confer resistance to heterologus potyviruses (no broad-range protection)

cDNA of satellite RNA

- Some RNA virus have small RNA molecules: satellites
- Depend on viral genomes for their replication
- Not necessary for viral functions
- Satellites either decrease or increase severity of disease produced by virus carrying it

Contd.

- cDNA copies of satellites that reduce disease severity have been integrated into host genomes

- Expression of satellite has been shown to reduce disease symptoms as well as virus accumulation

Defective viral genome

- Defective or deleted genomes of some RNA and DNA viruses disrupt replication of complete genomes of those viruses with which they are associated

- African cassava mosaic virus (ACMV): two single stranded DNA molecules A- and B-DNAs

Contd.

- In addition, 50% deleted B-DNA I also found associated

- Tobacco plants containing deleted B-DNA integrated in their genomes showed reduced systemic spread when infected with ACMV

Antisense RNA approach

- Produced by inverting cDNA copy of mRNA with respect to promoter in an expression vector
- Yields full-length complementary copy of m RNA sequence
- Interact with mRNA molecules by base-pairing to form double stranded RNA
- Tobacco: CMV

Ribozyme mediated approach

- RNA molecules that exhibit enzyme activities
- Hybrid RNA consisting of tobacco ringspot virus (TobRV) satellite RNA endoribonuclease catalytic sequences linked to antisense RNA specific of specific genes against they are targeted

Contd.

- **Strategy consists of:**

➢ Producing ribozyme specific to part of target virus genome

➢ To produce cDNA of this ribozyme

➢ To integrate it into host plant genome

Development of RT-PCR based method for selection of potato virus Y in tobacco and potato

- Top four leaves of two-month old green house grown tobacco plants of Havana 425 variety were mechanically infected with PVY

- Progressing from basal to apical direction first leaf was harvested after 1 week
- Second leaf after 2 weeks
- Third leaf after 3 weeks
- Fourth leaf after 4 weeks of infection

Contd.

- Leaf was used for double antibody sandwich (DAS) ELISA test, NASH and RT-PCR
- Potato plants cv. *Kufri Jyoti* from an infected field were also collected
- Tubers were subjected to methods of PVY detection
- ELISA was found to be effective in detecting PVY in mechanically infected tobacco plants maintained in green house

Contd.

- Nucleic Acid Spot Hybridization (NASH) was found equally effective as ELISA in detecting PVY level in mechanically infected and green house maintained tobacco plants
- Cloned coat protein gene of PVY was used as a probe
- RT-PCR was found to be highly efficient method of detection

Contd.

- RT-PCR consists of RT reaction at 60^{o}C for 30 minute
- Initial denaturation: 94^{o}C for 1 minute
- Followed by 94 ^{o}C for 30 sec, 54 ^{o}C for 1 minute and 72 ^{o}C for 1 minute
- 30 cycles
- RT-PCR was found to be more sensitive than ELISA and NASH

Contd.

- RT-PCR based protocol was developed and compared to NASH and ELISA
- Results clearly showed that all the three protocols can detect virus
- RT-PCR based protocol was more efficient and sensitive in detecting PVY from field samples

Contd.

- RT-PCR could detect presence of very small amount of infection in field samples which was not detected by ELISA or NASH

- Also helps to correctly identify resistant plants in population

Corrections

Virus resistant plants

➢ Coat protein mediated

- Papaya resistant to papaya ringspot virus, PRSV

- Variety Rainbow, yielded 112,000 kg/ha marketable fruits, compared to 5,600 kg/ha from non-transgenic lines

Contd.

- CP gene of Potato mosaic virus (PMV) strain N605 provides resistance in transgenic potato plants against this strain and also to related strain 0803

- Transgenic clones C2, C3, C4, C5, C6 and PT-6 of plum transformed with coat protein (CP) gene of *Plum pox virus* (PPV)

Contd

- Mutant replicases from Cucumber mosaic virus (CMV) subgroup I conferred high levels of resistance in tobacco plants to all subgroup I CMV strains

Viral movement proteins (MPs)

- Mutant MPs from PMV show resistance to several TMVs, Cauliflower mosaic virus (CaMV) and other viruses

- *Nicotiana occidentalis* plants expressing movement protein (P50) and partially functional deletion mutants (DeltaA and DeltaC) of Apple chlorotic leaf spot virus (ACLSV) showed resistance to Grapevine berry inner necrosis virus (GINV)

RNA silencing

- Pepper mild mottle virus (PMMoV) and Plum pox virus (PPV)

- Tomato plants expressing a single, small chimeric hairpin RNA comprised of 4Ngene segments (150-nt) of topsoviruses [TSWV, GRSV, tomato chlorotic spot virus (TCSV) and watermelon silver mottle virus (WSMoV) display broad-spectrum resistance against topsoviruses

Contd.

- Used single transgene with viral fragments from Watermelon silver mottle virus (WSMoV), Cucumber mosaic virus (CMV), Cucumber green mottle mosaic virus (CGMMV) and Watermelon mosaic virus (WMV) and transformed into watermelon

- Exposed to CMV, CGMMV and WMV

Contd

- *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus*(SPCSV), *Sweet potato virus G* (SPVG) and *Sweet potato mild mottle virus* (SPMMV)

- Coat protein gene segments from each of the four viruses mentioned were used to induce gene silencing in transgenic sweet potato

Potassium transport gene enhances resistance to soybean mosaic virus

- Altering the level of potassium can reduce the spread of viral diseases
- Potassium transporters are leading targets for breeding for virus resistance including soybean mosaic virus (SMV)
- Gene *GmAKT2* was significantly induced by SMV inoculation in resistant varieties

Contd.

- Overexpression of *GmAKT2* enhanced SMV resistance in soybean

- Manipulation of potassium transporter expression is a novel molecular approach for enhancing SMV resistance

Insect resistance

Contd.

Gene product	Target insects	Transformed plants
a-Amylase inhibitor of the common bean (aAI-Pv)	Coleoptera	Azuki bean61, pea62, tobacco63
a-Amylase inhibitor from cereals (WMAI-1)	Lepidoptera	Tobacco
Bifunctional inhibitor of a-amylases and serine proteinases (14K-CI)		Tobacco5
Lectins		
Snowdrop lectin (GNA)	Homoptera, Lepidoptera	Grapevine64, oilseed rape49, potato38,65–67, rice68, sweet potato51, sugarcanea, sunflowerb
Pea lectin (p-lec)	Homoptera, Lepidoptera	Potato69, tobacco70

Contd.

Gene product	Target insects	Transformed plants
Wheat germ agglutinin (WGA)	Lepidoptera, Coleoptera	Maize
Jacalin	Lepidoptera, Coleoptera	Maize
Rice lectin	Lepidoptera, Coleoptera	Maize
Chitinase		
Bean chitinase (BCH)	Homoptera, Lepidoptera	Potato
Tobacco anionic peroxidase	Lepidoptera, Coleoptera	Sweet gum71, tobacco71, tomato71
Tomato chitinase	Homoptera	Oilseed rape72
Tryptophan decarboxylase from *Catharanthus roseus* (TDC)	Homoptera	Tobacco

alpha-amylase inhibitors

- Introduction and expression of the bean a-AI gene in pea confers resistance to the bruchid beetles

Lectins

- Engineered plants showed resistance against brown plant hopper (*Nilaparvata lugens*) and green leaf hopper (*Nephotettix virescens*)
- Wheat germ agglutinin, pea lectin and rice lectin have been expressed in plants like tobacco, maize, and potato mainly against aphids
- Chickpea: garlic lectin gene (*asaf*): Aphids

Contd.

- Snowdrop lectin (*Galanthus nivalis* agglutinin) expressing plants are resistant to solitary bee *Osmia bicornis*

Thank You